

Confirmation of *N*-Nitrosodimethylamine and *N*-Nitrosopyrrolidine in Foods By Conversion to their Nitramines with Pentafluoroperoxybenzoic Acid

WALTER I. KIMOTO, LEONARD S. SILBERT, and WALTER FIDDLER

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118

A method is presented, wherein concentrated dichloromethane extracts from malt, beer, and nonfat dried milk powder containing *N*-nitrosodimethylamine (NDMA), and fried dry-cured and pump-cured bacon containing NDMA and *N*-nitrosopyrrolidine (NPYR) undergo 3N HClO₄-Cellite microcolumn cleanup followed by peroxidation with pentafluoroperoxybenzoic acid, a stable solid peroxyacid, to *N*-nitrodimethylamine and *N*-nitropyrrolidine, as an aid to nitrosamine confirmation. The nitramine-containing solution underwent further cleanup with 6N HClO₄-Cellite and acid alumina-6% H₂O microcolumns for subsequent analysis by gas chromatography interfaced with thermal energy analyzer and ⁶³Ni electron capture detectors. With a 20 g sample, 1 ppb NDMA and 2.5 ppb NPYR could be confirmed by this method. The extract can be concentrated further before analysis, so the detection level can be reduced to <1 ppb NDMA and 1 ppb NPYR.

The confirmation of volatile nitrosamines in food and nonfood products by mass spectrometry is currently considered the most reliable technique (1). Because of its cost, however, the mass spectrometer is not available to many investigators who perform nitrosamine analysis. Furthermore, nitrosamines are often detected in various substrates in quantities too low for mass spectral confirmation. For these reasons, alternative methods are needed which, though not as definitive as mass spectrometry, will confirm the presence of nitrosamines. Peroxidation of nitrosamines to their corresponding nitramines with trifluoroperoxyacetic acid, prepared by the reaction of

trifluoroacetic acid or anhydride with 30–90% hydrogen peroxide, was used in the past for detecting nitrosamines; this was because the electron capture (EC) detector showed greater sensitivity and selectivity for nitramines than detectors then available for nitrosamines (2–4). This peroxidation reaction has been applied, on a limited basis, as a confirmatory procedure by a few investigators, primarily for the detection of *N*-nitrosodimethylamine (NDMA) in nitrite-treated fish (2, 5), cured meat products (6), and ambient air and cigarette smoke (7).

The thermal energy analyzer (TEA), which shows a high degree of selectivity and sensitivity for detecting nitrosamines when interfaced to a gas chromatograph (GC), has simplified nitrosamine analysis and is used widely for this purpose. The TEA detector is also responsive to nitramines. Several investigators have reported nitramine to nitrosamine molar responses of 0.35 to 0.87, depending on the particular volatile nitramine, and a significantly different response for the same nitramine (8–10). Despite the variation in TEA response, in general, the retention times of the nitramines are longer than the corresponding nitrosamines. The recognition of the presence of the nitrosamine peak before peroxidation, and, of its reduction or disappearance afterward coupled with the simultaneous appearance of the nitramine peak, was recommended as an aid to confirmation of volatile nitrosamines in a recent IARC publication (11). While our research was in progress, Sen et al. (12) reported the trifluoroperoxyacetic acid peroxidation of nitrosamines to nitramines in various food products. In this study, the nitramines were detected by

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned. Received November 17, 1983. Accepted February 7, 1984.

GC-TEA at an elevated furnace temperature of 625°C rather than the range 450°–475°C, normally used for nitrosamine analysis.

The objectives of this investigation were to develop a reliable aid to nitrosamine confirmation by a peroxidation reaction that would minimize repeated use of concentrated hydrogen peroxide, and to use TEA for detecting nitramines under normal conditions so that routine nitrosamine analysis would not be disrupted by changes in furnace temperature.

METHOD

Reagents

(a) *Solvents*.—Glass-distilled dichloromethane (DCM; redistilled), hexane and pentane both passed through silica gel 60 columns (Burdick & Jackson Laboratories, Muskegon, MI 49442), and anhydrous ethyl ether (redistilled, or reflux with 3–5 g LiAlH₄/L for 2 h, distill, and store under nitrogen at –20°C; J. T. Baker Chemical Company, Phillipsburg, NJ 08860).

(b) *Sodium sulfate*.—Anhydrous, granular, reagent grade (Mallinckrodt, Inc., St. Louis, MO 63147).

(c) *Silica gel 60*.—70–230 mesh. Place in chromatographic column and wash with anhydrous ether (5 mL/g from unopened can) previously saturated with water. Reactivate by drying 14 h in 150°C oven (Brinkmann Instruments, Inc., Westbury, NY 11590).

(d) *Alumina*.—Acid, 100–200 mesh. Deactivate to activity 3 with 6% water, place in chromatographic column, and wash column with redistilled anhydrous ethyl ether (4 mL/g) for cleanup. Activate 14 h in 180°C oven, then deactivate to activity 3 with 6 g water/94 g alumina (Bio-Rad Laboratories, Rockville Center, NY 11571). *Caution*: Remove residual ethyl ether before activating alumina or silica gel 60 in the oven, to avoid potential explosive hazard.

(e) *Perchloric acid*.—70%. Dilute to 3N and 6N (Eastman Kodak Co., Rochester, NY 14650).

(f) *Celite 545*.—Heat 4h in 180°C oven (Fisher Scientific Co., Fair Lawn, NJ 07410).

(g) *Boiling chips*.—Carborundum, small size (or equivalent).

(h) *Lithium aluminum hydride*.—Used as obtained commercially (Alfa Products, Danvers, MA 01923).

(i) *Pentafluoroperoxybenzoic acid*.—React pentafluorobenzoic acid and 90% hydrogen peroxide in the presence of methanesulfonic acid and trifluoromethanesulfonic acid at 55°–65°C. Cool reaction mixture, extract with DCM, and concentrate extract. Recrystallize crude peroxyacid from DCM; peroxyacid will precipitate as long needles, with 99–100% peroxide content as determined by starch-I₂-thiosulfate method (14). Store peroxyacid at –20°C until use. *Caution*: 90% H₂O₂ is an extremely strong oxidizer and must be handled appropriately. A more detailed procedure for the preparation will be reported elsewhere (13).

(j) *N-Nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR)*.—Working standard, 0.1 ng/μL DCM. *Caution*: NDMA and NPYR are potent animal carcinogens and must be handled appropriately.

(k) *N-Nitrodimethylamine (DMN) and N-nitrosopyrrolidine (PYRN)*.—Prepare with trifluoroacetic acid and 30% hydrogen peroxide by the method of Emmons (15). Working standard, 0.1 ng/μL hexane.

(l) *Hydrogen peroxide*.—90% (FMC Corp., Philadelphia, PA 19103).

Apparatus

(a) *Disposable Pasteur pipets*.—Heavy wall, 5¾ in. length, 5.5 mm id (Fischer Scientific Co., Cat. No. 13-678-6A).

(b) *Evaporative concentrator*.—Kuderna-Danish, 250 mL with 4 mL concentrator tube and Snyder and micro-Snyder distilling columns (Kontes Glass, Vineland, NJ 08360).

(c) *Glass wool*.—Pyrex or equivalent.

(d) *Microflex vial*.—1 mL (Kontes Glass).

(e) *Gas chromatograph-thermal energy analyzer (GC-TEA)*.—Previously described (16), except reduce GC He flow rate to 17 mL/min and program oven temperature from 130° to 220°C at 4°C/min. Inject 8 μL sample at attenuation 8.

(f) *Gas chromatograph-⁶³Ni electron capture detector*.—Hewlett-Packard Model 5880A. GC instrument conditions: 1.83 m × 3.2 mm stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 Gas-Chrom P; 95% argon–5% methane carrier gas, 30 mL/min; injector 200°C; detector 300°C; column, 140°C for DMN, 180°C for PYRN. Inject 1 μL sample at attenuation 2⁴ or 16.

Samples

(a) *Cure-pumped bacon*.—Analyze 20 g fried samples for NDMA and NPYR by dry column method (17).

(b) *Dry-cured bacon*.—Analyze 10 g or 25 g fried samples for NDMA and NPYR by dry column (17), multidetectorial (18), and mineral oil distillation (19) methods.

(c) *Beer*.—Analyze 25 g or 50 g samples for NDMA by another dry column method (20).

(d) *Nonfat dried milk powder*.—Analyze 25 g samples for NDMA by a third dry column method (21).

(e) *Malt*.—Analyze samples for NDMA by DCM extraction (50 g) method, different dry column method (25 g), and mineral oil distillation (25 g) methods from those described above (22).

For the current study, one beer and all of the fried cured-pumped bacon samples had previously been analyzed by GC-low resolution mass spectrometry method (23) to ensure that nitrosamines were present.

Procedures

(a) *Preperoxidation cleanup*.—Place DCM extracts of food product (0.2–0.5 mL) in concentrator tube and, if necessary, concentrate to 0.2 mL and dilute with 4–5 mL pentane. Pack disposable Pasteur pipet containing glass wool plug with 3 cm layer of 3N HClO₄–Celite mixture. To prepare acid–Celite, place 1 g Celite 545 and 0.8 mL 3N HClO₄ in 30 mL beaker and mix with 3 mm od stirring rod 2 min. Wash column with 2 mL pentane, and then add food extract–pentane mixture. Wash column with ca 10 mL pentane, and place column on top of second pipet column containing glass wool plug and 0.5 cm layer of acid alumina (activity 3), washing column with 3 mL DCM before use. Elute nitrosamines from acid–Celite column with 4 mL DCM onto acid alumina column which will retain pigmented compounds. Collect eluate from alumina column in concentrator tube, add boiling chip, fit tube with micro-Snyder column, place in 70°C water bath, and concentrate eluate to ca 0.5 mL. Remove boiling chip and concentrate eluate to 0.2–0.3 mL, or to its starting volume with gentle stream of nitrogen if GC-TEA analysis is desired.

(b) *Peroxidation*.—Place eluate in 1 mL microflex vial containing 10–15 mg pentafluoroperoxybenzoic acid and reduce volume to ca 0.1 mL with stream of nitrogen. Cap vial, shake by hand to obtain homogeneous solution, and store in dark at ambient temperature for 4 h. Add ca 0.1 mL each of DCM and 1N NaOH, cap vial, shake vigorously by hand in rocking

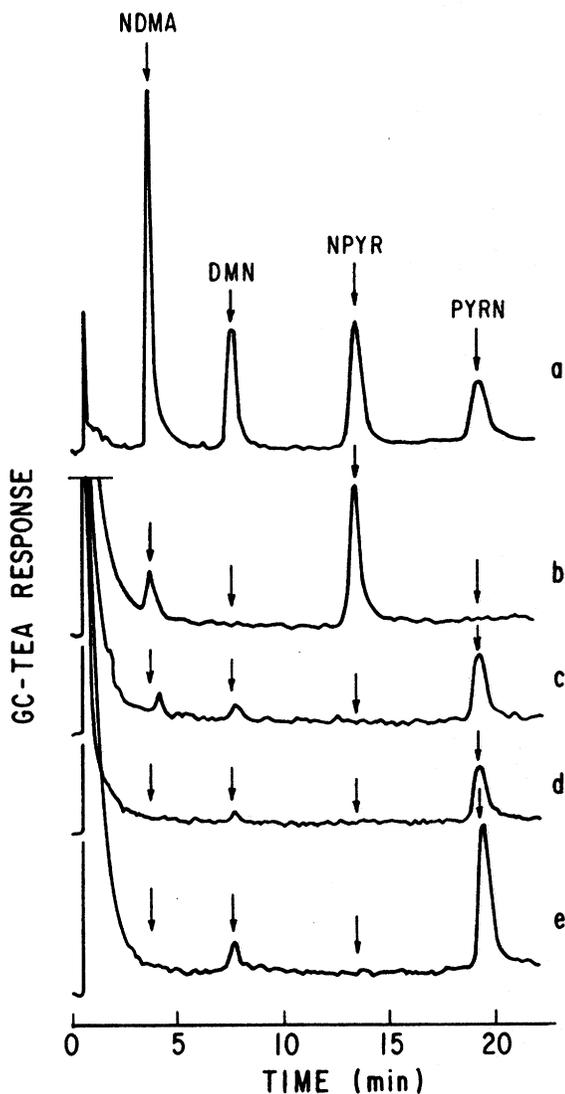


Figure 1. GC-TEA chromatogram of (a) 0.4 ng each of NDMA, DMN, NPYR, and PYRN standards; (b) fried cure-pumped bacon sample extract after acid-Celite column cleanup; (c) sample after peroxidation; (d) sample after acid-Celite and acid alumina cleanup, (e) sample concentrated to ca $\frac{1}{5}$ original volume.

motion for 10–15 s, and let mixture stand 10–15 min to permit phases to separate. Remove usually cloudy aqueous upper layer with pipet, add Na_2SO_4 , dilute extract with DCM, pass this through pipet column containing 1–2 cm layer of anhydrous Na_2SO_4 , and collect filtrate in concentrator tube. Wash vial twice with DCM and also pass washings through Na_2SO_4 column. Concentrate filtrate first on water bath and then with stream of nitrogen to 0.2 mL, or to same volume before peroxidation if GC-TEA analysis is desired.

(c) *Postperoxidation cleanup*.—Add 4–5 mL pentane to 0.2 mL concentrated extract, and pass mixture through pipet column containing glass wool plus 1 cm layer of Na_2SO_4 , glass wool, and 2 cm layer of mixture of 6N HClO_4 -Celite (0.8 mL/g). Wash column with 5 mL pentane, elute nitramines with 4 mL DCM, concentrate eluate on water bath to ca 0.5 mL, then add 4 mL pentane and pass eluate through 1.5 cm layer of acid alumina (activity 3) pipet column. Wash column with ca 6 mL pentane and elute nitramines with 3 mL LiAlH_4 -treated ethyl ether. Collect eluate in concentrator tube containing hexane equal to predetermined volume for concentrated eluate, then concentrate eluate in water bath to 0.4–0.5 mL, remove boiling chip, and reduce volume further with

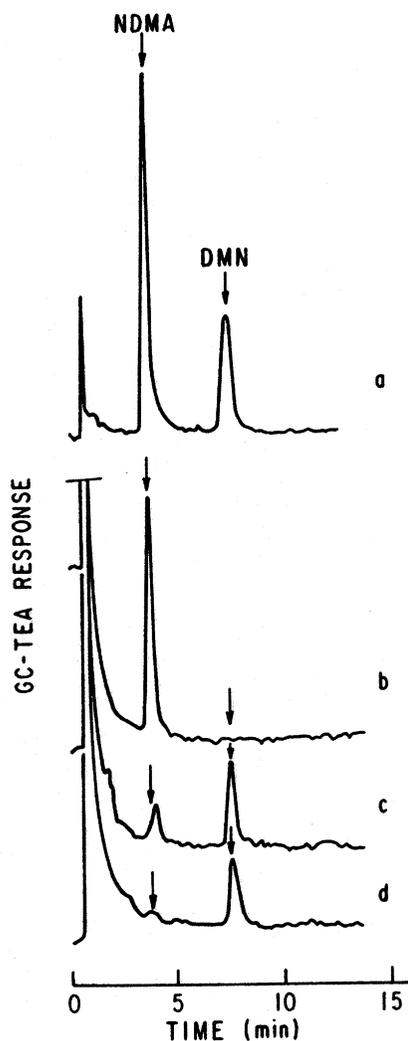


Figure 2. GC-TEA chromatogram of (a) 0.4 ng each of NDMA and DMN standards; (b) beer sample extract after acid-Celite cleanup; (c) sample after peroxidation; (d) sample after acid-Celite and acid alumina cleanup.

stream of nitrogen. Subject reagent and solvent blanks minus the food extracts to the same procedure to determine the presence of possible interfering peaks after GC detection.

Results and Discussion

Preliminary peroxidation reactions with the stable peroxyacid, *p*-nitroperoxybenzoic acid, indicated that it was unsuitable: Nitrosamine standards required several hours at 70°C for their conversion to nitramines. A stronger peroxyacid was therefore required. Pentafluoroperoxybenzoic acid was suitable because the peroxidation reaction could be run at room temperature. Many of the peroxidation reactions, including some of the products of these reactions, which are represented in Figures 1–4, were carried out after storage of pentafluoroperoxybenzoic acid at -20°C for more than a year. The stability of the peroxyacid appeared unaffected by longterm storage at -20°C . Certain food extracts yielded non-nitramine TEA-responsive peaks after peroxidation with pentafluoroperoxybenzoic acid, thereby requiring sample cleanup before peroxidation. The use of HClO_4 -Celite columns for cleanup was based on the results of investigations on the retention of various nitrosamines on Celite 545 con-

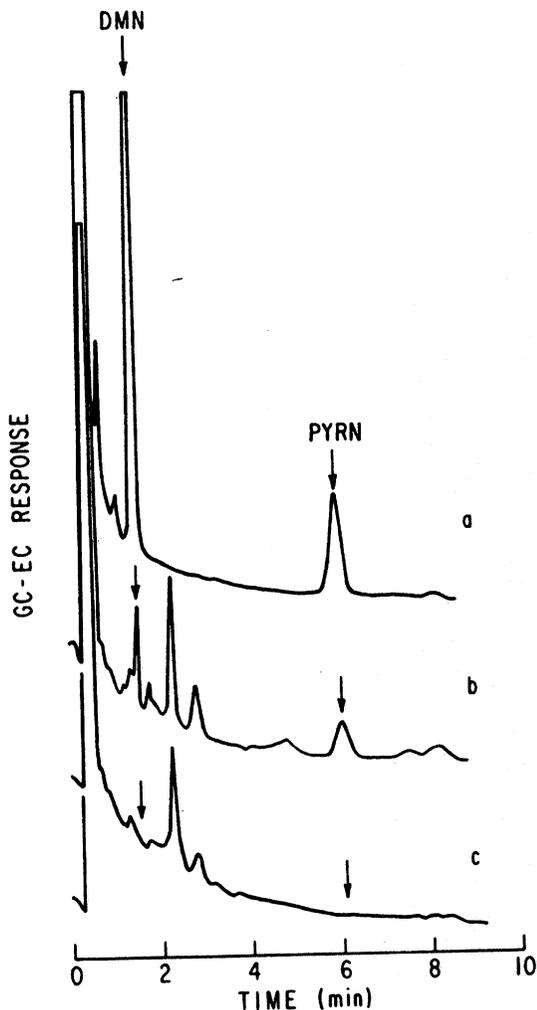


Figure 3. GC-EC chromatogram at column temperature of 180°C: (a) 0.1 ng each of DMN and PYRN standards; (b) fried cure-pumped bacon sample extract after peroxidation and cleanup steps; (c) blank.

taining different concentrations of HClO_4 (G. W. Harrington and H. M. Pylypiw, Jr, unpublished data, Temple University, Philadelphia, PA 1982). The HClO_4 -Celite gave better results in preliminary studies than the HCl -Celite column we used previously (24), and was therefore used for this study.

The GC-TEA chromatograms for the before and after peroxidation reaction of cure-pumped bacon and beer are shown in Figures 1 and 2, respectively. Figure 1b shows the relatively clean chromatogram of a bacon extract after acid-Celite cleanup. In contrast, the chromatogram of the original extract (not shown) contained a large initial peak, with a small apparent NDMA tailing peak, thereby making quantitation of NDMA difficult. The original extract contained 3.5 ppb NPYR and approximately 0.3 ppb NDMA as determined by GC-TEA analysis. The chromatogram resulting after the peroxidation reaction is shown in Figure 1c. In this chromatogram, the PYRN peak was prominent, whereas the DMN peak was very small, and a contaminant peak eluting slightly after NDMA, which was also present after the peroxidation reaction in other food extracts and the blanks, was removed by the postperoxidation cleanup procedure (Figure 1d). Acid-Celite cleanup of the peroxidized extract resulted in some loss of DMN and PYRN (Figure 1d). Concentrating the post-

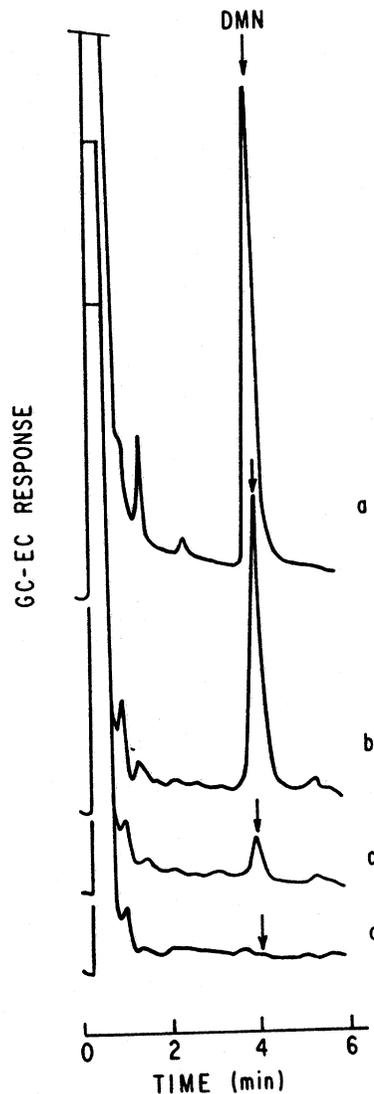


Figure 4. GC-EC chromatogram at column temperature of 140°C: (a) 0.1 ng DMN standard; (b) beer sample extract after peroxidation and cleanup steps; (c) fried cure-pumped bacon sample extract after peroxidation and cleanup steps; (d) blank.

peroxidation acid-Celite cleanup extract to approximately $\frac{1}{3}$ its volume increased the peak size of PYRN, and of DMN from very weak to prominent, but not the background level. The overall sensitivity was thereby increased (Figure 1e). The results for beer, in which the original NDMA content was 1.6 ppb, are shown in Figure 2. These results were similar to those for bacon in Figure 1.

For GC-TEA analysis, the acid-Celite cleanup step followed by peroxidation and washing the reaction mixture with 1N NaOH may be sufficient; reasonably clean chromatograms were obtained for bacon and beer extracts as indicated by Figure 1c and Figure 2c. When GC-EC analysis was performed, the reaction mixture needed to be cleaned up further with an acid-Celite column containing a stronger acid solution than that used for cleaning the preperoxidation extracts. This is necessary because the amine nitrogen in the nitramine is less basic than that of the corresponding nitrosamine. The acid alumina (activity 3) column was also needed to replace the DCM nitramine solvent with hexane-ethyl ether because the former produces about a 100 times greater EC detector response than the latter.

GC temperature programming was not possible with GC-EC analysis because of a rapid increase in the baseline with

an increase in the oven temperature. Two isothermal GC oven temperatures were used, 140°C for DMN and 180°C for PYRN. At 180°C, the retention times for DMN and PYRN were about 1.5 and 6 min, respectively. DMN and PYRN were detected in the cure-pumped bacon sample (Figure 3b), which was the same as the bacon sample represented in Figure 1, with no interfering peaks indicated in the blank (Figure 3c). At an oven temperature of 140°C, for the beer sample (Figure 4b), which was the same as the beer sample represented in Figure 2d, the DMN EC response was strong, but weak for the bacon sample (Figure 4c), whereas the blank contained no peaks in this same region (Figure 4d). This was similar to the relative TEA response for these same 2 samples (compare Figures 1d and 2d); therefore, the results for the GC-TEA and GC-EC were in agreement. Since the TEA and EC detectors operate on different principles, detection of nitramines on both detectors increased the confidence that nitrosamines were actually present.

For discussion and illustrative purposes, the beer and bacon samples were selected as representative examples because the NDMA in beer and NPYR in cure-pumped bacon had been previously confirmed by GC-MS (19). In addition, the 0.3 ppb apparent NDMA in bacon could be confirmed by this method as DMN only after concentration of the extract to increase sensitivity. Up to now, confirmation of 1 ppb NDMA was difficult. This method permitted confirmation of apparent 0.3 ppb NDMA in bacon since the sample extract could be reduced to increase the nitramine response without a corresponding increase in background response.

The method, as reported, may be limited to certain volatile nitrosamines because others will not be retained by the acid-Celite columns used. Among some of the other volatile nitrosamines reported in food and nonfood products, *N*-nitrosomorpholine can be analyzed by the method described in this paper, whereas *N*-nitrosodiethylamine (NDEA) and *N*-nitrosopiperidine (NPIP) require higher concentrations of HClO₄. For analyzing NDMA and/or NPYR in the presence of NDEA and/or NPIP, the effluent from the 3N HClO₄-Celite column has to be passed through a 6N HClO₄-Celite column, which retains NDEA and NPIP. After peroxidation the nitramines of NDEA and NPIP are retained by a 70% HClO₄-Celite column that is effective for cleanup before GC-EC analysis.

The lowest confirmatory levels for this method by GC-TEA were approximately 20 and 50 ng/mL in food extracts containing NDMA and NPYR, respectively, with a TEA signal-to-noise ratio of 4:1. For a 20 g sample, this is equivalent to 1 ppb for NDMA and 2.5 ppb for NPYR. The extracts can be concentrated as indicated by Figure 1e, so the actual NDMA and NPYR levels can be reduced to <1 ppb and 1 ppb, respectively. The overall recovery of nitrosamine in the concentrated DCM extract to nitramine including the cleanup steps was 50–60% by the GC-TEA analysis, based on the assumption of a stoichiometric conversion of nitrosamine to nitramine. Despite using only 1/8 of the total sample size for analysis, the GC-EC analysis has greater sensitivity for DMN and about the same for PYRN compared to the GC-TEA analysis. However, the GC-EC detector is less selective and therefore more responsive to contaminants than GC-TEA. Some food extracts gave TEA responsive peaks other than nitramines after peroxidation with pentafluoroperoxybenzoic acid when the cleanup procedure was omitted. In general, the use of cleanup procedures will decrease the possibility that the nitrosamine and nitramine peaks observed are due to contaminants. In our opinion, if the nitramine peak height is greater than 30% AUFS under the conditions reported (about 5 ppb NDMA or 10 ppb NPYR), the probability of this

and the larger parent nitrosamine peak being due to contaminants is greatly reduced. Therefore, the GC-TEA analysis alone is considered sufficient as a confirmatory aid. However, as the nitramine peak height becomes smaller than this arbitrary 30% level, the probability that the nitramine and the parent nitrosamine peaks are due to potential contaminants also increases. To compensate for this situation we recommend the use of both GC-TEA and GC-EC detection, in addition to the utilization of the previously reported ultraviolet photolytic technique applied directly to nitrosamine-containing solutions (25).

In conclusion, a large number of bacon, malt, nonfat dried milk powder, and beer samples containing varying levels of nitrosamines, analyzed by various isolation procedures commonly in use, were employed to demonstrate the general applicability of the nitramine confirmatory method. On a qualitative basis, analyses by both the GC-TEA and GC-EC techniques were in agreement for the presence of nitramine when these compounds could be detected by GC-TEA. Once prepared, the ease of handling pentafluoroperoxybenzoic acid makes this peroxyacid superior to the peroxidation reagents, hydrogen peroxide and trifluoroacetic acid, that have been commonly used.

Acknowledgments

We thank J. W. Pensabene of the Eastern Regional Research Center for the preparation of *N*-nitrodimethylamine and *N*-nitropyrrolidine, and the National Cancer Institute for the loan of a thermal energy analyzer under contract No. N01-CP-55715.

REFERENCES

- (1) Gough, T. A. (1978) *Analyst* **103**, 785–806
- (2) Sen, N. P. (1970) *J. Chromatogr.* **51**, 301–304
- (3) Telling, G. M. (1972) *J. Chromatogr.* **73**, 79–87
- (4) Walker, E. A., Castegnaro, M., & Pignatelli, B. (1975) *Analyst* **100**, 817–821
- (5) Gadbois, D. F., Ravesi, E. M., Lundstrom, R. C., & Maney, R. S. (1975) *J. Agric. Food Chem.* **23**, 665–668
- (6) Sen, N. P. (1972) *Food Cosmet. Toxicol.* **10**, 219–223
- (7) Cucco, J. A., & Brown, P. R. (1981) *J. Chromatogr.* **213**, 253–263
- (8) Fiddler, W., Doerr, R. C., & Piotrowski, E. G. (1978) *Environmental Aspects of N-Nitroso Compounds*, E. A. Walker, M. Castegnaro, L. Griquite, R. E. Lyle, & W. Davis (Eds.), Scientific Publication No. 19, International Agency for Research on Cancer, Lyon, France, pp. 33–39
- (9) Hotchkiss, J. H., Barbour, J. F., Libbey, L. M., & Scanlan, R. A. (1978) *J. Agric. Food Chem.* **26**, 884–887
- (10) Walker, E. A., & Castegnaro, M. (1980) *J. Chromatogr.* **187**, 229–231
- (11) Sen, N. P., Fiddler, W., & Webb, K. S. (1983) *Environmental Carcinogens Selected Methods of Analysis*, Volume 6, *N-Nitroso Compounds*, H. Egan, R. Preussmann, I. K. O'Neill, G. Eisenbrand, B. Spiegelhalder, & H. Bartsch (Eds.), Scientific Publication No. 45, International Agency for Research on Cancer, Lyon, France, pp. 499–503
- (12) Sen, N. P., Seaman, S., & Tessier, L. (1982) *N-Nitroso Compounds: Occurrence and Biological Effects*, H. Bartsch, I. K. O'Neill, M. Castegnaro, & M. Okada (Eds.), Scientific Publication No. 41, International Agency for Research on Cancer, Lyon, France, pp. 185–197
- (13) Silbert, L. S. (1984) *Tetrahedron Letters*
- (14) Swern, D. (1970) *Organic Peroxides*, Volume 1, Chapter 7, D. Swern (Ed.), Wiley Interscience, Division of John Wiley & Sons, New York, New York, pp. 497–500
- (15) Emmons, W. D. (1954) *J. Am. Chem. Soc.* **76**, 3468–3470
- (16) Pensabene, J. W., Fiddler, W., Miller, A. J., & Phillips, J. G. (1980) *J. Agric. Food Chem.* **28**, 966–970
- (17) Pensabene, J. W., Miller, A. J., Greenfield, E. L., & Fiddler, W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 151–156