

MEAT AND MEAT PRODUCTS

Dry Column-Thermal Energy Analyzer Method for Determining *N*-Nitrosopyrrolidine in Fried Bacon: Collaborative Study

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A dry column method for isolating *N*-nitrosopyrrolidine (NPYR) from fried, cure-pumped bacon and detection by gas chromatography-thermal energy analyzer (TEA) was studied collaboratively. Testing the results obtained from 11 collaborators for homogeneous variances among samples resulted in splitting the nonzero samples into 2 groups of sample levels, each with similar variances. Outlying results were identified by AOAC-recommended procedures, and laboratories having outliers within a group were excluded. Results from the 9 collaborators remaining in the low group yielded coefficients of variation (CV) of 6.00% and 7.47% for repeatability and reproducibility, respectively, and the 8 collaborators remaining in the high group yielded CV values of 5.64% and 13.72%, respectively. An 85.2% overall average recovery of the *N*-nitrosoazetidine internal standard was obtained with an average laboratory CV of 10.5%. The method has been adopted official first action as an alternative to the mineral oil distillation-TEA screening procedure.

The mineral oil distillation-thermal energy analyzer (TEA) procedure, developed by Fine et al. (1), is currently the most widely used method for the isolation and detection of volatile nitrosamines (NAs) in fried, cure-pumped bacon. This procedure, however, has several disadvantages; the 2 most serious are the lengthy analysis time and the potential for artifactual nitrosamine formation. We have reported the development and preliminary evaluation of a rapid, distillation-free, dry column chromatographic method for *N*-nitrosopyrrolidine (NPYR) in fried bacon, although other volatile NAs can also be determined (2). The critical evaluation of this method in our laboratory, as well as a limited interlaboratory study, demonstrated its ruggedness, reliability, and precision; the method was equivalent or superior to the mineral oil procedure (2). Performance of this method, herein referred to as the "ERRC" method, in providing rapid, inexpensive, and accurate NPYR determinations indicated its potential usefulness as a screening procedure. The ERRC method has the additional advantage of providing a cleaner sample than that obtained from the mineral oil procedure, thus permitting direct mass spectral confirmation—if sufficient concentrations of NAs are present—with a minimum of sample cleanup (3). We performed a multiple laboratory collaborative study to determine the accuracy and precision of the ERRC method within and among laboratories. The results of this study are reported here.

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N-Nitrosopyrrolidine in Fried Bacon Thermal Energy Analyzer Method First Action

(Caution: Nitrosamines are potential carcinogens. Exercise care in handling these materials.)

Principle

Ground sample is mixed with Na₂SO₄ and Celite and *N*-nitrosoazetidine (NAZET) is added as internal std. After transfer to acid Celite column and washing, *N*-nitrosopyrrolidine (NPYR) is eluted with CH₂Cl₂ and detected by GC with thermal energy analyzer.

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 CH₂Cl₂, then dry 4 h in 120° vac. oven before use.

(b) *Dichloromethane, isooctane, and n-pentane*.—Distd in glass (Burdick & Jackson Laboratories, Inc.).

(c) *Sodium sulfate*.—Anhyd., granular (Mallinckrodt No. 8024).

(d) *Phosphoric acid*.—6N. Ext once with equal vol. of CH₂Cl₂ to remove impurities.

(e) *N-Nitrosoazetidine (NAZET) internal std soln*.—Prep. stock soln 1.0 μg/mL in isooctane. Dil. to 0.10 μg/mL in CH₂Cl₂ before analysis.

(f) *N-Nitrosopyrrolidine (NPYR) std soln*.—Prep. single stock soln in isooctane contg 1.0 μg/mL each of NAZET and NPYR. Dil. to 0.10 μg/mL in CH₂Cl₂ before analysis.

Apparatus

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

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

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

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

(e) *Gas chromatograph-thermal energy analyzer*.—Varian Aerograph gas chromatograph Model 2700, or equiv., interfaced with thermal energy analyzer Model 502. Operating conditions: 2.7 m × 3.2 mm stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas-Chrom P; He carrier gas 35 mL/min; column 180° isothermal, injector 200°, TEA furnace, 450°; TEA vac. 1.5 mm; liq. N₂-alcohol cold trap.

Determination

Weigh 10 g Celite into 250 mL beaker. Add 10 mL 6N H_3PO_4 , ca 3 mL at a time, and stir Celite with small glass rod until mixt. is fluffy and uniform in texture. Using powd. funnel, pour acid-Celite into chromatc column contg glass wool plug at bottom. Insert tamping rod thru Celite and tamp from bottom up to ht of ca 25 mm. Accurately weigh 10.0 ± 0.1 g doubly ground fried bacon and quant. transfer to mortar. Add 1.0 mL internal std soln (equiv. to 10 ppb) to bacon sample, using 1.0 mL transfer pipet. Then add 25 g Na_2SO_4 and mix with pestle ca 30 s. Add 20 g Celite to mortar and grind 15–20 s until Celite is thoroly mixed with Na_2SO_4 and bacon. Then grind with moderate pressure for addnl 2 min. Quant. transfer free-flowing dry mixt. into chromatc column, and tamp with glass rod to total ht ca 100 mm. Add 30 g Na_2SO_4 to top of column. Rinse mortar and pestle with 10 mL pentane- CH_2Cl_2 (95 + 5), and add rinse to column, immediately followed by 90 mL same solv. Collect eluate in 100 mL graduate. When level of solv. in column drops so that it just touches top of Na_2SO_4 , add 125 mL CH_2Cl_2 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. When column stops dripping, remove KD flask, add 2 small boiling chips to flask, attach 3-section Snyder column, and conc. eluate to 4 mL on steam bath. Continue concn (add new boiling chip) to 1.0 mL with micro Snyder column in 70° H_2O bath. Note: Room temp. should be $<24^\circ$ during anal. of sample.

Inject 9.0 μ L NPYR std soln at lowest attenuation that yields signal at least $\frac{1}{2}$ full scale TEA response, and measure peak hts. Repeat to assure good reproducibility of retention time and response. Inject 9.0 μ L sample soln, and measure peak hts. For each injection, calc. R , ratio of NPYR peak ht to NAZET peak ht

$$NPYR, \mu\text{g/kg (ppb)} = (R/R') \times (C/W) \times 1000$$

where R and R' = ratio of NPYR peak ht to NAZET peak ht for sample and std, resp.; C = concn of NPYR in GC working std or $\mu\text{g/mL}$; W = wt of sample, g.

Collaborative Study

The 11 collaborators in this study operated independently and each laboratory was requested to use the same analyst for the full study. Two different sets of practice samples were sent to each collaborator before the start of the main study.

The collaborative study consisted of 5 duplicate samples of fried bacon, coded so that the duplicates were unknown to the analysts. Four samples containing normally incurred NPYR, and one sample of nitrite-free fried bacon, were ground twice through a 3 mm plate and mixed thoroughly, and 10.0 ± 0.1 g aliquots were sealed under vacuum in aluminum cans. Samples were stored in a freezer at -22°C for 24 h. The nitrite-free bacon sample was fortified with NPYR, equivalent to 5 ppb, prior to canning. The frozen samples and the nitrosamine standards were packed in Styrofoam shipping containers with 10 lb dry ice and shipped by air freight to each collaborating laboratory. The collaborators were instructed to let the samples thaw in a refrigerator and to prepare working concentrations of the nitrosamine standards in dichloromethane 24 h before the start of the assay. Each collaborator was requested to analyze the samples in a consistent order, i.e., 3 samples in duplicate on the first day and 2 samples in duplicate on the second day. The analysts were

requested to return the results and chromatograms to our laboratory after the analyses were completed.

Subsequent to the completion of the collaborative study, we found that the use of H_3PO_4 -Celite, instead of HCl -Celite, yielded eluates that were cleaner for the GC-TEA and mass spectral analysis. Five bacon samples containing 3–17 ppb NPYR and NAZET internal standard were extracted in duplicate, incorporating each acid into the method. The corrected NPYR (CNPYR) or % NAZET results obtained by the 2 acids showed no significant ($P < 0.05$) difference. In addition, 4 samples containing 5–13 ppb NPYR were analyzed in quadruplicate; the % NAZET recovery ranged from 87 to 101%. Repeatability values for CNPYR and % NAZET were 0.34 ppb and 4.37%, respectively; these results compare favorably with our earlier report (2).

We conclude, therefore, that H_3PO_4 -Celite is equivalent to HCl -Celite. The added advantage of using phosphoric acid, however, is that it yields fewer interfering compounds in the eluate, and is less volatile than HCl , thus, further reducing the possibility of artifactual NA formation on the column (2).

A typical chromatogram from a fried bacon extract is shown in Figure 1.

Results and Discussion

Results of this study were treated following procedures for determining outliers and for analysis of variance outlined by Youden and Steiner (4).

Raw data from the collaborative NPYR analyses are listed in Table 1. Eleven laboratories performed duplicate analyses on 5 samples ranging from nondetectable levels to approximately 19 ppb NPYR. Our earlier report of this method (2) recommended using corrected values in presenting NA data. Therefore, the raw data were corrected for recoveries of the internal standard (Table 2). The CNPYR values which were used for statistical analyses are presented in Table 3.

The rank sum test for the determination of outlying laboratories indicated that no laboratory possessed a systematic bias; therefore, none was excluded on the basis of this test.

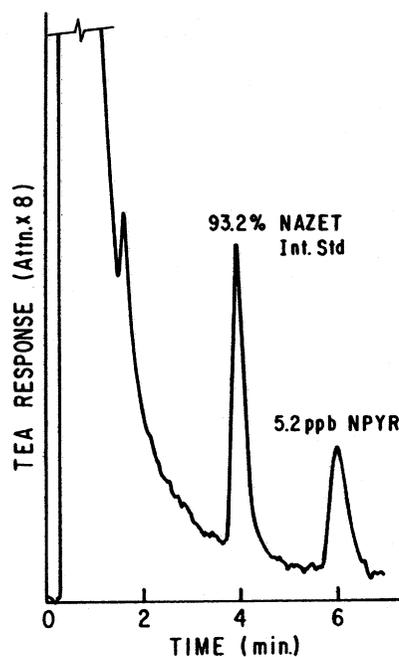


Figure 1. GC-TEA chromatogram from fried bacon extract.

Table 1. Collaborative results for N-nitrosopyrrolidine, ppb (uncorrected)

Coll.	Sample				
	A	B	C	D	E
1	13.18	ND	8.55	17.73	5.68
	13.64	ND	9.77	18.64	5.91
2	12.09	ND	8.34	19.53	5.53
	11.84	ND	8.57	17.00	5.31
3	13.62	ND	9.04	18.55	5.80
	14.64	ND	9.04	18.49	6.32
4	14.37	ND	10.40	16.25	6.53
	15.49	ND	10.31	17.43	6.37
5	10.21	ND	6.50	11.83	5.18
	8.78	ND	5.95 ^a	11.53	4.49
6	13.27	ND	8.69	17.88	5.58
	13.28	ND	9.68	18.37	6.67
7	12.62	ND	6.59	14.12	4.51
	10.46	ND	6.47	13.65	4.62
8	14.10	ND	9.07	19.65	6.77
	14.34	ND	9.63	21.65 ^a	6.70
9	16.50	ND	10.17	18.06	4.68
	16.33 ^a	ND	9.67	18.54	5.64
10	11.11	ND	7.39	13.04	4.13
	5.22 ^a	ND	6.96	11.96 ^a	4.13
11	12.09	ND	6.05	8.14	4.19
	10.93	ND	7.44 ^a	10.70	4.42 ^a
All values:					
\bar{X}	12.64		8.38	16.03	5.42
SD	2.57		1.46	3.55	0.91
CV, %	20.3		17.5	22.1	16.8
Results excluding outlying cells:					
\bar{X}	12.72		8.80	15.91	5.53
SD	1.76		1.24	3.38	0.88
CV, %	13.9		14.1	21.2	15.8

^aCell excluded, based on outlier tests of CNPYR values.

Dixon's test for outliers was then applied to individual results. Laboratories 5 and 11 were found to have outlying values for sample C, and Laboratory 11 was also determined to be an outlier for sample E. The test for variation between replicates showed that the results for sample A from Laboratories 9 and 10 and for sample D from Laboratories 8 and 10 exhibited significant variation between replicates.

An examination of the homogeneity of variance between samples indicated significant evidence of heterogeneity of variance. Nonzero sample levels with similar variances were separated into 2 groups, arbitrarily called high and low. The high group consisted of samples A and D and the low group, samples C and E. This eliminated the heterogeneity and allowed further analysis by individual groups.

Whenever an individual value or an entire cell was identified as an outlier, the laboratory was eliminated from the analysis of the groups in which the outlier occurred. This allowed the maximum amount of useful data to be incorporated into the statistical analysis, while simultaneously fulfilling the reference method (4) requirement of complete data cells for 2-way analysis of variance (ANOVA). Other models with unbalanced data structures were considered, but not used because the expected mean square terms, used to estimate variance components, are not well defined and are dependent on the data structure. This approach would lead to reproducibility estimates that are not well determined. Another alternative was to use a one-way ANOVA design, where one sample at a time is treated. This would result in reproducibility and repeatability estimates for each sample, but the reproducibility would not include a component corresponding to laboratory by sample interaction.

A preliminary report on the ERRC screening method (2) estimated repeatability and reproducibility values for NPYR in fried bacon as 0.71 and 1.03 ppb, respectively. These data

Table 2. Collaborative results for recovery of NAZET, %

Coll.	Sample					\bar{X}	SD	CV, %
	A	B	C	D	E			
1	89.1	101.8	88.8	96.4	98.2	95.8	4.94	5.2
	89.3	96.4	100.0	100.0	98.2			
2	94.7	109.8	93.3	101.5	97.7	96.7	5.64	5.8
	93.3	97.6	89.7	93.1	95.9			
3	74.3	83.2	89.7	82.1	90.1	86.6	5.80	6.7
	83.8	90.5	89.2	88.0	94.7			
4	91.8	89.8	101.1	93.5	102.1	97.3	5.03	5.2
	95.3	98.9	102.4	93.8	104.0			
5	77.9	52.2	76.3	78.1	78.9	75.0	8.39	11.2
	79.9	75.6	76.3 ^a	73.1	82.0			
6	71.4	79.4	97.9	81.2	83.7	84.9	9.39	11.1
	80.4	82.0	86.4	82.5	103.9			
7	78.3	90.4	65.0	75.5	69.2	74.4	8.15	11.0
	62.5	81.2	73.7	77.1	70.7			
8	82.2	65.7	89.6	62.5	120.4	91.1	19.70	21.6
	78.1	110.2	94.0	93.9 ^a	114.3			
9	64.0	85.5	90.7	76.5	83.5	83.2	8.46	10.2
	77.9 ^a	85.5	87.2	88.2	92.9			
10	75.2	82.7	70.7	67.7	75.2	73.9	5.45	7.4
	69.2 ^a	81.2	69.2	78.2 ^a	69.2			
11	71.9	75.4	77.2	47.4	93.0	78.8	15.81	20.1
	79.0	73.7	100.0 ^a	70.2	100.0 ^a			

^aCell excluded, based on outlier tests of CNPYR values.

Table 3. Collaborative results for N-nitrosopyrrolidine, ppb (corrected for NAZET recovery)

Coll.	Sample			
	A	C	D	E
1	14.79	9.63	18.39	5.78
	15.27	9.77	18.64	6.02
2	12.77	8.94	19.24	5.66
	12.69	9.55	18.26	5.54
3	18.33	10.08	22.59	6.44
	17.47	10.13	21.01	6.67
4	15.65	10.29	17.38	6.40
	16.25	10.07	18.58	6.13
5	13.11	8.52	15.15	6.57
	10.99	7.80 ^a	15.77	5.48
6	18.59	8.92	22.02	6.67
	16.52	11.20	22.27	6.42
7	16.12	10.14	18.70	6.52
	16.74	8.79	17.70	6.53
8	17.15	10.12	31.44	5.62
	18.36	10.24	23.06 ^a	5.86
9	25.78	11.21	23.61	5.60
	20.96 ^a	11.09	21.02	6.07
10	14.77	10.45	19.26	5.49
	7.54 ^a	10.06	15.29 ^a	5.97
11	16.82	7.84	17.17	4.51
	13.84	7.44 ^a	15.24	4.42 ^a
All values:				
\bar{X}	15.93	9.65	19.63	5.93
SD	3.64	1.07	3.70	0.62
CV, %	22.8	11.1	18.8	10.5
Results excluding outlying cells:				
\bar{X}	15.64	10.04	19.04	6.07
SD	2.20	0.71	2.55	0.43
CV, %	14.0	7.1	13.39	7.0

^aCell excluded, based on outlier tests.

were calculated from a limited intergovernmental 3-laboratory study. The present study extends the use of the method to include both government and industry laboratories, the primary users. The mean, standard deviation (SD), and CV values of samples before and after the removal of outlying cells are shown in Tables 1–3. NAZET recoveries (Table 2) over all laboratories ranged from 47.4 to 120.4% (\bar{X} = 85.2%). Among laboratories, SD and CV values of NAZET recovery ranged from 4.94 to 19.70% and from 5.2 to 21.6% (average CV = 10.5%), respectively. In CNPYR data, after outlier removal, had CV values ranging from 7.0 to 14.0% among sample levels (uncorrected NPYR, 13.9–21.2%). Collaborators' data on CNPYR from Laboratories 1–7 and 11 (high group) were tested by ANOVA (Table 4) for overall characteristics of the method. CNPYR data from Laboratories 1–4 and 6–10 (low group) were also tested by ANOVA (Table 5). Within-laboratory variation (repeatability) was 0.96 ppb (CV = 5.64%) for the high group and 0.48 ppb (CV = 6.00%) for the low group. Reproducibility values were estimated at 2.33 ppb (CV = 13.72%) for the high group and 0.60 ppb (CV = 7.47%) for the low group.

This study demonstrates that the ERRC method is a precise extraction procedure for NPYR in fried cure-pumped bacon. The procedure developed and reported here is simple, requiring only a few manipulative steps. However, the preparation and packing of the acid–Celite column is critical for high recovery and proficiency (2). Through experience with column packing, a chemist will achieve the necessary high recoveries and repeatable determinations. Also, these results

Table 4. Analysis of variance of CNPYR determinations for samples A and D (high group)

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio
Among laboratories	120.135	7	17.162	6.23*
Between sample levels	85.021	1	—	—
Lab. × level interact.	19.290	7	2.756	2.99*
Error	14.756	16	0.922	—
Total	239.202	31	—	—

* $P < 0.05$.

Repeatability = $\sqrt{MS_{error}} = \sqrt{0.922} = 0.960$; CV = 5.64%

Reproducibility = $\sqrt{\delta_o^2 + \delta_{LS}^2 + \delta_L^2} = \sqrt{5.441} = 2.333$; CV = 13.72%

where $\delta_o^2 = 0.922$; $\delta_{LS}^2 = 0.917$; $\delta_L^2 = 3.602$

are acceptable when compared with the results reported for the mineral oil distillation screening method. Greenfield et al. (5) obtained a repeatability value of 1.21 ppb (CV = 9.5%) and a reproducibility value of 1.84 ppb (CV = 14.4%) for NPYR in their collaborative study.

The mineral oil distillation-GC/TEA method has several disadvantages, including the lengthy analytical time involved. The ERRC method is significantly more rapid than the other method. In our previous paper (2), we also reported results demonstrating the artifactual production of NPYR as a result of the mineral oil distillation procedure when significant levels of nitrite were present in fried bacon before analysis. However, this is not a problem with the ERRC method. We now have further evidence to demonstrate the validity of this method. Morpholine, an amine whose rate of nitrosation is much greater than that for pyrrolidine, was added to nitrite-free bacon at the 0, 10, 50, and 100 ppm levels before analysis; no NMOR (N-nitrosomorpholine) was detected. No NMOR was detected by the ERRC method when 100 ppm morpholine was added to fried bacon cured conventionally with 120 ppm NaNO₂.

Recommendation

We recommend that the dry column screening method be adopted official first action as an alternative method to the mineral oil vacuum distillation method for the determination of NPYR in cooked bacon on the basis of the simplicity, high recovery, freedom from artifacts, and acceptable precision.

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Table 5. Analysis of variance of CNPYR determinations for samples C and E (low group)

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio
Among laboratories	3.352	8	0.419	<1
Between sample levels	144.174	1	—	—
Lab. × level interact.	3.919	8	0.490	2.09
Error	4.207	18	0.234	—
Total	152.651	35	—	—

Repeatability = $\sqrt{MS_{error}} = \sqrt{0.234} = 0.484$; CV = 6.00%

Reproducibility = $\sqrt{\delta_o^2 + \delta_{LS}^2 + \delta_L^2} = \sqrt{0.362} = 0.602$; CV = 7.47%

where $\delta_o^2 = 0.234$; $\delta_{LS}^2 = 0.128$; $\delta_L^2 = 0.602$

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REFERENCES

- (1) Fine, D. H., Rounbehler, D. P., & Oettinger, P. E. (1975) *Anal. Chim. Acta* **78**, 383-389
- (2) Pensabene, J. W., Miller, A. J., Greenfield, E. L., & Fiddler, W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 151-156
- (3) Kimoto, W. I., & Fiddler, W. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1162-1167
- (4) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
- (5) Greenfield, E. L., Smith, W. J., & Malanoski, A. J. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 1319-1332