Enzymatic Method for Low Alcohol Concentrations in Malt Beverages

Subcommittee Members: C. Bartnett, *Chairman*; S. Bock; C. Curran; L. Gerber; S. Giorno; T. Hassinger; P. Hedges; K. Kitahara, Brewers Association of Japan (BAJ); R. Maruyama; K. Nakatani (BAJ); T. Otterson; C. Sall; I. Shelton; C. Wehmeier; B. Williams; and J. Mueller (*ex officio*).

Keywords: Beer, No-alcohol, Nonalcoholic, Spectrophotometer

CONCLUSION

The repeatability and reproducibility coefficients of variation for all three sample pairs were acceptable.

RECOMMENDATIONS

1. The method, as given in the Appendix, is recommended for inclusion in *Methods of Analysis*.
2. Discharge the subcommittee.

The subcommittee was formed to investigate an enzymatic method for the determination of ethanol in low-alcohol malt beverages. When ethanol concentrations are low, it is difficult to accurately determine alcohol concentrations by distillation. The Boehringer Mannheim enzymatic method (3) is more sensitive at low alcohol concentrations and has been adopted by the European Brewery Convention (EBC) as an official method (4).

PROCEDURE

Three sample pairs were evaluated following the Youden unit block design (5). Sample pairs A/B and C/D were commercial products. Sample pair E/F represented malt beverages further diluted to achieve very low alcohol levels (less than 0.05% v/v). The EBC method (4) in principle, rather than the method supplied with the Boehringer Mannheim kit, was followed for this collaborative test.

RESULTS AND DISCUSSION

Fifteen collaborators participated in the current test. One participant had a defective kit (based on the response to the standard ethanol solution provided) and was excluded. The results of the remaining 14 collaborators are shown in Table I. Outliers were found in two of the three sample pairs. Results were statistically analyzed following the published procedures (5); the statistical results are shown in Table II.

The repeatability for sample pairs A/B and C/D was improved over last year's collaborative study on samples of similar ethanol content (2), although the reproducibility was not. The reproducibility coefficient of variation was 14.2, corresponding to 0.025% v/v ethanol, in the worst case. Both the repeatability and reproducibility errors were higher than those found in the EBC collaborative but were judged acceptably low.

LITERATURE CITED

1. American Society of Brewing Chemists. *Methods of Analysis*, 7th ed.

TABLE I
Ethanol Content by Enzymatic Method (% v/v)

Collaborator	Sample Pair A	B	Sample Pair C	D	Sample Pair E	F
1	0.44	0.44	0.19	0.18	0.033[a]	0.034[a]
2	0.47	0.48	0.18	0.18	0.045	0.035
3	0.42	0.42	0.19	0.17	0.041	0.033
4	0.48	0.44	0.19	0.23	0.045[a]	0.030[a]
5	0.44	0.45	0.18	0.20	0.043	0.035
6	0.36	0.36	0.13	0.12	0.040	0.031
7	0.50	0.51	0.30[a]	0.27[a]	0.052	0.046
8	0.43	0.44	0.32[a]	0.32[a]	0.040	0.030
9	0.42	0.43	0.17	0.17	0.039	0.033
10	0.40	0.41	0.17	0.17	0.038	0.031
11	0.40	0.41	0.17	0.19	0.043	0.036
12	0.47	0.43	0.13	0.18	0.049	0.039
13	0.38	0.38	0.21	0.21	0.034[a]	0.040[a]
14	0.42	0.39	0.17	0.17	0.039	0.033
Mean[b]	0.431	0.428	0.173	0.181	0.0426	0.0347
Grand mean[b]	0.429		0.177		0.0387	

[a]Outlier at $P \leq 0.01$ based on totals and/or differences (1).
[b]Calculated excluding outliers.

Statistical Analysis-4 Youden unit block collaborative testing procedure. The Society: St. Paul, MN, 1976.
2. American Society of Brewing Chemists. Report of Subcommittee on Ethanol in Low-Alcohol Beers by Enzymatic Method. *Journal* 48(4):156, 1990.
3. Boehringer Mannheim GMBH. *Methods of Enzymatic Food Analysis,* p. 17, 1984.
4. European Brewery Convention. *Analytica,* 4th ed. Method 9.2 Ethanol in alcohol-free and low alcohol beers. p. E153, 1987.
5. Guidelines for Collaborative Study Procedures. *J. Assoc. Off. Anal. Chem.* 71:161, 1988.

APPENDIX

ENZYMATIC METHOD FOR LOW ALCOHOL CONCENTRATIONS IN MALT BEVERAGES

This method utilizes a Boehringer Mannheim kit (2,3) for the determination of ethanol in low or no alcohol samples. This kit is sensitive at very low ethanol levels. Ethanol is oxidized to acetaldehyde by nicotinamide-adenine dinucleotide (NAD) in the presence of alcohol dehydrogenase under alkaline conditions. Acetaldehyde is oxidized to acetic acid and reduced NAD (NADH) in the presence of aldehyde dehydrogenase. The amount of NADH is proportional to the amount of ethanol and is determined spectrophotometrically by means of its ultraviolet absorbance at 340 nm.

Reagents

(a) *Boehringer Mannheim GMBH ethanol* (UV-method) enzymatic kit for the determination of ethanol in foodstuffs and other materials (Cat. No. 176 290) or equivalent. The kit should be stored at 4°C.
(b) *Redistilled or distilled deionized water.*
(c) *Sodium hydroxide solution,* 12.5%.

Apparatus

(a) *Spectrophotometer,* 340 nm.
(b) *Cuvettes,* glass or disposable acrylic, 1 cm, volume 4 ml.
(c) *Volumetric pipettes,* 3 and 10 ml.
(d) *Micropipettes,* 0.1 ml and 0.05 ml (or adjustable micropipette with disposable tips).
(e) *Volumetric flasks,* 100 ml.
(f) *Erlenmeyer flasks,* 250 ml.

TABLE II
Statistical Summary of Results[a]

Sample Pair	No. of Labs	Grand Mean	Repeatability s_r	cv_r	r_{95}	Reproducibility s_R	cv_R	R_{95}
A/B	14	0.429	0.013	3.1	0.038	0.039	9.1	0.109
C/D	12	0.177	0.015	8.4	0.041	0.025	14.2	0.071
E/F	11	0.039	0.0012	3.0	0.0033	0.0045	11.6	0.0126

[a]All calculations were made based on reference 5.

Method

Sample preparation. Attemperate the beer to 20°C and degas according to Beer-1. Adjust the pH of the samples to pH 8–9 by adding 12.5% sodium hydroxide solution (reagent c) dropwise. Dilute samples having less than 0.05% v/v ethanol 10-fold with redistilled or distilled deionized water (reagent b). Dilute samples having less than 0.5% v/v ethanol 100-fold by two successive 10-fold dilutions with redistilled or distilled deionized water (reagent b).

Procedure. Pipette the following into cuvettes, cover with stoppers or plastic film, and mix:

	Blank	Samples	Standard
Reaction mixture (kit)	3.00 ml	3.00 ml	3.00 ml
Redistilled water	0.10 ml	...	...
Diluted sample	...	0.10 ml	...
Ethanol standard (kit)	...	...	0.10 ml

After 3 min, read and record the absorbances of the blank (A_{B1}), the samples (A_{S1}), and the ethanol standard (A_{E1}) against redistilled or distilled deionized water (reagent b) at 340 nm. Add 0.05 ml of alcohol dehydrogenase from the kit (reagent a) to the blank, the samples, and the ethanol standard; then cover with stoppers or plastic film and mix. After 10 min, read the absorbances of the blank (A_{B2}), the samples (A_{S2}), and the ethanol standard (A_{E2}) against redistilled or distilled deionized water (reagent b) at 340 nm.

Calculation of Results

Calculate the absorbance difference for the blank ($A_{B2} - A_{B1} = \Delta A_B$), the samples ($A_{S2} - A_{S1} = \Delta A_S$), and the ethanol standard ($A_{E2} - A_{E1} = \Delta A_E$). Subtract the absorbance difference of the blank from the absorbance difference of the ethanol standard ($\Delta A_E - \Delta A_B = \Delta E$) and the samples ($\Delta A_S - \Delta A_B = \Delta S$). The performance of the method should be checked by measuring the recovery of ethanol standard and comparing with the amount stated on the bottle in the kit. The ethanol levels should be similar, or the samples should be reanalyzed.

$$\text{g/L Ethanol} = \frac{(3.15\text{ ml})(46.07\text{ g/mol})(\Delta)(F)}{[6{,}300\text{ mol/(L) (cm)}](1.0\text{ cm})(0.1\text{ ml})(2\text{ mol/mol})}$$
$$= (0.1152\text{ g/L})\,(\Delta X)\,(F)$$

3.15 = Final volume of the reaction mixture (ml)
46.07 = Molecular weight of ethanol (g/mol)
ΔX = Final absorbance of sample (ΔS) or ethanol (ΔE)
F = Initial sample dilution
6300 = Molar absorption coefficient of NADH at 340 nm [mol/(L)(cm)]
1.0 = Light path (cm)
0.1 = Volume of diluted sample (ml)
2 = 1 mol of ethanol results in 2 mol of NADH

$$\%\text{ v/v Ethanol} = \frac{\text{g/L Ethanol}}{(0.78816\text{ g/ml})(10)}$$

0.78816 = Density of ethanol at 20°C (g/ml)
10 = Conversion factor for g/L to percent

Report the results to two significant decimal places for samples greater than 0.05% v/v ethanol and three significant decimal places for samples less than or equal to 0.05% v/v ethanol.

Example

$A_{B1} = 0.198$
$A_{S1} = 0.211$
$A_{B2} = 0.342$
$A_{S2} = 0.458$
$A_{B2} - A_{B1} = 0.342 - 0.198 = 0.144\ (\Delta A_B)$
$A_{S2} - A_{S1} = 0.458 - 0.211 = 0.247\ (\Delta A_S)$
$\Delta A_S - \Delta A_B = 0.247 - 0.144 = 0.103\ (\Delta S)$
$(0.1152)(\Delta S)(F) = (0.1152)(0.103)(100) = 1.18656$ g/L

$$\frac{1.18656 \text{ g/L}}{(0.78816 \text{ g/ml})(10)} = 0.15\% \text{ v/v Ethanol}$$

Precision

Based on a collaborative study of samples having ethanol concentrations of 0.04–0.43% v/v (1), repeatability coefficients of variation of 3.0–8.4 and reproducibility coefficients of variation of 9.1–14.2 can be expected.

REFERENCES

1. American Society of Brewing Chemists. Report of Enzymatic Method for Low Alcohol Concentrations in Malt Beverages. *Journal* 49:185, 1991.
2. Boehringer Mannheim GMBH. *Methods of Enzymatic Food Analysis,* p. 17, 1984.
3. European Brewery Convention. *Analytica,* 4th ed. Method 9.2 Ethanol in alcohol-free and low alcohol beers. p. E153, 1987.