

**The Occurrence and Determination of N-Nitroso Compounds<sup>1,2,3</sup>****W. FIDDLER***U.S. Department of Agriculture, Eastern Regional Research Center,  
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**The Occurrence and Determination of N-Nitroso Compounds.** Fiddler, W. (1975). *Toxicol. Appl. Pharmacol.* 31, 352-360. This article reviews the occurrence of nitrosamines in food products, the methods used for their determination, and their associated problems. Low concentrations of nitrosamines (ppb) have been primarily confirmed in fish exposed to nitrite and in cured meats. Many early reports of nitrosamines were based on results obtained using nonspecific methods. At present, selective gas-liquid chromatographic detectors and mass spectrometry are commonly used for detecting and confirming the identity of volatile nitrosamines.

In 1964, a Norwegian report identified dimethylnitrosamine (DMNA) as the cause of hepatotoxic disorders in domestic animals which had been fed nitrite-treated herring meal (Ender *et al.*, 1964). This finding initiated the search for nitrosamines (NA's) in the human food supply. There followed reports of their presence in food products, e.g., on heated flour, cheese, milk (Hedler and Marquardt, 1968), alcoholic beverages (McGlashan *et al.*, 1968), mushrooms, fish, and cured meat samples (Ender and Ceh, 1968). These studies, however, were carried out using nonspecific detection methods. Therefore, there is doubt as to the authenticity of these findings. Mass spectrometry, with its great sensitivity and its ability to produce specific fragmentation patterns for a particular compound, is at present the best and most commonly accepted method of identifying trace amounts of NA's. After 1970, reports appeared in the literature in which the presence of NA's, primarily in fish and cured meats, was confirmed by mass spectrometry.

**NITROSAMINES IN FISH PRODUCTS**

Howard *et al.* (1970) of the U.S. Food and Drug Administration found 1-5 ppb apparent DMNA, which they could not confirm by mass spectrometry, in smoked, nitrite-treated chub. The Code of Federal Regulations (1972a) permits the use of NaNO<sub>2</sub> in combination with salt to aid in inhibiting the outgrowth of and toxin formation from *Clostridium botulinum*, Type E. Fazio *et al.* (1971a) confirmed the presence of 4-26 ppb DMNA in sable, salmon, and shad, including 4 ppb DMNA in two samples of raw sable. Current regulations (1972b) permit the use of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> salts in

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<sup>2</sup> Reference to brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

<sup>3</sup> Precautions should be exercised in the handling of nitrosamines since they are potential carcinogens.

these products, but limit the residual to 500 ppm and 200 ppm, respectively. In a survey for volatile nitrosamines in food products in the United Kingdom, DMNA was confirmed in 15 of 35 fish samples at concentrations ranging from 1 to 9 ppb (Crosby *et al.*, 1972). While the occurrence of DMNA in fresh fish is relatively low (3 samples of 15), the incidence in salted (2 of 2), baked (1 of 1) and fried (9 of 11) fish is more frequent. These results seem to implicate salt and heat treatment as possible factors in NA formation. A similar observation was reported by Fong and Chan (1973a) who found 10–1000 ppb DMNA in 16 of 17 samples of salt-dried fish purchased locally in Hong Kong. Upon examination, little or no  $\text{NO}_2^-$ , but small concentrations of  $\text{NO}_3^-$ , were found in the crude salts used in the preparation of these salt-dried products. In a follow-up article Fong and Chan (1973b) presented evidence that *Staphylococcus aureus* isolated from salt-dried fish reduced the  $\text{NO}_3^-$  present in the curing salt. The resulting  $\text{NO}_2^-$  was responsible for the formation of DMNA during storage.

Marine fish are particularly susceptible to NA formation because they are rich in amines. Specifically, trimethylamine oxide is present in relatively high concentrations and forms trimethylamine upon storage. Free dimethylamine also forms during storage (Reay and Shewan, 1949). Both these amines are known to yield DMNA with  $\text{NO}_2^-$  under even mildly acidic conditions (Fiddler *et al.*, 1972a). Therefore, care must be taken in the storage of fish, their exposure to  $\text{NO}_3^-$  and  $\text{NO}_2^-$  salts and the degree of contamination by  $\text{NO}_3^-$  reducing bacteria in order to prevent NA's from forming.

#### NITROSAMINES IN CURED MEATS

There have been several reports of DMNA, which were confirmed by mass spectrometry, in a variety of cured meat products (Crosby *et al.*, 1972; Fazio *et al.*, 1971b; Sen, 1972; USDA, 1972; Wasserman *et al.*, 1972). For the most part the occurrence of these positive samples was random. However, in the case of fried bacon, nitrosopyrrolidine (NO-Pyr) has been found in a very high percentage of samples tested, and in concentrations as high as 108 ppb (Crosby *et al.*, 1972; Fazio *et al.*, 1973; Sen *et al.*, 1973a). The fact that NO-Pyr is found in fried and not in raw bacon seems to indicate that it forms as a result of heating. We have found that the amount of NO-Pyr present is dependent on the temperature, time, and the cooking method (Fiddler *et al.*, 1973b; Pensabene *et al.*, 1974). Our laboratory has underway several studies to determine how NO-Pyr is formed and the effect of bacon processing and storage on its formation.

One method to either eliminate or reduce the amount of NO-Pyr and other NA's formed in cured meat products is by using sodium ascorbate or its isomer sodium erythorbate in concentrations greater than that now permitted, which is ca. 550 ppm (Code of Federal Regulations, 1973). While these reductants are permitted for use as cure color accelerators, we found they also inhibit the formation of NA's (Fiddler *et al.*, 1972b, 1973a). Another way would be to reduce the level of  $\text{NO}_2^-$  used in curing since the rate of NA formed is dependent upon the square of the molar concentration of  $\text{NO}_2^-$  (Mirvish, 1970). One might ask, why not remove  $\text{NO}_2^-$  completely to eliminate all NA's? The answer is, nitrite, as used in meat curing, has antibacterial activity, particularly against *Clostridium botulinum*, and reacts with meat constituents to give the characteristic flavor, color, antioxidative and textural qualities to cured meats.

During a survey of cured meat products in Canada it was observed that fairly high

levels of NO-Pyr (13–105 ppb) and lower concentrations of nitrosopiperidine (NO-Pip) (50–60 ppb) were confirmed in Mettwurst sausages of certain firms (Sen *et al.*, 1973b). A study as to the cause of these NA's by the same workers revealed that the meat-curing mixtures of one manufacturer contained both NA's, with NO-Pip present in a concentration as high as 25 ppm. There was a correlation between the age of the cure mixture and the amount of NA present. Further investigations indicated that amine components of the spices present reacted with  $\text{NO}_2^-$  to form the NA's. The authors suggest that paprika forms mainly NO-Pyr and DMNA while black pepper predominantly forms NO-Pip (Sen *et al.*, 1973b).

A survey of commercial, preblended cure mixtures in the United States by the Food and Drug Administration and USDA Animal, Plant Health Inspection Service also turned up positive samples containing NO-Pyr and NO-Pip. This finding resulted in a regulation which prohibits the use of these prepared cure premixes (Federal Register, 1973). Curing salts and spices have to be packaged separately in order to prevent NA formation.

### DETECTION OF NITROSAMINES

To date, all of the NA's reported in food products are classified as volatile. This property permits preliminary sample cleanup by distillation, followed by other isolation and separation procedures such as solvent partitioning and thin-layer, column, and gas-liquid chromatography. Since the concentrations, for the most part, are in the

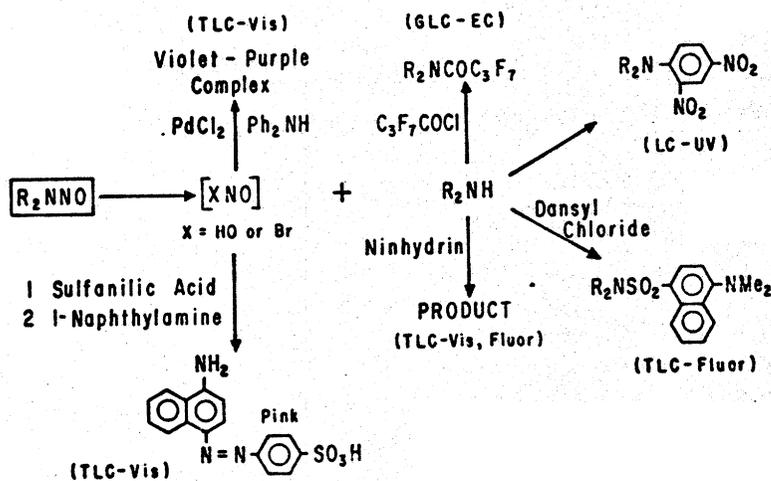


FIG. 1. Cleavage methods for the determination of nitrosamines.

nanograms NA per gram food, or ppb level, it is necessary to remove natural components which may interfere with the detection and positive identification of the NA(s) present.

Several methods have been used to detect NA's. In the principal ones, the NA is cleaved to generate nitrous acid or its derivative and secondary amine, is reduced to form an asymmetric hydrazine, is oxidized to form a nitramine or is kept intact.

Many of the earlier workers used the cleavage method exclusively (Fig. 1). In this procedure, thin-layer chromatographic (tlc) plates were subjected to uv light or HBr,

sprayed with Griess reagent (sulfanilic acid/1-naphthylamine)<sup>4</sup> and/or Preussmann reagent (PdCl<sub>2</sub>/diphenylamine) to give colored spots (Preussmann *et al.*, 1964). Several of the early claims of NA's in foods became questionable when it was later found that certain food components gave false positive results, particularly with the Preussmann reagent (Hedler and Marquardt, 1968; Möhler *et al.*, 1972; Sen *et al.*, 1969; Thewlis, 1968). The other product of the cleavage reaction is the secondary amine which has been detected by reaction with: ninhydrin to give visible or fluorescent tlc spots (Kröller, 1967; Sen *et al.*, 1969; Sen and Dalpe, 1972), heptafluorobutanoyl chloride to give a volatile amide sensitive to a glc electron capture (ec) detector (Alliston *et al.*, 1972), and 5-dimethylaminonaphthyl-1-sulfonyl chloride (dansyl chloride) to form a fluorescent sulfonamide (Eisenbrand and Preussmann, 1970). Recently the reaction of the secondary amine with 1-fluoro-2,4-dinitrobenzene has been proposed as a method of detection with liquid chromatographic separation (Cox, 1973). The primary limitation to these cleavage methods is that the NA is detected indirectly. Furthermore, it is very

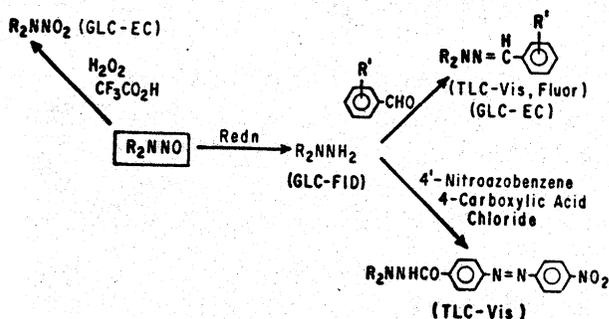


FIG. 2. Oxidation-reduction methods for the determination of nitrosamines.

important to remove free amines prior to cleavage of the NA and prevent formation of amines prior to derivatization in order to prevent erroneous results.

Other indirect methods include the oxidation and reduction of the NA shown in Fig. 2. Nitrosamine oxidation with trifluoroperacetic acid yields its corresponding nitramine which can be detected with a glc-ec detector (Reineccius and Coulter, 1972; Sen and Dalpe, 1972; Telling, 1972). This procedure is selective and sensitive—picograms of nitramine can be detected (Sen, 1970). While the oxidation reaction gives poor yields and is not reproducible for quantitative purposes, it offers an additional confirmatory procedure when used with other techniques.

Reduction of the NA to the asymmetric hydrazine and subsequent reaction with different aromatic aldehydes to form hydrazones is the basis for the most versatile reaction. Depending upon the aromatic aldehyde used, researchers have obtained colored (Möhler and Mayrhofer, 1968; Serfontein and Smit, 1967) and fluorescent (Yang and Brown, 1972) tlc spots, and volatile hydrazones which respond to a glc electron capture detector (Hoffmann and Vais, 1971). While the sensitivity of these detection methods is good, the selectivity has yet to be determined.

The most preferred techniques are those which detect and confirm the NA directly.

<sup>4</sup> *N*-1-naphthylethylenediamine is currently used since there is a question as to the carcinogenicity of 1-naphthylamine.

Of the direct methods reported, polarography has been responsible for at least two erroneous reports of NA's: in alcoholic beverages (McGlashan *et al.*, 1968) and in browning reaction products (Devik, 1967). Furfural (McGlashan *et al.*, 1970) and pyrazines (Kadar and Devik, 1970) respectively, were later found to give the same polarographic half-wave potential as NA's. While polarographic detection appears to be sufficiently sensitive, it is not specific for NA's in most food products without the removal of naturally occurring interfering components.

Gas-liquid chromatography is the most widely used and reliable means of detecting and quantitating NA's, particularly since glc columns have been used to aid in the separation of sample components. In order to increase the specificity of response to NA's, glc detectors have been used that are more selective than the conventional flame ionization detector (FID). Most examples from biological materials do not contain electron-capturing materials. A heptafluorobutyric acid anhydride adduct, which is electron-capture sensitive, has been used to detect NA's (Brooks *et al.*, 1972). This is in addition to forming the nitramine and heptafluorobutanamide, mentioned previously. The alkali flame ionization (AFID), Coulson electrolytic conductivity (CECD), and microcoulombic detectors are selective toward nitrogen-containing compounds. The CECD, in the pyrolytic mode, has limitations. It has been used for simple aliphatic NA's like DMNA, which give good detector responses (Johnson and Rhoades, 1972; Panalaks *et al.*, 1973; Sen *et al.*, 1973a), but alicyclic NA's like NO-Pyr do not respond as well (Essigmann and Issenberg, 1972). Other workers have used the CECD with apparent success in the reductive mode which is sensitive to the alicyclics (Crosby *et al.*, 1972). Experience in our laboratory using this detector for NO-Pyr in fried bacon was not satisfactory since quantitative reproducibility was difficult to maintain. The microcoulombic detector has had limited use to date (Newell and Siskin, 1972) and is similar to the CECD when operated in the reductive mode. Both these detectors detect ammonia produced by the catalytic reduction of the NA in the glc effluent stream. In one, ammonia is measured by a titration cell, and in the other a change in the electrolytic conductivity of water is measured. These detectors offer advantages for the large-scale screening of food products because of their selectivity and because the samples analyzed need a minimum of cleanup. If a positive sample is indicated, additional confirmatory procedures that require more extensive sample cleanup are necessary.

Investigations of NA's in cured meats in our laboratory have been carried out primarily with the AFID, a FID modified by placing a KCl-coated coiled Pt-Ir wire over the flame jet (Howard *et al.*, 1970). This detector is not as selective toward nitrogen-containing compounds as the other two detectors but it gives a good response to volatile NA's. An example of NO-Pyr found in fried bacon is shown in Fig. 3. The peak in the chromatogram at 14 min represents ca. 3.5 ng of NA. The use of this detector requires a very lengthy and rigorous clean-up procedure, an outline of which is shown in Fig. 4. This clean-up is also required so that additional confirmatory procedures can be performed on all peaks identified as NA's by retention time.

In general, all of the methods mentioned in this paper have been applied only to volatile NA's. The fact that a sample gives a colored tlc spot at a particular  $R_f$  value or glc peak at the same retention time as an authentic sample of NA, no matter how selective the detector used, does not prove that a NA is present. Far too many erroneous results have been reported. The initial findings must be confirmed. Many investigators

in the field claim that the best method presently available for the confirmation of NA's in food products in a combination of glc separation and mass spectrometry. Determining the presence of characteristic  $m/e$  fragments, particularly of the parent peak, by high-resolution mass spectrometry provides the greatest amount of specificity and selectivity.

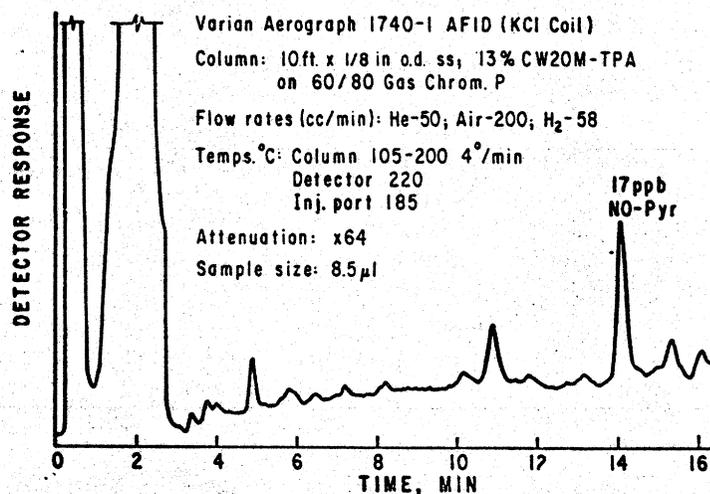


FIG. 3. Chromatogram of fried bacon sample using a gas-liquid chromatographic alkali flame ionization detector.

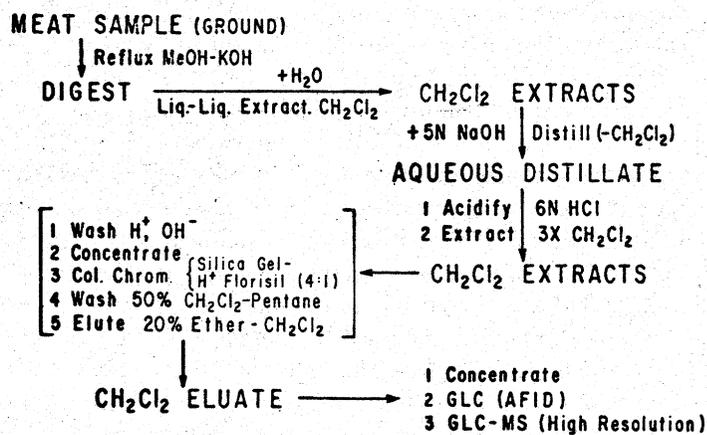


FIG. 4. Procedure for the isolation and identification of six volatile nitrosamines in cured meat products.

This, however, is not without error. Recently a silicon compound was reported that had a fragment peak which could not be differentiated from the parent peak of DMNA at a resolution of 1 in 12,000 (Dooley *et al.*, 1973; Gough and Webb, 1973). Therefore, care must be exercised and a number of different methods need to be utilized to give the best assurance that NA's are present.

In spite of all the possibilities for error, evidence to date indicates that low concentrations of NA's are present in some products in the food supply which have been exposed to  $\text{NO}_2^-$ . The major question which still needs to be answered is, what are the effects of these low concentrations of NA's on man?

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