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Cereal β-glucan quantification with calcofluor-application to cell culture supernatants

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ABSTRACT

The specific binding of the fluorescent dye calcofluor to cereal β-glucan results in increased fluorescence intensity of the formed complex and is in use for the quantification of β-glucan above a critical molecular weight (MW) by flow injection analysis. In this study, this method was applied in a fast and easy batch mode. In order to emphasize the spectral information of the emission spectra of the calcofluor/β-glucan complexes, derivative signals were calculated. A linear relationship was found between the amplitude of the second derivative signals and the β-glucan concentration between 0.1 and 0.4 μg/mL. The low detection limit of this new method (0.045 μg/mL) enabled its use to study the transport of cereal β-glucans over differentiated Caco-2 cell monolayers. Additionally, the method was applied to quantify β-glucan in arabinoxylan samples, which correlated well with data by an enzyme based method.

1. Introduction

Cereal β-D-glucans are linear mixed linked (1→3,1→4) D-glucose polymers (hereafter referred to as β-glucans), which are of importance for a wide range of subject areas. They occur as natural cell wall polysaccharides in a variety of cereals, with the highest contents found in barley and oat (Lazaridou, Biliaderis, & Izydorczyk, 2007). Their ability to form viscous solutions in water can lead to undesired effects, e.g. in poultry feed production where β-glucans may decrease nutrient digestibility, and during the brewing process where they may cause filtration problems (Saki et al., 2010). On the other hand, the ability of cereal β-glucan to form viscous solutions in the human small intestine is believed to be related to their beneficial effects on serum cholesterol and postprandial blood glucose levels in human nutrition (AbuMweis, Jew, & Ames, 2010; Behall, Scholfield, & Hallfrisch, 2006; Hallfrisch, Scholfield, & Behall, 2003; Wolever et al., 2010; Wood et al., 1994). In addition, cereal β-glucans have also been ascribed immune-modulating properties (Murphy et al., 2008; Volman et al., 2010; Yun, Estrada, Van Kessel, Park, & Laarveld, 2003). The potential uptake of cereal β-glucans from the intestine is an important question in the context of their possible immune-modulating effect. Barley β-glucan labelled with fluorescein dichlorotriazine, which covalently reacts with hydroxyl groups of β-glucan and also may cross-link the β-glucan chains, has been detected in spleen, lymph nodes and bone marrow of mice following oral administration (Hong et al., 2004). However, this labelling may change the properties of the polymer and its bioavailability. Specific and highly sensitive detection methods that do not require β-glucan derivatization may be helpful tools to investigate a possible uptake from the intestine, but are currently lacking.

Due to their wide range of interest, various methods for β-glucan quantification have been developed. The methods used today are essentially based on two different principles, enzymatic degradation of the β-glucan polymer (McCleary & Codd, 1991) or specific binding of the fluorescent dye calcofluor to the β-glucan chain (Wood, 1980). The necessary enzymes for cereal β-glucan quantification are commercially available as a kit from Megazyme (Megazyme International Ireland Ltd., Bray, Republic of Ireland). Since the enzymatic method is based on quantification of generated glucose, extraction and purification of β-glucan from grain and wort is necessary prior to the specific enzyme treatment, which makes the method relatively time consuming. In addition sample amounts of e.g. 500 mg barley flour are recommended for accurate results. This is unproblematic for routine flour/grain analysis, but it can be challenging for specific samples such as purity control of arabinoxylan samples extracted from β-glucan rich grains that are intended for specific biological tests. The other principal approach,

Abbreviations: DMEM, Dulbecco's modified eagle medium; TEER, transepithelial electrical resistance; FIA, flow injection analysis; RMSEC, root mean square error of calibration; PCA, principal component analysis; AS, analytical signal, defined as the sum of the absolute amplitude values of the second derivatives of the fluorescence emission spectra of the calcofluor/β-glucan complexes at 419, 433 and 448 nm; SEM, scanning electron microscopy; PBS, phosphate buffered saline; MW, molecular weight; M_w, weight average molecular weight.

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employing the calcofluor dye, has been primarily applied to quantify β-glucans in malt, wort and beer by the use of flow injection analysis (FIA) (Mekis, Pinter, & Bendek, 1987; Sendra, Carbonell, Gosalbes, & Todo, 1989). The sample is injected into the reagent stream and β-glucan binding to calcofluor results in an increased fluorescence intensity detected by a flow-through-cell fluorescence spectrophotometer. Either peak height or peak area is used for quantification by comparison with standard β-glucan solutions. Only cereal β-glucans with molecular weight (MW) above a critical value are fully detected by all calcofluor based methods (Gomez, Navarro, Carbonell, & Sendra, 2000; Kim & Inglett, 2006). This critical value has been reported to depend on the ionic strength of the measurement solution (Gomez et al., 2000; Kim & Inglett, 2006; Wood, 1980). If the measured samples contain β-glucan below a critical MW, the β-glucan content of the sample will be underestimated. However, since it is often the β-glucans with high MW that are of interest due to their viscous properties this limitation is of less importance.

Kim, Inglett, & Liu (2008) have modified the calcofluor-FIA method to be used in batch mode with a common fluorescence spectrophotometer. Here the calcofluor solution is titrated with the sample in the fluorescence spectrophotometer cuvette. The integrated fluorescence intensity is plotted against the volume of added substrate solution to obtain titration curves. Sample β-glucan concentrations are calculated by superimposing the titration curves of unknown sample onto that of a known sample and using the shift factor for β-glucan concentration calculation. The method was reported to have good correlation with the standard enzymatic method. However, the necessity to record titration curves adds to the time required for analysis of one sample. Schmitt and Wise (2009) have adapted the calcofluor method to a microplate format. Samples and standards are pipetted into microplate wells and calcofluor solution is rapidly added to all wells directly prior to fluorescence reading on a fluorescence plate reader by the use of an automatic multichannel pipettor. The method uses the emission at 420 nm of the calcofluor/β-glucan complex for β-glucan quantification.

None of the above mentioned methods has been optimized for the detection of low concentrations of cereal β-glucans. Based on the lowest standard concentrations reported for the batch mode titration and microplate format calcofluor methods, cereal β-glucan concentrations as low as 5 μg/mL (titration method) and 2.5 μg/mL (microplate method) can be detected. This sensitivity is too low to make those methods suitable for investigating a possible uptake of cereal β-glucans from the intestine. Based on the method by Schmitt and Wise (2009) and Kim et al. (2008) we have developed a technique for cereal β-glucan quantification with calcofluor dye that employs the use of a common fluorescence spectrophotometer, but without the requirement to record sample titration curves. The information in the whole emission spectra from 401 to 480 nm was used and combined with data analysis techniques to improve calibration. The method was applied to study the transport of cereal β-glucan preparations with different weight average molecular weights (M_w) over differentiated Caco-2 cell monolayers as an *in vitro* model of the intestinal epithelium and to determine the amount of co-extracted β-glucan in presumably pure arabinoxylan samples.

2. Materials and methods

2.1. β-glucan standards and carbohydrate samples

Cereal β-glucan standards of different M_w of 40, 123, 183, 245 and 359 kDa were obtained from Megazyme ((1,3)(1,4)-β-glucan, molecular weight standards, Megazyme). A commercial β-glucan standard for the calcofluor-FIA method (standard barley β-glucan for calcofluor/CFA; Megazyme) was used as a control for the proposed new methodology. Arabinoxylan fractions AX_Tyra and AX_Tyra_L were purified from the Norwegian barley variety Tyra and the β-glucan fraction BG_Olve was purified from the Norwegian barley variety Olve as previously described (Knutsen & Holtekjolen, 2007; Samuelsen, Rieder, Grimmer, Michaelsen, & Knutsen, 2011). Carbohydrates were stored in a desiccator under vacuum over di-phosphorus pentoxide to ensure complete dryness. Stock solutions of 1 mg/mL were prepared in distilled water by incubation in a boiling water bath for 15 min. Dilutions to working strength (10 μg/mL) were prepared with distilled water. The β-glucan content of the carbohydrate samples was determined by an enzymatic procedure using a combined treatment with cellulase (Megazyme) and β-D-glucosidase (Megazyme), followed by incubation with GOPOD reagent (glucose assay kit, Megazyme) and absorbance reading at 510 nm.

2.2. Reagent mixture preparation

Calcofluor was obtained from Megazyme and dispersed in 100 mM phosphate buffer pH 7 by stirring with a magnetic stirrer to a concentration of 100 μg/mL. Aliquots of the calcofluor solution were kept frozen at −18 °C and a new tube was thawed every day. calcofluor solutions were protected from light by aluminium foil throughout the preparation and experiments. Reagent mixtures were prepared in microtubes by combining 200 μL 0.5 M sodium phosphate solution pH 11, 50 μL 4.5 M NaCl solution, 500 μL serum free DMEM cell culture medium without phenol red, and 0–40 μL β-glucan working strength solution (10 μg/mL) with distilled water up to a total volume of 980 μL. The salt solution was added to the reagent mixtures to ensure a high ionic strength of the sample solution. High ionic strength has been reported to increase fluorescence intensity and to decrease the critical molecular weight below which the fluorescence of the calcofluor/β-glucan complex decreases considerably (Gomez et al., 2000; Kim & Inglett, 2006; Wood, 1980). For measurements of arabinoxylan solutions the cell culture medium was replaced with distilled water. This replacement did not affect the method. Directly before each measurement, 20 μL of calcofluor solution (100 μg/mL) was added to the sample solution, the tube was vortexed and the content transferred to a 1 mL quartz cuvette for front face fluorescence measurements. The final concentrations were 100 mM sodium phosphate solution pH 11 and 225 mM NaCl. The calcofluor concentration in the cuvette was 2 μg/mL and the concentrations of the β-glucan standards ranged from 0.1 to 0.4 μg/mL.

2.3. Instrumentation and data analysis

Fluorescence measurements were collected with a Perkin Elmer LS50B (Perkin Elmer Instruments LLC, Shelton, CT, USA) fluorescence spectrophotometer equipped with a variable-angle front-face accessory, to ensure that reflected light, scattered radiation, and depolarization phenomena were minimized. The angle of incidence, defined as the angle between the excitation beam and the perpendicular to the cell surface, was about 30°. Samples were placed in a 1-mL quartz cell (2 mm optical path length), and spectra were recorded at a set room temperature of 20 °C. Slits at excitation and emission monochromators were set at 10 and 5 nm, respectively. Acquisition speed was fixed at 500 nm/min, as a compromise between noise in the spectra and collection time. The excitation wavelength was set at 360 nm. Emission spectra were recorded between 390 and 500 nm, with wavelength increments of 0.5 nm. The spectral region of interest was 401–480 nm. All spectra were smoothed by the moving average method with a segment of 21 data points. Their second derivatives were calculated

by the Savitzky–Golay algorithm (Savitzky & Golay, 1964) with a wavelength increment of 1.5 nm and they were smoothed again, in the same conditions as the spectra. The Unscrambler X software package (CAMO PROCESS AS, Oslo, Norway) was employed for data treatment and analysis. On each measurement day, two calcofluor solution reference spectra were recorded and averaged. The second derivative of this calcofluor reference was subtracted from the second derivatives of the spectra of the calcofluor/β-glucan complexes obtained at the same day. For standard curve construction an analytical signal (AS) was calculated as the sum of the absolute amplitude values of the second derivatives of the fluorescence emission spectra of the calcofluor/β-glucan complexes at 419, 433 and 448 nm. All samples were measured in duplicates. For the construction of standard curves the spectra of the duplicates were averaged before further processing, while arabinoxylan sample and cell culture sample spectra were analysed without averaging.

2.4. Cell cultures

Caco-2 cells (a gift from Professor Kirsten Sandvig, Norwegian Radium Hospital) were grown in Dulbecco's modified eagle medium (DMEM) containing 10% heat inactivated fetal calf serum, 1% non-essential amino acids, 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were maintained at 37 °C with 5% CO_2 in a humidified incubator. Cells were plated in 12-well plates at a concentration of 3.0×10^5 cells/mL on Millicell hanging cell culture inserts (Pore size 0.4 μm, Millipore AS, Oslo, Norway). Medium was changed three times per week for 2 weeks. Differentiation of the cells was checked by scanning electron microscopy (SEM) and measurement of transepithelial electrical resistance (TEER) with a Millicell-ERS electrode (Millipore). TEER values were between 300 and 500 Ωcm^2 after 2 weeks incubation and SEM (data not shown) revealed the development of microvilli at the cell surfaces. For transport experiments, cells were washed once with sterile PBS and the medium was replaced by serum free DMEM without phenol red (1.3 mL basolateral). Serum free DMEM was added to freeze dried β-glucan aliquots to give concentrations of 1 mg/mL. The β-glucan concentration was checked with the new calcofluor method presented here before addition to the apical side of the cell-covered filters in triplicates. Serum free DMEM without phenol red was used as a control. Fluorescein-dextran 10 kDa (Sigma Aldrich, St. Louis, MO) was added to the apical side of each filter at a concentration of 1.85 mg/mL as a control for unspecific transport across the Caco-2 monolayer. TEER was determined at the start of the experiment and after 9 h incubation time. Aliquots of two times 100 μL of the basolateral medium were assayed for fluorescein content in a microplate fluorescence spectrophotometer (Fluorstar Optima, BMG Labtech, Ortenberg, Germany) and two times 500 μL of the basolateral medium were stored frozen for calcofluor fluorescence measurements. If not otherwise stated, all cell culture solutions were obtained from Invitrogen (Carlstad, CA).

2.5. Statistical analysis

The root mean square error of calibration (RMSEC) of the different standard curves was calculated as follows:

$$\mathrm{RMSEC} = \sqrt{\frac{(y - \hat{y})^2}{n}}$$

where $\hat{y}$, predicted β-glucan concentration; y, known β-glucan concentration and n, number of independent measurements.

Along with R^2, the RMSEC was used for model selection. The RMSEC is a measure of the calibration error and has the same unit as the response, which makes it easy to interpret.

Standard curves were validated on a test set of samples (25% of all samples). The relative error of each prediction was calculated as follows:

$$\text{Relative error}\,(\%) = \frac{\hat{y} - y}{y} \times 100$$

The relative errors of the models were calculated as the average of the absolute values of the relative errors for each prediction. To test for significance of differences between the actual and the predicted values, the average of the predictions (experimental average) was compared with the actual value for each concentration level. Significance testing was performed by *t*-test calculation at 95% confidence level. The limit of detection, which describes the lowest concentration level (or amount) of the target analyte that can be determined to be statistically different from an analytical blank, was calculated according to the Clayton and Long–Winefordner criteria (Clayton, Hines, & Elkins, 1987; Long & Winefordner, 1983).

Simple correlation analysis (Pearson correlation) of cell culture data (TEER, fluorescein dextran and β-glucan concentration, β-glucan molecular weight) was performed using Minitab (version 16; Minitab Inc., State College, PA, USA). Significant differences were declared at $p < 0.05$. Principal component analysis (PCA) of spectra obtained from the basolateral cell culture media was conducted on spectra subjected to second derivative treatment after subtraction of the calcofluor spectrum with the Unscrambler software package (CAMO PROCESS AS, Oslo, Norway).

3. Results and discussion

3.1. The use of second derivative emission spectra of the calcofluor/β-glucan complex improves calibration

A pre-experiment, testing the stability of the calcofluor/β-glucan complex, was carried out by recording fluorescence emission spectra 0, 1, 2 and 5 min after preparation of the calcofluor/β-glucan reagent mixture as described in Section 2.2. During this storage time, no signs of intensity loss could be observed.

The fluorescence emission spectra of calcofluor solutions as well as calcofluor/β-glucan complexes prepared with the M_w = 359 kDa β-glucan standard are shown in Fig. 1A. With increasing β-glucan concentrations, the intensity of the fluorescence signal of the complex increases and its characteristic double peak, which has been previously reported under alkaline conditions (Wood, 1982; Kim & Inglett, 2006), becomes more pronounced. The emission spectrum of the calcofluor solution alone shows a broad maximum at approximately 435 nm, while the emission spectra of calcofluor/β-glucan complexes present two maxima at 422 and 442 nm. The second derivatives of the emission spectra of the complex were explored in an attempt to emphasize the spectral information related to the complex between calcofluor and β-glucan and diminish the contribution of calcofluor. It should be noted that spectra may be treated with any programme able to calculate the second derivative and that most fluorescence spectrophotometers have pre-installed data analyses software capable of doing this.

Fig. 1B shows the second derivatives of the spectra from Fig. 1A with the spectrum of the pure calcofluor solution subtracted from the spectra of the calcofluor/β-glucan complexes. Depending on the β-glucan concentration, the minima at 419 and 448 nm and the maximum at 433 nm all show larger absolute values. For β-glucan quantification we have defined the analytical signal (AS) based on the second derivative emission spectra of the calcofluor-β-glucan complex (pure calcofluor spectra subtracted) by summing up the absolute signal values at 419, 433 and 448 nm.

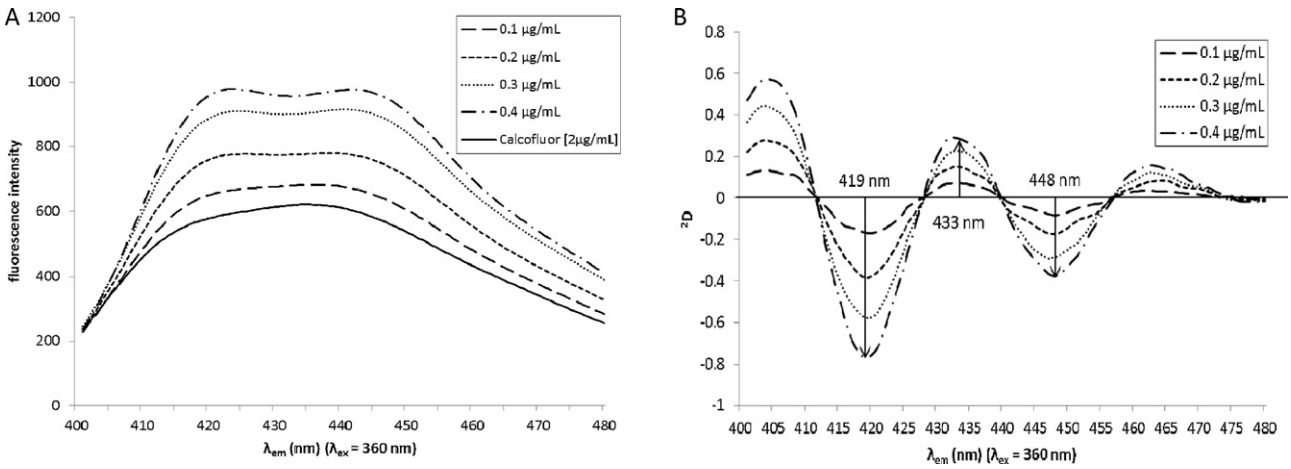

Fig. 1. Fluorescence emission of calcofluor and calcofluor/β-glucan complexes prepared with different concentrations (0.1–0.4 μg/mL) of the M_w = 359 kDa cereal β-glucan standard. (A) Emission spectra and (B) second derivatives of the emission spectra from A (calcofluor subtracted).

Existing quantification methods for β-glucan by calcofluor fluorescence either use the peak height at the first maximum of the original spectra (in our experiment at 422 nm) or the integrated fluorescence intensities over the whole emission range. Fig. 2A and B shows the differences in peak heights at 422 nm of calcofluor and calcofluor/β-glucan complex spectra plotted against the concentrations of the M_w = 359 kDa β-glucan standard. Fig. 2A shows data obtained at one measurement day, while Fig. 2B shows all the data obtained from 11 different measurement days. The respective AS-values of the calcofluor/β-glucan complex spectra are plotted against the concentrations of the M_w = 359 kDa standard in Fig. 2C (one day) and D (11 different days). Linear regression of the data obtained at the same day yielded a perfect straight line independent of the use of peak height, integrated fluorescence intensity (data not shown) or AS. However, as Fig. 2B and D illustrates there was an obvious day to day variation. The measurements were carried out over a long time period and several β-glucan stock solutions had to be prepared within this time. Later experiments by our group have shown that the preparation of such small amounts (10 mL) of relatively low concentrated (1 mg/mL) β-glucan stock solutions with exact concentrations is difficult. The used stock solutions may actually have varied slightly in β-glucan concentration explaining a part of the day to day variation. Furthermore a part of the observed day to day variation may be due to aggregate formation or changes in β-glucan conformation of the β-glucan stock solutions resulting in less soluble β-glucan available for interaction with calcofluor or small changes in specific interaction with calcofluor. This argument is strengthened by the fact that we observed

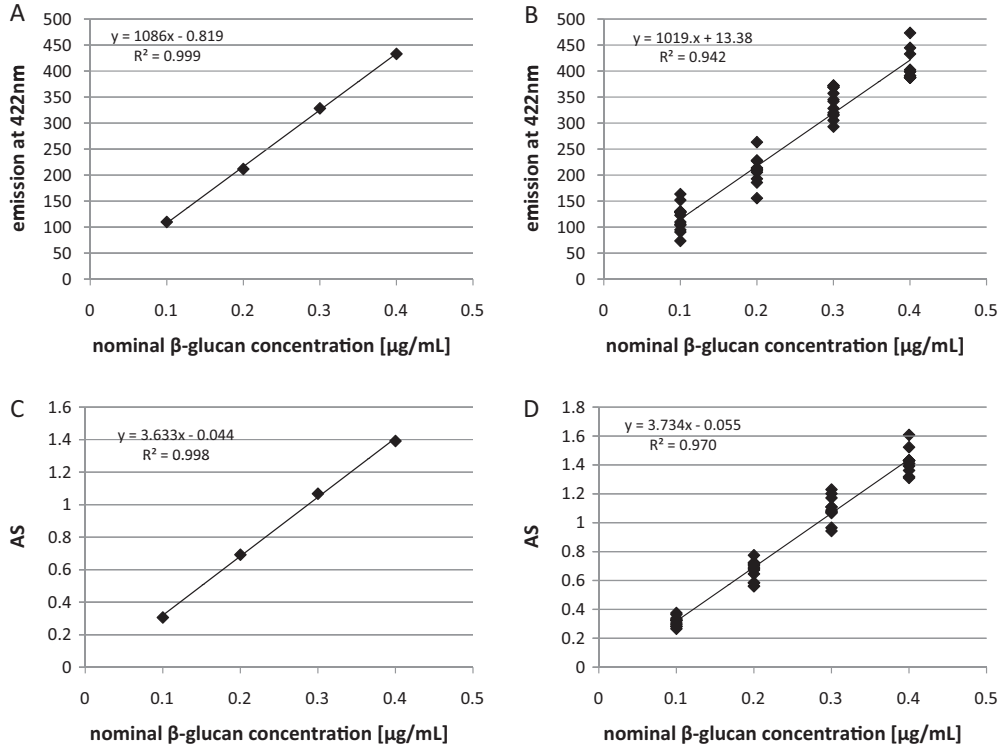

Fig. 2. Linear regression of the difference in fluorescence intensity at the first maximum of the original spectra (422 nm) of calcofluor and calcofluor/β-glucan complexes against the concentration of the M_w = 359 kDa β-glucan standard for one (A) and 11 measurement days (B) and linear regression of the analytical signals (AS) obtained from the second derivatives of calcofluor/β-glucan complex spectra against the concentration of the M_w = 359 kDa β-glucan standard for one (C) and 11 measurement days (D).

Table 1
Enzymatically determined β-glucan content of the different β-glucan preparations.

β-Glucan preparation	β-Glucan content based on dry weight [%]
40 kDa	83.9 ± 4.2
123 kDa	93.7 ± 1.4
183 kDa	93.5 ± 1.2
245 kDa	81.5 ± 3.0
359 kDa	91.6 ± 1.7
BG_Olve	76.8 ± 2.5

visible aggregate formation in a commercial β-glucan standard for FIA with calcofluor from Megazyme one or two weeks after reconstitution even though the standard was reconstituted according to manufacturer's instructions and it was claimed by the manufacturer that the solution would be stable for up to 3 months. To avoid the necessity of measuring a standard curve each day linear regression of the data obtained at different days was conducted (Fig. 2B and D). Fig. 2B shows the standard curve of the M_w = 359 kDa standard based on the differences in peak height of the calcofluor and calcofluor/β-glucan complex spectra. The standard curve has an R^2 of 0.94 and a root mean square error of calibration (RMSEC) of 0.027. However, a clear separation of the different β-glucan concentrations was not always possible. The R^2 of a linear regression based on the AS-values (Fig. 2D) was with 0.97 slightly improved. Fig. 2D shows a clear separation of the different β-glucan concentrations, which is also reflected in a lower RMSEC of 0.017 compared to the one of the model in Fig. 2B. Interestingly a calibration using a linear regression based on the integrated fluorescence intensities of the same calcofluor/β-glucan complex spectra (with pure calcofluor subtracted) yielded an R^2 of 0.93 and a RMSEC of 0.029 (regression not shown) and offered no improvement compared to the calibration based on the peak height differences. Therefore the AS has been employed in the present work for all further standard curves and β-glucan quantifications.

3.2. Molecular weight dependence of the fluorescence intensity of the calcofluor/β-glucan complex

It has been suggested that β-glucan molecules with MW above a critical value are fully detected by the calcofluor FIA method. Hence the fluorescence intensity of their complex with calcofluor is thought independent of MW (Gomez et al., 2000; Kim & Inglett, 2006). An investigation of cereal β-glucan standards with different M_w with our new method revealed an apparent deviation from these previous reports. As shown in Fig. 3A not only the β-glucan standards with lower M_w (40 kDa, 123 kDa) but also two of the β-glucan standards with higher M_w (245 kDa and BG_Olve, M_w approximately 600 kDa) showed a lower standard curve slope than the bulk of the β-glucan standards with higher M_w. The standard curve slopes in Fig. 3A were plotted under the assumption that the β-glucan standards are 100% pure β-glucan. However, determination of the β-glucan content of the different standards by an enzymatic method (as described in Section 2.1) revealed heterogeneity in β-glucan content amongst them (Table 1). The β-glucan concentrations in the standard curves were then subsequently corrected for the actual β-glucan content of the different preparations. As shown in Fig. 3B the response curves of the β-glucan preparations with higher M_w (M_w = 183 kDa and higher) became very similar after this correction. The slopes comparison test also showed no significant difference (at a confidence level of 95%) between the standard curve slopes of the M_w = 359 kDa standard and the ones of the BG_Olve and M_w = 245 kDa standard after correction for β-glucan content of the different β-glucan preparations. Correction of the β-glucan content had, however, no profound effect on the low slopes of the M_w = 123 kDa and M_w = 40 kDa standard curves.

The binding of calcofluor on β-glucan molecules has been reported to be enhanced with higher salt concentrations (Wu, Deng, Tian, Wang, & Xie, 2008). The critical MW value, below which the fluorescence intensity of the calcofluor/β-glucan complex becomes weaker, has also been reported to depend on the ionic strength of the substrate solution (Gomez et al., 2000; Kim & Inglett, 2006). The critical MW value or range is not known but has been estimated to be centred around degree of polymerization (DP) = 70 (corresponding to 11 kg/mol) in high ionic strength buffer (0.17 M NaCl) (Gomez et al., 2000). Kim and Inglett (2006) have also used cereal β-glucan M_w standards from Megazyme to investigate molecular weight dependence of fluorescence intensity of the calcofluor/β-glucan complex. They showed superposition of titration curves of β-glucan standards with M_w = 123, 183 and 359 kDa, but not 40 kDa in 100 mM phosphate buffer with pH 10. This is in contrast to the findings of our study in which also a lower fluorescence intensity of the calcofluor/β-glucan complex formed with the M_w = 123 kDa β-glucan standard was evident. Even though all experiments in this study were carried out in high ionic strength solution with pH 11, the applied β-glucan concentration range was much lower than the concentrations used in the above mentioned studies, which may explain this difference. No significant difference could be observed between the M_w = 359 kDa standard curve slope and the ones of the M_w = 245 kDa, M_w = 183 kDa, Olve_BG and FIA standard β-glucan after correction for the actual β-glucan content in the different standard preparations. This suggests that also for the low concentrations used in this study, there is a critical MW value or MW range above which the fluorescence intensity of the calcofluor/β-glucan complex is only dependent on the β-glucan concentration. This makes it possible to use a common standard curve for quantification of the higher M_w β-glucan preparations used in this study. Fig. 4 shows the common standard curve of all the β-glucan preparations with higher M_w. The R^2 of this common standard curve was 0.94, which is lower than that of the M_w = 359 kDa standard alone (see Section 3.1; Fig. 2D). Accordingly, the RMSEC was with 0.0235 slightly higher than that of the M_w = 359 kDa standard curve. This is not surprising since the common standard curve utilizes the corrected β-glucan concentrations, which are based on the enzymatic measurement of β-glucan in the samples. Any imprecision of the enzymatic quantification measurements will be added to the imprecision of the calcofluor method. The limit of detection of the common standard curve was 0.045 μg/mL according to the Clayton criterion and 0.027 μg/mL according to the Long–Winefordner criterion (Clayton et al., 1987; Long & Winefordner, 1983). Calculation of the limit of detection according to Clayton takes not only type I errors (reporting an analyte as present, when it is not) but also type II errors (reporting an analyte as not present, when it is) into account, which explains the obtained higher detection limit with the Clayton criterion compared to Long–Winefordner. The calculated detection limits are approximately 100 times lower than the lowest standard concentrations reported in previous methods (Kim et al., 2008; Schmitt & Wise, 2009). The common standard curve from Fig. 4 underestimates the β-glucan concentration of the M_w = 123 kDa standard by approximately 35%. The concentration of the M_w = 123 kDa standard in the cell culture supernatants (see Section 3.5) was therefore determined by using a separate standard curve solely based on this β-glucan preparation.

3.3. Validation of the obtained calibrations

For model validation a random test set of 25% of all measurements (a total of 28 measurements for the common standard curve and 11 for the M_w = 359 kDa standard curve) was excluded from model calibration. The linear regression models fitted with the remaining data were subsequently used to predict the test set data. The relative errors of the predictions were calculated as stated in

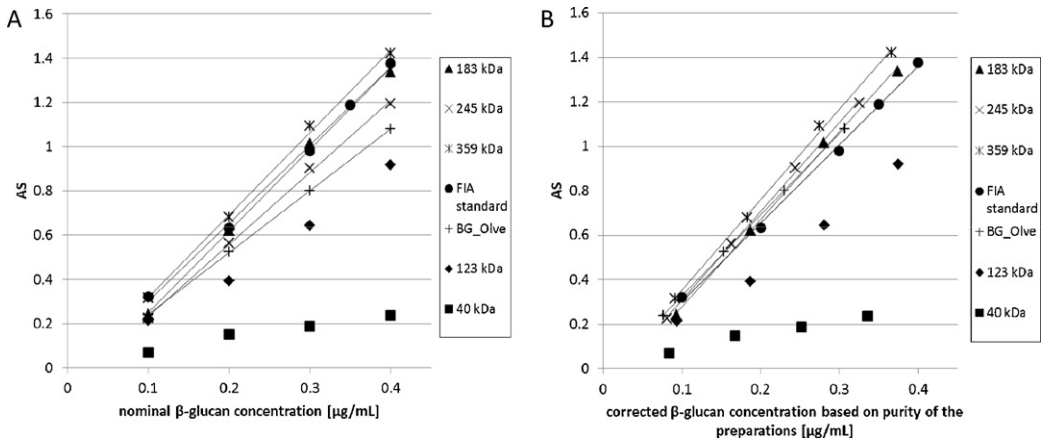

Fig. 3. Linear regression of the AS values calculated from the second derivatives of spectra of calcofluor/β-glucan complexes prepared with β-glucan preparations of different M_w against β-glucan concentration; averages of the AS values obtained at different measurement days are plotted instead of all separate measurements for a clear presentation. (A) Nominal β-glucan concentrations, 100% purity of the β-glucan preparations was assumed. (B) The nominal β-glucan concentrations have been corrected for the actual β-glucan content in the different preparations.

Section 2.5. The average relative error of the common standard curve was 9.9% while the M_w = 359 kDa standard curve exhibited a relative error of 6.1%. For both approaches the predicted β-glucan concentrations were not significantly different (at a confidence level of 95%) from the corrected nominal concentrations. As seen in Fig. 2B and D, there is a certain day to day variation of the new assay. More accurate results may be obtained by recording a standard curve for each day, which is subsequently used to quantify all samples measured at the same day. To assess the relative error of this approach standard curves of the M_w = 359 kDa β-glucan standard were determined at 4 different days and a nominal β-glucan concentration of 0.25 µg/mL was used for predictions each day with a relative error of 4.7%. However, if we assume that a part of the day to day variation is due to changes in the β-glucan stock solution this variation may underestimate the real error of the analysis.

3.4. β-Glucan quantification in arabinoxylan samples

The proposed, new quantification method was applied to determine the β-glucan content of two arabinoxylan samples extracted from the Norwegian barley variety Tyra. One of the samples, AX_Tyra_L, has been subjected to a lichenase treatment after extraction and purification to remove remaining β-glucan. The results from the new calcofluor method are compared with results by an enzymatic method in Table 2. The results for AX_Tyra are 15.1 and 16.7% β-glucan of dried material based on the calcofluor and the common enzymatic method, respectively. This is quite similar, while the β-glucan content for the lichenase treated sample, AX_Tyra_L, was considerably lower with the calcofluor method (0.24%), then with the enzymatic method (1.7%). This is probably due to the fact that the calcofluor method only determines β-glucan molecules of a certain size, above the critical MW value, while the enzymatic method has no such restriction. Obviously lichenase treatment did not result in complete degradation of β-glucan during the isolation of arabinoxylan and the remaining fragments were not completely removed during purification of the arabinoxylan. Some of the fragments seem to be large enough for calcofluor detection, while others are only detected with the enzymatic method. Nevertheless, the low amount of sample required for the proposed, new calcofluor method makes it a highly attractive approach to determine the content of co-extracted β-glucan or relatively large β-glucan fragments in special samples like arabinoxylan preparations extracted from β-glucan rich cereals like barely that are intended for special biological tests.

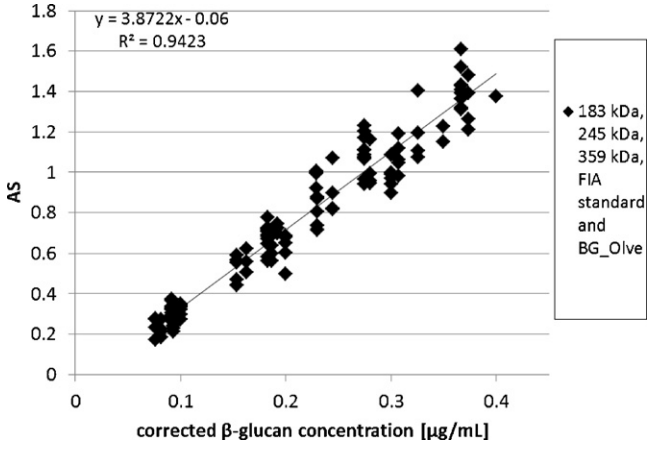

Fig. 4. Linear regression of the AS values calculated from the second derivatives of spectra of complexes of calcofluor with β-glucan preparations M_w = 183 kDa, M_w = 245 kDa, M_w = 359 kDa, FIA standard β-glucan and BG_Olve against corrected β-glucan concentrations.

3.5. Transport of cereal β-glucans across differentiated Caco-2 cell monolayers

The new calcofluor method was further used to determine the transport of cereal β-glucan preparations of different molecular weight over differentiated Caco-2 cell monolayers. Differentiated Caco-2 cell monolayers are often used as *in vitro* model for drug transport and have been shown to yield similar drug permeability coefficients for passive transport as those for human small intestine *in vivo* (Artursson & Karlsson, 1991; Artursson, Palm, & Luthman, 2001; Lennernas, Palm, Fagerholm, & Artursson, 1996). The M_w = 123 kDa standard was used as the β-glucan preparation with lowest M_w for the cell experiment since the low response of

Table 2
β-Glucan contents in two arabinoxylan samples determined with an enzymatic method and the proposed, new calcofluor method.

Sample	β-Glucan content enzyme method [% of dried material]	β-Glucan content calcofluor method [% of dried material]
AX_Tyra	16.70 ± 0.04	15.12 ± 0.03
AX_Tyra_L	1.7 ± 0.1	0.24 ± 0.006

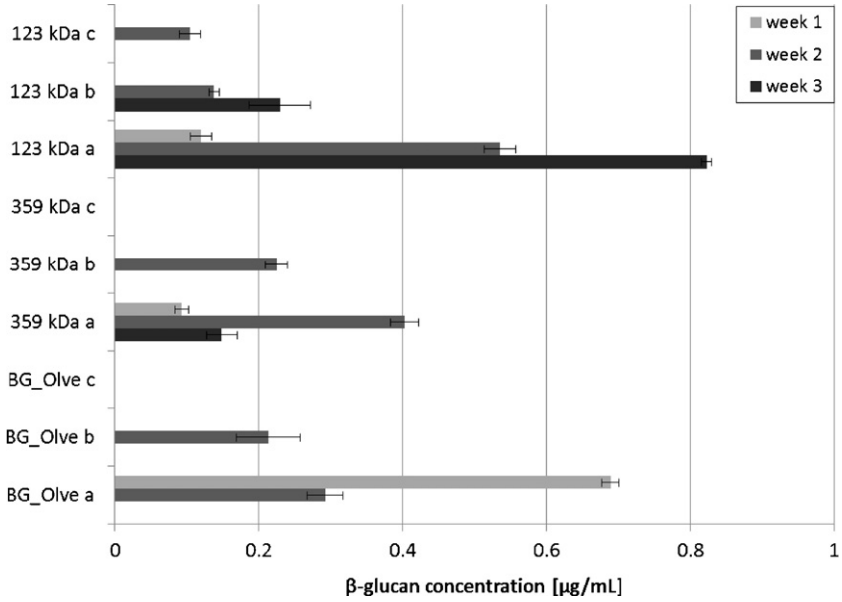

Fig. 5. β-Glucan concentrations in basolateral media of differentiated Caco-2 cell layers after 9 h incubation with cereal β-glucan preparations of different M_w (123 and 359 kDa and BG_Olve with ca. 600 kDa). Each β-glucan preparation was added to 3 monolayers (a–c) for each experiment week. Only concentrations above the detection limit are shown and data represent mean values ± standard deviation of duplicate measurements.

the M_w = 40 kDa standard calcofluor/β-glucan complex resulted in a much higher detection limit for this sample. The M_w = 359 kDa standard and BG_Olve (approximately 600 kDa M_w) were used as cereal β-glucan preparations of medium and high M_w, respectively. Each β-glucan was added to 3 monolayers in 3 independent experiments. During the 9 h incubation time no decrease in TEER over the cell layers was detected. Only 4 of the total 33 differentiated monolayers showed a TEER value below 300 Ωcm^2 after sample addition, probably due to disruption of the cell layer during washing or sample addition. These 4 monolayers were excluded from all further analysis. Cereal β-glucan concentrations were determined in the basolateral media in duplicate. For all control samples (apical addition of medium without β-glucan) and 11 of the cell layers exposed to β-glucan, the basolateral β-glucan concentration was below the limit of detection of the method (0.045 μg/mL). The results of the 13 other samples are presented in Fig. 5. β-glucan could be detected on the basolateral side of the Caco-2 cell layers after incubation with all three different β-glucan preparations. No correlation between the M_w of the β-glucan preparation and the basolateral β-glucan concentration could be found. The concentrations varied considerably among the different monolayers independent of the week in which the cell culture experiment had been performed. However, the number of monolayers with a basolateral β-glucan concentration above the limit of detection was highest for the M_w = 123 kDa standard, followed by M_w = 359 kDa and lowest for BG_Olve, which also had the highest M_w. Correlation analysis of the cell culture samples revealed no significant correlation between basolateral β-glucan concentration (if determinable) and TEER both at the beginning and the end of the experiment. There was a weak but non-significant correlation between basolateral β-glucan and fluorescein dextran concentration (0.560; p = 0.059). Basolateral fluorescein dextran concentration was significantly correlated with TEER at beginning and end of the experiment both for the monolayers with β-glucan concentrations above the limit of detection and for all other samples including controls.

The proposed, new calcofluor method enables the quantification of cereal β-glucans with pre-determined M_w and molecular distribution characteristics in cell culture samples above a concentration of 0.09 μg/mL (cell culture samples were diluted 1:1 during the assay due to buffer addition as described in section 2.2) with little variation between parallels (see standard deviations in Fig. 4). The reason for the varying basolateral β-glucan concentrations among the different monolayers is not known, but since there was no significant correlation between β-glucan concentration and TEER, and TEER values remained stable (above 300 Ωcm^2) throughout the experiment, the variation is unlikely to arise from punctures in the cell layer. Fluorescent labelled dextran is a common marker of paracellular transport across cell layers such as differentiated Caco-2 cells (Neuhaus et al., 2006). The low and non-significant correlations between β-glucan and fluorescein dextran concentration and TEER and β-glucan concentrations indicate that cereal β-glucans are probably not transported via the paracellular route across intact Caco-2 cell layers. Considering their size, this is not surprising. Instead, cereal β-glucans may be transported via transcytosis. Further experiments are needed to clarify the issue of cereal β-glucan transport across Caco-2 cell monolayers as well as the potential transport mechanism involved. The high amount of monolayers with basolateral β-glucan concentrations below the detection limit makes it difficult to draw conclusions regarding the transport of cereal β-glucans over differentiated Caco-2 cell monolayers from the present data. Fig. 6A shows the score plot of PCA of the second derivatives of spectra (401–480 nm) of all cell culture samples. The first pc which explains 97% of the total spectral variation separates the samples with β-glucan concentrations above the limit of detection according to the β-glucan concentration. The wavelength, which are most important for this separation can be deducted from the loadings plot of the first pc in Fig. 6B. The highest positive loadings can be observed at wavelength 419 and 448, while wavelength 404 and 433 contribute with the highest negative loadings. Except for the absolute values being opposite, which is due to the PCA scores being negative for the samples with highest β-glucan concentration, the loadings for the first pc correspond very well with the changes induced in the second derivatives of the spectra with higher β-glucan concentrations (see Fig. 1B), which form the base of the AS.

Even though the score plot does not show a completely clear separation between the control samples and the β-glucan samples with β-glucan concentrations below the limit of detection of the new calcofluor method, the β-glucan samples are located more to the left in the score plot. This indicates that those samples may

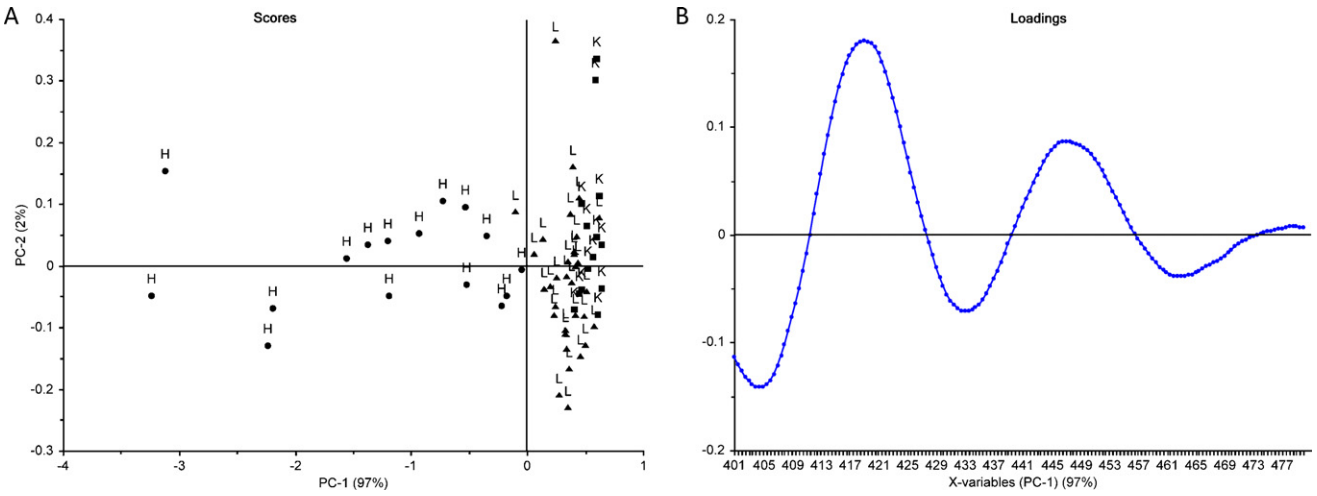

Fig. 6. (A) Score plot of the two first principal components of PCA on spectra from the cell culture experiment (second derivatives, pure calcofluor spectra subtracted). H: Samples with β-glucan concentration above the detection limit (0.045 μg/mL); K: control samples without apical β-glucan addition; L: apical β-glucan addition and basolateral β-glucan concentrations below the detection limit. (B) Loadings for the first principal component.

contain low concentrations of β-glucan. But the concentrations are too low to be accurately quantified with the current method. Longer incubation times in combination with lower amounts of basolateral medium may increase the basolateral β-glucan concentrations above the limit of detection of the new calcofluor method for a higher percentage of monolayers. This will potentially alleviate a final conclusion on the transport of cereal β-glucans over differentiated Caco-2 cell monolayers.

4. Conclusions

The new calcofluor batch method is a fast and easy method to quantify cereal β-glucan in very low concentrations that requires minimal sample amounts. No sample titration or the recording of a daily standard curve is required and the limit of detection is 0.045 μg/mL. Cereal β-glucans above a critical MW can be quantified with an average relative error of 10%. A common standard curve can be constructed for cereal β-glucans above a critical MW after the standards have been checked for their actual β-glucan contents. The method was successfully applied to determine cereal β-glucan content in special samples such as arabinoxylan preparations and cell culture samples.

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