ENVIRONMENTAL CARCINOGENS SELECTED METHODS OF ANALYSIS

Volume 1 – VOLATILE NITROSAMINES

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

WORLD HEALTH ORGANIZATION

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

ENVIRONMENTAL CARCINOGENS SELECTED METHODS OF ANALYSIS

VOLUME 1 - Analysis of Volatile Nitrosamines in Food

EDITOR-IN-CHIEF: H. EGAN

EDITORS

R. PREUSSMANN M. CASTEGNARO

E.A. WALKER A.E. WASSERMANN

TECHNICAL EDITOR FOR IARC

W. DAVIS

IARC Scientific Publications No. 18

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

LYON

1978

The International Agency for Research on Cancer (IARC) was established in 1965 by the World Health Assembly as an independently financed organization within the framework of the World Health Organization. The headquarters of the Agency are at Lyon, France, and it has Research Centres in Iran, Kenya and Singapore.

The Agency conducts a programme of research concentrating particularly on the epidemiology of cancer and the study of potential carcinogens in the human environment. Its field studies are supplemented by biological and chemical research carried out in the Agency's laboratories in Lyon and, through collaborative research agreements, in national research institutions in many countries. The Agency also conducts a programme for the education and training of personnel for cancer research.

The publications of the Agency are intended to contribute to the dissemination of authoritative information on different aspects of cancer research.

ISBN 978-92-832-2200-2

The authors alone are responsible for the views expressed in the signed articles in this publication.

PRINTED IN SWITZERLAND

CONTENTS

METHODS OF ANALYSIS

Foreword

During the last three decades, epidemiologists have collected and analysed a mass of data on cancer incidence in a large number of defined populations, and striking differences in cancer patterns have been demonstrated. The available evidence suggests that variations in the environment, directly or indirectly, are responsible for the observed differences in cancer incidence at certain sites. Analytical epidemiological studies are being carried out in many countries, the objectives of which are to identify specific carcinogens. Undoubtedly, "lifestyle" habits - smoking, immoderate drinking of alcoholic beverages, unbalanced diets - make a major contribution to cancer causation. However, an important role for carcinogenic chemicals present in very low concentrations in the environment and associated with pollution of air, food and water cannot be excluded.

In industrialized communities it has been estimated that from 3 to 5 per cent of all cancers in man may be due to direct industrial exposure. The long term effects on the general population of very low concentrations of chemical carcinogens, acting either singly or in combination, and entering the environment as natural or industrial pollutants, still remain to be assessed. However, monitoring both the carcinogenic "load" in the environment and secular trends in cancer incidence may make it feasible in the future to identify modifications in the level of environmental carcinogens. Such monitoring might open the way not only to the evaluation of hazards, but also to the possibilities for control and prevention.

The need for accurate data derived from reliable analytical techniques for measuring environmental carcinogens is of major importance in modern epidemiological research. I wish, therefore, to express my gratitude to all who have contributed towards the compilation of this manual of selected methods. With its publication, I hope that the Agency will contribute effectively to increasing the power of current research on the etiology of human cancer.

John HIGGINSON, M.D.

Director

International Agency
for Research on Cancer,
Lyon, France

INTRODUCTION

There are two broad avenues of approach to the identification of environmental carcinogens, the measurement of cancer incidence and the measurement of the carcinogens, known or putative, themselves. The International Agency for Research on Cancer is concerned with both of these and sees the publication of selected analytical methods as an important means of encouraging the generation of reliable and comparable data on the levels of selected carcinogens in the environment that may ultimately be shown to be associated with the incidence of a given cancer. In considering the substances to be included in the manual, the Editorial Board has looked in the first place at those chemicals which have been included in the IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, the publication of which commenced in 1972. The chemicals which have been evaluated in the Monographs were selected on the basis of evidence of human exposure, together with some experimental evidence of carcinogenicity, with or without evidence or suspicion of a human risk. A short priority list of chemicals, or groups of chemicals, to be treated in this manual, has been prepared on the basis of these evaluations, selecting those substances, or groups of substances, for which there was strong evidence of carcinogenicity in man or in experimental animals, and for which human exposure was likely to occur.

The volumes in preparation include vinyl chloride and the polycyclic aromatic hydrocarbons.

For the preparation of each volume a small Review Board, appointed by the Editorial Board, has undertaken the detailed evaluation of the literature and the main selection of methods of analysis to be included and, following detailed discussions with the Editorial Board, has provided the text upon which the volume is based. No attempt has been made to provide standard methods of analysis, but, wherever possible, methods have been selected for which reliable data have been established by collaborative or co-operative studies, preferably on an international basis.

The methods are presented in the recommended ISO format.

The Editorial Board recognizes that analytical chemistry is a subject which is constantly developing and that new or improved methods may supersede those now published. It therefore proposed to include such methods from time to time in future volumes of the Manual.

H. EGAN

Editor-in-Chief

MEMBERS OF THE EDITORIAL BOARD

OF

ENVIRONMENTAL CARCINOGENS - SELECTED METHODS OF ANALYSIS

C. AGTHE	Food Safety Division of Environmental Health, World Health Organization, Geneva, Switzerland
P. BOGOVSKI	Institute of Experimental and Clinical Medicine, Tallinn, Estonian SSR
E. BOYLAND	London School of Hygiene and Tropical Medicine, London, UK
W. DAVIS	Technical Editor, International Agency for Research on Cancer, Lyon, France
H. EGAN (Chairman and Editor-in-chief)	Laboratory of the Government Chemist, London, UK
L. GRICIUTE	Unit of Environmental Carcinogens, International Agency for Research on Cancer, Lyon, France
H. KUNTE	Institute of Hygiene, Johannes Gutenberg University, Mainz, FRG
R. PREUSSMANN	German Cancer Research Centre, Institute of Toxicology and Chemotherapy, Heidelberg, FRG
P.L. SCHULLER	National Institute of Public Health, Bilthoven, The Netherlands
V.B. VOUK	Control of Environmental Pollution and Hazards, Division of Environmental Health, World Health Organization, Geneva, Switzerland

E.A. WALKER	Unit of Environmental Carcinogens, International Agency for Research on Cancer, Lyon, France
L. STOLOFF	Food and Drug Administration, Department of Health, Education and Welfare, Washington, USA
R. TRUHAUT	Hospital of Ville-Evrard Neuilly s/Marne, France

RECOMMENDED ABBREVIATIONS

In an attempt to standardize the abbreviations used for *N*-nitroso compounds, recommendations made at the 5th Meeting on Analysis and Formation of *N*-Nitroso Compounds (Durham, New Hampshire, USA, 24-26 August 1977) have been followed in the present volume and are given below.

N-nitrosodimethylamine	- NDMA
N-nitrosodi- -butylamine	- NDBA
N-nitrosodi-isobutylamine	- NDi-BA
N-nitrosoethylmethylamine	- NEMA
N-nitrosopyrrolidine	- NPYR
N-nitrosopiperidine	- NPIP
N-nitrosomorpholine	- NMOR
Mononitrosopiperazine	- M-NPZ
Dinitrosopiperazine	- D-NPZ
N-nitrosoproline	- NPRO
N-nitrososarcosine	- NSAR
N-nitrosohydroxyproline	- NHPRO
N-nitrosopipecolic acid	- NPIC
N-nitrosohydroxypyrrolidine	- NHPYR
N-nitrosonornicotine	- NNN
N-nitrosodiethanolamine	- NDELA

When abbreviating nitrosureas, use -NU. For nitrosourethanes, use -NUT.

CARCINOGENICITY OF *N*-NITROSO COMPOUNDS

CARCINOGENICITY OF *N*-NITROSO COMPOUNDS AND THEIR POSSIBLE ROLE IN THE DEVELOPMENT OF HUMAN CANCER

Laima Griciute

N-Nitroso compounds, both in the form of nitrosamines as well as nitrosamides, have been shown, in animal experiments, to be among the most potent chemical carcinogens known to date. It wass only a little more than 20 years ago that Magee & Barnes (1956) first described the hepatocarcinogenicity of the simplest nitrosamine (*N*-nitrosodimethylamine) in the rat. During the relatively short time since that land mark, *N*-nitroso compounds have become the subject of intensive international research, with growing scientific as well as practical impact, since such compounds have been shown to occur in the human environment and therefore constitute a potential health risk for man.

Several reviews on the carcinogenicity of *N*-nitroso compounds are available (Druckrey et al., 1967; IARC, 1972; Barnes, 1967; Magee et al., 1976; Preussmann, 1975) and a revised IARC monograph in the series "evaluation of carcinogenic risk of chemicals to man" is being prepared, containing extensive information concerning *N*-nitroso compounds in the human environment. This monograph will be available early in 1978. For this reason, only a short summary of the more important data will be given here.

N-Nitroso compounds are among the most potent chemical carcinogens known at the present time. More than 120 different compounds have been tested in animal experiments and about 80% of them have been shown to be carcinogens. Any new compound of this group must therefore be considered a potential carcinogen until evidence to the contrary appears.

The more important representative of the *N*-nitrosamine group, such as *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA), have been tested in many animal species. NDEA has been shown to be carcinogenic in more than 20 of these, including subhuman primates. No animal species is known to be resistant to the potent carcinogenic effects of this compound.

Tumours can be induced in all important organs. Organ specificity in carcinogenesis is mainly governed by the chemical structure of the carcinogenic compound, but also depends on animal species and strain, dosage, mode of administration and duration of exposure. Organs affected include liver, lungs, kidney, oesophagus, stomach, urinary

bladder, brain and nervous system and blood-generating organs. Many of the tumours produced are similar to those in man.

Carcinogenic response is clearly dose-related. In chronic feeding studies, the lowest effective daily dose (in the diet) has been determined to be 2 ppm (mg/kg) for NDMA (Terracini et al., 1967), 0.75 ppm for NDEA (Druckrey et al., 1963) and 10 ppm for *N*-nitrosopyrrolidine (Preussmann et al., 1977). *N*-nitrosamines, therefore, can be active carcinogens at low doses in animals. It has been shown in several studies that a single dose can also be effective. Mohr & Hilfrich (1972), for example, injected s.c. single doses of *N*-nitrosodiethylamine in rats. Even at the very low dose of 1.25 mg/kg b.w. (0.3 mg/animal), typical kidney tumours were observed.

Certain *N*-nitroso compounds have been shown to be active in transplacental carcinogenesis (IARC Scientific Publication No 4, 1973). In a similar, but more far-reaching study, Tomatis et al. (1975), in a so-called multigeneration experiment, have shown increased tumour incidence in F_2 and F_3 generations after a short exposure *in utero* of the F_1 generation to *N*-nitrosomethylurea. F_2 and F_3 had no direct contact with the carcinogen. It can be expected that the fetus is 50 to 100 times more sensitive to induction of lesions by *N*-nitrosourea derivatives than is the adult organism.

In general, most *N*-nitroso compounds are mutagenic in different test systems (Montesano & Bartsch, 1976), but mutagenic activity does not always correspond to carcinogenic activity (Lijinsky & Elespuru, 1976).

At present, there is no direct and conclusive evidence for carcinogenicity of *N*-nitroso compounds in man. A recent report concerning two cancer patients, however, associates leukaemia and treatment with *N*-nitrosourea derivatives (Cohen et al., 1976). This is probably not conclusive, but is at least suggestive. Other indirect evidence is also available:

1. The same acute changes have been observed in persons working with NDMA in industry as in animals treated with this compound, e.g. centriglobular liver necrosis (Magee, 1973).
2. The *in vitro* metabolism of NDMA in rat and human liver, as measured by CO_2 formation, is similar (Montesano & Magee, 1974).
3. Proliferative changes, considered to be precancerous, have been observed in human embryo lung tissue cultures, following administration of *N*-nitrosoureas (Kolesnichenko, 1975).

The foregoing observations justify, to some extent, the uncertain extrapolation from animal data. No epidemiological evidence concerning the carcinogenicity of *N*-nitroso compounds in man is presently available,

but may arise in the future from studies of occupational exposure to *N*-nitrosamines (e.g. *N*-nitrosodiethanolamine in curring fluids).

Exposure of the general population to potentially carcinogenic *N*-nitroso compounds may arise from two sources:

1. Preformed *N*-nitroso compounds in different environmental media, such as food, air and water, as well as in tobacco smoke (Hecht et al., 1978; Hoffmann et al., 1974, 1976), certain cosmetic articles (Fan et al., 1977) and drugs (Eisenbrand et al., 1978).

2. *N*-nitroso compounds formed in the human gastrointestinal tract, or other sites, by the reaction of nitrosating agents, such as nitrite, nitrous gases and sometimes nitrate, on precursors such as secondary and tertiary amines.

Monitoring of human exposure to determine the total load of *N*-nitroso compounds must therefore take both sources into consideration.

The determination of preformed volatile *N*-nitrosamines is at present the most advanced aspect of the study of exogenous *N*-nitrosamines in the environment. The methods described in this volume, which have been drawn from those successfully employed in the collaborative studies conducted by the IARC, in conjunction with the International Union of Pure and Applied Chemistry (Castegnaro & Walker, 1978; Walker & Castegnaro, 1974, 1976), show the tremendous progress in this field from the very first attempts (Preussmann et al., 1964), and the subsequent methodical developments by different groups (Eisenbrand, 1974; Fine & Rounbehler, 1976; Gough & Woollam, 1976; Preussmann & Eisenbrand, 1971; Telling et al., 1974; Walker & Castegnaro, 1976; Walker et al., 1975). The present volume shows that we now have reliable and sensitive methods for the quantitative analytical determination of volatile *N*-nitrosamines. It must always be realized, however, that volatile exogenous *N*-nitrosamines are only a part of the total load of *N*-nitroso compounds. At present, most non-volatile derivatives cannot reliably be detected and determined, but methods are being developed and collaboratively tested for this purpose.

The amount of exposure to endogenous *N*-nitroso compounds formed in the gastrointestinal tract or other sites in the human body is completely unknown. The analytical determination of the precursors, also briefly dealt with in this volume, still presents many problems. Despite much progress, from the first observation (Sander, 1967) to the present time, endogenous human exposure cannot yet be quantified. However, a promising move toward the solution of this problem has recently been made by Fine and co-workers (1977), who determined *N*-nitrosamines in human biological material, such as blood.

Systematic quantitative information on exogenous *N*-nitroso compounds in the human environment is still scanty. The nitroso

compounds most often detected have been NDMA, NDEA, *N*-nitroso-pyrrolidine (NPYR) and *N*-nitrosopiperidine (NPIP). The main substrates were cured meat and fish, tobacco smoke, certain alcoholic beverages air and surface water. The quantities detected vary from 0 to 1.000 µg/kg, but in most case lie between 1 and 10 µg/kg (ppb). At present, it is generally agreed that the most suitable method for the identification of *N*-nitroso compounds is gas chromatography, combined with mass spectrometric confirmation. Nitrosamine-specific chemiluminescence detection (e.g. Thermal Energy Analyzer), however, will very likely be the method of choice in the future.

Since standardization of methods is always the first step toward the goal of obtaining reproducible and comparable data from trace analytical methods, the International Agency for Research on Cancer has undertaken the publication of this first volume of the Manuals of Selected Methods of Analysis for Environmental Carcinogens.

REFERENCES

Castegnaro, M. & Walker, E.A. (1978) *Collaborative studies on volatile and nonvolatile nitrosamines: a report of the current status of studies.* In: Walker, E.A., Griciute, L., Castegnaro, M & Lyle, R., eds, *Environmental Aspects of* N-*Nitroso Compounds*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* in press)

Cohen, R.J., Wiernik, P.K. & Walker, M.D. (1976) Acute nonlymphocytic leukemia associated with nitrosourea chemotherapy: report of two cases. *Cancer Treat. Rep. 60*, 1257-1261

Druckrey, H., Schildbach, A., Schmähl, D., Preussmann, R. & Ivankovic, S. (1963) Quantitative Analyse der Carcinogenen Wirkung von Diäthylnitrosamin. *Arzneim. Forsch., 13*, 841-846

Druckrey, H., Preussmann, R., Ivankovic, S. & Schmähl, D. (1967) Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch., 69*, 103-201

Eisenbrand, G. (1974) *Recent developments in trace analysis of volatile nitrosamines. A brief review.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 9), pp. 6-11

Eisenbrand, G., Spiegelhalder, B., Janzowski, C., Kann, J. & Preussmann, R. (1978) *Volatile and non volatile* N-*nitroso compounds in foods and other environmental media.* In: Walker, E.A., Griciute, L., Castegnaro, M. & Lyle, R., eds, *Environmental Aspects of* N-*Nitroso Compounds*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* in press)

Fan, T.Y., Goff, U., Song, L., Fine, D.H., Arsenault, G.P. & Biemann, K. (1977) *N*-Nitrosodiethanol in consumer cosmetics, lotions and shampoos. *Food Cosmet. Toxocil.* (in press)

Fine, D.H. & Rounbehler, D.P. (1976) *Analysis of volatile* N-*nitroso compounds by combined gas chromatography and thermal energy analysis.* In: Walker, E.A., Bogovski, P. & Griciute,L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 14), pp. 117-127

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

Gough, T.A. & Woollam, C.J. (1976) *Techniques for the screening of foodstuffs for volatile nitrosamines.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds. Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 14), pp. 85-96

Hecht, S.S., Chen, C.B. & Hoffmann, D. (1978) *Chemical studies on tobacco smoke. LVI. Tobacco-specific nitrosamines: Origines, carcinogenicity and metabolism.* In: Walker, E.A., Griciute, L., Castegnaro, M. & Lyle, R., eds, *Environmental Aspects of* N-*Nitroso Compounds*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* in press)

Hoffmann, D., Rathkamp, G. & Liu, Y.Y. (1974) *Chemical studies on tobacco smoke. XXVI. On the isolation and identification of volatile and non-volatile* N-*nitrosamines and hydrazines in cigarette smoke.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 9), pp. 159-165

Hoffmann, D., Hecht, S.S., Ornaf, R.M., Tso, T.C. & Wynder, E.L. (1976) *Chemical studies on tobacco smoke. XLII Nitrosonornicotine: presence in tobacco, formation and carcinogenicity.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 14), pp. 307-320

International Agency for Research on Cancer (1972) *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Vol. 1*, Lyon, International Agency for Research on Cancer, pp. 95-142

Kolesnichenko, J. (1975) Effect of *N*-nitrosomethylurea on organ cultures of human embryonal pulmonary tissue. *Voprosy Onkologii, VXXI, 2*, 79-84 (in Russian)

Lijinsky, W. & Elespuru, R.K. (1976) *Mutagenicity and carcinogenicity of* N-*nitroso derivatives of carbamate insecticides.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 14), pp. 425-428

Magee, P.N. (1973) Nitrosamines: ubiquitous carcinogens? *New Scientist, 59*, 432-434

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

Magee, P.N. & Barnes, J.M. (1967) Carcinogenic nitroso compounds *Adv. Cancer Res., 10*, 163-246

Magee, P.N., Montesano, R. & Preussmann, R. (1976) N-*Nitroso compounds and related carcinogens.* In: Searle, C.E., ed., *Chemical Carcinogens*, Washington, Am. Chem. Soc. Monograph No. 173, pp. 491-625

Mohr, U. & Hilfrich, J. (1972) Effect of a single dose of *N*-diethylnitrosamine on the rat kidney. *J. Natl. Cancer Inst., 49*, 1729-1731

Montesano, R. & Magee, P.N. (1974) *Comparative metabolism* in vitro *of nitrosamines in various animal species including man.* In: Montesano, R. & Tomatis, L., eds, *Chemical Carcinogenesis Essays*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 10), pp. 39-56

Montesano, R. & Bartsch, H. (1976) Mutagenic and carcinogenic *N*-nitroso compounds: Possible environmental hazards. *Mutat. Res., 32*, 179-228

Preussmann, R. (1975) *Chemische Carcinogene in der menschlichen Umwelt.* In: Grundmann, E., ed., *Handbuch der allgem. Pathol., Vol. VI/6l II*, Berlin-Heidelberg-New York, Springer-Verlag, pp. 421-594

Preussmann, R. & Eisenbrand, G. (1972) *Problems and recent results in the analytical determination of* N-*nitroso compounds.* In: Nakahara, W., Takayama, S., Sugimura, T. & Odashima, S., eds, *Topics in Chemical Carcinogenesis*, Tokyo, University of Tokyo Press, pp. 323-341

Preussmann, R., Neurath, G., Wulf-Lorentzen, G., Daiber, D. & Hengy, H. (1964) Anfärbemethoden und Dünnschichtchromatographie von organischen *N*-Nitrosoverbindungen. *Z. analyt. Chem.*, *202*, 187-192

Preussmann, R., Schmähl, D. & Eisenbrand, G. (1977) Carcinogenicity of *N*-nitrosopyrrolidine: Dose-response study in rats. *Z. Krebsforsch. 90-2*, 161-166

Sander, J. (1967) Kann Nitrit in der menschlichen Nahrung Ursache einer Krebsentstehung durch Nitrosaminbildung sein? *Arch. Hyg. Bakt.*, *151*, 22-28

Telling, G.M., Bryce, T.A., Hoar, D., Osborne, D. & Welti, D. (1974) *Progress in the analysis of volatile* N-*nitroso compounds.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso compounds in the environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 9), pp. 12-17

Terracini, B., Magee, P.N. & Barnes, J.M. (1967) Hepatic pathology in rats on low dietary levels of dimethylnitrosamine. *Br. J. Cancer*, *21*, 559-565

Tomatis, L., Hilfrich, J., Turusov, V. (1975) The occurrence of tumours in F_1, F_2 and F_3 descendants of BD rats exposed to *N*-nitrosomethylurea during pregnancy. *Int. J. Cancer*, *15*, 385-390

Tomatis, L. & Mohr, U., eds (1973) *Transplacental carcinogenesis*, Lyon, International AGency for Research on CAncer (*IARC Scientific Publications* No. 4)

Walker, E.A. & Castegnaro, M. (1974) *A report on the present status of a collaborative study of methods for the trace analysis of volatile nitrosamines.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Comp unds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 9), pp. 57-62

Walker, E.A., Castegnaro, M. & Pignatelli, B. (1975) Use of clean-up method to improve specificity in the analysis of foodstuffs for volatile Nitrosamines. *Analyst*, *100*, 817-821

Walker, E.A. & Castegnaro, M. (1976) *New data on collaborative studies on analysis of volatile nitrosamines.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 14), pp. 77-84

Walker, E.A. & Castegnaro, M. (1976) Problems in trace analysis of nitrosamines in foodstuffs. *Natl. Bur. Stand. Spec. Publ.*, *422* Vol. II, pp. 727-736

The following sections devoted to the different aspects of nitrosamine analysis were reviewed by: M. Castegnaro, W. Fiddler, P. Issenberg, S. Tannenbaum, G. Telling and E.A. Walker.

GENERAL REVIEW OF APPROACHES TO NITROSAMINE ANALYSIS

1. SAMPLING AND SAMPLES

T. Kawabata

1.1 *General remarks*

In many respects, the sampling procedure is as important as the analytical procedure employed. The results obtained from analysis are of little value if they are obtained from a test sample which does not accurately represent the average composition of the material analysed. Likewise, samples of a diet should be sufficient in number and variety to be statistically representative and to allow replication and confirmation. Nevertheless, no attempt will be made in this Manual to set down detailed instructions for sampling in environmental studies, as such a detailed approach is beyond its scope. However, some general guidelines for taking representative samples are given below.

1.2 *Definitions*

a. Sampling unit: the smallest quantity in which the product is available.

b. Lot: a clearly defined block of material comprised of units of a single type, class, size and composition manufactured, stored and shipped under essentially the same conditions and for the same period of time.

c. Sample: a sampling unit or collection of sampling units, selected at random, which is determined to be needed to ensure that the analytical results are representative of an entire lot.

d. Random selection: selection such that every sampling unit in a lot has an equal chance of being included in the sample.

1.3 *Sample preparation*

For small samples of food (i.e. less than 1 kg) a representative sample may be obtained by homogenizing, then taking a suitable aliquot. When a blender is used, care should be taken to keep the blending time to a minimum, to avoid loss of volatile nitrosamines due to localized heat which may be generated within the sample. For larger samples, where a lot cannot be thoroughly mixed, a composite sample of randomly selected units (e.g., a number of cans of food from a batch process) may be taken and suitably homogenized. Where a heterogeneous product is sampled, random selection is often impracticable. In this case, a representative sample may be obtained by separating the essential component parts, such as the crust and meat filling of a pie, and

taking from each an amount in proportion to its size. If different parts present different analytical clean-up problems, they should be analysed separately. An alternative approach is to take a number of parts which are considered to be representative of the whole sample. Whichever method of sample selection is used, the quantity must be sufficient so that, after mincing or grinding and finally mixing, duplicate samples may be taken. After preparation, samples not immediately analysed should be stored in airtight containers, preferably completely filled.

Special techniques for obtaining representative samples of cheese, animal foods, etc., can be found in the appropriate literature, e.g., Official Methods of Analysis of the Association of Official Analytical Chemists, 12th ed., 1975. Similarly, the determination of the number of samples required for a statistically correct demonstration of the presence or exact level of *N*-nitroso compounds for regulatory purposes is also beyond the scope of this Manual. For more information on this subject, readers are referred to ASTM Standards - E122-72, *Choice of Sample Size to Estimate the Average Quality of a Lot of Process* (1972) and E105-58, *Probability Sampling of Materials* (1958) and ISO/TC 69, *Applications of Statistical Methods* (ISO Technical Committee 69), ISO 2602-1973, ISO 2854-1976, ISO 2859-1974, ISO 3207-1975, ISO 3301-1975, ISO 3494-1976, ISO 3534-1977.

Attention is drawn to the need for careful reporting and labelling, particularly with regard to sample origin and conditions of transport, since these may later be found to be relevant to the formation of "artefact" nitrosamines.

When perishable samples (or samples which are not to be analysed immediately) are received by a laboratory, they should be stored at deep-freeze temperatures. Where applicable, representative ground or minced samples can be prepared and treated with methanolic potassium hydroxide solution to prevent the formation of *N*-nitroso compounds during storage and transit.

2. EXTRACTION AND CLEAN-UP METHODS

G.M. Telling

2.1 *Introduction*

The pattern of development of analytical methods for volatile *N*-nitrosamines closely follows that for other trace contaminants, such as pesticide residues. Interest in *N*-nitrosamine analysis became significant only in the late 1960s, when the problem was taken up independently by a number of laboratories. As a result, although the general approach was one of extraction, clean-up and determination, a considerable variety of techniques were employed. A major problem in *N*-nitrosamine analysis arises from the fact that the only common property of these compounds is a structural feature, namely the *N*-nitroso group, >N-N=O. This group may be attached to a large variety of moieties, R and R', which donate different physico-chemical properties to the individual molecules, $\begin{matrix} R \\ R' \end{matrix}\!\!> N\text{-}N{=}O$

Furthermore, our knowledge of the precursor amines, which give rise to the nitrosamines, is also limited. The analytical approach depends on whether or not the *N*-nitrosamine of interest is steam volatile. The chemical nature of volatile *N*-nitrosamines is now fairly well defined and various methods for their determination have been reported.

Collaborative studies organized by the International Agency for Research on Cancer (IARC) have shown that several methods are now available for the quantitative determination of a series of volatile *N*-nitrosamines added to luncheon meat at the 5-20 µg/kg level. The literature also contains a number of techniques for the analysis of food products, alcoholic drinks, air, water and biological samples, such as urine or blood. The present review covers the wide variety of techniques reported over the period 1966-75 and will compare those techniques which have become established and used in the third IARC Collaborative Study.

As in most other residue methods, analysis for volatile *N*-nitrosamines can be considered to consist of three stages, viz:

extraction
clean-up of extracts
determination.

It is not always possible to divide methods into these neat compartments, but they are useful in considering available techniques. In an ideal process, quantitative extraction of the desired component would be achieved without extraction of interfering material. Such a situation is never achieved in practice and the problem is one of

obtaining optimum extraction conditions. Normally, the more vigorous the extraction conditions employed, the larger the amount of co-extracted interfering material. This then necessitates more rigorous clean-up conditions. Similarly, clean-up should give quantitative removal of interfering material while retaining the species to be determined. This, again, is never achieved and conditions have to be optimized. The degree of clean-up required often depends on the final determinative step to be used. The introduction of selective systems, such as gas chromatography with high-resolution mass spectrometry (GC-MS) and, especially, the Thermal Energy Analyzer (TEA), have allowed much less rigorous clean-up techniques to be used.

2.2 *Extraction*

Techniques for extraction can be considered under three main headings:

2.2.1 *Direct solvent extraction*

A range of solvents for direct extraction of nitrosamines from food substrates has been reported, including water (Ender & Ceh, 1967), diethyl ether (Panalaks et al., 1973; Sen et al., 1969; Van Ginkel, 1970), acetonitrile/heptane (Eisenbrand et al., 1969) and dichloromethane (Heyns & Koch, 1971; Neurath et al., 1964b).

Most reported clean-up procedures now incorporate extraction into dichloromethane as one or more of the steps. Neurath et al. (1964b) examined the efficiency of various solvents and concluded that extraction was almost complete with dichloromethane. This was confirmed by Telling et al. (1971), who showed that when aqueous solutions of volatile nitrosamines are extracted with equal volumes of dichloromethane, more than 95% of the nitrosamine is found in the organic phase, except for *N*-nitrosodimethylamine (NDMA), where only 77% is transferred to the organic phase. The volume of dichloromethane required can be considerably reduced by the use of salting-out techniques (Crosby et al., 1972a).

Extraction of alcoholic beverages has posed analytical problems, since the lower homologues of dialkyl nitrosamines are freely soluble in water, ethanol and solvent mixtures. Direct distillation presents problems due to the formation of azeotropes. Castegnaro et al. (1974a) reported a successful technique for spirits, based on the adjustment of alcohol content to 55%, saturation with anhydrous magnesium perchlorate and extraction with dichloromethane.

Urine and faeces have been extracted successfully with dichloromethane, after being made alkaline (Kawabata & Miyakoshi, 1976; Telling et al., 1974).

2.2.2 *Digestion techniques*

The most vigorous extraction technique is based on digestion of solid samples with a mixture of methanol and potassium hydroxide for 3-5 hr. The digest is then extracted with dichloromethane, either in a separator (Howard et al., 1970) or by continuous liquid-liquid extraction (Fazio et al., 1972). Heating the sample with citrate-phosphate buffer (pH 7.0) to break down the sample and to coagulate protein has also been proposed (Iwaoka et al., 1974). The resulting digest is also extracted into dichloromethane. As will be seen in Table 1, these vigorous extraction conditions necessitate considerable subsequent clean-up of the extracts.

2.2.3 *Distillation methods*

The low molecular weight nitrosamines are all steam volatile, hence the first stage of many reported methods involves distillation. Neurath et al.(1964b) first reported the distillation of nitrosamines from meat under alkaline conditions and Eisenbrand et al. (1970) showed that nitrosamines could be successfully distilled from aqueous systems under alkaline, neutral and acid conditions. The use of acid conditions is somewhat suspect, however, as these may encourage the formation of nitrosamines as artifacts. The most commonly used alkaline distillation conditions are either strongly alkaline (sodium hydroxide, Neurath et al., 1964b) or mildly alkaline (potassium carbonate, Telling et al., 1971). The use of strongly alkaline distillation is preferred by Sen et al. (1974a), in order to avoid nitrosamine formation during analysis. Addition of up to 20% of sodium chloride to improve the distillation yield is frequently employed. Distillation from aqueous methanol has also been suggested (Walters et al., 1970).

Distillation may be carried out in steam in one (Eisenbrand et al., 1970b) or two (Crosby et al., 1972a) stages, or under vacuum (Neurath et al., 1964b). Diethyl ether extracts of flour have also been steam distilled, after addition of water (Sen et al., 1969). The conditions used for distillation can also vary widely (Eisenbrand et al., 1970) ranging from ambient conditions to reduced temperature and pressure; distillation at 50-60°C under reduced pressure has been used by Telling et al. (1971). Fine et al. (1975c) have modified the distillation processes by adding mineral oil and sodium hydroxide, to improve recoveries, especially of nitrosopyrrolidine (NPYR).

The dichloromethane extract from methanolic potassium hydroxide digestion has been mixed with alkali, the solvent removed and the residual aqueous solution steam distilled (Howard et al., 1970). In a similar manner, direct dichloromethane extracts have been distilled from sodium hydroxide and barium hydroxide solutions (Fazio et al., 1972).

Table 1. A summary of methods used in the third IARC Collaborative Study[a]

Collaborating Laboratory	21	4	1	5	15	16	9	13	3	18	20	14
Sample	Distil from mineral oil + NaOH	Steam distil from NaCl	Vacuum distil from NaCl + K_2CO_3	Steam distil from NaCl	Digest with methanol + KOH Extract into CH_2Cl_2 Distil from NaOH	Digest with methanol + KOH Extract into CH_2Cl_2 Distil from alkali	Extract with CH_2Cl_2 Steam distil from NaOH/NaCl Steam distil from tartaric acid + NaCl	Add K_2CO_3 extract with CH_2Cl_2 Steam distil from NaOH + $Ba(OH)_2$	Vacuum distil from NaCl + K_2CO_3	Extract alkaline slurry with CH_2Cl_2 Distil from KOH	Steam distil from NaCl Steam distil from tartaric acid	Steam distil from NaCl
Aqueous Distillate	Add HCl	Add NaCl + sulfuric acid		Add NaCL + sulfuric acid	Add HCl	Add HCl		Shake with ion exchange resin at pH 6.5	Add H_2SO_4	Add K_2CO_3	Add KOH	Add H_2SO_4
	Extract	Extract	Extract	Extract	Extract	Extract	Extract	Extract	Extract	Extract	Extract	Extract
Dichloro-methane extract		Wash with NaOH		Wash with NaOH	Wash with HCl Wash with NaOH	Wash with HCl Wash with NaOH	Use direct			Wash with glycine-HCl buffer		Wash with KOH
	Concentrate	Concentrate	Concentrate	Concentrate	Concentrate	Concentrate	Concentrate	Concentrate	Concentrate	Concentrate	Concentrate	Concentrate
Concentrated extract		Transfer into hexane	Transfer into hexane	Transfer into hexane	Chromato-graphy on Florisil + Silica gel column	Chromato-graphy on Silica gel or acid Celite column	Add Silica gel Extract from gel with ethyl acetate			Transfer into heptane Chromato-graphy on alumina column	Transfer into hexane Alumina column Oxidise Alumina column	Add pentane Alumina column Oxidise Alumina column
	Use direct							Use direct	Use direct			
Determinative technique	TEA	GC-MS	GC-MS	GC-MS	GC-MS	Thermionic + GC-MS confirmation	Thermionic before/after irradiation	Thermionic	Coulson or Denitrosa-tion	Coulson + TLC + GC-MS	EC-GLC of Nitramines	EC-GLC of Nitramines

[a] Walker, E.A., Bogovski, P. & Griciute, L., eds (1976) *Environmental N-Nitroso Compounds. Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications* No. 14)

A variety of distillation techniques has also been proposed for the analysis of alcoholic beverages. Sen & Dalpe (1972) used azeotropic distillation, while Crosby et al. (1972b) used a spinning-band column for fractional distillation. Williams et al. (1971) used normal vacuum distillation, followed by addition of water until a two-phase system with dichloromethane was obtained to overcome the effect of the ethanol. Castegnaro et al. (1974a) eliminate distillation by pre-saturating of the alcoholic beverage with anhydrous magnesium perchlorate and extracting directly.

2.4 *Clean-up*

Because of the effect of interfering materials on the efficiency of gas chromatographic systems and the limitations in selectivity of most detector systems, some clean-up is always necessary.

The extraction of distillates with dichloromethane is itself a useful clean-up step. If distillates are acidified prior to extraction (Goodhead & Gough, 1975), many basic materials are held back in the aqueous phase without any loss of nitrosamines. Washing dichloromethane extracts with acid (Fazio et al., 1972), hydrochloric acid/glycine buffer and/or alkali (Fazio et al., 1972; Marquardt & Hedler, 1966; Sen & Dalpe, 1972) have been reported to achieve significant clean-up of the organic phase, with no loss of nitrosamines. Washing with acetonitrile/heptane (Eisenbrand et al., 1969), and permanganate or bromine water (Crosby et al., 1972b) have also been reported.

A number of methods have used a second distillation to clean up primary distillates. Such a distillation may be a separate step or the two distillations may be linked together in a cascade process.

Thin-layer chromatography has been used by several workers. Marquardt and Hedler (1966) applied multiple TLC on silica gel plates to isolate nitrosamines from extracts of flour, but later workers (Sen et al., 1969) have found that losses of the more volatile nitrosamine are high under these conditions. Eisenbrand et al. (1970a) later developed a technique which overcame these objections, but thin-layer chromatography does not appear to be widely used.

A wide range of clean-up systems based on column chromatography has been reported. Column materials have included acid-treated Florisil (Wasserman et al., 1972), acid celite (Howard et al., 1970), alumina (Sen, 1970; Telling, 1972; Walker et al., 1975) and cellulose (Ender et al., 1964). The use of charcoal has been proposed by several workers, with subsequent recovery from the charcoal by elution with methanol (Walters et al., 1970) or by steam distillation (Ender & Ceh, 1968), but recoveries were usually low. The use of an ion-exchange resin has been advocated, either in column form (Sen et al., 1969) or by addition to the distillate. Criticism of the former technique has been made on the grounds that higher molecular weight

nitrosamines are not quantitatively eluted (Telling et al., 1971). If oxidation to nitramines is the basis of the determinative step, further clean-up on an alumina column is usually carried out (see section 3).

A clean-up technique based on liquid-liquid partition of citrate-phosphate buffer (pH 7.0) eluates obtained from column chromatography has been reported (Iwaoka et al., 1974), but does not appear to have been adopted by other workers.

2.5 *Comparison of extraction and clean-up techniques*

A critical evaluation based on a practical study of all the above ideas is impossible, but it is interesting to see which of them have survived in the form of an acceptable analytical method. As a follow-up to the Third IARC Collaborative Study (Walker & Castegnaro, 1976) on the determination of a range of *N*-nitrosamines added to luncheon meat at the 5-10 µg/kg level, participants were asked to submit details of the methods used. The twelve methods submitted at this stage are summarised in Table 1. These techniques represent extremes of separation conditions. All the methods gave acceptable results.

The relative merits of steam, compared with vacuum, distillation are probably a question of individual preference and experience. Acidification of distillates prior to extraction with dichloromethane appears to improve clean-up without loss of *N*-nitrosamines. The need of further clean-up stages, such as acid-alkali washing or column chromatography, appears to depend on the final detection step used.

3. FINAL CHROMATOGRAPHY AND DETECTION

N.P. Sen

3.1 In almost all cases, a chromatographic separation is part of the final determinative process. Chromatography and detection are therefore dealt with together in this section. A number of methods have been described.

3.2 *Polarographic detection*

Heath & Jarvis (1955) were the first to use polarography for detecting nitrosodimethylamine (NDMA) in animal tissues (limit of detection about 500 µg/l). Volatile nitrosamines could be distinguished on the basis of differences in half-wave potentials. Although several other workers (Devik, 1967; McGlashan et al., 1968; Walters et al., 1970) have used the technique, it is now realized that it is not sufficiently specific for the analysis of complex mixtures. Interference was shown to occur from furfural and pyrazines, common constituents of many foodstuffs (Kadar & Devik, 1970; McGlashan et al., 1970). The technique can be used for the analysis of nitrosamines in less complex systems, such as models for experiments on kinetics of formation. More recently, the use of differential pulse polarography has been investigated (Chang & Harrington, 1975).

3.3 *Spectrophotometric detection*

Most nitrosamines display a characteristic UV absorption spectrum, with a strong absorption band at or near a wavelength of 230 nm and a minor absorption band at a higher (330-350 nm) wavelength (Druckrey et al., 1967; Lijinsky et al., 1970; Moehler & Mayrhofer, 1968). Many workers have successfully exploited this characteristic for the measurement of nitrosamine concentrations in model systems (Eisenbrand et al., 1974; Mirvish et al., 1972) as well as in extracts of environmental samples containing fairly high levels of nitrosamines (Ender et al., 1967). Due to serious interference from other components absorbing in the same region of the spectrum, the technique is unsuitable for trace analysis of nitrosamines in food extracts or other environmental samples. For similar reasons, or lack of sensitivity, the IR and NMR techniques, although useful for characterizing pure nitrosamines (Levin et al., 1970; Rao, 1963), have found little use for trace analysis of nitrosamines in complex mixtures.

In some spectrophotometric methods, the nitrosamines are first split and the resulting nitrosating agent is then determined colorimetrically. Splitting can be brought about either by exposure to UV light (Daiber & Preussmann, 1964) or by treatment with hydrobromic acid in glacial acetic acid (Eisenbrand & Preussmann, 1970). Sander (1967) observed that if the irradiation is carried out with long wavelength (360 nm) UV light at an alkaline pH, the technique is more specific for nitrosamines. Fan & Tannenbaum (1971) have developed an automated technique based on this principle. However, inorganic and organic nitrite and C-nitro compounds interfere with the analysis (Walters et al., 1970). The most extensively studied colorimetric method is that of Eisenbrand & Preussmann (1970), in which the liberated nitrosating species, formed after treatment with hydrobromic acid in glacial acetic acid, is captured by diazotization with sulfanilic acid and subsequent coupling with N[-naphthyl(1)]-ethylenediamine. The technique has been found to be satisfactory for the analysis of 17 nitrosamines, with an average recovery of 99.5%. As it determines only nitrite, the method is incapable of distinguishing between different nitrosamines, but is suitable for group analysis. The same authors have developed an alternative technique for detecting and determining individual secondary amines produced by cleavage of the nitrosamines, to provide an estimate of the levels of individual nitrosamines in the sample (see 3.4, 3.6 and 5.3.2). The colorimetric method of Eisenbrand & Preussmann has been used for measuring nitrosamines in food extracts (Nagata & Mirna, 1974; Walters et al., 1974).

Nitrosamines may be reduced to asymmetric hydrazines or secondary amines and measured colorimetrically after suitable derivative formation (Ender & Ceh, 1968; Kadar & Devik, 1972; Neurath et al., 1964b; Serfontein & Hurter, 1966b). 5-Nit.o-2-hydroxybenzaldehyde derivatives have been used for hydrazines and 4'-nitro-4-azobenzoic acid chloride derivatives for secondary amines. These techniques are excellent for the analysis of nitrosamines in relatively simple solutions, but it is doubtful whether they will be reliable for the analysis of complex mixtures, such as food extracts at low µg/kg levels. However, with extensive clean-up, Kadar & Devik (1972) have used such techniques for measuring the levels of nitrosamines in cigarette smoke condensates.

3.4 *Thin-layer chromatography (TLC) methods*

TLC has been used by many workers for both qualitative and semi-quantitative determination of nitrosamines. The main advantages are simplicity and relatively low cost. The technique is, however, semi-quantitative and not sufficiently selective to distinguish nitrosamines of closely related structure.

Preussmann et al. (1964a) first reported a TLC method for detecting nitrosamines in µg quantities. The method used a diphenylamine spray reagent under UV light and formation of a coloured complex with

palladium chloride. Preussmann et al. (1964b) have also described another technique in which the nitrosamines are detected on TLC plates by spraying with Griess reagent followed by exposure to UV light. A positive response to both reagents is considered to be convincing evidence of the presence of a nitrosamine. In some methods (Kroeller, 1967; Sen et al., 1969), the secondary amine components, produced by splitting the nitrosamines under UV light, are detected by the classical ninhydrin reagent. The detection limit of the TLC methods ranges between 0.2 and 2 µg per compound, depending on the nature of the nitrosamines; the ninhydrin reagent is more sensitive than the other two reagents. Young (1976) has recently described a highly sensitive technique whereby nitrosamines can be detected on TLC plates as fluorescent spots after exposure to UV light followed by spraying with fluorescamine reagent. The detection limit varies between 10 and 500 ng.

The most commonly used developing solvent for TLC is *n*-hexane-diethyl-ether-dichloromethane (4:3:2), first reported by Preussmann et al. (1964a,b). By varying the composition of the solvent, it is possible to separate most of the commonly found nitrosamines, but in some cases, e.g. NDMA and nitrosopyrrolidine (NPYR), resolution is difficult.

In some methods, nitrosamines are chromatographed after derivatization. The derivatives include nitramines, obtained by oxidation of the nitrosamines (Sen & Dalpé, 1972); fluorescent dansyl derivatives of the secondary amines, obtained by hydrolysis of the nitrosamines (Eisenbrand, 1972), and fluorescent hydrazones, obtained by reduction of the nitrosamines and subsequent derivatization with an aldehyde (Yang & Brown, 1972). Alternatively, hydrazines, the reduction products of nitrosamines, can be treated with 4-nitroazobenzene-4'-carboxylic acid chloride and the resulting hydrazides analysed by two-dimensional TLC (Serfontein & Hurter, 1966b). The areas containing the fluorescent hydrazones can be scraped off the TLC plates, extracted and identified by their characteristic UV and mass spectra (Yang & Brown, 1972). Sen et al. (1973) observed that the ninhydrin reaction product of nitrosopyrrolidine (NPYR) on silica gel plates (MN Kieselgehl GHR) is highly fluorescent when viewed under UV light inside a dark viewer, giving a detection limit of 10 ng per spot. The technique has been successfully used for the semi-quantitative estimation of NPYR in a wide variety of foodstuffs (Iyengar et al., 1976; Panalaks et al., 1974; Sen et al., 1974c). Fishbein (1972) has published a review of various methods for the analysis of nitrosamines.

TLC methods are versatile and some of the techniques which have been proposed for the analysis of nitrosamines are very sensitive. However, little is known about their reliability when applied to environmental samples. Comparison of TLC data with that obtained by GC-MS analysis has shown that the TLC-fluorometric method of Sen et al. (1973) for NPYR is reliable (Foreman & Goodhead, 1975; Sen et

al., 1976b). This technique gave results with reasonable accuracy and precision in two IARC collaborative studies of spiked meat samples (Walker & Castegnaro, 1974, 1976). The disadvantage of the technique is that it is applicable only to the determination of NPYR. TLC methods based on detection by Griess reagent are not sufficiently sensitive to permit analysis of foodstuffs below 20 µg/kg levels. Some of the newer fluorescent techniques (Eisenbrand, 1972; Yang & Brown, 1972; Young, 1976) may be more suitable, if found to be reliable.

3.5 *High-pressure liquid chromatography (HPLC) methods*

HPLC may offer a rapid screening method for detecting nitrosamines. Prior to the development of a suitable interface with the thermal energy analyser detection system (see section 6), the main hindrance was the lack of sensitive and specific detectors. To overcome this, Cox (1973) reduced the nitrosamines to secondary amines and converted these to 2,4-dinitrophenyl derivatives, which have a strong absorption band in the 340–360 nm region. The technique was applied to the analysis of nitrosamines in pork luncheon meat and fried pig liver. Interfering compounds could be distinguished by carrying out a blank determination with the sample, omitting the reduction step. Further work, however, is needed to evaluate the method for application to a wide range of samples. Iwaoka & Tannenbaum (1976) have developed a detector based on the principle of photohydrolysis of *N*-nitroso compounds and colorimetric detection of the liberated nitrite by Griess reagent. This detector has been found to work satisfactorily when connected to an HPLC system.

3.6 *Gas-liquid chromatography (GC) methods*

GC is the technique most widely used for detecting volatile nitrosamines in food or other environmental materials. Its main advantages are high sensitivity, variety of methods of detection, accuracy and relatively rapid speed of analysis.

3.6.1 *Columns*

The most commonly used column for the gas chromatographic separation of nitrosamines is Carbowax 20M, coated on acid-washed silanized Chromosorb W or Celite and packed in a stainless steel or glass tubing (i.d. 2 mm or 4 mm) of varying length, usually between 2 and 3 m (Castegnaro et al., 1974a; Crosby et al., 1972; Issenberg & Tannenbaum, 1972; Sen et al., 1970; Telling et al., 1971). Several other stationary phases, such as Reoplex 400 (Sen et al., 1969), FFAP (Fiddler et al., 1971; Gough & Webb, 1972), SE-30 (Kroeller, 1967), Carbowax 1540 (Howard et al., 1970), BDS (Scanlan & Libbey, 1971) and Versamid (Kawabata et al., 1972), have also been

used successfully. Several investigators (Gough & Sugden, 1975; Heyns & Roeper, 1970; Stephany et al., 1976a) have found that capillary or SCOT columns, which give better peak resolution, provide additional specificity. Since most of the nitrosamines studied so far are thermally stable and chromatograph well in stainless steel columns, the use of glass columns does not seem to offer any particular advantage. Both temperature and pressure programming and isothermal mode of operations have been used (Foreman & Goodhead, 1975; Gough & Webb, 1973; Sen, 1974; Wasserman, 1974).

3.6.2 *Detectors*

Initially, the conventional flame-ionization detector (FID) was used, but it was soon realized that it lacked the necessary selectivity (Foreman et al., 1970; Kroeller, 1967; Sen et al., 1969; Serfontein & Hurter, 1966b). Nitrogen-specific detectors were then shown to be of value in overcoming this drawback. The alkali flame-ionization detector (AFID) and the Coulson electrolytic-conductivity detector (CECD) are the two major detectors of this type. The AFID, highly specific for nitrogen compounds, uses a ring or coil coated with $RbSO_4$ or KCl on or around the hydrogen flame (Aue et al., 1967; Howard et al., 1970; Kawabata et al., 1972). The sensitivity is greater than that of conventional FID, with a limit of detection for NDMA of about 4 ng. The AFID has been successfully used for routine screening of foodstuffs for the presence of nitrosamines (Fazio et al., 1971; Fiddler et al., 1971; Kawabata et al., 1972; Pensabene et al., 1974). The sensitivity of the detector may be affected by organochlorine solvents, the potassium chloride AFID being less affected than the rubidium sulfate AFID (Eisenbrand, 1974). Sensitivity is also affected by the condition of the salt tip (Gough & Sugden, 1973).

In the CECD system, the GC column effluent is mixed with a stream of hydrogen gas and passed through a hot quartz pyrolysis tube containing a nickel wire catalyst, where all nitrogen-containing organic compounds present are reduced to ammonia. The ammonia is then detected and quantitatively measured by its effect on the conductivity of water in a detector cell (Coulson, 1965). There is a modified CECD in which the ammonia is measured in a titration cell (Newell & Sisken, 1972).

Although the above detectors are useful for screening analyses, they are not specific for nitrosamines. Many other nitrogen, as well as phosphorus, compounds give positive response, especially with the AFID (Krejci & Dressler, 1970; Palframan et al., 1973). Rhoades & Johnson (1970) were the first to modify the operation of the CECD to increase its selectivity for nitrosamines. By pyrolysing nitrosamines, ammonia is formed on the hot surface of the quartz furnace tube in the absence of hydrogen gas and Ni catalyst. Under these pyrolytic conditions, only nitrosamines and simple amines are detected. Since most of the free amines are removed in the clean-up

process, the detector is highly selective for nitrosamines. Although CECD in the pyrolytic mode gives excellent results for simple dialkylnitrosamines, alicyclic nitrosamines, such as NPYR, do not break down efficiently enough to permit detection at low levels (Issenberg & Tannenbaum, 1972), but careful control of the pyrolysis temperature can improve this situation. In the pyrolytic mode of operation, the detector is not as sensitive as in the reduction mode (Palframan et al., 1973; Sen et al., 1974b). Various studies (Goodhead & Gough, 1975; Iyengar et al., 1976; Panalaks et al., 1973; Sen et al., 1976b) have shown that use of a GC-CECD system, either in the pyrolytic or reductive modes, can be very useful for preliminary screening of foodstuffs for the presence of nitrosamines.

Palframan et al. (1973) compared the performance characteristics of an AFID with that of a CECD and concluded that the sensitivity of the two detectors, for practical purposes, is about the same, but the latter is much superior with regard to selectivity for nitrogen compounds. CECD is also less susceptible to changes in operating conditions, such as sample and solvent size and fluctuations in gas flow rate, and is therefore more suitable for routine use. Fiddler (1975), on the other hand, prefers AFID to CECD because of the difficulties experienced in maintaining quantitative reproducibility with the latter detector.

A modified Hall detector (Hall, 1974) has been used successfully in the pyrolytic mode for the analysis of nitrosamines in a variety of foodstuffs, such as wheat, rice, bread, cured ham and fermented sausages (Eisenbrand et al., 1976; Rappardt et al., 1976). The principle of operation of the Hall detector is the same as that of the CECD, but the former is much more sensitive than the latter, mainly due to improved geometry of the electrolytic conductivity cell and other operating parameters (e.g. higher cell voltage). About 50 pg NDMA can easily be detected. The detection limits for nitrosopiperidine (NPIP) and NPYR were 0.5 and 1 ng respectively. These workers also concluded that the detector is more specific for nitrosamines in the pyrolytic than in the reductive mode. Issenberg & Sornson (1976) used a Hall detector (reductive mode) for monitoring the levels of nitrosamines in a laboratory atmosphere.

3.6.3 *Detection of derivatives*

Several investigators have attempted to increase the sensitivity and selectivity of the detection techniques by converting the nitrosamines to derivatives that are sensitive to electron capture (EC) detection (see section 5). Sen (1970) oxidized NDMA to *N,N*-dimethylnitramine by treating with peroxytrifluoroacetic acid. The EC detector was extremely sensitive to the nitramine derivative, about 8 pg being easily detected. After clean-up of the nitramine derivative on a basic alumina column, Sen (1970, 1972) was able to use the technique for confirming the presence of NDMA in a sample of nitrite-treated fish and several samples of cured meat

products. Althorpe et al. (1970) independently developed a similar technique and extended it to the detection of other volatile nitrosamines. More recently, Telling (1972) applied column chromatography, both for an initial clean-up of food sample distillate on alumina (neutral, activity 3) and for the final clean-up after oxidation (using mixed alumina/magnesium oxide). Recoveries at the 2.5 µg level were 77% for NDMA and 92–95% for NDEA, *N*-nitrosodiisobutylamine (NDi-BA), NDBA and NPYR. Final chromatograms were very clean, with the exception of spiced meat products. Telling et al. (1974), in a later investigation, further improved the sensitivity of the nitramine technique. A dual chromatographic procedure has also been described (Castegnaro et al., 1974a; Walker et al., 1975). Specificity was improved by GC-analysis of separate column fractions. Recoveries from spiked samples were normally greater than 60%. The scheme was used in the analysis of samples from a dietary survey. Other workers (Juszkiewicz & Kowalski, 1974; Kubacki & Borys, 1976) have also found the nitramine technique useful.

As described in section 5, nitrosamines can be reduced or cleaved to secondary amines, which then can be converted to electron-capturing derivatives and detected by GC. Secondary amines frequently occur in the environment. In addition, components or contaminants in foods may break down during a reduction step to yield secondary amines. These could lead to contamination of the final extract and to false positive results (see section 5). Eisenbrand et al. (1976) isolated the amines generated from nitrosamines on a hydrophilic, SE-cellulose, ion-exchange column; introduction of this step very effectively separated the amines from interfering compounds and provided clean analytical solutions.

3.7 *GLC-MS and GLC-TEA*

Mass spectrometric and chemiluminescence-based techniques for determining nitrosamines are dealt with in sections 4 and 6.

4. MASS SPECTROMETRIC TECHNIQUES

T.A. Gough

4.1 *Introduction*

It is generally recognised that the most reliable means of confirming the presence of volatile nitrosamines is by combined gas chromatography and high-resolution mass spectrometry (GC-MS). In addition to confirmation, quantification can also be based on high-resolution mass spectral data.

The mass spectrometry of nitrosamines is well documented and several papers describe in detail the fragmentation pathways of a number of nitrosamines (Collin, 1954; Saxby, 1972; Schroll et al., 1967). Low-resolution, electron-impact spectra have also been published (Mass Spectrometry Data Centre, 1971; Lijinsky et al., 1973; Pensabene et al., 1972) and recently Gadbois et al. (1975) and Gaffield et al. (1976) presented information on the chemical ionisation of nitrosamines.

Even after intensive clean-up, food extracts are still complex mixtures containing many potentially interfering substances. Analysis therefore entails the use of a GC separation of volatile nitrosamines and it is clearly more practical and less time consuming to carry out on-line GC-MS, rather than to trap constituents from the GC effluent for subsequentmass spectrometry.

4.2 *Techniques employing low-resolution mass spectrometry*

A GC-MS procedure for separating and detecting microgram amounts of some dialkyl and other volatile nitrosamines in standard mixtures was published by Heyns & Roper (1970), using 25 metre capillary columns, directly coupled to a mass spectrometer. In view of their limited sample capacity, capillary columns are less suitable than conventional packed columns for the detection of mg/l levels of nitrosamines in food extracts. For this reason, most workers use packed columns in combination with mass spectrometry. However, analysis of samples using low-resolution mass spectrometry requires a somewhat higher GC column efficiency than analysis using high-resolution mass spectrometry, as the separation of individual components is more important for identification using low resolution. Both the clean-up procedure and the GC conditions must therefore be selected to suit the mass spectrometry facilities available.

Using an Atlas CH4 mass spectrometer, a low-resolution mass spectral technique for the determination of nitrosamines has been developed by Fazio et al. (1971). A complete spectrum is run at

the appropriate retention time for the nitrosamine under study. The procedure was described for *N*-nitrosodimethylamine in fish (Fazio et al., 1971). Although the relative intensities of the parent ion (m/e 74) and fragment ions varied between standard solutions and spiked fish extracts, these variations were small and the method was considered to be reliable, as the mass spectrometry data was coupled with coincidence of retention times. The procedure was subsequently expanded to detect several other dialkyl and heterocyclic nitrosamines in cured meat products (Fazio et al., 1973).

Wasserman et al. (1972) have also used low-resolution spectra to study the occurrence of nitrosamines in frankfurters.

Low-resolution mass spectrometry, employing the LKB 9000 combined GC-MS instrument with a double-stage jet separator, was used by Williams and co-workers to study alcoholic beverages (Williams et al., 1971). The GC effluent was first monitored for the parent ions of *N*-nitrosodimethylamine and the diethylamine. When a positive response was obtained, complete spectra were run. Low-resolution spectra have been used for detecting *N*-nitrosodimethylamine in fish (Fong & Chan, 1973a) by monitoring the parent ion (m/e 74) and the NO^+ fragment (m/e 30), using a Hitachi RMS-4 instrument fitted with a glass frit separator. The same authors (Fong & Chan, 1973b) calculated the contents of *N*-nitrosodimethylamine on the basis of the parent ion intensities and GC peak areas. Low-resolution multi-ion monitoring of fragments m/e 74, 42 and 30 to detect *N*-nitrosodimethylamine in fish has been used by Gadbois et al. (1975). As fragments of mass 42 are among those most commonly encountered in mass spectrometry, mass 42 is of little diagnostic value in nitrosamine analysis.

N-nitrosodimethylamine determined in fish meal, using an alkali flame-ionization detector (AFID) was confirmed qualitatively from low-resolution spectra, obtained with the LKB instrument (Skaare & Dahle, 1975). This technique can produce erroneously high values if other nitrogen-containing compounds which respond to an AFID detector are co-eluted with the nitrosamine.

The LKB GC-MS system has also been used for the detection of *N*-nitrosopyrrolidine formed from proline and collagen, using the complete spectrum (Gray & Dugan, 1975). Model nitrosamine formation systems have also been studied, particularly by Scanlan and co-workers (Bills et al., 1973; Huxel et al., 1974; Warthesen et al., 1975), using complete spectra. These were obtained from Atlas CH4 and Finnigen 1015C spectrometers, the former with a membrane separator and the latter with a jet separator. Sen et al. (1972, 1973, 1974a,b) have employed low-resolution spectra, obtained using a Hitachi RMS-4 spectrometer with a glass frit separator, to identify *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine in bacon and other cured meat products.

An example of the use of high efficiency GC combined with a low-resolution procedure has been reported by Essigman & Issenberg (1972), who collected nitrosamines after a preliminary separation from extraneous material in a post GC column trap. The contents of the trap were vaporized onto a second column (usually an open tubular column) and passed into a Hitachi RMU-7 spectrometer through a glass frit separator. A total ion current response was obtained for the fraction vaporized from the trap. For those compounds with retention time equal to that of any of the nitrosamines, low-resolution spectra and specific ion monitoring were used. For *N*-nitrosodimethylamine, masses at m/e 74, 42 and 30 were scanned concurrently.

4.3 *Techniques employing high-resolution mass spectrometry*

The first reports on the use of high-resolution techniques for the detection of nitrosamines in foodstuffs were made at the IARC Conference on *N*-Nitroso Compounds, Analysis and Formation, in 1971. One paper (Crosby et al., 1972b) reported the monitoring of GC effluent for the parent ion of each nitrosamine at a resolution of 10,000 and the other (Osborne, 1972) the monitoring of the NO^+ ion at a resolution of 15,000. Detailed descriptions of both procedures have subsequently been published and are discussed below. The procedure developed by Telling et al. (1971) employed a packed, polar, GC column connected to an AEl MS902 high-resolution mass spectrometer with a glass frit separator. The NO^+ ion was monitored using the peak-matching facility of the spectrometer. A resolution of 15,000 is sufficient to resolve NO^+, with satisfactory sensitivity, from all other ions of m/e 30, with the exception of $C^{18}O^+$. Supporting evidence for the presence of nitrosamines from complete low-resolution spectra was not always satisfactory. As an example, the authors refer to hydroxy-acetone, which had a retention time similar to that of *N*-nitrosodimethylamine, the same molecular weight and a similar spectrum containing the $C^{18}O^+$ ion. When such interference was suspected, *N*-nitrosodimethylamine was confirmed by monitoring the parent ion (m/e 74) at high resolution. The NO^+-monitoring technique was used to examine a number of cured meat products, with a detection limit of 25 µg/kg with respect to the original sample. In a subsequent paper (Bryce & Telling, 1972), the multiplier resistor grid of the mass spectrometer was modified to effect a 10-fold sensitivity increase. The interpretation of low-resolution spectra after NO^+ monitoring was found to be difficult with some food extracts and parent or other abundant ion monitoring at high resolution was preferred. The detection limit of the method with these modifications, using 25 µl injection volumes, fell to 1 µg/kg. Total GC-MS analysis time per run for the steam-volatile nitrosamines (*N*-nitrosodimethylamine to *N*-nitrosodibutylamine, *N*-nitrosopyrrolidine) was 1 hour, but this could be halved by monitoring only for *N*-nitrosodimethylamine, -diethylamine and -pyrrolidine (Telling et al., 1974).

In the original method proposed by Gough & Webb (1972), a similar GC system was coupled to the mass spectrometer through a membrane separator. This has an efficiency greater than that of diffusion separators for the transfer of low molecular weight compounds, resulting in a lower limit of detection. The mass spectrometer employed was also an MS902, with both multiplier resistor and amplifier gain modifications. The GC-MS measurements were made directly on the parent ion of the nitrosamines, using the peak-matching facility of the instrument. Parent ion monitoring on the steam-volatile nitrosamines was more sensitive than NO^+ monitoring. The resolution employed was 7,000,or 12,000 in doubtful cases (particularly for *N*-nitrosodimethylamine in complex extracts). Using an injected volume of 5 µl, this gave detection limits of 1 µg/kg and 5 µg/kg of sample, respectively. Linearity of response to each parent ion over the range of 2-50 mg/l (injected on the GC) was established and the variation of mass spectrometer response for repeated analyses of standard solutions was measured. Subsequent papers from this group have concentrated on methods for minimising the total GC-MS analysis time, whilst maintaining the reliability of the technique. By using pressure-programmed GC, analysis time was 36 minutes for the steam-volatile nitrosamines. Pressure programming required an equilibration period between runs of only 3 minutes, whereas temperature programming needed 15 minutes (Gough & Webb, 1973). An alternative procedure, in which the chromatography was carried out isothermally and isobarically, does not require the construction of elaborate GC equipment (Gough & Sugden, 1975). Analysis time was minimised by the use of two columns in series, a packed column followed by a support-coated open tubular (SCOT) column. Short retention time dialkyl nitrosamines were passed through both columns. Longer retention time heterocyclic nitrosamines were passed only through the first column, giving an analysis time of 28 minutes. For normal GC sample injection volumes of 5 µl, a detection limit of 1 µg/kg was obtained, although this can be substantially improved in both of the above systems by injecting larger samples and utilising solvent venting facilities.

Other developments in mass spectrometric determination of nitrosamines include the use of alternative types of GC-MS interface. A silver frit device, which has better thermal stability than the membrane separator, has been used for the less volatile nitrosamines (Gough & Webb, 1974). A palladium-silver alloy tube, with hydrogen used as the carrier gas, has also been evaluated as a GC-MS interface. Quantitative transfer of dialkyl nitrosamines was achieved but, under some conditions, the heterocyclic nitrosamines were found to break down in the tube (Gough & Sawyer, 1974).

The reliability of high-resolution parent ion monitoring has been extensively studied (Goodhead & Gough, 1975) and a large number of cured meat and other products have been examined by this procedure (Crosby et al., 1972a; Gough & Goodhead, 1975; Gough & Walters, 1976; Gough et al., 1976a, 1977a,c; Mottram et al., 1975; Patterson

et al., 1976). Sen et al. (1976a,b) have used both NO^+ monitoring at a resolution of 5000 and parent ion monitoring at a resolution of 10,000 in recent work with a Varian MAT 311A spectrometer. Using high-resolution parent ion monitoring with GC temperature programming, a detection limit of 3 µg/kg for *N*-nitrosopyrrolidine and *N*-nitrosodimethylamine has been achieved by peak matching on a Dupont 21-492 instrument (Fiddler et al., 1975; Heisler et al., 1974; Kushnir et al., 1975; Pensabene et al., 1974). High-resolution parent ion monitoring has been employed to determine *N*-nitrosodimethylamine in gastric juice, using a CEC 21-110C spectrometer with peak matching (Lane et al., 1974). Stephany et al. (1976b) used single ion monitoring at a resolution of 4000 for meat samples, with two different isothermally-operated capillary columns. The detection limit for the method was 0.5 µg/kg of sample. A similar method was described by Groenen et al. (1976), using either a membrane or slit separator and a Varian 731 spectrometer. An alternative high-resolution procedure adopted by Crathorne et al. (1975) used a compound of the same nominal mass as the nitrosamines for accurate mass setting. This procedure, however, can only be used for the detection of one nitrosamine per GC injection. The mass spectrometer, a Hitachi RMU-71 fitted with a glass frit separator, was operated with a resolution of 7,500. High stability of the mass spectrometer is essential.

4.4 *Techniques employing mass spectrometry of derivatives*

Derivatives (see section 5) screened by electron capture can be checked directly by mass spectrometry. Using an LKB-9000, and more recently an MS902 spectrometer, Eisenbrand (1972) and Eisenbrand et al. (1976) monitored the m/e 169 ($C_3F_7^+$) ion or the parent ion to detect the heptafluorobutyramides derived from nitrosamines. Such a procedure has no particular advantage over direct examination of the nitrosamine extract when high-resolution facilities are available. The analysis of nitrosamines as nitramines has been studied by mass spectrometry. This method has two advantages, (a) the main part of the nitrosamine molecule is still intact and (b) concentration of the solutions is facilitated (Castegnaro, 1976; Castegnaro & Walker, 1977).

4.5 *Limitations of mass spectrometric determination of nitrosamines*

Theoretical considerations and practical experience indicate that, for food extracts, high-resolution parent ion monitoring is, in general, to be preferred to low-resolution mass spectrometry. However, it was observed both by Gough & Webb (1973) and by Dooley et al. (1973) that, even with high resolution, a potentially interfering fragment [$^{29}SiMe_3$] with a retention time close to that of *N*-nitrosodimethylamine may be encountered. Although a resolution of 70,000

is required for complete separation, a mismatch between the two compounds can be observed even at a resolution of 7,000. This, coupled with a small difference in GC retention time, should be sufficient to alert the analyst. Other potentially undesirable effects have recently been observed by Gough et al. (1977c) and arise from the method of displaying the high-resolution signal.

Co-eluted material of *any* mass may tend to suppress the mass spectrometer response and hence affect quantification (Gough & Webb, 1972). In an extreme case, complete suppression, resulting in a false negative for the nitrosamine, could result. Bryce and Telling (1972), Gough & Webb (1972) and Pensabene et al. (1974) describe the use of peak-matching in this connection. The mass region in the vicinity of the reference fragment (usually derived from a fluorinated hydrocarbon) and the nitrosamine fragment of the same nominal mass are alternatively scanned every few seconds and displayed on an oscilloscope. This enables the analyst to observe the reference peak as well as the rise and fall of the nitrosamine peak. Any suppression is therefore immediately apparent, as the reference peak is also suppressed. Some workers, although using high-resolution techniques, monitor only the precise mass of the parent ion and the peak-matching safeguard is thus lost. Precise mass spectrometry at high resolution demands a very stable instrument.

It must be acknowledged that the parent ion monitoring procedure, when used to detect a series of consecutively eluted nitrosamines, requires considerable skill. Not all commercially available multi-ion monitoring units are suitable for use at high resolution and, in any case, they cannot normally accommodate a wide mass range, such as is required for a series of nitrosamines (m/e 74 to 158), without loss of sensitivity at the higher mass. A unit for the automatic selection of accurate masses, using an MS9 spectrometer, is being developed by Hogg (1975) and may have application to nitrosamine analysis.

4.6 *Conclusions*

The most reliable procedure for quantitatively detecting nitrosamines is by mass spectrometry, using parent ion monitoring and peak matching at high resolution, after GC separation. High-resolution monitoring of NO^+ for all nitrosamines is less sensitive than parent ion monitoring, but has the advantage of ease of operation and, in principle, the ability to detect hitherto unrecognized nitrosamines. Low-resolution mass spectrometers are less costly and more widely available. Low-resolution multi-ion monitoring can be used successfully on relatively clean extracts, particularly nitrosamines having long GC retention times. Several different GC-MS separators may be used, each offering various advantages. For repetitive analyses, speed is desirable and dual-column isothermal or single-column pressure-programmed runs economise time.

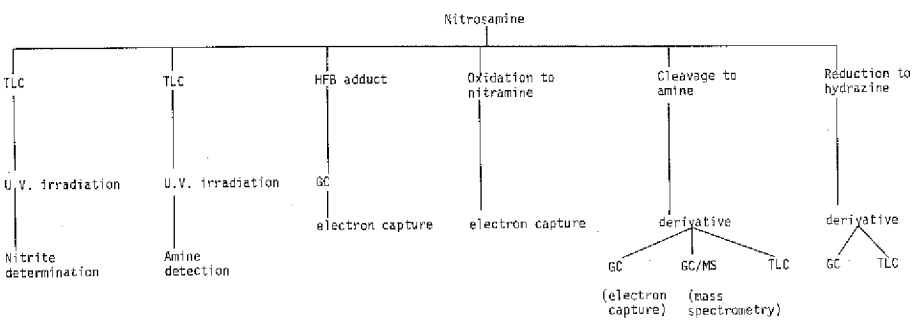
5. DERIVATIVE FORMATION

G. Eisenbrand

5.1 *Introduction*

When a sufficiently selective detector, such as a mass spectrometer or Thermal Energy Analyser, is not available, there are numerous ways in which the nitrosamine molecule may be chemically modified to give derivatives which can be determined with greater ease, sensitivity and specificity than the parent compound. Those reactions which have been utilized for analytical purpose are shown in Scheme 1.

SCHEME 1



5.2 *The reduction of nitrosamines to hydrazines*

The formation of hydrazines was one of the first chemical transformation techniques to be applied to the trace analysis of nitrosamines. Various reducing agents have been utilized, together with several different methods for making suitable derivatives from the generated hydrazine. Reduction of nitrosamines by lithium aluminium hydride in ether has been described by Schueler & Hanna (1951) and Poirier & Benington (1952). Neurath et al. (1964a,b) reduced

nitrosamines by this technique and condensed the resulting unsymmetrical hydrazines with 5-nitro-2-hydroxybenzaldehyde. The yellow Schiff bases were separated by thin-layer chromatography (TLC), eluted with methanol and quantitatively determined by UV-spectrometry. The sequence of analytical operations was difficult to standardize and yields obtained from recovery experiments with different nitrosamines added to smoke condensates (1 µg/cigarette) were not more than 20%.

Serfontein & Hurter (1966a,b) also used lithium aluminium hydride in ether to reduce nitrosamines and converted the resulting hydrazines with 4-nitroazobenzene-4'-carboxylic acid chloride into intensely coloured derivatives, which were then separated by multiple, two-dimensional, thin-layer chromatography. By this method, the authors detected *N*-nitrosopiperidine (NPIP), *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) in tobacco smoke condensates. However, no reliable determination of the amount of nitrosamines present could be made.

Ender & Ceh (1971) reduced nitrosamines in an aqueous acidic system, using granulated zinc and hydrochloric acid (15-20 hr) at room temperature; the resulting hydrazines were then reacted with 4-dimethylaminobenzaldehyde and the Schiff bases formed were determined colorimetrically at 458 nm. The authors claimed a quantitative yield for NDMA, NDEA, and *N*-nitrosoethylmethylamine (NEMA), and yields of about 60% for higher alkylnitrosamines; no recovery data were given for analyses of nitrosamines in foods.

Yang & Brown (1972) reduced nitrosamines by refluxing with lithium aluminium hydride in tetrahydrofuran (THF). They reported a 75-85% conversion to hydrazines for 18 different dialkyl, heterocyclic and aromatic nitrosamines and a nearly quantitative yield for the ensuing condensation with 9-anthraldehyde to fluorescent hydrazones. This procedure, however, has not been applied to trace analysis of nitrosamines in foods.

Hoffman and co-workers (1974) used diborane in ether as the reducing agent and converted the hydrazines formed to 3,5-dinitrobenzaldehyde hydrazones, which they determined by gas chromatography with electron-capture detection. Positive findings were confirmed by mass spectrometry. ^{14}C-NDMA was used as an internal standard for quantitative evaluations. No quantitative data are available which would allow direct comparison of the yields of hydrazines obtained by reduction with lithium aluminium hydride with those obtained by reduction with diborane. However, the use of a radioactively labelled internal standard makes the quantitative data reported by these authors more reliable than results obtained by comparison with external standards only.

5.3 *Methods using the cleavage of the N-NO bond*

5.3.1 *Determination of nitrite*

The N-NO bond can be cleaved by chemical agents or physical processes. The cleavage of the nitrosamine group was employed, in the first instance, to generate nitrite, which could then be quantitatively determined by simple colorimetric methods (Daiber & Preussmann, 1964; Sander, 1967). Eisenbrand and Preussmann (1970) later introduced hydrogen bromide in glacial acetic acid (HBr/HAc) for the quantitative removal of the nitroso group from the nitrosamine molecule under very mild conditions.

In the latter reaction, the nitrosylbromide released can also be trapped by diazotization and coupling to an azo dye; this colorimetric nitroso group determination was shown to give very consistent results and to be more sensitive than the earlier techniques, especially for compounds insoluble in water. Johnson & Walters (1971) showed that the method was specific for the *N*-nitroso group, giving no response for *N*-nitro, *C*-nitro and azoxy functions.

5.3.2 *Determination of amines*

The acid-catalyzed denitrosation with HBr/HAc, which was shown to be virtually quantitative for a wide range of nitrosamines, offers a very convenient way to generate secondary amines from nitrosamines, from which a number of derivatives may be obtained for analysis. Eisenbrand (1970, 1972) examined three different techniques of derivative formation from the amines:

1. Reaction with 1-dimethylaminonaphthalene-5-sulfonylchloride (dansylchloride) to form strongly fluorescent dansyl derivatives.
2. Formation of 4'-nitroazobenzenecarboxamides.
3. Reaction with heptafluorobutyrylchloride (HFB-Cl), with formation of the HFB-amides.

This work showed that, although in principle all three methods could be used, the HFB derivatives were most suitable. Since all HFB derivatives generate $C_3F_7^+$-fragments in the ion-source of a mass spectrometer, monitoring at m/e 169 can suffice for their determination in the gas chromatographic effluent. Chromatograms obtained by single-ion detection in a coupled gas chromatography/low-resolution mass spectrometry instrument were practically without interfering peaks. The yields of HFB derivatives from 0.5 µg each of NDMA, NDEA, *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA, *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR) were in the range of 72-78% (Eisenbrand et al., 1976). Detection limits were below 10 picograms. This method was successfully used in collaborative studies organized by IARC to determine contents of NDMA, NDEA, NPIP and NPYR, added to luncheon meat at levels of 5 and 20 µg/kg

(Walker & Castegnaro, 1976). It also was applied routinely in conjunction with other methods in screening samples of food for nitrosamines (Eisenbrand et al., 1976).

A disadvantage of methods which rely on the formation of amines is the risk of contamination by amines. Amines present in food must be removed by extracting with aqueous acid. This, however, provides no protection from amines present in the atmosphere of the laboratory, for instance, which may contaminate apparatus or samples (Bogovski et al., 1972). Blank experiments are thus required to correct the results. Glassware should be cleaned in chromate-sulfuric acid and stored in a desiccator. Solvents should be carefully purified before use to remove amines (Eisenbrand et al., 1976).

Klimisch & Stadler (1974a) reported the fluorimetric determination of NDMA and NPIP by acid-catalyzed denitrosation in HBr/HAc, followed by reaction of the resulting amines with 7-chloro-4-nitro-benzo-2-oxa-1, 3-diazole. The derivatives formed were separated by thin-layer chromatography and the fluorescence of the spots measured *in situ*. A limit of detection of 10^{-12} moles of amine was obtained with pure compounds; the method was not applied to the analysis of nitrosamines in other than standard solutions.

Cleavage of the N-NO bond by electrolysis in an alkaline medium has been carried out by Alliston et al. (1972), to generate secondary amines from nitrosamines. The amines were converted to HFB derivatives and determined by electron-capture, following gas chromatography. Extracts from food samples were divided into two aliquots, one being subjected to electrolysis while the other was used as a blank to correct for contamination by adventitious amines. Yields of HFB derivatives prepared from aqueous solutions of NDMA, NDEA, NDPA, NDBA, NPIP and NPYR (0.25-10 µg each) varied from 26 to 113%. The method has been used to determine the nitrosamine contents of cheese, meat products and fish.

Pailer & Klus (1971) analysed tobacco smoke condensates for nitrosamines by denitrosation with cuprous chloride in hydrochloric acid; they determined the resulting amines by GC-EC of the corresponding trifluoroacetyl derivatives. On the basis of retention times, they concluded that NDMA, nitrosoethylmethylamine, nitrosomethyl-isobutylamine and nitrosoethyl-*n*-propylamine were present in amounts of 0.2-0.7 µg/cigarette. No recovery data were given and the results were not confirmed by high-resolution MS.

Klus & Kuhn (1975) denitrosated *in situ* with titanium trichloride. The *N*-nitroso derivatives of the tobacco alkaloids nornicotine, anabasin and anatabin were separated by thin-layer chromatography (TLC), then sprayed with titanium trichloride in hydrochloric acid. The denitrosated alkaloids were subsequently separated by TLC in the perpendicular direction. The limit of detection was 0.5 µg/spot. Results were given only for pure compounds.

5.3.3 *Formation of nitramines*

Sen (1970) and Althorpe et al. (1970) independently developed methods for the analytical determination of nitrosamines, using their oxidation to nitramine (Emmons, 1954). Sen prepared dimethylnitramine by oxidation of NDMA with trifluoroacetic acid/50% hydrogen peroxide. He also isolated NDMA from a fish sample by thin-layer chromatography and converted it to the nitramine; 60% of the theoretical conversion yield was obtained. Althorpe et al. (1970) studied the conversion of a series of nitrosamines to nitramines by oxidation with peroxytrifluoroacetic acid. All nitrosamines gave 72 to 86% conversion to nitramines after 3.5 hr, with the exception of NPIP, which gave a yield of only 25%. The sensitivity of electron-capture detection was 100 to 1000 times greater than that of flame-ionisation detection.

Telling (1972) extended this investigation and found even higher yields at the 1 µg level (92-95%) but, again, *N*-nitrosopiperidine could not be detected by this method in amounts below 50 µg. According to these results, interference from oxidation products should not become significant until levels below 0.25 µg of a given nitrosamine are reached, except for *N*-nitrosodi-isobutylamine. NPIP was not included in these experiments. A comparison of results obtained by GC/high-resolution MS with those obtained by the nitramine technique showed good agreement. TLC of nitramines was also used for the determination of nitrosamines in alcoholic beverages (limit of detection 25 µg/l) (Sen & Dalpe, 1972).

Walker et al. (1975) found that the use of 50% hydrogen peroxide, instead of 85-90%, resulted in much higher yields of *N*-nitrosopiperidine than those obtained by Telling (1972). Confirmation of nitramines can be obtained by mass spectrometry (Castegnaro & Walker, 1977).

Castegnaro et al. (1974b) investigated an interesting method for preventing losses of nitrosamines by evaporation. The triethyloxonium salts of nitrosamines were formed in dichloromethane, the solvent removed on a water bath and the adducts oxidized to nitramines. In experiments with pure compounds, 70-90% recoveries were obtained from nitrosamines added at the 50 µg/kg level to aqueous-ethanolic mixtures. The same authors also tried a modified oxidation procedure in model experiments, using methylethylketone peroxide as the oxidizing agent (Castegnaro et al., 1972).

5.3.4 *Heptafluorobutyryl adducts*

Brooks et al. (1972) found that nitrosamines reacted with heptafluorobutyryl-anhydride (HFBA) or trifluoroacetic anhydride (TFA) in pyridine to form electron-capturing derivatives. Gough et al. (1975, 1976b) also studied the reaction with HFBA. Using GC/high-resolution MS, they were able to provide structural assignments. The reaction is of doubtful analytical value.

6. THE USE OF CHEMILUMINESCENCE-BASED DETECTORS

D.H. Fine

6.1 *Introduction*

In 1973, Fine & Rufeh (1974) proposed the use of chemiluminescence to detect nitrosamines as nitric oxide, after catalytic cleavage of the N-NO bond. Detectors for oxides of nitrogen based on chemiluminescence have become commercially available in recent years (Stevens & Hodgeson, 1973) and are both reliable and sensitive. The addition of a device to generate nitric oxide from a nitrosamine would thus provide, in principle, a very selective and sensitive nitrosamine detector. Such a system has been developed by Fine et al. (1975a,b) and is now commercially available. Gough et al. (1977b) have also described a simple, laboratory-constructed, detector system based on the same principles.

6.2 *General principles*

Liquid or gas samples are swept through a catalytic pyrolyser by a carrier gas, usually argon (nitrogen may be contaminated with nitric oxide and is unsuitable). *N*-nitroso compounds rupture at the N-NO bond, releasing NO. The yield of NO is stoichiometric. Solvent vapour, pyrolysis products and NO pass through a cold trap at -150°C which, in principle, removes all materials other than the permanent gases. The NO and the carrier gas then pass into a low-pressure reaction chamber , where the NO reacts with ozone.

The key reactions are:

$$RR'N\text{-}NO \xrightarrow{\text{Pyrolysis}} R + R' + N + NO$$

$$NO + O_3 \longrightarrow NO_2^* + O_2$$

$$NO_2^* \longrightarrow NO_2 + h\gamma$$

Excited NO_2^* decays to its ground state with the emission of light in the near infra-red region of the spectrum. The intensity of the emission is a measure of the amount of *N*-nitroso compound. The system is selective because it will respond only if a compound passes the following sequential tests:

1. pyrolyse within a few seconds on a catalytic surface at a moderate temperature, giving a product which
2. survives a cold trap at -150°C,

3. reacts with ozone at reduced pressure, giving a product which
4. emits light in the near infra-red region of the spectrum.
5. Steps 3 + 4 must be sufficiently rapid for emission to occur before the reactants leave the reaction chamber.

The Thermal Energy Analyser (TEA) (Fine & Rufeh, 1974) has been used as the detector for a gas chromatograph (GC-TEA) (Fine & Rounbehler, 1975), and a high-pressure liquid chromatograph (HPLC-TEA) (Fine et al., 1976a, 1977b). So far, the detector system of Gough et al. (1977b) has only been described for use with GC. *N*-nitroso compounds, organic nitrites and some nitrates, plus a few aliphatic *C*-nitroso compounds (Fine et al., 1975a; Stephany & Schuller, in press), are the only compounds known so far to give a response on the TEA. Because of this extremely high selectivity, it is possible to analyse *N*-nitroso compounds quantitatively at high sensitivity, even in the presence of many co-eluting compounds, which minimises clean-up procedures. At present, confirmation of positive data by high-resolution MS is desirable.

The detection limit of the TEA is of the order of 0.01-0.5 ng for NDMA. The detector of Gough et al is less sensitive by about two orders of magnitude.

6.3 *Applications*

The Thermal Energy Analyser has been employed for the determination of volatile nitrosamines in fish, bread, cheese, meat and fruit (Fine et al., 1975c, 1976a; Fine & Rounbehler, 1976; Gough et al., 1977c), alcohol (Gough, 1977), blood and urine (Fine et al., 1976b, 1977a; Gough et al., 1977c), oil and grease (Fine & Rounbehler, 1976) and soil (Fan et al., 1976a). The detector system of Gough has been evaluated in only one laboratory (Gough et al., 1977b) and used for screening foodstuffs and biological materials for the presence of volatile nitrosamines. As with the TEA, the quantitative results agreed well with those obtained by high-resolution, peak-matching mass spectrometry.

7. A BRIEF REVIEW OF ANALYTICAL METHODS FOR THE DETERMINATION OF AMINES

G.B. Neurath

7.1 *Introduction*

Ever since Sander (1971) demonstrated that nitrosamines could be formed in the human organism from their amine precursors, it has been evident that more information on the occurrence of amines in the human environment is essential.

The term amine covers a wide variety of compounds, which includes not only relatively simple organic bases but also alkaloids, biogenic amines, drugs, etc., of differing complexity and chemical and physical properties. Therefore, although the basic character of amines facilitates the initial steps in their isolation from an environmental medium, a wide variety of techniques are required for the end analysis of different amines.

This review is not intended to give a comprehensive survey of analytical methods for all types of amines. It is intended to indicate those that may be used for the relatively simple members of the class; in particular, those which may be encountered, often in only trace amounts, in the human environment as biogenic products, products of decomposition or pollutants of industrial origin. Furthermore, as this manual is devoted to the analysis of nitrosamines, attention is particularly directed to the secondary and tertiary amines which are precursors of nitrosamines. The references are mainly restricted to those of fairly recent origin, giving methods which by their general acceptance are considered to be reasonably reliable.

7.2 *General aspects*

Only primary and secondary amines form derivatives by substitution and this property may be employed to form derivatives suitable for detection and measurement. Primary amines generally are susceptible only to acylation, but secondary amines undergo, in addition, a number of useful condensation reactions, such as the formation of Schiff bases. Tertiary and quarternary amines normally do not dorm derivatives, except after some drastic changes in the molecule. Analysis of environmental amines is further complicated by differences in volatility, basicity, amphoteric character, etc., and although the methodology which has been developed during the last decade has taken advantage of these differences, there is no universal approach in the search for nitrosatable amines.

7.3 *Preliminary isolation techniques*

As a preliminary step, the sample is blended with water, acidified and extracted with a non-polar solvent, such as hexane, pentane or ether, to remove non-basic materials.

Volatile amines may be removed by steam distillation. After being made alkaline, the distillate is collected in an acid trap (Neurath & Schreiber, 1974 ; Neurath et al., 1966a,b; Singer & Lijinsky, 1976a,b; Stein von Kamienski, 1957; Steiner & Stein von Kamienski, 1953). Although little is known concerning the formation of amines during alkaline steam distillation, 19 amino acids have been tested as possible precursors by Thaler & Sturm (1971). Only cystein and cystine were found to release ammonia.

Frequently, solvent extraction from an alkaline solution is used to extract both nonvolatile and volatile amines. The amines may then be removed from solution employing ion-exchange resins. With this procedure, it is sometimes possible to effect the entire clean-up by means of an acid ion-exchange column (Pailer et al., 1967, 1969). Column chromatography may also be required to isolate ampholytes, such as amino acids, which are difficult to extract with solvents.

7.4. *Chromatographic techniques*

Chromatographic techniques are normally employed in the determinative step, but they also constitute an essential part of the clean-up process.

7.4.1 *Thin-layer chromatography*

Thin-layer chromatography (TLC) has been applied to the analysis of amines (as such) and of a variety of derivatives which can be selected to take advantage of particular properties, such as colour or fluorescence.

Hydrochlorides of primary, secondary and tertiary amines have been separated on buffered silica gel by Teichert et al. (1960) and could be detected in amounts of from.1-10 µg. Grasshoff (1965) carried out a similar separation on magnesium silicate layers. Ninhydrin has been used as a general reagent for the detection of primary amines, sodium nitroprusside for secondary amines and Dragendorff reagent for tertiary amines.

Good separation and high sensitivity on silica gel plates were achieved by Neurath & Doerk (1964), using the red coloured 4'-nitro-azo-benzyl-(4)-amides, which permitted detection of less than 1 µg of amine. The same derivatives were also found to be sufficiently volatile to permit gas-chromatographic separation and mass-spectrometric identification (Neurath & Lüttich, 1968). This technique was applied to the determination of amines in tobacco and tobacco smoke, in the

course of a search for nitrosatable precursors (Neurath et al., 1966a,b). The 4-dimethylamino-azo-benzoxamides have been used by Churacek et al. (1972) for a similar purpose.

Colorimetric detection of the acylated derivatives of primary and secondary amines is generally found to be more reliable than detection of the parent amines. Seiler & Wiechmann (1965, 1967) were the first to take advantage of the fluorescing properties of the 1-dimethylamino-naphthalene-5-sulfonamides (dansyl-derivatives) on silica gel. Under ultraviolet light (365 nm), as little as 10^{-10} moles are detectable.

These derivatives have been used by Gruger (1972) to determine volatile amines in marine fish and by Lakritz et al. (1975) to study the concentrations of spermine, spermidine, putrescine, cadaverine, histamine, tyramine, tryptamine and ethanolamine in fresh, cooked, smoke-cured and putrefied pork.

Klimisch & Stadler (1974a) described a microquantitative determination of aliphatic amines which employed the formation of fluorescent derivatives with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. An advantage of this method is that the reagent itself does not fluoresce. The derivatives were separated by TLC on polyamide-11-foils. A calibration curve was linear for quantities between 15 and 50 ng per spot. The method was applied to the determination of nitrosamines after acid-catalysed denitrosation to the corresponding amines (Klimisch & Stadler, 1974b).

The amino acid reagent 1-fluoro-2,4-dinitrobenzene was found to be suitable for amine analysis (Richardson, 1963) and the procedure was applied to TLC on silica gel in a study of the principal aliphatic secondary amines of Burley tobacco (Bush, 1970). The spots were analysed by extraction with acetone, followed by spectrophotometric measurement at 355 nm.

7.4.2 *Gas chromatography*

Direct gas chromatographic (GC) separation and determination of amines without formation of derivatives is generally unsatisfactory. Interactions between strong bases and active sites on the supports frequently cause peak tailing and even "ghost" effects are sometimes observed. Coating with alkali, which is often recommended, is not entirely satisfactory. The use of graphitized carbon black, thermally treated in a hydrogen stream and coated with basic compounds such as tetraethylene pentamine, is claimed to eliminate adsorption effects and give symmetrical peaks with amines (Di Corcia et al., 1970; Di Corcia & Samperi, 1974). The latter method was successfully used in the determination of aliphatic amines in aqueous solutions. Miller et al. (1972) utilized the technique for the determination of dimethyl-and trimethylamines in fish, using an alkali flame-ionization detector (AFID),which was both sensitive and selective for nitrogen compounds.

The main progress in analysis of amines by GC has been through the development of suitable derivatives. Van den Heuvel et al. (1964), in a comparative study of the trifluoroacetyl, pentafluoropropyonyl and heptafluorobutyryl derivatives for analysis of amines, showed that whilst the sensitivity to electron-capture detection increases with the number of fluorine atoms in the derivative, the separation factor tends to decrease. This result was subsequently confirmed by other workers and the trifluoroacetamides have since been chosen for many studies where both electron-capture and flame-ionization detection (FID) have been employed for the separation and determination of amines in complex mixtures: for example, the comprehensive investigation of the bases in tobacco and tobacco smoke (Irvine & Saxby, 1969; Pailer & Hübsch, 1966; Pailer et al., 1967, 1969).

Using GC and FID, the trifluoroacetamides have been employed for the detection of primary and secondary amines in fresh vegetables, preserves, mixed pickles, fish and fish products, bread, cheese, stimulants, feedstuffs and surface waters (Neurath & Schreiber, 1974; Neurath et al., 1976). Analysis of the trifluoroacetamides has also been used to identify several primary amines in Emmental cheese (Ney & Wirotama, 1972).

Heptafluorobutyryl derivatives have been used in combination with electron-capture detection by Walle & Ehrsson (1970) for picogram quantities of amino compounds and for the determination of nitrosamines after splitting them to give the secondary amines (Alliston et al., 1972; Eisenbrand, 1972). Heptafluorobutyryl-imidazole has been recommended as a reagent for amines by Staab & Walther (1960).

With the object of restricting analysis to the naturally occurring nitrosatable amines, Singer & Lijinsky (1976a,b) chose the classical Hinsberg method of forming the p-toluene-sulfonyl derivatives for the separation of the secondary from the accompanying primary and tertiary amines. Tertiary amines do not react and the products of primary amines are soluble in alkali,so that the secondary amine derivatives can easily be isolated. The p-toluene sulfonamides are readily separated by GC and have characteristic mass-spectrometric fragmentation patterns, which facilitate their identification. The method was applied to several foodstuffs (Singer & Lijinsky, 1976a) and to tobacco and cigarette smoke condensate (Singer & Lijinsky, 1976b). Quantitative determinations were carried out using AFID and qualitative determinations using a Coulson detector in the nitrogen-selective reduction mode.

Some interesting derivatization methods have been proposed recently but are not yet widely used. For example, Gejvall (1974) analysed the urethanes formed by reaction of amines with diethyl pyrocarbonate, using GC with AFID. The reaction of different isocyanates with amines from N,N'-di- and N,N', N'-tri-substituted ureas was studied by Nitsche et al. (1974), who found the tert-butyl and the 3-tri-fluoromethyl-phenylureas to be useful derivatives for the

GC analysis of primary and secondary amines. Electron-capture and nitrogen detectors, as well as mass spectrometry, have been used with these derivatives. The procedure was intended for use in studying the metabolism of dithiocarbamate fungicides and urea herbicides. Long-chain primary amines have been analysed by GC, after conversion to the dimethylamine derivatives (Metcalfe & Martin, 1972). According to the authors, excellent separation was obtained on silicone oil capillary columns.

7.6 *Conclusion*

A considerable variety of methods are available for the analysis of many environmental amines. While, in general, the basic nature of these compounds is useful in the preliminary isolation, the end analysis is most satisfactorily carried out on one or more of the numerous derivatives which are readily formed from amines.

8. A BRIEF REVIEW OF ANALYTICAL METHODS FOR THE DETERMINATION OF NITRATE AND NITRITE IN FOODSTUFFS

G.M. Telling

8.1 *Introduction*

A knowledge of nitrate and nitrite levels is of great importance in studies of *N*-nitrosamines. A wide range of methods has appeared in the literature and these have been reviewed by Taras (1958), Bolz (1973), Schuller & Veen (1967) and Usher & Telling (1975).

Methods of nitrate and nitrite analysis can be considered in terms of extraction, clean-up, determination and interference, as in the following sections.

8.2 *Extraction*

Aqueous extraction of substrates has been widely used. Alkaline or near-neutral conditions are preferred, as nitrites tend to be destroyed at pH less than 5. Both cold and hot water extraction techniques have been used, with and without the addition of alkaline buffer, for periods ranging from 5 min to 2 hr (Table 1).

Rammell & Joerin (1972) found that extraction with sodium hydroxide solutions gave higher recoveries of nitrite from cheese than were obtained by simple aqueous extraction. Elliot & Porter (1971) compared aqueous extractions from meat over a range of pH values and found recoveries of 44.2% at pH 3.5 and 97% at pH 5.5-6.5, but a decrease to 92% at pH 9.5.

It is important to recognise which form of nitrite a technique is designed to measure. When nitrate/nitrite is added to meat, some nitrite is eventually to be found in a bound form, e.g., nitrosothiols, nitrosylmyoglobin, etc. It can probably be assumed that techniques based on short extraction periods at neutral pH measure only free nitrite. Heavy metal ions, such as Hg^{2+}, Ca^{2+} or Ag^{+}, have been recognized to cause cleavage of the nitrosyl ion from nitroso compounds, especially nitrosothiols (Mirna, 1974). Hence, in the AOAC method (Official Methods of Analysis, 1965), which uses mercuric chloride as a clearing agent, some of the bound nitrite will be measured, as well as the free. However, Mirna & Hofmann (1969) have shown that, in neutral and alkaline media, degradation of nitrosothiols to NO^{+} occurs. Hence, such techniques will tend to measure both free and also some bound nitrite. Mirna (1974) reports that nitrosylmyoglobin is also measured, if 80% aqueous acetone is used for extraction, and that some nitrosylmyoglobin breaks down to give free nitrite when cured meats

are cooked in slightly alkaline solution. Under these circumstances, the general method of estimating recovery for an extraction technique by the addition of known amounts of nitrite to a substrate is subject to error, as no distinction is made between loss due to chemical binding and loss due to physical limitations of the technique.

Table 1. Typical extraction systems for nitrate/nitrite in foods

Substrate (wt)	Volume of extractant (ml)	Extraction procedure	Time (min)	Additives
Baby food (2g) (Kamm et al., 1965)	100	Shake mechanically	10	pH 9.6 buffer
Baby food (10 g) (Green, 1970)	190	Stand, occasional shaking	60	$CdCl_2$ + $BaCl_2$ pH 1.0
Meat (10-20 g) (Adriaanse & Robbers, 1969)	90	Shake mechanically	30	pH 9.6 buffer
Meat (10 g) (Fudge & Truman, 1973)	100	Agitate 15 min on boiling water bath	75	Saturated borax
Cheese (10 g) (McKay, 1974)	60	Agitate on water bath at 50°C	30	Sodium hydroxide
Meat (10 g) (Telling, unpublished)	70	Macerate hot	3-4	-
Meat (5 g) (Official Methods of Analysis, AOAC, 1965)	300	Stand on steam bath	120	$HgCl_2$
Meat (10 g) (Elliot & Porter, 1971)	50	Macerate hot	30	pH 5.6 buffer

8.3 *Clean-up*

Techniques for clean-up (which includes clearing) are based on one of two broad approaches:

(1) deproteinization, using reagents such as Carrez or potassium aluminium sulfate,

(ii) separation techniques, such as chromatography (e.g., ion exchange and gel filtration) or dialysis.

8.3.1 *Deproteinization*

The following are common deproteinizing/clearing agents: zinc sulfate + borax (Grau & Mirna, 1957); potassium aluminium sulfate (Follett & Ratcliff, 1963); zinc sulfate + alkali (Manning et al., 1968); mercuric chloride (Official Methods of Analysis, AOAC, 1965); zinc acetate + potassium ferrocyanide + sodium hydroxide (Becker, 1965); dialysed iron + alumina cream (BFMIRA, 1972); alumina cream (Kamm et al., 1965). All of these, however, have been criticised. The method of Grau & Mirna (1957) which employs zinc hydroxide (formed from zinc sulfate and borax) to precipitate protein from meat extracts at a pH of between 6.0 and 6.5, was criticised by Follett & Ratcliff (1963), on the grounds that insufficient pH control results in precipitation of zinc during or after passage down the cadmium column. Follett & Ratcliff (1963) overcame this problem by using potassium aluminium sulfate as a precipitant. In their method, the heated extract is buffered and saturated potassium aluminium sulfate is added. The pH is controlled within the correct limits of 5.5-6.5 by titrating the extract with potassium aluminium sulfate, using Bromocresol Purple as indicator. This precipitates aluminium hydroxide, bringing the pH of the solution close to the isoelectric point of the soluble protein, which is also precipitated.

Manning et al. (1968) found this method unsatisfactory when used for milk and milk products and investigated three classical clarifying agents, copper hydroxide, cadmium hydroxide and zinc hydroxide. They found zinc hydroxide, generated by mixing zinc sulfate and alkali, most satisfactory. McKay (1974) also used this system for cheese extracts, but Rammel & Joerin (1972) preferred Carrez reagents (zinc acetate and potassium ferrocyanide) to clarify this type of extract.

Carrez reagents have been the most popular clarifying agents for aqueous extracts of meat samples. Adriannse & Robbers (1969) found them satisfactory for meat and vegetable extracts, with the proviso that the zinc acetate reagent was added before the potassium ferrocyanide, to prevent reaction between the latter reagent and nitrite. Fudge & Truman (1973) also used Carrez reagents for meat products and suggested altering the amounts and mode of addition of the reagents, if samples produced cloudy filtrates, e.g., meat products containing cereals. However, altering the amounts or ratio of Carrez reagents may produce a precipitate during the reduction, presumably zinc hydroxide, which was found by Follet & Ratcliff (1963) to interfere at this stage. BFMIRA (1972) recommends two deproteinizing agents for meat extracts, saturated potassium aluminium sulfate or dialysed iron and alumina cream. The latter are more suitable for emulsions and products with high fat content, as fat is coprecipitated with protein and this facilitates subsequent filtration. With such products, however, the filtrate may remain cloudy and require a further treatment with

dialysed iron and alumina. Good recoveries (greater than 95%) were obtained for nitrate and nitrite added to meat over the range 50-500 mg/kg, using both these precipitation procedures.

Usher & Telling (1975) compared the efficiencies of five treatments, using measurements of nitrite recovery, total nitrogen, soluble protein nitrogen and non-protein nitrogen contents before and after treatment of the extracts. They reported that all reagents removed soluble protein to varying extents, but none significantly affected levels of non-protein nitrogen. In their view, the best method for deproteinization was to use Carrez reagents plus titration with sodium hydroxide until the solution became alkaline to phenolphthalein.

8.3.2 *Separation techniques*

Chromatographic techniques have been used in a few instances. Johnson (1969) reported that gel filtration on Sephadex G25 cleaned up a range of substrates sufficiently well to allow nitrite determination, although such extracts still contained materials which interfered in the reduction of nitrate to nitrite. Conversely, ion exchange on Amberline CG 400 gave solutions suitable for the determination of nitrate, but not nitrite. Thin layer chromatography has been used by Hatcher & Schall (1965) to clean up animal feeds.

8.3.3 *Dialysis*

Dialysis has been used to clean up blood (Litchfield, 1967), tobacco leaf extracts (Lowe & Hamilton, 1967), water and soil (Henriksen & Selmer-Olsen, 1970) and spinach extracts[1] in automated systems.

8.4 *Determination*

(a) *Nitrate*

A wide range of techniques has been reported, based upon one or another of the following reactions:

(i) nitration of chromatropic acid, 4-methyl umbelliferone, salicylic acid, phenol disulphonic acid or various xylenol isomers,

(ii) oxidation of brucine, diphenylamine, strychnine or strychnidine,

(iii) reduction of nitrate to nitrite.

---

[1] Wiersum, M.L., private communication.

Hatcher & Schall (1965) reported that, in the analyses of samples which contain water-soluble organic matter and low levels of nitrate, all the techniques based on reactions (i) and (ii) give erratic values, due to reduction of nitrate by organic matter in the presence of strong acids, and considerable charring, which may contribute colour or interfering organic anions. In methods based on oxidation, the colour developed did not always obey Beer's law and constant calibration of the standard curve was required. Other workers had also reported interference from nitrite, chloride and carbohydrate.

Reduction of nitrate to nitrite is the most commonly-used method. Reduction may be achieved with copper, zinc, hydrazine sulfate, cadmium powder or spongy cadmium. All these reagents have been successfully applied in automated systems, but spongy cadmium is generally considered to give the most consistent results.

With the publication of a satisfactory technique for the reduction of nitrate to nitrite by Grau & Mirna (1957), later modified by Follett & Ratcliff (1963), it became possible to determine simultaneously nitrate and nitrite in the same extract. In the Follett & Ratcliff (1963) method, the reduction is achieved by spongy cadmium in a specialized glass column with capillary inlet and outlet tubes. This column can be replaced by a 25 ml burette without any loss in reduction efficiency, although the life of the packing may be expected to be rather less than with the original apparatus. The optimum pH is 9.5-9.7 and the reduced nitrate is eluted from the column with 100 ml of distilled water at a flow rate of approximately 5 ml/min. The column has to be regenerated with 0.1 N hydrochloric acid after each reduction and must be pre-washed with 0.1 N hydrochloric acid before use, as the activity of the spongy cadmium deteriorates with time, even when stored under water. Various workers have reported that reduction by this technique is practically 100% complete.

Elliott & Porter (1971) proposed a rapid reduction technique whereby nitrate is reduced by shaking a meat extract with 1 g of spongy cadmium for 5 min at pH 9.6. Fudge & Truman (1973) found that recoveries of nitrate with this technique were low, indicating inefficient reduction. Increasing the amount of spongy cadmium used failed to improve recoveries. Usher & Telling (1975) have found that the main cause of reduced efficiency of reduction in meat was the presence of polyphosphate. In such cases, the polyphosphate interfered with the buffering effect, shifting the pH outside the 9.5-9.7 range. This problem could be overcome by using a buffer of twice the strength proposed by Follett & Ratcliff (1963). With the stronger buffer, however, the Orange I method could no longer be used and *N*-1-naphthylethylenediamine reagent was employed instead. When a large number of samples are to be analysed on a routine basis, the column technique is less time consuming, but otherwise the shaking method is found to have definite advantages.

(iv) Nitrate ion selective electrodes

The introduction of nitrate selective electrodes has made possible the direct determination of nitrate activities in water and in plant suspensions (Paul & Carlson, 1968; Milham et al., 1970). This method has been used with baby food (Westcott, 1971) and spinach extracts (Voogt, 1969). The nitrate electrode is non-specific and various other anions interfere. The influence of these anions must be eliminated. This is usually done with a suitable buffer solution, e.g., one containing silver to precipitate halides, aluminium to complex anions of organic acids and sulphamic acid to destroy nitrite. The precision of the method can be improved by carefully controlling the temperature and standardizing the rate of stirring (Milham et al., 1970). The procedure can be automated with a flow-through electrode.

(b) *Nitrite*

Methods generally used for the determination of nitrite in aqueous solution are based on diazotization of a primary amine, using nitrite as the source of nitrous acid, plus coupling with an aromatic compound bearing influential amino or hydroxyl substituents to form an azo colour, which can be measured spectrophotometrically. Although a wide range of amines are both potential reactants with nitrite and potential coupling agents, the commonly-used methods involve diazotization of sulfanilic acid (Adriaanse & Robbers, 1969; Bratton & Marshall, 1939; Follett & Ratcliff, 1963; Griess, 1879; Ilosvay, 1889), sulphanilamide (Shinn, 1941) and coupling of the diazo compound with 1-naphthylamine (Griess, 1879; Ilosvay, 1889; Kamm et al., 1965), 1-naphthol-7-sulfonic acid (Adriaanse & Robbers, 1969), 1-naphthol (Follett & Ratcliff, 1963) or *N*-1-naphthylethylenediamine (Bratton & Marshall, 1939; Shinn, 1941). The methods which involve the use of 1-naphthol or carcinogenic 1-naphthylamine are no longer generally acceptable.

Nitrite may also be determined indirectly by oxidation to nitrate with potassium permanganate and measurement of nitrate by one of the procedures described above.

8.5 *Interference*

Although the technique of nitrate reduction with spongy cadmium and nitrite determination by formation of a diazonium salt is now widely used, there are a number of associated problems which become important in the determination of low levels of nitrate and nitrite (10-20 mg/kg).

8.5.1 *Dilution effect*

When the methods are used to determine low levels of nitrite, there is a great temptation to achieve a greater final intensity of colour by using a larger volume of the extract. Nicholas & Fox (1973) studied the effect of dilution in the AOAC method (Official Methods of Analysis, AOAC, 1965). Using commercial frankfurters, they varied sample dilution from 1:2 to 1:1000 and found that the nitrite response decreased with increasing concentration of soluble solids. Their explanation was that an increasing ratio of water to meat results in an increasing ratio of total dissolved oxygen in the water to endogenous reductants in the meat; this resusts in more extensive oxidation of compounds which could later interfere in the colour reaction. Kamm et al. (1965) reported a similar pattern when analysing a range of baby foods.

8.5.2 *Interference from ascorbate*

A wide range of oxidising and reducing substances, of which ascorbate is the most important, will interfere in the determination of nitrate and nitrite. With deproteinizing systems which operate at acid pH values, ascorbate can completely remove nitrite from the test solution.

Fudge & Truman (1973) removed ascorbate interference with an activated charcoal (Darco 60), while BFMIRA (1972) prefer to remove ascorbate by titration with 0.1 N iodine.

Usher & Telling (1975) oxidize ascorbate with permanganate under alkaline conditions, removing excess permanganate with 0.1 N arsenic trioxide.

Nicholas & Fox (1973) claim that the 2 hour extraction used in the AOAC method (Official Methods of Analysis, AOAC, 1965) obviates the need to eliminate ascorbate.

8.5.3 *Interference from sulfur dioxide*

The presence of sulfur dioxide can also interfere in the determination of nitrite, but Usher & Telling (1975) suggest that, since its use in meat products is very restricted, this effect is unlikely to be important.

8.5.4 *The meat blank*

When determining nitrate and nitrite in meat, there are typical blank values associated with the reagents and the reduction column (e.g., 5 mg/kg). These blanks are constant and present no particular problem.

Of much greater importance is the so-called meat blank. When an extract of uncured meat is reduced with spongy cadmium and subjected to a diazo coupling method, an apparent nitrate value is obtained. This was first reported by Grau & Mirna (1957), who quoted 10 mg/kg of

potassium nitrate as a meat blank. This finding was confirmed by Follett & Ratcliff (1963) and by Mohler (1964), who found meat blanks of 13-30 mg/kg, expressed as potassium nitrate. More recently, BFMIRA has reported meat blanks of up to 80 mg/kg.

The blank correction of Follett & Ratcliff (1963) requires that part of the deproteinised extract be passed through a column of an anion-exchange resin (Deacidite FF) to remove nitrate and nitrite. The column is then eluted and the eluate is reduced and coloured to give an accurate value for the meat blank. This value must be subtracted from the total apparent nitrate. The significance of such a meat blank obviously becomes greater as the level of nitrate decreases. Follett & Ratcliff suggested that this meat blank was probably associated with amino acids, but other work (Usher & Telling, 1975) appears to contradict this proposal.

The various problems of dilution effect, interference from reducing agents and the meat blank indicate that, although reduction of nitrate to nitrite with spongy cadmium and subsequent determination of nitrite by formation of a diazo compound is the method generally favoured, the technique is not ideal and is suspect for the determination of low (10 mg/kg) levels of nitrate and nitrite in food samples.

8.6 *Conclusions*

The accurate determination of nitrate and nitrite in foodstuffs still poses analytical problems. The severity of the extraction procedure will determine whether free or free plus bound nitrate/nitrite is extracted. Once in solution, free nitrite is readily determined by diazotization and coupling reactions. The determination of nitrate is more difficult. The reduction of nitrate to nitrite is not easy to control; standard methods using spongy cadmium give rise to variable sample blanks and are affected by co-extracted materials, even in clear solutions. Classical methods for the determination of nitrate are generally unreliable and subject to interference.

REFERENCES

Adriaanse, A. & Robbers, J.E. (1969) Determination of nitrite and nitrate in some horticultural and meat products and in samples of soil. *J. Sci. Food Agric., 20*, 321-325

Alliston, T.G., Cox, B.G. & Kirk, R.S. (1972) The determination of steam-volatile *N*-nitrosamines in foodstuffs by formation of electron-capturing derivatives from electrochemically derived amines. *Analyst, 97*, 915-920

Althorpe, J., Goddard, D.A., Sissons, D.J. & Telling, G.M. (1970) The gas chromatographic determination of nitrosamines at the picogram level by conversion to their corresponding nitramines. *J. Chromatogr., 53*, 371-373

Association of Official Agricultural Chemists (1965) *Meat and meat products, Chapter 23.* In: Horwitz, W., ed, *Official Methods of Analysis*, AOAC, p. 347

Aue, W.A., Gehrke, C.W., Trindle, R.C., Stalling, D.L. & Ruyle, C.D. (1967) Application of the alkali-flame detector to nitrogen-containing compounds. *J. Gas Chromatogr., 5*, 381-382

Becker, K.F. (1965) Nitrat- und Nitritgehalt in Spinat. *Bundesgesundheitsblatt, 17*, 246-248

B.F.M.I.R.A. (1972) *Determination of nitrate and nitrite in meat products.* In: Evans, G.G., ed., *Technical circular No. 508*, pp. 1-12

Bills, D.D., Hildrum, K.I., Scanlan, R.A. & Libbey, L.M. (1973) Potential precursors of *N*-nitrosopyrrolidine in bacon and other fried foods. *J. Agric. Food Chem., 21*, 876-877

Bogovski, P., Castegnaro, M. & Walker, E.A. (1972) *The inhibiting effect of tannins on the formation of nitrosamines.* In: Bogovski, P., Preussmann, R. & Walker, E.A., eds, *N-Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 3*), pp. 127-129

Boltz, D.F. (1973) Recent developments in methods for determination of anions (section V). *Crit. Rev. Anal. Chem., 3*, 147-149

Bratton, A.C. & Marshall, E.K. (1939) A new coupling component for sulfanilamide determination. *J. Biol. Chem., 128*, 537-550

Brewer, P.G. & Riley, J.P. (1965) Automatic determination of nitrate in sea water. *Deep Sea Res.*, *12*, 765-772

Brooks, J.B., Alley, C.C. & Jones, R. (1972) Reaction of nitrosamine with fluorinated anhydrides and pyridine to form electron-capturing derivatives. *Anal. Chem.*, *44*, 1881-1884

Bryce, T.A. & Telling, G.M. (1972) Semiquantitative analysis of low levels of volatile nitrosamines by gas chromatography - mass spectrometry. *J. Agric. Food Chem.*, *20*, 910-911

Bush, L. (1970) Principal aliphatic secondary amines of Burley tobacco. *Beitr. Tabakforsch.*, *5*, 275-278

Castegnaro, M. (1976) Analyse des composés N-nitroso volatils dans les boissons alcooliques. Doctoral thesis, University of Lyon, pp. 70-94

Castegnaro, M. & Walker, E.A. (1977) *Developments in nitrosamine analyses.* In: Tinbergen, B.J. & Krol, B., eds, *Proc. Second Int. Symp. Nitrite Meat Prod., Zeist*, Wageningen, PUDOC, pp. 187-190

Castegnaro, M., Pignatelli, B. & Walker, E.A. (1972) *Investigation of the use of 2-butanone peroxide in oxidation of nitrosamines for gas chromatography.* In: Bogovski, P., Preussmann, R. & Walker, E.A., eds, N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 3*), pp. 87-89

Castegnaro, M., Pignatelli, B. & Walker, E.A. (1974a) A rapid method for the semi-quantitative determination of volatile *N*-nitrosamines in alcoholic beverages. *Analyst, 99*, 156-162

Castegnaro, M., Pignatelli, B. & Walker, E.A. (1974b) *An investigation of the possible value of oxonium salt formation in nitrosamine analysis.* In: Bogovski, P. & Walker, A.E., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 45-48

Chang, S.K., Harrington, G.W. (1975) Determination of dimethylnitrosamine and nitrosoproline by differential pulse polarography. *Anal. Chem.*, *47*, 1857-1860

Churacek, J., Pechova, H., Tocksteinova, D. & Zikova, Z. (1972) Separation and identification of primary and secondary aliphatic amines as p-(N,N-dimethylamino)-benzene-p-azo-benzamides by paper and thin-layer chromatography. *J. Chromatogr.*, *72*, 145-152

Collin, J. (1954) Spectrométrie de masse de dérivés azotés dialkyl nitrosamines et nitrosodérivés. *Bull. Soc. Roy. Sci. Liège*, *23*, 201-220

Coulson, D.M. (1965) Electrolytic conductivity detector for gas chromatography. *J. Gas Chromatogr., 3*, 134-137

Cox, G.B. (1973) Estimation of volatile N-nitrosamines by high-performance liquid chromatography. *J. Chromatogr., 83*, 471-481

Crathorne, B., Edwards, M.W., Jones, N.R., Walters, C.L. & Woolford, G. (1975) Use of isomers in the detection and estimation of volatile nitrosamines by combined high-resolution mass spectrometry - gas chromatography. *J. Chromatogr., 115*, 213-217

Crosby, N.T., Foreman, J.K., Palframan, J.F. & Sawyer, R. (1972a) Estimation of steam-volatile *N*-nitrosamines in foods at the 1 µg/kg level. *Nature (London), 238*, 342-343

Crosby, N.T., Foreman, J.K., Palframan, J.F. & Sawyer, R. (1972b) *Determination of volatile nitrosamines in food products at the µg/kg level.* In: Bogovski, P., Preussmann, R. & Walker, E.A., eds, N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 3*), pp. 38-42

Daiber, D. & Preussmann, R. (1964) Quantitative colorimetrische Bestimmung organischer N-nitroso-Verbindungen durch photochemische Spaltung der Nitrosaminbindung. *Z. Anal. Chem., 206*, 344-352

Devik, O.G. (1967) Formation of N-nitrosamines by the Maillard reaction. *Acta Chem. Scand., 21*, 2302-2303

Di Corcia, A. & Samperi, R. (1974) Gas chromatographic determination at the parts-per-million level of aliphatic amines in aqueous solution. *Anal. Chem., 46*, 977-981

Di Corcia, A., Fritz, D. & Bruner, F. (1970) Use of graphitized carbon black for linear gas-liquid-solid chromatography of polar low boiling compounds. *Anal. Chem., 42*, 1500-1504

Dooley, C.J., Wasserman, A.E. & Osman, S. (1973) A contaminant in N-nitrosodimethylamine confirmation by high-resolution mass spectrometry. *J. Food Sci., 38*, 1096

Druckrey, H., Preussmann, R., Ivankovic, S. & Schmaehl, D. (1967) Organotrope Carcinogene Wirkungen bei 65 verschiedenen N-nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch., 69*, 103-201

Eisenbrand, G. (1970) Zur Spurenanalytik cancerogener Nitrosamine. Doctoral Thesis, University of Freiburg, pp. 110-127

Eisenbrand, G. (1972) *Determination of volatile nitrosamines at low levels in food by acid catalysed denitrosation and formation of derivatives from the resulting amines.* In: Bogovski, P., Preussmann, R. & Walker, E.A., eds, N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 3*), pp. 64-70

Eisenbrand, G. (1974) *Recent developments in trace analysis of volatile nitrosamines: a brief review.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 6-11

Eisenbrand, G. & Preussmann, R. (1970) Eine neue Methode zur kolorimetrischen Bestimmung von Nitrosaminen nach Spaltung der *N*-Nitrosogruppe mit Bromwassersotff im Eisessig. *Arzneim. Forsch., 20*, 1513-1517

Eisenbrand, G., Marquardt, P. & Preussmann, R. (1969) Trace analysis of *N*-nitroso compounds. I. Liquid-liquid distribution in aceto-nitrile-heptane as clean-up method. *Z. Anal. Chem., 247*, 54-55

Eisenbrand, G., Spaczynski, K. & Preussmann, R. (1970a) Spureanalyse von *N*-Nitroso Verbindungen. III. Quantitative Dünnschicht-chromatographie von Nitrosaminen. *J. Chromatogr., 51*, 503-509

Eisenbrand, G., von Hodenberg, A. & Preussmann, R. (1970b) Trace analysis of *N*-nitroso compounds. II. Steam distillation at neutral, alkaline and acid pH under reduced and atmospheric pressure. *Z. Anal. Chem., 251*, 22-24

Eisenbrand, G., Ungerer, O. & Preussmann, R. (1974) *Formation of N-nitroso compounds from agricultural chemicals and nitrite.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 71-74

Eisenbrand, G., Von Rappardt, E., Zappe, R. & Preussmann, R. (1976) *Trace analysis of volatile nitrosamines by a modified nitrogen-specific detector in pyrolytic mode and by ion-specific determination of heptafluorobutyramides in a gas chromatography-mass spectrometry system.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 65-75

Elliott, R.J. & Porter, A.G. (1971) A rapid cadmium reduction method for the determination of nitrate in bacon and curing brines. *Analyst, 96*, 522-527

Emmons, W.D. (1954) Peroxytrifluoroacetic acid. I. The oxidation of nitrosamines to nitramines. *J. Am. Chem. Soc., 76*, 3468-3470

Ender, F. & Ceh, L. (1967) Occurrence and determination of nitrosamines in foodstuffs for human and animal nutrition. Alkylierend wirkende Verbindungen. *Second Conference on Tobacco Research*, Freiburg, Wissenschaftliche Forschungstelle in Verband der Cigaretten-Industrie, pp. 83-89

Ender, F. & Ceh, L. (1971) Conditions and chemical reaction mechanisms by which nitrosamines may be formed in biological products with reference to their possible occurrence in food products. *Z. Lebensm. Unters. Forsch., 145*, 133-142

Ender, F., Havre, G.N., Helgebostad, A., Koppang, N., Madsen, R. & Ceh, L. (1964) Isolation and identification of a hepatotoxic factor in herring meal produced from sodium nitrite preserved herring. *Naturwissenschaften, 24*, 637-638

Ender, F., Havre, G.N., Madsen, R., Ceh, L. & Helgebostad, A. (1967) Studies on conditions under which *N*-nitroso-dimethylamine is formed in herring meal produced from nitrite-preserved herring. *Z. Tierphysiol., 22*, 181-189

Essigmann, J.M. & Issenberg, P. (1972) Gas chromatographic determination of volatile nitrosamines in foods. *J. Food Sci., 37*, 684-688

Fan, S., Fine, D.H., Ross, R., Rounbehler, D.P., Silvergleid, A. & Song, L. (1976a) Determination of *N*-nitroso pesticides in air, water and soil (abstracted paper) 172nd American Chemical Society National Meeting, San Francisco, California

Fan, T.Y. & Tannenbaum, S.R. (1971) Automatic colorimetric determination of *N*-nitroso compounds. *J. Agric. Food Chem., 19*, 1267-1269

Fan, T.Y., Morrison, J., Rounbehler, D.P., Ross, R., Fine, D.H., Miles, W. & Sen, N.P. (1976b) *N*-nitrosodiethanolamine in synthetic cutting fluids, a part per hundred impurity. *Science, 196*, 70-71

Fazio, T., Damico, J.N., Howard, J.W., White, R.H. & Watts, J.O. (1971) Gas chromatographic determination and mass spectrometric confirmation of *N*-nitrosodimethylamine in smoke-processed marine fish. *J. Agric. Food Chem., 19*, 250-253

Fazio, T., Howard, J.W. & White, R. (1972) *Multidetection method for analysis of volatile nitrosamines in foods.* In: Bogovski, P., Preussmann, R. & Walker, E.A., eds, *N-Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 3*), pp. 16-24

Fazio, T., White, R.H., Dusold, L.R. & Howard, J.W. (1973) Nitrosopyrrolidine in cooked bacon. *J. Assoc. Off. Anal. Chem., 56*, 919-921

Fiddler, W. (1975) The occurrence and determination of *N*-nitroso compounds. *Toxicol. Appl. Pharmacol., 31*, 352-360

Fiddler, W., Doerr, R.G., Ertel, J.R. & Wasserman, A.E. (1971) Determination of *N*-nitrosodimethylamine in ham by gas-liquid chromatography with an alkali flame ionization detector. *J. Assoc. Off. Anal. Chem., 54*, 1160-1163

Fiddler, W., Feinberg, J.I., Pensabene, J.W., Williams, A.C. & Dooley, C.J. (1975) Dimethylnitrosamine in souse and similar jellied cured-meat products. *Food Cosmet. Toxicol., 13*, 653-654

Fine, D.H. & Rufeh, F. (1974) *Description of the thermal energy analyser for* N-*nitroso compounds.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 40-44

Fine, D.H. & Rounbehler, D.P. (1975) Trace analysis of volatile *N*-nitroso compounds by combined gas chromatography and thermal energy analysis. *J. Chromatogr., 109*, 271-279

Fine, D.H. & Rounbehler, D.P. (1976) *Analysis of volatile* N-*nitroso compounds by combined gas chromatography and thermal energy analysis.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 117-127

Fine, D.H., Rufeh, F. & Lieb, D. (1974) Group analysis of volatile and non-volatile *N*-nitroso compounds. *Nature (London), 247*, 309-310

Fine, D.H., Rufeh, F., Lieb, D. & Rounbehler, D.P. (1975a) Description of the thermal energy analyzer (TEA) for trace determination of volatile and non-volatile *N*-nitroso compounds. *Anal. Chem., 47*, 1188-1191

Fine, D.H., Lieb, D. & Rufeh, F. (1975b) Principle of operation of the thermal energy analyzer for the trace analysis of volatile and non-volatile *N*-nitroso compounds. *J. Chromatogr., 107*, 351-357

Fine, D.H., Rounbehler, D.P. & Oettinger, P.E. (1975c) A rapid method for the determination of sub-part per billion amounts of *N*-nitroso compounds in foodstuffs. *Anal. Chim. Acta, 78*, 383-389

Fine, D.H., Huffman, F., Rounbehler, D.P. & Belcher, N.M. (1976a) *Analysis of* N-*nitroso compounds by combined high-performance liquid chromatography and thermal energy analysis.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 43-50

Fine, D.H., Rounbehler, D.P. & Sen, N.P. (1976b) A comparison of some chromatographic detectors for the analysis of volatile *N*-nitrosamines. *J. Agric. Food Chem., 24*, 980-984

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

Fine, D.H., Rounbehler, D.P., Silvergleid, A. & Ross, R. (1977b) *Trace analysis of polar and a polar* N-*nitroso compounds by combined high-performance liquid chromatography and thermal energy analysis.* In: Tinbergen, B.J. & Krol, B., eds, *Proc. Second Int. Symp. Nitrite Meat Prod., Zeist*, Wageningen, PUDOC, pp. 191-198

Fishbein, L. (1972) *Chromatography of Environmental Hazards.* Vol. 1. Elsevier Publishing Co., Amsterdam, London, New York, pp. 37-65

Follett, M.J. & Ratcliff, P.W. (1963) Determination of nitrite and nitrate in meat products. *J. Sci. Food Agric., 14*, 138-144

Fong, Y.Y. & Chan, W.C. (1973a) Dimethylnitrosamine in Chinese marine salt fish. *Food Cosmet. Toxicol., 11*, 841-845

Fong, Y.Y. & Chan, W.C. (1973b) Bacterial production of dimethylnitrosamine in salted fish. *Nature (London), 243*, 421-422

Foreman, J.K. & Goodhead, K. (1975) The formation and analysis of *N*-nitrosamines. *J. Sci. Food Agric., 26*, 1771-1783

Foreman, J.K., Palframan, J.F. & Walker, E.A. (1970) Gas chromatographic determination of *N*-alkyl nitrosamines. *Nature (London), 225*, 544

Fudge, R. & Truman, R.W. (1973) The nitrate and nitrite contents of meat products - A survey by public analysts' laboratories in South Wales and South West of England. *J. Assoc. Public Anal., 11*, 19-27

Gadbois, D.F., Ravesi, E.M., Lundstrom, R.C. & Maney, R.S. (1975) *N*-nitrosodimethylamine in cold smoked sablefish. *J. Agric. Food Chem., 23*, 665-668

Gaffield, W., Fish, R.H., Holmstead, R.L., Poppiti, J. & Yergey, A.L. (1976) *Chemical ionisation-mass spectrometry of nitrosamines.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 11-20

Gejvall, T. (1974) Gas chromatographic analysis of amines separated as urethane derivatives. *J. Chromatogr., 90*, 157-161

Goodhead, K. & Gough, T.A. (1975) The reliability of a procedure for the determination of nitrosamines in food. *Food Cosmet. Toxicol., 13*, 307-312

Gough, T.A. (1977) A search for volatile nitrosamines in East African spirit. *Gut, 18*, 301-302

Gough, T.A. & Goodhead, K. (1975) Occurrence of volatile nitrosamines in spice premixes. *J. Sci. Food Agric.*, *26*, 1473-1478

Gough, T.A. & Sawyer, R. (1974) *Analysis of volatile nitrosamines: progress in gas chromatography-mass spectrometry techniques.* In: Bogovski, P. & Walker, E.A., eds, *N-Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 18-21

Gough, T.A. & Sugden, K. (1973) A study of the stability of a nitrogen-selective thermionic detector. *J. Chromatogr.*, *86*, 65-71

Gough, T.A. & Sugden, K. (1975) Dual column gas chromatographic system for use in mass spectral determination of nitrosamines. *J. Chromatogr.*, *109*, 265-269

Gough, T.A. & Walters, C.L. (1976) *Volatile nitrosamines in fried bacon.* In: Bogovski, P. & Walker, E.A., eds, *Environmental N-Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 196-203

Gough, T.A. & Webb, K.S. (1972) The use of a molecular separator in the determination of trace constituents by combined gas chromatography and mass spectrometry. *J. Chromatogr.*, *64*, 201-210

Gough, T.A. & Webb, K.S. (1973) A method for the detection of traces of nitrosamines using combined gas chromatography and mass spectrometry. *J. Chromatogr.*, *79*, 57-63

Gough, T.A. & Webb, K.S. (1974) The trace detection of some non-volatile nitrosamines by combined gas chromatography and mass spectrometry. *J. Chromatogr.*, *95*, 59-63

Gough, T.A., Sugden, K. & Webb, K.S. (1975) Pyridine catalyzed reaction of volatile *N*-nitrosamines with heptafluorobutyric anhydride. *Anal. Chem.*, *47*, 509-512

Gough, T.A., Goodhead, K. & Walters, C.L. (1976a) Distribution of some volatile nitrosamines in cooked bacon. *J. Sci. Food Agric.*, *27*, 181-185

Gough, T.A., Pringuer, M.A., Sugden, K., Webb, K.S. & Simpson, C.F. (1976b) Pyridine catalyzed reaction of *N*-nitrosodimethylamine with heptafluorobutyric anhydride. *Anal. Chem.*, *48*, 583-585

Gough, T.A., McPhail, M.F., Webb, K.S., Wood, B.J. & Coleman, R.F. (1977a) An examination of some foodstuffs for the presence of volatile nitrosamines. *J. Sci. Food Agric.*, *28*, 345-351

Gough, T.A., Webb, K.S. & Eaton, R.F. (1977b) A simple chemiluminescent detector for the screening of foodstuffs for the presence of volatile nitrosamines. *J. Chromatogr.*, *137*, 293-303

Gough, T.A., Webb, K.S., Pringuer, M.A. & Wood, B.J. (1977c) A comparison of various mass spectrometric and a chemiluminescent method for the estimation of volatile nitrosamines. *J. Agric. Food Chem., 25*, 663-667

Grasshoff, H. (1965) Thin layer chromatography of amines. *J. Chromatogr., 20*, 165-167

Grau, R. & Mirna, A. (1957) Über die Bestimmung von Nitrit, Nitrat und Kochsalz in Fleischwaren und Laken. *Z. Anal. Chem., 158*, 182-189

Gray, J.I. & Dugan, L.R. (1975) Formation of *N*-nitrosopyrrolidine from proline and collagen. *J. Food Sci., 40*, 484-487

Green, M.S. (1970) Notes on clearing methods for the determination of nitrate and nitrite in canned baby foods. *J. Assoc. Public Anal., 8*, 48-50

Griess, P. (1879) Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt ueber einige Azoverbindungen. *Ber. Dtsch. Chem. Ges., 12*, 426-428

Groenen, P.J., Jonk, R.J.G., Van Ingen, C. & Ten Noever de Brauw, M.C. (1976) *Determination of eight volatile nitrosamines in thirty cured meat products with capillary gas chromatography - high resolution mass spectrometry.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental N-Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 321-331

Gruger, E.H., Jr (1972) Chromatographic analyses of volatile amines in marine fish. *J. Agric. Food Chem., 20*, 781-785

Hall, R.C. (1974) A highly sensitive and selective micro-electrolytic conductivity detector for gas chromatography. *J. Chromatogr., Sci., 12*, 152-160

Hatcher, D.W. & Schall, E.D. (1965) Determination of nitrate in feeds. *J. Assoc. Off. Anal. Chem., 48*, 648-653

Heath, D.F. & Jarvis, J.A.E. (1955) The polarographic determination of dimethylnitrosamine in animal tissue. *Analyst, 80*, 613-616

Heisler, E.G., Siciliano, J., Krulik, S., Feinberg, J. & Schwartz, J.H. (1974) Changes in nitrate and nitrite content and search for nitrosamines in storage-abused spinach and beets. *J. Agric. Food Chem., 22*, 1029-1032

Henriksen, A. & Selmer-Olsen, A.R. (1970) Automatic methods for determining nitrate and nitrite in water and soil extracts. *Analyst, 95*, 514-518

Heyns, K. & Koch, H. (1971) Zur Frage der Enstehung von Nitrosaminen bei der Reaktion von Monosacchariden mit Aminosauren. *Z. Lebensm. Unters. Forsch., 145*, 76-84

Heyns, K. & Roper, H. (1970) Ein spezifisches analytisches Nachweisverfahren fuer Nitrosamine durch Kombination von Gaschromatographie mit der Massenspektrometrie. *Tetrahedron Lett., 10*, 737-740

Heyns, K. & Roper, H. (1971) Ein spezifisches analytisches Trenn- und Nachweisverfahren für Nitrosamine durch Kombination von Capillargaschromatographie und Massenspektrometrie. *Z. Lebensm. Unters. Forsch., 145*, 69-75

Hoffmann, D., Rathkamp, G. & Liu, Y.Y. (1974) *Chemical studies on tobacco smoke. XXVI. On the isolation and identification of volatile and non-volatile* N-*nitrosamines and hydrazines in cigarette smoke.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 159-165

Hogg, A.M. (1975) A digital mass readout for magnetic sector mass spectrometers. *Proc. Am. Soc. Mass Spectrom.*, pp. 256-258

Howard, J.W., Fazio, T. & Watts, J.O. (1970) Extraction and gas chromatographic determination of *N*-nitrosodimethylamine in smoked fish: application to smoked nitrite-treated chub. *J. Assoc. Off. Anal. Chem., 53*, 269-274

Huxel, E.T., Scanlan, R.A. & Libbey, L.M. (1974) Formation of *N*-nitrosopyrrolidine from pyrrolidine ring containing compounds at elevated temperatures. *J. Agric. Food Chem., 22*, 698-700

Ilosvay, M.L. (1889) L'acide azoteux dans la salive et dans l'air exhalé. *Bull. Soc. Chim. Paris, 2*, 388-391

International Agency for Research on Cancer (1976) *Recommendations of a working conference on* N-*nitroso compounds in the environment.* In: Walker, E.A., Bogovski, P. & Griciute,L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 506-507

Irvine, W.J. & Saxby, M.J. (1969) Gas chromatography of primary and secondary amines as their trifluoroacetyl derivatives. *J. Chromatogr., 43*, 129-131

Issenberg, P. & Sornson, H. (1976) *A monitoring method for volatile nitrosamine levels in laboratory atmospheres.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*) pp. 97-108

Issenberg, P. & Tannenbaum, S.R. (1972) *Approaches to the determination of volatile and non-volatile* N-*nitroso compounds in foods and beverages.* In: Bogovski, P., Preussmann, R. & Walker, E.A., eds, N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 3*), pp, 31-37

Iwaoka, W. & Tannenbaum, S.R. (1976) *Photohydrolytic detection of* N-*nitroso compounds in high-performance liquid chromatography.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 51-56

Iwaoka, W.T., Weisman, M.S. & Tannenbaum, S.R. (1974) *A solvent-partitioning, high-speed, liquid chromatographic procedure for clean-up in the analysis of nitrosamines.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 32-35

Iyengar, J.R., Panalaks, T., Miles, W.F. & Sen, N.P. (1976) A survey of fish products for volatile *N*-nitrosamines. *J. Sci. Food Agric.*, *27*, 527-530

Johnson, D.B. (1969) Gel filtration in sample preparation for nitrite assay. *Ir. J. Agric. Res.*, *8*, 444-446

Johnson, E.M. & Walters, C.L. (1971) The specificity of the release of nitrite from *N*-nitrosamines by hydrobromic acid. *Anal. Lett.*, *4*, 383-386

Juszkiewicz, T. & Kowalski, B. (1974) *Passage of nitrosamines from rumen into milk in goats.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 173-176

Kadar, R. Devik, O.G. (1970) Pyrazines as interfering substances in the determination of nitrosamines in roasted foods. *Acta Chem. Scand.*, *24*, 2943-2948

Kadar, R. & Devik, O.G. (1972) The possible occurrence of nitrosamines in tobacco smoke. *Beitr. Tabakforsch.*, *6*, 117-119

Kamm, L., McKeown, G.G. & Morrison Smith, D. (1965) Food additives: new colorimetric method for the determination of the nitrate and nitrite content of baby foods. *J. Assoc. Off. Agric. Chem.*, *48*, 892-897

Kawabata, T. & Miyakoshi, S. (1976) *Microbial formation of* N-*nitroso-pyrrolidine from* N-*nitrosoproline.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 261-266

Kawabata, T., Matsui, M., Ishibashi, T. & Nakamura, M. (1972) Analysis of *N*-nitrosamines by gas chromatography. *Jpn Anal.*, *21*, 1326-1332

Klimisch, H.J. & Stadler, L. (1974a) Fluorimetrische Bestimmung von Nitrosaminen nach saurekatalysierter Denitrosierung und Derivatisierung mit 7-Chlor-4-nitrobenzo-2-oxa-1,3-diazol. *J. Chromatogr.*, *90*, 223-225

Klimisch, H.J. & Stadler, L. (1974b) Mikroquantitative Bestimmung von Aliphatischen Aminen mit 7-Chlor-4-nitrobenzo-2-oxa-1,3-diazol. *J. Chromatogr.*, *90*, 141-148

Klus, H. & Kuhn, H. (1975) Reaktionschromatographischer Nachweis einiger *N*-Nitrosamine der Tabakalkaloide. *J. Chromatogr.*, *109*, 425-426

Krejci, M. & Dressler, M. (1970) Selective detectors in gas chromatography. *Chromatogr. Rev.*, *13*, 1-59

Kroeller, E. (1967) Untersuchungen zum Nachweis von Nitrosaminen in Tabakrauch und Lebensmitteln. *Dtsch. Lebensm. Rundsch.*, *63*, 303-305

Kubacki, S.J. & Borys, A. (1976) *Method for the determination of nitrosopyrrolidine in meat and meat products at the 1 µg/kg level by means of gas chromatography - electron capture detection.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental N-Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 109-116

Kushnir, I., Feinberg, J.I., Pensabene, J.W., Piotrowski, E.G., Fiddler, W. & Wasserman, A.E. (1975) Isolation and identification of nitrosoproline in uncooked bacon. *J. Food Sci.*, *40*, 427-428

Lakritz, L., Spinelli, A. & Wasserman, A. (1975) Determination of amines in fresh and processed pork. *J. Agric. Food Chem.*, *23*, 344-346

Lane, R.P., Rice, R.H. & Bailey, M.E. (1974) Gas chromatographic - Mass spectrometric determination of *N*-nitrosodimethylamine formed in synthetic and human gastric juice. *J. Agric. Food Chem.*, *22*, 1019-1023

Levin, I.W., Milne, G.W.A. & Axenrod, T. (1970) Raman and infrared spectra of dimethylnitrosamine. *J. Chem. Phys.*, *53*, 2505-2512

Lijinsky, W., Keefer, L. & Loo, J. (1970) The preparation and properties of some nitrosoamino acids. *Tetrahedron*, *26*, 5137-5153

Lijinsky, W., Christie, W.H. & Rainey, W.T. (1973) Mass spectra of *N*-nitroso compounds. Oak Ridge National Laboratory Report ORNL-TM-4359, pp. 1-106

Litchfield, M.H. (1967) The automated analysis of nitrite and nitrate in blood. *Analyst*, *92*, 132-136

Lowe, R.H. & Hamilton, J.L. (1967) Rapid method for determination of nitrate in plant and soil extracts. *J. Agric. Food Chem., 15*, 359-361

McGlashan, N.D., Walters, C.L. & McLean, A.E.M. (1968) Nitrosamines in African alcoholic spirits and oesophageal cancer. *Lancet, ii*, 1017

McGlashan, N.D., Patterson, R.L.S. & Williams, A.A. (1970) *N*-nitrosamines and grain-based spirits. *Lancet, ii*, 1138

McKay, A.J. (1974) Determination of nitrate and nitrite in cheese. *Aust. J. Dairy Technol., 29*, 34-36

Manning, P.B., Coulter, S.T. & Jenness, R. (1968 Determination of nitrate and nitrite in milk and dry milk products. *J. Dairy Sci., 51*, 1725-1730

Marquardt, P. & Hedler, L. (1966) Uber das vorkommen von Nitrosoaminen in Weizenmehl. *Arzneim. Forsch., 16*, 778-779

Mass Spectrometry Data Centre (1971) Spectra nos 3829-3847, 3868-9, 5881-5897, Her Majesty's Stationery Office, London

Metcalfe, L.D. & Martin, R.J. (1972) Gas chromatography of positional isomers of long chain amines and related compounds. *Anal. Chem., 44*, 403-405

Milham, P.J., Award, A.S., Paull, R.E. & Bull, J.H. (1970) Analysis of plants, soils and waters for nitrate by using an ion-selective electrode. *Analyst, 95*, 7571-757

Miller, A., Scanlan, R.A., Lee, J.S. & Libbey, L.M. (1972) Quantitative and selective gas chromatographic analysis of dimethyl- and trimethylamine in fish. *J. Agric. Food Chem., 20*, 709-711

Mirna, A. (1974) *Determination of free and bound nitrite.* In: Krol, B.J. & Tinbergen, B.J., eds, *Proc. First Int. Symp. Nitrite Meat Prod., Zeist*, Wageningen, PUDOC, pp. 21-28

Mirna, A. & Hoffmann, K.K. (1969) Behaviour of nitrite in meat products. I. Reaction of nitrite with sulfhydryl compounds. *Fleischwirtschaft, 49*, 1361-1363

Mirvish, S., Wallcave, L., Eagen, M. & Schubik, P. (1972) Ascorbate-nitrite reaction: possible means of blocking the formation of carcinogenic *N*-nitroso compounds. *Science, 177*, 65-68

Mohler, K. (1964) Reaktionsprodukte von Nitrit und Nitrat in Fleischerzeugnissen. I. Mitteilung zur Bestimmung von Nitrit und Nitrat. *Z. Lebensm. Unters. Forsch., 125*, 337-340

Mohler, K. & Mayrhofer, L. (1968) Nachweis und Bestimmung von Nitrosaminen in Lebensmitteln. *Z. Lebensm. Unters. Forsch.* *135*, 313-318

Mottram, D.S., Patterson, R.L.S., Rhodes, D.N. & Gough, T.A. (1975) Influence of ascorbic acid and pH on the formation of *N*-nitrosodimethylamine in cured pork containing added dimethylamine. *J. Sci. Food Agric.*, *26*, 47-53

Nagata, Y. & Mirna, A. (1974) Einfluss der Verar beitung auf die Bildung von Nitrosaminen in Fleischwaren. *Fleischwirtschaft*, *54*, 1781-1786

Neurath, G. & Doerk, E. (1964) Identifizierung und quantitative Bestimmung einzelner primärer und sekundärer Amine aus Gemischen als 4-Nitro-azobenzolcarbonsäure-(4)-amide. *Chem. Ber.*, *97*, 172-178

Neurath, G. & Lüttich, W. (1968) Gas chromatographic separation of 4-Nitroazobenzene-4-Carboxamides of primary and secondary amines. *J. Chromatogr.*, *37*, 205-212

Neurath, G.B. & Schreiber, O. (1974) *Investigations on amines in the human environment.* In: Bogovski, P. & Walker, E.A., eds, *N-Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 211-214

Neurath, G., Piermann, B. & Wichern, H. (1964a) Zur Frage der *N* nitrosoverbindungen in Tabakrauch. *Beitr. Tabakforsch.*, *2*, 311-319

Neurath, G., Piermann, B.& Duenger, M. (1964b) Identifizierung von *N*-Nitroso Verbindungen und asymmetrischen Hydrazinen als 5-Nitro-2-hydroxybenzal-Derivate und Anwendung im Mikromabstab. *Ber.*, *97*, 1631-1636

Neurath, G., Dünger, M., Gewe, J., Lüttich, W. & Wichern, H. (1966a) Untersuchung der Flüchtigen Basen des Tabakrauches. *Beitr. Tabakforsch.*, *3*, 563-570

Neurath, G., Krull, A., Pirmann, B. & Wandrey, K. (1966b) Untersuchung der Flüchtigen Basen des Tabaks, II. *Beitr. Tabakforsch.*, *3*, 571-576

Neurath, G., Dünger, M., Pein, F.G., Ambrosius, D. & Schreiber, O. (1977) Primary and secondary amines in the human environment. *Food Costmet. Toxicol.*, *15*, 275-282

Newell, J.E. & Sisken, H.R. (1972) Determination of nitrosodimethylamine in the low parts per billion. *J. Agric. Food Chem.*, *20*, 711-713

Ney, K.H. & Wirotama, I.P.B. (1972) Unsubstituierte aliphatische Monocarbonsäuren und flüchtige aliphatische Monoamine im Emmentaler Käse. *Z. Lebensm. Unters. Forsch., 149*, 347-349

Nicholas, R.A. & Fox, J.B. (1973) Critical evaluation of the AOAC method of analysis for nitrite in meat. *J. Assoc. Off. Anal. Chem., 56*, 922-925

Nitsche, I., Selenka, F. & Ballschmiter, K. (1974) Zum Metabolismus von Dialkyldithiocarbamaten. I. Mitt. Bestimmung der beim Abbau entstehenden Amine durch Umsetzung mit Isocyanaten und gaschromatographische Identifizierung der gebildeten Harnstoffderivate. *J. Chromatogr., 94*, 65-73

Osborne, D.R. (1972) *Use of vacuum distillation and gas chromatography - mass spectrometry for detection of low levels of volatile nitrosamines in food.* In: Bogovski, P., Preussmann, R. & Walker, E.A., eds, *N-Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 3*), pp. 43-47

Pailer, M. & Hübsch, W.J. (1966) Bestimmung von primären und sekundären Aminen in Form von Amiden mit Hilfe der Gaschromatographie auf gepackten und Kapillarsäulen. *Monatsh. Chem., 97*, 1541-1553

Pailer, M. & Klus, H. (1971) Die Bestimmung von Nitrosaminen im Zigarettenrauchkondensat. *Fachliche Mitt. Oesterr. Tabakregie, 12*, 203-211

Pailer, M., Hübsch, W.J. & Kuhn, H. (1967) Untersuchung der aliphatischen und aromatischen primären und sekundären Amine des Zigarettenrauches mit Hilfe der Gaschromatographie und Massenspektrometrie. *Fachliche Mitt. Oesterr. Tabakregie, 7*, 109-118

Pailer, M., Vollmin, J., Karnincic, C. & Kuhn, H. (1969) Uber das Vorkommen von primären und sekundären Aminen im Zirarrenrauch. *Fachliche Mitt. Oesterr. Tabakregie, 10*, 165-168

Palframan, J.F., Macnab, J. & Crosby, N.T. (1973) An evaluation of the alkali flame ionisation detector and the Coulson electrolytic conductivity detector in the analysis of *N*-nitrosamines in foods. *J. Chromatogr., 76*, 307-319

Panalaks, T., Iyengar, J.R. & Sen, N.P. (1973) Nitrate, nitrite and dimethylnitrosamine in cured meat products. *J. Assoc. Off. Anal. Chem., 56*, 621-625

Panalaks, T., Iyengar, J.R., Donaldson, B.A., Miles, W.F. & Sen, N.P. (1974) Further survey of cured meat products for volatile *N*-nitrosamines. *J. Assoc. Off. Anal. Chem., 57*, 806-812

Patterson, R.L.S., Taylor, A.A., Mottram, D.S. & Gough, T.A. (1976) Localised occurrence of *N*-nitrosopyrrolidine in fried bacon. *J. Sci. Food Agric., 27*, 257-260

Paul, J.L. & Carlson, R.M. (1968) Nitrate determination in plant extracts by the nitrate electrode. *J. Agric. Food Chem., 16*, 766-768

Pensabene, J.W., Fiddler, W., Dooley, C.J., Doerr, R.C. & Wasserman, A.E. (1972) Spectral and gas chromatographic characteristics of some *N*-nitrosamines. *J. Agric. Food Chem., 20*, 274-277

Pensabene, J.W., Fiddler, W., Gates, R.A., Fagan, J.C. & Wasserman, A.E. (1974) Effect of frying and other cooking conditions on nitrosopyrrolidine formation in bacon. *J. Food Sci., 39*, 314-316

Poirier, R.H. & Benington, F. (1952) Reduction of *N*-nitrosodiphenylamine to *unsym*-diphenylhydrazine by lithium aluminum hydride. *J. Am. Chem. Soc., 74*, 3192

Preussmann, R., Daiber, D. & Hengy, H. (1964a) A sensitive colour reaction for nitrosamines on thin-layer chromatograms. *Nature (London), 201*, 502-503

Preussmann, R., Neurath, G., Wulf-Lorentzen, G., Daiber, D. & Hengy, H. (1964b) Anfaerbemethoden und Dünnschicht-Chromatographie von organischen *N*-Nitrosoverbindungen. *Z. Anal. Chem., 202*, 187-192

Rammell, C.G. & Joerin, M.M. (1972) Determination of nitrite in cheese and other dairy products. *J. Dairy Res., 39*, 89-94

Rao, C.N.R. (1963) *Chemical applications of infrared spectroscopy.* New York, Academic Press, chap. IV, p. 275

Rhoades, J.W. & Johnson, D.E. (1970) Gas chromatography and selective detection of *N*-nitrosamines. *J. Chromatogr. Sci., 8*, 616-617

Richardson, M. (1963) Spectrophotometric micro-estimation of some simple plant amines. *Nature (London), 197*, 290-291

Sander, J. (1967) Eine Methode zum Nachweis von Nitrosaminen. *Hoppe Seylers Z. Physiol. Chem., 348*, 852-854

Sander, J. (1971) Untersuchungen über die Entstehung Kanzerogener Nitrosoverbindungen im Magen von Versuchstieren und ihre Bedeutung für den Menschen. *Arzneim. Forsch., 21*, 1572-1580, 1707-1713, 2034-2039

Saxby, M.J. (1972) Mass spectrometry of volatile derivatives. V. *N*-dialkyl-nitrosamines. *J. Assoc. Off. Anal. Chem., 55*, 9-12

Scanlan, R.A. & Libbey, L.M. (1971) *N*-nitrosamines not identified from heat induced D-glucose/L-alanine reactions. *J. Agric. Food Chem., 19*, 570-571

Schroll, G., Cooks, R.G., Klemmensen, P. & Lawesson, S.O. (1967) The mass spectra of nitroso compounds. *Ark. Kemi, 28*, 413-422

Schueler, F.W. & Hanna, C. (1951) A synthesis of unsymmetrical dimethylhydrazine using lithium aluminum hydride. *J. Am. Chem. Soc.*, *73*, 4996

Schuller, P.L. & Veen, E. (1967) Preservatives: A review of methods of analysis. *J. Assoc. Off. Anal. Chem.*, *50*, 1127-1145

Seiler, N. & Wiechmann, M. (1965) Zum Nachweis von Aminen im 10^{-10} Mol-Maßstab. Trennung von 1-Dimethylaminonaphthalin-5-sulfonsäure-amiden auf Dünnschichtchromatogrammen. *Experientia*, *21*, 203-204

Seiler, N. & Wiechmann, M. (1967) Zur Chromatographie einiger 1-dimethylaminonaphthalin-5-sulfonyl-derivate auf Kieselgel G-Schichten. *J. Chromatogr.*, *28*, 351-362

Sen, N.P. (1970) Gas-liquid chromatographic determination of dimethyl-nitrosamine as dimethylnitramine at picogram levels. *J. Chromatogr.*, *51*, 301-304

Sen, N.P. (1972) The evidence for the presence of dimethylnitrosamine in meat products. *Food Cosmet. Toxicol.*, *10*, 219-223

Sen, N.P. (1974) *Nitrosamines.* In: Liener, I.E., ed., *Toxic Constituents of Animal Foodstuffs.* New York, Academic Press, p. 131

Sen, N.P. & Dalpe, C. (1972) A simple thin-layer chromatographic technique for the semi-quantitative determination of volatile nitrosamines in alcoholic beverages. *Analyst.*, *97*, 216-220

Sen, N.P., Smith, D.C., Schwinghamer, L. & Marleau, J.J. (1969) Diethylnitrosamine and other *N*-nitrosamines in foods. *J. Assoc. Off. Anal. Chem.*, *52*, 47-52

Sen, N.P., Smith, D.C., Schwinghamer, L. & Howsam, B. (1970) Formation of nitrosamines in nitrite-treated fish. *Can. Inst. Food Technol.*, *3*, 66-69

Sen, N.P., Schwinghamer, L.A., Donaldson, B.A. & Miles, W.F. (1972) *N*-nitrosodimethylamine in fish meal. *J. Agric. Food Chem.*, *20*, 1280-1281

Sen, N.P., Donaldson, B., Iyengar, J.R. & Panalaks, T. (1973) Nitrosopyrrolidine and dimethylnitrosamine in bacon. *Nature (London)*, *241*, 473-474

Sen, N.P., Donaldson, B.A., Charborneau, C. & Miles, W.F. (1974a) Effect of additives on the formation of nitrosamines in meat curing mixtures containing spices and nitrite. *J. Agric. Food Chem.*, *22*, 1125-1130

Sen, N.P., Iyengar, J.R., Donaldson, B.A. & Panalaks, T. (1974b) Effect of sodium nitrite concentration on the formation of nitrosopyrrolidine and dimethylnitrosamine in fried bacon. *J. Agric. Food Chem.*, *22*, 540-541

Sen, N.P., Iyengar, J.R., Donaldson, B., Panalaks, T. & Miles, W.F. (1974c) *The analysis and occurrence of volatile nitrosamines in cured meat products.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 49-52

Sen, N.P., Donaldson, B., Seaman, S., Iyengar, J.R. & Miles, W.F. (1976a) Inhibition of nitrosamine formation in fried bacon by propyl gallate and L-ascorbyl palmitate. *J. Agric. Food Chem.*, *24*, 397-401

Sen, N.P., Iyengar, J.R., Miles, W.F. & Panalaks, T. (1976b) *Nitrosamines in cured meat products.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 333-342

Serfontein, W.J. & Hurter, P. (1966a) A method for identifying small amounts of nitrosamines in biological material. *Nature (London)*, *209*, 1238-1239

Serfontein, W.J. & Hurter, P. (1966b) Nitrosamines as environmental carcinogens. II. Evidence for the presence of nitrosamines in tobacco smoke condensates. *Cancer Res.*, *26*, 575-579

Shinn, M.B. (1941) Colorimetric method for determination of nitrite. *Ind. Eng. Chem. Anal. Ed.*, *13*, 33-35

Singer, G.M. & Lijinsky, W. (1976a) Naturally occurring nitrosatable compounds. I. Secondary amines in foodstuffs. *J. Agric. Food Chem.*, *24*, 550-553

Singer, G.M. & Lijinsky, W. (1976b) Naturally occurring nitrosatable amines. II. Secondary amines in tobacco and cigarette smoke condensate. *J. Agric. Food Chem.*, *24*, 553-555

Skaare, J.V. & Dahle, H.K. (1975) Gas chromatographic determination and mass spectrometric confirmation of *N*-nitrosodimethylamine in fish meal. *J. Chromatogr.*, *111*, 426-429

Staab, H.A. & Walther, G. (1960) *N*-(trifluoroacetyl) Imidazole. *Angew. Chem.*, *72*, 35

Stainton, M.P. (1974) Simple efficient reduction column for use in the automated determination of nitrate in water. *Anal. Chem.*, *46*, 1616

Steiner, M. & Stein von Kamienski, E. (1953) Der papierchromatographische Nachweis primärer, sekundärer und tertiärer Alkylamine in Pflanzen. *Naturwissenschaften*, *40*, 483-484

Stein von Kamienski, E. (1957) Untersuchungen über die Flüchtigen Amine der Pflanzen. *Planta*, *50*, 315-330

Stephany, R. & Schuller, P.L. (1977) *How specific and sensitive is the thermal energy analyzer?* In: Tinbergen, B.J. & Krol, B., eds, *Proc. 2nd Int. Symp. Nitrite Meat Prod., Zeist*, Wageningen, PUDOC, pp. 249-255

Stephany, R.W., Freudenthal, J., Egmond, E., Gramberg, L.G. & Schuller, P.L. (1976a) Mass spectrometric quantification of traces of volatile *N*-nitrosamines in meat products. *J. Agric. Food Chem., 24*, 536-539

Stephany, R.W., Freudenthal, J. & Schuller, P.L. (1976b) *Quantitative and qualitative determination of some volatile nitrosamines in various meat products.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental N-Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 343-354

Stevens, R.K. & Hodgeson, J.A. (1973) Applications of chemiluminescent reactions to the measurement of air pollutants. *Anal. Chem., 45*, 443*R*-449*R*

Takahashi, T. (1973) Process for production of tungsten oxide catalyst. *Bull. Pat. Office* (Japan) Publication No. 20711

Taras, M.J. (1958) In: Boltz, D.F., ed., *Colorimetric determination of non metals.* New York, Interscience publishers, pp. 124-129

Teichert, K.H., Mutschler, E. & Rochelmeyer, H. (1960) Beiträge zur analytischen Chromatographie. *Dtsch. Apoth. Ztg, 100*, 283-286

Telling, G.M. (1972) A gas-liquid chromatographic procedure for the detection of volatile *N*-nitrosamines at the ten parts per billion level in foodstuffs after conversion to their corresponding nitramines. *J. Chromatogr., 73*, 79-87

Telling, G.M., Bryce, T.A. & Althorpe, J. (1971) Use of vacuum distillation and gas chromatography-mass spectrometry for determination of low levels of volatile nitrosamines in meat products. *J. Agric. Food Chem., 19*, 937-940

Telling, G.M., Bryce, T.A., Hoar, D., Osborne, D. & Welti, D. (1974) *Progress in the analysis of volatile N-nitroso compounds.* In: Bogovski, P. & Walker, E.A., eds, *N-Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 12-17

Thaler, H. & Sturm, W. (1971) Verhalten von Aminosäuren und Amiden bei der Bestimmung von Ammoniak in Lebensmitteln. *Z. Lebensm. Unters. Forsch., 145*, 263-270

Terrey, D.R. (1966) An automatic absorptionmetric method for the determination of nitrate. *Anal. Chim. Acta, 34*, 41-45

Usher, C.D. & Telling, G.M. (1975) Analysis of nitrate and nitrite in foodstuffs: a critical review. *J. Sci. Food Agric., 2*, 1793-1805

Van Ginkel, J.G. (1970) Nitrosamines in cheese. *Annu. Bull. Int. Dairy Fed., Part 6*, 45 p.

Van den Heuvel, W.J.A., Gardiner, W.L. & Horning, E.C. (1964) Characterization and separation of amines by gas chromatography. *Anal. Chem., 36*, 1550

Von Rappardt, E., Eisenbrand, G. & Preussmann, R. (1976) Selective detection of *N*-nitrosamines by gas chromatography using a modified microelectrolytic conductivity detector in the pyrolytic mode. *J. Chromatogr., 124*, 247-255

Voogt, P. (1969) Die Bestimmung von Nitrat im Spinatmittels einer Nitratselektiven Elektrode. *Dtsch. Lebensm. Rundsch., 65*, 196-198

Walker, E.A. & Castegnaro, M. (1974) *A report of the present status of a collaborative study of methods for the trace analysis of volatile nitrosamines.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 57-62

Walker, E.A. & Castegnaro, M. (1976) *New data on collaborative studies on analysis of volatile nitrosamines.* In: Walker, E.A., Bogovski, P. & Griciute, L., eds, *Environmental* N-*Nitroso Compounds: Analysis and Formation*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 14*), pp. 77-83

Walker, E.A., Castegnaro, M. & Pignatelli, B. (1975) Use of a clean-up method to improve specificity in the analysis of foodstuffs for volatile nitrosamines. *Analyst, 100*, 817-821

Walle, T. & Ehrsson, H. (1970) Quantitative gas chromatographic determination of picogram quantities of amino and alcoholic compounds by electron capture detection. I. Preparation and properties of the heptafluorobutyryl derivatives. *Acta Pharm. Suec., 7*, 389-406

Walters, C.L., Johnson, E.M. & Roy, N. (1970) Separation and detection of volatile and non-volatile *N*-nitrosamines. *Analyst, 95*, 485-489

Walters, C.L., Newton, B.E., Parke, D.V. & Walker, R. (1974) *The precursors of* N-*nitroso compounds in foods.* In: Bogovski, P. & Walker, E.A., eds, N-*Nitroso Compounds in the Environment*, Lyon, International Agency for Research on Cancer (*IARC Scientific Publications No. 9*), pp. 223-228

Warthesen, J.J., Scanlan, R.A., Bills, D.D. & Libbey, L.M. (1975) Formation of heterocyclic *N*-nitrosamines from the reaction of nitrite and selected primary diamines and amino acids. *J. Agric. Food Chem., 23*, 898-902

Wasserman, A.E. (1974) *Trace nitrosamine analysis.* In: Morton, I. & Rhodes, D.N., eds, *The Contribution of Chemistry to Food Supplies*, International Union of Pure and Applied Chemistry and International Union of Food Science and Technology, London, Butterworths, pp. 227-246

Wasserman, A.E., Fiddler, W., Doerr, R.C., Osman, S.F. & Dooley, C.J. (1972) Dimethylnitrosamine in Frankfurters. *Food Cosmet. Toxicol., 10*, 681-684

Westcoot, C.C. (1971) A simplified method for determining nitrates in food. *Food Technol., 25*, 49-50

Williams, A.A., Timberlake, C.F., Tucknott, O.G. & Patterson, R.L.S. (1971) Observations on the detection of dimethyl- and diethyl-nitrosamines in foods with particular reference to alcoholic beverages. *J. Sci. Food Agric., 22*, 431-434

Yang, K.W. & Brown, E.V. (1972) A sensitive analysis for nitrosamines. *Anal. Lett., 5*, 293-304

Young, J.C. (1976) Detection and determination of *N*-nitrosamines by thin-layer chromatography using fluorescamine. *J. Chromatogr., 124*, 17-28

METHODS OF ANALYSIS

It is clear from the preceding sections that there are numerous approaches to the analysis of volatile nitrosamines. Even within such a limited area as sample extraction, different techniques have been employed, each of which could affect the choice of method for clean-up and even for final detection. As attention to detail is essential if results are to be reproducible, the selected methods which follow have been presented precisely as they were applied. No attempt has been made to combine similar methods, since the combination would have to be checked experimentally. Even apparently minor modifications of analytical procedures may lead to unexpected difficulties.

The methods were selected as generally representative of those shown to be reliable in the IARC collaborative studies. They are also illustrative of techniques in use in a number of countries and reflect instrumentation and materials available. The present selection, however, does not imply that these are the only useful methods. Collaborative studies will continue and may lead to the adoption of standardized methods. In any case, the manual will be brought up to date when necessary.

METHOD 1 – ANALYSIS OF VOLATILE *N*-NITROSAMINES IN FOOD USING FLAME THERMIONIC DETECTION AND MASS SPECTROMETRY CONFIRMATION

T. FAZIO, J.W. HOWARD & D. HAVERY

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of volatile *N*-nitrosamines in cold cuts, sausages, infant foods, canned meats, bacon, hams and other pork products, miscellaneous beef products, fish, chicken, spices, dried blood, vegetable oils and cheeses.

The method has a limit of detection of 10 micrograms per kilogram (µg/kg) in a 25 g sample. The confirmation by mass spectrometry (MS) requires 0.1 µg *N*-nitrosamine

2. REFERENCES

Fazio, T., Howard, J.W. & White, R. (1972) *IARC Scientific Publications No. 3*, 16-24

Howard, J.W., Fazio, T. & Watts, J.O. (1970) *J. Assoc. Off. Anal. Chem.*, *53*, 269-274

3. DEFINITION

Volatile *N*-nitrosamine contents of meat, cheese and other products: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

The analytical scheme includes an initial digestion of the food in methanolic potassium hydroxide; liquid-liquid extraction of the *N*-nitrosamines in dichloromethane; transfer of the *N*-nitrosamines to an alkaline solution by distillation of the dichloromethane, followed by distillation of the *N*-nitrosamines from the aqueous solution; acidification of the distillate; extraction of the *N*-nitrosamines

from the acid solution with dichloromethane; acid and alkaline washing of the extract; concentration of the washed extract; column chromatography on silica gel, concentration of the eluate containing *N*-nitrosamines; gas-chromatography (GC) with a modified thermionic detector. Confirmation requires further clean-up by column chromatography on an acid Celite column and GC with the modified thermionic detector, followed by verification of the identity of the suspected *N*-nitrosamine by mass spectrometry (MS).

5. HAZARDS

N-nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

Methanol	Analytical grade
Dichloromethane	Burdick & Jackson "Distilled in Glass"
n-Pentane	Burdick & Jackson "Distilled in Glass"
Diethyl ether	E-138, Fisher Scientific Co. or equivalent
Perfluorokerosene (PFK)	As supplied for mass spectrometry
Perfluorotributylamine	As supplied for mass spectrometry

---

[1] Steere, N.V. (1974) Safety in the Chemical Laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] *National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens.* Department of Health, Education and Welfare Publication (NIH), 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Potassium hydroxide	Analytical grade
Silicon carbide grains	Fisher "Boileezers" B-365
Calcium sulfate anhydrous	Hammond Drierite Co. (10-20 mesh)
Barium hydroxide	Analytical grade
Sodium sulfate	Analytical grade - anhydrous granular
Silica gel	0.05-0.2 mm for chromatography, 70-325 mesh (E 7734, Merck or equivalent)
Celite 545	NAW, Fisher Scientific or equivalent (heat in muffle furnace overnight at 700°C)
Nitrogen	Ultra high purity
Helium	Ultra high purity
Sodium hydroxide 5N	
Hydrochloric acid 6N	
N-nitrosamine standard solutions	For each *N*-nitrosamine a standard solution (A) 2 g/l is prepared in dichloromethane and diluted to 100 mg/l (B). Solution (C) is then prepared in dichloromethane to contain all the *N*-nitrosamines of interest, each at 0.5 mg/l. All standard solutions are stored in a refrigerator at 11°C and renewed periodically depending on their response to GC analysis (approximately at 2 year intervals).

NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material, a test must be performed using the procedure described in section 9, but omitting the sample.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

Liquid-liquid extractor	Kontes (K-001596-175, or equivalent

[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Evaporative concentrator (K-D)	Kuderna-Danish, 250 and 500 ml capacity with 24/40 column connection and 19/22 lower joint (K-570001). Concentrator tube - size 425, 19/22 joint, 4 ml capacity with 0.1 ml subdivisions from 0 to 2 (K-570050) and 19/22 pennyhead stopper (K-850500) calibrated before use. Distillation column - Snyder, 24/40 joints, 3 sections (6630-02, Ace Glass, Inc., Vineland, N.J., or equivalent)
Funnel-Buchner	60 ml capacity with coarse porosity disk (K-420280)
Chromatographic column	Chromaflex, size 222, 14.5 mm i.d. x 250 mm (K-420280)

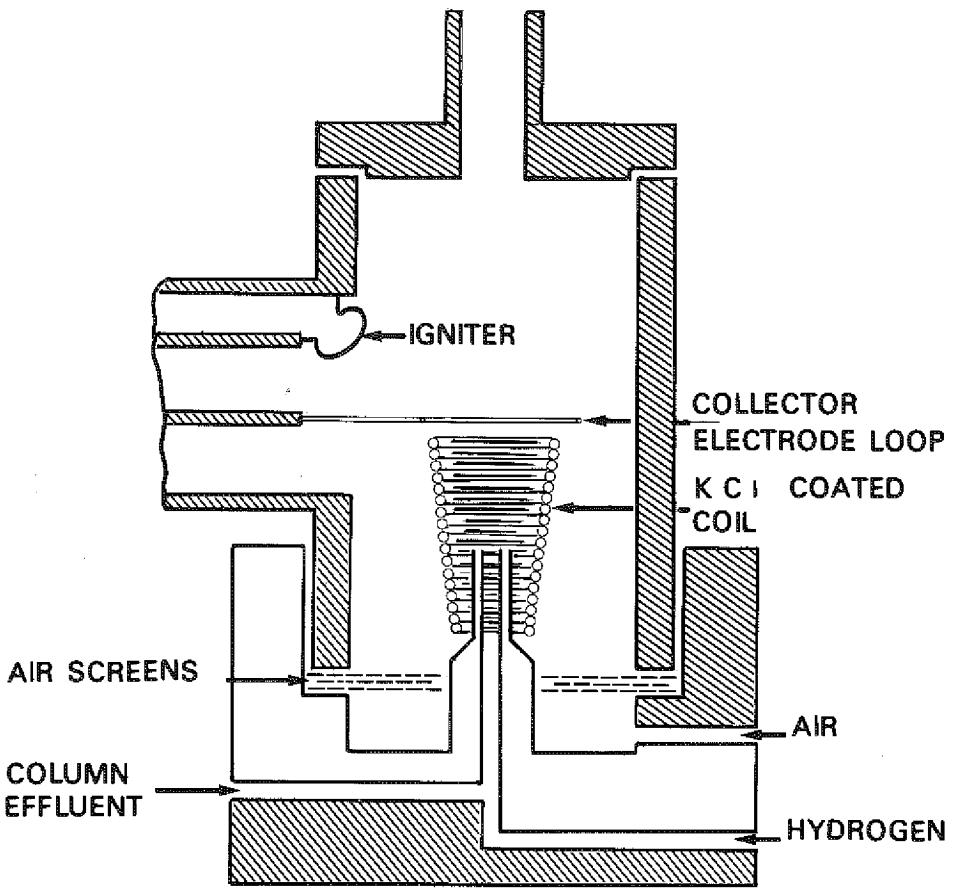
(Glassware catalogue numbers refers to Kontes Glass Co., Vineland, NJ 08360, USA, unless otherwise stated)

Gas chromatograph	Barber-Coleman model 5000, or equivalent, fitted with a modified KCl thermionic detector (see Fig. 1).
Mass spectrometer A	Varian MAT CH-4B interfaced to a Varian 1740 GC using a double stage Llewellyn separator, with automatic electronic gating for removal of the solvent, and a splitter allowing 1 part to be detected with the flame-ionization detector and 10 parts to go through the gating valve into the mass spectrometer.
Data system A	Varian data machines V-76 with 65K words of semiconductor memory, a 4.5M word disc, two 800 BPI 9 track tape drives, an ASR-33, a Statos 21 and a Tektronix 4010 display terminal.
Mass spectrometer B	Finnigan 3300 F electron-impact quadrupole mass spectrometer, interfaced to a Finnigan 9500 GC through a Gohlke all-glass jet separator.
Data system B	Finnigan model 6100.

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and changes in composition are prevented (see Chapter 1, Sampling and Samples).

FIG. 1. MODIFIED THERMIONIC DETECTOR



9. PROCEDURE

NOTE: *N*-nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Digestion*

9.1.1 Weigh 100 g of representative sample and place in 1000 ml round-bottomed flask.

9.1.2 Add 200 ml methanol, 15 g potassium hydroxide and silicon carbide grains. Shake to mix.

9.1.3 Place in heating mantle, insert condenser, reflux at a slow rate initially and gradually increase to a rapid rate for 2 hours.

9.1.4 Cool to room temperature and filter through a fluted filter paper or wad of glass wool into a 500 ml glass stoppered flask.

9.1.5 Take two separate aliquots of filtered digest (9.1.4), each equivalent to 25 g of sample. For determination of recovery, add 0.5 ml of standard *N*-nitrosamines solution C to one of the aliquots. Treat each aliquot separately according to instructions 9.2.1 to 9.6.9.

9.2 *Liquid-liquid extraction of digest*

9.2.1 Place wad of glass wool in bottom of liquid-liquid extractor, over aperture to siphon arm. Add 25 g anhydrous calcium sulfate and 100 ml purified dichloromethane.

9.2.2 Attach 250 ml boiling flask, containing 50 ml purified dichloromethane and silicon carbide grains, to siphon arm of extractor.

9.2.3 Add 75 ml of distilled water to extractor, followed by one aliquot of sample filtrate (9.1.5), and finally rinse aliquot container with 50 ml water and add to extractor.

9.2.4 Fit dispersion tube, cooled coiled water condenser and heating mantle.

9.2.5 Extract for 3 hours.

9.3 *Distillation of liquid-liquid extract*

9.3.1 Remove 250 ml boiling flask containing dichloromethane extract 9.2.5.
Add 75 ml 5 N sodium hydroxide and 4 g barium hydroxide.

9.3.2 Connect distillation head, condenser and adapter.

9.3.3 Carefully distil off dichloromethane and discard.

9.3.4 Increase variable heat control and collect 70 ml of aqueous distillate in 250 ml separation funnel, fitted with a 30 mm diameter glass funnel containing a small pad of glass wool.

9.4 *Solvent extraction of distillate*

9.4.1 To the distillate (9.3.4) add 10 ml of 6 N hydrochloric acid, 70 ml of purified dichloromethane and shake for 3 min. Separate organic and aqueous phases.

9.4.2 Repeat dichloromethane extraction twice.

9.4.3 Combine dichloromethane extracts in 500 ml separation funnel.

9.4.4 Add 50 ml 6 N hydrochloric acid and shake for 1 min.

9.4.5 Draw off lower layer into 500 ml separation funnel containing 50 ml 5 N sodium hydroxide.

9.4.6 Wash aqueous hydrochloric acid layer twice with 25 ml dichloromethane by shaking 2 min.

9.4.7 Add each wash to dichloromethane extract (9.4.5).

9.4.8 Shake funnel containing sodium hydroxide-dichloromethane.

9.4.9 Allow layers to separate.

9.4.10 Draw off lower layer and pass through 35 g anhydrous sodium sulfate (held in 60 ml coarse sintered-glass funnel and pre-washed with 25 ml dichloromethane) into 500 ml K-D evaporator. Wash anhydrous sodium sulfate with 25 ml dichloromethane and add to K-D.

9.4.11 Wash alkaline layer from 9.4.9 with 25 ml dichloromethane by shaking 30 sec and pass the dichloromethane through sodium sulfate. Add to K-D.

9.5 *Concentration of extract*

9.5.1 Add silicon carbide grains, attach 3-section Snyder column and carefully concentrate to approximately 4 ml on a steam bath.

9.5.2 Remove apparatus from bath, cool and drain any remaining solvent in distilling column and concentrator into concentrator tube.

9.5.3 Carefully concentrate solution in concentrator tube to 1 ml under gentle stream of nitrogen.

9.6 *Column chromatographic clean-up of concentrate*

9.6.1 Add 5 g of silica gel to chromatographic column containing a glass wool plug and pack with gentle tapping.

9.6.2 Place a small piece of glass wool on top.

9.6.3 Wash column with 50 ml of *n*-pentane.

9.6.4 Stop flow when liquid is approximately 1 mm above the adsorbent.

9.6.5 Quantitatively transfer the 1 ml dichloromethane extract from 9.5.3, using approximately 20 ml *n*-pentane in small portions (4 x 5 ml).

9.6.6 Allow extract and each rinse to flow into column, stopping when the level of liquid is 1 mm above adsorbent.

9.6.7 Wash column with 200 ml 25% dichloromethane in *n*-pentane (flow rate approximately 60 drops/min) and discard.

9.6.8 Elute with 125 ml 30% anhydrous diethyl ether in dichloromethane at the above flow rate.

9.6.9 Collect eluate in 250 ml K-D and concentrate to 0.50 ml as described above (9.5). Stopper; retain for GC analysis.

9.7 *GC analysis*

9.7.1 GC conditions:

Column: Glass packed with 10% Carbowax 1540 + 5% potassium hydroxide on 100/120 mesh Chromosorb W/HP
Length: 3 m I.D.: 4 mm

Carrier gas: Argon, 70 ml/min

Column temperature: programmed 80–180°C at 5°C/min

Injection port temperature: 200°C

Detector temperature: 205°C

Hydrogen: 40 ml/min

Air: 400 ml/min

Electrometer setting: 100 (4x) equivalent to 4×10^{-9} A full scale deflection.

9.7.2 GC procedure:

9.7.2.1 Inject 8 µl *N*-nitrosamine standard solution C into GC and carry out chromatographic analysis.

9.7.2.2 Using the same conditions, inject 8 µl concentrated sample solution (9.6.9) and carry out chromatographic analysis.

9.7.2.3 When a sample shows peak(s) for *N*-nitrosamine(s), check identity by MS.

9.8 *GC-MS confirmation*

9.8.1 Column chromatographic clean-up of concentrate 9.6.9 on acid Celite:

9.8.1.1 Add 10 ml 6 N hydrochloric acid in approximately 3 ml portions to 6 g Celite with vigorous stirring.

9.8.1.2 Pack mixture into 22 x 300 mm chromatographic column on top of glass wool plug and compress to approximately 5 cm.

9.8.1.3 Place small glass wool plug on top of adsorbent.

9.8.1.4 Quantitatively transfer remaining sample concentrate (9.6.9) to acid Celite column, using 20 ml of *n*-pentane in 5 ml portions and collecting *n*-pentane wash in 500 ml K-D.

9.8.1.5 After column has drained, add 180 ml *n*-pentane, again draining column completely into the K-D.

9.8.1.6 Add 50 ml dichloromethane and collect eluate in a separate 250 ml K-D.

9.8.1.7 Concentrate each eluate (*n*-pentane and dichloromethane) to 0.50 ml as previously described (9.5).

9.8.2 Obtain GC data as described above (9.7.1-9.7.2).

9.8.3 Further concentrate the above eluates (from 9.8.1.7) to approximately 0.1 ml under a gentle stream of nitrogen. Stopper and retain for GC/MS confirmation.

9.8.4 GC-MS (method A)

9.8.4.1 Mass spectrometer A operating conditions:

Resolution: 600
Electron energy: 70eV
Emission: 100 µA
Accelerating voltage: 3 kV
Source and interface connecting tube temperatures: 180°C
GC/MS interface temperature: 80°C.

9.8.4.2 GC conditions:

Column: 10% Carbowax 20 M + 5% potassium hydroxide on 100/120 mesh Chromosorb W/HP
Length: 1.9 m I.D.: 4 mm
Carrier gas: Helium, 20 ml/min
Column temperature: isothermal 190°C
Injection port temperature: 200°C.

9.8.4.3 GC-MS procedure

9.8.4.3.1 The MS and data system are interconnected with the sequential switching unit and, with both systems operating under normal conditions, PFK is introduced into the MS to calibrate the mass scale of the data system.

9.8.4.3.2 One of two possible GC operating conditions are chosen:
(a) if the sample contains NDMA and/or *N*-nitrosoethylmethylamine (NEMA), together with any other *N*-nitrosamines, a programmed GC run is used for the standard sample; 80-200°C at 6°C/min.
(b) if the sample does not contain NDMA and/or NEMA, then the GC is run isothermally at 190°C.

9.8.4.3.3 5 µl of the appropriate standard solution(s) B of the *N*-nitrosamine(s) to be analysed is injected into the GC (500 ng of each component on column) and, after the solvent has been vented, the electronically controlled gating valve is opened.

9.8.4.3.4 The MS is put into cyclic scan and all data is collected by the data system.

9.8.4.3.5 Reconstructed mass spectrum-chromatogram for characteristic fragments is plotted and background-substracted spectra made where the ion current tracing for these fragments was at maximum.

9.8.4.3.6 The sample of interest is then run on the GC/MS/data system under conditions identical to those used for the standard. The injection volume is usually between 20 and 30 µl for the sample. The response observed for the sample must be consistent in all respects with that of the standard run, to be sure of the identity of the *N*-nitrosamine.

9.8.5 GC-MS (method B)

9.8.5.1 Mass spectrometer B operating conditions:

Resolution: 1000
Electron Energy: 70 eV
Emission: 500 µA
Separator and transfer line temperature: 200°C
Manifold temperature: 60°C.

9.8.5.2 GC conditions:
Column: 10% carbowax 20 M + 5% potassium hydroxide on 100/120 mesh Chromosorb W/HP
Length: 1.9 m I.D.: 4 mm
Carrier gas: Helium, 25 ml/min
Column temperature: isothermal 190°C or temperature programmed as in 9.8.5.3.2
Injection port temperature: 200°C.

9.8.5.3 GC-MS procedure:

9.8.5.3.1 The MS/data system is calibrated by introducing perfluorotributylamine into MS under normal operating conditions.

9.8.5.3.2 One of two possible GC operating conditions are chosen:
(a) if the sample contains NDMA and/or NEMA, together with any other *N*-nitrosamines, a programmed GC run is used for the standard sample; 80-200°C at 6°C/min.
(b) if the sample does not contain NDMA and/or NEMA, the GC is run isothermally at 190°C.

9.8.5.3.3 1 µl of the appropriate standard solution B of the *N*-nitrosamine(s) to be analysed is injected into the GC (100 ng of each component on column) and, after the solvent has been vented, the column effluent is allowed to enter the MS.

9.8.5.3.4 The MS is repeatedly scanned under computer control and all data is stored by the data system.

9.8.5.3.5 Approximately one minute after the last *N*-nitrosamine has been eluted, data acquisition is terminated and the GC effluent is again diverted away from the MS.

9.8.5.3.6 Ion profiles are extracted from the data to determine the elution times of *N*-nitrosamines in the sample. For example, in the case of *N*-nitrosopyrrolidine, the intensities of m/e 30, 41, 43, 69 and 100 are plotted as function of spectrum number. A maximum in all these ion profiles at the same spectrum number may indicate the presence of *N*-nitrosopyrrolidine.

A background-subtracted plot of this spectrum is then compared to the spectrum of *N*-nitrosopyrrolidine under the same analytical conditions.

10. METHOD OF CALCULATION

V = Total sample volume = 0.50 ml
X = Peak height of standard (9.7.2.1)
Y = Peak height of sample (9.7.2.2)
C = Concentration of standard = 0.5 ng/µl
W = Peak height of spiked sample (9.9.4)
Z = *N*-nitrosamine content of sample

Thus,

$$Z = \frac{YVC}{W-Y} = \frac{250Y}{W-Y} \text{ ng/25 g} = \frac{10Y}{W-Y} \text{ µg/kg or ppb}$$

and

$$\% \text{ recovery} = 100\left(\frac{W - Y}{X}\right)$$

As this value does not vary widely, recovery tests are made every 10-15 samples.

NOTE: If analysis of spiked sample is not carried out, an approximate result (lower limit) may be obtained from

$$Z = \frac{YVC}{X} = \frac{10\ Y}{X} \text{ µg/kg or ppb.}$$

11. REPEATABILITY AND REPRODUCIBILITY

11.1 *Repeatability*

At the 10 µg/kg level, the repeatability of this method is ± 6%.

11.2 *Reproducibility*

No data available.

12. SCHEMATIC REPRESENTATION OF PROCEDURE

Sample
↓
Digestion in methanol-KOH
↓
Filtration
↓
Liquid-liquid extraction of filtrate with CH_2Cl_2
↓
Add 75 ml 5 N NaOH, $Ba(OH)_2$ solution
↓
Distil

CH_2Cl_2 layer discarded

Collect 70 ml aqueous distillate
↓
Add 10 ml 6 N HCl
↓
Extract 3 times by CH_2Cl_2 (3 x 70 ml)
↓
Wash combined CH_2Cl_2 by 6 N HCl
↓
Wash combined CH_2Cl_2 by 5 N NaOH
↓
Re-extract NaOH layer by CH_2Cl_2; combine CH_2Cl_2 extracts
↓
Dry over Na_2SO_4
↓
Concentrate in K-D with Snyder column to 4 ml
↓
Concentrate to 1 ml under nitrogen stream
↓
Column chromatography clean-up on silica gel (5 g)
Wash column with 200 ml CH_2CL_2-*n*-pentane (25-75)
Elute with 125 ml diethyl ether-CH_2Cl_2 (30-70)
↓
Recover diethyl ether-CH_2Cl_2 in K-D
↓
Concentrate to 0.50 ml
↓
GC screening using thermionic detector

Positive results are checked as follows:

Column chromatography on acid Celite
Elution by *n*-pentane (200 ml)
then by CH_2Cl_2 (50 ml)
↓
Recover separately *n*-pentane and CH_2Cl_2 in K-D
↓
Concentrate each eluate to 0.5 ml → GC screening
↓
Further concentrate each eluate to 0.1 ml (N_2 stream)
↓
GC-MS confirmation.

13. ORIGIN OF THE METHOD

Department of Health, Education and Welfare
Public Health Service
Food and Drug Administration
Washington DC 20204
USA

Contact point: Dr D.C. Havery.

METHOD 2 – ANALYSIS OF VOLATILE *N*-NITROSAMINES IN FOOD USING FLAME THERMIONIC DETECTION AND MASS SPECTROMETRY CONFIRMATION

T. KAWABATA, M. NAKAMURA, M. MATSUI & T. ISHIBASHI

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of volatile *N*-nitrosamines in food. The method is particularly suitable for the determination of *N*-nitrosodimethylamine (NDMA) content of various fish products and has a limit of detection of 1 microgram/kilogram (µg/kg) in a 100 g sample (0.1 ng of NDMA). The confirmation by mass spectrometry (MS) requires 4 to 10 ng of *N*-nitrosamine.

2. REFERENCES

Kawabata, T., Matsui, M., Ishibashi, T. & Nakamura, M. (1972) *Jpn. Analyst, 21*, 1326-1332

Kawabata, T., Nakamura, M., Matsui, M. & Ishibashi, T. (1974) *Bull. Jpn. Soc. Sci. Fish., 40*, 79-85

Kawabata, T., Nakamura, M., Matsui, M. & Ishibashi, T. (1974) *Bull. Jpn. Soc. Sci. Fish., 40*, 87-96

Kawabata, T. (1974) *IARC Scientific Publications No. 9*, 154-158

3. DEFINITION

Volatile *N*-nitrosamine contents of foods: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

N-Nitrosamines are extracted from an alkaline slurry of the sample with dichloromethane in a blender. They are then transfered to a

barium hydroxide-sodium chloride solution and steam distilled. The distillate is purified using one of the two following methods: (a) ion-exchange chromatography on Amberlite CG 120 and extraction of *N*-nitrosamines from the eluate into dichloromethane; (b) extraction of *N*-nitrosamines from the distillate into dichloromethane and column chromatography on acid-treated Celite 545. The purified extract is concentrated and analysed by gas-chromatography (GC), using flame thermionic detection. The presence of *N*-nitrosamines is confirmed by MS.

5. HAZARDS

N-nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

Dichloromethane	Wako Pure Chemicals, certified for pesticide residue analysis
n-Pentane	Wako Pure Chemicals, analytical grade
Potassium carbonate	Wako Pure Chemicals, analytical grade

---

[1] Steere, N.V. (1974) Safety in the Chemical Laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] *National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens.* Department of Health, Education and Welfare Publication (NIH), 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Sodium chloride	Wako Pure Chemicals, analytical grade
Barium hydroxide	Wako Pure Chemicals, analytical grade
Sodium sulfate, anhydrous	Wako Pure Chemicals, analytical grade
Potassium di-hydrogen-phosphate	Wako Pure Chemicals, analytical grade
Di-sodium hydrogen-phosphate	Wako Pure Chemicals, analytical grade
Ion-exchange resin	Amberlite CG-120, type 1 (100-200 mesh). The resin is immersed in water, treated twice with 2 N hydrochloric acid, washed till neutral, then buffered twice with 20-25 ml of phosphate buffer (pH 6.5).
Celite 545	Johns Manville, heated at 700°C for 24 hours, and stored in a desiccator before use. Acid-treated Celite is obtained by adding 4 ml 6 N hydrochloric acid to 6 g Celite just before use.
Hydrochloric acid (6N & 2N)	
Phosphate-buffer solution (pH 6.5)	
N-Nitrosamine standard solutions	Standard solution A: A stock standard solution in dichloromethane is prepared containing 1 g/l of each of the *N*-nitrosamines of interest and diluted as appropriate (9.3.2.1). Standard solution B: A stock internal standard solution of NDi-BA in dichloro-methane (B) is prepared containing 1 g/l and diluted as appropriate (9.2.1.8, 9.3.2.2). Standard solutions are stored in a refrigerator at 4°C in brown bottles with teflon-lined caps. They are renewed every two months.

NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material a test must be performed using the procedure described in section 9, but omitting the sample.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

Blender	Nippon Seiki & Co., 500 ml stainless steel cup (maximum speed 20,000 rpm)
Buchner-Funnel	60 ml capacity with course porosity disc.
Evaporative concentrator (K-D)	Kuderna-Danish flask - 500 ml, with 4 ml graduated concentrator tube. Snyder, 3 sections, distillation column
Rotary evaporator	Nakajima Seisakusho fitted with 500 ml flask with 4 ml graduated tube attached and connected to an aspirator.
Clean-up column	Fritted glass funnel 2.5 x 2.4 cm I.D. (for ion exchange) 25 mm I.D. x 20 cm glass chromatographic column with fritted glass disc (for celite).
Gas chromatograph	Shimadzu GC-5 APF equipped with an alkali flame-ionization detector (KBr mono-crystal on detector tip)
Gas chromatograph -mass spectrometer	Shimadzu LKB-9000 GC-MS GC detector: total ion monitor.

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented (see Chapter 1, Sampling and Samples).

9. PROCEDURE

NOTE: *N*-Nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Extraction and steam distillation*

9.1.1 Weigh 100 g of representative sample into a 500 ml stainless-steel blender cup.

---

[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

9.1.2 Add 200 ml dichloromethane and 5 g potassium carbonate. Blend for 4 min at high speed.

9.1.3 Filter the dichloromethane extract through the Buchner funnel or, alternatively, separate the extract by centrifugation (1500 g).

9.1.4 Repeat extraction twice with 200 ml dichloromethane.

9.1.5 Evaporate the combined dichloromethane extracts to about 20-30 ml using an aspirated rotary evaporator (see Note on Procedure, section 13.1) or K-D.

9.1.6 Transfer the concentrated extract into a 500 ml boiling flask.

9.1.7 Add 20 g barium hydroxide, 20 g sodium chloride and 100 ml distilled water.

9.1.8 Connect distillation head, condenser and receiver.

9.1.9 Steam distill and collect about 150 ml of aqueous distillate for all *N*-nitrosamines except *N*-nitrosopyrrolidine (NPYR) or 300 ml (for NPYR).

9.2 *Column chromatographic clean-up*

The aqueous distillate is cleaned up in one of the following two ways, depending on the nature of the sample (see Note on Procedure, section 13.2).

9.2.1 Using ion exchange column:

9.2.1.1 Remove dichloromethane from distillate (9.1.9) by placing the flask on a water bath at 40-50°C and blowing nitrogen through capillary tube until only aqueous distillate remains.

9.2.1.2 Adjust pH of the aqueous distillate to 6.5 using phosphate buffer solution.

9.2.1.3 Pass the distillate through an ion-exchange resin buffered at pH 6.5 and held in a fritted glass funnel.

9.2.1.4 Collect effluent in a 500 ml separation funnel.

9.2.1.5 Add 150 ml dichloromethane and shake for 2 min.

9.2.1.6 Separate lower layer and repeat dichloromethane extraction.

9.2.1.7 Pass combined dichloromethane extracts through 35 g anhydrous sodium sulfate (pre-wet with dichloromethane) held in a fritted glass funnel, and collect in 500 ml K-D.

9.2.1.8 Add 1 ml NDi-BA internal standard (1 mg/l in dichloro dichloromethane.

9.2.1.9 Concentrate to 1 ml. Stopper, retain for GC analysis.

9.2.2 Using Celite column:

9.2.2.1 Extract the aqueous distillate (9.1.9) with 2 portions 150 ml dichloromethane.

9.2.2.2 Pass combined dichloromethane extracts through 35 g anhydrous sodium sulfate (pre-wet with dichloromethane), held in a fritted glass funnel, into a 500 ml K-D or aspirated rotary evaporator with a 4 ml concentrator tube attached.

9.2.2.3 Evaporate to about 1 ml with either K-D or rotary evaporator

9.2.2.4 Add 6 g of acid treated Celite to the chromatographic column and pack with gentle tapping to give a column height of about 50 mm.

9.2.2.5 Transfer the concentrated extract to the chromatographic column.

9.2.2.6 Wash the column with 200 ml *n*-pentane and discard eluate.

9.2.2.7 Elute the *N*-nitrosamines from the column with 50 ml dichloromethane.

9.2.2.8 Add 1 ml of NDi-BA standard solution (1 mg/l) to the eluate.

9.2.2.9 Concentrate to about 1 ml in K-D or rotary evaporator.

9.3 *GC analysis*

9.3.1 GC conditions:

Either of the following two columns may be employed.

Column: (a) glass packed with 25% PEG 6000 on chromosorb W (60-80 mesh).
Length: 2.5 m I.D.: 3 mm

(b) glass packed with 20% Versamid 900 on Chromosorb W (60-80 mesh)
Length: 2.5 m I.D.: 3 mm

Carrier gas: nitrogen, 40 ml/min for the PEG column and 60 ml/min for the Versamid column.

Column temperature: isothermal 140°C

Injection port temperature: 180°C

Detector temperature: 180°C

Hydrogen: 30 ml/min

Air: 800 ml/min

9.3.2 Preparation of calibration curves:

9.3.2.1 Using standard solution A, prepare a series of diluted standards containing each *N*-nitrosamine at 1, 2, 3 and 4 mg/l respectively.

9.3.2.2 Using standard solution B, prepare a dilute internal standard containing 2 mg/l NDi-BA.

9.3.2.3 To 1 ml of each dilute standard from 9.3.2.1, add 1 ml of dilute internal standard from 9.3.2.2.

9.3.2.4 Carry of GC analysis of each dilluted standard from 9.3.2.3, using a 5 µl injection.

9.3.2.5 Repeat GC analysis 5 times in all for each member of the dilution series.

9.3.2.6 Measure peak areas for each *N*-nitrosamine on the gas chromatogram and calculate the ratio of the peak area of each *N*-nitrosamine to that of the internal standard. For each *N*-nitrosamine, plot calibration curves of peak area ratio (*N*-nitrosamine/internal standard) *versus* weight ratio (*N*-nitrosamine/internal standard) using average values from the five analyses.

9.3.3 GC procedure:

Inject 5 µl of the concentrated distillate (9.2.1.9 or 9.2.2.9) into GC. When a sample shows *N*-nitrosamine peak, check its identity by MS.

9.4 *GC-MS confirmation*

9.4.1 GC conditions:

Either of the following two columns may be employed:

Column: (a) glass packed with 25% PEG 6000 on Chromosorb W (60-80 mesh)
Length: 3 m I.D.: 3 mm

(b) glass packed with 20% Versamid 900 on Chromosorb W (60-80 mesh)
Length: 3 m I.D.: 3 mm

Carrier gas: Helium, 30 ml/min

Column temperature: Isothermal 130°C or programmed 60-160°C at 4°C/min

Injection port temperature: 200°C

Separator temperature: 250°C

9.4.2 MS conditions:

Electron energy: 70 eV

Accelerating voltage: 8 Kv

Resolution: 1200

A nitrosamine is confirmed when the molecular ion (M^+) given below is detected.

N-Nitrosamine	Molecular ion peak (M^+) m/e
NDMA	74
NDEA	102
NDPA	130
NDBA	158
NPYR	100
NPIP	114

10. METHOD OF CALCULATION

For the quantification of *N*-nitrosamines in an unknown sample, the method of calculation is as follows:

(a) measure area of *N*-nitrosamine peak and of the internal standard peak;

(b) calculate ratio of areas of *N*-nitrosamine peak to internal standard peak;

(c) obtain, from the calibration curve, the weight ratio (A) corresponding to the peak area ratio of the *N*-nitrosamine in the unknown sample.

$$Z = A \times \frac{W'}{W}$$

where Z = concentration
A = weight ratio of the *N*-nitrosamine to the internal standard
W' = weight of internal standard added to the unknown sample (1 µg)
W = weight of sample (100 g)

therefore:

Z = (A/100) µg/100 g
= (A/10) µg/kg

NOTE: The value for Z may be corrected using an average value for recovery for a given type of sample (see 12.1).

11. REPEATABILITY AND REPRODUCIBILITY

11.1 *Repeatability*

For NDMA, the coefficient of variation at the 1 µg/kg level is 8%.

11.2 *Reproducibility*

No data available.

12. RECOVERIES

12.1 *Recovery test of NDMA added to fish-ham samples*

Amount of NDMA added	Recovery of NDMA (%)			Average
0 ppb (control)	n.d.			
10 ppb	61.3	64.1	61.3	62.2
25 ppb	61.6	56.0	63.8	60.5
50 ppb	58.3	56.3	58.0	57.5

n.d.: not detected (less than 1 ppb). Size of sample analysed: 100 g.

13. NOTE ON PROCEDURE

13.1 To obtain reduced pressure in the rotary evaporator an aspirator is employed. The temperature of the bath is 25-30°C.

13.2 Clean-up method 9.2.1 is preferably applied for nitrite-treated and salted fish raw products and fish eggs, while clean-up method 9.2.2 is mainly applied to fish ham, fish sausage and cured meat products, such as ham, sausage and bacon.

14. SCHEMATIC REPRESENTATION OF THE PROCEDURE

Sample (100 g)
↓
Add K_2CO_3 (5 g) CH_2Cl_2 (200 ml)
↓
Extract in a blender
↓
Repeat extraction twice
↓
Bulk CH_2Cl_2 extracts
↓
Evaporate under reduced pressure at 25-30°C to ≃ 20-30 ml
↓
Add 10 g ($Ba(OH)_2$, 10 g NaCl and 100 ml H_2O
↓
Steam distill
↓
Collect distillate CH_2Cl_2 + 150 (or 300) ml H_2O

Clean-up on ion-exchange column

Remove CH_2Cl_2
↓
Adjust pH to 6.5
↓
Chromatograph (ion-exchange, Amberlite CG-120, buffered at pH 6.5)
↓
Extract eluate with CH_2Cl_2 (2 x 150 ml)
↓
Combine CH_2Cl_2 extracts
↓
Dry on 35 g anhydrous Na_2SO_4

Clean-up on Celite column

Extract with CH_2Cl_2 (2 x 150 ml)
↓
Dry CH_2Cl_2 extract on 35 g anhydrous Na_2SO_4
↓
Evaporate in vacuum to ≃ 1 ml
↓
Chromatograph on Celite 545 column (add 4 ml 6 N HCl to 6 g Celite 545, and pack in a glass column with fritted plug.)
↓
Wash with 200 ml *n*-pentane
↓
Elute with 40 ml CH_2Cl_2

Add internal standard (1 µg NDi-BA)
↓
Evaporate under reduced pressure at 25-30°C or K-D, to 1 ml
↓
GC analysis using an AFID (KBr mono-crystal) detector
↓
Examine positives by GC-MS

15. ORIGIN OF THE METHOD

National Institute of Health
Department of Biomedical Research on Food
2-10-35 Kamiosaki
Shinagawa-ku
Tokyo
141 Japan

Contact point: Dr T. Kawabata

METHOD 3 – ANALYSIS OF VOLATILE *N*-NITROSAMINES IN FOOD USING FLAME THERMIONIC DETECTION AND MASS SPECTROMETRY CONFIRMATION

W. FIDDLER, J.W. PENSABENE, J.C. DOOLEY & A.E. WASSERMAN

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in meat and cheese products.

The method has a limit of detection of 0.5 microgram per kilogram (µg/kg) in a 25 g sample. The confirmation by mass spectrometry (MS) requires 3 µg *N*-nitrosamine.

2. REFERENCES

Fazio, T., Howard, J.W. & White, R. (1972) *IARC Scientific Publications No. 3*, 16-24

Fiddler, W., Pensabene, J.W., Fagan, J.C., Thorne, E.J., Piotrowski, E.G. & Wasserman, A.E. (1974) *J. Food Sci., 39*, 1070-1071

3. DEFINITION

Volatile *N*-nitrosamine contents of meat and cheese products: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

N-Nitrosamines are extracted with dichloromethane from a methanolic-alkaline digest of the foodstuff to be analysed, the extract is distilled and the distillate washed with hydrochloric acid and sodium hydroxide. The dichloromethane solution is concentrated to 1 ml and contaminating material removed by passage through a column containing acid-treated Florisil and silica gel. The concentrated sample is analysed quantitatively by gas-chromatography (GC), with flame thermionic detection. The presence of *N*-nitrosamines is confirmed by MS.

5. HAZARDS

N-Nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

Methanol	Baker, redistilled from Zinc dust
Dichloromethane	Burdick & Jackson "distilled in glass"
n-Pentane	Fisher "certified for pesticide residue analysis"
Diethyl ether	Burdick & Jackson "distilled in glass"
Perfluorokerosene (PFK)	As supplied for mass spectrometry
Xylene	Analytical grade
Potassium hydroxide	Analytical grade
Silicon carbide grains	Fisher "Boileezers" B-365
Calcium sulfate	Hammon Drierite Co. (8 mesh)
Barium hydroxide	Analytical grade
Sodium sulfate	Hammon Drierite Co. (8 mesh)
Silica gel	Merck E 7734 (# 68-01-41)
Florisil	60-100 mesh (F-100) washed with methanol then dichloromethane and dried in vacuum oven

---

[1] Steere, N.V. (1974) Safety in the chemical laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. *Department of Health, Education and Welfare Publication (NIH)*, 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Nitrogen	Ultra high purity
Helium	Ultra high purity
Sodium hydroxide 5N	
Hydrochloric acid 6N	
N-nitrosamine standard solutions	For each *N*-nitrosamine a standard solution (A) 100 mg/l is prepared in dichloromethane and diluted to 5 mg/l (B). Solution C is then prepared to contain all the *N*-nitrosamines of interest, each at 0.5 mg/l. All standard solutions are kept in a refrigerator at 4°C in brown bottles with teflon-lined caps and renewed every two months.

NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material a test must be performed using the procedure described in section 9, but omitting the sample.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

Liquid-liquid extractor	Kontes (K001596-175) or equivalent
Evaporative concentrator (K-D)	Kuderna Danish, 250 and 500 ml capacity Concentrator tube - 4 ml capacity graduated Kontes (K-570050) or equivalent Snyder (3 sections) and Micro-Snyder distillation columns
Clean-up column	Chromatographic column (14.5 x 250 mm), with teflon stopcock (water cooled)
Gas chromatograph	Varian-Aerograph Model 1740-1, KCl-coated coil, alkali flame-ionization detector
Gas chromatograph/ Mass spectrometer	Varian Model 1720, flame-ionization detector, effluent splitter: 1:1 coupled to DuPont Model 21-492 mass spectrometer, using a GC/MS interface: SS J. & T.-type.

[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented (see Chapter 1, Sampling and Samples).

9. PROCEDURE

NOTE: *N*-Nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Digestion*

9.1.1 Weigh 100 g of representative sample into a 1000 ml round-bottomed flask.

9.1.2 Add 200 ml methanol, 30 g potassium hydroxide and silicon carbide grains. Shake to mix.

9.1.3 Insert condenser, place on heating mantle and reflux for 3-5 hours to digest.

9.1.4 After cooling to room temperature take two separate aliquots of digest, each equivalent to 25 g of sample. For determination of recovery, add 1.0 ml of *N*-nitrosamine standard solution C to one of the aliquots. Treat each aliquot separately according to the following instructions (9.2.1 to 9.6.9):

9.2 *Liquid-liquid extraction of digest*

9.2.1 Place wad of glass wool over aperture in bottom of liquid-liquid extractor. Add 30 ml water, 25 g calcium sulfate and 100 ml dichloromethane, then add one of the aliquots from 9.1.4.

9.2.2 Attach to siphon arm, 250 ml boiling flask containing 50 ml dichloromethane and silicon carbide grains.

9.2.3 Add sufficient water to begin to displace dichloromethane into boiling flask.

9.2.4 Fit dispersion tube, coiled water condenser and heating mantle.

9.2.5 Extract for 5 hours.

9.3 *Distillation of liquid-liquid extract*

9.3.1 Add 75 ml 5 N sodium hydroxide and 8 g barium hydroxide to 250 ml boiling flask containing dichloromethane extract 9.2.5

9.3.2 Connect distillation head, foam trap condenser and receiver.

9.3.3 Distil off dichloromethane and discard.

9.3.4 Collect 60-65 ml aqueous distillate in 250 ml separation funnel.

9.4 *Solvent extraction of distillate*

9.4.1 Add 5 ml 6 N hydrochloric acid, 70 ml dichloromethane to distillate from 9.3.4 and shake for 3 min. Separate organic and aqueous phases.

9.4.2 Repeat twice the dichloromethane extraction of the aqueous phase from 9.4.1

9.4.3 Combine the three dichloromethane extracts in a 500 ml separation funnel.

9.4.4 Add 50 ml 6 N hydrochloric acid and shake for 1 min.

9.4.5 Separate lower layer (dichloromethane) into 500 ml separation funnel containing 50 ml 5 N sodium hydroxide.

9.4.6 Wash aqueous hydrochloric acid layer twice with 25 ml dichloromethane by shaking for 1 min and separating (washings).

9.4.7 Add dichloromethane washings to dichloromethane extracts and sodium hydroxide solution (9.4.5) and shake for 1 min.

9.4.8 Draw off dichloromethane layer. Pass through 35 g anhydrous sodium sulfate (pre-wet with dichloromethane), held in a fritted glass funnel, into a 500 ml KD evaporator with concentrator tube attached.

9.4.9 Wash alkaline layer from 9.4.7 with 25 ml dichloromethane by shaking for 30 seconds. Separate and transfer dichloromethane fraction to the K-D

9.5 *Concentration of extract*

9.5.1 Add silicon carbide grains to the K-D, attach 3-section Snyder column and concentrate to approximately 4 ml in a steam bath.

9.5.2 Continue concentration (removal of dichloromethane) to 1 ml with a Micro-Snyder column in a water bath at 70°C.

9.6 *Column-chromatographic clean-up of concentrate*

9.6.1 Add 1 g acidified Florisil (10 g + 8 ml 6 N hydrochloric acid) to chromatographic column containing a glass wool plug.

9.6.2 Add 4 g silica gel to column and pack with gentle tapping.

9.6.3 Place a small piece of glass wool on top.

9.6.4 Wash column with 50 ml *n*-pentane.

9.6.5 Stop flow when liquid is approximately 1 mm above the adsorbent.

9.6.6 After diluting the 1 ml dichloromethane extract concentrate (9.5.2) in 20 ml *n*-pentane, transfer quantitatively in small portions to the column. Stop flow as in 9.6.5.

9.6.7 Wash the column with 200 ml dichloromethane:*n*-pentane (1:1) (flow rate approximately 20 drops/min) and discard eluate.

9.6.8 Elute with 125 ml diethyl ether:dichloromethane (1:5) at the same flow rate.

9.6.9 Collect eluate in 250 ml K-D and concentrate to 1.0 ml (total sample volume) as described in 9.5.1 and 9.5.2. Stopper; retain for GC analysis.

9.7 *GC analysis*

9.7.1 GC conditions:

Column: stainless steel packed with 13% Carbowax 20 M-TPA on 60/80 mesh Gas Chrom. P.
Length: 3.5 m o.d.: 3.2 mm

Carrier gas: Helium, 50 ml/min

Column temperature: Programmed 105-200°C at 4°C/min

Injection port temperature: 185°C

Detector temperature: 220°C

Hydrogen: 58 ml/min

Air: 200 ml/min

9.7.2 GC-procedure:

9.7.2.1 Inject 8 µl of *N*-nitrosamine standard solution C into GC and carry out chromatographic analysis

9.7.2.2 Using the same conditions, inject 8 µl of concentrated sample solution (9.6.9) and carry out chromatographic analysis.

9.7.2.3 When a sample shows *N*-nitrosamine peak, check its identity by MS, after concentration to 0.1-0.2 ml under gentle stream of nitrogen.

9.8 *MS confirmation*

9.8.1 GC conditions:

Column: 3% SE 30 on 100/120 mesh Varaport 30
Length: 1.8 m i.d.: 3 mm

Carrier gas: Helium, 30 ml/min

Column temperature: Programmed 100-200°C at 6°C/min

Injection port temperature: 200°C

Detector temperature: 200°C

GC-MS interface temperature: 200°C

9.8.2 MS conditions:

The MS is adjusted for resolution of 12,000, based on appropriate reference peak. Reference peaks include:

Compound	Exact mass	Reference	$\frac{\Delta M}{M}$
N-nitrosodimethylamine (NDMA)	74.04800	69.99857(PFK)	.057850
N-nitrosodiethylamine (NDEA)	102.07931	99.99361(PFK)	.020858
N-nitrosopyrrolidine (NPYR)	100.06366	99.99361(PFK)	.000700
N-nitrosomorpholine (NMOR)	116.05857	106.07825(xylene)	.094084
N-nitrosopiperidine (NPIP)	114.07931	106.07825(xylene)	.075426
N-nitrosodibutylamine (NDBA)	158.14190	154.99201(PFK)	.020323
N-nitrosodipropylamine (NDPA)	130.11060	118.99201(PFK)	.093440
**N*-nitrosoproline methyl ester (NPROMe)	158.06914	154.99201(PFK)	.019853
*$C_6H_{10}O_2N^+$ ion from NPROMe	128.07115	118.99201(PFK)	.076300

* Both ions used to determine presence of this compound.

Electron energy: 70 eV

Accelerating voltage: 1800 V

Filament current: 200 μA

MS is operated in peak matching mode with the reference peak set on switch position "A" and the sample peak on switch position "B".

Sensitivity of the GC-MS combination is determined by running a standard solution of the *N*-nitrosamine to be confirmed and is checked daily at the start of operation and whenever two consecutive samples are found to contain no *N*-nitrosamine.

$$\text{sensitivity} = \frac{\text{peak height (mv)}}{\text{ng of } N\text{-nitrosamine injected}}$$

The GC-MS interface includes a 1:1 splitter, which allows half of the injected *N*-nitrosamine to enter the MS.

9.8.3 GC-MS procedure:

9.8.3.1 With instruments properly adjusted, inject 1 µl of *N*-nitrosamine standard solution A into the GC and look for signal on MS to ascertain that the instruments are responding.

9.8.3.2 Inject 2 µl *N*-nitrosamine standard solution B into the GC. The effluent from the column is directed to the flame-ionisation detector. Exactly one minute before desired *N*-nitrosamine peak elutes from the GC, open the GC-MS interface valve. Determine sensitivity as described above (9.8.2).

9.8.3.3 Close interface valve.

9.8.3.4 Heat column at elevated temperature (not exceeding that used for "conditioning" the column) for 5-15 min.

9.8.3.5 Cool column oven to 100°C.

9.8.3.6 Inject into GC a volume of test solution (9.7.2.3) equivalent to approximately 10 ng *N*-nitrosamine. One minute before *N*-nitrosamine peak elutes from GC, open interface valve.
For confirmation, peak in test solution must be superimposed over the reference peak on the oscilloscope and the GC retention times must coincide.

9.8.3.7 For semi-quantitative analysis, measure the peak height from the test solution in millivolts and compare with the height of the known standard (9.8.3.2).

10. METHOD OF CALCULATION

V = Total sample volume = 1.0 ml
X = Peak height of standard (9.7.2.1)
Y = Peak height of sample (9.7.2.2)
C = Concentration of standard = 0.50 µg/ml
W = Peak height of spiked sample (9.7.2.2)
Z = Amount of *N*-nitrosamines in 1 kg, therefore

$$Z = \frac{1000}{25}\left(\frac{YVC}{W-Y}\right) = \left(\frac{20\ Y}{W-Y}\right) \quad \mu g/kg \text{ or ppb.}$$

$$\% \text{ recovery} = \left(\frac{W-Y}{X}\right)100$$

NOTE: If spiked aliquot (9.1.4) has not been analysed, a lower limit for Z may be obtained from

$$Z = \frac{1000}{25}\left(\frac{YVC}{X}\right) = \left(\frac{20\ Y}{X}\right) \ \mu g/kg$$

11. REPEATABILITY AND REPRODUCIBILITY

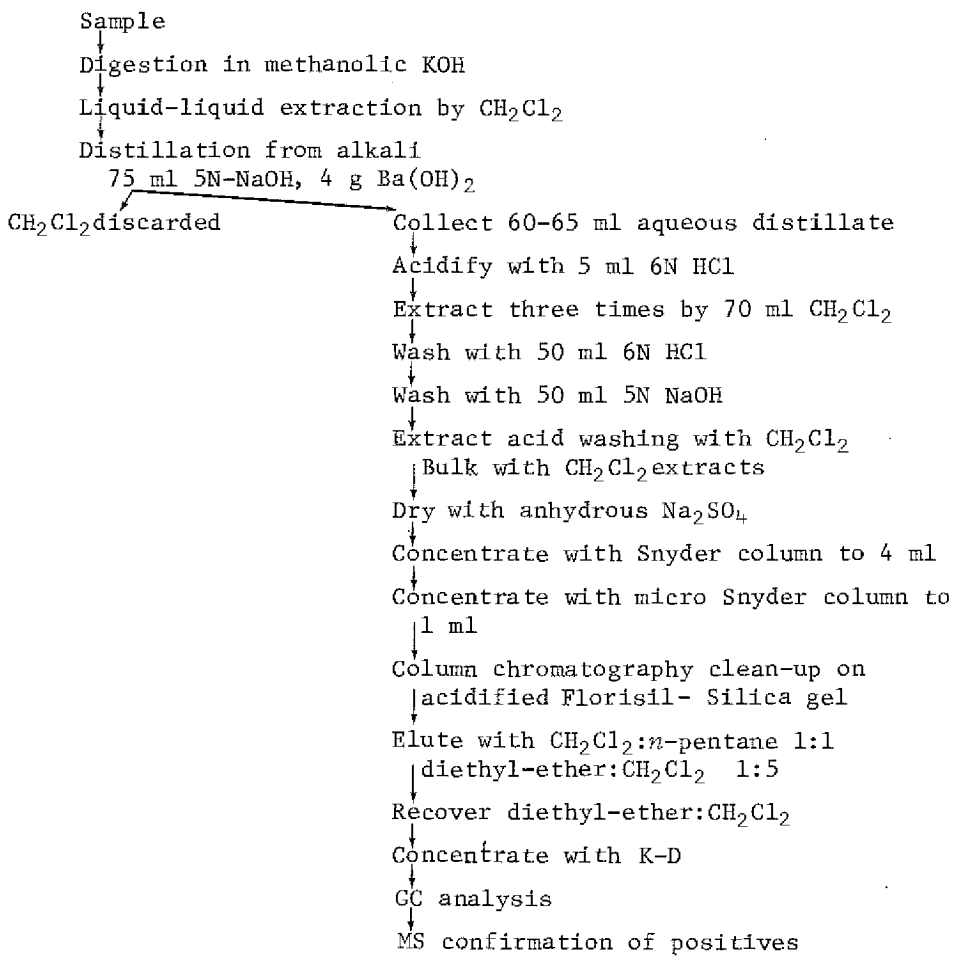
11.1 *Repeatability*

At the 10 µg/kg level, Z is repeatable to within about ± 2 µg/kg.

11.2 *Reproducibility*

In the same range, the method is reproducible to within approximately 2-3 µg/kg.

12. SCHEMATIC REPRESENTATION OF THE PROCEDURE



13. ORIGIN OF THE METHOD

United States Department of Agriculture
600 E Mermaid Lane
Philadelphia PA 19118
USA

Contact point: Dr W. Fiddler

METHOD 4 – ANALYSIS OF VOLATILE *N*-NITROSAMINES USING GAS CHROMATOGRAPHY WITH COULSON DETECTOR: ESTIMATION OF *N*-NITROSOPYRROLIDINE BY THIN-LAYER CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

N.P. SEN

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in meat products, including cooked bacon and cooked bacon fat. The method can be applied to other types of foods, such as cheese, fish, fish meal, vegetables, alcoholic beverages, soya bean oil, wheat flour, spices and spice-nitrite curing mixture.

The gas chromatography (GC) method, with the Coulson electrolytic-conductivity detector, has a limit of detection of 1 ng for *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA). For high molecular weight *N*-nitrosamines and cyclic *N*-nitrosamines such as *N*-nitrosopiperidine (NPIP), the limit is 5 ng.

The thin-layer chromatography (TLC) method for NPYR, with fluorimetric estimation, has a limit of detection of 10 ng.

2. REFERENCES

Fine, D.H., Rounbehler, D.P. & Sen, N.P. (1976) *J. Agric. Food Chem.*, *24*, 980-984 (Tables I & II)

IARC collaborative study data:

IARC Scientific Publications No. 9, p. 61, Table 3, Laboratory No. 18

IARC Scientific Publications No. 14, p. 79, Table 2, Laboratory No. 18, Coulson and TLC

Iyengar, J.R., Panalaks, T., Miles, W.F. & Sen, N.P. (1976) *J. Sci. Food Agric.*, *27*, 527-530 (Table I)

Panalaks, T., Iyengar, J.R. & Sen, N.P. (1973) *J. Assoc. Off. Anal. Chem.*, *56*, 621-625

Sen, N.P., Donaldson, B., Seaman, S., Iyengar, J.R. & Miles, W.F. (1976) *J. Agric Food Chem.*, *24*, 397-401 (Table III)

3. DEFINITION

Volatile *N*-nitrosamine contents of meat products: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as micrograms per kilogram (µg/kg) (parts per billion).

4. PRINCIPLE

An alkaline slurry of the sample is extracted with dichloromethane and an aliquot of the extract is used for *N*-nitrosamine analysis. The *N*-nitrosamines are separated from interfering compounds by distillation from an alkaline solution, followed by an acid wash (to remove amine components) and chromatography on a basic alumina column. The levels of all the *N*-nitrosamines, except NPYR, are estimated by GC, using a Coulson electrolytic-conductivity detector (pyrolytic mode), and that of NPYR by thin layer chromatography using fluorimetric detection.

5. HAZARDS

N-nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

---

[1] Steere, N.V. (1974) Safety in the chemical laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. *Department of Health, Education and Welfare Publication (NIH)*, 75-900 (1975)

6. REAGENTS[1]

Dichloromethane *n*-Heptane *n*-Pentane *n*-Hexane Diethyl-ether	All solvents are of analytical grade and distilled from an all-glass apparatus. Anhydrous solvents are dried over anhydrous sodium sulfate.
Sodium sulfate (anhydrous)	Analytical grade (powdered and granular)
Silicon carbide grains	Fisher's "Boileezers" B-365
Potassium carbonate	Analytical grade
Basic alumina	Aluminium oxide (without binder), Woelm basic, activity grade 1, for chromatography (Alupharm Chemicals). Heat alumina at 500°C for 5 hours, cool in a desiccator, add 3% water, mix well in a stoppered flask and store the stoppered flask inside a desiccator over silica gel.
Antifoam "A"	Dow Corning Silicones Inter-America Ltd.
Silica gel plates	MN-Silica gel G-HR for TLC (Macherey, Nagel & Co, Germany). Note: other brands may not be suitable. Prepare plates 0.5 mm thickness, dry at 90°C for 45 min and store in a dry box.
Potassium hydroxyde 3 N	
Potassium carbonate solution	20% in distilled water
Glycine-hydrochloric acid buffer	Dissolve 22 g glycine in 200 ml 1 N hydrochloric acid, dilute to 1000 ml with water and adjust pH to 2.1 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide.
Ninhydrin solution	0.3% in 95% ethanol, store at 4°C.

[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

N-Nitrosamine standard solutions	Solution A: contains 10 mg/l of each of the *N*-nitrosamines of interest in dichloromethane . Solution B: contains 1 mg/l of each of the *N*-nitrosamines of interest in dichloromethane. All standard solutions are stored at -20°C. The stoppered containers should be placed inside a second vessel containing some dichloromethane (as the atmosphere in the latter is saturated with respect to dichloromethane, evaporation is prevented. Concentrated ($\geq$ 1000 mg/l) stock solutions are renewed once a year, diluted standards ($\simeq$ 1-10 mg/l) once a month.

NOTE: To ensure the absence of traces of *N*-nitrosamines or other interfering material, a blank test must be performed using the procedure described in section 9, but omitting the sample.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

Blender	Stainless-steel blender with variable speed
Distillation apparatus	All-glass flash evaporator with provision for circulating ice-cold water through the condenser
Evaporative concentrator (K-D)	Kuderna-Danish Flask (a) 35 ml micro concentration flask, graduated to 1.0 ml (Fig. 1) (b) 1000 ml flask graduated to 20.0 ml (Fig. 2) Snyder columns (a) large, with 3 sections and 24/40 joints; (b) micro, with 3 sections and 14/20 joints (Kontes Glass Co.).

[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Chromatography column	Glass, with teflon stopcock. Length: 24-25 cm; o.d.: 1-1.2 cm.
Gas chromatograph	Varian Aerograph model 2700, connected to a Coulson detector (Tracor Inc.) operating in the pyrolytic mode (i.e without catalyst or hydrogen gas).
UV viewing chamber	Chromato-Vue (Ultraviolet Products Inc., San Gabriel, California). Use the top short-wave lamp as well as the bottom transilluminator for viewing purposes.
UV irradiation lamp	General Electric G15T8 germicidal lamp used without filter and enclosed in a wooden box.
Separatory funnels	1000 ml or 500 ml, with teflon stoppers and stopcocks (do not use grease)

FIG. 1

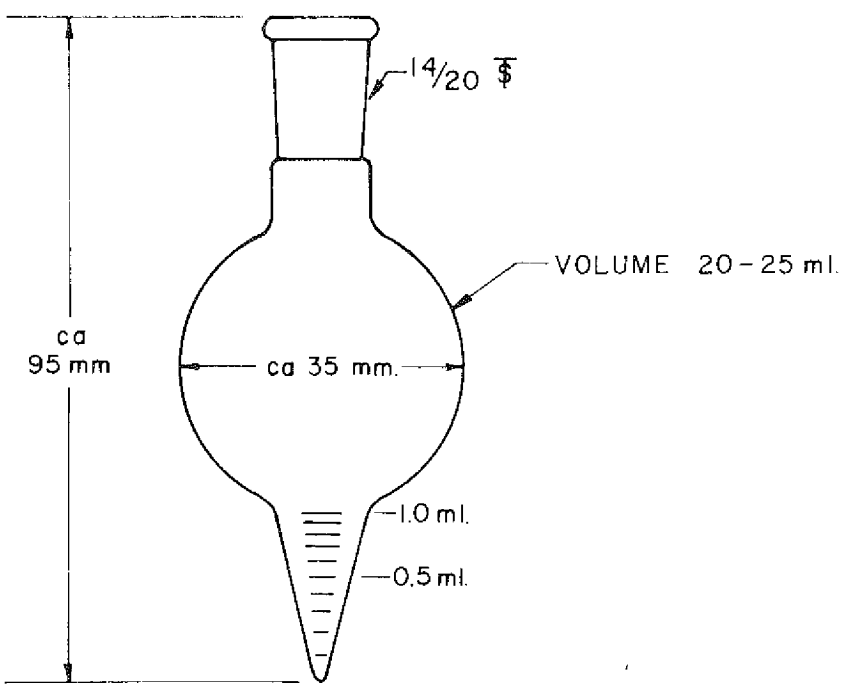
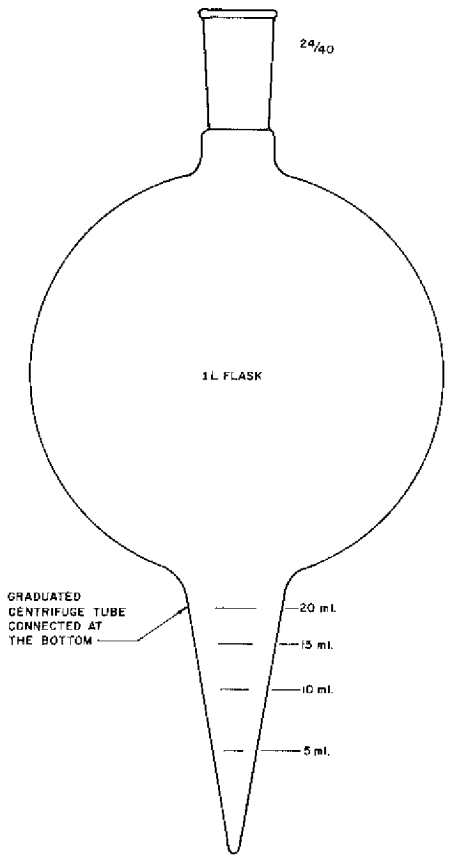


FIG. 2



8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented (see Chapter 1, Sampling and Samples).

9. PROCEDURE

NOTE: *N*-Nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Extraction*

9.1.1 Depending on the kind of sample, weigh 25-100 g (see Note on Procedure, 12) and transfer quantitatively to the blender. Rinse sample container with 40-60 ml dichloromethane and add rinsings to the sample.

9.1.2 Add 50-60 ml 3 N potassium hydroxide to the sample, mix well with a spatula. Add 600 ml dichloromethane and blend for 5 min at low speed.

9.1.3 Add 100-150 g anhydrous sodium sulfate (granular) and blend for 5 min at low speed.

9.1.4 Carefully decant and filter extract through glass wool, using enough to ensure retention of all solid particles, and collect filtrate in a 2000 ml volumetric flask.

9.1.5 Repeat extraction of the sample residue in the blender with 600 ml dichloromethane for 8-10 min. If emulsion forms, it may be broken by sprinkling about 20 g anhydrous sodium sulfate on the surface. Filter as above (9.1.4).

9.1.6 Repeat 9.1.5 twice, but using less dichloromethane (e.g., 400 ml), if necessary so that the final volume of filtrate does not exceed 2000 ml.

9.1.7 Make up volume to 2000 ml with dichloromethane and mix well.

9.2 *Distillation and extraction*

9.2.1 Place a 600 ml aliquot of the extract from 9.1.7 in a 2000 ml distilling flask. Add 200 ml 3 N potassium hydroxide, a few silicon carbide boiling chips and about 1 g antifoam "A".

9.2.2 Distil under vacuum until all dichloromethane is distilled off. Discard dichloromethane distillate.

9.2.3 Continue vacuum distillation, using a water bath at about 45°C, and collect about 170 ml aqueous distillate (during distillation, ice-cold water must be passed through the condenser and the collection flask must be immersed in ice).

9.2.4 Rinse condenser with 200 ml dichloromethane and use the rinsing for the first extraction in step 9.2.5.

9.2.5 Add 34 g potassium carbonate to distillate and mix. Extract the solution with two 170 ml portions of dichloromethane. Discard the aqueous phase.

9.2.6 Combine the two dichloromethane extracts in a separation funnel and wash by shaking with 50 ml of glycine buffer, then with 50 ml 20% potassium carbonate solution. Discard the aqueous phase in each case.

9.2.7 Dry the organic layer over powdered anhydrous sodium sulfate in a glass-stoppered Erlenmeyer flask for 30 min. Shake occasionally.

9.2.8 Filter through Whatman No. 1 filter paper and collect filtrate in a 1000 ml concentration flask (K-D) (Fig. 2).

9.3 *Concentration and clean-up on alumina column*

9.3.1 Add 1 or 2 "Boileezers" to the flask (9.2.8), fit Snyder column and concentrate dichloromethane extract to 15 ml by heating the flask in a water bath (50°C).

9.3.2 Add 20.0 ml anhydrous *n*-heptane through the top of the Snyder column, continue heating until volume is reduced to 20-21 ml (during the last stage, raise water bath temperature to 80-90°C).
Overheating or prolonged heating at 90°C will result in excessive losses of *N*-nitrosamines.

9.3.3 Remove flask from water bath and rinse Snyder column with 1 ml anhydrous *n*-heptane. Add rinsing to concentrated solution from 9.3.2.

9.3.4 Prepare an alumina column by filling three-quarters of the chromatography column with anhydrous *n*-pentane, then adding sufficient alumina to obtain a column 3.5 to 4 cm high.

9.3.5 Pass *n*-heptane solution of sample extract through column at a flow rate of about 1 ml/min; discard effluent.

9.3.6 Wash column with 50 ml anhydrous *n*-pentane (2 ml/min); discard washing.

9.3.7 Elute column with 50 ml anhydrous dichloromethane (2 ml/min). Collect eluate in a 125 ml Erlenmeyer flask (24/40 joint).

9.3.8 Add one or two "Boileezers" and concentrate eluate to about 10 ml by heating in a water bath at 60°C (use a Snyder column).

9.3.9 Quantitatively transfer concentrate to a micro concentration flask, fit micro Snyder column and concentrate to 0.8 ml. (Final volume of concentrates should not be less than 0.8 ml at any stage. Do not use any silicon carbide grains in the micro flask).

9.3.10 Make up volume to 1.0 ml with dichloromethane and use aliquots for GC or TLC analysis.

9.4 *GC analysis*

9.4.1 GC conditions:

Column: stainless steel packed with 10% Carbowax 20 M on 60-80 mesh Chromosorb W, HMDS-treated.
Length: 2m; O.D.: 3.2 mm.

Carrier gas: Helium, 30 ml/min

Column temperature: isothermal 90°C

Injector temperature: 190°C

Transfer line temperature: 200°C

The effluent from the column must be vented to atmosphere for the first 3 min after each injection.

9.4.2 Procedure:

9.4.2.1 Inject 5 µl of *N*-nitrosamine standard solution A (10 mg/l) and check the detector sensitivity. Change Coulson furnace temperature (by 50°C at a time) and determine the optimum furnace temperature.

9.4.2.2 Inject 5-10 µl of *N*-nitrosamine standard solution B (1 mg/l). (5 ng of each *N*-nitrosamine should give easily distinguishable peaks. If not, adjust water flow rate through the Coulson cell to obtain the required sensitivity).

9.4.2.3 Inject 4-5 µl of the concentrated meat extract (9.3.10). (For good quantitative comparisons, the peaks obtained with the meat extract should be approximately the same size (± 20%) as those obtained with the standards. If not, inject varying amounts of *N*-nitrosamine standard solution B until the respective peak areas are similar in size).

9.5 *TLC analysis for* N-*nitrosopyrrolidine*

9.5.1 Spot aliquots of 10 µl and 100 µl of the concentrated extract (9.3.10) and 10, 20 and 40 µl of *N*-nitrosamine standard solution B on the TLC plate. (Do not blow air)

9.5.2 Develop plate in *n*-hexane:diethyl-ether:dichloromethane (4:3:2) until the solvent front advances about 15 cm. (The tank must be lined with filter paper and well saturated with the solvent system)

9.5.3 Take out plate and spray with 30% acetic acid until *thoroughly wet* (use a fine spray bottle).

9.5.4 Irradiate plate under GE-germicidal lamp for 10 min.

9.5.5 Let plate dry at room temperature for 1 hour.

9.5.6 Spray plate with ninhydrin solution until plate is *thoroughly wet*.

9.5.7 Place plate flat on its back inside an oven (90-100°C) and heat for 15-30 min.

9.5.8 Take out plate, cool for 2-3 min and place inside viewing chamber. NPYR should appear as orange fluorescent spots (other *N*-nitrosamines do not fluoresce).

9.6 *Analysis of spiked sample*

Using the same procedure (9.1.1 to 9.5.8), repeat analysis with the same sample spiked at the 10 or 25 µg/kg level with all the *N*-nitrosamines of interest.

10. METHOD OF CALCULATION

10.1 *GC method*

The amount of *N*-nitrosamine present in the extract is calculated by comparison of the peak areas obtained with the standard solution and the sample extract.

10.1.1 The content of *N*-nitrosamine, x, (not corrected for recovery) is given by:

$$Z_x = \frac{1000\ Vv_s C_s}{Wv_e} \left(\frac{A_e}{A_s}\right)_x \quad \mu g/kg$$

where

V = Volume of concentrated extract (9.3.10) (ml)

v_s = Volume of standard solution B injected (µl)

v_e = Volume of concentrated extract injected (µl)

C_s = Concentration of standard solution B (µg/ml)

W = Weight of sample (9.1.1) (g)

A_e = Peak area of *N*-nitrosamine, x, from concentrated extract (9.3.10)

A_s = Peak area of *N*-nitrosamine, x, from standard solution B

10.1.2 The content of *N*-nitrosamine, x, in the spiked sample is given by:

$$Z_{sx} = \frac{1000\ Vv_s C_s}{Wv_e} \left(\frac{A_{se}}{A_s}\right)_x \quad \mu g/kg$$

where

A_{se} = Peak area of *N*-nitrosamine, x, from concentrated extract of spiked sample

The other symbols are defined in 10.1.1.

10.1.3 The recovery of *N*-nitrosamine, x, is given by:

$$R_x = \frac{W(Z_{sx} - Z_x)}{10\ VC}\ \%$$

where

V = Volume of spike added (ml)
C = Concentration of standard spike solution (µg/ml)
W = Weight of spiked sample (g)

10.1.4 The content of *N*-nitrosamine, x, corrected for recovery, is given by:

$$Z_{xc} = \frac{100\ Z_x}{R_x}\ \mu g/kg$$

10.2 *TLC method*

The NPYR content is estimated by comparing the size and intensity of the spots obtained with the sample extract with those obtained with the standard solution. Failure to detect 10-20 ng of NPYR in a standard spot will suggest that the procedure is not working and should be repeated.

11. REPEATABILITY AND REPRODUCIBILITY

11.1 *Repeatability*

No data are available for the whole method.

GC-Coulson: ± 5% for repeated injection of the same solution or extract.

TLC-Fluorimetric: ± 20% for repeated spotting of the same solution or extract and comparison with a standard spotted beside it.

11.2 *Reproducibility*

No data available.

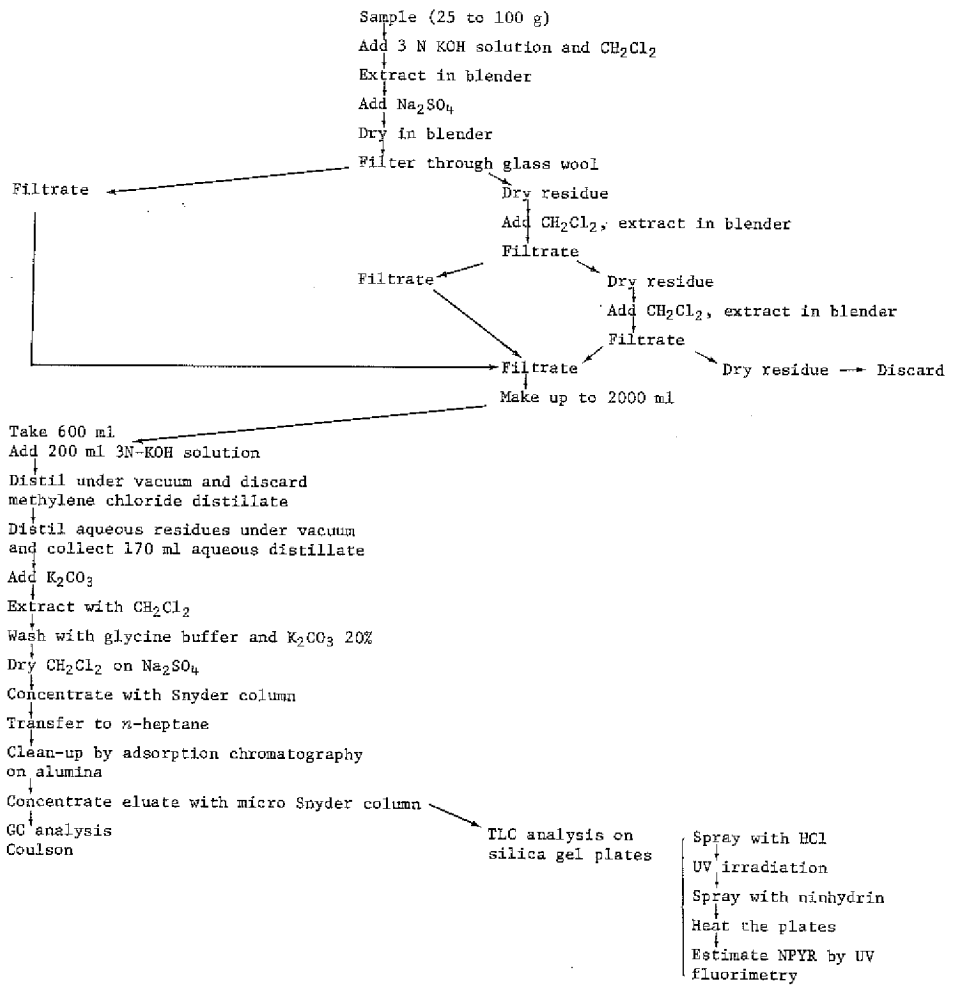
12. NOTE ON PROCEDURE

The following rough guide may be used to choose the weight of sample to be employed in 9.1.1:

- 25 g for samples which give high background interference (e.g., fish meal, mixture of spices and nitrite)
- 50 g for samples which give moderate background interference (e.g., fried bacon, cooked bacon fat)
- 100 g for samples which give low background interference (e.g., meat, fish, cheese, vegetables, etc.)
- 25-50 g for samples rich in *N*-nitrosamines.

If enough sample is not available, weigh 25-100 g sample, extract as described in 9.1 using proportionately smaller amounts of reagents and solvents. Then, in step 9.2.1, use the total extract obtained in 9.1.7.

13. SCHEMATIC REPRESENTATION OF PROCEDURE



14. ORIGIN OF THE METHOD

Health and Welfare Canada
Health Protection Branch
Food Research Division
Tunney's Pasture
Ottawa, Ontario K1A 0L2
Canada

Contact point: Dr N.P. Sen

METHOD 5 – DETERMINATION OF VOLATILE *N*-NITROSAMINES IN FOOD BY CHEMILUMINESCENCE USING THE THERMAL ENERGY ANALYSER

D.H. FINE

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in a wide range of foodstuffs, drinks, biological materials, pesticides, cutting fluids and cosmetics.

The method as described has a limit of detection of 5 micrograms per kilogram (µg/kg) in a 20 g sample, but this can be lowered to 0.05 µg/kg in a 20 g sample by further concentration of the extract.

2. REFERENCES

Fine, D.H., Rufeh, F. & Lieb, D. (1974) *Nature, 247*, 309-310

Fine, D.H. & Rounbehler, D.P. (1975) *J. Chromatogr., 109*, 271-279

Fine, D.H., Rufeh, F., Lieb, D. & Rounbehler, D.P. (1975) *Anal. Chem., 47*, 1188-1191

3. DEFINITION

Volatile *N*-nitrosamine contents: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

The procedure includes vacuum distillation of the *N*-nitrosamines from sodium hydroxide and mineral oil, acidification of the distillate, extraction of the *N*-nitrosamines into dichloromethane and gas-liquid chromatography (GC), using a thermal energy analysis detector (TEA).

5. HAZARDS

N-nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

Mineral oil	Commercial grade
Dichloromethane	Burkick & Jackson Laboratories Inc.
Sodium hydroxide 0.1N	
Hydrochloric acid 0.1N	
N-Nitrosamine standard solutions	(a) External standard, 1mg/l of any *N*-nitrosamine in dichloromethane. (b) Internal standard; a mixed solution in water containing 1 mg/l of each of the *N*-nitrosamines of interest. More concentrated stock solutions may be prepared and diluted as required. Both stock and diluted standard solutions should be renewed every two months.

NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material, a test must be performed using the procedure described in section 9, but omitting the sample.

---

[1] Steere, N.V. (1974) Safety in the chemical laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. *Department of Health, Education and Welfare Publication (NIH)*, 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

Distillation apparatus (Fig. 1),

Any single-column, temperature-programmable gas chromatograph,

Thermal Energy Analyser (TEA) (Fig. 2), Thermo Electron Corporation.

FIG. 1. DISTILLATION APPARATUS

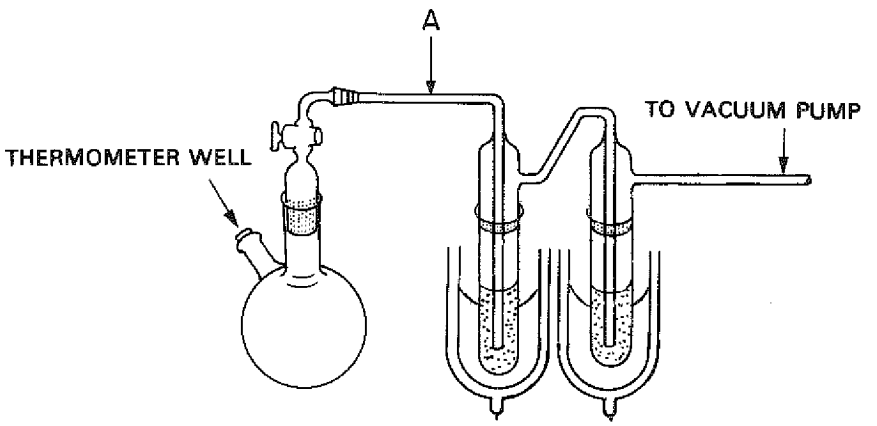
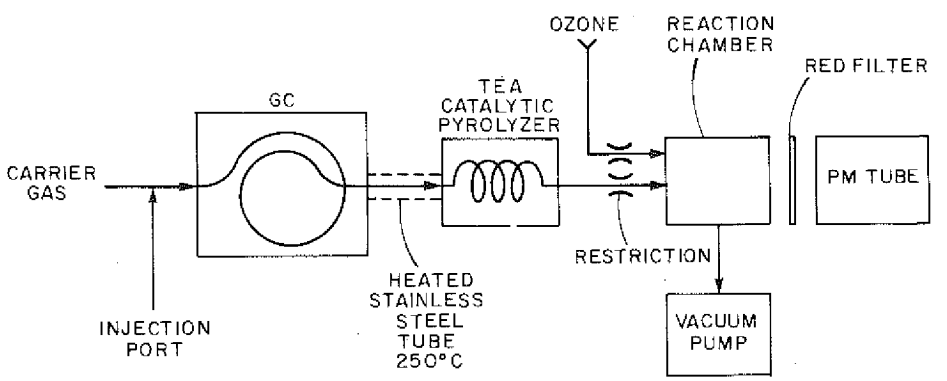


FIG. 2. THERMAL ENERGY ANALYSER



[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented; see Chapter 1, Sampling and Samples.

9. PROCEDURE

NOTE: *N*-Nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Distillation from mineral oil*

9.1.1 Weigh 25 g of food sample.

9.1.2 Cut into slices or mince in a food grinder.

9.1.3 Place in a 500 ml round-bottomed flask (see Note on Procedure section 13).

9.1.4 Add 20 ml crude mineral oil and 4 ml 0.1N sodium hydroxyde.

9.1.5 Connect greased adapters and thermometer to the flask (Fig. 1), connect adapter to traps and connect traps to vacuum pump manifold.

9.1.6 Immerse traps in liquid nitrogen in a Dewar flask.

9.1.7 Open system to vacuum pump and adjust pressure to 2 torr.

9.1.8 Heat round-bottomed flask slowly.

9.1.9 Stop the distillation when the thermometer in the vapour phase indicates 110°C (about 40 min).

9.1.10 Release vacuum, remove Dewar and allow traps to reach room temperature.

9.2 *Solvent extraction and concentration*

9.2.1 Transfer the melted distillate to a 125 ml separation funnel.

9.2.2 Rinse tube A (Fig. 1) and the trap with water and add rinsing to the distillate.

9.2.3 Add 4 ml 0.1 N hydrochloric acid and 10 ml dichloromethane; shake for 3 min.

9.2.4 Repeat dichloromethane extraction twice.

9.2.5 Combine dichloromethane extracts and dry over ≈ 10 g sodium sulfate. Rinse sodium sulfate with ≈ 10 ml dichloromethane and add rinsing to the extracts.

9.2.6 Add 150 µl isooctane.

9.2.7 Concentrate to 500 µl in a concentrator tube fitted with a Snyder column in a water bath at 58°C.

9.3 *GC-TEA analysis*

9.3.1 GC conditions:

Column: stainless steel tube, packed with 10% Carbowax 20 M on Chromosorb W, 80/100 mesh
Length: 3.5 m i.d.: 3 mm

Carrier gas: Argon, 10 ml/min at 100 psi (7 atmos.)

Column temperature: programmed 140 to 210°C at 5°C/min

9.3.2 TEA conditions:

Pressure: 0.4 torr.

Oxygen flow rate: 5-10 ml/min.

9.3.3 Inject 5 µl of standard solution (a) and measure the peak area for the *N*-nitrosamine.

9.3.4 Inject 5 µl of concentrated extract (9.2.7) and measure the peak area for all compounds corresponding to the retention time of a *N*-nitrosamine. Calculate *N*-nitrosamine contents according to 10.1.

9.3.5 When recovery correction is desired (see 13), inject 5 µl of concentrated spiked sample extract and measure the peak area for all compounds corresponding to the retention time of a *N*-nitrosamine. Calculate *N*-nitrosamine content according to 10.2.

10. METHOD OF CALCULATION

The *N*-nitrosamine contents are calculated by comparing each peak area obtained with the concentrated sample (or spiked sample) extract with that obtained with the standard solution (a)

10.1 The apparent[1] content, Z_x, of *N*-nitrosamine, x, in the sample is

$$Z_x = \frac{v_s A_x M_x C_a V_e}{v_e A_s M_s W} \quad \mu g/kg$$

[1] Not corrected for recovery.

10.2 The apparent[1] content, Z_{xs}, of *N*-nitrosamine, x, in the spiked sample is:

$$Z_{xc} = \frac{v_s A_{xs} M_x C_a V_{es}}{v_{es} A_s M_s W} \quad \mu g/kg$$

10.3 The recovery, R_x, of *N*-nitrosamine, x, during analysis is:

$$R_x = \frac{100\ W}{V_s C_b} \left(Z_{xs} - Z_x \right) \quad \%$$

10.4 The corrected content, Z_{xc}, of *N*-nitrosamine x in sample is:

$$Z_{xc} = \frac{100\ Z_x}{R_x}$$

where

v_s = Volume of standard *N*-nitrosamine solution (a) injected (µl)

$C_{a,b}$ = Concentration of standard *N*-nitrosamine solution (a), (b), (mg/l)

M_x = Molecular weight of *N*-nitrosamine x

M_s = Molecular weight of *N*-nitrosamine in standard solution (a)

W = Weight of sample (g)

A_x = Peak area of *N*-nitrosamine, x, from extract (mm^2)[2]

A_s = Peak area from standard *N*-nitrosamine solution (a) (mm^2)[2]

Axs = Peak area of *N*-nitrosamine, x, from spiked sample extract (mm^2)[2]

v_e = Volume of extract (9.2.7) injected (µl)

V_e = Volume of concentrated extract (9.2.7) (µl)

v_{es} = Volume of spiked sample extract (9.2.7) injected (µl)

---

[1] Not corrected for recovery.

[2] The area of each recorded peak must be multiplied by the appropriate attenuation factor.

V_{es} = Volume of spiked sample extract (9.2.7) (µl)

V_s = Volume of spike [standard solution (b)] added at step 9.1.3 (µl)

11. RECOVERIES

Some experimental recoveries of *N*-nitrosamines from foodstuffs at the sub 5 µg/kg concentration level

	NDMA	NDEA	NDPA	NDBA	NPYR
Canned tuna fish	71%	90%		98%	100%
Canned corned beef	75%	90%		95%	100%
Fried bacon			100%		
Soya bean oil			100%		

12. REPEATABILITY AND REPRODUCIBILITY

12.1 *Repeatability*

At the 5 µg/kg level, the coefficient of variation of the results is < 10% for NDMA and < 5% for NDEA, NDBA, NPIP and NPYR.

At the 2.5 µg/kg level, the coefficient of variation of the results for NDMA is < 10%.

12.2 *Reproducibility*

No data available.

13. NOTE ON PROCEDURE

When corrections for incomplete recovery of *N*-nitrosamines in the concentrated extract (9.2.7) are required, the Internal Standard Method may be used. In this case the sample is spiked with the mixed standard *N*-nitrosamine solution at step 9.1.3 and procedures 9.1 and 9.2 are then carried out as described. An unspiked extract is also prepared as described in 9.1 and 9.2. Both spiked and unspiked samples must be analysed.

14. SCHEMATIC REPRESENTATION OF PROCEDURE

Sample
↓
Mincing
↓
Addition of NaOH solution (4 ml 0.1 N NaOH) and mineral oil (20 ml)
↓
Vacuum distillation
↓
Acidification of distillate (4 ml 0.1 N HCl)
↓
Extraction with CH_2Cl_2 (3 × 10 ml)
↓
Concentration
↓
GC-TEA analysis

15. ORIGIN OF THE METHOD

Thermo Electron Research Center
85 First Avenue
Waltham MA 02154
USA

Contact point: Dr D. Fine

METHOD 6 – MASS SPECTROMETRIC DETERMINATION OF VOLATILE *N*-NITROSAMINES AFTER SCREENING BY THE COULSON ELECTROLYTIC DETECTOR

T. GOUGH & K. WEBB

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in meat products, fish and related products, cheese, milk and related dairy products, eggs, vegetables, fresh and canned fruits, soups, bread and pastry-based foods, rice, nuts, frying oils, beverages, salad constituents and cooked complete meals, such as mixed grills, stews and casseroles.

The method has a limit of detection of 0.1 to 5 micrograms per kilogram (µg/kg) in 250 g, depending on mass spectrometry (MS) conditions and the particular *N*-nitrosamine.

2. REFERENCES

Gough, T.A. & Webb, K.S. (1973) *J. Chromatogr.*, *79*, 57-63

Gough, T.A. & Sugden, K. (1975) *J. Chromatogr.*, *109*, 265-269

Gough, T.A. & Webb, K.S. (1972) *J. Chromatogr.*, *64*, 201-210

3. DEFINITION

Volatile *N*-nitrosamine contents of foods: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

N-nitrosamines are steam distilled from an aqueous slurry of the sample and extracted from the distillate with dichloromethane. The dichloromethane extract is concentrated to a small volume. The concentrated extract is screened by gas-liquid chromatography (GC), using a Coulson electrolytic-conductivity detector operating in the reductive mode. Any apparent positive results for *N*-nitrosamines are then

confirmed by combined GC and MS. Two GC systems are described, one using pressure programming and the other column switching.

5. HAZARDS

N-nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

Dichloromethane	Analytical grade, fractionally redistilled, a middle cut being taken.
n-hexane	Spectrosol grade or equivalent
Perfluoro-tri-*n*-butylamine	
Sodium chloride	Analytical grade
Antifoam tablets	Antifoam 'S' (Thompson & Capper Ltd, Liverpool)
Sodium sulfate	Anhydrous, granular, analytical grade
Sulfuric acid, 10 N	
Sodium hydroxide, 1.5 N	

---

[1] Steere, N.V. (1974) Safety in the chemical laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. *Department of Health, Education and Welfare Publication (NIH)*, 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

N-Nitrosamine standard solutions	Solution (a); 10 mg/l of each of the *N*-nitrosamines of interest, in hexane. Solution (b); 10 mg/l of NDPA in water. The standard solutions are stored in a dark room at 10°C and renewed approximately every 6 months. New standards are checked against old standards.

NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material, a test must be performed using the procedure described in section 9, but omitting the sample.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

Distillation apparatus	Distillation flask 1000 ml, receiver 500 ml
Evaporative concentrator (K-D)	Kuderna-Danish Flask 500 ml, Concentrator tube 10 ml
Gas chromatographs	1. For use with Coulson detector: Varian 1200, fitted with a Coulson electrolytic-conductivity detector operating in the reductive mode. 2. For use with the mass spectrometer: System A: Philips Research Model, fitted with a pressure-programming facility which is laboratory built (Gough & Webb, 1973). Omission of this facility will result in substantially longer analysis times. System B: Pye Model 104, fitted with a column-switching facility (Gough & Sugden, 1975).

[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

GC-MS interface	Silicone membrane separator, constructed in the laboratory (Gough & Webb, 1972), operated at 145°C with the transfer line to the MS held at 160°C.
Mass spectrometer	AEI MS902, fitted with peak-matching facilities and operated at a resolution of 7000 or 12000, at 10% valley.

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented (See Chapter 1, Sampling and Samples).

9. PROCEDURE

NOTE: *N*-Nitrosamines are degraded by U-V light and exposure of such extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Distillation, extraction and concentration*

9.1.1 Comminute 250 g of food and slurry in a total of about 250 ml of water, including estimated moisture originally present in the sample. Place in a 1000 ml round-bottomed concentration flask.

9.1.2 Add 100 g of sodium chloride and 2 antifoam tablets, if necessary.

9.1.3 Add 250 µl of the internal standard solution (b).

9.1.4 Steam distil the mixture and collect the first 400 ml of distillate. Transfer distillate to a 500 ml separation funnel.

9.1.5 Add 80 g of sodium chloride and 4 ml of 10 N sulfuric acid.

9.1.6 Extract acidified distillate with 4 x 40 ml of dichloromethane. Combine dichloromethane extracts.

9.1.7 Wash combined extracts with 70 ml 1.5 N sodium hydroxide solution.

9.1.8 Dry organic layer over 40 g anhydrous sodium sulfate.

9.1.9 Place dried extract in K-D and reduce volume to 2.5 ml by evaporation in a water bath at 46°C.

9.1.10 Add 800 µl of *n*-hexane and continue evaporation to about 250 µl.

9.1.11 Measure volume with a 500 µl-capacity syringe.

9.1.12 Transfer extract to a glass vial fitted with a septum cap and store at 10°C.

9.2 *Preliminary screening of extracts by GC with Coulson detection*

9.2.1 GC conditions:

Column: stainless steel packed with 15% FFAP on Diatomite CLQ, 80-100 BS mesh.
Length: 6.3 m i.d.: 1.8 mm

Carrier gas: Helium, 25 ml/min

Column temperature: isothermal 140°C

Injection port temperature: 140°C

Transfer line temperature: 180°C

9.2.2 Coulson detector conditions:

Hydrogen flow rate: 60 ml/min

Venting block temperature: 180°C

Furnace temperature: 700°C

9.2.3 Procedure:

Inject a 5 µl aliquot of the extract (9.1.12) on the gas chromatograph and compare the retention times of the peaks obtained with those of the standard *N*-nitrosamines (see Notes on Procedure, 13.1).

9.3 *GC-MS confirmation and quantification (System A)*

9.3.1 GC conditions:

Column: 2 columns in series,
a) stainless steel, packed with 15% Carbowax 20M on 80/100 BS mesh Chromosorb W/AW, DMCS-treated. Length: 2.4 m i.d.: 1.8 mm
b) stainless steel, packed with 5% Carbowax 20 M on 80/100 BS mesh Chromosorb W/AW, DMCS-treated. Length: 5.4 m i.d.: 1.8 mm

Carrier gas: Helium

Column temperature: isothermal 145°C

Injection port temperature: 160°C

9.3.2 MS conditions:

Accelerating voltage: 8 KV

Trap current: 100 µA

Electron beam energy: approximately 70 eV

Multiplier voltage: -2KV

9.3.3 Procedure:

9.3.3.1 Introduce perfluoro-tri-*n*-butylamine, as a reference compound, into the MS via the cold inlet system.

9.3.3.2 Set the MS to monitor the NDMA parent ion, using the reference ion at m/e 74 and the mass ratio, as shown in Notes on Procedure, 13.2.

9.3.3.3 Display on oscilloscope in peak-matching mode.

9.3.3.4 Adjust the carrier gas flow-rate to 6 ml/min.

9.3.3.5 Inject a 5 µl aliquot of the mixed standard *N*-nitrosamine solution (a); vent solvent for 5 min.

9.3.3.6 When NDMA elutes from the GC, record the maximum peak height of the parent ion observed on the MS oscilloscope.

9.3.3.7 Reset the MS magnet current to monitor the parent ion of NDEA, using the data in Notes on Procedure, 13.2, and record the height of this peak.

9.3.3.8 One minute after the elution time of NDEA, increase the GC gas flow-rate to 25 ml/min, using the programming unit.

9.3.3.9 Reset the MS to monitor the parent ions of NDPA, NDBA, NPIP and NPYR, recording the peak heights, as before.

9.3.3.10 Repeat procedure 9.3.3 with 5 µl of the food extract (9.1.12) in step 9.3.3.5.

9.3.3.11 Repeat procedure 9.3.3 with standard *N*-nitrosamine solution (a) to determine whether any change in sensitivity has occurred during the injection of the sample.

9.4 *GC-MS confirmation and quantification (System B)*

9.4.1 GC conditions:

Column: a) stainless steel, packed with 15% Carbowax 20M on 80/100 BS mesh Chromosorb W/AW, DMCS-treated. Length: 1.6 m i.d.: 1.8 mm

b) SCOT, containing Carbowax 20M
Length: 30 m i.d.: 0.5 mm

Columns a) and b) are connected in series, through two Carle microvalves, type 2011 P.

Carrier gas: Helium, 4 ml/min

Column temperature: isothermal 140°C

9.4.2 MS conditions:

Accelerating voltage: 8 KV

Trap current: 100 µA

Electron beam energy: 70 eV

Multiplier voltage: -2 KV

9.4.3 Procedure:

9.4.3.1 Introduce perfluoro-tri-*n*-butylamine, as a reference compound, into the MS via the cold inlet system.

9.4.3.2 Set the MS to monitor the parent ion of NDMA, using the reference ion at m/e 74 and the mass ratio as shown in Notes on Procedure, 13.2.

9.4.3.3 Display on oscilloscope in peak-matching mode.

9.4.3.4 Adjust carrier gas flow-rate to 4 ml/min.

9.4.3.5 Inject a 5 µl aliquot of the standard *N*-nitrosamine solution (a) on the GC and vent solvent for 2 min.

9.4.3.6 When the NDMA elutes from the GC, record the maximum peak height of the parent ion observed on the MS oscilloscope.

9.4.3.7 Reset the MS magnet current to monitor the parent ions of NDEA and NDPA, using the data of Notes on Procedure, 13.2. Record the heights of these peaks.

9.4.3.8 Three minutes after the elution of NDPA, switch the microvalves to bypass the SCOT column.

9.4.3.9. Reset the MS magnet current to monitor the parent ions of NPIP and NPYR.

9.4.3.10 Repeat procedure 9.4.3 with 5 µl of the food extract (9.1.12) in step 9.4.3.5.

9.4.3.11 Repeat procedure 9.4.3, with the standard *N*-nitrosamine solution (a).

10. METHOD OF CALCULATION

10.1 The content, Z_x, of *N*-nitrosamine, x, in the test sample is

$$Z_x = \frac{10^5 V C_x}{M R_x} \ \mu g/kg$$

where

C_x = Concentration of *N*-nitrosamine, x, in concentrated extract (9.1.12) (measured by MS calibrated with standard solution) (µg/ml)

V = Volume of the concentrated extract (9.1.11) (ml)

M = Mass of sample (g)

R_x = Recovery of *N*-nitrosamine x (%)

10.2 *Correction factor for recovery* R_x

$$R_x = \frac{\left(\begin{matrix}\text{Actual recovery of} \\ \text{internal standard (NDPA)}\end{matrix}\right)\left(\begin{matrix}\text{Mean recovery of} \\ \text{N-nitrosamine x}\end{matrix}\right)}{\left(\begin{matrix}\text{Mean recovery of} \\ \text{internal standard (NDPA)}\end{matrix}\right)}$$

NOTE: This correction factors implies that the deviation (%) of the actual recovery from the mean recovery is the same in sign and magnitude for all the *N*-nitrosamines in a particular type of sample. It is also assumed that endogenou endogenous NDPA may be neglected.

11. RECOVERY

The actual recovery is the recovery of the 2.5 µg NDPA spike (9.1.3) from the sample of food and is given by $R_{NDPA} = (100 C_{NDPA} V / 2.5)\%$. The mean recovery of standard is the mean obtained from at least ten samples of the same type. The mean recovery of the *N*-nitrosamine (x) being measured is obtained from at least ten samples of the same type, spiked with the given *N*-nitrosamine. In our laboratory (section 15), some values for cured meats are:

N-nitrosamine	Mean recovery %
NDMA	68
NDEA	80
NDPA	80
NDBA	75
NPIP	82
NPYR	60

12. REPEATABILITY AND REPRODUCIBILITY

12.1 *Repeatability*

Repeatability of MS measurements: ± 10%

Repeatability of recovery measurements: ± 10%

With daily calibration checks, the quantitative results for *N*-nitrosamines in the food sample are repeatable to within ± 20%.

12.2 *Reproducibility*

No data available.

13. NOTES ON PROCEDURE

13.1 *Retention data*

N-Nitrosamine	Preliminary screening		GC-MS confirmation retention time	
	Retention time	Kovats index	System A	System B
NDMA	10 min	1312	11 min	7 min
NDEA	13 min	1390	15 min	9 min
NDPA	21 min	1523	20 min	15 min
NDBA	43 min	1687	30 min	—
NPIP	51 min	1715	32 min	21 min
NPYR	58 min	1738	36 min	26.5 min

13.2 *Mass spectral data*

N-nitrosamine	Molecular weight	Base peak (m/e)	Relative intensity of parent ion[a]	Reference mass	Ratio
NDMA	74.0480	74	100.00	73.9968	1.000692
NDEA	102.0793	102	100.00	100.9984	1.010702
NDPA	130.1106	70	24.73	130.9920	0.993272
NDBA	158.1419	84	14.74	156.9969	1.007293
NPIP	114.0793	42	91.26	113.9967	1.000725
NPYR	100.0637	41	92.86	99.9936	1.000701

[a] Intensity of base peak = 100

14. SCHEMATIC REPRESENTATION OF PROCEDURE

Mince sample

↓

Add 250 ml H_2O, 100 g NaCl, 2 tablets antifoam and 250 µl internal standard solution

↓

Steam distill (400 ml)

↓

Add 80 g NaCl and 4 ml 10 N - H_2SO_4 to distillate

↓

Extract with 4 x 40 ml CH_2Cl_2

↓

Combine extracts

↓

Wash combined extract with 70 ml 1.5 N - NaOH solution

↓

Dry organic layer on Na_2SO_4

↓

Concentrate to ≃ 250 µl

↓

Analyse by GC (Coulson detector)

↓

Confirm and quantify by GC-MS

15. ORIGIN OF THE METHOD

Laboratory of the Government Chemist
Cornwall House
Stamford Street
London, SE1 9NQ
UK

Contact point: Dr T.A. Gough

METHOD 7 – MEAT AND MEAT PRODUCTS – MASS SPECTROMETRIC DETERMINATION OF VOLATILE *N*-NITROSAMINES

R.W. STEPHANY, J. FREUDENTHAL & P.L. SCHULLER

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in meat and meat products.

The method has a limit of detection of 0.1-0.2 micrograms per kilogram (µg/kg), depending on mass spectrometer (MS) conditions.

2. REFERENCES

Cox, G.B. (1973) *J. Chromatogr.*, *83*, 471-481

Stephany, R.W., Freudenthal, J., Egmond, E., Granberg, L.G. & Schuiler, P.L. (1976) *J. Agric. Food Chem.*, *24*, 536-539

Stephany, R.W. (1977) *Proc. of the 2nd International Symposium on Nitrite in Meat Products, Zeist, The Netherlands*, 7-10 September 1976, PUDOC, Wageningen, pp. 239-248

3. DEFINITION

Volatile *N*-nitrosamine contents of meat and meat products: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

N-nitrosamines are steam distilled from a test portion of the sample and extracted from the acidified distillate with dichloromethane. The extract is washed with alkali and concentrated. *n*-Hexane is added to the extract during evaporation of the dichloromethane.

The concentrated extract is examined by gas chromatography/mass spectrometry (GC-MS).

5. HAZARDS

N-nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

All reagents must be of analytical purity.

Water	Distilled or equivalent purity
Dichloromethane	Distilled shortly before use
n-Hexane	
N-Nitrosamines	Obtained commercially and used without further purification
Sodium chloride	
Antifoam "S" tablets	Thompson & Capper Ltd, Liverpool, UK
Sodium sulfate	Anhydrous
Boiling chips	Pumice
Sulfuric acid 5.0 M	
Sodium hydroxide 1.5 M	

---

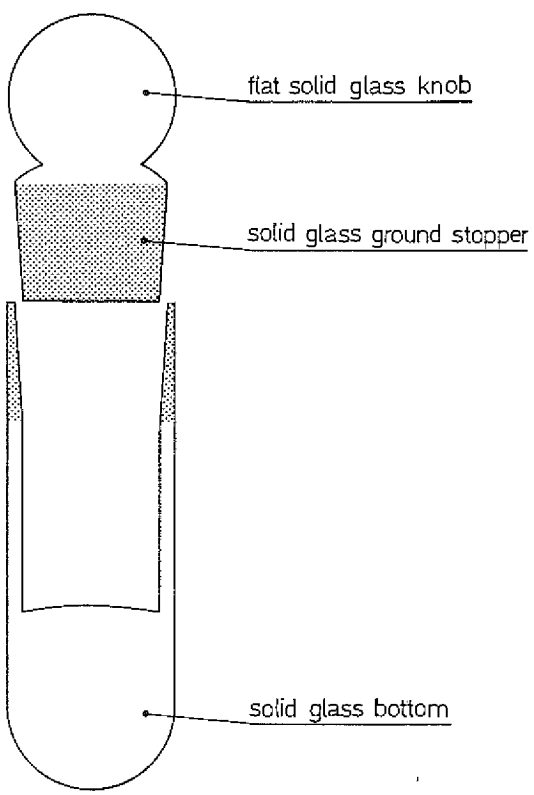
[1] Steere, N.V. (1974) Safety in the chemical laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. *Department of Health, Education and Welfare Publication (NIH)*, 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Helium	
Glass wool	
N-nitrosamines standard solutions	Solution A, 100 mg/l of each of the *N*-nitrosamines of interest in dichloromethane. Solution B, 10 mg/l of each of the *N*-nitrosamines of interest in dichloromethane. All the standard solutions are stored at 4°C in the dark. Under these conditions, it is recommended to renew the standard solutions twice a month. For the preparation of *N*-nitrosamines' standard solution A it is recommended to use a mini weighing bottle (Fig. 1) according to the prescription in Notes on Procedure 12.1.

FIG. 1. WEIGHING BOTTLE, MINIATURE TYPE



NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material, a test must be performed using the procedure described in section 9, but omitting the sample.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

Mechanical meat mincer	Laboratory size, fitted with a perforated plate with holes not exceeding 4 mm in diameter.
Meat chopper	Braun Minipimer
Distillation equipment	SVL-steam, 2000 ml vessel (Sovirel, cat. No. 4.296-65), with lid (No. 4.926-01) Heating device e.g., type Isomantle MBS 2L, Isopad Ltd.
Evaporative concentrator (K-D)	Kuderna-Danish evaporator K 570000, 250 ml Concentrator tube: 10 ml calibrated (Kontes Glass Company).
Reacti-vials	1 ml equipped with mininert valves (Pierce Chemical Company)
Gas chromatograph	Varian 1740
Mass spectrometer	Varian Mat CH 5 single-focussing or Varian Mat 731 double-focussing

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented; (see Chapter 1: Sampling and Samples).

---

[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

9. PROCEDURE

NOTE: *N*-Nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Test portion*

9.1.1 Weigh to the nearest 0.1 g, about 250 g (= m_1) of a twice-minced representative sample and transfer it quantitatively to the steam distillation vessel.

9.1.2 Add 100 g of sodium chloride to the test portion and sufficient water to bring the total amount of water (including the moisture in the test portion) to about 250 g.

9.1.3 Add two antifoam "S" tablets and homogenize thoroughly with the meat chopper. Rinse the meat chopper with a few ml of water and add rinsings to the distillation vessel.

9.2 *Steam distillation*

Steam distil until 400 ml of distillate are collected in a receptacle immersed in an ice-water mixture, adjusting the rate of distillation to maintain a constant volume in the distillation vessel.

9.3 *Solvent extraction of distillate*

9.3.1 Add 80 g of sodium chloride and 4 ml of 5 M sulfuric acid to the distillate. Shake to dissolve.

9.3.2 Transfer quantitatively into a 500 ml separation funnel fitted with a teflon stopcock. Extract with 40 ml of dichloromethane.

9.3.3 Repeat the extraction 3 times and combine the 4 extracts in a 500 ml separation funnel.

9.3.4 Wash the combined dichloromethane extracts with 70 ml of 1.5 M sodium hydroxide.

9.3.5 Collect the organic layer and dry it by adding approximately 10 g of anhydrous sodium sulfate and leaving to stand for at least 60 min. or overnight.

9.3.6 Filter the dry extract through glass wool in a K-D evaporator. Rinse the sodium sulfate with 10-20 ml dichloromethane and collect rinsings in the same K-D.

9.4 *Concentration of extract*

9.4.1 Immerse K-D evaporator in a 50°C water bath and concentrate filtrate to approximately 2 ml.

9.4.2 Cool the graduated tube of the K-D evaporator in a mixture of water and ice. Disconnect the concentrator and quickly add 0.8 ml of *n*-hexane to the cold extract.

9.4.3 Connect the concentrator again and continue the evaporation until 0.25 ml of extract remains. Cool to room temperature.

9.4.4 Measure the volume (V_1) of the extract with a graduated syringe (Hamilton or equivalent).

9.4.5 Transfer a measured volume (approximately half) of the extract into a reacti-vial (see Notes on Procedure, 12.2). If only non corrected *N*-nitrosamine contents are required, transfer all the extract in a reacti-vial

9.5 *Spiking of concentrated extract*

(Required only for the explicit determination of *N*-nitrosamine recoveries)

9.5.1 Transfer quantitatively the remaining part of the extract from 9.4.3 to a second reacti-vial.

9.5.2 Add 10.0 µl (V_5) of *N*-nitrosamine standard solution A.

9.5.3 Homogenize by smooth swirling. Final volume = V_3 (see Notes on Procedure, 12.2).

9.6 *Extraction of a spiked test portion*

9.6.1 Weigh to the nearest 0.1 g about 250 g (m_2) of the test sample and transfer quantitatively to the steam distillation vessel.

9.6.2 Carry out the procedure described in 9.1.2 and 9.1.3.

9.6.3 Using a graduated syringe (Hamilton or equivalent), add 250 µl (V_4) of *N*-nitrosamine standard solution B to the homogenized contents of the steam distillation vessel.

9.6.4 Homogenize again with the meat chopper. Rinse chopper into distillation vessel with a few ml of water.

9.6.5 Carry out the procedure as described in 9.2 to 9.4.3, inclusive.

9.6.6 Measure the volume (V_2) of the extract with a graduated syringe and transfer it into a third reacti-vial (see Notes on Procedure, 12.2).

9.7 *Gas chromatography - mass spectrometry quantification*

9.7.1 GC conditions:

Column: (a) All glass capillary coated with OV 101
Length: 34 m i.d.: 0.45 mm

(b) All glass capillary coated with UCON 50 HB-5100
Length: 30 m i.d.: 0.45 mm

Carrier gas: Helium, 4 ml/min

Injector temperature: 250°C

Column temperature: As appropriate to *N*-nitrosamines analysed and column used. (See Notes on Procedure, 12.3 for typical values).

Injected volume: 0.2-1.0 µl

9.7.2 MS conditions:

Resolution: 4000 at 10% valley (Ref. 3)

Electron energy: 70 eV

Electron current: 200 µA

Pumping speed at the ion source is 400 l/sec with a turbo-molecular pump. The GC is directly coupled to the MS and the ion current signal is recorded on a 10 mV full-scale deflection recorder. The mass spectrometry is performed with single ion detection.

N-nitrosodimethylamine	(m/e)	= 74.048
N-nitrosodiethylamine	(m/e)	= 102.079
N-nitrosodibutylamine	(m/e)	= 84.081
N-nitrosopyrrolidine	(m/e)	= 100.064
N-nitrosopiperidine	(m/e)	= 114.079

9.8 *GC-MS procedure*

9.8.1 Determine accurately the GC retention time for a *N*-nitrosamine peaks with a stopwatch.

9.8.2 Measure the heights (x) of the MS peaks from the extract of the test portion (9.4.4) and from the spiked test portion (9.6.6)

9.8.3 Measure also the height of the MS peak from the spiked extract of test portion (9.5.3) if the recovery is required.

9.8.4 When a positive response (> 1 µg/kg) is found using GC column (a), confirm using GC column (b).

10. METHOD OF CALCULATION

10.1 N-*Nitrosamine content*

Calculate each *N*-nitrosamine content, T, to the nearest 0.1 µg/kg according to the equation

$$T = \left(\frac{x_1 V_1}{x_2 V_2 m_1 - x_1 V_1 m_2} \right) T_1 V_4 \quad \mu g/kg$$

where

- T = *N*-Nitrosamine content in µg *N*-nitrosamine per kg test sample
- x_1 = MS peak height per µl of extract of test portion (9.4.4)
- x_2 = MS peak height per µl of extract of spiked test portion (9.6.6) using the same column as for x_1
- V_1 = Volume of extract of test portion (9.4.4) in ml
- V_2 = Volume of extract of spiked test portion (9.6.6) in ml
- V_4 = Volume of *N*-nitrosamine standard solution B (9.6.3) in ml
- m_1 = Mass of test portion in kg (9.1.1)
- m_2 = Mass of spiked test portion in kg (9.6.1)
- T_1 = Concentration of *N*-nitrosamine standard solution B (mg/l)

10.2 *Recoveries*

Calculate for each *N*-nitrosamine the percentage recovery (R) to the nearest percent according to the equation.

$$R = 100 \left[\frac{x_2 V_2 V_1 \quad T_2 V_5}{x_3 V_3 V_1 (T_1 V_4 + T m_2) - x_2 V_2 (V_3 - V_5) T m_1} \right]$$

where

- T_2 = Concentration of standard solution A in mg/l
- x_3 = MS peak height per µl spiked extract of test portion 9.5.3
- V_3 = Volume of spiked extract of test portion (9.5.3) in ml
- V_5 = Volume of *N*-nitrosamine standard solution A in ml

The other symbols are defined in 10.1.

11. REPEATABILITY AND REPRODUCIBILITY

11.1 *Repeatability*

The difference between the results of two complete determinations, carried out in rapid succession by the same analyst, should not be greater than 20% of the *N*-nitrosamine contents.

11.2 *Reproducibility*

No data available.

12. NOTES ON PROCEDURE

12.1 *Preparation of* N-*nitrosamines' standard solution A*

Weigh the empty stoppered mini weighing bottle to the nearest 0.1 mg. Pipette 10 µl of *N*-nitrosamine in this bottle using a disposable micropipette, stopper immediately and reweigh to the nearest 0.1 mg. Unstopper bottle in the neck of a one mark wide neck volumetric flask already filled up with dichloromethane and drop bottle and stopper into the solvent. Shake to homogenize.

12.2 If immediate GC/MS analysis of the extracts is not possible, the reacti-vials containing the extracts should be stored at -70°C.

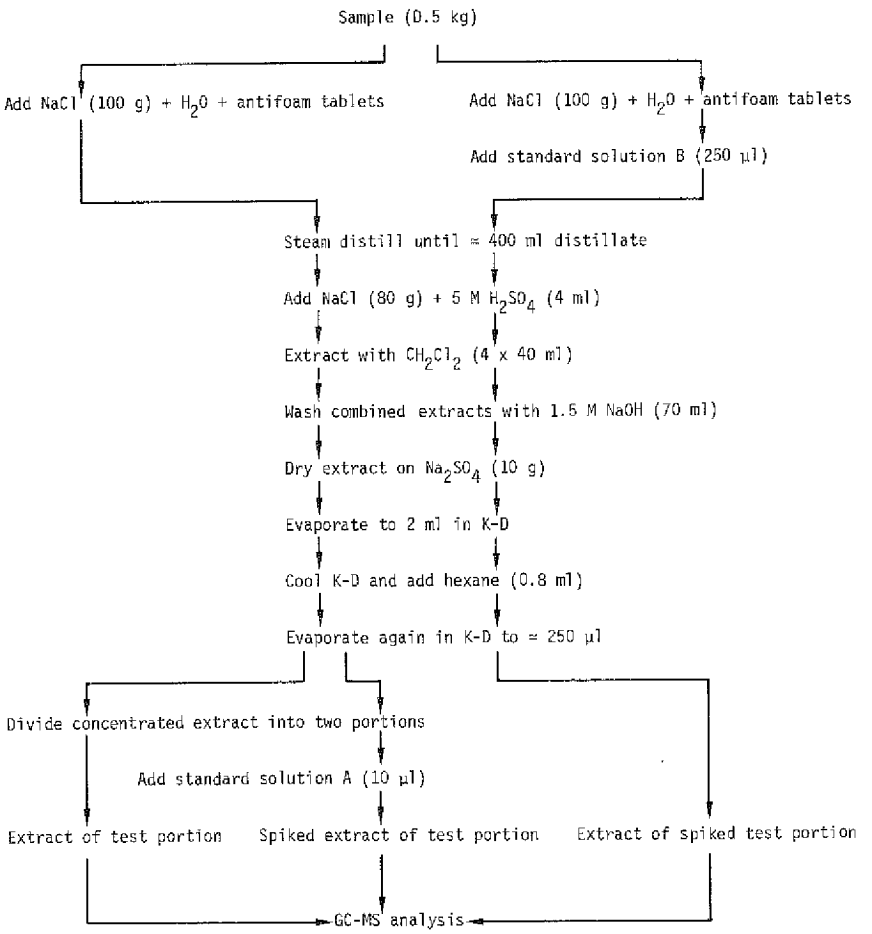
12.3

Compound	Ucon 50 HB-5100 column (b)		OV-101 column (a)	
	Retention time	T, °C	Retention time	T, °C
N-nitrosodimethylamine	2 min 47 sec	70	1 min 42 sec	100
N-nitrosodiethylamine	4 min 48 sec	70	2 min 20 sec	100
N-nitrosodibutylamine	6 min 20 sec	110	5 min 49 sec	125
N-nitrosopyrrolidine	5 min 04 sec	70	2 min 40 sec	125
N-nitrosopiperidine	4 min 40 sec	110	2 min 26 sec	140

For each *N*-nitrosamine to be determined a separate set of GC-MS runs (9.8.2 and eventually 9.8.3) is required (see Reference 2 and 3).

13. SCHEMATIC REPRESENTATION OF PROCEDURE

SCHEMATIC REPRESENTATION OF PROCEDURE



14. ORIGIN OF THE METHOD

Rijks Instituut voor de Volksgezondheid
Antonie van Leeuwenhoeklaan 9
Postbus 1
Bilthoven
The Netherlands

Contact point: Dr R.W. Stephany or Dr P.L. Schuller

METHOD 8 – MASS SPECTROMETRIC DETERMINATION OF VOLATILE *N*-NITROSAMINES IN FOOD

G.M. TELLING, D.R. HOAR & T.A. BRYCE

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in meat products, raw and cooked bacon, fats and oils, raw meats, a wide range of raw vegetables and various dishes consisting of vegetables cooked with cheese, eggs, etc. It has also been applied successfully to fish products, with some alteration of the method.

This method has a limit of detection of 1-10 micrograms per kilogram (µg/kg) in a 250 g sample, depending on mass spectrometer (MS) conditions.

2. REFERENCES

Bryce, T.A. & Telling, G.M. (1972) *J. Agric. Food Chem.*, *20*, 910-911

Telling, G.M., Bryce, T.A. & Althorpe, J. (1971) *J. Agric. Food Chem.*, *19*, 937-940

Telling, G.M., Bryce, T.A., Hoar, D., Osborne, D. & Weti, D. (1974) *IARC Scientific Publications No. 9*, 12-17

3. DEFINITION

Volatile *N*-nitrosamine contents of meat and other products (see Section 1): the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

N-Nitrosamines are distilled from an alkaline suspension of the sample to which a suitable internal standard has been added. The distillation is performed at 60°C under reduced pressure for $1\frac{1}{2}$ hours and at 75°C for a further $\frac{1}{2}$ hour. The distillate is extracted with dichloromethane and the extract is concentrated to 1 ml. After transfer into 0.25 ml *n*-hexane, the sample is examined by gas-liquid chromatography (GC)/high-resolution mass spectrometry (MS).

Use of a steam distillation technique under normal pressure is preferable for analysis of fish products (see Special Case, section 14).

5. HAZARDS

N-Nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

Dichloromethane	Analytical grade, redistilled by taking $2\frac{1}{2}$ l of solvent in a suitable flask, discarding the first 50 ml distillate, collecting the next 2100 ml and discarding the last fraction.

---

[1] Steere, N.V. (1974) Safety in the Chemical Laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] *National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens*. Department of Health, Education and Welfare Publication (NIH), 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

n-hexane	Spectrosol grade or equivalent
Perfluorotributylamine	As supplied for mass spectrometry
Sodium chloride	Analytical grade
Potassium carbonate	Analytical grade
N-nitrosamine standard solutions:	Solution A, 10 mg/l of each of the *N*-nitrosamines of interest (including the internal standard) in dichloromethane. Solution B, 1 mg/l of a *N*-nitrosamine suitable for use as an internal standard[1] (NDEA or NDBA have been found to be suitable for a wide range of samples) in distilled water.

NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material, a test must be performed using the procedure described in section 9, but omitting the sample.

7. APPARATUS[2]

Usual laboratory equipment and the following items:

Distillation apparatus	As shown in Figure 1 using joints fitted with FIVAC teflon sleeves
Separation funnels	500 ml with teflon stoppers and taps
Evaporator (K-D)	Kuderna-Danish 500 ml capacity Concentrator tube calibrated to 250 µl
Gas chromatograph/Mass Spectrometer	Pye 104 (or equivalent) coupled by a Watson-Biemann fritted-glass separator to a mass spectrometer (e.g., A.E.I. MS 902) capable of peak switching at a resolution of 11-12000 and using an oscilloscope fitted with a graticule.

---

[1] A suitable *N*-nitrosamine for this purpose is one that is present in negligible amounts in the sample to be analysed.

[2] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

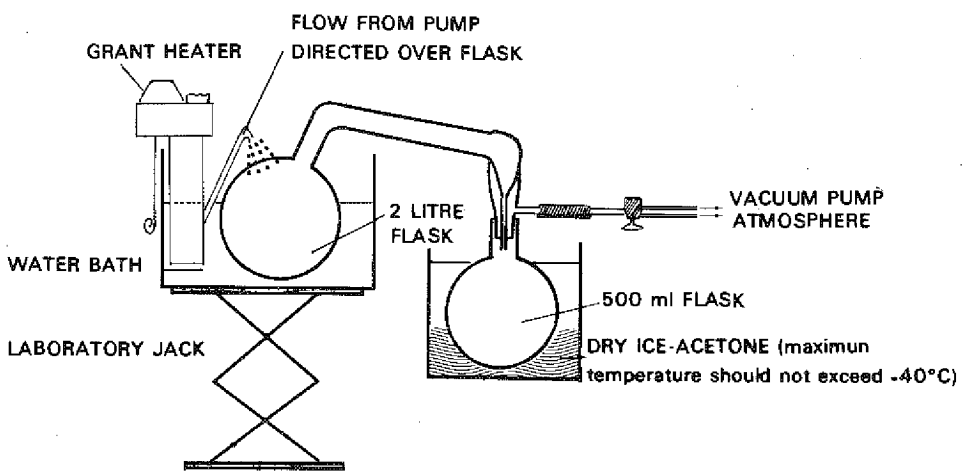
8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented (see Chapter 1, Sampling and Samples).

9. PROCEDURE

NOTE: *N*-Nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

FIG. 1. MODIFIED APPARATUS FOR DISTILLATION OF NITROSAMINES



9.1 *Distillation of alkaline suspension*

9.1.1 Weigh 250 g of a representative sample into a 2000 ml round-bottomed flask.

9.1.2 Add 250 ml water, 50 g sodium chloride and 10 g potassium carbonate. Mix well.

9.1.3 Add 2.5 ml of a 1 mg/l solution of internal standard (*N*-nitrosamine standard solution B, normally NDEA or NDBA).

9.1.4 Connect the flask to the distillation bridge. Place in water bath held below 10°C.

9.1.5 Apply vacuum until sample froths gently.

9.1.6 Heat water bath to 60°C.

9.1.7 Apply vacuum periodically to maintain boiling.

9.1.8 When side-arm is dry (≈ 1½ hours), raise temperature of water bath to 75°C.

9.1.9 Continue distillation for a further 30 min.

9.1.10 Release vacuum and measure the volume of distillate (see notes on procedure, 13.1).

9.1.11 Wash condenser, adding washings to distillate.

9.2 *Solvent extraction of distillate*

9.2.1 Transfer distillate + washings to a 500 ml separation funnel.

9.2.2 Add 250 ml dichloromethane and shake well.

9.2.3 Transfer dichloromethane layer to a 500 ml K-D evaporator.

9.2.4 Add 250 ml dichloromethane to aqueous layer remaining in separation funnel. Shake well.

9.2.5 Transfer dichloromethane layer to the same K-D.

9.3 *Concentration of extract*

9.3.1 Add 1 anti-bumping granule and fit a cone to the K-D.

9.3.2 Evaporate solvent in 60°C water bath, ensuring that the level of solvent in the evaporator is always above the level of water in the bath.

9.3.3 When volume of solvent is less than 20 ml, remove cone and continue evaporation.

9.3.4 When volume of solvent is less than 5 ml, remove evaporator bulb and replace with Quickfit cone.

9.3.5 Continue evaporation until solvent volume is 1 ml.

9.3.6 Remove tube from water bath and add 0.5 ml *n*-hexane.

9.3.7 Continue evaporation until volume is 0.25 ml.

9.3.8 Stopper tube and retain for GC-MS analysis.

9.4 *GC-MS analysis*

9.4.1 GC conditions:

Column: 10% Carbowax 20 M on 100/120 mesh Celite
Length: 5.5 m I.D.: 4 mm

Carrier gas: Helium at 2.2 bars (32 psi) inlet pressure

Column temperature: 120-200°C at programmed rate of 2°C/min.

Under these conditions the relative retention times are as follows:

NDMA: 1.00; NDEA: 1.35; NDPA: 2.00; NDi-BA: 2.15; NDBA: 2.90; NPIP: 3.05; NPYR: 3.15.

9.4.2 MS conditions and procedure:

Follow the appropriate instructions laid down in the users' manual of the instrument.

Filament current 0.9A (A.E.I. MS 902)

Electron energy 70 eV

9.4.2.1 Inject the reference compound (perfluorotributylamine) into the gallium inlet.

9.4.2.2 Using the nominal m/e 69 peak of the reference compound, set the resolution to 11-12000.

9.4.2.3 Find the reference peak at nominal m/e 70 for the determination of *N*-nitrosodiemthylamine (NDMA).

9.4.2.4 Display the peak on the lower mass trace on the oscilloscope screen.

9.4.2.5 Put the Mass Ratio controls to the correct ratio for monitoring NDMA on the second (higher mass) trace on the oscilloscope screen. The relevant data for the various *N*-nitroso compounds are:

Compound		Molecular or fragment ion	Reference ion (m/e)	Mass ratio
N-Nitrosodimethylamine	(NDMA)	74.048010	69.99857	1.057850
N-Nitrosodiethylamine	(NDEA)	102.079308	99.99361	1.020858
N-Nitrosodipropylamine	(NDPA)	130.110607	118.99201	1.093440
N-Nitrosodi-iso-butylamine	(NDi-BA)	84.081320	80.99521	1.038102
N-Nitrosodibutylamine	(NDBA)	84.081320	80.99521	1.038102
N-Nitrosopiperidine	(NPIP)	114.079308	113.99668	1.000725
N-Nitrosopyrrolidine	(NPYR)	100.063659	99.99361	1.000701

See Notes on Procedure, 13.2

9.4.3 GC-MS procedure:

9.4.3.1 Set the column temperature to 120°C. Ensure that the valve between interface and spectrometer is closed. Turn the source high tension and electron beam supplies off. Turn the accelerating voltage down to 2 KV.

9.4.3.2 Inject 20 µl of *N*-nitrosamine standard solution A into GC.

9.4.3.3 Start the GC temperature programme at 2°C/min up to 200°C.

9.4.3.4 When the solvent peak has been eluted, open the valve between the interface and the MS (split ratio 5:1 in favour of MS).

9.4.3.5 Turn on the source high tension and electron beam supplies and turn the accelerating voltage to 8 KV. Check the appearance of the reference peak at nominal m/e 70 on the oscilloscope trace.

9.4.3.6 Watch for the appearance of NDMA on the higher mass trace and take a reading "x" of the peak height with reference to the graticule.

9.4.3.7 Find reference peak m/e 100 and adjust mass ratio for NDEA.

9.4.3.8 Read height of NDEA peak on oscilloscope screen.

9.4.3.9 Repeat 9.4.3.7 and 9.4.3.8 for each of the remaining *N*-nitrosamines in turn.

9.4.3.10 Reset the spectrometer for NDMA, turn down accelerating voltage to 2 KV and turn off source high tension and electron beam supplies.

9.4.3.11 Turn off valve between interface and spectrometer.

9.4.3.12 Cool the GC column to 100°C.

9.4.3.13 When the chromatograph is stable, inject 25 µl of test solution (9.3.8) and repeat stages 9.4.3.3 to 9.4.3.12 (see Notes on Procedure, 13.3).

10. METHOD OF CALCULATION

10.1 *Calculation of* N-*nitrosamine (NA) content (assuming 100% recovery)*

X = Peak height of NA in standard solution A (9.4.3.2)
Y = Peak height of NA in concentrated test solution (9.3.8)
V_1 = Volume of standard solution A injected = 20 µl (9.4.3.2)
C = Concentration of standard solution A = 0.01 µg/µl
V_2 = Volume of concentrated test solution injected = 25 µl (9.4.3.13)

C_2 = Concentration of test solution injected
V_3 = Total volume of concentrated test solution = 250 µl (9.3.7)

Thus, amount of NA in 250 g sample = C_2V_3, and

Z = NA content in 1 kg of sample = $4\ C_2V_3$

$$= 4\left(\frac{YC_1V_1}{XV_2}\right)V_3$$

For the values of C and V given above,

$$Z = \frac{8Y}{X}\ \mu g/kg$$

10.2 *Correction factor for incomplete recovery*

When a suitable internal standard has been added (see 9.1.3), the corrected NA content, T, may be calculated as follows:

for a given *N*-nitrosamine, NA_i,

$$T_i = Z_i f_i$$

where the correction factor is,

$$f_i = \left(\frac{\text{mean \% recovery of internal standard}}{\text{\% recovery of internal standard}}\right)\left(\frac{100}{\text{mean \% recovery of } NA_i}\right)$$

The % recovery of internal standard, R_s, is given by:

$$R_s = 100\left(\frac{YC_1V_1V_3}{XV_2C_sV_s}\right)$$

where X and Y are the peak heights of the internal standard in 9.4.3.2 and 9.3.8, respectively, and C_s and V_s are the concentration and volume of the internal standard added in 9.1.3. The other symbols are defined in 10.1.

11. RECOVERIES

Percentage recoveries at step 9.3.8 for a range of *N*-nitroso compounds added to meat at step 9.1.3 (10 µg/kg level) are as follows:

	Maximum	Mean	Minimum
N-nitrosodimethylamine	100	88	66
N-nitrosodiethylamine	106	90	76
N-nitrosodibutylamine	62	55	48
N-nitrosodi-isobutylamine	72	68	65
N-nitrosopyrrolidine	84	60	42

12. REPEATABILITY AND REPRODUCIBILITY

12.1 *Repeatability*

For a series of luncheon meat samples spiked at the 10 µg/kg level with 5 *N*-nitrosamines, the coefficients of variation are as follows (%):

	NDMA	NDEA	NDi-BA	NDBA	NPYR
Series 1	14.5	12.5	3	14.6	24.6
Series 2	7.1	11.8	4.7	11.9	14.2
Series 3	6.3	4.4	3.3	14.2	17.1

12.2 *Reproducibility*

No data available.

13. NOTES ON PROCEDURE

13.1 The recovery of NPYR depends on the volume of distillate collected.

For a non-aqueous sample, such as bacon fat, a minimum of 95% of the water added at step 9.1.2 should be recovered in the distillate.

For food samples which normally have a moisture content, all the water added at step 9.1.2 plus ≈ 30% of the nominal moisture content of the sample should be recovered in the distillate.

13.2 The MS902 instrument is only capable of single-ion monitoring so that manual resetting of the reference ion and mass ratio are necessary to monitor different *N*-nitrosamines as they elute. This resetting procedure takes a finite time and, when *N*-nitrosamines elute from the GC column with similar retention times, e.g., NDPA and NDi-BA, resetting of the instrument to a different reference ion is not possible. For extracts containing complex mixtures of *N*-nitrosamines, it is therefore necessary to perform repeat GC injections in order to analyse for all components using the fragment and reference ions given above.

Alternatively, by suitable choice of ions, it is possible to analyse for mixtures of *N*-nitrosamines in a single GC run. This is achieved for the above *N*-nitroso compounds by using the alternative fragment and reference ions listed below. Use of the less intense fragment ions at nominal m/e 115 in the spectra of NDi-BA and NDBA results in a 5x loss of sensitivity for these *N*-nitrosamines.

Compound	Molecular or fragment ion (m/e)	Reference ion (m/e)	Mass ratio
NDMA	74.048010	69.99857	1.057850
NDEA	102.079308	99.99361	1.020858
NDPA	130.110607	113.99668	1.141354
NDi-BA	115.087133	113.99668	1.009566
NDBA	115.087133	99.99361	1.150945
NPIP	114.079308	99.99361	1.140866
NPYR	100.063659	99.99361	1.000701

13.3 The GC conditions can be modified for different groups of *N*-nitroso compounds. For a mixture of NDMA, NDEA and NPYR, the GC is run isothermally at 130°C until after the elution of NDMA, when the column temperature is turned directly up to 205°C. The standard solution used in this case contains only 5 mg/l of each *N*-nitroso compound, as the sensitivity of the method is considerably increased.

14. SPECIAL CASE

For fish products, replace 9.1.1 to 9.1.11 by the following:

1. Weigh 250 g macerated or minced fish in a 2000 ml, 2-necked, round-bottomed flask.
2. Add 50 g sodium chloride, 10 g potassium carbonate and 50 ml de-ionized water.
3. Add 2.5 ml of a 1 µg/ml solution of a suitable internal standard (see section 6).

4. Connect the flask to the distillation apparatus.
5. Pass ice water through the condenser and cool the 500 ml receiver in an ice bath.
6. Start distillation, collect more than 200 ml.
7. Stop distillation.
8. Wash condenser, adding washing to the distillate.

15. SCHEMATIC REPRESENTATION OF PROCEDURE

Sample
↓
Add H_2O, NaCl, K_2CO_3 and internal standard
↓
Vacuum distill
↓
Extract distillate twice with 250 ml CH_2Cl_2
↓
Concentrate in K-D to 1 ml
↓
Add 0.5 ml *n*-hexane
↓
Concentrate to 0.25 ml
↓
GC/High-resolution MS analysis

16. ORIGIN OF THE METHOD

Unilever Research
Colworth Laboratory
Sharnbrook, Beds MD44 1LQ
UK

Contact point: Dr G.M. Telling

METHOD 9 – ANALYSIS OF VOLATILE *N*-NITROSAMINES IN FOOD BY ELECTRON CAPTURE AND COULSON DETECTION OF THEIR *N*-NITRAMINE DERIVATIVES

E.A. WALKER, M. CASTEGNARO & B. PIGNATELLI

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in meat, meat products, cooked dishes, food ingredients, bread and cereals, vegetables and animal foods. It has also been applied with some modifications to a large variety of alcoholic drinks with low or high alcohol content.

The method has a limit of detection of 1 microgram per kilogram (µg/kg) in a 50 g sample.

2. REFERENCES

Emmons, N.D. (1954) *J. Am. Chem. Soc., 76*, 3468-3470

Castegnaro, M. & Walker, E.A. (1976) *Proc. 2nd Symp. Nitrite in Meat Products, Zeist, The Netherlands*, 7-10 September 1976, PUDOC, Wageningen, pp. 187-190

Walker, E.A. & Castegnaro, M. (1976) *Natl Bur. Stand. (U.S.) Spec. Publ., 422*, Accuracy in trace analysis: sampling, sample handling and analysis. *Proc. 7th IMR Symp., 7-11 October 1974*, Gaithersburg, Md., pp. 727-736

Walker, E.A., Castegnaro, M. & Pignatelli, B. (1975) *Analyst, 100*, 817-821

Telling, G.M. (1972) *J. Chromatogr., 73*, 79-87

3. DEFINITION

Volatile *N*-nitrosamine contents in food: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

A minced sample is suspended in water saturated with sodium chloride. A cascade-type distillation in two stages (steam distillation from neutral solution and distillation from acid solution) is performed. The distillate is made alkaline and *N*-nitrosamines are extracted with dichloromethane. The extract is concentrated and the *N*-nitrosamines are transferred to 5 ml *n*-hexane. *N*-Nitrosamine purification and separation is then carried out by adsorption chromatography. *N*-Nitrosamines in the eluate (divided into a first and a second fraction) are oxidized to *N*-nitramines, which are extracted into dichloromethane. The two extracts are separately concentrated and the *N*-nitramines, in *n*-hexane, are separated by adsorption chromatography. Successive fractions are collected and analysed by gas chromatography, using electron-capture detection. Fractions giving peaks for *N*-nitramines are combined and *N*-nitramines are confirmed by gas chromatography with Coulson electrolytic-conductivity detection.

5. HAZARDS

N-nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

---

[1] Steere, N.V. (1974) Safety in the chemical laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. *Department of Health, Education and Welfare Publication (NIH)*, 75-900 (1975)

6. REAGENTS[1]

All chemicals should be of analytical grade.

Dichloromethane	Distilled from potassium carbonate in glass
n-hexane	
n-pentane	Distilled in glass
Diethyl ether	Dried with sodium
Trifluoroacetic acid	Distilled in glass
Hydrogen peroxide	50% Koch Light or 85% Air Liquide
Anti-bumping granules	Silicon carbide
Sodium chloride	
Potassium hydroxide	
Sodium sulfate	
Tartaric acid	
Aluminium oxide	60 Active, Basic (type E), Merck ref. 1067 or Prolabo equivalent
Aluminium oxide	90 Active, Neutral Merck ref. 1077
Potassium carbonate	≈ 20% solution in distilled water
N-Nitrosamine standard solutions	Solution (a); contains 50 mg/l of each of the *N*-nitrosamines of interest in dichloromethane. Solution (b); 50 mg/l of NDPA (internal standard) in dichloromethane. A more concentrated stock solution containing 500 mg/l of each *N*-nitrosamine may be employed and diluted 1 → 10 as required. Stock solution is generally renewed twice a month.

NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material a test must be performed using the procedure described in section 9, but omitting the sample.

---

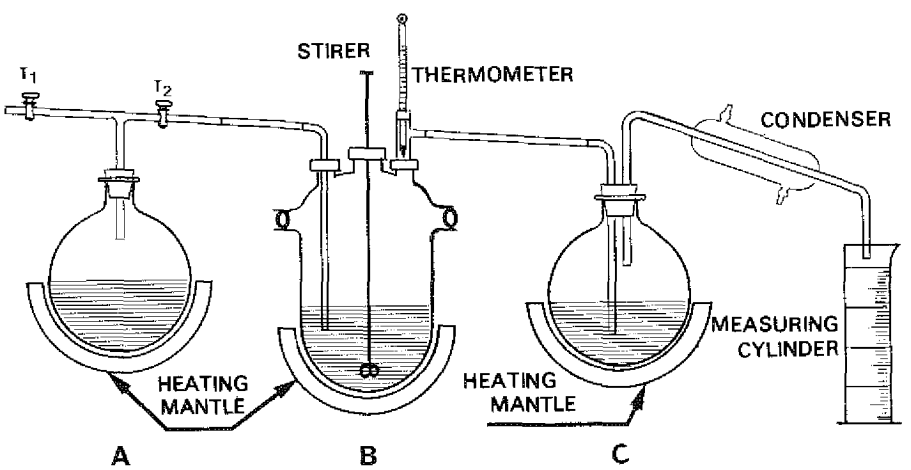
[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

Distillation	Standard Sovirel equipment, parts No. 4296-54 and 4296-01 modified with an SVL 22 with corresponding joints (See Fig. 1, part B).
Evaporative concentrator (K-D)	Kuderna Danish flask (250 ml), graduated concentrator tube (2.5 ml), Snyder column (3 sections).
Gas chromatographs	Pye 104, fitted with electron-capture detector (Ni63, 10 mCi) Tracor, fitted with Coulson electrolytic-conductivity detector (reductive mode).

FIG. 1



[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented (see Chapter 1, Sampling and Samples).

9. PROCEDURE

NOTE: *N*-Nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Distillation, extraction and concentration*

9.1.1 Weigh 50 g of a representative sample and add to 200 ml water and 84 g sodium chloride in flask B (Fig. 1). Add 10 µl internal standard solution (b).

9.1.2 To flask C (Fig. 1), add 200 ml water, 3 g tartaric acid and 84 g sodium chloride.

9.1.3 Open T_1 (Fig. 1) and regulate heat to flask A (containing 200 ml of water) for continuous steady boiling. At the same time start to heat flasks B and C.

9.1.4 Close T_1, open T_2 (Fig. 1) and adjust rate of boiling in B and C to maintain the liquid level constant in each flask.

9.1.5 Collect 250 ml of distillate in a 250 ml cylinder (D, Fig. 1).

9.1.6 To the distillate, add 14 g potassium hydroxide, shake and leave to stand for half an hour.

9.1.7 Transfer the solution to a 500 ml separation funnel, add 100 ml redistilled dichloromethane, shake for 3 min and allow to separate.

9.1.8 Collect dichloromethane layer in a 500 ml separation funnel.

9.1.9 Extract alkaline distillate with 2 further 100 ml portions of dichloromethane.

9.1.10 Combine the dichloromethane extracts in the 500 ml separation funnel (9.1.8), add 15-20 g anhydrous sodium sulfate and shake for 1 min.

9.1.11 Collect dried dichloromethane extract in a 500 ml round-bottomed flask. Wash the sodium sulfate with 20 ml dichloromethane and combine with the extract.

9.1.12 Concentrate the combined extracts to approximately 10 ml in a K-D on a water bath at 50°C. Add 6 ml *n*-hexane and further concentrate to approximately 4 ml.

9.1.13 Adjust to 5 ml with *n*-hexane.

9.2 *Adsorption chromatography of* N-*nitrosamines*

9.2.1 Prepare an adsorption chromatography column in accordance with the instructions given in Notes on Procedure (13.1).

9.2.2 Add the *n*-hexane solution (9.1.13) to the top of the column and run off the liquid from the bottom until the upper liquid level coincides with the upper surface of the column (5 ml of liquid are collected).

9.2.3 Elute the column successively with 25 ml of a 25% solution of diethyl ether in *n*-pentane, 15 ml of a 50% solution of diethyl ether in *n*-pentane, 15 ml of a 75% solution of diethyl ether in *n*-pentane and finally with 15 ml 100% diethyl ether.

9.2.4 Collect separately the first 25 ml (fraction A) and the succeeding 55 ml (fraction B).

9.3 *Oxidation of* N-*nitrosamines to* N-*nitramines*

9.3.1 To each fraction A and B, add a mixture containing 9 ml trifluoracetic acid and 7 ml hydrogen peroxide (85% or 50%)(see Notes on Procedure, 13.2).

9.3.2 Agitate $3\frac{1}{2}$ hours in a shaker.

9.3.3 Cool the solution in an ice bath and by dropwise addition of ≃ 150 ml of a 20% solution of potassium carbonate, adjust the pH to between 10 and 11.

9.3.4 Transfer to a 250 ml separation funnel and extract with 50 ml dichloromethane.

9.3.5 Repeat the extraction with a further volume of 50 ml dichloromethane and combine the extracts.

9.3.6 Transfer the combined extracts to a 250 ml separation funnel and add approximately 15 g anhydrous sodium sulfate.

9.3.7 Shake vigorously for 1 min and run the dichloromethane layer into a 250 ml beaker, wash the sodium sulfate with 20 ml dichloromethane and combine the washing with the previous extracts.

9.3.8 Reduce the volume of the combined solution to about 7 ml on a water bath at 50°C.

9.3.9 Add 2 ml *n*-hexane and further reduce the volume to about 1 ml, taking care not to reduce the volume below 0.5 ml.

9.3.10 Adjust the volume to 5 ml with *n*-pentane.

9.4 *Adsorption chromatography of* N-*nitramine extract*

9.4.1 Prepare an adsorption chromatography column as indicated in Notes on Procedure, 13.1.

9.4.2 Add the *n*-pentane solution (9.3.10) to the column and run off solvent until the upper level coincides with the upper surface of the column.

9.4.3 Elute the column successively with 25 ml 5% diethyl ether in *n*-pentane, 30 ml 25% diethyl ether in *n*-pentane, 10 ml 80% diethyl ether in *n*-pentane and 10 ml 100% diethyl ether. Discard the first 20 ml then collect in 5 ml fractions. Retain for GC analysis.

9.5 *Preparation of standard* N-*nitramines solution*

9.5.1 With 10 µl of *N*-nitrosamine standard solution (a), carry out procedures 9.2.1 to 9.4.3. Retain the separate 5 ml fractions for GC analysis.

9.5.2 After GC analysis of individual fractions, (9.6.1-9.6.2.1) combine all 5 ml fractions showing a positive peak for *N*-nitramines and concentrate (water bath, 50°C) to 5 ml.

9.6 *Gas chromatographic quantification of* N-*nitramines*

9.6.1 Gas chromatographic conditions:

Column: glass packed with 10% Carbowax 20 M on Chromosorb W, 80/100 mesh.
Length: 3 m i.d.: 4 mm

Carrier gas: Nitrogen (high purity) 50 ml/min

Scavenge flow on detector: Nitrogen (high purity) 10 ml/min

Injection port and column temperature: isothermal 140°C

Detector temperature: 210°C

9.6.2 Procedure:

9.6.2.1 Carry out GC analysis with 5 µl aliquots of each of the fractions 1-6 from fraction A and with each of the fractions 3-10 from fraction B (see section 15) (These fractions and the distribution of the *N*-nitramines may not correspond exactly. The distribution of *N*-nitrodipropylamine gives a useful indication).

9.6.2.2 Combine all 5 ml fractions showing a positive peak for *N*-nitramines. Concentrate (water bath, 50°C) to 5 ml.

9.6.2.3 Inject 5 µl of concentrate from 9.6.2.2 on GC. Measure peak height, h_x, for each *N*-nitramine, x.

9.6.2.4 Inject 5 µl of concentrate standard solution from 9.5.2. Measure peak height, h_{sx}, for each *N*-nitramine, x.

9.7 *GC-Coulson detector confirmation of* N-*nitramines*

9.7.1 Gas chromatographic conditions:

Column: glass packed with 15% FFAP on Chromosorb W 80/100 mesh, DMCS-treated.
Length: 2 m i.d.: 4 mm

Carrier gas: Argon (high purity) 50 ml/min

Injection port temperature: 160°C

Column temperature: isothermal 140°C

Transfer line temperature: 200°C

9.7.2 Coulson detector operating conditions:

Gas reactant: hydrogen (high purity) 80 ml/min

Scavenge flow: 50 ml/min

Block temperature: 200°C

Pyrolyser temperature: 620°C

Pyrolysis tube: quartz (i.d.: 1 mm; o.d.: 6.2 ml) connected to the cell with Teflon tubing (i.d.: 0.5 mm)

9.7.3 Procedure:

9.7.3.1 Concentrate sample from 9.6.2.2 to 500 µl. Inject 10-20 µl.

9.7.3.2 Vent out solvent peak (≃ 4 min).

9.7.3.3 Record chromatogram.

9.7.3.4 Concentrate standard solution from 9.5.2 to 500 µl. Inject 10 µl. Vent out solvent peak and record chromatogram.

9.7.3.5 Confirm identity of peaks obtained with unknown (9.6.2.2) by comparing retention times with those obtained with standard (9.5.2).

10. METHOD OF CALCULATION

10.1 *Content of N-nitrosamine*

Calculate the content of *N*-nitrosamine, x, in µg per kg of test sample as follows:

$$Z_x = \frac{10^5 CV}{MR_x}\left(\frac{h_x}{h_{sx}}\right)$$

where

C = Concentration of *N*-nitrosamine in standard solution (a) (µg/ml)

V Volume of standard solution (a) (9.5.1) (ml)

h_x Peak height of *N*-nitramine, x, from 5 µl of sample 9.6.2.2 (mm)

h_{sx} Peak height of *N*-nitramine, x, from 5 µl of standard 9.5.2 (using same attenuation as for h_x) (mm)

R_x Recovery of *N*-nitrosamine (nitramine) x (%)

M Mass of sample (9.1.1) (g)

10.2 *Actual recovery of internal standard (NDPA)*

Since the amount of the NDPA spike (0.5 µg) in 9.1.1 is the same as the amount of NDPA in the standard (9.5.1), and the final volumes of concentrate (5 ml) and the injected volumes (5 µl) are the same in both cases, the actual recovery of NDPA at step 9.1.13 is given simply by

$$R_{NDPA} = 100\left(\frac{h_x}{h_{sx}}\right) \%, \text{ where x = NDPA}$$

(It is assumed that the endogenous NDPA content of the sample is negligible.)

10.3 *Recovery, R_x, to be applied in 10.1*

$$R_x = \frac{R_{NDPA} \; \langle R_x \rangle}{\langle R_{NDPA} \rangle} \; \%$$

where

$\langle R_x \rangle$ = Mean recovery of *N*-nitrosamine, x, for the given type of sample

$\langle R_{NDPA} \rangle$ = Mean recovery of NDPA for the same type of sample.

NOTE: The foregoing calculation assumes that the recovery after adsorption chromatography (9.2), the oxidation yield (9.3) and the recovery after chromatography (9.4) are identical for a given *N*-nitrosamine (nitramine) in the sample and in the standard solution (a). It is assumed, in addition, that, for any particular analysis,

$$\frac{R_x}{\langle R_x \rangle} = \frac{R_{NDPA}}{\langle R_{NDPA} \rangle}$$

These assumptions have proved to be sufficiently valid for the sample types covered by Scope and Field of Application (1).

11. REPEATABILITY AND REPRODUCIBILITY

11.1 *Repeatability*

Standard deviation at 10 µg/kg level should not exceed 1 µg/kg.

11.2 *Reproducibility*

No data available for reproducibility

12. RECOVERY

For the sample types covered by scope and field of application (1), the recoveries are 85 ± 15% for NDMA, NDEA, NDPA, NDBA, NMAA and NPIP, and 55 ± 15% for NPYR.

13. NOTES ON PROCEDURE

13.1 *Adsorption chromatography of* N-*nitrosamines and* N-*nitramines*

Preparation of the column: Wash 100 g of basic and neutral alumina with a 500 ml portion of diethyl-ether in *n*-pentane and two 500 ml portions of diethyl ether, then activate for 3 hours at 240°C. Prepare basic alumina by addition of 1.9 g distilled water per 100 g dried alumina and leaving to equilibrate overnight under gentle shaking. Similarly, prepare neutral alumina by addition of 6 g distilled water per 100 g alumina. Just cover three grams of the basic alumina with *n*-pentane and wash by gentle agitation for half an hour. Add the washed alumina to the glass column and run off solvent (*n*-pentane) from the bottom of the column until the top of the solvent layer is level with the upper surface of the alumina. Repeat the above procedure with 3 g neutral alumina. Finally, add 1 g of anhydrous sodium sulfate to the top of the column. The column is then ready for use. Use freshly prepared columns for each separation.

13.2 *A comparison of conversion yields of* N-*nitrosamines to* N-*nitramines using 85% and 50% hydrogen peroxide (procedure 9.3.1 and 9.3.2, using 0.5 µg of each* N-*nitrosamine in 100 ml, 50 ml or 20 ml of dichloromethane)*[a]

	Conversion (%)[a]						
	85% hydrogen peroxide				50% hydrogen peroxide		
	Solvent volume (mls)				Solvent volume (mls)		
	0[b]	100	50	20	100	50	20
NDMA	82	81	79	83,5	58	62	58
NDEA	84	86	84	85,5	107	75	74
NDPA	87	106	90	88,5	87	84,5	74
NDBA	86	81	81	83	81	81	70
NPNA	91	93	91	89	81	80	72
NPIP	52	57	53	47	83	74	68
NPYR	88	83	88	88	98	93	93

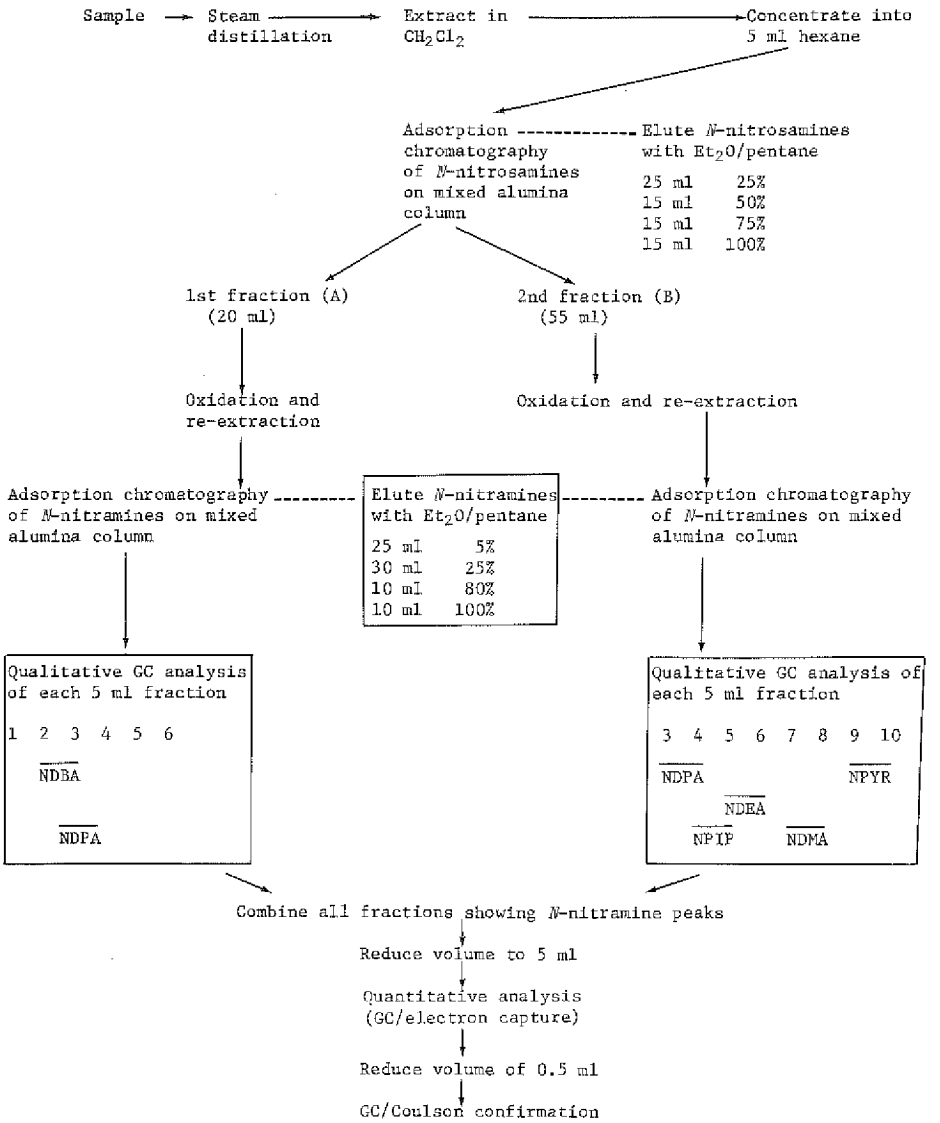
[a] Similar results have been obtained using varying amounts of diethyl ether in pentane.

[b] Using the method of G.M. Telling (1972).

14. SPECIAL CASE

For analysis of volatile *N*-nitrosamines in alcoholic beverages, step 9.2 is omitted and step 9.1 is replaced by extraction of the spirit (saturated with magnesium perchlorate) with 3 x 50 ml dichloromethane. The oxidation is performed on the extract, concentrated to approximately 100 ml.

15. SCHEMATIC REPRESENTATION OF PROCEDURE



16. ORIGIN OF THE METHOD

Unit of Environmental Carcinogens
International Agency for Research on Cancer
150 cours Albert-Thomas
69372 Lyon
France

Contact point: Mr E.A. Walker

METHOD 10 – ANALYSIS OF VOLATILE *N*-NITROSAMINES IN MEAT PRODUCTS USING OXIDATION OF *N*-NITROSAMINES TO *N*-NITRAMINES AND ELECTRON-CAPTURE DETECTION OF *N*-NITRAMINES

S.J. KUBACKI & A. BORYS

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in meat and meat products.

The method has a limit of detection of 1 microgram per kilogram (µg/kg) in a 250 g sample.

2. REFERENCES

Althorpe, J., Goddard, D.A., Sissons, D.J. & Telling, G.M. (1970) *J. Chromatogr.*, *53*, 371-373

Kubacki, S.J. & Borys, A. (1976) *IARC Scientific Publications No. 14*, 109-116

Kubacki, S.J. & Danielewska, B. (1975) *Pr. Inst. Lab. Badaw. Przem. Spozyw.*, *23*, 409

Sen, N.P. (1970) *J. Chromatogr.*, *51*, 301-303

Telling, G.M. (1972) *J. Chromatogr.*, *73*, 79-87

3. DEFINITION

Volatile *N*-nitrosamine contents of meat and meat products: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (parts per billion).

4. PRINCIPLE

The method is based on steam distillation of *N*-nitrosamines from a neutral medium, their extraction from the distillate in dichloromethane, clean-up of the extract on an alumina column, oxidation of *N*-nitrosamines to *N*-nitramines with peroxytrifluoroacetic acid, additional clean-up on a magnesium oxide/alumina column, qualitative and quantitative estimation

of the *N*-nitramines by gas chromatography (GC), using an electron-capture detector.

5. HAZARDS

N-Nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

Dichloromethane	
n-pentane	
Diethyl ether	
Hydrogen peroxide	85-90%, Laport Industries Ltd.
Trifluoroacetic anhydride	Koch - Light
Sodium chloride	Analytical grade
Anhydrous sodium sulfate	Analytical grade
Alumina	Neutral, De Haen AG. Activate for 3 hours at 300°C (see 12.1) and deactivate by addition of 6 ml of water to 94 g alumina in a suitable Erlenmeyer flask. Stopper the flask tightly and shake 2 hr to equilibrate.

[1] Steere, N.V. (1974) Safety in the chemical laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. *Department of Health, Education and Welfare Publication (NIH)*, 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Magnesium oxide	BDH Chemicals Ltd. Activated for 2 hr at 150°C (see section 12.1).
Calcium carbonate	Powdered, analytical grade
Sulfuric acid 3 M	
Potassium hydroxide solution 5% (w/v)	
N-nitrosamine standard solution	Prepare in dichloromethane a mixed solution of the following *N*-nitrosamines:

N-nitrosodimethylamine	(NDMA)	100 mg/l
N-nitrosodiethylamine	(NDEA)	200 mg/l
N-nitrosodipropylamine	(NDPA)	400 mg/l
N-nitrosodibutylamine	(NDBA)	800 mg/l
N-nitrosopyrrolidine	(NPYR)	1200 mg/l
N-nitrosodiamylamine	(NDAA)	1600 mg/l

Oxidizing mixture	Carefully add 0.4 ml hydrogen peroxide to 4-5 ml dichloromethane in a 10 ml volumetric flask. Slowly add 2.5 ml trifluoroacetic anhydride, swirl gently and allow to cool in an ice bath for 5 min. Allow the contents of the flask to attain room temperature, then dilute to 10 ml with dichloromethane. Prepare this solution daily

NOTE: To ensure the absence of traces of *N*-nitrosamines or interfering material a test must be performed using the procedure described in section 9, but omitting the sample. Background at retention times of *N*-nitramines should not exceed 1% full scale deflection.

7. APPARATUS[1]

Usual laboratory equipment and the following items:

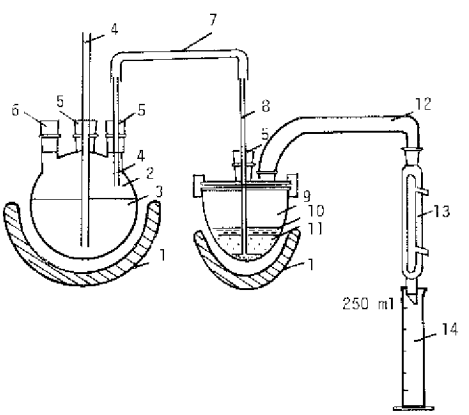
Steam distillation apparatus (Fig. 1)

---

[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Evaporator (K-D)	Kuderna-Danish 250 ml and 500 ml, with concentrator tube (approximately 5 ml)
Liquid chromatography column	Glass column 300 x 10 mm I.D. Quickfit cat. No. CR 12/30
Gas chromatograph	Pye-Unicam Ltd., series 104 with electron-capture detector (Ni-63, 10mC)

FIG. 1. DISTILLATION APPARATUS



1. ELECTRICALLY HEATED MANTLES
2. ROUND-BOTTOM FLASK (2 l CAPACITY)
3. DISTILLED WATER
4. GLASS TUBE (9 mm. i.d.)
5. QUICKFIT CONE WITH SCREW THREAD ADAPTER
6. GLASS STOPPER
7. PLASTIC TUBE
8. GLASS TUBE WITH HOLES AT THE END FOR STEAM DISTRIBUTION
9. DISTILLATION VESSEL
10. LAYER OF SOLUTION
11. SAMPLE
12. GLASS CONNECTOR
13. WATER CONDENSER
14. GRADUATED CYLINDER (250 ml CAPACITY)

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented; see Chapter 1: Sampling and Samples.

9. PROCEDURE

NOTE: *N*-nitrosamines are degraded by U-V light and exposure of extracts or standard solutions to sources such as sunlight should be avoided.

9.1 *Steam distillation*

9.1.1 Weigh approximately 300 g of meat or meat product. Comminute and mince by passing twice through a meat mincer.

9.1.2 Weigh 250 g of the minced meat in a 2000 ml round-bottomed flask and add 100 g sodium chloride.

9.1.3 Assemble the steam distillation apparatus as shown in Fig. 1. Place a 250 ml graduated cylinder at the outlet of the condenser. Steam distil for two hours to obtain 250 ml distillate. In order to avoid overheating the sample, do not start heating distillation flask before the steam has reached the sample and some water condensed. During the distillation, the level of water in the distillation vessel (Fig. 1) should be as close as possible to the surface of the sample.

9.1.4 Add 25 g anhydrous sodium sulfate and 3 ml of 3 M sulfuric acid to the distillate and transfer completely to 500 ml separation funnel.

9.2 *Solvent extraction of distillate*

9.2.1 Extract distillate 4 times with separate 50 ml portions of dichloromethane, using the first portion, divided into 3 parts, to rinse the cylinder.

9.2.2 Transfer each dichloromethane extract to a 500 ml separation funnel containing 50 ml 5% potassium hydroxyde solution.

9.2.3 Wash each extract separately with the same 50 ml portion of 5% potassium hydroxide solution.

9.2.4 Dry each extract by passing through 15 g anhydrous sodium sulfate in a sintered-glass funnel.

9.3 *Concentration of extract*

9.3.1 Collect dried dichloromethane extracts in a 500 ml K-D evaporator.

9.3.2 Rinse the sintered-glass funnel and sodium sulfate with dichloromethane and add rinsings to the K-D.

9.3.3 Place K-D in a water bath at 50°C and concentrate to 4-5 ml.

9.3.4 Disconnect the Snyder column and continue concentration to approximately 1 ml in a water bath at 40°C.

9.3.5 Add 4 ml *n*-pentane to the concentrate.

9.4 *Adsorption chromatographic clean-up*

9.4.1 Prepare alumina column (see Notes on Procedure, 12.2).

9.4.2 Transfer *n*-pentane:dichloromethane concentrate to the column, then run off solvent from bottom of column until the liquid level is approximately 1 mm above the sodium sulfate layer.

9.4.3 Rinse concentrator tube with *n*-pentane and proceed as in 9.4.2.

9.4.4 Rinse the walls of the column and run off solvent until liquid layer is about 1 mm above sodium sulfate layer.

9.4.5 Wash the column with 50 ml dichloromethane:*n*-pentane (1:99) (flow rate approximately 60 drops/min) and discard eluate

9.4.6 Elute the *N*-nitrosamines with 50 ml dichloromethane:*n*-pentane (1:1) (flow rate approximately 60 drops/min) into a K-D.

9.4.7 Concentrate the eluate as in 9.3.3 and 9.3.4.

9.5 *Oxidation of* N-*nitrosamines to* N-*nitramines*

9.5.1 Add 0.2 ml oxidizing solution to the concentrate.

9.5.2 Allow to stand $3\frac{1}{2}$ hours in the dark.

9.5.3 Add 4 drops distilled water, 0.25 g calcium carbonate and shake for 1 min (use of an ultrasonic mixer is preferable).

9.5.4 Add 0.25 g anhydrous sodium sulfate and shake until water is completely absorbed.

9.5.5 Add 4 ml *n*-pentane and keep for adsorption chromatography.

9.6 *Adsorption chromatographic clean-up of extract containing* N-*nitramines*

9.6.1 Prepare alumina-magnesium oxide column (see Notes on Procedure, 12.3).

9.6.2 Proceed as in 9.4.2, 9.4.3 and 9.4.4.

9.6.3 Wash the column with diethyl ether:*n*-pentane (1:99) (flow rate approximately 60 drops/min) and discard eluate.

9.6.4 Elute *N*-nitramines with 50 ml diethyl ether:*n*-pentane (1:3) Collect eluate in a K-D.

9.6.5 Concentrate the eluate to about 1 ml as in 9.3.3 and 9.3.4, and note final volume to within 0.01 ml.

9.7 *GC analysis*

9.7.1 GC conditions:

Column: glass packed with 10% PEG 20M on Diatomite C AW, DMCS-treated 100-120 mesh.
Length: 2.7 m I.D.: 4 mm

Carrier gas: Argon, 40 ml/min.

Column temperature: isothermal 175°C.

Detector temperature: 225°C

Pulse: 500 µs

9.7.2 Inject 5 µl of sample and carry out chromatographic analysis (measure retention times and peak areas).

9.7.3 Inject 5 µl of the appropriate *N*-nitramine solution and carry out chromatographic analysis.

10. METHOD OF CALCULATION

10.1 Calculate the concentration, Z_x, of the *N*-nitrosamine, x, from the following formula:

$$Z_x = \frac{400\ A_x CV}{R_x A_s} \quad \mu g/kg$$

where

A_x = Peak area of the *N*-nitramine, x, from 5 µl of concentrated sample;
A_s = Peak area of the same *N*-nitramine from 5 µl of standard solution;
C = Concentration of the *N*-nitramine in the standard solution in µg/ml;
V = Total volume of concentrated eluate (9.6.5) in ml;
R_x = Mean recovery of *N*-nitramine x (%).

10.2

10.2 The mean recovery of the *N*-nitramine derived from a given *N*-nitrosamine, x, is obtained by analysis of a minimum of ten spiked and unspiked samples of the same type, by the procedure described in 9.1.1 to 9.7.3. The spike should be added at step 9.1.2.

11. REPEATABILITY AND REPRODUCIBILITY

11.1 *Repeatability*

The coefficient of variation using this method is about 10%.

11.2 *Reproducibility*

No data available.

12. NOTES ON PROCEDURE

12.1 Magnesium oxide and alumina should be washed with *n*-pentane and diethyl ether or dichloromethane before activation. Sodium sulfate and sodium carbonate should also be washed in the same way.

12.2 Add about 15 ml *n*-pentane to the column, then slowly add 4 g activated alumina. Remove solvent from the bottom of the column, using slight vacuum, until the liquid level is approximately 5 mm above the adsorbent layer. Rinse the walls of the column, using 5 ml *n*-pentane, and remove solvent as before. Add 2 g anhydrous sodium sulfate to the column, followed once more by a rinse with 5 ml *n*-pentane. The liquid level should finally be 1 mm above the sodium sulfate layer.

12.3 Add about 15 ml *n*-pentane to the column, then slowly add 4 g activated alumina. Remove solvent from the bottom of the column, using slight vacuum, until the liquid level is approximately 5 mm above the adsorbent layer. Rinse the walls of the column, using 5 ml *n*-pentane, and remove solvent as before. Add 2 g activated magnesium oxide, followed by another rinse with 5 ml *n*-pentane. Remove the latter as before, then add 2 g anhydrous sodium sulfate to the column. Rinse the walls of the column again with 5 ml *n*-pentane. The liquid level should finally be 1 mm above the sodium sulfate layer.

12. SCHEMATIC REPRESENTATION OF PROCEDURE

Sample
↓
Mince
↓
Steam distillation
↓
Extraction with CH_2Cl_2 (4 x 50 ml)
↓
Drying of extracts on Na_2SO_4
↓
Concentration to 4-5 ml with K-D
↓
Further concentration to 1 ml
↓
Adsorption chromatography of *N*-nitrosamines on alumina
(a) *n*-pentane:CH_2Cl_2 (99:1) ⟶ discarded
(b) *n*-pentane:CH_2Cl_2 (1:1)
↓
Concentration of the eluate (b) to 1 ml
↓
Oxidation of *N*-nitrosamines to *N*-nitramines
↓
Neutralization
↓
Adsorption chromatography of *N*-nitramines on alumina:magnesium oxide
(a) diethyl ether-*n*-pentane (99:1) → discarded
(b) diethyl ether:*n*-pentane (1:3)
↓
Concentration of the eluate (b)
↓
GC with electron-capture detection

13. ORIGIN OF THE METHOD

Department of Instrumental Analysis
Institute of the Fermentation Industry
Warsaw 12
Rakowiecka 36
Poland

Contact point: Dr S.J. Kubacki

METHOD 11 – ANALYSIS OF VOLATILE *N*-NITROSAMINES IN FOOD USING NITROGEN-SPECIFIC DETECTION AND ION-SPECIFIC DETERMINATION OF HEPTAFLUOROBUTYRAMIDES BY GAS CHROMATOGRAPHY/ LOW-RESOLUTION MASS SPECTROMETRY

G. EISENBRAND & R. PREUSSMANN

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of the contents of various volatile *N*-nitrosamines in meat and meat products, cooked dishes, food ingredients, cereals, vegetables, cheese and animal foods. Using a 100 g sample, the method has a limit of detection of 1 microgram/kg (µg/kg) with both detection systems.

2. REFERENCES

Eisenbrand, G. (1970) Ph D. Thesis, University of Freiburg, Germany (1)

Eisenbrand, G. (1972) *IARC Scientific Publication No. 3*, pp. 64-70 (2)

Eisenbrand, G., Rappard, E. v., Zappe, R. & Preussmann, R. (1976) *IARC Scientific Publication No. 14*, pp. 65-75 (3)

Hall, R.C. (1974) *J. Chromatogr. Sci., 12*, 152-160 (4)

Rappard, E. v., Eisenbrand, G. & Preussmann, R. (1976) *J. Chromatogr., 124*, 247-255 (5)

Rhoades, J. & Johnson, D. (1970) *J. Chromatogr. Sci., 8*, 616-617 (6)

Telling, G.M. (1972) *J. Chromatogr., 73*, 79-87 (7)

3. DEFINITION

Volatile *N*-nitrosamine contents of foods: the *N*-nitrosamine contents determined according to the procedure described in this method and expressed as µg/kg (ppb).

4. PRINCIPLE

N-Nitrosamines are distilled under reduced pressure from a moderately alkaline suspension of food. The distillate is acidified to pH 1 and the *N*-nitrosamines are extracted in dichloromethane. After addition of *n*-hexane, the organic phase is concentrated to 1 ml and the concentrate analysed by two different methods. One method consists of direct gas chromatography using an electrolytic-conductivity detector in the pyrolytic mode (ref. 6.3); the other method consists of acid-catalyzed denitrosation of nitrosamines, formation of their corresponding heptafluorobutyramides and detection of these derivatives using their $C_3F_7^+$ and molecular ion by gas chromatography/mass spectrometry (GC-MS) (ref. 7,3,5).

5. HAZARDS

N-Nitrosamines are carcinogens and every possible precaution must be taken to avoid human exposure. All operations involving handling of *N*-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box. Rubber surgical gloves, which are frequently employed, do not give complete protection. They should be removed and disposed of immediately after use and not worn for long periods. Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

General outlines[1] and stringent requirements[2] for handling chemical carcinogens are available.

6. REAGENTS[3]

Dichloromethane	Analytical grade, passed through a column of granular calcium oxide and then distilled.
Methanol	Analytical grade, passed through an Amberlyst 15/silica-gel 60 column and then distilled.

---

[1] Steere, N.V. (1974) Safety in the Chemical Laboratory CXVI - Occupational safety and health standards adopted for fourteen carcinogens. *J. Chem. Educ.*, *51* (6), A322-A325

[2] *National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens.* Department of Health, Education and Welfare Publication (NIH), 75-900 (1975)

[3] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

n-Pentane	Analytical grade } Passed through a silica gel 60 column and distilled.
n-Hexane	Analytical grade }
Perfluorokerosene (PFK)	As supplied for mass spectrometry
Potassium carbonate	Analytical grade
Sodium chloride	Analytical grade
Sodium sulfate, anhydrous	Analytical grade
Sodium carbonate	Analytical grade
Sulfuric acid (5N)	
Hydrochloric acid (4N)	In 50% aqueous methanol
Sodium bicarbonate (1 M)	
Hydrogen bromide in dichloromethane (5%)	Bubble hydrogen bromide gas from a cylinder into ice-cooled dichloromethane until correct weight obtained.
Heptafluorobutyryl chloride (HFB-Cl) reagent	0.5% freshly distilled HFB-Cl Merck Darmstadt in *n*-pentane (Store in a dessicator over P_2O_5)
Standard *N*-nitrosamine solutions:	A. mixed solution containing 1 mg/l of each of the *N*-nitrosamines of interest in dichloromethane. B. 1 mg/l of *N*-nitrosodipropylamine (NPYR) in dichloromethane
Standard HFB-derivative solutions: (see 13 - Note on Procedure)	C. Mixed solution containing 0.5 mg/l of each of the HFB-derivatives of interest in pentane. D. 0.5 mg/l of DP-HFB in pentane.

NOTE: To ensure the absence of traces of nitrosamines or interfering material a test must be performed using the procedure described in section 9, but omitting the sample.

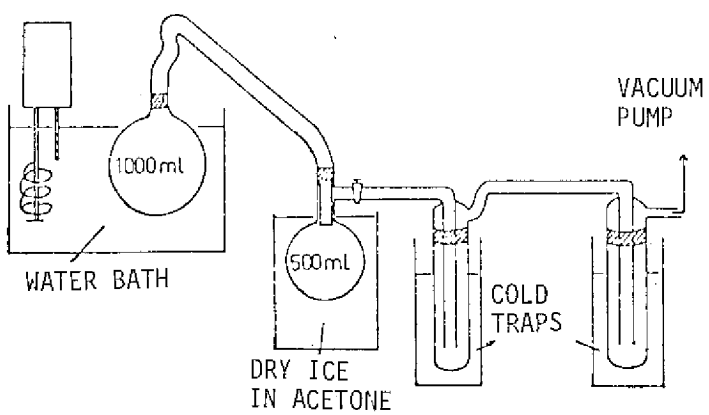
7. APPARATUS[1]

NOTE: Glassware used for the HFB-derivative method should be cleaned in potassium dichromate-sulfuric acid, rinsed with distilled water and stored in a dessicator over P_2O_5

Usual laboratory equipment and the following items:

Distillation apparatus	Joints of polished glass (see Fig. 1)
Evaporative concentrator (K-D)	Kuderna-Danish flask 100 ml, Concentrator tube calibrated to 1 ml, Snyder distillation column
Clean-up column	200 x 5 mm i.d. glass chromatography column

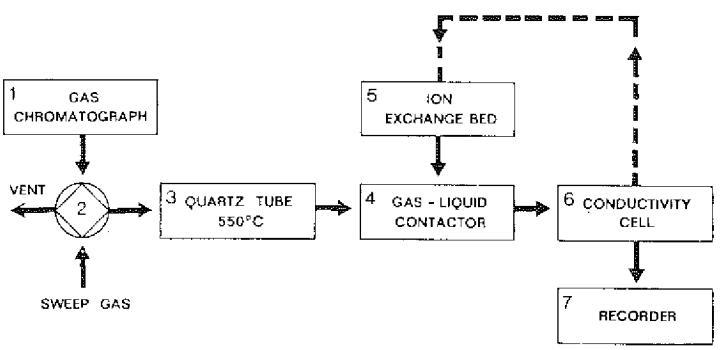
FIG. 1. DISTILLATION APPARATUS



[1] Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the company and/or product by the International Agency for Research on Cancer to the exclusion of others which may also be suitable.

Gas chromatograph	Hewlett Packard 7620, connected via heated outlet (200°C) to a modified nitrogen-specific detector.
Detector	(see Fig. 2) low-dead-volume electrolytic-conductivity cell from a Hall detector (Tracor Instruments), connected to a modified pyrolysis furnace from a Coulson detector (Tracor Instruments). The effluent from the gas chromatograph (1) enters the quartz tube in the pyrolysis furnace (3) via a four port valve (2). Pyrolysis products are swept by the carrier gas into the gas-liquid contactor (4), where they are dissolved in deionized water which is continually recycled through an ion-exchange column (5). The signal from the conductivity cell (6) is amplified and recorded (7).

FIG. 2. BLOCK DIAGRAM OF THE MODIFIED MICROELECTROLYTIC-CONDUCTIVITY DETECTOR



GC/MS combination	LKB 9000 with Multiple-ion-detection unit (MID), set for ion-specific detection at m/e 169 and m/e of the parent ions of the various HFB-amides.

8. SAMPLING

Proceed from a representative sample and store in such a way that deterioration and change in composition are prevented (see Chapter 1, Sampling and Samples).

9. PROCEDURE

9.1 *Vacuum distillation*

9.1.1 Weigh 100 g of a representative sample.

9.1.2 Add 12 g potassium carbonate, 60 g sodium chloride and 100 ml water and homogenize in a blender (3 min).

9.1.3 Transfer homogenate into 1000 ml round-bottomed flask with polished glass joint. Rinse blender with 100 ml water, add the washings to the homogenate.

9.1.4 Add 1 ml NDPA standard solution B. Connect flask to distillation bridge of distillation apparatus with polished glass joints (Fig. 1). Seal joints by wrapping with PTFE tape.

9.1.5 Immerse flask up to polished joint in a water bath at 3-5°C.

9.1.6 Apply vacuum until sample froths gently.

9.1.7 Heat water bath to 60°C within 1 hr.

9.1.8 Distil in a closed system, applying vacuum only for short periods to maintain boiling at 60°C.

9.1.9 Distil until no more distillate is produced and distillation bridge is dry ($2\frac{1}{2}$-3 hr).

9.1.10 Release vacuum

9.1.11 Wash condenser with a minimum of water, adding the washings to the distillate.

9.2 *Liquid-liquid extraction*

9.2.1 Acidify distillate to pH 1 with 5 N sulfuric acid.

9.2.2 Extract for two hours with about 60 ml dichloromethane in a continuous liquid-liquid extractor.

9.2.3 Shake the extract with 1/10 of its volume of water.

9.2.4 Discard water and dry the dichloromethane phase by shaking it with sodium sulfate (2-3 g) in the separation funnel.

9.2.5 Drain into K-D evaporator.

9.3 *Concentration of extract*

9.3.1 Concentrate in K-D to about 8 ml (water bath at 50°C).

9.3.2 Rinse the tube with 2 ml *n*-hexane and concentrate to 3 ml

9.3.3 Further concentrate to 1 ml by a gentle stream of nitrogen. Take 0.40 ml aliquot for denitrosation and heptafluorbutyramide (HFB) formation.
Keep the remainder for analysis by GC, with nitrogen-specific conductivity detector.

9.4 *GC analysis using modified nitrogen-specific conductivity detector*

9.4.1 GC-conditions

Column: stainless steel packed with 3% Carbowax 20 M terephthalate on 100/120 mesh Gaschrom Q.
Length: 3 m; O.D.: 3.2 mm

Carrier gas: Helium, 36 ml/min.

Column temperature: isothermal for 4 min at 130°C, then programmed at 8°C/min up to 200°C.

Injection port temperature: 200°C.

Transfer line to detector: glass-lined steel capillary (Scientific Glass Engineering Co. (SGE), I.D., 0.7 mm; Length, 30 cm.

9.4.2 Conductivity detector conditions:

Temperature of transfer line: 200°C

Temperature of vent block: 270°C

Pyrolyzer temperature: 550°C

Pyrolysis tube : Quartz; I.D., 1.0 mm; O.D., 6 mm; Length, 30 cm. The end of the tube contains a plug of 100 mg potassium carbonate between 5 mm plugs of silanized glass wool.

Connection to conductivity cell: Teflon tubing, O.D., 1.0 mm; I.D.: 0.6 mm; Length, 1 cm.

Conductivity cell water flow: 0.85 ml/min.

Background conductivity: about 1 μmho.

Sweep gas: Helium, 36 ml/min.

Attenuation: between 1 and 4.

9.4.3 Procedure:

9.4.3.1 Inject 2-5 µl of concentrated sample extract (9.3.3), the column being connected to the vent via the four-way venting valve.

9.4.3.2 After elution of solvent peak, connect the column to the detector and record chromatogram.

9.4.3.3 Repeat 9.4.3.1 and 9.4.3.2 with 5 µl of *N*-nitrosamine standard solution A.

9.4.3.4 Repeat 9.4.3.1 and 9.4.3.2 with 5 µl NDPA standard solution B.

9.5 *Derivative formation*

9.5.1 Mix 0.40 ml aliquot of concentrate (9.3.3) with 0.4 ml of 5% hydrogen bromide in dichloromethane

9.5.2 Allow to stand for 15 min.

9.5.3 Evaporate to dryness in vacuo at 30°C and remove residual traces of hydrogen bromide by stream of nitrogen.

9.5.4 Take up the residue in 0.5 ml methanol.

9.5.5 Prepare column (5 mm I.D.) of 200 mg SE-cellulose (Serva, Heidelberg); activate with 1 ml of 4 N hydrochloric acid in 50% aqueous methanol and wash with 2 ml portions of 50% aqueous methanol until washings are neutral (normally 4 washings are sufficient).

9.5.6 Transfer methanol solution (9.5.4) to column and wash with 1.5 ml methanol.

9.5.7 Elute the amines from the column with 2 ml 4 N hydrochloric acid in 50% aqueous methanol.

9.5.8 Evaporate to dryness in vacuo at 60°C.

9.5.9 Add 0.5 ml dichloromethane and 1 ml *n*-pentane and shake.

9.5.10 Add 1.5 ml water and shake.

9.5.11 Remove the organic layer by means of a Pasteur pipette.

9.5.12 Re-extract aqueous phase with 1 ml *n*-pentane and discard *n*-pentane; if necessary, repeat *n*-pentane extraction until *n*-pentane remains colourless.

9.5.13 Add 0.5 ml *n*-pentane, 1 ml 0.5% solution of HFB-chloride in *n*-pentane and 0.5 ml 1 M sodium bicarbonate solution. Stir magnetically for 15 min.

9.5.14 Add 0.5 ml 5 N sodium hydroxide and stir for another 5 min.

9.5.15 Discard the aqueous layer.

9.5.16 Add anhydrous sodium carbonate (100 mg) and stir.

9.5.17 Transfer *n*-pentane solution to 2 ml volumetric flask, wash the residual sodium carbonate with *n*-pentane and use the washings to make up to volume.

9.5.18 Retain for gas chromatography/mass spectrometry.

9.6 *Blank correction for derivative*

Obtain a blank correction for the derivative solution (9.5.17) by substituting an equal volume of *n*-hexane for the sample concentrate in the sequence of operations 9.5.1 to 9.5.17. Substract peak-heights of HFB derivatives obtained with the blank from those obtained with the sample (see 9.7).

9.7 *Gas chromatography/Mass spectrometry of HFB derivatives*

9.7.1 GC conditions:

Use alternatively column (a) or (b)

(a) Column: glass, packed with 3% OV 1 on Gaschrom Q 100/120 mesh. Length: 3 m; I.D., 2 mm.

Carrier gas: Helium, 20 ml/min.

Column temperature: isothermal 1 min at 110°C, then programmed at 15°C/min up to 160°C.
Isothermal at 130°C for separation of Di-*n*-propyl HFB (DP-HFB) and Pyrrolidyl-HFB (PYR-HFB)

(b) Column: glass, packed with 3% QF 1 on Gaschrom Q 100/120 mesh. Length: 3 m; I.D., 2 mm.

Carrier gas: Helium, 20 ml/min

Column temperature: isothermal at 130°C.

9.7.2 Mass spectrometry conditions:

Accelerating voltage: 3.5 KV

Filament current: 60 µA

Electron-beam energy: 70 eV

Separator temperature: 220°C

Ion source temperature: 250°C

9.7.3 Procedure:

9.7.3.1 Adjust the MS to monitor the GC effluent at m/e 169 ($C_3F_7^+$), using PFK as calibration standard. Adjust the slits to obtain a resolution of about 500.

9.7.3.2 Inject 2 µl sample (9.5.18)

9.7.3.3 Vent out the solvent peak.

9.7.3.4 Record chromatogram of gas chromatograph effluent on U-V oscillographic recorder.

9.7.3.5 If the chromatogram shows indications of the presence of HFB-derivatives, readjust the instrument to monitor the corresponding molecular ions. Several m/e values can be monitored simultaneously by sequential variation of the accelerating voltage, using the multiple-ion-detection unit.

9.7.3.6 Inject 2 µl HFB-derivative standard solution C.

9.7.3.7 Repeat 9.7.3.3 to 9.7.3.5

9.7.3.8 Inject 2 µl DP-HFB standard solution D.

9.7.3.9 Repeat 9.7.3.3 to 9.7.3.5.

10. METHOD OF CALCULATION

Results of both analytical methods are calculated by comparing a given peak height from sample with that of the corresponding peak from the standard solution.

10.1 *GC with nitrogen-specific detection*

$$Z_X = 1000 \frac{H_X C_{SE} H_{DPE} Wv_{SE}}{H_{SE} C_{DPE} H_{DPI} Mv_{DPE}} \quad \mu g/kg$$

where

Z_X = Concentration of *N*-nitrosamine, X, in sample (µg/kg)

H_X = GC peak height of *N*-nitrosamine, X, in the sample (9.4.3.2) (mm)

C_{SE} = Concentration of, X, in external standard, A (mg/l)

H_{SE} = GC peak height of, X, in external standard (9.4.3.3) (mm)

C_{DPE} = Concentration of NDPA external standard, B (mg/l)

H_{DPE} = GC peak height of NDPA external standard (9.4.3.4) (mm)

W = Amount of NDPA added as an internal standard (9.1.4) (µg)

H_{DPI} = GC peak height of NDPA internal standard (9.4.3.2) (mm)

M = Mass of sample analysed (9.1.1) (g)

v_{SE} = Volume of external standard injected (9.4.3.3) (µl)

v_{DPE} = Volume of NDPA external standard injected (9.4.3.4) (µl)

10.2 *HFB-Method (GC-MS)*

$$Z_X = 1000 \frac{H_X C_{SE} H_{DPE} F_{DP} W v_{SE}}{H_{SE} C_{DPE} H_{DPI} F_X M v_{DPE}}$$

where

Z_X = Concentration of *N*-nitrosamine, X, in sample (µg/kg)

H_X = MS peak height of X-HFB in external standard, C (mg/l)

v_{SE} = Volume external standard injected (9.7.3.6) (µl)

v_{DPE} = Volume DP-HFB external standard injected (9.7.3.8) (µl)

H_{SE} = MS peak height of X-HFB in external standard (9.7.3.6) (mm)

C_{DPE} = Concentration of DP-HFB external standard, D (mg/l)

H_{DPE} = MS peak height of DP-HFB external standard (9.7.3.8) (mm)

F_{DP} = Molar correction factor = NDPA/DP-HFB

W = Amount of NDPA added as an internal standard (9.1.4) (µg)

H_{DPI} = MS peak height of DP-HFB from NDPA internal standard (9.7.3.2) (mm)

F_X = Molar correction factor = nitrosamine X/X-HFB

M = Mass of sample analysed (9.1.1) (g)

NOTE: In 10.1 and 10.2, the equations for Z are corrected for incomplete recovery of endogenous *N*-nitrosamines in the concentrated extract (9.3.3) by assuming that the recovery of all of the endogenous *N*-nitrosamines is the same as that of the internal standard, NDPA, added at step 9.1.4. In addition, it is assumed that the amount of endogenous NDPA in the sample (9.1.1) is insignificant compared to the amount added at step 9.1.4.

11. REPEATABILITY AND REPRODUCIBILITY

11.1 *Repeatability*

11.1.1 GC with nitrogen-specific detection (9.4)

Chromatographic results for *N*-nitrosamines:

$\bar{X}_1$ = mean retention time (sec);
$\bar{X}_2$ = mean peak area (mm^2);
S_1 and S_2 = standard deviations;
V_1 and V_2 = coefficients of variation
P = 100 (peak area of given compound)/(peak area of NDMA);
M = 100 (mol. wt. of NDMA)/(mol. wt. of given compound).

N-nitrosamines	$\bar{X}_1$	S_1	V_1	$\bar{X}_2$	S_2[a]	V_2	P	M
NDMA	113	0.44	0.39	120	7.3	6.1	100	100
NDEA	145	0.44	0.30	106	6.7	6.3	87	73
NDPA	230	0.5	0.22	100	4.9	4.9	82	57
NDBA	451	0.5	0.11	64	2.0	3.1	53	47
NPIP	506	0.33	0.07	32	1.0	3.2	26	65
NPYR	561	0.53	0.09	17	1.3	7.7	14	74

[a] Standard deviation with a flame-ionisation detector was 1 for NDMA.

The coefficient of variation for recovery of NDPA (added as internal standard) for the whole analytical procedure, including clean-up and nitrogen-specific detection, was 18.5% in 11 consecutive determinations analysing different food types (grain, bread, anchovis paste, ham, salami-sausage).

11.1.2 HFB-Method

Overall yields (% of theoretical) of HFB-amides from 0.5 µg of a given nitrosamine

Compound	Mean yield %	No. of determinations	Standard deviation %
NDMA	72.3	19	6.4
NDEA	77.6	19	6.3
NDPA	77.1	10	6.4
NDBA	75.8	10	6.8
NPIP	77.3	18	5.5
NPYR	75.1	11	8.2

The coefficient of variation for recovery of NDPA (added as an internal standard) for the whole analytical procedure, including clean-up and HFB-derivative formation, was 16.9% in 11 consecutive determinations, analysing different food types (grain, bread, anchovis paste, ham, salami sausage).

11.2 *Reproducibility*

No data available

12. RECOVERY

At the 10 ppb level, mean recoveries for the internal standard NDPA were found to be 70% by nitrogen-specific detection and 68% by the HFB-method, in the range of samples mentioned in I (Scope and Field of Application).

13. NOTE ON PROCEDURE

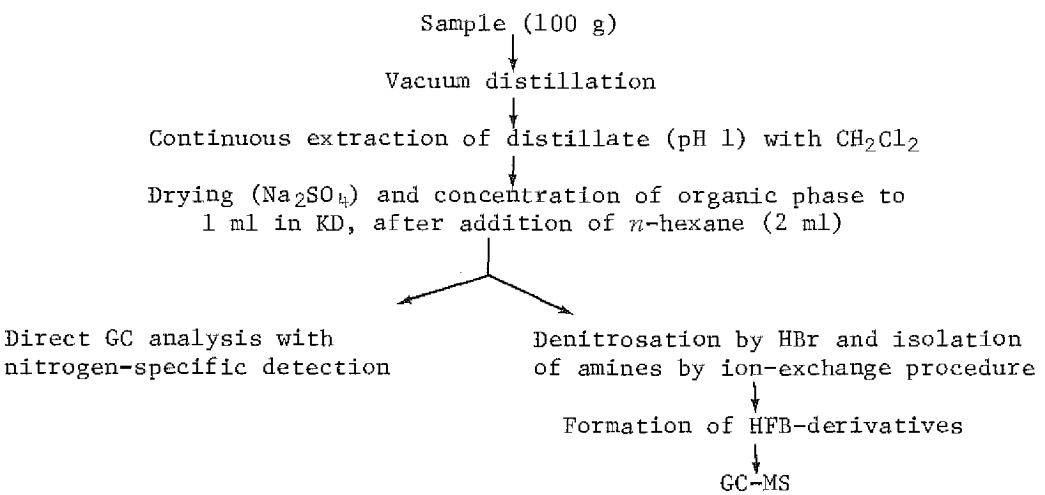
Synthesis of HFB-amides: Add a solution of the respective free amine in diethylether dropwise to a stirred, ice-cooled solution of HFB-chloride (34 mmole) in diethylether (20 ml) until the solution remains alkaline. Let stand at room temperature for 12 hr. Alternatively dissolve an amine hydrochloride (mmole) in pyridine (20 ml) and add the solution to an ice-cooled solution of HFB-chloride in pyridine (20 ml); reflux for 1 hr; add 50 ml of dichloromethane after the reaction mixture has cooled down.

Wash the solutions with 2 N HCl, saturated aqueous $NaHCO_3$ then with water until neutrality. Remove water by filtration over Na_2SO_4 and distill the organic solvent off. Purify the crude HFB-amides by fractional distillation under reduced pressure through a short Vigreux column. Confirm identity and purity by gaschromatography, mass spectrometry and elemental analysis.

Properties of HFB-amides

HFB-amide of	Mol. formula Mol. weight	Elemental analysis C	H	N	Boiling point Torr (uncorr.)	
Dimethylamine	$C_6H_6NOF_7$ (241.116)	calc. 29.89 found 30.04	2.51 2.67	5.81 5.63	51.0°	13
Diethylamine	$C_8H_{10}NOF_7$ 269.17	calc. 35.69 found 35.40	3.75 3.77	5.21 5.52	68.5°	14
Di-*n*-propylamine	$C_{10}H_{14}NOF_7$ 297.218	calc. 40.41 found 40.31	4.75 4.70	4.71 5.02	82.0°	11
Di-*n*-butylamine	$C_{12}H_{18}NOF_7$ 325.268	calc. 44.31 found 44.03	5.58 5.61	4.31 4.40	104.5°	11
Pyrrolidine	$C_6H_3NOF_7$ 267.152	calc. 35.96 found 35.54	3.02 3.05	5.24 5.03	87.0°	12
Piperidine	$C_7H_5NOF_7$ 281.178	calc. 38.44 found 38.32	3.59 3.64	4.98 5.23	87.5°	13
Methyl-ethylamine	$C_5H_4NOF_7$ 255.132	calc. 32.95 found 32.92	3.16 3.28	5.49 5.33	68.5°	13
Methyl-*n*-propylamine	$C_6H_6NOF_7$ 269.17	calc. 35.69 found 35.91	3.75 3.89	5.21 5.04	73,8°	15
Methyl-*n*-pentylamine	$C_8H_{10}NOF_7$ 297.218	calc 40.41 found 40.70	4.75 4.89	4.71 4.62	92.5°	11
Di-*n*-pentylamine	$C_{12}H_{22}NOF_7$ 353.327	calc. 47.59 found 47.76	6.28 6.26	3.96 4.12	76.0°	0.25
Di-*i*-pentylamine	$C_{12}H_{22}NOF_7$ 353.327	calc. 47.59 found 47.91	6.28 6.58	3.96 4.01	98.0°	3

14. SCHEMATIC REPRESENTATION OF PROCEDURE



15. ORIGIN OF METHOD

Deutsches Krebsforschungszentrum
Institut fur Toxikologie und Chemotherapie
Im Neuenheimer Feld 280
6900 Heidelberg
FRG

Contact point: Dr G. Eisenbrand

PUBLICATIONS OF THE INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

SCIENTIFIC PUBLICATIONS SERIES

No. 1 LIVER CANCER (1971)
176 pages US$ 10.00; Sw. fr. 30.–

No. 2 ONCOGENESIS AND HERPESVIRUSES (1972)
Edited by P.N. Biggs, G. de Thé & L.N. Payne
515 pages US$ 25.00; Sw. fr. 100.–

No. 3 N-NITROSO COMPOUNDS – ANALYSIS AND FORMATION (1972)
Edited by P. Bogovski, R. Preussmann & E.A. Walker
140 pages US$ 6.25; Sw. fr. 25.–

No. 4 TRANSPLACENTAL CARCINOGENESIS (1973)
Edited by L. Tomatis & U. Mohr
181 pages US$ 12.00; Sw. fr. 40.–

No. 5 PATHOLOGY OF TUMOURS IN LABORATORY ANIMALS. VOLUME I TUMOURS OF THE RAT PART 1 (1973)
Editor-in-Chief V.S. Turusov
216 pages US$ 15.00; Sw. fr. 50.–

No. 6 PATHOLOGY OF TUMOURS IN LABORATORY ANIMALS. VOLUME I TUMOURS OF THE RAT PART 2 (1976)
Editor-in-Chief V.S. Turusov
315 pages US$ 35.00; Sw. fr. 90.–

No. 7 HOST ENVIRONMENT INTERACTIONS IN THE ETIOLOGY OF CANCER IN MAN (1973)
Edited by R. Doll & I. Vodopija
464 pages US$ 40.00; Sw. fr. 100.–

No. 8 BIOLOGICAL EFFECTS OF ASBESTOS (1973)
Edited by P. Bogovški, J.C. Gilson, V. Timbrell & J.C. Wagner
346 pages US$ 32.00; Sw. fr. 80.–

No. 9 N-NITROSO COMPOUNDS IN THE ENVIRONMENT (1974)
Edited by P. Bogovski & E.A. Walker
243 pages US$ 20.00; Sw. fr. 50.–

No. 10 CHEMICAL CARCINOGENESIS ESSAYS (1974)
Edited by R. Montesano & L. Tomatis
230 pages US$ 20.00; Sw. fr. 50.–

No. 11 ONCOGENESIS AND HERPESVIRUSES II (1975)
Edited by G. de Thé, M.A. Epstein & H. zur Hausen
Part 1, 511 pages, US$ 38.00; Sw. fr. 100.–
Part 2, 433 pages, US$ 30.00; Sw. fr. 80.–

No. 12 SCREENING TESTS IN CHEMICAL CARCINOGENESIS (1976)
Edited by R. Montesano, H. Bartsch & L. Tomatis
666 pages US$ 48.00; Sw. fr. 120.–

No. 13 ENVIRONMENTAL POLUTION AND CARCINOGENIC RISKS (1976)
Edited by C. Rosenfeld & W. Davis
454 pages US$ 20.00; Sw. fr. 50.–

No. 14 ENVIRONMENTAL N-NITROSO COMPOUNDS – ANALYSIS AND FORMATION (1976)
Edited by E.A. Walker, P. Bogovski & L. Griciute
512 pages US$ 45.00; Sw. fr. 110.–

No. 15 CANCER INCIDENCE IN FIVE CONTINENTS, VOL. III (1976)
Edited by C.S. Muir, J. Waterhouse, P. Correa & J. Powell
584 pages US$ 40.00; Sw. fr. 100.–

No. 16 AIR POLLUTION AND CANCER IN MAN (1977)
Edited by U. Mohr, D. Schmähl & L. Tomatis
331 pages US$ 35.00; Sw. fr. 90.–

No. 17 DIRECTORY OF ON-GOING RESEARCH IN CANCER EPIDEMIOLOGY (1977)
Edited by C.S. Muir & G. Wagner
599 pages US$ 10.00; Sw. fr. 25.–

No. 19 ENVIRONMENTAL ASPECTS OF N-NITROSO COMPOUNDS (1978)
Edited by E.A. Walker, M. Castegnaro, L. Griciute & R.E. Lyle
566 pages US$ 50.00; Sw. fr. 100.–

IARC MONOGRAPHS ON THE EVALUATION OF THE CARCINOGENIC RISK OF CHEMICALS TO MAN

Volume 1, 1972

Some inorganic substances, chlorinated hydrocarbons, aromatic amines, N-nitroso compounds, and natural products
184 pages US$ 4.20; Sw. fr. 12.–

Volume 2, 1973

Some inorganic and organometallic compounds
181 pages US$ 3.60; Sw. fr. 12.–

Volume 3, 1973

Certain polycyclic aromatic hydrocarbons and heterocyclic compounds
271 pages US$ 5.40; Sw. fr. 18.–

Volume 4, 1974

Some aromatic amines, hydrazine and related substances, N-nitroso compounds and miscellaneous alkylating agents
286 pages US$ 7.20; Sw. fr. 18.–

Volume 5, 1974

Some organochlorine pesticides
241 pages US$ 7.20; Sw. fr. 18.–

Volume 6, 1974

Sex hormones
243 pages US$ 7.20; Sw. fr. 18.–

Volume 7, 1974

Some anti-thyroid and related substances, nitrofurans and industrial chemicals
326 pages US$ 12.80; Sw. fr. 32.–

Volume 8, 1975

Some aromatic amines
357 pages US$ 14.40; Sw. fr. 36.–

Volume 9, 1975

Some aziridines, N-, S- and O-mustards and selenium
268 pages US$ 10.80; Sw. fr. 27.–

Volume 10, 1976

Some naturally occurring substances
353 pages US$ 15.00; Sw. fr. 38.–

Volume 11, 1976

Cadmium, nickel, some epoxides and miscellaneous industrial chemicals, and general considerations on volatile anaesthetics
306 pages US$ 14.00; Sw. fr. 34.–

Volume 12, 1976

Some carbamates, thiocarbamates and carbazides
282 pages US$ 14.00; Sw. fr. 34.–

Volume 13, 1977

Some miscellaneous pharmaceutical substances
255 pages US$ 12.00; Sw. fr. 30.–

Vol. 14, 1977

Asbestos
106 pages US$ 6.00; Sw. fr. 14.–

Volume 15, 1977

Some fumigants, the herbicides 2,4-D and 2,4,5-T, chlorinated dibenzodioxins and miscellaneous industrial chemicals
354 pages US$ 20.00; Sw. fr. 50.–

Volume 16, 1977

Some aromatic amines and related nitro compounds – Hair dyes, colouring agents and miscellaneous industrial chemicals
400 pages US$ 20.00; Sw. fr. 50.–

Volume 17, 1978

Some N-Nitroso compounds
365 pages US$ 20.00; Sw. fr. 50.–

WHO/IARC publications may be obtained, direct or through booksellers, from:

ALGERIA: Société Nationale d'Edition et de Diffusion, 3 bd Zirout Youcef, ALGIERS

ARGENTINA: Carlos Hirsch SRL, Florida 165, Galerías Güemes, Escritorio 453/465, BUENOS AIRES

AUSTRALIA: *Mail Order Sales:* Australian Government Publishing Service Bookshops, P.O. Box 84, CANBERRA A.C.T. 2600; *or over the counter from* Australian Government Publications and Inquiry Centres *at:* 113–115 London Circuit, CANBERRA CITY A.C.T. 2600; Shop 42, The Valley Centre, BRISBANE, Queensland 4000; 347 Swanston Street, MELBOURNE VIC 3000; 309 Pitt Street, SYDNEY N.S.W. 2000; Mt Newman House, 200 St. George's Terrace, PERTH WA 6000; Industry House, 12 Pirie Street, ADELAIDE SA 5000; 156–162 Macquarie Street, HOBART TAS 7000 — Hunter Publications, 58A Gipps Street, COLLINGWOOD VIC 3066

AUSTRIA: Gerold & Co., Graben 31, 1011 VIENNA I

BANGLADESH: WHO Representative, G.P.O. Box 250, DACCA 5 — The Association of Voluntary Agencies, P.O. Box 5045, DACCA 5

BELGIUM: Office International de Librairie, 30 avenue Marnix, 1050 BRUSSELS — *Subscriptions to World Health only:* Jean de Lannoy, 112 rue du Trône, 1050 BRUSSELS

BRAZIL: Biblioteca Regional de Medicina OMS/OPS, Unidade de Venda de Publicações, Caixa Postal 20.381, Vila Clementino, 04023 SÃO PAULO, S.P.

BURMA: *see* India, WHO Regional Office

CANADA: *Single and bulk copies of individual publications (not subscriptions):* Canadian Public Health Association, 1335 Carling Avenue, Suite 210, OTTAWA, Ont. K1Z 8N8. *Subscriptions: Subscription orders, accompanied by cheque made out to the* Royal Bank of Canada, OTTAWA, Account World Health Organization, *should be sent to the* World Health Organization, P.O. Box 1800, Postal Station B, OTTAWA, Ont. K1P 5R5. *Correspondence concerning subscriptions should be forwarded to the* World Health Organization, Distribution and Sales, 1211 GENEVA 27, Switzerland

CHINA: China National Publications Import Corporation, P.O. Box 88, PEKING

COLOMBIA: Distribuidora Ltd, Pío Alfonso García, Carrera 4a, Nos 36–119, CARTAGENA

CZECHOSLOVAKIA: Artia, Ve Smeckach 30, 111 27 PRAGUE 1

DENMARK: Ejnar Munksgaard, Ltd, Nørregade 6, 1164 COPENHAGEN K

ECUADOR: Librería Científica S.A., P.O. Box 362, Luque 223, GUAYAQUIL

EGYPT: Nabaa El Fikr Bookshop, 55 Saad Zaghloul Street, ALEXANDRIA

EL SALVADOR: Librería Estudiantil, Edificio Comercial B No 3, Avenida Libertad, SAN SALVADOR

FIJI: The WHO Representative, P.O. Box 113, SUVA

FINLAND: Akateeminen Kirjakauppa, Keskuskatu 2, 00101 HELSINKI 10

FRANCE: Librairie Arnette, 2 rue Casimir-Delavigne, 75006 PARIS

GERMAN DEMOCRATIC REPUBLIC: Buchhaus Leipzig, Postfach 140, 701 LEIPZIG

GERMANY, FEDERAL REPUBLIC OF: Govi-Verlag GmbH, Ginnheimerstrasse 20, Postfach 5360, 6236 ESCHBORN — W. E. Saarbach, Postfach 101610, Follerstrasse 2, 5 COLOGNE 1 — Alex. Horn, Spiegelgasse 9, Postfach 3340, 6200 WIESBADEN

GREECE: G. C. Eleftheroudakis S.A., Librairie internationale, rue Nikis 4, ATHENS (T. 126)

HAITI: Max Bouchereau, Librairie "A la Caravelle", Boîte postale 111-B, PORT-AU-PRINCE

HONG KONG: Hong Kong Government Information Services, Beaconsfield House, 6th Floor, Queen's Road, Central, VICTORIA

HUNGARY: Kultura, P.O.B. 149, BUDAPEST 62 — Akadémiai Könyvesbolt, Váci utca 22, BUDAPEST V

ICELAND: Snaebjörn Jonsson & Co., P.O. Box 1131, Hafnarstraeti 9, REYKJAVIK

INDIA: WHO Regional Office for South-East Asia, World Health House, Indraprastha Estate, Ring Road, NEW DELHI 110002 — Oxford Book & Stationery Co., Scindia House, NEW DELHI 110000; 17 Park Street, CALCUTTA 700016 (Sub-Agent)

INDONESIA: M/s Kalman Book Service Ltd, Jln. Cikini Raya No. 63, P.O. Box 3105/Jkt., JAKARTA

IRAN: Iranian Amalgamated Distribution Agency, 151 Khiaban Soraya, TEHERAN

IRELAND: The Stationery Office, DUBLIN 4

ISRAEL: Heiliger & Co., 3 Nathan Strauss Street, JERUSALEM

ITALY: Edizioni Minerva Medica, Corso Bramante 83–85, 10126 TURIN; Via Lamarmora 3, 20100 MILAN

JAPAN: Maruzen Co. Ltd, P.O. Box 5050, TOKYO International 100–31

KENYA: The Caxton Press Ltd, Gathani House, Homa Bay Road, P.O. Box 41742, NAIROBI

KUWAIT: The Kuwait Bookshops Co. Ltd, Thunayan Al-Ghanem Bldg, P.O. Box 2942, KUWAIT

LAO PEOPLE'S DEMOCRATIC REPUBLIC: The WHO Representative, P.O. Box 343, VIENTIANE

LEBANON: Documenta Scientifica/Redico, P.O. Box 5641, BEIRUT

LUXEMBOURG: Librairie du Centre, 49 bd Royal, LUXEMBOURG

MALAYSIA: The WHO Representative, Room 1004, Fitzpatrick Building, Jalan Raja Chulan, KUALA LUMPUR 05–02 — Jubilee (Book) Store Ltd, 97 Jalan Tuanku Abdul Rahman, P.O. Box 629, KUALA LUMPUR 01–08 — Parry's Book Center, K. L. Hilton Hotel, Jln. Treacher, P.O. Box 960, KUALA LUMPUR

MEXICO: La Prensa Médica Mexicana, Ediciones Científicas, Paseo de las Facultades 26, Apt. Postal 20-413, MEXICO CITY 20, D.F.

MONGOLIA: *see* India, WHO Regional Office

MOROCCO: Editions La Porte, 281 avenue Mohammed V, RABAT

MOZAMBIQUE: INLD, Caixa Postal 4030, MAPUTO

NEPAL: *see* India, WHO Regional Office

NETHERLANDS: N. V. Martinus Nijhoff's Boekhandel en Uitgevers Maatschappij, Lange Voorhout 9, THE HAGUE 2000

NEW ZEALAND: Government Printing Office, Mulgrave Street, Private Bag, WELLINGTON 1, *Government Bookshops at:* Rutland Street, P.O. Box 5344, AUCKLAND; 130 Oxford Terrace, P.O. Box 1721, CHRISTCHURCH; Alma Street, P.O. Box 857, HAMILTON; Princes Street, P.O. Box 1104, DUNEDIN — R. Hill & Son, Ltd, Ideal House, Cnr Gillies Avenue & Eden St., Newmarket, AUCKLAND 1

NIGERIA: University Bookshop Nigeria Ltd, University of Ibadan, IBADAN

NORWAY: Johan Grundt Tanum Bokhandel, Karl Johansgt. 43, 1010 OSLO 1

PAKISTAN: Mirza Book Agency, 65 Shahrah Quaid-E. Azam, P.O. Box 729, LAHORE 3

PHILIPPINES: World Health Organization, Regional Office for the Western Pacific, P.O. Box 2932, MANILA — The Modern Book Company Inc., P.O. Box 632, 926 Rizal Avenue, MANILA

POLAND: Składnica Księgarska, ul Mazowiecka 9, 00052 WARSAW *(except periodicals)* — BKWZ Ruch, ul Wronia 23, 00840 WARSAW *(periodicals only)*

PORTUGAL: Livraria Rodrigues, 186 Rua do Ouro, LISBON 2

REPUBLIC OF KOREA: The WHO Representative, Central P.O. Box 540, SEOUL

SINGAPORE: The WHO Representative, 144 Moulmein Road, G.P.O. Box 3457, SINGAPORE 11 — Select Books (Pte) Ltd, 215 Tanglin Shopping Centre, 2/F, 19 Tanglin Road, SINGAPORE 10

SOUTH AFRICA: Van Schaik's Bookstore (Pty) Ltd, P.O. Box 724, PRETORIA 0001

SPAIN: Comercial Atheneum S.A., Consejo de Ciento 130–136, BARCELONA 15; General Moscardó 29, MADRID 20 — Librería Díaz de Santos, Lagasca 95, MADRID 6; Balmes 417 y 419, BARCELONA 6

SRI LANKA: *see* India, WHO Regional Office

SWEDEN: Aktiebolaget C. E. Fritzes Kungl. Hovbokhandel, Regeringsgatan 12, 10327 STOCKHOLM

SWITZERLAND: Medizinischer Verlag Hans Huber, Länggass Strasse 76, 3012 BERNE 9

THAILAND: *see* India, WHO Regional Office

TUNISIA: Société Tunisienne de Diffusion, 5 avenue de Carthage, TUNIS

TURKEY: Librairie Hachette, 469 av. de l'Indépendance, ISTANBUL

UNITED KINGDOM: H. M. Stationery Office: 49 High Holborn, LONDON WC1V 6HB; 13a Castle Street, EDINBURGH EH2 3AR; 41 The Hayes, CARDIFF CF1 1JW; 80 Chichester Street, BELFAST BT1 4JY; Brazennose Street, MANCHESTER M60 8AS; 258 Broad Street, BIRMINGHAM B1 2HE; Southey House, Wine Street, BRISTOL BS1 2BQ. *All mail orders should be sent to* P.O. Box 569, LONDON SE1 9NH

UNITED STATES OF AMERICA: *Single and bulk copies of individual publications (not subscriptions):* WHO Publications Centre USA, 49 Sheridan Avenue, ALBANY, NY 12210. *Subscriptions: Subscription orders, accompanied by check made out to the* Chemical Bank, New York, Account World Health Organization, *should be sent to the* World Health Organization, P.O. Box 5284, Church Street Station, NEW YORK, NY 10249. *Correspondence concerning subscriptions should be forwarded to the* World Heath Organization, Distribution and Sales, 1211 GENEVA 27, Switzerland. *Publications are also available from the* United Nations Bookshop, NEW YORK, NY 10017 *(retail only), and single and bulk copies of individual* International Agency for Research on Cancer *publications (not subscriptions) may also be ordered from the* Franklin Institute Press, Benjamin Franklin Parkway, Philadelphia, PA 19103

USSR: *For readers in the USSR requiring Russian editions:* Komsomolskij prospekt 18, Medicinskaia Kniga, Moscow — *For readers outside the USSR requiring Russian editions:* Kuzneckij most 18, Meždunarodnaja Kniga, Moscow G-200

VENEZUELA: Editorial Interamericana de Venezuela C.A., Apartado 50785, CARACAS 105 — Librería del Este, Apartado 60337, CARACAS 106

YUGOSLAVIA: Jugoslovenska Knjiga, Terazije 27/II, 11000 BELGRADE

Special terms for developing countries are obtainable on application to the WHO Representatives or WHO Regional Offices listed above or to the World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland. Orders from countries where sales agents have not yet been appointed may also be sent to the Geneva address, but must be paid for in pounds sterling, US dollars or Swiss francs.

Price: Sw. fr. 90.— US $ 45.00 Prices are subject to change without notice. IARC/1/78