

Rapid Dry Column Method for Determination of *N*-Nitrosopyrrolidine in Fried Bacon

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A rapid method has been developed for the determination of *N*-nitrosopyrrolidine (NPYR) in fried bacon at less than the 1 ppb level. Ground fried bacon is mixed with anhydrous sodium sulfate and Celite by using a glass mortar and pestle. This dry mixture is then added to a chromatographic column containing a layer of acid-Celite. The column is washed with pentane-dichloromethane, and the nitrosamines are eluted with pure dichloromethane. The eluate is concentrated, and the nitrosamines are quantitated by using a gas chromatograph interfaced with a thermal energy analyzer. Recovery of the nitrosamine internal standard, *N*-nitrosoazetidine, added at the 10 ppb level, was over 90%. The results obtained by this method are in good agreement with the mineral oil distillation procedure currently used in the FSIS monitoring program. Because 25 samples can be analyzed per day per person, this simple screening procedure offers advantages over other methods.

In 1978, the USDA Food Safety and Quality Service (now Food Safety and Inspection Service (FSIS)) established a 10 ppb violative level for volatile nitrosamines in fried, cure-pumped bacon (1) because this product consistently contained *N*-nitrosopyrrolidine (NPYR) and, to a lesser extent, *N*-nitrosodimethylamine (NDMA). Since then, the mineral oil distillation-gas chromatographic (GC) thermal energy analyzer (TEA) screening method developed by Fine et al. (2) has been the most widely used procedure for determining volatile nitrosamines in cured meat products. Their method involves vacuum-distillation of the nitrosamines from a mixture of comminuted sample, mineral oil, and a small amount of base, followed by extraction of the aqueous distillate with dichloromethane (DCM), and concentration of the DCM before detection and quantitation. This procedure is used by FSIS in their monitoring program. Samples presumably in violation are then analyzed by the more broadly applicable and lengthy FDA mul-

tidetection procedure, which involves sample digestion in methanolic potassium hydroxide, liquid-liquid extraction of the nitrosamines into DCM, distillation from base, acidification of the aqueous distillate, extraction with DCM, concentration before detection by GLC with alkali flame ionization detection (AFID), and a column chromatographic cleanup step for subsequent GLC/mass spectrometric confirmation (3). In the original multidetection method, the nitrosamines were detected by GLC, using a modified thermionic or alkali flame ionization detector (4). Havery et al. (3) analyzed 18 fried bacon samples and generally found good agreement between NPYR values obtained by the GLC/AFID, GLC/TEA, and mineral oil distillation-GLC/TEA procedures. Greenfield et al. (5) recently carried out a 9-laboratory collaborative study on fried bacon fortified with 6 volatile nitrosamines at 6 levels ranging from 0 to 17 ppb and 10 ppb *N*-nitrosodipropylamine internal standard, using the mineral oil distillation-GLC/TEA procedure.

However, the mineral oil distillation-GLC/TEA and other published procedures (6) have several disadvantages. The most important of these is the lengthy analytical time involved. A more rapid method therefore is needed for the routine determination of volatile nitrosamines in fried bacon. We have developed a rapid dry column method based on the principle employed by Maxwell et al. (7) for isolating lipids from muscle and adipose tissue.

METHOD

Note: Nitrosamines are potential carcinogens. Exercise care in handling these materials.

Reagents

(a) *Celite* 545.—Not acid-washed (Fisher Scientific Co.). Run reagent blank before start of sample analysis, particularly if new bottle of Celite is used. If interfering chromatographic products are noted, prewash Celite twice with dichloromethane, then dry 4 h in 120°C vacuum oven before use.

(b) *Dichloromethane* (DCM) and *n*-pentane.—

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Distilled in glass (Burdick & Jackson Laboratories, Inc.).

(c) *Hydrochloric acid*.—6N. Extract once with equal volume of DCM to remove impurities.

(d) *Sodium sulfate*.—Anhydrous, granular (Mallinckrodt No. 8024).

(e) *Internal standard solution*.—0.10 μg *N*-nitrosoazetidine (NAZET)/mL DCM.

(f) *N-Nitrosopyrrolidine (NPYR) and NAZET GLC working standard*.—Each 0.10 μg /mL DCM.

Apparatus

Usual laboratory equipment and the following items:

(a) *Mortar and pestle*.—Glass, 473 mL (16 oz), A. H. Thomas Co.

(b) *Chromatographic column*.—Glass, 350 \times 32 mm id with 60 \times 6 mm id drip tip.

(c) *Evaporative concentrator*.—Kuderna-Danish (KD), 250 mL; concentrator tube, 4 mL, graduated; Snyder (3-section) and micro Snyder distilling columns (Kontes Glass Co.).

(d) *Tamping rod*.—Glass, 450 mm long with 12 mm diam. disc prepared by glassblower.

(e) *Gas chromatograph-thermal energy analyzer*.—Varian Aerograph gas chromatograph Model 2700, or equivalent, interfaced with a thermal energy analyzer Model 502. Operating conditions: 2.7 m \times 3.2 mm stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas-Chrom P; helium carrier gas 35 mL/min; column 180°C isothermal, injector 200°C, TEA furnace, 450°C; TEA vacuum 1.5 mm; liquid nitrogen-ethanol cold trap.

Procedure

(a) *Sample analysis*.—Weigh 10 g Celite into 250 mL beaker. Add 10 mL 6N HCl,² ca 3 mL at a time, and stir Celite with small glass rod until mixture is fluffy and uniform in texture. Using a powder funnel, pour acid-Celite into chromatographic column containing glass wool plug at bottom. Insert tamping rod through Celite and tamp from bottom up to achieve height of ca 25 mm. Accurately weigh 10.0 \pm 0.1 g doubly ground fried bacon and quantitatively transfer sample to mortar. Add 1.0 mL internal standard solution (equivalent to 10 ppb) to bacon sample, using 1.0 mL transfer pipet. Then add 25 g Na₂SO₄ and mix with pestle ca 30 s. Add 20 g Celite to mortar and grind 15–20 s until Celite is thoroughly mixed with Na₂SO₄ and bacon. Then, grind with moderate pressure for an ad-

ditional 2 min. Quantitatively transfer free-flowing dry mixture into chromatographic column, and tamp with glass rod to achieve total height of ca 100 mm. Add 30 g Na₂SO₄ to top of column. Rinse mortar and pestle with 10 mL pentane-DCM (95 + 5), and add rinse to column, immediately followed by 90 mL of same solvent. Collect eluate in 100 mL graduated cylinder. When level of solvent in column drops so that it just touches top of Na₂SO₄, add 125 mL DCM at one time. After 85 mL of wash eluate has been collected, discard and change receivers. Collect remaining eluate in 250 mL KD flask equipped with 4 mL concentrator tube. (Some samples yield turbid effluent; this is normal.) When column stops dripping, remove KD flask, add 2 small boiling chips to flask, attach 3-section Snyder column, and concentrate eluate to 4 mL on steam bath. Continue concentration (add new boiling chip) to 1.0 mL with micro Snyder column in 70°C water bath. Note: Room temperature should be <24°C during analysis of sample.

(b) *Nitrosamine determination*.—Inject 9.0 μL GLC working standard at lowest attenuation that yields signal at least one-third full scale TEA response, and measure peak heights. Repeat to assure good reproducibility of retention time and response. Inject 9.0 μL concentrated nitrosamine-containing sample, measure response of the 2 nitrosamines, and calculate NPYR in ppb, using following formula:

$Z = YACV \times 1000 / XBW$, where $Z = \mu\text{g}$ NPYR/kg (ppb); $V =$ total volume of sample = 1.0 mL; $X =$ peak height of NPYR in standard; $Y =$ peak height of NPYR in sample; $C =$ concentration of standard = 0.10 $\mu\text{g}/\text{mL}$; $A = \mu\text{L}$ of standard injected; $B = \mu\text{L}$ of sample injected; $W =$ weight of sample analyzed = 10.0 \pm 0.1 g.

Statistical Analysis

One-tailed paired *t*-tests or analyses of variance were performed on the measured nitrosamine according to methods described by Snedecor and Cochran (8). Where only the statistical summary is presented, the raw data are available on request. The uncorrected NPYR data were reported as measured and the corrected NPYR data were adjusted for the recovery of the internal nitrosamine standard. For statistical purposes, NPYR data were reported to 2 decimal places.

Results and Discussion

The recovery of 14 volatile nitrosamines added to nitrosamine-free fried bacon was determined

² Author's note: Phosphoric acid (6N) has replaced hydrochloric acid.

Table 1. Recovery of volatile nitrosamines at the 10 ppb level from nitrosamine-free bacon

N-Nitroso compound	Rec., %			
	Range	Mean (n = 4)	SD	CV, %
Dimethylamine	100.0–104.0	101.40	1.89	1.86
Methylethylamine	87.7–94.7	91.43	3.75	4.10
Diethylamine	69.7–77.3	74.20	3.38	4.55
Methylpropylamine	42.7–62.7	52.28	8.23	15.75
Ethylpropylamine	NR ^a	—	—	—
Dipropylamine	NR	—	—	—
Ethylbutylamine	NR	—	—	—
Propylbutylamine	NR	—	—	—
Methylamylamine	NR	—	—	—
Azetidine	90.7–95.7	93.20	2.17	2.33
Dibutylamine	NR	—	—	—
Piperidine	62.5–74.1	68.08	4.77	7.01
Pyrrrolidine	103.6–110.0	105.43	3.57	3.38
Morpholine	94.1–104.1	98.08	4.32	4.41

^a NR = no recovery.

Table 2. Statistical analysis on repeatability of ERRC method

Sources of variation	df	NPYR, uncorrected			NPYR, corrected			% Recovery of NAZET		
		SS	MS	F	SS	MS	F	SS	MS	F
N-Nitrosopyrrolidine	10	463.10	46.31		472.53	47.25		1607.61	160.76	
Error	23	3.09	0.13	344.77**	3.03	0.13	359.16**	404.47	17.59	9.14**
Total	33	466.19	—		475.54	—		2012.09	—	
Repeatability ^a			0.37 ppb			0.36 ppb			4.19%	

** P < 0.01.

^a Repeatability = $\sqrt{MS_{error}}$.

to assess the applicability of our procedure, referred to here as the ERRC method (Table 1). The 2 nitrosamines that commonly occur in fried bacon, NDMA and NPYR, as well as N-nitroso-methylethylamine, -azetidine, -morpholine, were recovered at a mean level >90%. The mean recovery of the other nitrosamines varied from 0 to 74%. The mean recovery for N-nitrosopiperidine, which is occasionally found in fried bacon, was 68%. Because N-nitrosodipropylamine (NDPA), the internal standard in the mineral oil method, was not recovered, NAZET was selected as the internal standard. The possibility of this 4-membered heterocyclic nitrosamine being naturally present or formed in food products was considered unlikely. Also, preliminary evidence indicates a highly significant correlation ($r^2 = 0.925$, $P < 0.01$) between NAZET and NPYR recoveries. Although the ERRC method is applicable to all the volatile nitrosamines detected in fried bacon, this study was restricted to the determination of NPYR, because it is found in the highest concentration and is most likely to exceed the violative level.

Eleven bacon samples were analyzed, 6 in quadruplicate and 5 in duplicate, to determine within-laboratory repeatability of the ERRC

Table 3. Determination of N-nitrosopyrrolidine (ppb) in fried bacon by the ERRC and mineral oil-TEA methods

Sample No. ^a	Mineral oil			ERRC		
	Uncorr.	Corr.	NDPA, % rec.	Uncorr.	Corr.	NAZET, % rec.
1	5.28	6.29	84.0	4.64	5.29	87.7
	5.45	5.85	93.2	4.40	5.13	85.8
2	17.75	22.05	80.5	16.46	21.57	76.3
	14.84	20.14	73.7	15.82	19.36	81.7
3	6.16	6.88	89.5	5.29	6.71	78.9
	6.58	6.38	103.2	5.43	6.52	83.3
4	6.33	6.01	105.4	4.13	4.64	89.1
	4.94	5.26	93.9	4.19	4.83	86.8
5	15.14	15.92	95.1	16.23	16.70	97.2
	13.87	14.47	95.8	15.25	16.93	90.1
6	7.14	7.01	101.8	6.85	7.42	92.3
	6.08	6.31	96.3	6.71	7.10	94.5
7	13.16	12.70	103.6	13.08	13.57	96.4
	13.61	13.24	102.8	13.69	13.69	100.0
8	5.71	5.50	103.9	5.22	6.40	81.6
	5.84	5.62	103.9	5.37	5.91	90.8

^a Duplicate determinations.

Table 4. Statistical analysis on determination of *N*-nitrosopyrrolidine in fried bacon by the ERRC and mineral oil-TEA methods

Determination	No. of detns	Mean (\bar{X})		SD between samples		Results between methods		Min. oil vs ERRC <i>t</i>
		Min. oil (\bar{x}_1)	ERRC (\bar{x}_2)	Min. oil (s_1)	ERRC (s_2)	$\bar{x}_1 - \bar{x}_2$	$s_1 - s_2$	
NPYR, uncorr. (ppb)	16	9.25	8.92	4.63	5.22	0.33	-0.59	0.342
NPYR, corr. (ppb)	16	9.98	10.11	5.80	6.02	-0.13	-0.22	-0.211
% Rec. of int. std ^a	16	95.40	88.38	8.83	6.53	7.12	2.30	1.532

^a NAZET for ERRC method, NDPA for mineral oil method.

method. Determinations of NPYR ranged from 2.23 to 16.93 ppb, corrected (1.78 to 16.23 ppb, uncorrected) and recovery of the NAZET internal standard ranged from 73.7 to 101.8% with a mean of 91.3%. Analysis of variance of the results (Table 2) indicated that repeatability of NPYR determination is 0.36 ppb (0.37 uncorrected) and standard deviation of recovery of NAZET standard is 4.19%.

A ruggedness test of the ERRC method for the determination of 1.5 and 6.0 ppb NPYR, conducted by using different grinding, packing, and solvent elution steps of the procedure specified in the experimental section, indicated that results were not significantly different. However, the column-packing step of the procedure did lead to significant differences in the determinations of 1.5 ppb NPYR. When columns were packed too tightly, determinations varied 16% (23%, uncorrected) at 1.5 ppb NPYR compared with 0.3% (1.0%, uncorrected) at 6.0 ppb NPYR.

Comparative analysis of the ERRC and mineral oil methods was obtained with duplicate determinations of 8 samples of fried bacon containing from 4 to 20 ppb NPYR (Table 3). The 2 methods require a different internal standard because NDPA which is used in the mineral oil procedure, is not recoverable by this procedure, and NAZET, which is used in this procedure, sometimes decomposes during the distillation step of the mineral oil procedure. Statistical analysis of these determinations indicated that the results were equivalent (Table 4). Means of NPYR determinations with this method were 1.3% higher (3.6% lower, uncorrected) and standard deviation of the determinations was 3.7% higher (11.3%, uncorrected) than with the mineral oil reference method. Recovery of internal standard averaged 7.5% lower and varied 35% less than with the reference method. From the mean differences and standard deviations of determinations and recovery of internal standard, a *t*-test ($P = 0.05$) indicated that results with the 2 methods were not significantly different.

Determination of NPYR tends to be higher with the mineral oil method than with the multidetection method (3, and unpublished data). We therefore undertook a study to determine if NPYR was produced as an artifact during analysis. From 0 to 100 ppm sodium nitrite (NaNO_2) was added to nitrite-free bacon, and then determined by both the ERRC method and the mineral oil procedure. With increasing levels of NaNO_2 , NPYR increased in the mineral oil procedure; up to 4 ppb was found when 50 ppm sodium nitrite was added (Table 5). No NPYR was detected when the samples were analyzed by the ERRC method. High residual nitrite in fried bacon could result from undercooking, which may be caused by slice thickness or compositional factors that affect the rate of frying. This bacon, when analyzed by the mineral oil procedure could produce more NPYR as an artifact. Because of this possibility, several investigators now add sodium ascorbate and/or α -tocopherol before distillation to avoid artifactual nitrosamine formation in the mineral oil method (unpublished). This precaution is not necessary with the ERRC method.

A limited interlaboratory study using the ERRC method was conducted on fried bacon containing 0-50 ppb normally incurred NPYR. Samples were analyzed in 3 laboratories; how-

Table 5. Effect of added sodium nitrite on *N*-nitrosopyrrolidine formation, determined by the mineral oil and ERRC methods^a

Sample No.	NaNO_2 added, ppm	NPYR, ppb	
		Min. oil	ERRC
1	0	0.23	ND ^b
2	10	0.99	ND
3	25	1.34	ND
4	50	3.58	ND
5	100	4.84	ND

^a Two separate experiments in duplicate.

^b ND = none detected.

Table 6. Interlaboratory collaborative study on determination of *N*-nitrosopyrrolidine (ppb, corrected) in fried bacon by ERRC method

Sample No. (dupls)	Analyst 1		Analyst 1A		Analyst 2		Analyst 3	
	NAZET, % rec.	NPYR	NAZET, % rec.	NPYR	NAZET, % rec.	NPYR	NAZET, % rec.	NPYR
1	108.2	27.73	89.5	30.21	97.7	30.71	88.8	31.85
	103.4	27.61	91.7	31.07	74.1	33.32	84.9	29.20
2	102.3	10.76	89.5	10.39	103.5	8.75	82.5	11.33
	102.3	10.83	95.8	10.72	82.4	9.48	93.6	10.66
3	100.0	8.92	85.3	8.92	103.5	8.75	91.1	8.79
	100.0	9.08	91.6	8.80	78.3	8.39	82.2	8.65
4	108.0	N.D.	96.8	N.D.	92.0 ^a	N.D.	92.3	N.D.
	109.1	N.D.	96.9	N.D.	91.8	N.D.	96.1	N.D.
5	100.0	48.82	98.9	45.57	100.0	47.01	92.2	49.12
	94.6	47.80	100.0	47.32	95.7	44.92	92.4	46.66
6	91.3	2.86	95.6	2.36	82.6	2.35	87.3	2.60
	90.3	2.61	92.8	2.61	85.7	2.70	85.9	2.76
7	86.8	7.90	89.0	8.23	91.3	7.36	82.9	7.91
	96.7	7.60	97.6	8.23	104.8	7.34	77.4	8.23
8	91.4	6.69	97.6	6.61	88.1	4.19	85.0	6.88
	98.9	6.18	95.2	6.10	100.0	4.92	83.3	6.60

^a Average used for statistical purposes, no internal standard added.

ever, in Laboratory 1, 2 different analysts performed the assay. Because each analyst worked independently, the data were treated statistically as if from 2 separate laboratories. Corrected results of the study (Table 6) were treated statistically because the variation was less than in the uncorrected data. The average within-laboratory recoveries with standard deviation for the internal standard were: 1A, $99.0 \pm 6.7\%$; 1B, $94.0 \pm 4.2\%$; 2, $92.0 \pm 9.5\%$; 3, $87.4 \pm 5.2\%$. The analysis of variance on the corrected results is shown in Table 7. A significant ($P < 0.01$) difference between the bacon samples was observed with an *F*-test as expected because fried bacon samples with a wide NPYR range were intentionally used. No significant laboratory effect nor laboratory \times sample interaction was indicated by the analysis of variance. The standard deviations for reproducibility and repeatability, determined as prescribed by Steiner (9), were 1.03 and 0.71, respectively. This compares favorably with the values of 1.34 and 1.04 for reproduc-

ibility and repeatability obtained in the recent collaborative study (5) on the FSIS mineral oil procedure. A collaborative study of the ERRC method involving a larger number of laboratories is planned.

In conclusion, we are reporting a method for the determination of NPYR in fried bacon, which is rapid, less susceptible to artifactual nitrosamine formation, and quantitatively as good as the currently employed method. With the ERRC method, 25 or more samples per analyst per day can be analyzed with limited glassware, thereby significantly reducing the cost of analysis.

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Table 7. Analysis of variance on interlaboratory collaborative study

Variation	df	SS	MS	<i>F</i>
Sample	7	14635.9	2090.8	4182.5**
Laboratory	3	3.9	1.3	<1
Lab \times sample	21	35.1	1.7	3.3
Error	32	16.0	0.5	
Total	63	14690.9		

** $P < 0.01$.

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