A Simple Screening Method for Determination of Volatile Nitrosamines in Fried Bacon Rasher and Cook-out Fat

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A method is described for the rapid screening of cooked bacon rasher for volatile nitrosamines. The analysis is based on fluorescence densitometry of the nitrobenzooxadiazole amines produced by reaction of the amines liberated from nitrosamines by hydrogen bromide in glacial acetic acid or preferably hydrogen bromide in dichloromethane with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The NBD amines are separated by thin-layer chromatography on silica gel. The limit of detection is in the order of 1 μg/kg (ppb) using a 20-g sample. The technique has been compared with a gas chromatograph–thermal energy analyzer method and showed excellent agreement.

Recently, we described the analysis of volatile nitrosamines in bacon cook-out fat (Cross et al., 1978) and showed a good correlation between our method and a GC–MS method (Bryce and Telling, 1972; Telling et al., 1974). In that publication we described the analysis of both the nitrite and amine portions produced by splitting the nitrosamines with hydrogen bromide in glacial acetic acid (Eisenbrand and Preussman, 1970). One procedure could then be used to substantiate the other. The modified Griess reaction used for determining nitrite did not lend itself to the analysis of small samples and, in addition, was found to be very sensitive to interferences, particularly when bacon rasher was being analyzed and the amount of nitrosamine present was less than 5 μg/kg (ppb). On the other hand, the determination of the amine portion by fluorescence densitometry of its derivative with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) after thin-layer chromatographic separation was extremely sensitive and not subject to the interference. Because we were primarily interested in the analysis of fried bacon, we decided to eliminate the analysis of the nitrite portion of the molecule. This allowed us to simplify the method considerably. The modified procedure described here can be applied to samples as small as 10–20 g of cooked bacon.

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. The gloves should not be re-used.

Apparatus. Thin-layer densitometer, Vitatron TLD-100 or equivalent; Polytron Homogenizer, Brinkmann Instruments.

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

Cooking of Bacon. Ten slices of bacon are fried for 12 min in an electric frying pan starting with the pan at room temperature. The thermostat is set at 172 °C. After 4 min, the bacon is turned frequently until the frying period is complete. The rasher is removed from the pan and patted dry on paper towelling to remove excess cook-out fat. This frying procedure results in maximum measurable nitrosamine in both the rasher and in the cook-out fat (Bharucha et al., 1979).

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Separation of the Nitrosamines from the Bacon Rasher. Place the fried bacon rasher in a polyethylene bag and cover the bag with dry ice. Pulverize the frozen sample in the bag to obtain a well-mixed sample. Accurately weigh a 20-g sample of the bacon rasher into a 500-mL round-bottomed flask and add 60 mL of cold 3 N sodium hydroxide in water. Homogenize the sample in the flask for 30 s using a Polytron homogenizer at maximum speed. Rinse the probe with 5–10 mL of distilled water and transfer the washings to the sample flask. Connect the flask to a closed vacuum system with a 250-mL round-bottomed flask for receiver. The distillation apparatus is shown in Figure 1. Immerse the receiver in a dry ice–acetone bath and apply vacuum to the system by means of a water aspirator until the contents of the distilling flask are boiling vigorously. Close the vacuum line and add water at 60–65 °C to the constant temperature bath up the neck of the distilling flask. After 15 min open the vacuum line briefly to confirm that the low pressure is being maintained. Allow the distillation to proceed for a total of 30 min. Remove the receiver from the apparatus and melt the frozen distillate. Acidify the solution to pH 1 with concentrated sulfuric acid. Filter the solution through a small glass wool plug in the neck of a funnel into a 125-mL separatory funnel. Extract the solution with dichloromethane (3 × 30 mL). Carefully separate the layers so that no aqueous layer drains from the funnel. Combine the dichloromethane extracts in a second 125-mL separatory funnel. Backwash the dichloromethane solution with 10% sulfuric acid (1 × 5 mL). Dry the dichloromethane layer with about 5 g of anhydrous sodium sulfate and about 100 mg of anhydrous calcium chloride. Filter the solution into a 250-mL 24/40 conical flask, fit a Snyder column, and evaporate the dichloromethane on a hot plate until about 5 mL of solution remain. Remove the flask from the hot plate and allow the column to drain into the flask. Transfer the solution to a 25-mL pear-shaped flask using a Pasteur pipet. Add a boiling chip and attach a standard inner joint. Immerse the flask to the liquid level in a 60 °C water bath and continue the evaporation until the flask just goes dry. Rinse the column into the flask with 0.2 mL of glacial acetic acid. Remove the column and add 0.2 mL of 3% hydrogen bromide in glacial acetic acid. Stopper the flask and allow it to stand at room temperature for at least 15 min. The splitting reaction may be left overnight with no deleterious effects. Transfer the reaction mixture to a 25-mL round-bottomed flask and evaporate the solution carefully and completely to dryness on a rotary evaporator using a water bath at 50–60 °C. Wash the residue in the flask once with 0.2 mL of distilled hexane. Discard the hexane. Transfer the residue to a glass-stop-

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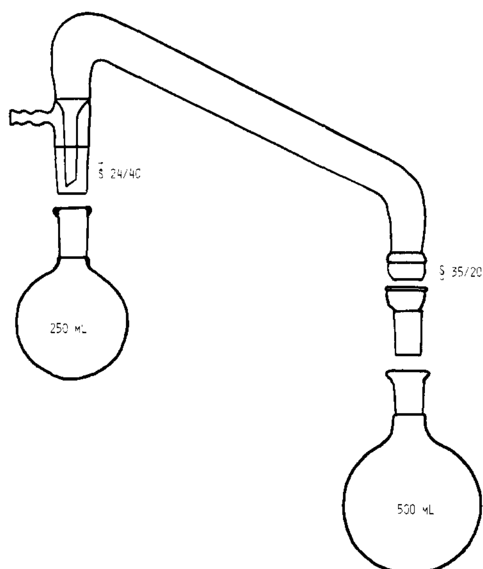

Figure 1. The closed system vacuum distillation apparatus.

pered, calibrated 1-mL test tube using small quantities of methanol. Use only about 0.5 mL of methanol for the transfer. Evaporate the methanol on a hot water bath using a gentle stream of nitrogen.

As an alternative and preferable procedure which avoids the transfer of the split solution, the splitting reaction may be done in the pear-shaped flask with hydrogen bromide in dichloromethane (Eisenbrand et al., 1976). In this method, evaporate the methylene chloride solution in the pear-shaped flask fitted with the inner joint until 1 mL of solvent remains. Add 1 mL of a 5% solution of hydrogen bromide in dichloromethane and allow the reaction to proceed in the stoppered flask as above. Evaporate the dichloromethane on a 60 °C water bath until the flask goes dry. Do not fit the inner joint to the flask. In order to be certain that all hydrogen bromide has been removed, blow nitrogen over the dry residue for about 1 min. Wash the residue with hexane. Transfer the residue from the pear-shaped flask to the 1-mL test tube and proceed with the derivatization.

Formation of the NBD Derivatives. Dissolve the residue in the tube in 40 μL of methanol. Add 40 μL of a 0.05% solution of NBD-Cl in methanol and 20 μL of freshly prepared 0.2 M sodium bicarbonate. Immerse the tube just above the liquid level in a 55 °C water bath for 1 h. Evaporate the solvents on a hot water bath using a stream of nitrogen.

Thin-Layer Chromatography. Add a predetermined volume of dichloromethane in hexane (1:4) (about 200 μL if the nitrosamine content is less than 10 ppb) and spot three times on a 20 × 20 cm TLC plate along with standards derivatized at the same time as the sample. Apply 5 μL to each spot. Develop the plate once for a distance of 12 cm in a saturated tank containing 1:1 cyclohexane-/ethyl acetate. Dry the plate with an air gun and spray it heavily (but not till translucent) with a solution of mineral oil in hexane (2:1) (Uchiyama and Uchiyama, 1978). Alternatively, the plate may be dipped in a 30% solution of mineral oil in hexane. More reproducible intensity measurements across the plate are obtained with this modification. When the plate is treated with mineral oil the fluorescence intensity is increased nearly tenfold and is stable for at least a day. Scan the plate on a Vitatron TLD-100 flying spot densitometer in the fluorescence mode with a 366-nm primary filter and a 546-nm secondary filter. Calculate the amount of nitrosamine present on the

Table I. A Comparison of Nitrosamine Determination by TLD and TEA

	nitrosamines, ppb[a]			
	NDMA		NPYR	
sample	TEA	TLD	TEA	TLD
1	0.4	4.0	4.2	5.3
1A[b]	0.2	1.5	0.5	0.5
2	1.1	1.9	8.1	7.4
2A	0.3	1.2	0.7	1.1
3	0.1	1.2	4.6	6.3
3A	0.1	0.6	0.3	0.2
4	0.6	0.8	3.0	2.0
4A	0.1	0.4	ND	0.2
5	4.5	4.8	29.0	27.1
6	10.0	9.0	4.9	4.3
7	0.7	0.7	2.0	1.2
7A	0.3	0.3	ND	ND
8	1.0	0.8	2.5	1.5
8A	0.4	0.3	ND	ND
	$r = 0.92$		$r = 0.994$	

[a] No correction applied for nonquantitative recovery. [b] Samples designated A were treated with antinitrosamine agent.

basis of a 100% conversion of the amine to the NBD derivative. Average the values found for the three spots.

The method described above can be applied directly to cooked bacon rasher. When cook-out fat is to be analyzed, the double-distillation technique described in our earlier publication (Cross et al., 1978) can be used. An aliquot of the steam distillate from sodium chloride solution corresponding to 20 g of fat is diluted with water to 60 mL and sodium hydroxide is added to give a 3 N solution for the closed system vacuum distillation.

RESULTS AND DISCUSSION

By dispensing with the analysis of the nitrite released from the nitrosamines by hydrogen bromide in glacial acetic acid, it was possible to make several simplifications to our analytical procedure (Cross et al., 1978). (a) It permitted the elimination of the silica gel column clean-up step which was rather laborious and time-consuming and had been necessary only for the colorimetric procedure. (b) The sample size could be decreased by a factor of about 10. (c) As a result of the smaller sample size, it was possible to use less solvent and reagent. (d) Since the nitrosyl bromide was not to be measured, the alternative splitting reaction with hydrogen bromide in dichloromethane could be used.

The elimination of the column clean-up step may allow amines to pass through the isolation procedure. For this reason the extraction of the acidified distillate with dichloromethane must be done very carefully. When both dimethylamine and pyrrolidine were added to the distillate at levels of 500–1000 ppm it was found that they were completely retained by the acidified aqueous solution.

The recoveries of dimethylnitrosamine (NDMA) and nitrosopyrrolidine (NPYR) added to bacon rasher before the homogenization step at levels between 5 and 10 ppb were examined. Twenty samples were analyzed and gave recoveries of 77% for NDMA with a standard deviation of 11.8 and 79% for NPYR with a standard deviation of 12.0. In other experiments in which the spiking was done at different stages of the analysis, it was determined that the major loss of NPYR occurred during the vacuum distillation, while the major loss of NDMA occurred at the extraction step. The recovery of NPYR was not improved by increasing the distillation time to 45 min. The recovery of NDMA could probably be improved by adding sodium sulfate to the distillate, thereby obtaining a more favorable

Table II. A Comparison of Nitrosamine Determination by TLD and TEA

	nitrosamines, ppb[a]			
	NDMA		NPYR	
sample[b]	TEA	TLD	TEA	TLD
2AR	4.3	3.1	19	15
2BR	2.1	2.3	1.9	1.5
2CR	1.9	1.8	12	9.1
2DR	1.1	1.2	0.7	0.4
2AF	13	12.1	40	46
2BF	2.0	1.7	1.7	1.3
2CF	4.4	5.7	38	43
2DF	3.6	1.1	1.6	1.8
3AR	3.9	3.5	25	24
3BR	3.6	3.1	7.7	4.7
3CR	1.2	2.0	6.2	5.4
3DR	0.5	1.0	0.4	0.2
3AF	9.1	10.7	55	53
3BF	3.9	3.1	2.9	2.0
3CF	4.1	5.4	23	27
3DF	3.5	4.2	0.8	0.9
	$r = 0.93$		$r = 0.99$	

[a] No corrections applied for nonquantitative recovery.
[b] R, rasher sample; F, cook-out fat sample.

partition coefficient between dichloromethane and the aqueous phase. It was felt that a slight increase in recovery would not outweigh the possibility of contamination from the sodium sulfate.

The recovery data for nitrosamines from fat were obtained using lard as the base oil. Both NDMA and NPYR were spiked into the fat at levels of 6 and 12 ppb. Thirteen samples were analyzed. They gave recoveries of 83% for NDMA with a standard deviation of 10.9 and 70% for NPYR with a standard deviation of 7.9.

Through the cooperation of Hormel Research Laboratories, we compared our simplified analytical procedure with the semiofficial gas chromatograph–thermal energy analyzer method which is in use in many laboratories on this continent and in Europe. Several bacon extracts from 20-g samples of fried bacon were prepared and divided into two equal portions before treatment with either hydrogen bromide in acetic acid or hydrogen bromide in dichloromethane. One half of each extract was analyzed at Hormel Research Laboratories using the GC–TEA method which detects the nitric oxide removed catalytically from the nitrosamines after separation by gas chromatography (Fine and Rounbehler, 1975). The other half of the extract was denitrosated and analyzed in our laboratories by the above-described thin-layer densitometric method. The results obtained by our TLD method in which hydrogen bromide in acetic acid was used for the denitrosation are compared with those obtained by the TEA method in Table I. The agreement in most cases is remarkably good, particularly in the case of NPYR. The correlation coefficients for NPYR and NDMA were 0.994 and 0.92, respectively.

Table II likewise compares results obtained on eight samples of fried bacon rasher and eight samples of the corresponding cook-out fat by the two procedures with the difference that herein the splitting on nitrosamines was carried out with hydrogen bromide in dichloromethane instead of acetic acid. The correlation coefficients for NDMA and NPYR were found to be 0.93 and 0.987, respectively. Again, excellent agreement between results was obtained by the current method and the semiofficial GC–TEA procedure.

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