A FLUORIMETRIC METHOD FOR MEASURING 1,3:1,4- β -GLUCAN IN BEER, WORT, MALT AND BARLEY BY USE OF CALCOFLUOR

by

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A convenient method for determining mixed linked 1,3:1,4- β -glucans in beer, wort, malt and barley is described. The fluorochrome Calcofluor, specific for β -glucosidic linkages, is used to stain these materials. The fluorescence intensity of the final suspension is measured and the result compared with traditional β -glucan measurements.

The correlation between fluorescence intensity and $(\text{NH}_4)_2\text{SO}_4$ precipitation was very good for beer and wort. The correlation between the fluorescence method and an enzymatic method used for malt and barley was somewhat poorer.

1. INTRODUCTION

In brewing, filtration time and haze formation are important factors which determine the economy of the brewing process and the quality of beers produced. In this connection β -glucans derived from endosperm cell walls have been intensively studied because they may impair filtration of wort and beer when dissolved as large molecules (8, 12, 13). Partly degraded β -glucans, however, seem to be beneficial for beer quality parameters such as foam stability and

flavour (11). Haze formation may occur in connection with β -glucan precipitation during cold storage of beer (16). To avoid the troublesome influence of β -glucans in wort and beer, the β -glucan content of the wort during mashing must be kept low. Well modified malt with high β -glucanase content and low content of β -glucan as well as a suitable adjunct are the prerequisites for an optimal mash. As a result, selection of malting barley lines with low β -glucan content has been undertaken.

Abbreviation: Mixed-linked 1,3:1,4- β -glucans, in the present communication referred to as β -glucans.

Thus at all stages in production, from the breeding of malting barleys to delivery of the beer, there is a need for quick and accurate methods for determining β -glucan levels. Only with such methods available will it be possible in practice to control the β -glucan level. At present, however, most methods are either laborious (6, 14) or imprecise (3). In beer and wort the amount of β -glucan is now normally determined by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (14). For malt and barley the most precise method seems to be a direct enzymatic one (6) employing digestion with a specific enzyme and measurement of the hydrolysis product, glucose, that is released.

A recent method (2) permits a morphological quantification of the degree of endosperm cell wall breakdown based on the ability of the fluorochrome Calcofluor to stain β -glucan (17). In the present paper, fluorescence measurements of Calcofluor-stained β -glucans in beer, wort, malt and barley are compared to established methods.

2. MATERIALS AND METHODS

2.1. Material

Beer – The investigations were carried out on 10 samples, six from the production lines of The United Breweries and four from the pilot brewery at the Carlsberg Research Laboratory. K. E. ERDAL and B. S. ENEVOLDSEN, Department of Brewing Chemistry, kindly supplied the beer samples.

Wort – The wort derived from Nortal malt was made at the Central Laboratory of the Carlsberg Brewery by J. C. LORENZEN according to the analysis committee of the European Brewery Convention (5). The malt samples used for the wort represent various degrees of modification.

Malt – The malt samples consisted of green and kilned malt from a pilot malting of Nortal used in a previous study (2).

Barley – The experiments were performed on barley grown at the Crop Research Division (D.S.I.R.) Research Farm, Lincoln, New Zealand during the period September 1977 – February 1978. The barley genotypes Minerva, Zephyr, Lami, Proctor, Triumph, Nirasaki and Risø 1508 were selected to provide a wide range of β -glucan content. The total β -glucan content ranged from 2.6 % to 6.36 % dry weight (1).

β -glucan – Purified barley β -glucan was purchased from Biocon Biochemicals Ltd., Cork, Eire.

All results are based on duplicate determinations.

2.2. Determination of β -glucan

2.2.1. Total β -glucan

The total β -glucan content in flour derived from barley and malt was measured as described by ANDERSON et al. (6). The method involves enzymatic hydrolysis of β -glucan with a specific enzyme – 1,3:1,4- β -D-glucanase (EC 3.2.1.73) followed by extraction and hydrolysis of liberated oligo-saccharides. The glucose released was measured by a glucose oxidase-peroxidase reagent (Boehringer Mannheim GmbH, Mannheim, W. Germany).

2.2.2. Soluble β -glucan

The method of PREECE and MACKENZIE (14) modified by ERDAL and GJERTSEN (9) has been used for the determination of soluble β -glucan in beer and wort. The β -glucans were precipitated by 30 g $(\text{NH}_4)_2\text{SO}_4$ per 100 ml beer or wort at 0 °C for 1 hour, centrifuged and washed twice with 20 % ethanol. The precipitate was then redissolved in 1 N H_2SO_4 by boiling for 15 min. Determination of the amount of reduced carbohydrates in the solution was carried out with phenol and sulphuric acid. Absorption was read at 490 nm with glucose as standard.

2.3. Viscosity determination

Wort viscosity was measured with a Haake Kugelfall Viskosimeter (Haake, K. G., Berlin-Steglitz, W. Germany).

2.4. Calcofluor method for β -glucan determination

2.4.1. Preparation of beer and wort

One ml wort or decarbonated beer was mixed vigorously with 1 ml 0.1 % aqueous Calcofluor for 1 min. Addition of 8 ml 96 % ethanol gave a turbid solution, which was mixed 15 sec on a Whirlimixer (Fisons Scientific Apparatus, Leicestershire, England), then centrifuged for 10

min at 4000 rpm. The precipitate was washed with 3×5 ml 80% ethanol followed again by centrifugation.

To insure that the excess Calcofluor was washed out, a Polytron mixer (Kinematica, Luzern, Switzerland) was used before centrifugation at each stage. This operation also preserves the colloidal nature of the precipitate. For fluorescence measurement, 5 and 10 ml of 75% fluorescence free glycerol (E. Merck, Darmstadt, W. Germany) was added to the precipitate of beer and wort, respectively, and the suspension mixed vigorously in the Polytron mixer. The 75% glycerol was used to avoid sedimentation in the suspension. Samples were placed in 10×10 mm quartz cuvettes and fluorescence spectra obtained from 370–550 nm with an excitation wavelength of 363 nm (Figure 2).

2.4.2. Preparation of malt and barley

Malt and barley were milled in an Udy cyclon mill (Udy Analyzer Co. Boulder, Col. USA) through a 0.4 mm sieve and the flour thoroughly mixed after milling. A subsample of about 100 mg of this coarse flour was milled in a ball mill for 15 min to obtain a homogeneous fine flour. The sample was dried in a vacuum oven at 50 °C to constant weight and stored in a dessicator until use. Ten mg dry weight was taken for the fluorescence measurement.

The sample was stained with 1 ml 0.1% aqueous Calcofluor and mixed 1 min in a Wirlimixer. Four ml 96% ethanol was added, and after mixing the sample centrifuged at 4000 rpm for 10 min. The precipitate was washed with 3×5 ml 80% ethanol. Ten ml 75% glycerol was added, and the suspension mixed with the Whirlimixer and transferred to a cuvette as in 2.4.1.

2.4.3. Preparation of purified β -glucan

Purified β -glucan was treated essentially as described for malt and barley.

2.4.4. Equipment for fluorescence measurement

All experiments were carried out with the system shown in Figure 1. The main equipment of the system was a scanning spectrofluorome-

Figure 1. Equipment for fluorescence measurement (simplified diagram).

ter, Jasco FP 550 (Japan spectroscopic Co. Ltd.) with two monochromators to perform excitation and emission spectra. The light source was a 150 W Xenon lamp. Specifications of the fluorescence unit were: Wavelength range 220–850 nm, wavelength accuracy ± 2.0 nm and reproducibility ± 2.0 nm. In all the investigations the spectral slits were kept at 5 nm on both the excitation and emission side.

The output signal (Figure 1) from the spectrofluorometer was transferred to a recorder via a low pass filter to improve the signal to noise ratio. The signal entering the recorder was also connected to an analog-digital converter and further through a BCD-interface to a Hewlett Packard 9825A calculator. This facility permits collection of a whole spectrum on a data tape. The spectrofluorometer was set to signals in the range of 0–2000 mv.

2.5. Quantitative fluorescence measurement model

Two empirical laws have been formulated concerning absorption intensity. LAMBERT's law states that the fraction of incident light absorbed is independent of the intensity of the source. BEER's law states that absorption is proportional to the number of absorbing molecules. A

combination of these laws is given in equation [1]:

$$\text{Log}_{10} \left(\frac{I_0}{I} \right) = L \cdot C \cdot \zeta \quad [1]$$

I_0 : Intensity of incident light, I : Intensity of transmitted light, L : Path length, ζ : Extinction coefficient, C : Concentration.

In spectroscopy the amount of absorbed light is equal to the difference between incident and transmitted light $I_{\text{abs}} = I_0 - I$. However, in the case of fluorescence, a fraction of the absorbed light is emitted. Thus we arrive at the following equation [2], derived from [1]:

$$F = I_0 \cdot Q_{\text{rel}} (1 - 10^{-\zeta \cdot L \cdot C}) \quad [2]$$

F : Intensity of fluorescence emission; Q_{rel} : Quantum yield (Quant emitted/Quant absorbed).

The relationship between fluorescence and the concentration of a given fluorochrome provides a curve which approaches an asymptote equal to $I_0 \cdot Q_{\text{rel}}$. In practice, however, many fluorochromes reach a maximum value and then decrease due to an increasing quenching effect.

In dilute solution or suspensions with weakly absorbing molecules, equation [2] may be approximated to a linear equation [3]:

$$F = I_0 \cdot Q_{\text{rel}} \cdot 2.303 \cdot \zeta \cdot C \cdot L \quad [3]$$

which for many applications is a good model for the relationship between fluorescence and concentration of a fluorescent compound.

A more exact linear calibration curve relating fluorescence and concentration can be obtained for an extended range of concentration with a transformation of the non-linear equation [2] to equation [4]:

$$C = 1/\zeta \cdot \log_{10} (1 - F/I_0 Q_{\text{rel}}) \quad [4]$$

for $L = 1$

For the present experiments $K = I_0 Q_{\text{rel}}$ and ζ were estimated with the aid of linear regression. The optimal values for $K = I_0 Q_{\text{rel}}$ and ζ were estimated with an iterative algorithm (7) programmed at the HP 9825A computer. The criterion used for optimal solution was maximal correlation between concentration and the logarithmic expression of equation [4] and the result expressed with an optimal value for $K = I_0 Q_{\text{rel}}$ and corresponding correlation coefficient (R^2).

Fluorescence intensity was read at 420 nm in all experiments. Transformation of data according to equation [4] was only used for beer and malt samples where a sufficient number of analyses were collected.

Figure 2. Excitation (----) and emission (—) spectra of Calcofluor stained β -glucan in a 75% glycerol suspension.

3. RESULTS

3.1. Excitation and emission spectra

Beer, wort, malt and barley stained with Calcofluor all shared the same characteristic absorption band with a maximum at 363 nm (Figure 2). A similar absorption band was observed for purified β -glucan stained with Calcofluor. The corresponding fluorescence spectrum exhibited a maximum emission at 420 nm with a shoulder at 445 nm (Figure 2). The same fluorescence profile was observed for Calcofluor in 75% glycerol indicating that precipitates must be carefully washed to remove excess Calcofluor. The position and shape of the curves were found to be strongly dependent on experimental conditions. For example, if the stained β -glucans were maintained in an aqueous suspension instead of 75% glycerol, a chemical shift towards the red region was observed. In this case, excitation and emission maxima shifted to 417 nm and 480 nm, respectively.

The fluorescent complex showed a weak fading effect. If a sample was repeatedly exposed to light at 363 nm for 3 min only a slight decrease in fluorescence intensity was observed after 4 cycles of exposure.

Figure 3. The relationship between fluorescence intensity and $(\text{NH}_4)_2\text{SO}_4$ precipitated β -glucan for 10 samples of beer.

Figure 4. Fluorescence intensity, transformed according to equation [4] and related to $(\text{NH}_4)_2\text{SO}_4$ precipitated β -glucan for 10 samples of beer.

R^2 = correlation coefficient, F = fluorescence intensity (mv).

3.2. Soluble β -glucan

3.2.1. Beer

The relationship between β -glucan content and fluorescence intensity in beer (Figure 3) was in accordance with the theoretical equation [2] (see section 2.5) showing an asymptotic approach to an upper limit. The linear correlation coefficient for data used for Figure 3 was $R^2 = 0.987$ with an intercept of 143.0 mv., confirm-

ing a non-linear relationship between β -glucan content and fluorescence intensity. Transformation of the data according to equation [4], Figure 4, improved the linear correlation coefficient to $R^2 = 0.998$. The optimal solution was reached at $I_0 \cdot Q_{\text{rel}} = 1431$ mv.

Using this model the β -glucan content of individual beers was estimated and compared with $(\text{NH}_4)_2\text{SO}_4$ precipitated β -glucan (Table I).

Table I

Fluorescence intensity, logarithm of fluorescence intensity and estimated β -glucan content compared to measured.

Samples	Fluorescence intensity (F) (mv)	-Log (1-F/1431)	β -glucan ^c estimated (mg · l ⁻¹)	β -glucan 30 % $(\text{NH}_4)_2\text{SO}_4$ (mg · l ⁻¹)
007-143a	1035	0.558	478 ± 14	475
007-144-5a	917	0.445	384 ± 7	390
108-12b	588	0.230	205 ± 1	200
21/4-012b	418	0.150	138 ± 1	141
007-140a	404	0.144	133 ± 2	134
091-164a	373	0.131	122 ± 2	125
28/4-013b	386	0.137	127 ± 5	124
5/5-014b	406	0.145	134 ± 1	123
12/5-015b	352	0.123	115 ± 0	116
19/5-016b	297	0.101	97 ± 1	104

a: Pilot brewery beer; b: Beer from production, c: Estimate from iterative linear model.

Figure 5. The relationship between $(\text{NH}_4)_2\text{SO}_4$ precipitated β -glucan and fluorescence intensity for 5 wort samples derived from malt samples with different degrees of modification.

R^2 = correlation coefficient.

Figure 7. The relationship between total β -glucan and fluorescence intensity for 13 malt samples of the barley genotype, Nortal.

○ = freeze-dried green malt samples with different degrees of modification, ● = kilned malt samples.

The high correlation between the two measurements (columns 3 and 4) was supplemented by high reproducibility with an average deviation less than 1.5 % (Table I, column 3).

3.2.2. Wort

Five worts derived from Nortal malts of widely differing degrees of modification were tested. The linear correlation was high R^2 =

Figure 6. The logarithmic relationship between viscosity and fluorescence intensity for the 5 wort samples of Figure 5.

R^2 = correlation coefficient.

Figure 8. The relationship between total β -glucan and fluorescence intensity for 7 barley varieties.

R^2 = correlation coefficient.

0.995, between the $(\text{NH}_4)_2\text{SO}_4$ precipitation and the Calcofluor methods of β -glucan analysis (Figure 5). The slope of the curve was flatter for wort than for beer (Figure 3), and showed a greater intercept, indicating a possible difference between the two methods of β -glucan analysis applied to wort as compared with beer. Good correlation ($R^2 = 0.983$) was also found between the logarithm of wort viscosity on the one hand and $(\text{NH}_4)_2\text{SO}_4$ precipitated β -glucan and Calcofluor bound β -glucan on the other (Figure 6).

3.3. Total β -glucan

3.3.1. Malt

Eleven freeze-dried green malt samples with increasing degrees of modification and two kilned samples, one kilned for 11 hours at 50 °C and another receiving a full kilning treatment, were analyzed for total β -glucan by the Calcofluor and the enzymatic methods. There was good linear relationship between the two methods ($R^2 = 0.95$, Figure 7) with a slight deviation for two samples which were least modified. If these two samples were omitted, the curve was found to be in agreement with equation [2] as in the case of beer. A transformation of this curve by equation [4] improved the correlation coefficient to $R^2 = 0.99$ with an upper limit $I_0 \cdot Q_{\text{rel}}$ at 748 mv.

The kilned malt samples fit the same curve as the green malt samples indicating that the kilning procedure did not change the affinity of Calcofluor for β -glucan in kilned malt. The accuracy of the Calcofluor β -glucan analysis decreased in malt with an average deviation of 7.5 % compared with less than 1.5 % in beer.

3.3.2. Barley

Seven barley varieties with widely different β -glucan contents as analyzed with the enzymatic method were tested for comparison with the Calcofluor method to determine total β -glucan content. The relationship between the two measurements of β -glucan (Figure 8) was similar to that found for malt but the correlation coefficient was lower, $R^2 = 0.86$.

4. DISCUSSION

In measuring soluble β -glucans, precipitation with 30 % $(\text{NH}_4)_2\text{SO}_4$ followed by hydrolysis and determination of the released glucose has been the preferred method since it was introduced by PREEZE and MACKENZIE in 1952 (14). The analysis is quite laborious and takes about a day. An analogous method has been described (8) using cold 71 % ethanol for precipitation. In the brewing industry, viscosity of wort and beer are important parameters (12, 15) influencing filtration rates. Viscosity measurements are often but not always well correlated to β -glucan content.

In barley and malt, total β -glucan have been estimated with enzymatic digestion methods. FLEMMING et al. (10) used the difference between the glucose content of a total digest and an amyloglucosidase digest as an estimate of β -glucan. A more exact method is the direct enzymatic method of ANDERSON et al. (6) previously discussed. Both methods are laborious, with a capacity of 6–10 samples per day with a processing period of 4–5 days.

In plant breeding the need for more convenient methods of analyzing barleys for β -glucans has resulted in the development of indirect measurements as exemplified by measurements of the viscosity of an acid extract (1), and of near infrared reflectance (3) where some wavelengths apparently are correlated to β -glucan content.

The method presented in this communication was carried out using the fluorochrome Calcofluor, which has been shown by WOOD and FULCHER (17) to cause preferential precipitation of oat gum and barley β -glucan in aqueous solutions containing protein, pentosan and starch. In the same study qualitative analysis indicated that Calcofluor had a weak affinity for polysaccharides containing pentose and galactose, whereas a dye complex formation was observed for cellulose (β -1,4-glucan).

The complex formed between Calcofluor and β -glucosidic bonds is utilized in this study as the basis for a convenient and reproducible method for the determination of β -glucan by fluorescence. Quantitative fluorescence measurements traditionally employ separation and extraction yielding a solution of one or a few components for analysis (4). In the present study, however, it has been shown that reproducible fluorescence measurements of Calcofluor stained β -glucan in

multicomponent colloid suspensions are well correlated with current β -glucan analyses. Especially in beer, the correlation between the fluorescence and β -glucan content determined by $(\text{NH}_4)_2\text{SO}_4$ precipitation was excellent, indicating very little unspecific dye binding of Calcofluor. In contrast to this, the lower correlation coefficient with respect to malt and barley might be due to dye binding of Calcofluor to non β -glucan compounds such as β -glucosidic linkages from cellulose.

There is an incomplete and scattered knowledge of the specificity and molecular size limits of the present precipitation methods. CRABB and BATHGATE (8) described that cold 71% ethanol precipitates β -glucans in wort that are greater than 2000 daltons while 30% $(\text{NH}_4)_2\text{SO}_4$ precipitates β -glucans greater than 50,000 daltons. The need for further basic studies with regard to the problem of β -glucan determinations in wort is exemplified by the low correlation coefficient ($R^2 = 0.64$) obtained by Scott (15) between wort viscosity and $(\text{NH}_4)_2\text{SO}_4$ precipitable β -glucans. SCOTT used malt from different barley varieties while wort used in the present study was derived from a single genotype of barley.

The results presented in this communication point to the feasibility of a method for β -glucan determination which is rapid, reproducible, and easy to handle as an alternative to present, more laborious methods. It is however necessary to study the Calcofluor method as well as the current reference methods for β -glucan analysis further to define their dependence on contaminating compounds and on the distribution of β -glucan molecular weight classes.

We plan to develop the Calcofluor method for routine use in determining β -glucan in beer and wort in the daily work of the brewery.

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