

Determination of Extractable, Apparent Total *N*-Nitroso Compounds in Cured-Meat Products

WALTER FIDDLER, JOHN W. PENSABENE, ROBERT C. DOERR, and ROBERT A. GATES

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Service, 600 E Mermaid Ln, Philadelphia, PA 19118

The modification of a newly developed method for determination of apparent total *N*-nitroso compounds by chemical denitrosation and chemiluminescence detection of nitric oxide (thermal energy analysis) is described. The minimum level of reliable measurement was 0.1 ppm, and the repeatability of the method was 0.2 ppm, based on the response of *N*-nitrosoproline (NPro). Seventy-three samples of cured-meat products, including frankfurters, bacon, and ham, were examined; 50 samples contained less than 1 ppm. The largest amounts, up to 24.8 ppm, were detected in canned corned beef. This method has several advantages over other methods.

Since the discovery of carcinogenicity of *N*-nitrosodimethylamine (NDMA; 1), extensive work has been performed to determine the role of nitrosamines in causing human cancer. As a result, nitrosamines have been analyzed extensively in foods and beverages (2). Techniques for the determination of parts-per-billion levels of volatile nitrosamines in foods are now well established (3). Development of analytical methods for nonvolatile nitrosamines, however, has been slow and restricted primarily to nitrosoamino acids, nitrosamines containing hydroxyl groups, or a combination. Volatile derivatives of nonvolatile nitrosamines are generally made to permit direct analysis by gas-liquid chromatography (GLC) with chemiluminescence (thermal energy analyzer, TEA) detection of nitric oxide and confirmation by gas chromatography/mass spectrometry (GC/MS) (4). Despite the ability to interface a liquid chromatograph to a TEA (5), this technique has not been widely adopted for analysis of nonvolatile nitrosamines in food products because of problems with aqueous mobile phases.

Another problem has been lack of information on the identity of nitrosamines likely to be encountered. For this reason, several attempts have been made to determine the amount of "total" nitroso groups that will estimate the sum of the volatile

and nonvolatile nitrosamine contents of food products. Daiber and Preussmann (6) proposed the use of UV light to selectively cleave the N-NO bond to liberate nitrite, which could be detected colorimetrically after addition of the Griess reagents. This technique takes advantage of the fact that nitrosamines are photolabile. Eisenbrand and Preussmann (7) used chemical denitrosation with hydrogen bromide. Released nitrite was analyzed spectrophotometrically by the same method.

Walters et al. (8) used TEA for chemiluminescence detection of nitrosyl halide generated by hydrogen bromide in glacial acetic acid. The main advantage of this method is that food can be analyzed directly, without the need to extract the nitrosamines from the sample. However, this method has several disadvantages. The most important is its inability to ensure the response is only from *N*-nitroso compounds and not from some other nitric oxide compounds (9). Recognition of the nonspecificity of this "total" *N*-nitroso method led several investigators to add "apparent" to the term "total *N*-nitroso compounds" (ATNC).

Despite its shortcomings, the Walters method provided some valuable information on the possible *N*-nitroso compounds in a variety of foods and beverages, including fried bacon and beer (10, 11). Variations and improvements of this method for analysis of environmental samples (12), gastric juice (13, 14), cutting oils (15), and feces (16) were reported.

In 1990, Havery (17) reported an improved detection method for determination of nonvolatile nitrosamines. It uses a postcolumn system to denitrosate the nitrosamine with hydrogen iodide in an aqueous mobile phase. The technique can be used without an LC column to estimate the ATNC content of samples. Reanalysis with a column can then help identify the compounds responsible for the nitric oxide response. Application of this method to sample analysis was not given. We investigated the Havery method for determination of ATNC because it offers some potential advantages over other available methods, particularly its ability to detect labile nitrosamines. In this paper, we describe our modification of this method and the results obtained from a survey of different types of cured-meat products.

METHOD

Reagents

(*Caution: N-Nitrosamines are potential carcinogens. Exercise care in handling these compounds.*)

Received January 9, 1995. Accepted by JL March 29, 1995.

Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

(a) *Acetonitrile (AcCN)*.—LC grade (Burdick & Jackson, Muskegon, MI).

(b) *Sulfuric and glacial acetic acids*.—Analytical reagent grade (Mallinckrodt, Inc., Paris, KY).

(c) *Potassium iodide (KI)*.—Granular, ACS grade (Fisher Scientific, Malvern, PA).

(d) *Sulfamic acid*.—Used without further purification (Pfaltz & Bauer, Inc., Waterbury, CT).

(e) *N-Nitrosamines*.—*N*-Nitrosoproline (NPro), *N*-nitrosothiazolidine-4-carboxylic acid (NTHZC), *N*-nitrosarcosine (NSar), *N*-nitrosohydroxyproline (NHPro), *N*-nitrosomethylurea (NMU), and *N*-nitrosomethylacetamide (NMA) were synthesized from their corresponding parent compounds and sodium nitrite under acidic conditions and purified by either vacuum distillation or recrystallization according to the general procedure described previously. Their identities and purities were established by MS (18). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NNMG) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

(f) *LC working standard solution*.—NPro in AcCN at a concentration of 1 $\mu\text{g}/\text{mL}$.

(g) *Meat products*.—Random samples were obtained from local retail outlets. Grind samples through a $1/16$ in. plate before analysis and store in a -20°C freezer until analyzed. For the cooking experiment, split frankfurters in half lengthwise and broil in a preheated electric oven 4.5 in. from the heating element for 3 then 2 min per side; fry bacon slices in preheated electric frying pan at 375°C for 3 min per side.

Apparatus

(a) *Homogenizer*.—Tissumizer Model SDT-1810 equipped with a 10N shaft (Teckmar Co., Cincinnati, OH).

(b) *Refrigerated centrifuge*.—Sorvall Model RC-5B (DuPont Instruments, Wilmington, DE).

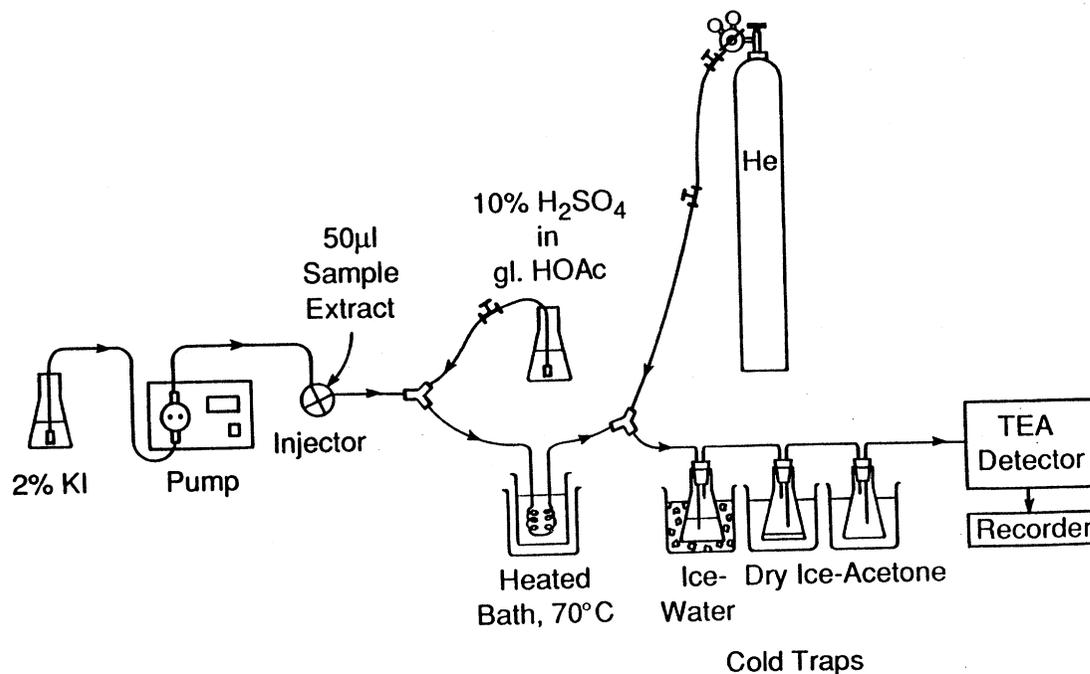
(c) *Mixer*.—Lab-Line Super-Mixer (Lab-Line Instruments, Melrose Park, IL).

(d) *LC injection valve*.—Rheodyne Model 7125 (Keystone Scientific, Bellefonte, PA).

(e) *Detection system*.—A diagram of the system is shown in Figure 1. An Altex Model 332 pump (Beckman Instruments, San Ramon, CA) was interfaced to a TEA Model 502 chemiluminescence detector (Thermedics, Inc., Woburn, MA). The mixing valve was the same as that described by Havery (17). The TEA operating conditions, the Teflon tubing reaction coil system, the solvent trapping system, and the fitting adapted to aspirate the sulfuric-acetic acid were the same as described previously (17).

Determination of ATNC

A schematic of the extraction procedure is shown in Figure 2. Weigh 10.0 g comminuted sample into a 35 mL plastic centrifuge tube, add 15 mL acetonitrile, and homogenize for 5 min at medium speed. Centrifuge the sample for 45 min at 15 000 rpm at 2°C . Decant the supernatant into a 25 mL graduated cylinder and measure the volume of the sample extract (top layer, if 2 layers are present). Take a 2.0 mL aliquot, add 0.6 mL 10% sulfamic acid in 1N sulfuric acid, and mix with the mixer for 5 s. Let phases separate. Use a 100 μL syringe to take 50 μL sample from the top layer and inject into a stream of 2% KI (flow rate of 1.5 mL/min) via the Rheodyne valve. Aspirate 10% sulfuric acid in glacial acetic acid into the stream, then pass that mixture through the reaction coil immersed in a 70°C water bath. The nitric halide or oxide resulting from the denitrosation reaction is swept through one ice water and 2 dry ice-



isopropyl alcohol cold traps by a stream of helium sufficient to maintain a TEA vacuum of 1.0 mm Hg. Analyze each sample in duplicate. Calculate the average response based on peak heights of 4 determinations of both the NPro standard and the cured-meat sample extract. This procedure was carried out by injecting 50 μ L NPro standard directly into the solvent stream without sulfamic acid treatment. Calculate the amount of ATNC in the sample as follows:

$$\text{ppm (as NPro)} = \frac{PH_{\text{sample}}}{PH_{\text{std}}} \times \frac{1 \mu\text{g NPro}_{\text{std}}}{\text{mL}} \times \frac{V_{\text{sample}}}{10 \text{ g sample}}$$

where PH_{sample} = peak height of the sample, PH_{std} = peak height of the standard, and V_{sample} = volume of the sample extract in milliliters.

The minimum level of reliable measurement was 0.1 ppm (signal-to-noise ratio, 2:1), and the repeatability of the method was 0.2 ppm.

Results and Discussion

Other researchers (8, 15, 19) indicated the necessity of removing residual nitrite present in the samples before analysis

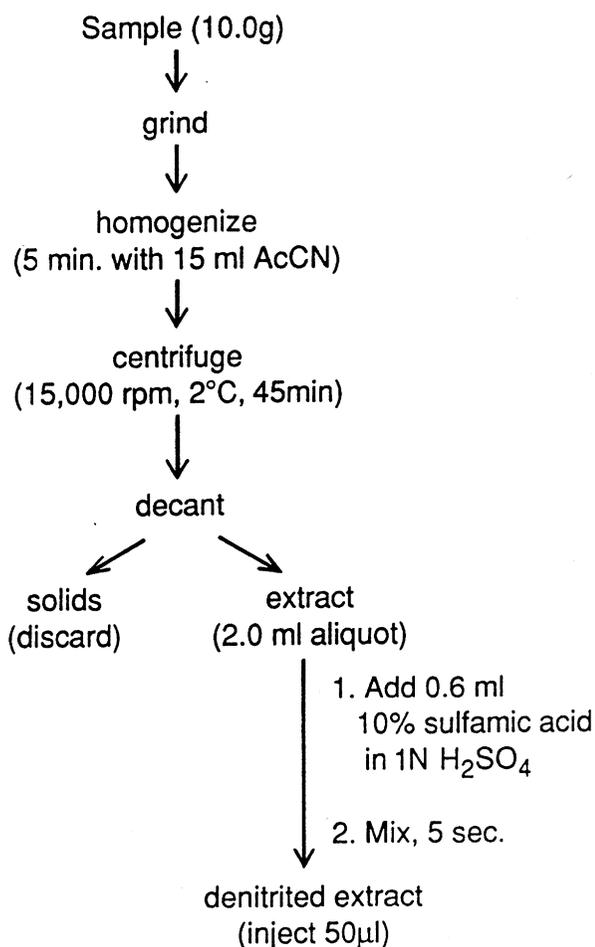


Figure 2. Schematic sample preparation procedure for determination of ATNC.

because it interferes with quantitation of ATNC by giving a false-positive response. Under our experimental conditions, the nitrite response was 60% of that obtained for NPro on an equimolar basis. In preliminary experiments, extracts from 7 samples of commercial frankfurters gave ATNC responses ranging from 0.6 to 22.0 ppm (calculated as NPro). This result paralleled the residual nitrite content. This result was obtained even after addition of 0.5 mL 10% sulfamic acid in 1N sulfuric acid, a treatment that destroyed 50 ppm NaNO_2 in an aqueous solution at room temperature after 2 min. This procedure had no effect on NPro, NTHZC, or NSar and resulted in only 11% decomposition of NHPro.

To determine whether the TEA response was due to residual nitrite, more sulfamic acid was added. Each addition resulted in a decrease in peak size. Heating the sample after sulfamic acid addition eliminated the response completely. This treatment destroyed both the nitrite and other *N*-nitroso compounds that might have been present. Attempts to modify the sulfamic acid method by raising the pH of the sample extract and by passing the extract through a strong anion-exchange cartridge were not satisfactory in eliminating all the nitrite. One approach to solving this problem was to extract the nitrosamines from the sample with an organic solvent, thereby leaving the nitrite behind in the aqueous layer. Methanol extraction of a ground frankfurter fortified with 2 ppm NNMG, NMU, or NMA gave low recoveries.

Ethyl acetate extraction resulted in highly variable recoveries, but AcCN was effective for the nitrosamines studied. Only a small amount of residual nitrite was taken up by AcCN. Walters et al. (20) recommended that, whenever possible and particularly when large amounts of nitrate are present, extraction of *N*-nitroso compounds be carried out with a water-immiscible solvent. Even though AcCN and water are miscible in the absence of the sample, 2 layers are formed when the sulfuric acid-sulfamic acid solution is added. In our experiments, the layers were noticeable because of sample pigments present in the top layer. The rapid and mild conditions, used for nitrite removal, described in the *METHOD* section did not compromise the acid-labile nitrosamines. Because of the formation of the 2 layers, the nitrosamines in the upper AcCN layer had only limited exposure to the acid at the interface.

AcCN extraction of NPro from 6 ham samples fortified at 1 ppm and analyzed in duplicate gave an average recovery of 65.9%. NTHZC, NSar, NMU, NNMG, NMA, and NHPro extractions gave similar results. For these analyses, a 2% aqueous KI system was used before injection instead of 10% KI postinjection as reported by Havery (17). This protocol gave a less noisy signal and a higher response for a nitrosamine standard than that obtained by adding both 6% KI and acid postinjection, the conditions that previously gave us the best results. This improvement suggests that the denitrosation reaction is more efficient with KI added before rather than after injection with the acid. A linear response was found for 25 to 600 ng of the reference standard NPro ($r^2 = 0.9393$). This amount is equivalent to 0.7 to 17.0 ppm in a 10 g sample.

Seventy-three samples of uncooked cured meats, representing a wide variety of product types, were analyzed. Results

are shown in Table 1. Most of the samples, with few exceptions, contained less than 1 ppm ATNC (as NPro). For example, a mean value of 0.3 ppm ATNC was obtained for the most popular cured-meat product, frankfurters. The frankfurters analyzed included 12 made with pork or beef or a combination, 6 with chicken, and 5 with turkey. No significant difference was noted among the franks made with different meats. The 10 samples of bacon, from 6 different manufacturers, also contained less than 1.0 ppm ATNC. One of these samples was a poultry bacon analogue product. Bacon was previously associated (21, 22) with formation of NDMA and *N*-nitrosopyrrolidine as a result of frying. Fermented products comprised "hard" salamis ($n = 4$), a similar heavily smoked product called Lebanon bologna, a pepperoni, and a dried-beef stick snack item. The highest ATNC levels in this group were found in the beef stick and the salami products. Two salami products contained 2.4 and 2.5 ppm.

These findings suggest that beef-containing products may have the highest ATNC content. For this reason, 5 samples of dried beef from 4 manufacturers were tested; 2 had ATNC values of 1.2 and 3.2 ppm. Products designated "other" included typical deli luncheon meats such as bologna, salami, pepper loaf, corned beef, and souse, which is a specialty item in the Pennsylvania area. The highest values in this group were found in one of the salamis and the corned beef. Results for the deli corned beef luncheon meat suggest that canned corned beef should also be tested for ATNC. Even though both products are called "corned beef," they are processed differently. Deli corned beef is usually made from an intact brisket or muscle and cured by an immersion process yielding a sliceable pink product. With canned corned beef, the nitrite-containing meat is retorted in the can; the result is a fibrous, red product. Canned corned beef is not made in the United States; this product is usually produced in Argentina and Brazil but marketed under the house and brand names of several major U.S. companies. Unlike other product types examined, none of the 10 samples of canned corned beef contained less than 1 ppm ATNC (mean value, 7.9 ppm).

Most striking were 2 samples containing over 10 ppm ATNC: 12.6 and 24.8 ppm. Massey et al. (10) found 600–

1400 μg (N-NO)/kg in 5 unspecified canned products. This range is equivalent to 2.0–4.6 ppm when the calculation is based on the NPro response. By contrast, in a survey of 6 canned hams, only 1 sample was in excess of 1 ppm ATNC (1.6 ppm). Four conventionally smoked, boneless hams produced in elastic rubber netting gave similar results, with highest value at 1.1 ppm. Also, higher amounts of nitrosamines could be present in these hams because of components in the rubber nettings (23).

To study the effect of home cooking on ATNC, 26 of the same frankfurter and bacon samples were analyzed after cooking. After taking the 0.2 ppm repeatability (24) of the method into consideration, only 4 samples showed an increase in ATNC after cooking. A slight increase, from 0.7 to 1.1 ppm, was noted in one chicken-containing frankfurter of the 16 broiled frankfurters tested. Broiling was selected because it yielded the highest levels of NDMA in frankfurters (25). Of the 10 fried bacon samples, 1 showed a slight increase and 2 showed significant increases in ATNC, from 0.7 to 2.8 and from 0.9 to 1.9 ppm, respectively. The latter product was a bacon analogue product.

In a survey of foods and beverages, Massey et al. (10) found 470–6000 μg (N-NO)/kg (equivalent to 1.5–19.6 ppm calculated by our method) in 5 samples of uncooked bacon. From 360 to 2400 μg (N-NO)/kg (equivalent to 1.2–7.9 ppm) was found in the cooked product. In this case (10), the homogenized sample was shaken in ethyl acetate containing 1% α -tocopherol before reflux. Then acetic acid was added to release nitric oxide from acid-labile compounds like nitrite and nitrosothiols. Addition of HBr in acetic acid then cleaved the *N*-nitroso-containing compounds.

More recently, the same researchers (26) reported 430–6800 μg (N-NO)/kg (equivalent to 1.4–22.3 ppm NPro) in fried bacon ($n = 26$). The sample was blended with aqueous sulfamic acid before chemical denitrosation. The difference between these and differences in our results could be due to variations in amounts of ingredients and processing and frying conditions used in the United Kingdom and in the United States. In addition, UK producers use meat from the swine's back for bacon, whereas those in the United States typically use meat

Table 1. Apparent total *N*-nitroso content of cured-meat products

Product	<i>n</i>	Range, ppm ^a	Mean ^a	No. samples in range ^b			
				ND–1.0	1.1–5.0	5.1–10.0	>10
Frankfurters	23	ND–0.8	0.27	23	—	—	—
Bacon	10	ND–1.0	0.50	10	—	—	—
Salami (fermented)	8	0.2–2.5	1.31	3	5	—	—
Dried beef	5	0.3–3.2	1.17	3	2	—	—
Corned beef (canned)	10	3.9–24.8	7.88	—	3	5	2
Ham (canned)	6	0.2–1.6	0.79	5	1	—	—
Ham (netted)	4	0.8–1.1	0.93	3	1	—	—
Other	7	0.2–2.4	1.22	3	4	—	—

^a Calculation based on response of NPro.

^b ND, not detected; —, $n = 0$.

from the belly. Another major difference is that the 2 methods detect different sources of nitric oxide. The thermal instability of some *N*-nitroso compounds in heated ethyl acetate and the direct use of acetic acid and sulfamic acid may prevent analysis of heat- and acid-labile nitrosamines that our method attempts to analyze by first extracting the sample with AcCN. Unlike other methods, where direct contact between sulfamic acid and nitrite occurs, the AcCN solvent extract in our method only has minimal contact at the aqueous interface that is sufficient to destroy any small amount of residual nitrite present. Because there are no flasks, condensers, or other glassware to disassemble, a large number of samples can be analyzed daily. The only disadvantage may be that the method is restricted to *N*-nitroso compounds soluble in AcCN. The limitations associated with this solvent cannot be assessed until the compounds responsible for the TEA response are elucidated.

None of the available ATNC methods gives information on individual nitrosamines, but all provide data that may be useful in identifying products that have the potential for containing high concentrations of *N*-nitroso compounds. The results obtained for canned corned beef suggest that this product warrants further examination for components responsible for the high ATNC. This method could also provide information on the nature of nitrosamine precursors, similar to that reported by Massey et al. (26). For example, total *N*-nitroso compound levels in fried bacon were higher than combined levels of simple volatile nitrosamines, nitrosoamino acids, and other nitrosamines that currently can be analyzed. They also found (26), on average, that only 16% of the total ANTC is accounted for by the sum of individual nitrosamines analyzed. Tricker et al. (27) showed that 50% of the total *N*-nitroso compound content is associated with an insoluble protein fraction in adipose tissue that is responsible for *N*-nitrosopyrrolidine in fried bacon. Other compounds giving an ATNC response may have the ability to transnitrosate secondary amines, amino derivatives, and amides. A good example of this process is the nitrite-lipid reaction product derived from bacon fat (28).

Conclusions

The reported method can identify products that may contain high levels of *N*-nitroso compounds. Results for canned corned beef suggest that this product warrants further examination of components responsible for the high ATNC values not observed for other products. This method for total *N*-nitroso compounds has a number of advantages over other reported methods: detection of thermally labile compounds, minimal loss of acid-labile nitrosamines, and high sample throughput.

References

- (1) Magee, P.N., & Barnes, J.M. (1956) *Br. J. Cancer* **10**, 114–122
- (2) Hotchkiss, J.H. (1989) *Cancer Surveys* **8**, 295–321
- (3) Hotchkiss, J.H. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1037–1054
- (4) Sen, N.P., & Kubacki, S.J. (1987) *Food Addit. Contam.* **4**, 357–383
- (5) Fine, D.H., Huffman, F., Roundbeher, D.P., & Belcher, N.M. (1976) in *Environmental N-Nitroso Compounds: Analysis and Formation*, A.E. Walker, P. Bogovski, & L. Gričute (Eds), IARC Sci. Publ., Lyon, France, No. **14**, pp. 43–50
- (6) Daiber, D., & Preussmann, R. (1964) *Z. Anal. Chem.* **206**, 344–352
- (7) Eisenbrand, G., & Preussmann, R. (1970) *Arzneim. Forsch.* **20**, 1513–1517
- (8) Walters, C.L., Downes, M.J., Edwards, M.W., & Smith, P.L.R. (1978) *Analyst* **103**, 1125–1133
- (9) Massey, R.C., Bayley, J.M., Key, P.E., McWeeney, D.J., & Knowles, M.E. (1984) *Food Addit. Contam.* **1**, 237–244
- (10) Massey, R.C., Key, P.E., McWeeney, D.J., & Knowles, M.E. (1984) *Food Addit. Contam.* **1**, 11–16
- (11) Massey, R.C., & Key, P.E. (1989) *Food Addit. Contam.* **6**, 453–458
- (12) Drescher, G.S., & Frank, C.W. (1978) *Anal. Chem.* **50**, 2118–2121
- (13) Bavin, P.M.G., Darkin, D.W., & Vaney, N.J. (1982) in *N-Nitroso Compounds: Occurrence and Biological Effects*, H. Bartsch et al. (Eds), IARC Sci. Publ., Lyon, France, No. **41**, pp. 337–343
- (14) Pignatelli, B., Richard, I., Bourgade, M.C., & Barsch, H. (1987) *Analyst* **112**, 945–949
- (15) Cox, R.D., & Frank, C.W. (1982) *Anal. Chem.* **54**, 557–559
- (16) Rowland, I.R., Granily, T., Bockman, O.C., Key, P.E., & Massey, R.C. (1991) *Carcinogenesis* **12**, 1395–1401
- (17) Havery, D.C. (1990) *J. Anal. Toxicol.* **14**, 181–185
- (18) Pensabene, J.W., Fiddler, W., Dooley, C.J., Doerr, R.C., & Wasserman, A.E. (1972) *J. Agric. Food Chem.* **20**, 274–277
- (19) Waters, C.L., Hart, R.J., & Perse, S. (1978) *Z. Lebensm.-Unters.-Forsch.* **167**, 315–319
- (20) Walters, C.L., Smith, P.L.R., & Reed, P.I. (1984) *IARC Sci. Publ. No.* **20**, 113–120
- (21) Fazio, T., White, R.H., Dusold, L.R., & Howard, J.W. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 919–921
- (22) Sen, N.P., Donaldson, B., Iyengar, J.R., & Panalaks, T. (1973) *Nature* **241**, 473–474
- (23) Sen, N.P., Baddoo, P.H., & Seaman, S.W. (1987) *J. Agric. Food Chem.* **35**, 346–350
- (24) Youden, W.J., & Steiner, E.M. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA, pp. 80–81
- (25) Fiddler, W., Pensabene, J.W., Gates, R.A., Hale, M., & Jahncke, M. (1992) *J. Food Sci.* **57**, 569–571
- (26) Massey, R.C., Key, P.E., Jones, R.A., & Logan, G.L. (1991) *Food Addit. Contam.* **8**, 585–598
- (27) Tricker, A.R., Perkins, M.J., Massey, R.C., & McWeeney, D.J. (1985) *Z. Lebensm.-Unters.-Forsch.* **180**, 379–383
- (28) Hotchkiss, J.H., Vecchio, A.J., & Ross, H.D. (1985) *J. Agric. Food Chem.* **33**, 5–8