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Determination of β-(1–3),(1–4)-D-glucans in barley by reversed-phase high-performance liquid chromatography

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Abstract

An HPLC method for the determination of β-glucan in barley was developed. The β-glucan was hydrolysed with lichenase [*endo*-β-(1–3),(1–4)-D-glucan-4-glucanhydrolase from *Bacillus subtilis*] to oligosaccharides, which were analysed by reversed-phase HPLC using water as the mobile phase at a flow-rate of 0.7 ml/min. The separation of the oligosaccharides was performed in a C_{18} stainless-steel column (Spherisorb ODS-2) with 5-μm particles in less than 10 min, with a refractive index detection.

1. Introduction

In the past, the use of barley in monogastric diets was restricted because of some negative aspects. This cereal shows great variability owing to variety, location and climate; in addition, barley has a lower energy value than corn. In any case, the main problem is the relatively high level of β-(1–3),(1–4)-D-glucan, also named mixed β-glucans, a type of polysaccharide that constitutes 70–75% of the endosperm cell wall [1,2]. Barley β-glucans, which are found at concentrations between 2 and 8%, increase the viscosity of the intestinal content in broilers [3], which in turn interferes with the digestion and absorption of nutrients [4]. This polysaccharide reduces productive parameters in broilers and creates health and management problems, especially of the litter [5,6]. On the other hand, the β-glucans are included in the soluble dietary fibre fraction of cereals that in human nutrition participates in the glucoregulation and produces a decrease in serum cholesterol levels in humans.

The mixed β-glucans were first described by Kjeldahl in 1881. These polysaccharides were shown to be linear, unbranched and composed of β-D-glucopyranose units joined by (1–3)-(30%) or β-(1–4)-glycosidic (70%) bonds [7,8]. Selective enzymolysis of mixed β-glucan with cellulase (EC 3.2.1.4) or laminarinase (E.C. 3.2.1.39) indicates that most of the polymer (85–95%) is composed of two main structural units, (1–3)-linked cellotriosyl and (1–3)-linked cellotraosyl units [9,10], and in the polysaccharides the (1–3) linkages occur singly [10–12]. Methylation analysis suggests that a small proportion (5–15%) of the polymer may consist of longer cellulose-like

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sequences (4–11 units) of (1–4)-linked glucose residues [8,10,13].

Several methods have been developed to determine this polysaccharide: selective precipitation of β-glucans extracted with 20–30% ammonium sulfate [14,15]; total acid hydrolysis of β-glucans extracted with water and determination of free glucose [16–18]; enzymatic methods, based on the specific hydrolysis of β-glucans by enzymes with β-glucanase activity, and determination of free glucose [19–24]; and measurement of fluorescence produced by the specific binding of high-molecular mass β-glucans to some dyes by means of flow-injection analysis (FIA) [25–27]. The enzymatic method developed by McCleary and Glennie-Holmes [23] and the FIA-Calcofluor method described by Jorgensen and Aastrup [25] have been accepted as official methods by the European Brewery Convention.

The current interest in β-glucans as a source of soluble dietary fibre for human nutrition and the problems that these polysaccharides produce in broiler nutrition have generated the need for an analytical method that is as simple and rapid as possible. These characteristics could be accomplished by high-performance liquid chromatography.

HPLC has been used extensively in the separation and characterization of food carbohydrates [28,29]. Because of the absence of a chromophore in the molecule of carbohydrates, the quantification of these compounds can be achieved by fluorescence or absorbance detection after derivatization of the carbohydrates [8,30–32] or performing the separation by reversed-phase (RP) HPLC with refractive index (RI) detection [33–37]. RP-HPLC with RI detection has been demonstrated to be a rapid and convenient method to analyse the carbohydrate syrups obtained by hydrolysis of starch, cellulose and inulin. The chromatographic systems used can be distinguished as three types: (1) a strong cation exchanger as the stationary phase and water as the eluent [38,39]; (2) a propylaminosilane-modified silica as the stationary phase and acetonitrile–water as the eluent [40–43]; (3) an octadecylsilane-modified silica as the stationary phase and water as the eluent [44–46]. This last method combines two important advantages, namely a pressure-stable stationary phase and a non-toxic, cheap eluent.

The objective of this study was to develop a simple and rapid method for the determination of barley β-glucans using HPLC of oligosaccharides released by *endo*-β-(1–3),(1–4)-D-glucan-4-glucanohydrolase (lichenase, EC 3.2.1.73). Lichenase specifically cleaves the β-(1–4)-linkage of a 3-O-substituted D-glucopyranose residue in the polysaccharide to give 3-O-β-cellobiosyl-D-glucose and 3-O-β-cellotriosyl-D-glucose as the major products [47]. The separation of these oligomers is carried out with water as the mobile phase at a flow-rate of 0.7 ml/min, an octadecylsilane-modified silica column and RI detection. This separation method permits the determination of β-glucans with the same accuracy as enzymatic or FIA-Calcofluor methods, and an HPLC analysis of a barley hydrolysate can be performed in 10 min.

2. Experimental

2.1. Equipment

A Perkin-Elmer liquid chromatograph (LC-410) equipped with a refractive index detector (LC-25) was used. Separation was carried out using a 5-μm Spherisorb ODS-2 reversed-phase column (250 × 4.6 mm I.D.). Chromatographic data were collected and recorded using a Perkin-Elmer LCI-100 integrator.

2.2. Reagents and standards

Lichenase, *endo*-β-(1–3),(1–4)-D-glucan-4-glucanohydrolase (EC 3.2.1.73) (from *Bacillus subtilis*), 50 U/ml, was used to hydrolyse barley β-glucan. Pure barley β-glucan (Sigma, St. Louis, MO, USA) and barleys with known β-glucan content (Megazyme, NSM, Australia) were used as standards. Sodium phosphate buffer (20 m*M*, pH 6.5) was prepared by dissolving 3.12 g of sodium dihydrogenphosphate dihydrate (analytical-reagent grade) in water, adjusting the

pH with 100 m*M* sodium hydroxide and diluting to 1 l. Ethanol (50%, v/v) was of analytical reagent-grade.

For mobile phase preparation, water, methanol and acetonitrile of HPLC grade (Carlo Erba, Milan, Italy) and 96% sulfuric acid (analytical-reagent grade) (Merck, Darmstadt, Germany) were used.

All solutions were prepared with HPLC-grade water. All standards and solvents were filtered with a 0.45-μm membrane and deoxygenated by helium bubbling.

2.3. Chromatographic conditions

The composition of the mobile phase was varied in experiments carried out for optimization; water at a flow-rate of 0.7 ml/min was selected as the final mobile phase. All samples were filtered with 0.45-μm nylon membrane disc filters (25 mm diameter) and the sample volume injected was 6 μl. The column was operated between 18 and 22°C. Since matrix effects were not found, quantification was carried out by the external standard method. The external standard used was a lichenase hydrolysate of pure β-glucan or a lichenase hydrolysate of a barley with known β-glucan content.

2.4. Sample preparation procedure

For the chromatographic determination of barley β-glucans, the polysaccharide was firstly hydrolysed with lichenase and the oligosaccharides released were separated with RP-HPLC. The barley was ground in a Cyclotec mill to pass a 0.5-mm sieve. The meal (0.5 g) was suspended in 1 ml of 50% ethanol and 5 ml of 20 m*M* sodium phosphate buffer (pH 6.5) in a boiling water-bath. The hydrolysis of β-glucan was carried out with 200 μl of lichenase solution at 40°C for 1 h. After 24 ml of water had been added, the solution was vigorously vortex mixed and centrifuged at 5000 *g* for 15 min. The supernatant was filtered with a 0.45-μm nylon membrane disc filter and 6 μl were injected for the chromatographic analysis.

Various barleys grown in Spain from three consecutive harvests were analysed.

2.5. Chemical methods used as comparisons with HPLC

The β-glucan content of 27 barleys was determined with the described RP-HPLC method and the results were compared with those obtained with the enzymatic McCleary and Glennie-Holmes method [23] and with the FIA-Calcofluor method developed by Jorgensen and Aastrup [25].

3. Results and discussion

Fig. 1 shows the chromatograms obtained by injection of lichenase hydrolysates of (a) a reagent blank solution, (b) a standard barley β-glucan, (c) a barley with known β-glucan content not treated with lichenase and (d and e) two barleys with known β-glucan content treated with lichenase. As can be seen in Fig. 1b, the hydrolysis of pure β-glucan led to the appearance of two different peaks, the first at about 7.03 min and the second at 8.10 min. These peaks also appear in chromatograms of barley hydrolysates, but only if lichenase is incorporated, producing the specific release of β-oligosaccharides from glucans (Fig. 1c, d and e). Chromatograms were recorded for up to 2 h, but in all cases only peaks before 10 min were obtained. The first and second peaks identified as β-oligosaccharides from β-glucans can be assigned to a cellotriosyl residue and a cellotetraosyl residue, respectively, in accordance with studies by Parrish et al. [9] and Woodward et al. [10]. The total area of both peaks showed a linear relationship with the β-glucan content present in the injected solutions ($r = 0.993$), allowing its quantification.

In barley chromatograms, peaks also appear between 2.12 and 4.69 min that have been identified as malto-oligosaccharides, mainly maltose and maltotriose, arising from partial hydrolysis of the starch present in the cereal. During the optimization steps, the amount of

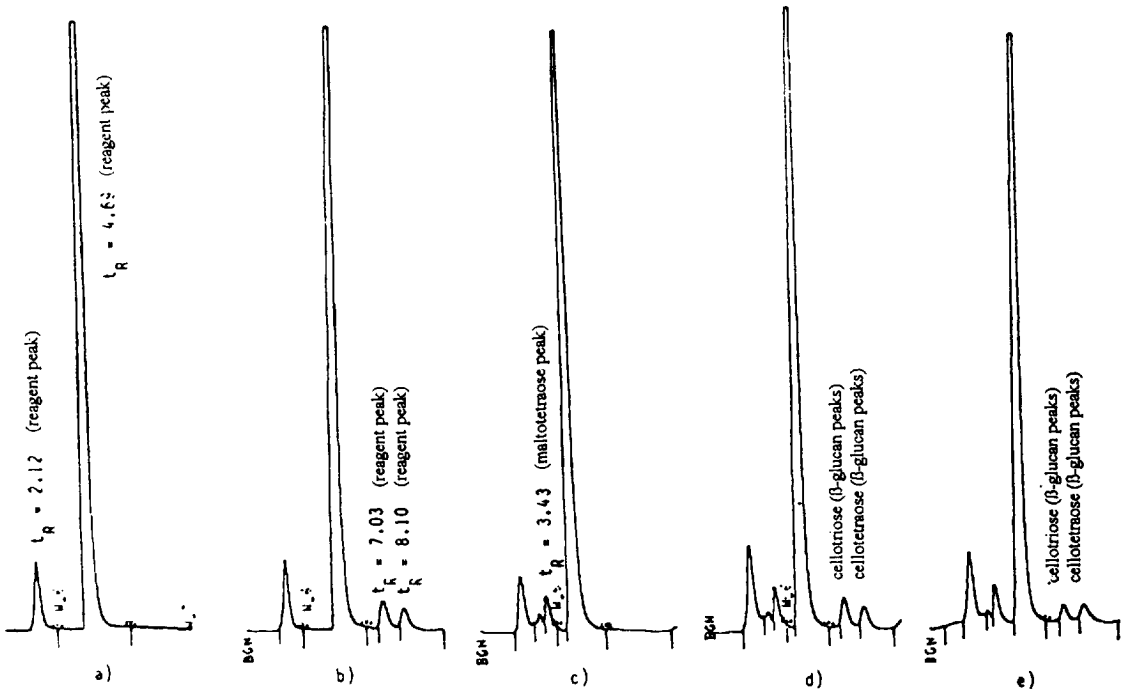

Fig. 1. Chromatograms obtained under the optimum experimental conditions (0.5 g of barley sample, mobile phase water at flow-rate of 0.7 ml/min) of (a) blank reagent solution, (b) extract of pure β-glucan treated with lichenase, (c) extract of barley not treated with lichenase and (d and e) extracts of barleys with known β-glucan content (4.4 and 3.2%, respectively) treated with lichenase.

sample used varied between 0.5 and 2.0 g. A linear relationship was found between the total area of the two peaks and the mass of sample ($r = 0.996$), but solutions containing 2 g developed a high viscosity, which made their handling difficult. A mass of 0.5 g of sample was selected as the optimum.

Several mobile phases were tried in order to obtain the best resolution of β-glucan peaks: water, water–methanol (95:5), water–acetonitrile (95:5) and sulfuric acid (from $3 \cdot 10^{-3}$ to $0.5 \cdot 10^{-2}$ M) in water, and at different flow-rates. The best separation was achieved with water at a flow-rate of 0.7 ml/min ($R_s = 0.96$).

The possible presence of interferences due to the barley background was examined by comparison of the linear equations for results obtained by the standard additions procedure on pure β-glucan in barley samples and by the analysis of various amounts of barley samples, quantified by the external standard method (Fig. 2). The analysis of covariance revealed that there were no statistically significant differences between the curves and the external standard

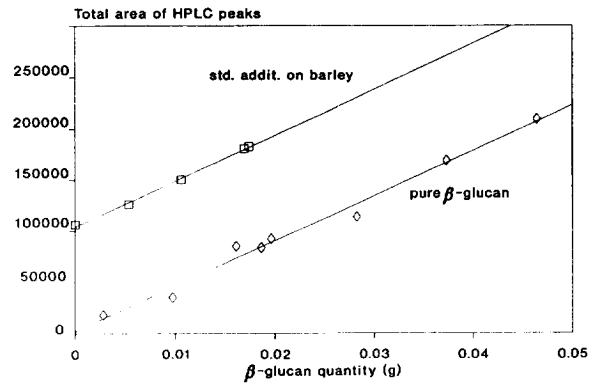

Fig. 2. Linearity of chromatographic response of β-glucan content in barley when measured by (◇) the external standard method ($r = 0.993$) or by (□) standard additions of β-glucan to barley ($r = 0.9986$).

method of quantification was adopted owing to its simplicity. With the standard addition procedure, recoveries of β-glucan between 95 and 105% were obtained.

The detection limit of the HPLC method was determined taking into account that the analyte concentration in the sample solution could be not free from error, and analysing increasingly diluted solutions of barleys until the measurement of peaks was no longer possible. A regression band was obtained; the intercept on the ordinate gave the range of responses that can be obtained for a sample without analyte and was associated with a range of concentrations. Below this range a sample cannot be quantified. This procedure was performed on two barley varieties, Barbarrosa (5.19% β-glucan) and Beka (3.00% β-glucan). The estimated limits of detection were 2.53 and 4.54 mg of β-glucan in solution, respectively, which correspond to about 1.5% of β-glucan in barley. As the level usually found in this cereal varies between 2 and 8%, the described procedure is inadequate for analysing many low-glucan barleys. However, in this case, a less diluted hydrolysate can be prepared in order to enhance the detection and quantification limits.

The repeatability ($r = 2\sqrt{2}s_r$; s_r = standard deviation) of the HPLC method was obtained by the determination of the β-glucan content in nine replicates of a standard barley under homogeneous conditions. The results are presented in Table 1. The β-glucan content of the standard barley used in this study was previously determined by an enzymatic method [23] and by the FIA-Calcofluor method [25] in two different laboratories. Table 2 shows the values obtained in the determination of the β-glucan content of the same barley performed on 12 different days by three different operators, in order to calculate the value of the between-day reproducibility ($R = 2\sqrt{2}s_R$; s_R = standard deviation) of the HPLC method. The method showed a repeatability of 0.28 and a reproducibility of 0.51.

The results for β-glucan content obtained with the described HPLC method in 27 barley samples were compared with those obtained using the enzymatic method of McCleary and Glennie-Holmes [23]. A statistically significant linear equation ($r = 0.910$) was obtained between them, as can be seen in Fig. 3. Both methods gave the same accuracy.

The β-glucan content of ten barley samples was also determined by the FIA-Calcofluor method [25] and a linear relationship was observed between the values obtained with this method and the HPLC procedure (Fig. 4, $r = 0.924$).

Table 1
Study of within-day repeatability of HPLC method applied to barley

Replicate	Sample mass (g)	Sample mass (g/dm)[a]	Total area	Amount of β-glucan (g)	β-Glucan (%/dm)[a]
1	0.5010	0.4526	95299	0.0170	3.75
2	0.5039	0.4552	94375	0.0168	3.69
3	0.5050	0.4562	92434	0.0165	3.61
4	0.5019	0.4534	91065	0.0162	3.58
5	0.5017	0.4532	96033	0.0171	3.77
6	0.5010	0.4526	104072	0.0185	4.10[b]
7	0.5037	0.4550	94915	0.0169	3.71
8	0.5048	0.4560	89466	0.0159	3.49
9	0.5079	0.4588	96114	0.0171	3.73
					Mean: 3.67
					S.D.: 0.10

[a] Expressed on a dry-matter basis.
[b] Rejected for calculations (4*d* criterion and 95% confidence level).

Table 2
Study of between-day reproducibility of HPLC method applied to barley

Replicate	Date of analysis	Total area	Sample mass (g)	Amount of β-glucan (g)	β-Glucan (%/dm)[a]
1	7.2.92	78 060	0.5040	0.0188	4.12
2	10.2.92	92 957	0.5018	0.0171	3.77
3	12.2.92	71 207	0.5077	0.0168	3.66
4	14.2.92	76 435	0.5045	0.0164	3.60
5	17.2.92	77 737	0.5091	0.0164	3.56
6	2.3.92	83 190	0.5077	0.0172	3.75
7	3.3.92	101 133	0.5077	0.0177	3.86
8	4.3.92	103 811	0.5077	0.0182	3.97
9	12.3.92	102 458	0.5057	0.0171	3.75
10	19.3.92	91 626	0.5060	0.0171	3.75
11	7.4.92	106 317	0.5032	0.0156	3.48
12	9.4.92	110 000	0.5050	0.0174	3.80
					Mean: 3.76
					S.D.: 0.18

[a] Expressed on a dry-matter basis.

4. Conclusions

A reliable method for the determination of barley β-glucans consisting of hydrolysis of the polysaccharide with lichenase and the determination of released oligosaccharides by reversed-phase HPLC was developed. The separation is performed in 10 min and the procedure yields accurate results and shows a good correlation with conventional methods.

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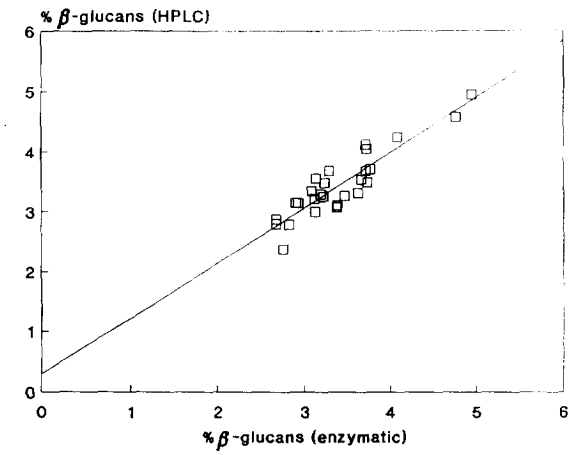

Fig. 3. Validation of HPLC method vs. enzymatic method ($n = 27$ samples, $r = 0.910$, range = 2.67–4.94%).

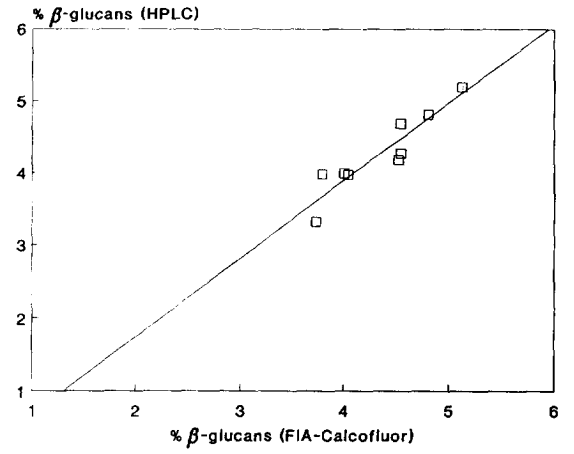

Fig. 4. Validation of HPLC method vs. FIA-Calcofluor method ($n = 10$ samples, $r = 0.924$, range = 3.73–5.12%).

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