N-Nitrosamines in Malt and Beer

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Key words: *Dichloromethane extraction, Distillation, Hall™ electrolytic conductivity detector, N-Nitrosodimethylamine, Thermal Energy Analyzer™.*

CONCLUSIONS

No significant differences were found between the mean results of the four beer methods under evaluation. The coefficients of variation for combined-laboratory errors were, in general, lower for Methods I (distillation) and IV (Celite) than for II (direct dichloromethane [DCM] extraction) and III (Preptube™). A significant difference was found between calculation procedure 1 (external standard curve) and calculation procedure 2 (standard addition curve) for the A-B sample pair but not the C-D sample pair with calculation procedure 2 giving higher results. Based on the amounts of NDMA added to the beers, calculation procedure 2 generally gave results closer to theoretical values.

Significant differences were found between the mean results of the four malt methods. Methods III and IV (Preptube extraction of aqueous extracts of ground or whole malt) gave mean results that were significantly different from Malt I (vacuum distillation) and Malt II (DCM extraction of an aqueous extract) but were not significantly different from each other. None of the methods showed consistently better precision for both sample pairs.

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RECOMMENDATIONS

1. Beer Methods I (distillation) and IV (Celite) are recommended for inclusion in the ASBC "Methods of Analysis" as a reference method and screening method, respectively. Calculation procedure 2 is recommended for both methods.
2. The subcommittee should evaluate the Celite method for malt and compare it to the vacuum distillation and direct DCM extraction methods.

This subcommittee was formally organized in January 1980 and completed its first round of collaboration in August 1980. A preliminary report on the collaborative study for beer was issued at the 46th Annual Meeting and a final report covering both malt and beer was submitted in September (1). Based on the conclusions reached and recommendations for further studies, the subcommittee planned to reevaluate two of the malt and beer methods involving either distillation or direct extraction, and to investigate a third method involving the Preptube. Several other studies were added later based on the recommendations of collaborators involved in methods development. These studies included the use of Celite to isolate NDMA from beer,[1] and the Preptube extraction of an aqueous extract of whole malt rather than milled malt (4).

[1]J. H. Hotchkiss, D. Havery, and T. Fazio. A Rapid Method for the Estimation of *N*-Nitrosodimethylamine in Malt Beverages. Presented at AOAC Meeting, Washington, D.C., Oct. 21, 1980.

PROCEDURE

Two pairs of test malts and two pairs of test beers were sent to each collaborator. The beers were prepared in a pilot brewery and were "spiked" with NDMA at levels of 1.0, 1.5, 4.0, and 5.0 μg/L for samples A through D, respectively. The malts were obtained from brewery blends selected on the basis of preliminary analysis and containing a range of approximately 3 to 10 μg/kg NDMA. The design of the study conformed to the Youden Unit Block method (5).

Collaborators were also sent standard solutions containing NDMA and *N*-nitrosodipropylamine (NDPA) at certified concentrations in ethanol and Preptubes. The NDPA was used as an internal standard. Additionally, collaborators were sent an unspiked beer labeled "Z" to be used for their standard addition curves.

The beer methods evaluated were: Beer I, distillation of beer followed by DCM extraction of the distillate; Beer II, direct DCM extraction of beer; Beer III, Preptube extraction of beer followed by DCM elution of the Preptube; Beer IV, Celite 545 extraction of beer followed by DCM elution of the Celite. Beer I and II are essentially the same methods as evaluated in the first collaboration except that 50 ml of beer was used instead of 25 ml in order to increase the sensitivity of the methods. For Beer III, only 15 ml of beer was used due to limitations of the Preptube; and for Beer IV, 25 ml of beer was used.

The malt methods evaluated were: Malt I, vacuum distillation of a Waring-Blendor ground malt and water slurry followed by DCM extraction of the distillate; Malt II, DCM extraction of a 70°C for 1 hr aqueous extract of Waring-Blendor ground malt; Malt III, Preptube extraction of the aqueous extract prepared in Malt II followed by DCM elution of the Preptube; Malt IV, Preptube

TABLE I
***N*-Nitrosodimethylamine (NDMA) (μg/L) Found in Spiked Pilot Beer Samples—Method I: Distillation**

	Samples							
	Calculation Procedure 1[a]				Calculation Procedure 2[b]			
Collaborator	A	B	C	D	A	B	C	D
1	1.09	1.73	3.90	4.15	1.58	2.27	4.80	5.05
2	1.19	1.68	3.62	4.46	1.17	1.66	3.58	4.42
3	1.48	2.01	4.70	5.67	1.43	1.95	4.56	5.50
4	1.22	1.48	3.98	4.76	1.36	1.62	4.19	5.01
5	1.02	1.45	4.08	4.89	--	--	--	--
6	0.96	1.34	3.36	3.87	--	--	--	--
7	--	--	--	--	1.32	1.78	4.11	4.81
9	1.25	1.54	3.70	3.92	1.16	1.46	4.51	4.86
13	1.10	1.29	3.36	4.07	1.28	1.54	4.09	4.94
14	--	--	--	--	4.34[c]	8.34[c]	--	--
Mean[d]	1.16	1.56	3.84	4.47	1.33	1.75	4.26	4.94
Grand mean[d]	1.36		4.16		1.54		4.60	
Theoretical NDMA[e]	1.26	1.76	4.26	5.26	1.26	1.76	4.26	5.26
% NDMA recovered	92.1	88.6	90.1	85.0	105.6	99.4	100.0	93.9

[a]External standard curve and correction for internal standard recovery.
[b]Standard addition curve based on ratio of NDMA to internal standard.
[c]Outlier according to Dixon's test, $P = 0.05$ (2).
[d]Means do not include values for sample pairs containing outliers.
[e]NDMA added plus NDMA in base beer "Z."

TABLE II
***N*-Nitrosodimethylamine (NDMA) (μg/L) Found in Spiked Pilot Beer Samples—Method II: Dichloromethane Extraction**

	Samples							
	Calculation Procedure 1[a]				Calculation Procedure 2[b]			
Collaborator	A	B	C	D	A	B	C	D
1	1.04	1.46	3.80	4.19	1.22	1.67	4.24	4.67
2	1.08	1.66	3.56	4.73	1.13	1.67	3.41	4.48
3	1.20	1.66	3.67	4.87	1.51	2.01	4.17	5.46
4	1.25	1.63	3.73	4.67	1.47	1.91	4.34	5.43
5	1.41	1.68	3.78	4.59	--	--	--	--
6	1.10	1.47	3.35	4.61	1.17	1.57	3.64	5.02
8	1.30	1.40	4.84	6.50	1.43	1.51	2.83	3.53
9	1.01	1.25	3.45	4.66	1.14	1.38	3.53	4.70
10	1.04	1.79	3.56	3.36	1.00	2.06	4.40	4.14
12	4.85[c]	2.91[c]	3.09	4.83	3.60[c]	1.70	2.26	3.49
13	1.50	1.92	4.60	5.63	1.68	2.10	5.07	6.38
14	0.69	1.46	4.48	5.24	1.07	1.70	4.18	4.81
Mean[d]	1.15	1.58	3.83	4.82	1.28	1.76	3.82	4.74
Grand mean[d]	1.36		4.32		1.52		4.28	
Theoretical NDMA[e]	1.26	1.76	4.26	5.26	1.26	1.76	4.26	5.26
% NDMA recovered	91.3	89.8	89.9	91.6	101.6	100.0	89.7	90.1

[a]External standard curve and correction for internal standard recovery.
[b]Standard addition curve based on ratio of NDMA to internal standard.
[c]Outlier according to Dixon's test, $P = 0.05$ (2).
[d]Means do not include values for sample pairs containing outliers.
[e]NDMA added plus NDMA in base beer "Z."

extraction of a 70°C for 1 hr aqueous extract of whole malt followed by DCM extraction of the Preptube. Malt I and II are essentially the same methods as evaluated in the first collaboration except that for Malt II, a more concentrated aqueous extract was prepared and 50 ml of "wort" was extracted rather than 25 ml resulting in a 4× increase in method sensitivity.

For all of the methods, the DCM was concentrated to 1.0 ml and an aliquot was injected into a gas chromatograph equipped with either a Hall™ electrolytic conductivity detector modified for nitrosamine analysis or a Thermal Energy Analyzer. Also, for all methods, two calibration/calculation procedures were compared. These were: calculation procedure 1, external NDMA standard curve and correction for internal standard recovery, and calculation procedure 2, standard addition curve using the ratio of the NDMA peak height or area to the NDPA (internal standard) peak height or area. For beers, the internal standard was added prior to distillation or extraction. For malts, the internal standard was added to the malt/water slurry for Malt I or to the 70°C aqueous extract for Malt II, III, or IV.

RESULTS AND DISCUSSION

Fifteen collaborators submitted results for one or more of the methods. The results for beer are tabulated in Table I through IV and the results for malt in Tables V through VIII. Statistical summaries are given in Tables IX and X.

An analysis of variance on all beer data except for outliers showed no significant differences between methods ($P = 0.05$). A significant difference was observed between calculation procedures for the A-B pair but not the C-D pair. Calculation procedure 2 gave higher mean values in all instances except for the C-D sample pair using Beer Method II.

The combined-laboratory errors in terms of coefficients of variation (c.v.) ranged from 7.2 to 22.8. None of the methods or

TABLE III
***N*-Nitrosodimethylamine (NDMA) (μg/L) Found in Spiked Pilot Beer Samples—Method III: Preptube**

	Samples							
	Calculation Procedure 1[a]				Calculation Procedure 2[b]			
Collaborators	A	B	C	D	A	B	C	D
1	1.32	1.76	3.84	4.25	1.20	1.65	3.93	4.35
2	1.55	2.36[c]	4.76	5.33	1.47	2.01	4.07	4.56
3	1.45	1.94	4.58	5.37	1.45	1.95	4.67	5.46
4	1.06	1.64	3.18	4.53	0.95	1.62	4.02	4.86
5	1.38	1.74	3.53	5.23	--	--	--	--
6	1.23	1.72	3.93	4.41	1.10	1.58	3.81	4.30
7	--	--	--	--	1.28	1.55	3.72	4.67
8	3.41[d]	3.77[d]	4.85	3.40	1.86	2.50	4.99	5.43
13	1.12	1.58	3.48	4.05	1.78	2.34	4.55	5.24
14	--	--	2.26	3.32	1.66	1.17	2.88	3.54
Mean[e]	1.26	1.73	3.82	4.43	1.42	1.82	4.07	4.71
Grand mean[e]	1.50		4.13		1.62		4.39	
Theoretical NDMA[c]	1.26	1.76	4.26	5.26	1.26	1.76	4.26	5.26
% NDMA recovered	100.0	98.3	89.7	84.2	112.7	103.4	95.5	89.5

[a]External standard curve and correction for internal standard recovery.
[b]Standard addition curve based on ratio of NDMA to internal standard.
[c]NDMA added plus NDMA in base beer "Z."
[d]Outlier according to Dixon's test, $P = 0.05$ (2).
[e]Means do not include values for sample pairs containing outliers.

TABLE IV
***N*-Nitrosodimethylamine (NDMA) (μg/kg) Found in Spiked Pilot Beer Samples—Method IV: Celite**

	Samples							
	Calculation Procedure 1[a]				Calculation Procedure 2[b]			
Collaborators	A	B	C	D	A	B	C	D
1	1.31	1.66	3.66	4.89	1.28	1.63	3.63	4.86
2	1.13	1.71	3.65	4.74	1.20	1.78	3.75	4.83
3	1.15	1.62	4.03	4.93	1.23	1.71	4.18	5.08
4	1.11	1.80	4.10	5.28	1.27	1.93	4.03	5.11
5	1.03	1.44	3.16	3.88	--	--	--	--
6	1.19	1.56	2.99	3.03	1.18	1.59	3.20	3.24
9	--	--	--	--	0.92[c]	1.45	3.59	5.45
10	1.74	2.13[c]	4.77	4.94	1.20	1.54	3.61	3.79
11	1.33	1.76	3.89	4.64	--	--	--	--
13	1.13	1.41	3.24	3.69	1.39[d]	1.72	3.94	4.46
Mean[e]	1.17	1.62	3.72	4.45	1.23	1.70	3.74	4.60
Grand mean[e]	1.40		4.08		1.46		4.17	
Theoretical NDMA[d]	1.25	1.75	4.22	5.21	1.25	1.75	4.22	5.21
% NDMA recovered	93.6	92.6	88.2	85.4	98.4	97.1	88.6	88.3

[a]External standard curve and correction for internal standard recovery.
[b]Standard addition curve based on ratio of NDMA to internal standard.
[c]Outlier according to Dixon's test, $P = 0.05$ (2).
[d]NDMA added plus NDMA in base beer "Z."
[e]Means do not include values for sample pairs containing outliers.

TABLE V
N-Nitrosodimethylamine (NDMA) (μg/kg) Found in Malt (Not Spiked)—Method I: Vacuum Distillation

	Samples							
	Calculation Procedure 1[a]				Calculation Procedure 2[b]			
Collaborator	A	B	C	D	A	B	C	D
1	5.80	5.24	2.11	1.27	7.61	6.94	3.01	2.01
2	7.38	5.74	3.13	2.53	9.17	7.07	3.68	2.89
3	6.73	5.34	2.81	1.31	9.03	7.26	4.00	2.12
4	8.04	5.79	2.97	2.45	10.76	7.69	3.87	3.22
5	7.79	6.50	3.79	2.57	--	--	--	--
6	5.53	4.41	3.26	2.81	--	--	--	--
7	--	--	--	--	--	--	2.91	2.93
8	10.50	7.62	2.72	2.08	11.02	8.14	3.88	3.25
13	5.92	5.08	3.05	3.19	7.30	6.44	4.40	4.53
Mean[c]	7.21	5.72	2.98	2.28	9.15	7.26	3.68	2.99
Grand mean[c]	6.46		2.63		8.20		3.34	

[a]External standard curve and correction for internal standard recovery.
[b]Standard addition curve based on ratio of NDMA to internal standard.
[c]Means do not include values for sample pairs containing outliers.

TABLE VI
N-Nitrosodimethylamine (NDMA) (μg/kg) in Malt (Not Spiked)—Method II: Dichloromethane Extraction of Wort

	Samples							
	Calculation Procedure 1[a]				Calculation Procedure 2[b]			
Collaborator	A	B	C	D	A	B	C	D
1	11.03	6.73	4.48	4.23	12.86	7.60	4.65	4.39
2	10.39	8.29	3.58	3.04	10.79	8.61	3.82	3.29
3	8.36	5.81	2.70	1.88	9.62	6.75	3.25	2.36
4	9.50	7.34	3.77	3.27	9.84	7.49	3.60	3.03
5	9.93	5.64	4.78	3.07	--	--	--	--
6	9.73	4.86	3.35	3.05	10.62	5.35	3.45	3.30
8	6.80	5.11	2.87	2.87	6.38	5.29	3.89	3.89
10	5.44	3.38	2.29	2.48	22.06[c]	11.16	5.46	5.76
12	6.98	4.54	4.90	2.75	8.64	4.28	3.54	0.11
13	8.40	5.68	3.06	2.09	9.32	6.12	3.02	1.87
15	--	--	--	--	7.05	5.66	3.38	2.82
Mean[d]	8.66	5.74	3.58	2.87	9.46	6.35	3.81	3.08
Grand mean[d]	7.20		3.23		7.90		3.44	

[a]External standard curve and correction for internal standard recovery.
[b]Standard addition curve based on ratio of NDMA to internal standard.
[c]Outlier according to Dixon's test, $P = 0.05$ (2).
[d]Means do not include values for sample pairs containing outliers.

TABLE VII
N-Nitrosodimethylamine (NDMA) (μg/kg) in Malt (Not Spiked)—Method III: Preptube Extraction of Wort

	Samples							
	Calculation Procedure 1[a]				Calculation Procedure 2[b]			
Collaborator	A	B	C	D	A	B	C	D
1	12.27	8.13	5.24	5.05	10.75	7.20	4.88	4.72
2	11.41	9.54	3.73	3.59	11.05	9.54	4.41	4.18
3	8.77	6.65	2.84	1.97	11.08	7.96	3.85	2.79
4	8.49	7.67	5.74	5.84	9.77	7.06	4.58	4.51
5	9.80	7.34	6.20	2.73	--	--	--	--
6	11.41	6.20	4.27	3.33	11.80	6.43	4.37	3.51
7	--	--	--	--	10.71	7.16	2.88	3.20
8	4.89[c]	4.25	2.76	2.92	6.60	4.86	3.24	3.75
13	9.16	7.29	4.19	3.12	7.28	5.64	2.87	1.99
Mean[d]	10.19	7.55	4.37	3.57	9.88	6.98	3.88	3.58
Grand mean[d]	8.87		3.97		8.43		3.73	

[a]External standard curve and correction for internal standard recovery.
[b]Standard addition curve based on ratio of NDMA to internal standard.
[c]Outlier according to Dixon's test, $P = 0.05$ (2).
[d]Means do not include values for sample pairs containing outliers.

calculation procedures showed c.v. less than 10 for both sample pairs. In general, however, the c.v. were lower for Methods I and IV than for II and III.

The actual addition levels of NDMA to the beers were 1.0, 1.5, 4.0, and 5.0 μg/L for beers A, B, C, and D, respectively. The base beer to which the NDMA was added contained 0.26 μg/L as determined by averaging the data obtained by collaborators for the "Z" beer used for the standard addition curves. Thus, the theoretical values for beers A through D were the addition levels plus 0.26 μg/L or 1.26, 1.76, 4.26, and 5.26 μg/L, respectively.

Calculation procedure 2 resulted in generally higher recoveries and values closer to theoretical than did calculation procedure 1. All methods showed acceptable recoveries when calculation procedure 2 was used with results ranging from a low of 88 to a high of 113% of theoretical.

Comments from collaborators regarding the beer methods indicated a distinct preference for Methods III, Preptube, and IV, Celite, because they were less time-consuming, less cumbersome than the distillation method, and did not have the problem of emulsion formation as did the direct DCM extraction method. Several collaborators, however, noted problems with the Preptube method, which could not be used by collaborators with Hall detectors because of the presence of interfering peaks. The Preptube method also lacked the sensitivity of the other methods since sample size is limited to 15 ml unless more than one Preptube is used. Additionally, a background NDMA peak was sometimes eluted from the Preptubes. No problems were reported with the Celite method.

Based on a combination of favorable precision, reasonable accuracy, simplicity, and speed, Method IV, Celite, appears to be the preferred method for screening purposes. Since Method I, distillation, has the advantage of resulting in an extract that is more suitable for mass spectral confirmation of the presence of NDMA, it is the preferred reference method. Calculation procedure 2 is more accurate than calculation procedure 1 based on recoveries of NDMA added to the beers.

TABLE VIII
***N*-Nitrosodimethylamine (NDMA) (μg/kg) in Malt (Not Spiked)—Method IV: Preptube Extraction of Wort from Whole Malt**

	Samples							
	Calculation Procedure 1[a]				Calculation Procedure 2[b]			
Collaborator	A	B	C	D	A	B	C	D
1	10.69	6.73	3.73	3.32	8.62	5.63	3.35	3.05
2	13.13	8.05	4.32	3.47	12.56	8.12	5.01	4.09[c]
3	9.42	6.16	4.68	2.82	8.76	5.92	4.65	3.08
4	8.66	5.36	3.46	1.65	9.24	5.96	4.06	2.11
5	5.81	5.38	2.78	1.88	--	--	--	--
6	8.53	7.51	4.79	2.77	8.85	7.81	4.95	2.92
7	--	--	--	--	10.58	7.34	3.61	3.08
13	8.62	8.54	4.76	3.55	6.85	6.74	3.41	2.24
Mean[d]	9.27	6.82	4.07	2.78	9.35	6.79	4.00	2.75
Grand mean[d]	8.04		3.43		8.07		3.38	

[a]External standard curve and correction for internal standard recovery.
[b]Standard addition curve based on ratio of NDMA to internal standard.
[c]Outlier according to Dixon's test, $P = 0.05$ (2).
[d]Means do not include values for sample pairs containing outliers.

TABLE IX
Statistical Summary for Beer

Sample Pair	Method[a]	Calculation Procedure[b]	No. of Labs.	Grand Mean[c]	Laboratory Error			c.v.[f]	Calculated F[d]	Critical F[g]
					Within[d]	Between[d]	Combined[e]			
	I	1	8	1.36	0.107	0.171	0.202	14.8	6.124	3.787
A-B		2	7	1.54	0.114	0.192	0.223	14.4	6.687	4.284
	II	1	11	1.36	0.145	0.148	0.207	15.2	3.089	2.978
		2	10	1.52	0.181	0.151	0.236	15.5	2.391	3.179
	III	1	6	1.50	0.051	0.128	0.138	9.2	13.646	5.050
		2	9	1.62	0.250	0.272	0.369	22.8	3.351	3.438
	IV	1	8	1.40	0.093	0.081	0.124	8.9	2.506	3.787
		2	6	1.46	0.091	0.052	0.105	7.2	1.651	5.050
	I	1	8	4.16	0.198	0.495	0.533	12.8	13.517	3.787
C-D		2	7	4.60	0.191	0.310	0.364	7.9	6.282	4.284
	II	1	12	4.32	0.374	0.540	0.656	15.2	5.169	2.818
		2	11	4.28	0.352	0.743	0.822	19.2	9.928	2.978
	III	1	9	4.13	0.626	0.507	0.806	19.5	2.312	3.438
		2	9	4.39	0.136	0.604	0.619	14.1	40.643	3.438
	IV	1	9	4.08	0.304	0.579	0.654	16.0	8.259	3.438
		2	8	4.17	0.421	0.381	0.568	13.6	2.636	3.787

[a]Method I: Distillation; Method II: DCM Extraction; Method III: Preptube; Method IV: Celite.
[b]Calculation procedure 1: external standard curve; calculation procedure 2: standard addition curve.
[c]Grand Mean = $(\overline{A} + \overline{B})/2$ or $(\overline{C} + \overline{D})/2$.
[d]Calculated per Youden and Steiner (5).
[e]Combined-laboratory error (S_c) calculated from within-laboratory error (S_r) and between-laboratory error (S_b); $S_c = \sqrt{S_r^2 + S_b^2}$.
[f]Coefficient of variation of S_c = c.v. = $100(S_c/\text{Grand mean})$.
[g]Critical F from tables of F distribution (3) at $P = 0.05$.

TABLE X
Statistical Summary for Malt

Sample Pair	Method[a]	Calculation Procedure[b]	No. of Labs.	Grand Mean[c]	Laboratory Error			c.v.[f]	Calculated F[d]	Critical F[g]
					Within[d]	Between[d]	Combined[e]			
	I	1	8	6.46	0.535	1.234	1.344	20.8	11.634	3.787
A-B		2	6	8.20	0.706	0.932	1.169	14.3	4.489	5.050
	II	1	10	7.20	0.799	1.407	1.618	22.5	7.200	3.179
		2	9	7.90	1.101	1.287	1.694	21.4	3.732	3.438
	III	1	7	8.87	1.067	0.754	1.306	14.7	1.999	4.284
		2	8	8.43	0.922	1.408	1.683	20.0	5.669	3.787
	IV	1	7	8.04	1.364	1.209	1.822	22.7	2.571	4.284
		2	7	8.07	1.044	1.001	1.447	17.9	2.837	4.284
	I	1	8	2.63	0.353	0.475	0.592	22.5	4.620	3.787
C-D		2	7	3.34	0.475	0.521	0.705	21.1	3.410	4.284
	II	1	10	3.23	0.523	0.589	0.787	24.4	3.537	3.179
		2	10	3.44	0.736	0.931	1.186	34.4	4.202	3.179
	III	1	8	3.97	0.835	0.960	1.272	32.0	3.641	3.787
		2	8	3.73	0.409	0.754	0.858	23.0	7.780	3.787
	IV	1	7	3.43	0.433	0.631	0.765	22.3	5.236	4.284
		2	6	3.38	0.513	0.251	0.571	16.9	1.479	5.050

[a] Method I: Vacuum distillation; Method II: DCM Extraction of Wort; Method III: Preptube Extraction of Wort; Method IV: as III but wort from whole malt.
[b] Calculation procedure 1: External standard curve; calculation procedure 2: standard addition curve.
[c] Grand mean = $(\overline{A} + \overline{B})/2$ or $(\overline{C} + \overline{D})/2$.
[d] Calculated per Youden and Steiner (5).
[e] Combined-laboratory error (S_c) calculated from within-laboratory error (S_r) and between-laboratory error (S_b); $S_c = \sqrt{S_r^2 + S_b^2}$.
[f] Coefficient of variation of S_c + c.v. = 100(Sc/Grand mean).
[g] Critical F from tables of F distribution (3) at $P = 0.05$.

An analysis of variance on all malt data except for outliers showed a significant difference between the four malt methods ($P = 0.05$). According to Duncan's multiple range test, Methods I and II were significantly different from each other and from methods III and IV but no significant difference was found between III and IV, both of which were Preptube methods (ground vs whole malt). A significant difference was found between the calculation procedures for the A-B pair but not the C-D pair. Calculation procedure 2 resulted in higher mean values for Methods I and II but not for III and IV.

The combined-laboratory errors in terms of c.v. for the malt methods ranged from 14.3 to 35.9. None of the methods showed consistently better precision than the others for both sample pairs. Since the malts were not spiked and actual NDMA levels are not known, no judgment can be made regarding accuracy of the methods.

Comments from collaborators indicated some charring problems with Method I in addition to it being cumbersome and time-consuming. Problems with the Preptube method were identical to those encountered with beer. Some collaborators again objected to the direct DCM extraction method because of emulsion problems.

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APPENDIX A
N-NITROSAMINES IN BEER BY DISTILLATION
(Beer Method I)

Note: Refer to safety and general precautions *prior to starting* (see Appendix C).

Reagents

(a) *Barium hydroxide*, $Ba(OH)_2$ analytical reagent grade.
(b) *Boileezers*, Fisher Scientific Co., Cat. No. B-365, or equivalent.
(c) *Sodium carbonate*, Na_2CO_3, analytical reagent grade.
(d) *Dichloromethane*, CH_2Cl_2, distilled in glass, Burdick and Jackson, or equivalent (hereafter referred to as DCM).
(e) *Sodium sulfate*, anhydrous, Na_2SO_4, analytical reagent grade.
(f) *Dry nitrogen*, N_2, ultra-high purity.
(g) *Standards*: *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodipropylamine (NDPA) each 100 μg/ml from Thermo Electron Corp., 115 Second Ave., Waltham, MA 02154, or equivalent.
(h) *Internal standard solution*, NDPA at 100 ng/ml in ethanol prepared by diluting a *portion* of the 100 μg/ml standard.[1]
(i) *Ethanol*, anhydrous reagent grade.
(j) *Water*, distilled in glass (H_2O put through deionizer may contain background nitrosamines).

Apparatus

(a) *Pipettes*, volumetric, assorted.
(b) *Distilling flasks*, round-bottom, 1-L with connecting adapter and Graham condenser set vertically.
(c) *Heating mantles* for 1-L flasks.
(d) *Variable transformers* for heating mantles.
(e) *Separatory funnels*, 250 ml.
(f) *Fritted glass funnels*, 60 ml.
(g) *Evaporative concentrator*, Kuderna-Danish, 250-ml capacity, 24/40 standard taper column connection, 19/22 lower standard taper joint. Concentrator tube size 425, 19/22 standard taper joint, 4-ml capacity, graduated, with 19/22 standard taper stopper. Snyder distillation column three sections, size 121 with 24/40 standard taper joint. (Available from Kontes, SGA Scientific, and others.)
(h) *Water bath*, 60°C.
(i) *Syringes*, 10-μl for gas chromatography.
(j) *Gas chromatograph* interfaced with TEA Model 502 analyzer or Tracor gas chromatograph with Hall electrolytic

[1] Note that the standards supplied by Thermo Electron Corp. do not necessarily contain 5.0 ml, so an aliquot should be removed from the vial for futher dilution.

conductivity detector and nitrosamine kit.
(k) *Gas chromatographic column* capable of baseline separation of NDMA and NDPA.
(l) *Flasks*, Erlenmeyer and volumetric, assorted.

Method

Decarbonate approximately 55 ml of beer by equilibrating to room temperature in a 125-ml Erlenmeyer flask and shaking until gas evolution stops. Transfer 50.0 ml of beer into 1-L round-bottom distillation flask containing 8 g $Ba(OH)_2$ and Boileezers. Add 1.0 ml of internal standard solution. Distill slowly (variable transformer setting 60%), collecting approximately 48 ml[2] in an ice-cooled 250-ml separatory funnel. Add 0.4 g Na_2CO_3. Extract four times with 20-ml portions of DCM, shaking each for 1 min. Pool extracts in a second 250-ml separatory funnel. Pass extract through 30 g Na_2SO_4 (held in a 60-ml fritted glass funnel prewetted with DCM) into a 250-ml Kuderna-Danish evaporator with 4-ml concentrator tube attached. Wash Na_2SO_4 with 15 ml DCM and add to evaporator flask. Add one Boileezer and Snyder column and carefully concentrate to 4 ml in a 60°C water bath. Further concentrate to 1.0 ml under a gentle stream of N_2 at room temperature (this final concentration should take about 30 min).[3] Inject aliquot into the gas chromatograph using either the GLC/TEA or GLC/HECD conditions.

A reagent blank should be prepared and chromatographed along with the samples. To prepare the reagent blank, substitute 4% v/v ethanol in distilled (not deionized) water for the beer and proceed as above. If the reagent blank shows a peak for NDMA, the DCM should be checked by concentrating 95 ml to 1 ml and chromatographing. If the DCM does not show an NDMA peak, other reagents should be checked. Reagents showing background nitrosamines should not be used.

Gas Chromatography

The following give examples of columns and conditions suitable for nitrosamine separation. Variations in columns and conditions are acceptable and the choice is left to the operator.

GLC/TEA Conditions
Gas chromatograph interfaced with TEA analyzer
Column: 6 ft × 6 mm i.d. glass packed with 10% Carbowax 20 M + 5% KOH on Anakrom AB, 100–120 mesh
Column temperature: 145°C
Injection port temperature: 200°C
Carrier gas: He at 35 ml/min
TEA Conditions
Furnace temperature: 475°C
Vacuum with oxygen: 1.0 torr
Trap temperature: −120 to −130°C
GLC/HECD Conditions
Gas chromatograph: Tracor 560/700A equipped with nitrosamine detector kit
Column: 6 ft × 6 mm i.d. glass packed with 15% LAC-2R-446 on Chromosorb® W, AW, 80–100 mesh
Column temperature: 140°C
Carrier gas: He at 20 ml/min
Injection port temperature: 200°C
Hall inlet temperature: 250°C
Hall reactor temperature: 700°C
Hydrogen flow: 50 ml/min
Electrolyte: 50% v/v n-propanol in water
Electrolyte flow: 0.5 ml/min

[2]Separatory funnel should be marked at 48 ml.
[3]T. Fazio, D. C. Havery, and J. W. Howard. Determination of Volatile *N*-Nitrosamines in Foodstuffs. II. A Continued Survey of Foods and Beverages. Presented at 6th International Meeting on Analysis and Formation of N-Nitroso Compounds, Budapest, Hungary, Oct. 16–19, 1979.

Calibration and Calculation

Prepare beer containing 0, 0.5, 1.0, 2.5, and 5.0 ppb (μg/L) of added NDMA as follows:

Decarbonate two 12-oz. bottles of a beer containing negligible NDMA content by equilibrating to room temperature in a 1-L Erlenmeyer flask and shaking until gas evolution stops. This is the base beer.

Prepare NDMA dilute standards from the 100 μg/ml standard using ethanol for dilution as follows:

Dilution A = dilute 1.0 ml of 100 μg/ml to 10 ml
Dilution B = dilute 5.0 ml of A to 100 ml = 500 ng/ml
Dilution C = dilute 5.0 ml of B to 10 ml = 250 ng/ml
Dilution D = dilute 2.0 ml of B to 10 ml = 100 ng/ml
Dilution E = dilute 1.0 ml of B to 10 ml = 50 ng/ml

Add one ml of the following to 100-ml volumetric flasks: ethanol, dilution E, dilution D, dilution C, and dilution B, and dilute to volume with previously decarbonated base beer. These samples contain 0, 0.5, 1.0, 2.5, and 5.0 ppb (μg/L) of added NDMA, respectively. Analyze each sample as previously described under **Method.**

Measure the peak height (or area) of the NDMA and NDPA (internal standard) peaks on the chromatograms. For each chromatogram, determine the ratio of the NDMA peak to the NDPA peak:

$$R = \frac{\text{peak height (or area) NDMA}}{\text{peak height (or area) NDPA (Int. std.)}}$$

Subtract R for the 0 ppb addition sample from the R values obtained for the other four chromatograms. Prepare a standard curve by plotting ppb added NDMA vs R values for each NDMA addition level (after subtraction of R for 0 ppb).

Calculate the slope and intercept of the regression line using the method of least squares where X = NDMA (μg/L) and Y = R value.

Calculation of unknowns: Measure peak height (or area) for the NDMA and NDPA peaks and calculate R as above. Determine μg/L in the beer by calculation using the regression equation and solving for X as follows:

$$\mu g/L = \frac{R - \text{intercept}}{\text{slope}}$$

Report results to one decimal place.

APPENDIX B
N-NITROSAMINES IN BEER BY CELITE ADSORPTION
(Beer Method IV)

NOTE: Refer to safety precautions *prior to starting* (see Appendix C).

Reagents

(a) *Celite 545* (not acid washed), Fisher Scientific Co., Cat. No. C-212. Fire contents of each bottle for 16 hr at 700°C before use.
(b) *Dichloromethane*, CH_2Cl_2, distilled in glass, Burdick and Jackson, or equivalent (hereafter referred to as DCM).
(c) *Sodium sulfate*, anhydrous granular, reagent grade.
(d) *Ethanol*, anhydrous, National Distillers and Chemical Corp., New York, NY 10016, or equivalent.
(e) *Standards: N*-nitrosodimethylamine (NDMA) and *N*-nitrosodipropylamine (NDPA) each 100 μg/ml from Thermo

Electron Corp., 115 Second Ave., Waltham, MA 02154, or equivalent.

(f) *Internal standard solution*, NDPA at 100 ng/ml in ethanol prepared by diluting a *portion* of the 100 μg/ml standard.[4]
(g) *Boiling chips*, Carborundum, small size, or equivalent.
(h) *Dry nitrogen*, N_2, ultra-high purity.
(i) *Ethanol*, 4% v/v prepared in glass-distilled water.

Apparatus

(a) *Pipettes*, volumetric, assorted.
(b) *Beakers*, 600 ml.
(c) *Evaporative concentrator*, Kuderna-Danish. See Beer Method I, apparatus (g).
(d) *Tamping rod*, 19-mm diameter disk.
(e) *Glass wool*, Pyrex or equivalent.
(f) *Chromatographic column*, glass, 28 mm i.d. × 400 mm long with stopcock.
(g) *Powder funnel.*
(h) *Syringes*, 10-μl for gas chromatography.
(i) *Gas chromatograph* interfaced with TEA Model 502 analyzer *or* Tracor gas chromatograph with Hall electrolytic conductivity detector and nitrosamine kit.
(j) *Gas chromatographic column* capable of baseline separation of NDMA and NDPA.
(k) *Flasks*, Erlenmeyer, 125 ml.
(l) *Balance* (±0.1 g).

Method

Weigh 25 g ± 0.1 g beer into tared 600-ml beaker. Add 1.0 ml of internal standard solution and 25 g Celite. Stir mixture until uniform (approximately 30 sec). Mixture will not pour but will appear light and fluffy. Place small glass wool plug in bottom of the chromatographic column and cover with 20 g of sodium sulfate. Place tamping rod and a powder funnel in the column with end of tamping rod extending into the column through the funnel and tamp the mixture, a little at a time, to a depth of 8–10 cm. Add 75 ml DCM to the beaker, swirl with the spatula, and pour through the funnel before removing the tamping rod. Adjust stopcock so that DCM flows at a rate of 1–2 ml/min into a Kuderna-Danish evaporator with 4 ml concentrator tube attached. Allow column to run dry. Approximately 35 ml of DCM will be recovered. Add 3 small boiling chips, fit the Kuderna-Danish with the distilling column, and concentrate to approximately 4 ml in a 60°C water bath. Allow column to drain and further concentrate to 1.0 ml under a gentle stream of N_2 at room temperature (this final concentration should take about 30 min). Inject aliquot into the gas chromatograph using either the GLC/TEA or GLC/HECD conditions.[5] A reagent blank should be prepared along with the samples and checked (see Beer Method I discussion under **Method**). The blank for this method is 25 ml of 4% ethanol (v/v) in distilled (not deionized) water in place of the beer.

[4]Note that the standards supplied by Thermo Electron Corp. do not necessarily contain 5.0 ml, so an aliquot should be removed from the vial for further dilution.

Gas Chromatography

As for Beer Method I.

Calibration and Calculation

As for Beer Method I except use 25 g beer as per this method (Beer IV).

[5]J. H. Hotchkiss, D. Havery, and T. Fazio. A Rapid Method for the Estimation of *N*-Nitrosodimethylamine in Malt Beverages. Presented at AOAC Meeting, Washington, D.C., Oct. 21, 1980.

APPENDIX C
PRECAUTIONS IN *N*-NITROSAMINE ANALYSIS

Safety Precautions

1. Nitrosamines are considered potent carcinogens. EXTREME CARE should be exercised in handling nitrosamines or solutions of nitrosamines. Skin contact should be avoided.
2. Mechanical pipetting aids should be used for all pipetting procedures.
3. All samples containing nitrosamines should be properly labeled as "spiked with nitrosamines" or "not for consumption," or with other adequate warning.

General Precautions

1. All glassware used for nitrosamine analyses should be thoroughly and routinely cleaned with Chromerge (or equivalent) and thoroughly rinsed with distilled water and dichloromethane.
2. Some nitrosamines degrade upon exposure to UV light. Prolonged exposure to fluorescent lights should be avoided unless lights are covered with yellow translucent shields to filter out UV light. Alternatively, sample containers can be covered with foil or other suitable material to provide protection from light.
3. Store standards and dichloromethane extracts in a freezer in amber bottles or foil-covered containers.