N-Nitrosamines in Malt and Beer

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CONCLUSIONS

No significant differences were found among the mean results of the three malt methods under evaluation. Method I (vacuum distillation) showed relatively low variability between laboratories for both sample pairs. Method III (Celite column extraction) showed lower variability within laboratories for both sample pairs than Method I or Method II (DCM extraction of ASBC wort). None of the methods showed consistently lower combined-laboratory error for both sample pairs.

RECOMMENDATIONS

1. Method I (vacuum distillation) is recommended for inclusion in the ASBC "Methods of Analysis" as a reference method.
2. The subcommittee should continue to evaluate screening methods as they evolve and compare them to the vacuum-distillation method. For example, a headspace GLC/TEA procedure has been proposed for volatile nitrosamines in malt,[1] and the Institute of Brewing is proposing an additional procedure.

This subcommittee was formally organized in January, 1980, and has completed two rounds of collaboration. Based on these studies, the Society has adopted reference and screening methods for determining *N*-nitrosodimethylamine (NDMA) in beer (1). A calculation procedure using a standard addition curve based on the ratio of NDMA to internal standard, *N*-nitrosodipropylamine (NDPA), has also been recommended. Studies this year were to reevaluate two of the malt methods and to investigate a third involving a Celite column extraction.

[1]Donley et al. Presented at the 48th Annual ASBC Meeting, Kansas City, MO, May, 1982.

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PROCEDURE

Two pairs of test malts, labeled A through D, were sent to each collaborator. The malts were from Great Western Malting Co. and were selected on the basis of preliminary analysis and contained approximately 3 and 10 μg/kg NDMA. An electric-dried malt labeled 'Z' was to be used for the standard addition curves. Collaborators were also sent standard solutions of 100 mg/ml NDMA and NDPA in ethanol. The NDPA was used as an internal standard. The design of the study conformed to the Youden Unit Block method (4).

The malt methods evaluated were: Method I, vacuum distillation of a ground malt, water, and oil slurry followed by dichloromethane (DCM) extraction of the distillate; Method II, DCM extraction of 70°C for 1 hr aqueous extract of ground malt; and Method III, DCM elution of a column packed with ground malt, water, and Celite mixture. Methods I and II were the same methods evaluated in the 1981 collaborative study.

For all methods, the DCM was concentrated to 1.0 ml and an aliquot injected into a gas chromatograph equipped with either a Hall™ electrolytic conductivity detector (HECD) modified for nitrosamine analysis or a Thermal Energy Analyzer™ (TEA). A standard addition curve using the ratio of NDMA peak height or area to NDPA peak height or area was used to calculate the mean values for NDMA. The internal standard was added to the malt/water/oil or Celite mixture for Methods I and III or the aqueous extract for Method II.

RESULTS AND DISCUSSION

Sixteen collaborators submitted results for one or more of the methods. Results are tabulated in Table I, and a statistical summary is given in Table II. Fifteen of the collaborators

performed the analyses using the TEA for nitrosamine detection. The sole collaborator using an HECD analyzed samples only by Method II; therefore it is only appropriate to evaluate these methods in terms of their suitability for use with GLC/TEA instrumentation.

Student *t*-test analysis of all data except outliers showed no significant differences between mean results ($P \leq 0.05$). The combined-laboratory errors in terms of coefficient of variation (c.v.) ranged from 13.8 to 28.1%, with no consistent trend for both pairs in any method. As expected, the c.v. generally was lower for the more concentrated sample pair for all methods. For both sample pairs, within-laboratory error was lowest for Method III.

Coefficients of variation from this study were comparable to results obtained for Methods I and II from the 1981 collaborative study. Based on a more acceptable between-laboratory error for Method I as compared to the other two methods, Method I is preferred for a reference method. Although Method I produced a c.v. of 28%, a c.v. of this magnitude is not unusual for methods in the μg/kg range. In addition, the extract obtained in Method I is the cleanest, and hence the most suitable, for mass spectral confirmation of NDMA. The current FDA regulation requires mass spectral confirmation of NDMA (3). Due to relatively large between-laboratory errors, neither Method II nor III is suitable for an official reference method. Either method could be effectively used as a rapid screening procedure in a given laboratory, with the qualification that results be periodically compared with results from the reference method. Further collaborative studies, however, are recommended before an official screening method is selected.

Several negative comments were made about each of the three methods. Several collaborators commented that the concentrated samples from the Celite method were dark yellow, contained a precipitate, and produced appreciable solvent tailing. Nevertheless, the data obtained using Method III suggest that these drawbacks did not seriously interfere with quantitation of NDMA. Preferences were indicated by different collaborators for each of the three methods without one emerging as a distinct favorite.

LITERATURE CITED

1. American Society of Brewing Chemists. Report of Subcommittee on *N*-Nitrosamines in Malt and Beer. *Journal* 39:99, 1981.
2. Dixon, W. J., and Massey, F. J., Jr. Introduction to Statistical Analysis, p. 470. McGraw-Hill: New York, 1969.
3. Federal Register, 46:39218, 1981.
4. Youden, W. J., and Steiner, E. H. Statistical Manual. Assoc. Off. Anal. Chem.: Washington, DC, 1975.

TABLE I
***N*-Nitrosodimethylamine (μg/kg) in Malt**

	Method I Vacuum Distillation				Method II Dichloromethane Extraction of ASBC Wort				Method III Celite Column Extraction			
Collaborator	A	B	C	D	A	B	C	D	A	B	C	D
1	2.51	2.48	8.13	11.56	...	...	...	...	3.65	3.32	12.84	13.32
2	3.58	3.44	7.38	8.31	3.91	3.61	6.28	6.90	3.83	3.43	7.44	8.54
3	...	...	...	...	2.72	2.83	6.26	7.61	...	...	...	...
4	3.79	4.16	9.63	10.06	4.00	4.23	8.53	10.05	3.66	3.69	8.78	9.70
5	4.50	4.57	10.31	9.57	4.92	5.00	8.71	9.98	4.45	4.49	9.57	9.78
6	2.33	1.52	10.20	11.18	3.63	3.73	10.16	10.84	2.28	2.24	14.89	14.93
7	3.98	3.78	9.70	14.11	6.96	6.23	12.96	11.70	6.87[a]	4.36	10.75	12.95
8	7.13[a]	3.88	9.79	10.17	5.26	5.01	9.00	9.00	4.25	3.81	8.91	8.72
9	3.24	4.09	9.59	9.81	5.67	5.94	11.25	10.85	3.72	3.46	9.95	10.27
10	...	...	...	...	3.35	3.46	7.36	10.68	...	...	...	...
11	...	...	...	...	5.76	6.33	9.97	10.13	4.62	4.06	10.57	9.00
12	2.63	2.44	8.87	9.18	3.14	3.26	8.23	8.10	2.00	2.05	8.69	8.28
13	...	...	...	...	3.84	4.21	9.57	10.12	...	...	...	...
14	3.91	3.95	9.94	8.63	4.06	4.33	7.72	6.97	3.50	3.41	9.95	10.68
15	2.39	2.21	11.24	10.76	2.65	3.07	9.74	9.32	2.68	2.62	12.78	12.38
Mean[b]	3.29	3.26	9.53	10.30	4.28	4.37	8.98	9.45	3.51	3.33	10.43	10.71
Grand mean[b]	3.28		9.91		4.33		9.21		3.42		10.57	

[a] Outlier according to Dixon's test, $P \leq 0.05$.
[b] Means do not include values for sample pairs containing outliers.

TABLE II
Statistical Summary for *N*-Nitrosodimethylamine in Malt

Sample Pair	Method[a]	No. of Labs.	Grand Mean[b]	Laboratory Error			c.v.[e]	Calculated F[c]	Critical F[f]
				Within[c]	Between[c]	Combined[d]			
A-B	I	10	3.28	0.302	0.856	0.908	27.7	17.03	2.98
	II	14	4.28	0.236	1.194	1.217	28.1	52.40	2.48
	III	11	3.42	0.154	0.789	0.804	23.5	53.33	2.79
C-D	I	11	9.91	1.211	0.627	1.364	13.8	1.54	2.98
	II	14	9.21	0.819	1.477	1.689	18.3	7.51	2.58
	III	12	10.57	0.663	2.044	2.148	20.3	20.03	2.79

[a] Method I: Vacuum distillation; Method II: DCM extraction of wort; Method III: Celite column extraction.
[b] Grand mean = $(\bar{A} + \bar{B})/2$ or $(\bar{C} + \bar{D})/2$.
[c] Calculated per Youden and Steiner (3).
[d] Combined-laboratory error (S_c) calculated from within-laboratory error (S_r) and between-laboratory error (S_b); $S_c = \sqrt{S_r^2 + S_b^2}$.
[e] Coefficient of variation of S_c = c.v. = 100 (S_c/Grand mean).
[f] Critical F from tables of F distribution (2) at $P \leq 0.05$.

APPENDIX A
N-NITROSAMINES IN MALT BY VACUUM DISTILLATION
(Malt Method 1)

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

Reagents
(a) *Ammonium sulfamate*, analytical reagent grade.
(b) *Sulfuric acid*, 1*N*.
(c) *Mineral oil*, pharmaceutical grade.
(d) *Sodium hydroxide*, 1.5*N*.
(e) *Sodium sulfate*, anhydrous, analytical reagent grade.
(f) *Dichloromethane*, CH_2Cl_2, distilled in glass, Burdick & Jackson or equivalent (hereafter referred to as DCM).
(g) *Nitrogen gas* (N_2), dry, ultra-high purity.
(h) *Bioleezers*, Fisher Scientific Cat. No. B-365, or equivalent.
(i) *Standards*: *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodipropylamine (NDPA) each 100 μg/ml, Thermal Electron Corp., or equivalent.
(j) Internal standard solution (I.S.), NDPA at 300 ng/ml in ethanol prepared by diluting a portion of the 100 μg/ml standard.
(k) *Ethanol*, anhydrous, reagent grade.
(l) *Water*, distilled in glass (water put through deionizer may contain background nitrosamines).

Apparatus
(a) *Balance*, analytical.
(b) *Waring Blendor* (or equivalent).
(c) *Distillation flask*, round-bottom, 500 ml with thermometer well as per Fig. 1.
(d) *Thermometer*, centigrade, for distillation flask.
(e) *Heating mantle* for distillation flask.
(f) *Vacuum pump* as per Fig. 1.
(g) *Vacuum gauge* as per Fig. 1.
(h) *Vapor traps* and connecting glassware as per Fig. 1.
(i) *Bath* to contain liquid N_2 as per Fig. 1.
(j) *Liquid* N_2.
(k) *Pipettes*, volumetric, assorted.
(l) *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).
(m) *Water bath*, 60° C.
(n) *Syringe*, 10-μl, for gas chromatography.
(o) *Gas chromatograph*, interfaced with TEA model 502 analyzer.

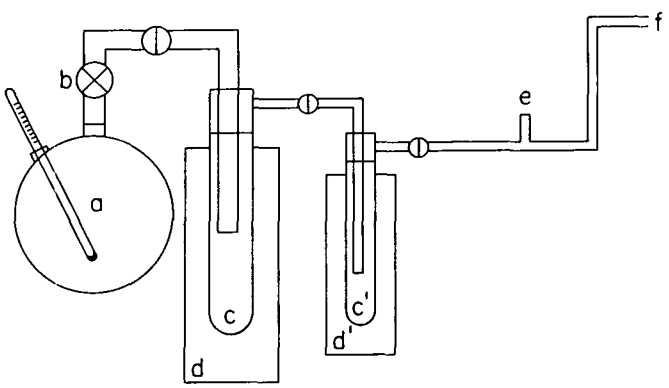

Fig. 1. Apparatus for vacuum distillation. **a**, Round-bottom flask; **b**, vacuum stopcock; **c**, vapor traps; **d**, liquid N_2 bath; **e**, to vacuum gauge; **f**, to vacuum pump.

(p) *Gas chromatographic column* capable of baseline separation of *N*-nitrosodimethylamine and *N*-nitrosodipropylamine.
(q) *Separatory funnels*, 250 ml.
(r) *Buchner* or *fritted glass funnels*, 60-ml capacity.

Method
Weigh 30 g of malt and grind in Waring Blendor for 30 sec at low speed. Transfer 25 g (± 0.05 g) ground malt to a 500-ml round-bottom distillation flask fitted with a thermometer well (see Fig. 1). Add 1.0 ml I.S., 2.5 g ammonium sulfamate dissolved in 20 ml of 1*N* H_2SO_4, 2 ml of ethanol, and 25 ml of mineral oil (pH of slurry should be about 2). Prepare apparatus for vacuum distillation as shown in Fig. 1. Apply a vacuum greater than 100 μ while heating the flask to 100° C over a 1-hr period. Collect the distillate in the two vapor traps connected in series and cooled in liquid N_2. Then thaw the distillate in each trap, combine, rinse each trap with 3 × 20 ml distilled H_2O and combine washings with distillate. Acidify to pH 2 with H_2SO_4, saturate with sodium sulfate, and transfer to a separatory funnel with washings. Rinse each vapor trap with 20 ml of DCM and transfer to separatory funnel. Shake contents of separatory funnel for 1 min, allow phases to separate, and draw off DCM layer. Further extract the aqueous phase with 2 × 20 ml of DCM. Pool the DCM extracts, wash with 1 × 20 ml 1.5*N* NaOH (discard washings), and dry by passing through 30 g of Na_2SO_4 contained in a fritted glass funnel into a Kuderna-Danish evaporator with 4-ml concentrator tube attached. Wash Na_2SO_4 with 15 ml DCM and add to evaporator flask. Add boileezers and Snyder column and carefully evaporate 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). Inject an aliquot into the gas chromatograph using GLC/TEA conditions. A reagent blank should be prepared along with the samples and checked.

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

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

Dilution A = 1.0 ml of 100 μg/ml to 10 ml
Dilution B = 5.0 ml of A to 100 ml = 500 ng/ml
Dilution C = 7.5 ml of B to 10 ml = 375 ng/ml
Dilution D = 5.0 ml of B to 10 ml = 250 ng/ml
Dilution E = 5.0 ml of D to 10 ml = 125 ng/ml
Dilution F = 5.0 ml of E to 10 ml = 62.5 ng/ml

Using malt Z, prepare and extract five samples according to **Method**, but substitute the following for the 2-ml ethanol addition before distillation:

Sample 1: 2 ml ethanol = 0 ppb added to malt
Sample 2: 1 ml ethanol + 1 ml dilution F = 2.5 ppb added to malt

Sample 3: 1 ml ethanol + 1 ml dilution E = 5.0 ppb added to malt
Sample 4: 1 ml ethanol + 1 ml dilution D = 10.0 ppb added to malt
Sample 5: 1 ml ethanol + 1 ml dilution C = 15.0 ppb added to malt

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

$$R = \frac{\text{peak value for NDMA}}{\text{peak value for NDPA}}$$

Subtract the R value for the 0 ppb addition 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 added). Calculate the slope and intercept of the regression line using the method of least squares where X = ppb (μg/kg) of NDMA and Y = R value.

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

$$\text{ppb } (\mu g/kg) = \frac{R - \text{intercept}}{\text{slope}}$$

Report results to one decimal place.

REFERENCE

1. Hotchkiss, J. H., Barbour, J. R., and Scanlan, R. A. *J. Agric Food Chem.* 28:678, 1980.

APPENDIX B
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 ultraviolet light. Prolonged exposure to fluorescent lights should be avoided unless lights are covered with yellow translucent shields to filter out ultraviolet 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.