# Naturally Occurring Nitrosatable Compounds. I. Secondary Amines in Foodstuffs

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A procedure is described for the analysis of naturally occurring secondary amines. Identification of tosylamide derivatives by GLC–MS is carried out after steam distillation and cleanup. Morpholine and dimethylamine are ubiquitous. Piperidine and pyrrolidine are found in plant-derived material. Other amines are found in smaller quantities.

In recent years there has been considerable interest in *N*-nitroso compounds because of their potent widespread carcinogenicity. The importance of this biological activity has engendered numerous analytical studies of the natural occurrences of these compounds, especially *N*-nitrosamines, and a variety of procedures for their isolation, separation, identification, and quantification have been described (International Agency for Research on Cancer, 1971, 1974) and reviewed (Mirvish, 1975; Scanlan, 1975).

The principal conclusion of most of these studies has been that *N*-nitrosamines do occur in a variety of materials, but at very low (parts per billion) levels [with the single exception of chewing tobacco, in which 89 ppm of *N'*-nitrosonornicotine was found (Hoffman et al., 1974)]. The significance of these small amounts of nitrosamines in human cancer is not known.

A number of investigators have shown that nitrosamines can be formed in vivo from ingested amines and nitrite (Sander et al., 1968; Greenblatt et al., 1972; Mysliwy et al., 1974). Moreover, the same tumors are produced in experimental animals that are fed amine plus nitrite as in those fed the corresponding nitrosamine (Sander and Bürkle, 1969; Taylor and Lijinsky, 1975; Ivankovic and Preussman, 1970). The in vivo production of nitrosamines, then, may be an extremely important source of carcinogens for man, especially in view of the current extensive use of appreciable amounts of sodium nitrite in preserved meats and the widespread natural occurrence of amines in the human diet.

The true importance of in vivo nitrosation is difficult to assess, however, because of the paucity of information on the nature and distribution of even the simple nitrosatable secondary aliphatic amines. Consequently, we have undertaken to develop analytical procedures for the determination of nitrosatable amines in a variety of foodstuffs and related materials. We report here our method and findings on secondary amines. A subsequent paper will deal with tertiary amines.

A variety of procedures are described in the literature for analyzing amines, including TLC of *p*-(*N*,*N*-dimethylamino)benzene-*p'*-azobenzamides (Churacek et al., 1972), 2,6-dinitrophenylhydrazonepyruvamides (Schwartz and Brewington, 1967), 5-dimethylamino-1-naphthalenesulfonamides (dansyl-amides) (Gruger, 1972), or 3,5-dinitrobenzamides (Wick et al., 1967); paper chromatography of the free amines (Slaughter and Uvgard, 1971) or of derivatives (Churacek et al., 1972); and gas chromatography of the free amines (Gruger, 1972; Wick et al., 1967; Slaughter and Uvgard, 1971; Miller et al., 1973) or of various derivatives (Irvine and Saxby, 1969a,b; Mosier et al., 1973; Neurath et al., 1966; Pailer et al., 1967; Bush, 1970). While this is not a complete compilation, it is indicative of the procedures available when we began our work. A number of these methods had even been applied to the types of materials in which we were interested, e.g. fish and fish protein concentrate (Wick et al., 1967; Miller et al., 1973), beer (Slaughter and Uvgard, 1971), tobacco leaf (Irvine and Saxby, 1969a,b; Bush, 1970), and cigarette smoke condensate ("tar") (Neurath et al., 1966; Pailer et al., 1967).

None was suited to our use, however. Because we are interested in precursors to carcinogenic nitrosamines, we sought a procedure which would allow the separation of primary amines from our analytical sample, since on nitrosation primary amines are diazotized and deaminated rather than yielding stable nitrosamines. The method of Schwartz and Brewington (2,6-dinitrophenylhydrazonepyruvamides) (Schwartz and Brewington, 1967) appeared promising, especially since we had available a nitrogen-specific Coulson electrolytic conductivity detector. Unfortunately, these derivatives were not sufficiently thermally stable for gas chromatographic analysis.

In contrast, the classical Hinsberg procedure (*p*-toluenesulfonamides) has proven very useful for our purposes in several respects. While not completely infallible [we do find some primary amine derivatives occasionally in the secondary amines fraction, and in our work on tobacco smoke condensate (Singer and Lijinsky, 1976), we also find some of the nicotine appearing in the secondary amine fraction] this procedure does provide a relatively clean "secondary amine fraction". (In referring to a "primary" or "secondary amine fraction", it should be understood that this fraction contains the *p*-toluenesulfonamides of the amines.) Derivatizing the amines in this manner reduces their volatility severely and allows their ready analysis by GLC and/or GLC–MS. Moreover, the *p*-toluenesulfonamides have very characteristic mass spectral fragmentation patterns which greatly facilitate identification of the GLC eluates (Singer et al., 1975).

## EXPERIMENTAL SECTION

All solvents and reagents were of reagent grade and were used as received. Melting points are uncorrected.

GLC analyses (quantitative and qualitative) were performed on a 10 ft × 2 mm i.d. glass column packed with 5% SE 30 on Gas-Chrom Q (80–100 mesh) with an oven temperature programmed from 150 to 200 °C at 2 °C/min and a helium flow rate of 60 ml/min. For quantitative determinations the entire effluent was led to the flame ionization detector, but for qualitative injections, half of the effluent was led to a Coulson detector operated in the usual nitrogen-selective reductive mode, and both detector outputs were recorded simultaneously.

Quantification was in some instances by the hand-measuring triangulation procedure and in others by computerized data acquisition, peak measurement (PSCAN), and data reduction (GCRPT) using BASIC programs written specifically for our PDP-11/20 RSTS system. The ana-

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Table I. Amines in Fish

| Substance | Dimethylamine | | Di-*n*-propylamine | | Morpholine | |
|---|---|---|---|---|---|---|
| | μmol/100 g | ppm | μmol/100 g | ppm | μmol/100 g | ppm |
| Canned tuna | 51 | 23 | | | ⩽0.7 | ⩽0.6 |
| Frozen ocean perch | 400 | 180 | | | 10 | 9 |
| Frozen cod | 1640 | 740 | | | tr[a] | tr[a] |
| Spotted trout[b] | 15 | 7 | 0.4 | 0.4 | 7 | 6 |
| Small mouth bass[c] | 250 | 110 | 0.2 | 0.2 | ⩽0.8 | ⩽0.7 |
| Salmon[d] | 180 | 82 | | | 1.2 | 1.0 |

[a] tr = trace, <0.3 μmol/100 g, <0.3 ppm. [b] One sample, caught locally. [c] Caught in local lake. [d] From Columbia River, Oregon. Shipped frozen; analyzed within 96 h of catching.

lytical solutions contained two internal standards, acenaphthene and 1-benzyloxynaphthalene, and the calculations included correction by relative molar response factors which had previously been determined for each *p*-toluenesulfonamide. Figure 1 is a chromatogram of a test mixture of tosylamides.

For the GLC–MS experiments, the conditions were the same except that the flow rate was reduced to 30 ml/min. The effluent was split, with approximately 80% being led through a porous SS tube separator. The mass spectra were obtained on a single-focusing mass spectrometer [30.5-cm radius, 90° magnetic sector] constructed in the Analytical Chemistry Division of ORNL. The spectra were low resolution ($M/\Delta M < 1000$) recorded with an oscillographic recorder. The accelerating voltage was 4 kV, and the ionizing voltage was 35 or 70 eV (no appreciable differences in fragmentation patterns were found between these two voltages). The source-can pressure was maintained at less than $5 \times 10^{-6}$ Torr uncorrected.

Samples for analysis were purchased locally or were caught locally (trout and bass). Perishable fish and meat were stored at −7 °C. Otherwise all samples were analyzed as received.

The *p*-toluenesulfonamides for use as authentic standards were prepared by standard procedures (Shriner et al., 1956; Vogel, 1957) and had melting points or boiling points in accord with literature values.

Since the initial portion of the isolation procedure differed for each type of material, typical analyses are described below.

**Ham and Fish.** Approximately 100 g of the material was cut from the frozen stored sample (fish samples were skinned before analysis), cubed, and placed in a blender with weighed amounts of water (ca. 200 ml) and 6 N HCl (ca. 100 ml) plus 20 μl of [$^{14}$C]dimethylamine ([$^{14}$C]DMA) solution (1470 dpm). After pureeing, the mixture was transferred to a tared flask with the help of a known amount of rinse water. The exact amount of the analysis sample was then calculated by difference.

The mixture was basified to pH 10 with 20% NaOH and 50 ml of saturated $Ba(OH)_2$ (to help control foaming) and was distilled with steam into a flask containing 5 ml of 2 N HCl. Approximately 0.5 l. was collected. After overnight continuous extraction with ether (to remove steam-volatile neutral compounds), the aqueous solution was concentrated to less than 3 ml (in vacuo) on the rotary evaporator (bath temperature ca. 55 °C).

The amines were derivatized by boiling this concentrate with *p*-toluenesulfonyl chloride (0.60 g) and 20% NaOH (20 ml) under reflux and a dry ice–acetone trap. Acidification to pH 1 with 6 N HCl and continuous extraction with ether for 1 h left only the tertiary amines in the aqueous solution. The ether solution, containing the primary and secondary *p*-toluenesulfonamides, was evaporated to dryness under an $N_2$ stream, and the residue was boiled under reflux with 20% KOH (10 ml). Continuous extraction for 1 h with ether removed the secondary amine *p*-toluenesulfonamides. This ether solution was evaporated to dryness under an $N_2$ stream and taken up in 5.0 ml of ethanol containing the two internal standards for subsequent GLC analysis. An aliquot of this solution was counted to determine the [$^{14}$C]DMA recovery, and the GLC values for DMA were then corrected accordingly.

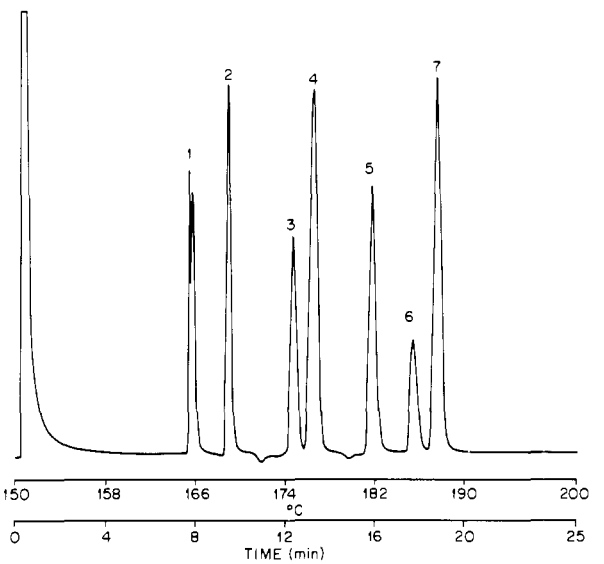


Figure 1. Chromatogram of test mixture of tosylamides on 10 ft × 2 mm i.d. 5% SE 30/Gas-Chrom Q (80–100), 150 to 200 °C at 4 °C/min: peak 1, dimethylamine; peak 2, methylethylamine; peak 3, ethylisopropylamine; peak 4, methylbutylamine; peak 5, di-*n*-propylamine; peak 6, morpholine; peak 7, piperidine and $\Delta^3$-piperidine.

If desired, the primary *p*-toluenesulfonamides may be recovered by acidification of the aqueous solution followed by ether extraction.

**Wine and Beer.** Each beverage (generally 2 l.) was acidified to pH 1, tagged with [$^{14}$C]DMA, concentrated to about 300 ml, and treated as above.

**Coffee and Tea.** Weighed amounts were tagged with [$^{14}$C]DMA, directly distilled with steam, and processed as described above.

**Frankfurters.** The acidified pureed mixture was distilled with steam to remove steam-volatile acids and neutral compounds. About 1 l. was collected. The mixture was then made basic and processed as usual.

**Water.** Water (0.50 l.) was basified and distilled into a flask containing 5 ml of 2 N HCl, and the distillate was processed as usual.

## RESULTS AND DISCUSSION

The results of the analyses are shown in Tables I (fish), II (meats), and III (beverages). The values for DMA are corrected for the recovered [$^{14}$C]DMA, but in general these recoveries were good (75–100%). The values for the other

**Table II. Amines in Meats**

| Substance | Dimethylamine | | Di-*n*-propylamine | | Pyrrolidine | | Morpholine | | Piperidine | |
|---|---|---|---|---|---|---|---|---|---|---|
| | μmol/100 g | ppm | μmol/100 g | ppm | μmol/100 g | ppm | μmol/100 g | ppm | μmol/100 g | ppm |
| Baked ham | 5 | 2 | tr[a] | tr[a] | tr[a] | tr[a] | 0.6 | 0.5 | 0.2 | 0.2 |
| Frankfurters | 2 | 1 | | | | | 0.5 | 0.4 | | |

[a] tr = trace, <0.1 μmol/100 g, <0.1 ppm.

**Table III. Amines in Beverages**

| Substance | Dimethylamine | | Methylethylamine | | Methyl-*n*-butylamine | | Pyrrolidine | | Morpholine | | Piperidine | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | μmol/100 g | ppm | μmol/100 g | ppm | μmol/100 g | ppm | μmol/100 g | ppm | μmol/100 g | ppm | μmol/100 g | ppm |
| Evaporated milk | 7 | 3 | | | 0.3 | 0.2 | 1 | 0.7 | 0.2 | 0.2 | 1.5 | 0.3 |
| Whole milk | 0.8 | 0.2 | | | | | | | | | 1.3 | 0.11 |
| Coffee[a,b] | 4 | 2 | ⩽0.8 | ⩽0.5 | | | 8 | 6 | 1 | 1 | 1 | 1 |
| Tea[b] | 1.6 | 0.7 | | | | | | | tr[c] | tr[c] | | *d* |
| Canned beer | 1.3 | 0.6 | | | | | | | 0.5 | 0.4 | | |
| Bottled beer | 1.6 | 0.7 | | | | | tr[c] | tr[c] | ⩽0.3 | ⩽0.2 | | |
| Wine | 0.2 | 0.07 | | | | | 0.09 | 0.06 | ⩽0.8 | ⩽0.7 | | |

[a] Average of two brands. [b] Per dry weight. [c] tr = trace, <0.1 μmol/100 g, <0.1 ppm. [d] Plus three other unidentifiable trace-level amines.

**Table IV. Reproducibility of Procedure**

| Substance | Determination no. | Dimethylamine μmol/100 g | Dimethylamine ppm |
|---|---|---|---|
| Canned tuna | 1 | 52.1 | 23.5 |
| | 2 | 47.0 | 21.2 |
| | 3 | 53.7 | 24.2 |
| | | 51.5 ± 4.2 (8.2%) | 23.2 ± 1.9 (8.2%) |
| Cod | 1 | 1621 | 731 |
| | 2 | 1653 | 745 |
| | | 1637 ± 23 (1.4%) | 738 ± 10 (1.3%) |

amines are uncorrected and should be assumed to be minimum values, but probably within 50% of the true level.

To determine the extent of recovery of the less-volatile amines, 100-ml water samples were spiked with morpholine and subjected to the isolation–analysis procedure. Recoveries were 45–67% of the morpholine and 90% of added [$^{14}$C]DMA.

The results presented in Tables I, II, and III are average values of several determinations each. The only exception is the value reported for trout, for which only enough material was available for one determination.

Two examples of the precision of the procedure outlined here are given in Table IV. In the first, two cans of tuna were mixed and homogenized, and then portions were taken for triplicate analyses. The values for DMA are grouped within 8%. Similar duplicate analyses on part of a cake of frozen cod fillets had precision better than 2%.

The minimum detectable levels may be illustrated by considering, for example, the morpholine value for frankfurters. The value of 0.5 μg/100 g represents 10 ng in the 1-μl injection of 50 μg in the entire sample. The practical measurable value is about one-third of this, although lower levels are detectable and identifiable by retention times, if not by GLC–MS. For the beverages that are concentrated during the analysis, the practical measurable level in the sample is, accordingly, about another sixfold lower, i.e., ca. 8 μg total or 4 ppb.

Because of their preponderance, methylamines have been the most thoroughly investigated amines in fish. In general, previous investigations have considered the levels of DMA to be a measure of frozen storage decomposition. In contrast, we are concerned with the amine content of

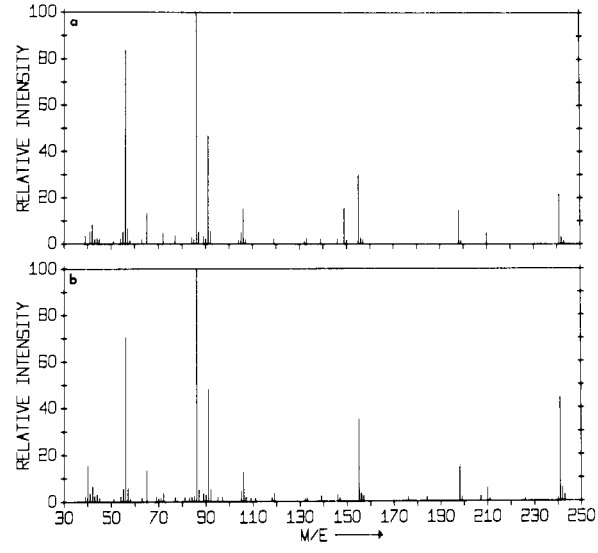


**Figure 2.** Mass spectra of (a) *N*-tosylmorpholine authentic sample and (b) *N*-tosylmorpholine isolated from ham.

samples that might be prepared for human consumption.

The levels of DMA in our fish samples (Table I) varied more than 100-fold, but only part of this may be due to frozen-storage decomposition. The trout sample, which had the lowest level we found, was analyzed within 3 days of being caught, while the samples with the highest levels, ocean perch and cod, were purchased frozen, and, thus, their storage time is unknown. There are also strong species variations, since the salmon and bass samples were analyzed within days of catching and contain intermediate levels.

Note that our DMA values for salmon are appreciably higher than those reported by Gruger (1972), but our values for cod are in accord with Castell's (Castell et al., 1970).

Initially, we were surprised to find a variety of simple cyclic amines in several foodstuffs. It seems reasonable, however, that these may all derive from plant alkaloid sources or, in the ham, may reflect the spices used in curing or processing.

Much more surprising was the near ubiquity of morpholine in our samples. There does not appear to be any previous report in the literature on naturally occurring

morpholine. Our identification is based not only on retention time and "spike" experiments, but also on comparison of GLC–MS spectra with a spectrum of authentic material (Singer et al., 1975) (Figure 2). We could find no evidence of morpholine in any water source available to us (detection level was about 0.07 ppb); therefore morpholine was not being introduced in water used during the analysis nor as a contaminant of our reagents. Moreover, it was present in samples of fish from local fresh water (including one of the local water sources checked), as well as in all the commercially prepared samples.

The morpholine is apparently not formed artifactually during our isolation procedure since none was detectable when the steam-distillation pot contained, for example, diethanolamine, choline, lecithin, etc. The natural source of the morpholine is still not clear. It should be noted that its ubiquitous occurrence is relevant to our concern about in vivo formation of nitrosamines. *N*-Nitrosomorpholine is produced in good yield at stomach pH, both in vitro (Fan and Tannenbaum, 1973) and in vivo (Sander and Bürkle, 1969; Sander et al., 1968), and is a potent liver carcinogen in the rat and mouse (Bannasch and Müller, 1964).

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# Naturally Occurring Nitrosatable Amines. II. Secondary Amines in Tobacco and Cigarette Smoke Condensate

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The previously described method for isolation and identification of naturally occurring secondary amines has been applied to tobacco and cigarette smoke condensate ("tar"). Pyrrolidine and dimethylamine are the predominant amines found in both substances.

The high incidence of human lung cancer which has been correlated with cigarette smoking cannot be accounted for by the amounts of known carcinogens which have been identified in cigarette smoke condensate ("tar"). A possible source of other carcinogens is the in vivo nitrosation of the amines from the mainstream smoke to produce carcinogenic *N*-nitrosamines. These compounds have been detected in cigarette smoke condensate (Roades and Johnson, 1972; Neurath, 1969; McCormick et al., 1973; Wynder and Hoffmann, 1968), but only at the parts per billion level. [*N*-Nitrosonornicotine has been found in chewing tobacco at a much higher level (Hoffmann et al., 1974).] It has been established that an appreciable portion of the inhaled smoke is swallowed and retained in the

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