J. Agric. Food Chem., Vol. 26, No. 3, 1978 **657**

Determination of Volatile *N*-Nitrosamines in Bacon Cook-Out Fat by Nitrite Release and Thin-Layer Chromatography of Fluorescent Amine Derivatives

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A method is described for the identification and determination of volatile *N*-nitrosamines in bacon cook-out fat. The technique is based on separation by double distillation, clean-up on a column of silica gel, determination by cleavage with HBr in glacial acetic acid, and subsequent colorimetric estimation of nitrite released and/or thin-layer chromatography of the 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) derivatives of the corresponding amines. Lower limits of detection of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) are in the order of 5 μg/kg (ppb). The technique has been compared with a high-resolution gas chromatography–mass spectrometry method and gave comparable results.

A number of methods are now available for the determination of volatile *N*-nitrosamines in foodstuffs and these have been reviewed recently (Foreman and Goodhead, 1975). In spite of the fact that the need for screening methods has been repeatedly emphasized (Goodhead and Gough, 1975), work on this type of method has been somewhat limited. This has been due, in no small measure, to the fact that it is now generally considered that unambiguous results can be obtained only by the use of high-resolution gas chromatography–mass spectrometry (GC–MS) (Walker et al., 1976). However, the cost of such equipment puts it beyond the reach of many laboratories and, for repeated analysis of a particular substrate, screening methods have an important part to play.

We wish to describe our experience with two non-GC–MS methods which have been combined to give a technique capable of accurately determining volatile *N*-nitrosamines in fried bacon cook-out fat. The technique combines those of measuring nitrite released from *N*-nitrosamines with hydrobromic acid in acetic acid (Eisenbrand, 1970) and the corresponding amines by fluorescent densitometry of their 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) derivatives (Klimisch and Stadler, 1974a,b). The combined method has been compared with a high-resolution GC–MS technique (Bryce and Telling, 1972; Telling et al., 1974) for the analysis of fried bacon cook-out fat and has shown excellent agreement. With suitable modifications to the clean-up procedure, this method can be applied to a wide range of other substrates.

EXPERIMENTAL SECTION

Important Safety Note. *N*-Nitrosamines have been shown to be highly carcinogenic compounds in test animals and all experimental work should be done in a well-ventilated area. Safety gloves should be worn whenever *N*-nitrosamines are being handled.

Apparatus. Thin-layer densitometer, Vitatron TLD-100; visible spectrophotometer, Beckman DK-2.

Reagents. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (Aldrich Chemical Co.); Merck precoated TLC plates, silica gel 60, 0.25 mm thickness.

Cooking of Bacon. Samples of bacon and cook-out fat were prepared either by frying for 12 min in an electric frying pan with the thermostat set at 172 °C and draining fat from rashers, or by grilling for 6 min with fat reaching a temperature of 170–200 °C.

Extraction and Cleanup. Weigh 200 g of cook-out fat into a 1-L round-bottomed flask and add a solution of 100 g of sodium chloride in 500 mL of water. Connect the flask in a standard distillation system and distill at atmospheric pressure until 440 mL of distillate have been collected. Transfer the distillate to a second 1-L round-bottom flask, add 48 g of sodium hydroxide, and allow to dissolve, keeping the system cool at all times.

Connect the flask in a closed vacuum system with a 1-L collecting flask immersed in a dry ice–acetone bath. Apply vacuum to the system by means of a water aspirator until the liquid in the distillation flask begins to boil. Close the vacuum system and immerse the distilling flask in a water bath at 65 °C. Maintain these conditions until about 65 mL of liquid remains and the temperature of the flask surface begins to rise (about 1 h).

Melt the distillate, adjust pH to 2 with 10% v/v sulfuric acid, and add 35 g of sodium sulfate. Extract with three 100-mL portions of dichloromethane and dry the combined extracts over a mixture of 25 g of anhydrous sodium sulfate plus 200 mg of anhydrous potassium carbonate. Transfer the solution to a 500-mL conical flask, fit a Snyder column, and evaporate the dichloromethane on a hot plate until the volume is reduced to about 20 mL. Transfer the flask to a water bath at 65 °C so that the bottom just touches the water. Continue the concentration while adding 8 mL of hexane in small portions through the Snyder column. When the top of the column shows no further refluxing, rinse the column with 2 mL of hexane.

Prepare 2.2 g of silica gel-H as a 6.5 × 1 cm column in hexane and top with a 1-cm layer of anhydrous sodium sulfate. Transfer the hexane concentrate to the top of the column, rinsing with a further 5 mL of hexane. When the level of hexane drops to the sodium sulfate layer, place a suitable receiver under the column and elute with 25 mL of pentane–diethyl ether–dichloromethane (10:3:2) at a rate of 4 mL min^{-1} under slight pressure. Discard the eluate. Elute with 25 mL of the same solvents in a ratio 5:3:2 into a 25-mL pear-shaped flask. Fit the flask with a micro-Snyder column and remove the solvent by evaporation on a water bath at 65 °C. Just before the flask goes dry, remove from the water bath and rinse the Snyder column with 2 × 0.5 mL of glacial acetic acid. Transfer the acetic acid solution to a glass-stoppered 10-mL cylinder and make up to 2 mL with glacial acetic acid. Add a solution of 3% hydrogen bromide in acetic acid to give a volume of 4.0 mL. Stopper the cylinder, mix well, and allow to stand at room temperature for 15 min.

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0021-8561/78/1426-0657$01.00/0 © 1978 American Chemical Society

Determination. (*a*) *Nitrite Content.* Transfer 2 mL of test solution to a 5-mL volumetric flask containing 2 mL of a 1:1 mixture of 1% sulfanilic acid in 30% v/v acetic acid and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 30% acetic acid, mix, and dilute to mark with the same mixed reagent. Allow to stand for 15 min at room temperature and measure absorbance at 550 nm in a 1-cm cell against a reagent blank.

Determine the nitrite content from a standard curve prepared from known amounts of *N*-nitrosopyrrolidine (NPYR) by the above method. Alternatively, use silver nitrite as the standard. Calculate the concentration of the *N*-nitrosamines in the original sample in micromoles × 10^{-2}/kilogram. In the case of NPYR this corresponds to micrograms/kilogram since its molecular weight is 100.

(*b*) *Amine Content.* Transfer the remaining 2 mL of test solution to a 25-mL round-bottomed flask and evaporate to dryness in vacuo. Transfer the residue to a 1-mL glass-stoppered tube with small volumes of methanol and remove the methanol under a stream of nitrogen on a water bath at 65 °C. Add 125 μL of methanol, 125 μL of 0.05% w/v NBD-Cl in methanol, and 60 μL of 0.1 M sodium bicarbonate, stopper the tube, and heat for 1.5 h in 55 °C water bath (Klimisch and Stadler, 1974a). Evaporate the contents of the tube to dryness under a stream of nitrogen and make up to a predetermined volume with dichloromethane in hexane (1:4). Prepare fresh standards from *N*-nitrosamines by the same procedure each day. Spot the amine–NBD derivatives plus standards on a 20 × 20 cm Merck precoated silica gel 60 TLC plate, layer thickness 0.25 mm. Develop once with cyclohexane–ethyl acetate (1:1). Immediately after development, air-dry the plates and scan on a Vitatron TLD flying spot densitometer in the fluorescence mode with a 366-nm primary filter and a 546-nm secondary filter. Remove the prediaphragm of the measuring lens to increase the sensitivity in the fluorescence mode. Calculate the quantities of *N*-nitrosamine present on the basis of a 100% conversion of the amine to the NBD derivative.

RESULTS AND DISCUSSION

Double Distillation. In our work on volatile *N*-nitrosamines in very fatty samples we found it necessary to take advantage of a double distillation to provide extracts of satisfactory purity for gas chromatographic or colorimetric quantitation. The work of Walters et al. (1970) showed that 70% of NDMA and 82% of NPYR could be recovered from 20% w/v sodium chloride solution, by atmospheric pressure distillation to one-half volume. In the presence of bacon fat we found that increased recoveries (95%) of each nitrosamine could be obtained by distilling 85% of the added water. The salt crystallizes out when about 50% of the water has been removed. The pot temperature is constant at near 115 °C until about 400 mL of distillate has been collected. The temperature then rises steadily to about 120 °C when the distillation is discontinued.

The second distillation (in vacuum) from 3 N sodium hydroxide was suggested by the work of Ender and Ceh (1971). The use of a closed vacuum system utilizing a large temperature gradient between the distilling flask and receiver was an important modification which decreased the time required for distillation, and, in addition, removed acidic materials.

Ender and Ceh reported repeated distillations to half volume because they required the final concentrate in a small amount of water. Since the determinative step of Eisenbrand and Preussmann was to be used there was no necessity for repeated distillation.

Table I. R_f Values of NBD-Amines on Silica Gel 60

Parent Amine	R_f value	
	System 1[a]	System 2[b]
Dimethylamine	0.16	0.25
Diethylamine	0.35	0.41
Di-*n*-propylamine	0.58	0.67
Pyrrolidine	0.27	0.35
Piperidine	0.42	0.47
Morpholine	0.19	0.18

[a] System 1: cyclohexane–ethyl acetate (1:1). [b] System 2: hexane–chloroform–diethyl ether–acetic acid (33:33:33:1).

It was well known that *N*-nitrosamines could be extracted from acidic aqueous medium to eliminate interference from basic components (Tannenbaum et al., 1970; Crosby et al., 1972).

Cleanup on a Silica Gel Column. Neutral contaminants in the dichloromethane extract were found to move rapidly on the 10:3:2 TLC system described by Sen (Sen et al., 1969). A further cleanup was therefore possible by using the small particle silica gel as adsorbent in a column.

The column clean-up procedure that we developed was tested with standard NDMA and NPYR. A mean recovery of each *N*-nitrosamine of 96% with a low of 93% and a high of 97% was obtained. The recovery tests included not only the column step but also the subsequent concentration through the micro-Snyder column and color development reactions since the quantities were determined by the colorimetric procedure.

Determination. Eisenbrand and Preussmann (1970) first described a method for the quantitative splitting of the nitroso group from *N*-nitrosamines by hydrogen bromide in acetic acid and colorimetric determination of the liberated nitrite.

In much of our early work we successfully used this technique for the determination of *N*-nitrosamines prepared as above from fried bacon and its cook-out fat. It was noted, however, that the color development reaction between sulfanilic acid and *N*-1-naphthylethylenediamine dihydrochloride is very sensitive not only to the amounts but also to the nature of impurities. Thus, for example, we found that smoke constituents in bacon hindered color development and, in extreme cases, completely inhibited it. To overcome this problem, we attempted to quantitate the amine portion left after the cleavage reaction. Eisenbrand (1972) recommended reaction of the amines with heptafluorobutanoyl chloride and measurement of the resulting derivatives by gas chromatography.

The dimethylamine derivative is extremely volatile but, in our hands, the method did not give quantitative data for this compound due to the presence of coeluting impurities.

We therefore examined the preparation of fluorescent amine derivatives, as described by Klimisch and Stadler (1974a), by reaction of the amines with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The NBD derivatives were subsequently separated by TLC. In their original work, Klimisch and Stadler used polyamide sheets to separate derivatized standards and showed that a linear calibration curve was obtained for quantities between 15 and 150 ng per spot. They also described the separation of the NBD derivatives on silica gel but noted that the NBD derivatives were less stable on silica gel than on polyamide. We found that precoated silica gel plates were much superior to in-laboratory prepared plates in that the derivatives were more stable on them. The fluorescence measurements of all the standards remain constant for the

Table II. A Comparison of the Described Extraction and Clean-Up Method plus Colorimetric Determination of Released Nitrite with the Unilever Method Using High-Resolution Gas Chromatography-Mass Spectrophotometry

	N-Nitrosamines, μmol × 10^{-2}/kg						
	Described method[c]				Unilever method		
	Colorimetric	GC-MS detn			GC-MS detn		
Sample[e]	detn	NDMA	NPYR	Total	NDMA	NPYR	Total
1	43	3	33	36	8	40	48
2	26	d	d	d	4	13	17
3	46	3	27	30	8	49	57
4	46	7	45	52	7	52	59
5	19	d	d	d	4	18	22
6	15	d	d	d	4	13	17
7	43	d	d	d	4	39	43
8	41	3	38	41	7	36	43
9	0	ND	Tr	Tr	Tr	1	1
10	0	ND	Tr	Tr	Tr	1	1
11	0	ND	Tr	Tr	d	d	d
12	0	ND	1	1	Tr	Tr	1
13	4	Tr	5	5 to 6	Tr	5	5 to 6
14	4	1	8	9	Tr	5	5 to 6
15	2	Tr	3	3 to 4	1	5	6
16	3	Tr	3	3 to 4	Tr	5	5 to 6

[a] Tr = 0.1-1.0 μg/kg. [b] ND = less than 0.1 μg/kg. [c] Corrected for 80% recovery through extraction and cleanup. [d] Not analyzed. [e] Samples within the brackets are duplicates.

Table III. A Comparison of Colorimetric and Thin-Layer Densitometric (TLD) Determinations on a Common Cleaned-Up Extract

	Nitrosamines, μmol × 10^{-2}/kg[a]			
	TLD			Colorimetric
Sample	NDMA	NPYR	Total	Total
11A[b]	26	36	62	65
11B	3	1	4	2
11C	3	4	7	5
11D	1	2	3	3
15A	17	44	61	55
15B	10	11	21	18
15C	2	6	8	12
15D	1	1	2	2
16A	15	27	42	38
16B	4	5	9	10
16C	2	2	4	7
16D	5	6	11	9
17A	10	30	40	35
17B	3	5	8	8
17C	1	2	3	4
17D	4	6	10	9
18A	19	37	56	46
18B	5	6	11	10
18C	2	6	8	9

[a] Corrected for 80% recovery extraction and clean-up procedure. [b] Samples A are controls; samples B, C, and D are test samples with additives.

time required to measure the plate. When silica gel plates prepared in the laboratory were used a steady, slow, decrease in intensity of fluorescence was observed.

On Merck silica gel 60 TLC plates only one development was necessary to obtain separation of the amines of interest. The R_f values we found for the two systems described by Klimisch and Stadler for silica gel are given in Table I. After separation by TLC, the NBD derivatives corresponding to 5 ng of either NDMA or NPYR gave full-scale deflection of the Vitatron recorder.

Known amounts of NDMA and NPYR added to lard or bacon fat at 10, 25, and 50 μg/kg gave mean recoveries of 85 and 78%, respectively. The ranges of recovery values were 79 to 88% and 71 to 84%, respectively, measured by the colorimetric procedure.

Collaborative Studies. As part of a collaborative program of work between Canada Packers and Unilver Ltd., an opportunity arose to compare the results obtained by the technique developed (as described above), and used at Canada Packers, with those obtained by the Unilever Research method based on the use of high-resolution gas chromatography–mass spectrometry with peak matching (Telling et al., 1974).

A series of Canadian and U.K. bacon samples were fried and grilled, as described earlier, and analyzed by the two techniques. In addition, some of the final test solutions produced by the presently described method were also analyzed by Unilever using their high-resolution GC-MS technique with peak matching. These results are summarized in Table II. In the first four samples enough bacon was cooked to give sufficient fat only for a single determination by each of the methods. Each of these four samples is therefore different. Enough of the later samples (5–16) was cooked to give fat for duplicate determinations by both methods.

Samples 9–16 were prepared in such a way as to produce bacon fats containing volatile N-nitrosamines at the 1–5 μg/kg level, thus allowing the method to be proved at or

Table IV. A Comparison of GC-MS, TLD, and Colorimetric Methods for Analysis of Nitrosamines in Bacon Fat

	Nitrosamines, μmol × 10^{-2}/kg						
	TLD		GC-MS		Total		
Sample	NDMA	NPYR	NDMA	NPYR	TLD	GC-MS	Colorimetric
1	3, 14[a]	36, 34[a]	7	36	39, 48[a]	43	36, 32[a]
2	1, 2[a]	2, 2[a]	1	3	3, 4[a]	4	2, 1[a]
3	26	41	11	53	67	64	50
4	3	3	2	7	6	9	4

[a] Duplicate analyses of the same lot of cook-out fat.

near detection limits. Agreement between the two methods is good. When the level of *N*-nitrosamine in a 200 g sample of fat is below about 5 μg/kg, the colorimetric method may give a false negative result. The limit of detection of the colorimetric method is therefore in the order of 5 μg/kg. The GC–MS results on sample 2 are low, probably due to a poor recovery of distillate.

In Table III, the results of a comparison of the colorimetric and thin-layer densitometric (TLD) methods are given. In these experiments half the final eluate was used for measurement of liberated nitrite and half for the measurement of amines. On the basis of these 19 samples, a correlation coefficient of 0.99 was calculated for the two methods. Table IV compares the results obtained by the GC–MS technique with those obtained with the currently described method using the colorimetric and TLD techniques.

The limited number of samples analyzed does not permit a statistical evaluation of these results but, when taken in conjunction with Tables II and III, results show that the combined methods described form a valid alternative to the GC–MS method for measuring volatile *N*-nitrosamines in cooked bacon fat.

LITERATURE CITED

Bryce, T. A., Telling, G. M., *J. Agric. Food Chem.* **20**, 910–911 (1972).

Crosby, N. T., Foreman, J. K., Palframan, J. F., Sawyer, R., *Nature (London)* **238**, 342–343 (1972).

Eisenbrand, G., Preussmann, R., *Arzneim. Forsch.* **20**, 10, 1513–1517 (1970).

Eisenbrand, G., IARC Scientific Publications No. 3, "*N*-Nitroso Compounds Analysis and Formation", Bogovski, P., Preussmann, R., Walker, E. A., Ed., International Agency for Research on Cancer, Lyon France, 1972, pp 64–70.

Ender, F., Ceh, L., *Z. Lebensm.-Unters. Forsch.* **145**, 133–142 (1971).

Foreman, J. K., Goodhead, K., *J. Sci. Food Agric.* **26**, 1771–1783 (1975).

Goodhead, K., Gough, T. H., *Food Cosmet. Toxicol.* **13**, 307–312 (1975).

Klimisch, H. J., Stadler, L., *J. Chromatogr.* **90**, 141–148 (1974a).

Klimisch, H. J., Stadler, L., *J. Chromatogr.* **90**, 223–225 (1974b).

Sen, N. P., Smith, D. C., Schwinghamer, L., Marleau, J., *J. Assoc. Off. Anal. Chem.* **52**, 47–52 (1969).

Tannenbaum, S. R., Issenberg, P., Rogers, J., Fan, T. Y., presented at Third International Congress of Food Science and Technology, Washington, D.C., 1970.

Telling, G. M., Bryce, T. A., Hoar, D., Osborne, D., Welti, D., IARC Scientific Publications No. 9, "*N*-Nitroso Compounds in the Environment", Bogovski, P., Walker, E. A., Ed., International Agency for Research on Cancer, Lyon, France, 1974, 12–17.

Walker, E. A., Bogovski, P., Griciute, L., Ed., Reports and Recommendations, IARC Scientific Publications No. 14, International Agency for Research on Cancer, Lyon, France, 1976, p 497.

Walters, C. L., Johnson, E. M., Ray, N., *Analyst (London)* **95**, 485–489 (1970).

Received for review August 10, 1977. Accepted January 7, 1978.

Behavior and Fate of [^{14}C]Maleic Hydrazide in Tobacco Plants

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The behavior and fate of [4,5-^{14}C]- and [3,6-^{14}C]maleic hydrazide (MH) have been studied in selected varieties of flue-cured and burley tobacco. Foliar-absorbed [^{14}C]MH was translocated rapidly to actively growing tissues in a "source-to-sink" pattern. Twenty-eight days after treatment, 30–40% of the absorbed [^{14}C]MH was translocated to the roots and released into the nutrient solution, 12–22% remained in the plant, 14–18% was extracted as methanol-soluble metabolites, and 25–35% remained in the roots and other tissues as a methanol-insoluble residue. Only 2% of the absorbed ^{14}C was evolved as $^{14}CO_2$. Degradation of the MH heterocyclic ring structure was not a significant metabolic pathway. The major methanol-soluble metabolite in foliar tissues was isolated and identified as the β-D-glucoside of MH. Unchanged MH was isolated and identified as a hydrolysis product following acid and base treatment of isolated methanol-insoluble residues. The distribution of ^{14}C in methanol-soluble and methanol-insoluble residue fractions from freshly harvested young tobacco plants was not altered greatly when the tobacco was flue-cured or air-cured under laboratory conditions.

Maleic hydrazide (1,2-dihydro-3,6-pyridazinedione) is used extensively as a systemic plant growth regulator for the control of axillary bud and sucker growth in tobacco (Tso, 1972). It is applied to the upper portion of the tobacco plant within 24 h after excising the inflorescence (topping). Under physiological conditions, maleic hydrazide (MH) exists as the phenolic tautomer, 6-hydroxy-3-(2*H*)-pyridazinone (Miller and White, 1956) and is apparently quite stable (Smith et al., 1959; Noodén, 1970).

Numerous reports of MH residues in tobacco (Haeberer et al., 1974; Lane, 1965; Hoffman et al., 1962; Liu and Hoffmann, 1973; Davis et al., 1974; Cheng and Steffens, 1976), limited information about MH metabolism in tobacco, and recent concern about the possible health effects of MH residues in tobacco products (Liu and Hoffmann, 1973) prompted the present investigation on the behavior and fate of MH in the tobacco plant. Specific objectives were: (1) to determine quantitatively the behavior and fate

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