β-Glucan in Congress Wort by Fluorescence Method

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CONCLUSIONS

1. Ruggedness testing demonstrated that several factors significantly affected the results of the fluorescence method for β-glucan in congress wort.
2. The fluorescence method for β-glucan in congress wort showed high repeatability and reproducibility coefficients of variation for all seven sample pairs.
3. The repeatability and reproducibility coefficients of variation were not substantially improved over the previous year's subcommittee results (2) and in the absence of a standard reference method were considered acceptable.

RECOMMENDATIONS

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

This subcommittee was charged with the determination of (1-3),(1-4)β-glucan in congress wort using the dye-binding character of the fluorochrome Calcofluor and flow injection analysis (4-7).

PROCEDURE

Collaborators received five malt sample pairs labeled G/I, C/J, A/D, E/B, and F/H and three autoclaved congress wort pairs labeled N/P, K/M, and L/O prepared from malt sample pairs G/I, A/D, and F/H, respectively. Each laboratory was requested to prepare a wort from each sample using the wort preparation method in Malt-4 (1). All eight sample pairs were then analyzed using a standardized collaborative protocol as well as an in-house protocol for flow injection analysis. The Youden unit block (1) experimental design was used.

RESULTS AND DISCUSSION

Ruggedness Testing

The design and the results of the ruggedness test of the fluorescence method are shown in Table I, and the main effects of the results are summarized in Table II. Six of the seven experimental factors considered—buffer, β-glucan standard, excitation wavelength, emission wavelength, irradiation of Calcofluor, and Calcofluor concentration—were found to have a significant effect at the 95% confidence level. The other factor tested—flow rate—had no significant effect at the 95% confidence level.

Collaborative Testing

Youden unit block treatment. Fourteen collaborators submitted

TABLE I
Design and Results of Flow Injection Assay Ruggedness Test, β-Glucan (mg/L)

	Factors[a]							Test Results			
Trial	A	B	C	D	E	F	G	Replicates		Totals	Differences
1	−	−	−	−	+	+	+	234	229	463	5
2	+	−	−	+	+	−	−	221	209	420	2
3	−	+	−	+	−	+	−	261	263	524	−2
4	+	+	−	−	−	−	+	214	215	429	−1
5	−	−	+	+	−	−	+	288	279	567	9
6	+	−	+	−	−	+	−	205	196	401	9
7	−	+	+	−	+	−	−	180	198	378	−18
8	+	+	+	+	+	+	+	211	215	426	−4

[a] A = buffer, B = β-glucan standard, C = excitation wavelength, D = emission wavelength, E = irradiation, F = flow rate, G = Calcofluor concentration.

TABLE II
Summary of Main Effects of Flow Injection Assay Ruggedness Test[a]

Factor	Levels Evaluated		Main Effect[b]	Confidence Interval[c]
	Low	High		
A Buffer	Phosphate	Glycine	−32.0*	−38.7 to −25.3
B β-Glucan standard	Biocon	Sigma	−11.8*	−18.5 to −5.1
C Excitation wavelength	350 nm	360 nm	−8.0*	−14.7 to −1.3
D Emission wavelength	420 nm	430 nm	34.4*	27.7 to 41.1
E Irradiation of Calcofluor	No	Yes	−29.3*	−36.0 to −22.6
F Flow rate	2.0 ml/min	3.0 ml/min	2.5	−4.2 to 9.2
G Calcofluor concentration	0.0015%	0.0025%	20.3*	13.6 to 27.0

[a]All calculations were made according to reference 1.
[b]* = Significant at the 95% confidence level.
[c]Confidence limits were ±6.67 for all factors.

TABLE III
β-Glucan in Congress Wort by Fluroescence Method (mg/L)

Collaborator	Sample Pairs G	I	Sample Pairs C	J	Sample Pairs A	D	Sample Pairs E	B	Sample Pairs F	H	Sample Pairs N	P	Sample Pairs K	M	Sample Pairs L	O
1	104	84	126[a]	127[a]	142[a]	163[a]	165	177	374[a]	332[a]	21[a]	84[a]	165	162	346	257
2	103[a]	105[a]	116	91	157	114	156	220	295	253	140	95	163	132	329	265
3	82	61	99	97	130	127	126	163	304	227	109	80	153	134	227	71
4	69	51	103	96	109	99	121[a]	121[a]	247[a]	263[a]	71	39	0[a]	81[a]	255	11
5	91	54	99	92	136	113	118	164	301	207	72[a]	220[a]	89	63	43[a]	167[a]
6	63	47	83	63	92	77	77	122	175	158	30[a]	50[a]	89	73	180	149
7	86	64	89	82	99	86	104	145	177	175	77	55	93	81	177	148
8	84	71	125	97	124	129	169	188	358	250	110	79	27[a]	142[a]	370[a]	25[a]
9	81	37	50[a]	35[a]	48[a]	38[a]	74	118	179	135	80	43	73[a]	50[a]	193	159
10	37[a]	35[a]	88	45	69	76	65[a]	105[a]	176	135	78	59	110	103	240	189
11	83	58	97	80	105	95	119	152	209	181	77	57	123	96	231	152
12	71	47	88	81	116	85	127	150	232	183	68	48	93	69	40[a]	153[a]
13	124[a]	105[a]	165[a]	126[a]	196[a]	166[a]	189[a]	206[a]	427[a]	350[a]	125	90	1,731	473	73	228
14	82	46	143	102	134	152	128	191	261	220	101	66	151	134	276	219
Mean[b]	82.4	56.3	102.8	84.2	115.6	104.8	123.9	162.7	242.5	193.1	94.2	64.6	127.5	108.5	254.3	168.1
Grand mean[b]	69.4		93.5		110.2		143.3		217.8		79.4		118.0		212.5	

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

results for the collaborative (Table III) and in-house methods. Wort sample pair L/O exhibited a visible precipitate and for that reason was omitted from statistical analysis. Outliers were identified using Dixon's outlier test (1) on all sample pairs. The results of the Youden unit block statistical analysis are summarized in Table IV. The repeatability coefficients of variation for the collaborative method ranged from 5.3 to 11.2 and were higher than those from the individual in-house methods, which ranged from 4.7 to 8.3 (data not shown). This indicates a certain degree of operator comfort with the in-house methods. A *t* test showed no difference between the means of the in-house and collaborative methods. The reproducibility coefficients of variation ranged from 17.7 to 29.1 and were higher than expected. However, the poor reproducibility is believed to be due to nonstandard equipment used by the collaborators in the study. Six different types of flow injection equipment were identified among the collaborators. Four of the 14 collaborators were identified as having flow injection equipment manufactured by the same company. Although four collaborators are considered inadequate for valid statistical analysis, the results indicate that improvements in precision may be possible. In the absence of a standard reference method or standard equipment, the high repeatability and reproducibility coefficients of variation were considered acceptable, providing that these are noted in the method.

Common β-glucan standard. Seven of the 14 collaborators were identified as using the same lot of β-glucan from one supplier; therefore, the statistics for these laboratories were examined separately. The reproducibility coefficients of variation for the seven laboratories with a common β-glucan standard were higher than for all 14 laboratories, ranging from 20.1 to 37.0 (data not shown).

TABLE IV
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}
G/I	11	68.8	6.9	10.1	19.4	12.2	17.7	34.0
C/J	11	93.5	10.1	10.9	28.4	17.8	19.0	49.7
A/D	11	110.3	12.4	11.2	34.6	24.3	22.1	68.1
E/B	11	143.3	11.8	8.3	33.2	30.9	21.6	86.6
F/H	11	217.8	22.6	10.4	63.3	54.6	25.1	153.0
N/P	11	79.4	6.0	7.6	16.8	21.7	27.4	60.9
K/M	11	118.0	6.3	5.3	17.5	34.3	29.1	96.2

[a]All calculations were made based on reference 3.

This indicates that using a common β-glucan standard did not improve the precision of the method.

Comparison of Malt-4 (congress worts) and autoclaved worts. A statistical comparison of sample pairs G/I and A/D (congress worts prepared in the collaborators' laboratories) with corresponding wort sample pairs N/P and K/M (prepared in a single laboratory and sent to collaborators) is shown in Table V. A comparison of *t* values indicated no significant difference in the means of the two methods of wort sample preparation.

LITERATURE CITED

1. American Society of Brewing Chemists. *Methods of Analysis,* 7th ed. Malt-4 Extract; 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

TABLE V
Comparison of Malt-4 (Congress Worts) and Autoclaved Worts[a]

Variable	β-Glucan Pair G/I N/P	β-Glucan Pair A/D K/M
Malt-4 mean, $\bar{x}$	68.8	110.3
Autoclaved mean, $\bar{x}$	79.4	118.0
Difference of means	10.6	7.7
t value[b]	1.32	0.58
Degrees of freedom	10	10

[a]All calculations were made according to reference 1.
[b]No significant difference at 95% confidence level.

β-Glucan in Barley, Malt, and Congress Wort by Fluorescence. *Journal* 48:145,1990.
3. Guidelines for Collaborative Study Procedures. *J. Assoc. Off. Anal. Chem.* 71:161, 1988.
4. Jensen, S. A., and Aastrup, S. A Fluorimetric method for measuring 1,3:1,4-β-glucan in beer, wort, malt and barley by use of Calcofluor. *Carlsberg Res. Commun.* 46:87-95, 1981.
5. Jorgensen, K. Quantification of high molecular weight (1-3)(1-4)-β-D-glucan using Calcofluor complex formation and flow injection analysis. I. Analytical principle and standardization. *Carlsberg Res. Commun.* 53:277-285, 1988.
6. Jorgensen, K. and Aastrup, S. Quantification of high molecular weight (1-3)(1-4)-β-D-glucan using Calcofluor complex formation and flow injection analysis. II. Determination of total-β-glucan content of barley and malt. *Carlsberg Res. Commun.* 53:287-296, 1988.
7. Mekis, E., Pintér, G., Béndek, G. Modified fluorimetric flow-injection-analysis (FIA) method for the determination of (1-3)(1-4)-β-D-glucan. *J. Inst. Brew.* 93:396-398, 1987.

APPENDIX
β-GLUCAN IN CONGRESS WORT BY FLUORESCENCE

The method exploits the ability of the fluorochrome Calcofluor to bind to (1-3)(1-4)-β-D-glucan and flow injection analysis to quantify β-glucan in congress wort (1).

Reagents
(a) *Barley β-glucan* (from a uniform source).
(b) *Sodium hydroxide* (1.0*N*).
(c) *Stock glycine buffer* (0.1*M* glycine to pH 9.0 with 1.0*N* NaOH).
(d) *Calcofluor (Cellufluor)*, Polysciences Inc., Warrington, PA, or equivalent (kept in dessicator at room temperature).
(e) *Ethanol* (reagent grade).

Apparatus
(a) *Flow injection analysis equipment* (flow rate 2.0–3.0 ml/min).
(b) *Fluorescence detector* (emission wavelength, 420 nm; excitation wavelength, 365 nm).
(c) *Volumetric flasks* (250, 500, 1,000, and 2,000 ml).
(d) *Erlenmeyer vacuum flask* (500 ml).
(e) *Vacuum aspirator* (with water trap).
(f) *Membrane filter assembly.*
(g) *Membrane filters* (polycarbonate 3 μm).
(h) *Automatic pipettor* (1.0 and 5.0 ml).

β-Glucan Preparation and Standardization Method
A 300-mg/L β-glucan stock standard is prepared as follows: Slurry 0.0750 g of β-glucan, db (reagent *a*) with 5.0 ml of ethanol (reagent *e*) in a 250-ml volumetric flask. Add 100 ml of distilled water to the flask and heat with stirring to 60–70°C on a hot plate to solubilize. Once dissolved, cool the flask to room temperature (≈22°C) and bring to volume. Stock must be stored at 4°C and is good for one week. A standard curve of 50, 100, 150, 200, 250, or 300 mg/L will be prepared from serial dilutions of the stock with distilled water as the diluent.

Calcofluor Preparation
Add 0.0125 g of Calcofluor (reagent d) to a 500-ml volumetric flask and bring to volume with 0.1*M* glycine buffer (reagent c) to make a 0.0020% (w/v) Calcofluor solution. Stir for 10 min at room temperature. The solution should be protected from light in an amber bottle to minimize photodegradation. Vacuum filter through a 3-μm membrane filter. Do not irradiate the Calcofluor solution as called for in some procedures.

Flow Injection Method
Condition the instrumentation as outlined by the manufacturer. Calibrate the instrument with the six-point standard β-glucan curve prepared in the Standardization Method. Prepare congress wort samples according to *Methods of Analysis*, Malt-4 (2), and inject wort undiluted (see Notes) according to the manufacturer's instructions.

Calculation of Results
The results described in mg/L β-glucan are predicted from the linear relationship of β-glucan concentration and fluorescence intensity calculated from either peak height or area. The values obtained will be reported as is, and any dilutions made to the samples will be accounted for by multiplying the result by the appropriate dilution factor. For example, the results in mg/L of a wort diluted 1:1 with distilled water will be multiplied by a dilution factor of 2.

Precision
Based on a collaborative study (3), one can expect repeatability coefficients of variation within a single laboratory of 8.3–11.2% and reproducibility coefficients of variation comparing two or more laboratories of 17.7–25.1%.

Notes
1. For wort β-glucan concentrations in excess of 200 mg/L, dilute wort 1:1 with distilled water and multiply the resulting concentration by 2 to determine the final concentration.
2. The results of the ruggedness testing (3) emphasize that flow injection method conditions must be accurately controlled and listed when comparing results from different laboratories. Those factors having the most significance (in order of decreasing significance) are: emission wavelength, buffer, no Calcofluor irradiation, Calcofluor concentration, uniform glucan standard, and excitation wavelength.

REFERENCES

1. European Brewery Convention. *Analytica*, 4th ed. Supplement. In press.
2. American Society of Brewing Chemists. *Methods of Analysis*, 7th ed. Malt-4 Extract. The Society: St. Paul, MN 1976.
3. American Society of Brewing Chemists. Report of Subcommittee on β-Glucan in Congress Wort by Fluorescence Method. *Journal* 49:187-189, 1991.