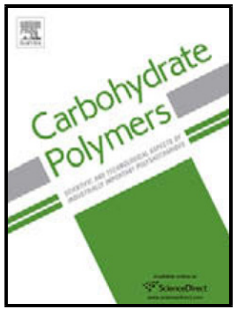

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Quantification of 1,3-β-$_D$-glucan from yeast added as a functional ingredient to bread

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Highlights

- Yeast β-glucan added to bread can be quantified by the modified aniline blue assay
- The presence of cereal 1,3-1,4-β-$_D$-glucans did not interfere with assay performance
- Detection levels are below the expected addition levels in bread products
- The effect of food processing on 1,3-β-G may be studied with the presented assay

Abstract

Due to their immunomodulatory effect, 1,3-β-G from yeast are used as functional ingredients, but reliable methods for their detection in foods are lacking. We have adapted a method based on fluorescence detection with aniline blue to quantify the amount of five commercial yeast β-glucan preparations added to crisp or yeast-leavened bread. This assay detected yeast β-glucan preparations added to different breads with an average recovery of 90, 96, 99 and 105%, while one of the preparations was overestimated, with an average recovery of 157%. The presence of cereal 1,3-1,4-β-$_D$-glucans did not interfere with assay performance. The addition

of 1,3-β-G at 0.2 and 0.5 g/100 g is low compared to the recommended dose of 1,3-β-G per serving demonstrating assay sensitivity. However, more research is needed to fully understand 1,3-β-G conformation/structure on aniline blue interaction as well as the effect of baking on structure and dissolution properties of yeast β-glucans.

Abbreviations

1,3-β-G, generic term used to describe all polysaccharides predominantly consisting of 1,3-linked β-$_D$-glucose in the main chain, including branched 1,3-1,6-β-glucans from yeast and mushroom cell walls.

Keywords

Yeast β-glucan, 1,3-β-glucan, quantification, aniline blue , FT-IR

1. INTRODUCTION

Glucans with a backbone of exclusive repeating units of (1→3)-β-$_D$-glucose (1,3-β-G) are ubiquitous in the environment. They have either a structural, storage or extracellular function in certain higher plants, yeasts, algae, mushrooms, fungi, brown seaweeds and bacteria. The 1,3-β-G derived from yeast and mushroom cell walls are branched polysaccharides containing side chains of varying length of β-1,6 or β-1,3 linked glucose attached to the backbone at the 6 position (Lowman et al., 2011; Manners, Masson & Patterson, 1973; Ohno, 2007). The 1,3-β-G from yeast and mushrooms are associated with a number of health benefits including certain immunomodulatory effects (Soltanian, Stuyven, Cox, Sorgeloos & Bossier, 2009; Thompson, Oyston & Williamson, 2010). This group of branched polysaccharides is sometimes referred to as 1,3-1,6-β-$_D$-glucans, but will hereafter simply be referred to as 1,3-β-

G. In particular 1,3-β-G derived from yeast are increasingly added as immunomodulating, functional ingredients to a wide range of foods. Yet at present there is a lack of available analytical methods to specifically measure how much added 1,3-β-G a particular 'ready-to-eat' food contains. From a commercial, regulatory and consumer acceptance perspective such an analytical method is highly desirable.

There is a wide range of different analytical methods for the quantification of 1,3-β-G, including the branched 1,3-β-G from fungi. However, most of these methods have been developed for the quantification of 1,3-β-G in yeast or mushroom preparations, but have not been applied, and may in fact not be applicable, to food products. There are two major problems for quantification of 1,3--β-G in food products. Firstly, interference of other substances especially other glucose polymers for example α-glucans such as starch, and mixed linkage 1,3-1,4-β-glucans from cereals that are naturally present in high amounts in many food products. Secondly, the necessity to quantify very small amounts of 1,3-β-G. This is because addition levels of 1,3-β-G in commercial products will be minimum doses that are economically viable for food production and at the same time are sufficient to confer the food product a functional health benefit. The suggested functional doses of 1,3-β-G are generally as low as 250 – 900 mg per day (Samuelsen, Schrezenmeir & Knutsen, 2014).

Acid hydrolysis (Freimund, Janett, Arrigoni & Amado, 2005) followed by determination of released glucose are not applicable as an analytical tool for 1,3-β-G in food products, mainly due to interference by other glucose polymers. A more specific approach is the use of different enzymes to degrade 1,3-β-G to glucose. Since 1,3-β-G are difficult to solubilize, the enzymatic approach contains an initial alkali swelling, followed by a two-step enzymatic digestion. In the first step, lyticase is used to further solubilize 1,3-β-G`s. This is followed by treatment with exo-1,3,-β-D-glucanase and β-glucosidase to completely and selectively convert and solubilize glucose from both β-1-3 and β-1-6 linked elements to glucose

(Danielson, Dauth, Elmasry, Langeslay, Magne & Will, 2010). This method works well for quantification of 1,3-β-G`s in commercial β-glucan preparations. Due to the high cost of lyticase its widespread adoption is limited, and an alternative enzyme mixture has been developed as part of a commercial yeast β-glucan determination kit (Megazyme). Both these enzymatic digestion methods are not selective for β-glucans from yeast and mushrooms since mixed linkage 1,3-1,4 β-glucans from cereals are also converted to glucose and included in the quantification. However, this interference is not the only hindrance for the application of these enzymatic approaches to food products. To determine very low amounts of 1,3-β-G in a complex matrix these assays lack both sensitivity and precision (own, unpublished results).

Interference of other substances and sensitivity problems are also a hindrance for the application of another approach using ^{1}HNMR and an internal standard to quantify 1,3-β-G directly solubilized in DMSO (Lowman & Williams, 2001) to food products.

Other assays for 1,3-β-G's exploit its ability to activate the coagulation cascade within amoebocytes derived from the hemolymph of horseshoe crabs (*Limulus Polyphemus*). 1,3-β-G's induce clot formation via specific interaction with the serine protease zymogen, factor G. Such assays are commercially available as clinical diagnostic tools of invasive fungal infections in humans (Hope, Walsh & Denning, 2005; Obayashi et al., 1995). Endotoxin from the cell walls of gram negative bacteria also activates the clotting cascade but via another serine protease zymogen, factor C. To avoid interference of endotoxin, alternative assays where factor C has been selectively removed from the limulus amoebocyte lysate can be used and are commercially available (For example Glucatell Associates of Cape Cod Inc.). However, these type of assays are expensive, time consuming and difficult to apply to food products.

Other workers have used fluorescence spectroscopy, using the dye Congo Red to specifically quantify 1,3-β-G's in their triple helical conformation such as when they are dissolved in water at room temperature (Nitschke, Modick, Busch, von Rekowski, Altenbach & Molleken, 2011). Yet again a lack of specificity, especially in a plant food matrix, is a problem because Congo Red also interacts in the same manner with cellulose and cereal mixed linked 1,3-1,4-β-glucan (Wood, 1980). This is particularly a problem for widely eaten cereal based foods such as bread.

Another approach that does not suffer to the same extent from the above mentioned drawbacks is the selective interaction of of 1,3-β-G's with the fluorochrome sodium 4,4`-[carbonylbis-(benzene-4,1-diyl)bis(imno)]bisbenzene-sulphonate (Evans, Hoyne & Stone, 1984; Koenig, Rühmann, Sieber & Schmid, 2017; Wood & Fulcher, 1984; Young & Jacobs, 1998). This fluorochrome is present in commercial preparations of aniline blue (Evans, Hoyne & Stone, 1984). It has been suggested that interaction with aniline blue requires 1,3-β-G in the single stranded form (Young & Jacobs, 1998) . The single stranded form of 1,3-β-G`s prevail under conditions of alkali denaturation (>0.25 M NaOH) (Sletmoen & Stokke, 2008) and an alkali denaturation treatment at elevated temperature (80 ºC) is usually used in applications of aniline blue for quantification of 1,3-β-G`s (Ko & Lin, 2004; Shedletzky, Unger & Delmer, 1997). However, aniline blue has also been successfully used for 1,3-β-G quantification without the use of alkali treatment (Koenig, Rühmann, Sieber & Schmid, 2017). The application of the aniline blue assay has been proposed as a promising specific assay to quantify the amount of 1,3-β-G's extracted from six categories of raw foods (Ko & Lin, 2004). However, this particular study suffered from a lack of validation to verify that the substance quantified in the food was actually only 1,3-β-G. No 'spike-recovery' tests were carried out and the effects of food processing where not assessed. Thus, confidence in that current assay format is undermined.

Faced with the challenge to determine how much 1,3-β-G are added as ingredients in bakery products we have further adapted an assay based on the established aniline blue approach. To have a reliable assay, a number of issues were addressed including preclusion of background interference from other types of β-glucans and 1,3--β-G's naturally present in common leavening agents such as yeast.

2. Material and Methods

2.1 Materials and Reagents

Commercial yeast β-glucan preparations were obtained from different producers. These comprised dispersible Wellmune (Wellmune WGP, F3005 Lot 11119-011, Biothera, Eagan, MN, USA (this product is now sold by Kerry Inc., Naas, Ireland)), a water soluble yeast β-glucan (soluble Wellmune, Lot: 122111-016, Biothera), NBG particulate (NBG batch15-BP-015, Biotec Betaglucans, Tromsø, Norway), Yestimun (Yestimune Yeast Bgl 42200P-030 batch 6612, Leiber GmbH, Bramsche, Germany) and Immiglucan (Immitec, Tønsberg, Norway). A shiitake polysaccharide was extracted as previously described (Govers, Tomassen, Rieder, Ballance, Knutsen & Mes, 2016) and obtained from Food & Biobased Research, Wageningen (Wageningen University and Research Centre, Wageningen, Netherlands). Common reagent grade chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, Darmstadt, Germany) and water soluble aniline blue (1B 501) was obtained from Chroma-Gesellschaft Schmid GmbH & Co (Chroma-Gesellschaft Schmid GmbH & Co, Muenster, Germany). Commercial wheat flour without added ascorbic acid was purchased from Lantmannen Cerealia (hvetemel industri, Lantmannen Cerealia, Oslo, Norway), oatbran concentrate containing 14% mixed linkage 1,3-1,4-β-glucans was obtained from Swedish oat fiber (Oatbran 14 bakery, Swedish oat fiber, Bua, Sweden) and dry yeast was obtained from

Idun (Idun, Oslo, Norway). Aniline blue dye mix solution was prepared by mixing 492 mL 1M glycine/NaOH buffer, pH 9.5 with 333 mL 0.1% aqueous aniline blue and 174 mL 1N HCl.

2.2 Characterization of yeast β-glucan preparations

FT-IR spectroscopy of the samples was used to evaluate differences in major constituents (carbohydrates, fat, protein) and overall composition of the samples. Samples were analysed in powder form using a Nicolet™ iS™ 5 spectrometer (ThermoFisher Scientific, Waltham, MA USA) equipped with a iD7 Single-Bounce ATR. Spectra were acquired with a resolution of 4 cm^{-1} based on 16 scans. Before each sample measurement, a background spectrum was obtained and all samples were measured in triplicates. All spectra were EMSC-processed (Afseth & Kohler, 2012) using The Unscrambler® X v10.4 (CAMO Software AS, Oslo, Norway).

For ^{1}HNMR analysis, samples (8 mg), previously subjected to vacuum drying over P_2O_5 were initially dispersed in 0.7 mL 0,25M LiCl in Me_2SO-*d*6 in a 2 mL Precellys cell tube containing ceramic beads and subjected to shaking at 6000 rpm for 2 times at 20 sec in a Precellys Evolution shaking device (Bertin technologies: Bordeaux, France) . Samples were then incubated in a boiling water bath for 1hr and centrifuged in a Micro Star 17 (WWR) centrifuge at maximum speed. The solutions were then transferred to a new tube and left for > 24 hrs. Although no precipitation was visible, the samples were centrifuged again at maximum speed and 0.6 mL was transferred to the NMR tube and added 0.1 mL of D_2O.

All NMR experiments were performed at 70°C using a 400 MHz Bruker AVANCE spectrometer equipped with a 5 mm probe (Bruker Corporation, Billerica, MA, USA). Proton spectra were acquired using 30°-pulse, a relaxation delay of 1.0 sec and 64 scans. FID's were

exponentially multiplied with a line broadening factor of 0.3 Hz before applying Fourier transformation. Chemical shifts were referenced to the DMSO signal at 2.51 ppm. Integrations were done manually after baseline correction with Whittaker Smoother function with a default filter of 2.43 hertz.

The content of 1,3-β-G and α-glucan was determined specifically. Two different methods were employed to measure the content of 1,3-β-G in different yeast preparations. An acid hydrolysis followed by derivatization of released monosaccharides and their quantification as alditol acetates by GC-FID was performed to quantify glucose expressed as polysaccharide equivalents (Englyst & Cummings, 1984), which was assumed to derive from 1,3-β-G. An enzymatic method (enzymatic yeast β-glucan assay kit, K-EBHLG, Megazyme) was also used to quantify the amount of 1,3-β-G in the different yeast preparations. The α-glucan content in the samples was analysed by an enzymatic approach. The method involved swelling in 2M KOH on ice for 20 min, followed by neutralization and incubation with amyloglucosidase. The generated glucose was measured by using a glucose oxidase/peroxidase system (Megazyme).

2.3 Preparation of test foods

Yeast leavened breads and crisp breads were prepared with 85% wheat flour and 15% oatbran concentrate. All breads contained therefore 2.1% mixed linkage 1,3-1,4-β-glucans from cereals. Different yeast β-glucan preparations were added to the dough to achieve addition levels of 0.5 and 0.2 (1 and 0.5 for soluble Wellmune) mg β-glucan preparation per 100 mg dry matter. On flour basis (14% moisture), 1% dry yeast, 1.5% NaCl, 1% fat (vegetable fat and oil, A/S Pals, Oslo, Norway) and 68% water were added to the dough of the yeast

leavened breads, while the dough for the crisp breads was prepared with 1% NaCl and 58% water.

Doughs were kneaded at 126 rpm for 270 sec in a Newport Scientific Dough lab (Perten Instruments, Stockholm, Sweden) using the 300 g bowl at 22ºC to achieve a dough temperature of 27 +/- 1 ºC. Doughs for yeast leavened breads were fermented at 27 ºC and 70% RH for 1 h, divided into 150 g dough pieces, molded into steel forms and rested at 30ºC for 45min. Doughs for unleavened crisp breads were rolled to a thickness of 0.6mm and pricked before baking. Breads were baked in a rotating hearth oven (Revent type 626 G EL IAC, Revent international, Väsby, Sweden) for 20 min (yeast-leavened breads) or 15 min (crisp breads). Immediately after the yeast-leavened breads were put into the oven the temperature was reduced from 240 to 220 ˚C and steam (from 2 L water) was injected. Baking temperature for the crisp breads was 190 ºC without steam injection.

In order to understand a potential effect of baking on detectability of yeast β-glucan from added yeast, breads were prepared with 2% dry yeast and 6% fresh yeast. These breads contained only wheat flour (no oatbran concentrate) and water addition was 60% based on flour. Dough samples were taken after mixing, fermentation and proofing in addition to the final baked product. In addition to that, breads were prepared with dispersible Wellmune (0.5 mg/100mg dry matter), but without yeast. The processing steps remained the same and samples were withdrawn after mixing, fermentation, proofing and baking to investigate the effect of processing on the re-detection of 1,3-β-G from β-glucan preparations.

2.4 Extraction method

Breads were freeze dried and all samples were milled on a laboratory hammer mill (Retsch, Model ZM100, Retsch GmbH, Haan, Germany) to pass a 0.5 mm screen. Aliquots of 50 mg were accurately weighed into 50 mL centrifuge tubes. A spike sample was included for

soluble Wellmune by adding 50 µL of a 10 mg / mL aqueous soluble Wellmune sample to two test tubes containing 50 mg freeze dried yeast leavened bread prepared with 1mg/100mg soluble Wellmune (total spike amount = 500 µg soluble Wellmune). Samples were incubated in a boiling water bath for 5min after addition of 10 mL 80% aqueous ethanol. The supernatant was removed after centrifugation at 2000 rpm for 10 min (Heraeus Multifuge 4 KR, DJB Labcare Ltd., Newport Pagnell, UK). After addition of 20 mL 5mM $CaCl_2$ and 10 µL thermostable α-amylase (Termamyl 120L, Novozymes A/S, Denmark) samples were incubated in a boiling water bath for 60 min. Tubes were cooled to RT and 30 mL ethanol were added. Samples were left in the freezer overnight and supernatants, containing low molecular weight carbohydrates such as maltodextrins, were removed after centrifugation at 2000 rpm for 10 min (Heraeus). The pellet, containing the β-glucan, was dried completely under vacuum before the addition of 1 mL DMSO containing 0.25M LiCl. Samples were incubated in a boiling water bath for 60 min, cooled, centrifuged (4000 rpm, 10 min, Hereaus) and aliquots of up to 100 µL were used in the aniline blue assay. All extractions were performed in duplicates. The extraction method and modified aniline blue assay are summarized in figure 1.

2.5 Aniline blue assay

The applied aniline blue assay was based on a method by Ko and Lin (2004) and Shedletzky et al. (1997) (Ko & Lin, 2004; Shedletzky, Unger & Delmer, 1997). In an initial protocol an aliquot of standard or sample in a total of 1280 µL aqueous solution was mixed with 120 µL 6M NaOH and incubated at 80 ºC for 30 min to denature the 1,3-β-G triple helices (Young & Jacobs, 1998). Tubes were cooled on ice and 2600 µL aniline blue dye mix solution was added before incubation at 50 ºC for 30 min to form the 1,3-β-G- aniline blue complex. Tubes were left over night at RT to further decolorize the unbound aniline blue dye. During the course of this work, the protocol was modified. The alkaline treatment at 80 ºC resulted in a

brown colour of the bread extracts, presumably due to caramelization of glucose and other carbohydrates generated during the extraction of the bread samples that included treatment with thermostable α-amylase. This brown colour then interfered with fluorescence measurements. Instead, standards and pellets after bread extraction were dissolved/extracted with DMSO containing 0.25M LiCl as described below. This process opens up the triple helical structures of 1,3-β-G and makes the alkali treatment step unnecessary (Yanaki, Tabata & Kojima, 1985). Aliquots of standards or samples in DMSO of up to 100 µL were mixed with DMSO containing LiCl to a final volume of 100 µL DMSO. Subsequently 1180 µL water, 2600 µL dye mix and 120µL 6M NaOH were added to the mixture and tubes were incubated at 50 ºC for 30 min. The heating at 80 ºC in alkaline solution could thus be omitted and browning reactions avoided. After resting overnight, fluorescence emission spectra (410 to 600nm) of the solutions were obtained with a spectrofluorometer (FluoroMax4, Horiba, Kyoto, Japan) set at an excitation wavelength of 398 nm. Samples were placed in a 4 mL quartz cell and spectra were recorded at an angle of 90º and a set temperature of 20 ºC. Slits at excitation and emission monochromaters were set at 2 and 1 nm, respectively. Corrected fluorescence signals (S1c/R1c) at 500 nm were used to construct calibration curves and calculate 1,3-β-D glucan concentrations. All standards and samples were measured in duplicates.

2.6 Statistics

One way ANOVA of 1,3-β-G recovery data was performed using minitab. The significance level was set at 0.05.

3. Results and Discussion

3.1 Characterization of yeast β-glucan preparations

In general, the FT-IR spectra of the different yeast β-glucan preparations were quite similar and only minor differences were observed (Figure 2). None of the yeast β-glucan preparations showed distinct/obvious bands at 1740 cm^{-1} (C=O stretching mode in ester bonds) or 1650 cm^{-1} (amide I band: mainly based on C=O stretch in amide bonds) in the FT-IR spectra, indicating the absence of considerable amounts of fat and protein, respectively. The carbohydrate ring vibrations around 1200-1000 cm^{-1} (mainly C-O-C, C-O, and C-C stretching modes) were the most dominant feature for all samples. Additionally, all samples showed a band at 889 cm^{-1} characteristic for β-linkage in the anomeric carbon (Kacurakova, Capek, Sasinkova, Wellner & Ebringerova, 2000; Synytsya & Novak, 2014). However, in contrast to the other samples, Immiglucan and Yestimun showed a shoulder at 930 cm^{-1} and 850 cm^{-1}, which indicates the presence of carbohydrate polymers with α-linkages in addition to the majority of β-linked polymers. Furthermore, the spectrum derived from NBG differed from the others in the region between 1600 and 1300 cm^{-1}, with two small but distinct peaks at 1577 cm^{-1} and 1371 cm^{-1}, which probably originate from sodium citrate in the sample as citric acid is used during manufacturing of NBG (personal communication with Biotec Betaglucans).

Table 1 shows the 1,3-β-G content of the different yeast β-glucan preparations used in this study. For dispersible Wellmune and NBG similar amounts of 1,3-β-G were obtained with both analytical methods. The amount of 1,3-β-G in soluble Wellmune was slightly lower with the enzymatic method than with the acid hydrolysis method. For Yestimun and Immiglucan this difference was more pronounced. This is likely related to the significant amounts of α-glucan found in Yestimun and Immiglucan samples (Table 1). Alpha-glucans are not hydrolyzed in the 1,3-β-G enzymatic assay kit, but generate glucose after acid hydrolysis thus explaining the differences in 1,3-β-G contents measured by the two different methods. It is

possible that the α-glucans present are glycogen, as glycogen can be associated with β-glucans in the yeast cell wall (Arvindekar & Patil, 2002).

^{1}H NMR analysis confirmed the presence of α-1-4 glucose with resonances in the 5.00 -5.35 region. Based on the integral ratio of anomeric resonances, these non-β-glucan constituents were present in both the Yestimune and the Immiglucan samples on a level >10%. Due to co-occurring anomeric signals from other unidentified carbohydrates in this region, no further quantification of α-glucan content in the samples with NMR was aimed at the present. For the other three β-glucan preparations, such defined resonances of α-anomeric protons were for all practical purposes absent (spectra not shown).

3.2 Assay development

Calibration curves for three different β-glucan preparations (two from yeast and one from Shiitake mushroom) were first prepared with the alkaline aniline blue method. Soluble Wellmune standard was dissolved in water, while dispersible Wellmune and Shiitake polysaccharide were suspended in 1M NaOH by stirring overnight. Linear calibration curves were obtained for all three samples in the range of 0.7 to 12.5 µg/mL (Figure 3 A). Although not a primary task in this work this confirms that the assay not only detects 1,3-β-G from yeast, but also 1,3-β-G from mushrooms, which have a slightly different structure (Ohno, 2007). It should however be noted that the fluorescence of the soluble Wellmune sample was dramatically lower than for the other two samples. This might be due to the low molecular weight of this water soluble β-glucan preparation (see below). Later, calibration curves were prepared by dissolving the β-glucan preparations in DMSO containing 0.25M LiCl. Omitting the alkaline heating step of the aniline blue assay dramatically increased fluorescence of the soluble Wellmune/aniline blue complex (Figure 3 B). Soluble Wellmune is a water soluble

yeast β-glucan preparation with a relatively low molecular weight, roughly two peaks corresponding to 36% (M_w 6670) and 63.5% (M_w 86200) of the total area were identified in the molecular weight distribution of soluble Wellmune (Govers, Tomassen, Rieder, Ballance, Knutsen & Mes, 2016). Alkaline treatment of this sample may have resulted in extensive degradation of the sample and a consequent decrease in fluorescence of the 1,3-β-G/ aniline blue complex. It is not known if a certain size of the 1,3-β-G is required for interaction with aniline blue, but several authors have described a decrease of fluorescence intensity of aniline blue/1,3-β-glucan complex with decreasing degree of polymerization of the 1,3-β-glucan (Adachi, Ohno, Yadomae, Suzuki, Sato & Oikawa, 1988; Englyst & Cummings, 1984). The lower fluorescence signal derived from low molecular weight soluble Wellmune compared to dispersible Wellmune also without alkaline treatment (Figure 3 B) confirms this.

DMSO in the assay mixture reduces the fluorescence of the 1,3-β-G/aniline blue complex in a dose dependent manner (data not shown). Concentrations as low as 1.25% of the final assay mixture slightly reduced the fluorescence of a standard 1,3-β-G (dispersible Wellmune), while DMSO concentrations of 10% resulted in an approximately halved fluorescence signal. To avoid differences in fluorescence based on different DMSO concentrations in the assay, all assay mixtures were standardized to a DMSO content of 2.5% corresponding to a total volume of 100µL DMSO in 4mL assay mixture (see figure 1). This concentration was chosen to ensure a low quenching effect and at the same time high enough volumes for correct pipetting of viscous DMSO extracts.

Calibration curves of the different yeast β-glucan preparations were constructed after dissolution/extraction in DMSO containing 0.25M LiCl. The concentrations of the preparations were corrected with their actual content of 1,3-β-G measured by the enzymatic method (Table1), rather than simply using the weight concentrations of the different commercial powdered preparations. The corrected calibration curves of NBG, dispersible

Wellmune and Yestimun then were superimposed, while Immiglucan and soluble Wellmune calibration curves were respectively located above and below (Figure 4). The higher fluorescence signals obtained with Immiglucan may be due to errors of determining total 1,3-β-G contents with the enzymatic method, differences in dissolution of the 1,3-β-G in DMSO, differences in 1,3--β-G structure or a contribution of other substances than 1,3-β-G to fluorescence. For practical reasons the calibration curve constructed for dispersible Wellmune was used for all further analysis of the bread samples prepared with NBG, Yestimun and dispersible Wellmune. Calculated 1,3-β-G concentrations were converted to contents of NBG, Yestimun and dispersible Wellmune by using the actual contents of 1,3-β-G in the different preparations determined by the enzymatic method (Table 1). For soluble Wellmune and Immiglucan individual calibration curves were constructed based on the weight of the preparations only.

3.3 Detection of 1,3-β-G in test breads and assay performance

The re-detection of the different yeast β-glucan preparations at two different doses and in two different types of bread is summarized in Table 2. The recovery of added material ranged between 63.7% for the higher dose (1 mg /100 mg) soluble Wellmune in crisp bread to 165% for the lower dose (0.2 mg / 100 mg) Yestimun added to yeast leavened bread. In general, no differences in recovery were observed for bread and crisp bread samples ($p=0.98$). The type of yeast β-glucan preparation, on the other hand, significantly influenced the recovery level ($p<0.001$). While dispersible Wellmune and Immiglucan showed recovery levels around 100%, range 89-104% with average 96% and range 95.1-104.1% with average 99.3% respectively, the other samples were either over- or underestimated. Especially breads prepared with Yestimun were overestimated (average 157%), but also breads with NBG (average 115%) were slightly overestimated.

Soluble Wellmune showed the lowest recovery range of 63.7-83.9% (average 75.1%), which may be due to a certain loss of soluble Wellmune during the extraction procedure. During extraction, samples are precipitated with 60% aqueous ethanol. Due to the low M_w of parts of the soluble Wellmune it is likely that a part of the sample is not precipitated. Extractions were therefore also performed with a spike control of soluble Wellmune as described earlier. The average spike recovery of soluble Wellmune was 83.5%, indicating that indeed a small part of the sample is lost during extraction. The recovery values for the different bread samples were then corrected for the average spike recovery of soluble Wellmune (Table 2). This improved the recovery of soluble Wellmune in the bread samples significantly. 96 and 100.5% of added soluble Wellmune was re-detected in the yeast leavened bread. However, for the crisp breads corrected recovery values were still low (76.3 and 86.9%). This may be due to a degradation of soluble Wellmune or the formation of complexes which reduce its ability to interact with aniline blue. In a crisp bread some part of the cereal material on the surface, including the added β-glucan, will be significantly over-baked, which might influence the extractability. The overestimation of Yestimun in all bread samples will be discussed in section 3.4.

There was an indication of different recovery between dose levels ($p= 0.054$), with higher recoveries for the lower dose levels. This may be related to the background fluorescence of breads and crisp breads prepared without the addition of yeast β-glucan preparations (Table 3). For the lower dose of 0.2mg/100mg this background has a higher influence (percent wise) on the total measured fluorescence signal as for the higher dose of 0.5mg/100mg, which may explain the generally slightly higher recovery levels for the lower dose.

The lower the addition level of yeast β-glucan, the lower will the differences in fluorescence between breads without added β-glucan and breads with β-glucan become. It is possible to correct for this background by subtracting the fluorescence of the control bread from the sample. However, for real commercial samples such a control (e.g. identical breads without

added β-glucan) will hardly be available and we have thus decided not to perform this type of correction. Nevertheless, the assay was able to clearly discriminate between breads prepared without, and breads prepared with, yeast β-glucan preparations at addition levels as low as 0.2 mg/100 mg dry matter. The reference amount customarily consumed (RACC) for yeast leavened bread is 50 g per serving. The moisture content of white bread is approximately 40%. One serving of bread therefore equals 30 g dry matter and a dose level of 0.2 mg/100 mg therefore corresponds to 60 mg β-glucan preparation per serving. Recommended daily dosages of yeast β-glucan preparations are between 250 and 900 mg (Samuelsen, Schrezenmeir & Knutsen, 2014). It can therefore be assumed that additions levels to bread are likely higher than 0.2 mg/100 mg in order to achieve doses with beneficial health effects. The presence of mixed linkage cereal 1,3-1,4-β-G did not interfere with the assay even though they were present at much higher (2.1 mg /100 mg; 10 fold higher) levels than the yeast-β-glucan preparations in all breads.

3.4 Effect of processing

The yeast leavened breads had a higher background fluorescence than the crisp breads, which were prepared without yeast (Table 3). The amount of dry yeast added to the breads (1% based on flour weight) corresponds to approximately 0.2 mg /100 mg dry matter assuming an average β-glucan content of 20% in the dry yeast (Cell walls account for approximately 30% of yeast dry weight and approximately 70% of the cell walls are β-glucan (Nguyen, Fleet & Rogers, 1998). The reactivity of dry yeast was therefore tested in the aniline blue assay. However, very weak fluorescence signals were achieved (Table 3). This is presumably due to the low extractability of 1,3-β-G from the native yeast cell wall, which limits interaction with aniline blue. However, it might be possible that 1,3-β-G is released from the yeast cell wall during the different steps of bread production. Figure 5A shows the background fluorescence of breads/doughs prepared with extra high amounts of yeast (2% dry yeast or 6% fresh yeast

based on flour) after different processing steps expressed as 1,3-β-G. There was no systematic increase in the detectability of 1,3-β-G during processing and no differences between the use of dry or fresh yeast. There are two possible explanations for these observations. Either the portion of 1,3-β-G that is released from yeast during baking may be released quite fast already during the mixing step, or the higher background fluorescence of yeast leavened bread may be related to other components than yeast. .The baking process may potentially influence the added yeast β-glucan preparations, for example by complex formation with other compounds in the dough or potential degradation as has been shown for cereal derived mixed linkage 1,3-1,4-β-G (Rieder, Ballance, Lovaas & Knutsen, 2015). The effect of the different baking steps on recovery of yeast β-glucan in dispersible Wellmune was therefore investigated. Figure 5B shows a slight decrease in recovery of 1,3-β-G during fermentation and proofing. However, the differences were relatively small and it is therefore unlikely that baking has a dramatic effect on 1,3-β-G from added dispersible Wellmune. This may nevertheless be different for different yeast β-glucan preparations and our recovery data indicate that the crisp bread process may negatively affect soluble Wellmune detectability with aniline blue.

Baking may also have affected 1,3-β-G from added Yestimun. Yestimun is prepared from spent brewer`s yeast, while all the other yeast β-glucan preparations in this study were prepared from baker`s yeast ((Stier, Ebbeskotte & Gruenwald, 2014), and personal communication)). It is difficult to state the structural differences between 1,3-β-G purified from baker`s and brewer`s yeast in general since these are also influenced by the extraction/purification method and the type of strain (sometimes proprietary) of the yeast. However, the structural differences are usually related to the degree of branching and the ratio of β-1,6 to β-1,3 linkages. Yestimun has been reported to contain an average of 22% β-1,6 linkages, while this ratio has been found to be in the range of 10-18% for dispersible Wellmune (Stier et al., 2014). It is therefore possible that the baking process influenced the

structure of 1,3-β-G in Yestimun or its solubility in DMSO in a different way than with dispersible Wellmune or the other yeast beta-glucan preparations. Potential changes occurring during baking may therefore be one of the reasons for the overestimation of Yestimun in all bread samples. In the end the baking process may for example open up structures, thereby increasing interaction with aniline blue and subsequent fluorescence. Further experiments are required to answer these important questions, and increase the understanding and control of the 1,3-β-G structure.

The modified aniline blue assay with DMSO detects most yeast β-glucan preparations added to different breads reasonably well.

The assay can be performed in the presence of high amounts (10fold) of mixed linkage 1,3-1,4-β-D glucans without the necessity for corrections. The detection levels presented here are realistic since they are below the expected addition levels in bread products. The modified aniline blue assay therefore presents a useful tool to ensure correct addition of yeast β-glucan preparations for manufactures of both yeast β-glucan preparations and bread products as well as regulatory agencies. The presented extraction procedure has been specifically developed for bread products, but may well be adapted for other food products. Due to its high sensitivity, the assay may also be a useful tool to investigate the effect of different types of food processing on 1,3-β-G from yeast preparations for example with regards to detectability and bioavailability.

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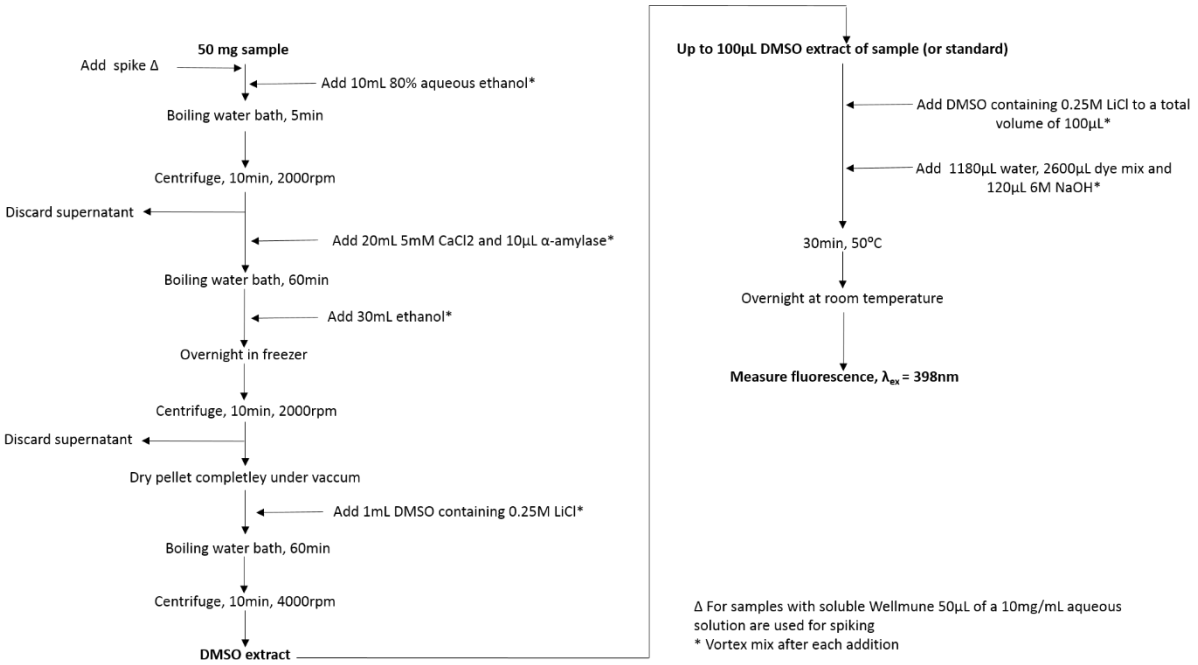


Figure 1: *Illustration of extraction process and aniline blue detection method*

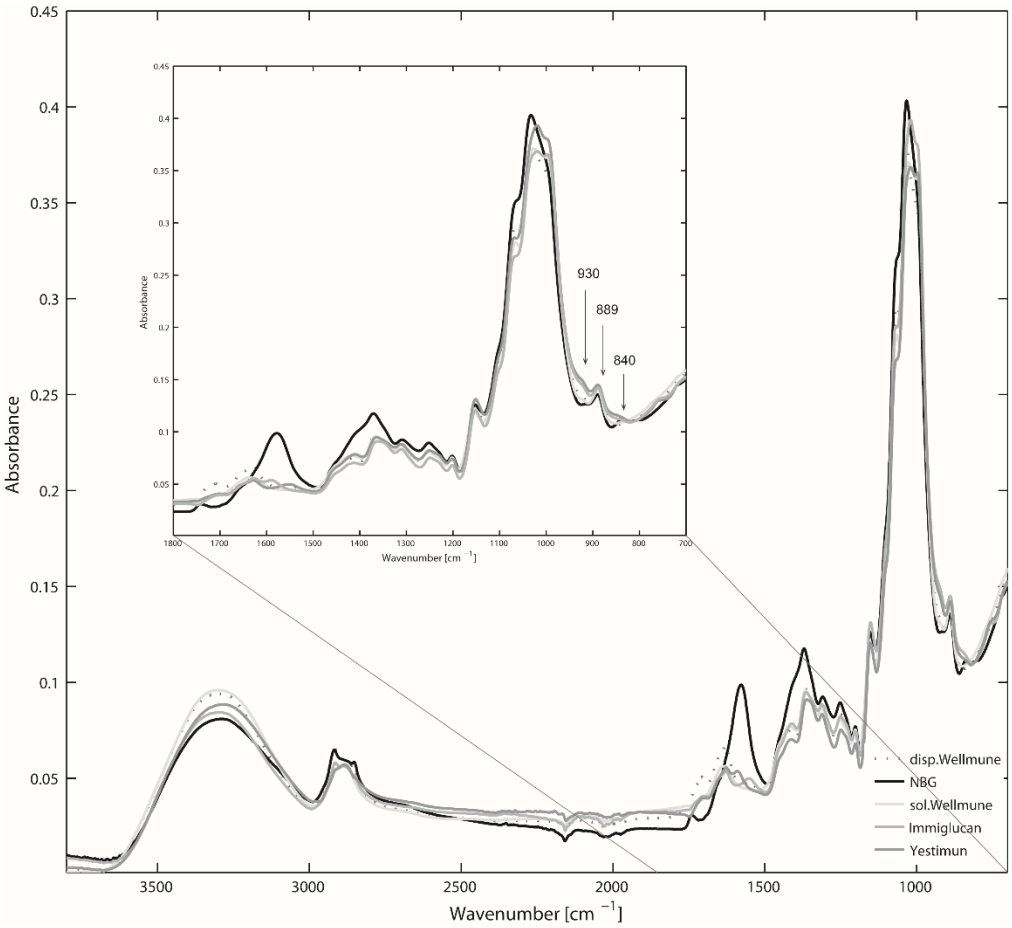
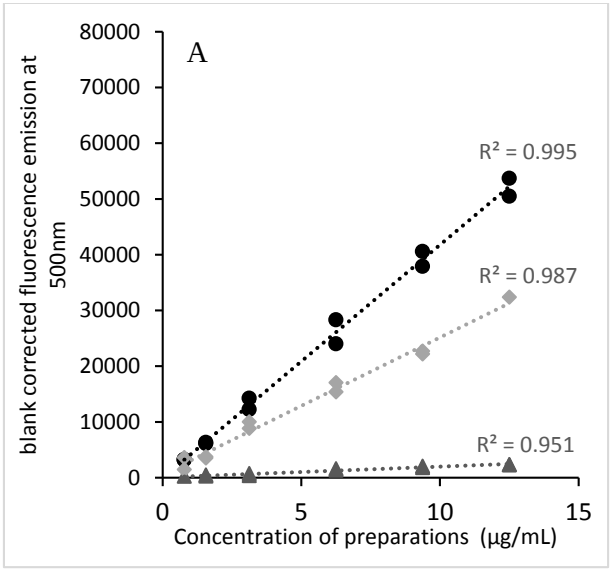


Figure 2: *FT-IR spectra of the different yeast β-glucan preparations*



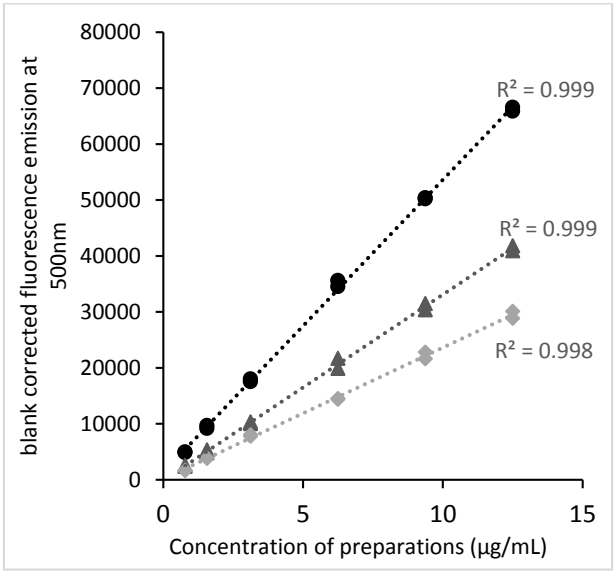


Figure 3: *Calibration curves of dispersible Wellmune (black dots), soluble Wellmune (dark grey triangles) and Shiitake polysaccharide (light grey diamonds) prepared with the classic alkaline aniline blue assay (A) and the modified DMSO based aniline blue assay (B).*

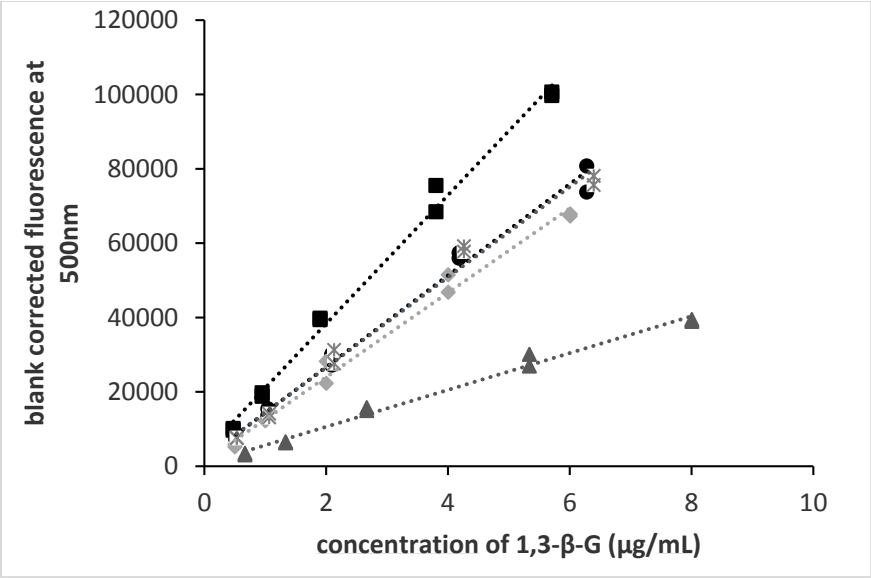


Figure 4: *Calibration curves of dispersible Wellmune (black dots), Immiglucan (black squares), NBG (grey diamons), Yestimun (grey stars) and soluble Wellmune (grey triangles). Concentrations have been corrected for the actual content of 1,3-β-G in the preparations measured by the enzymatic method (Megazyme yeast β-glucan assay kit).*

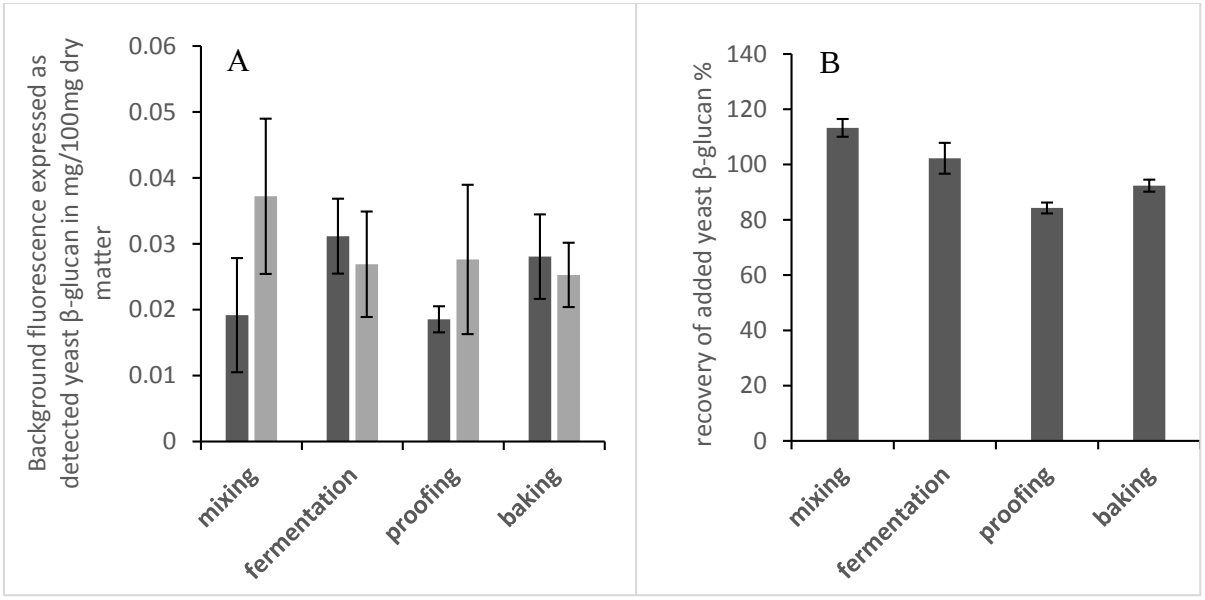


Figure 5: *Effect of different processing steps during baking on the aniline blue fluorescence from added dry or fresh yeast (A) and dispersible Wellmune (B).* ***A****: yeast amounts were increased to 2% dry yeast (dark grey) and 6% fresh yeast (light grey).* ***B****: doughs/breads were prepared without added yeast, but with the addition of 0.5 mg/100 mg dispersible Wellmune*

Table 1: *Content of 1,3-β-G in different yeast β-glucan preparations measured by acid hydrolysis as alditol acetates of glucose or with an enzymatic assay kit. Both expressed as g /100 g fresh weight.*

β-glucan preparation	1,3-β-G with acid hydrolysis (g /100 g)	1,3-β-G with enzymatic assay kit (g /100 g)	α-glucan enzymatic (g /100 g)
Soluble Wellmune	90.6 +/- 2.5	85.4 +/- 0.4	0.7 +/- 0.01
Dispersible Wellmune	66.3 +/- 2.9	67.0 +/- 0.5	0.2 +/- 0.01
NBG	61.9 +/- 0.3	64.1 +/- 0.6	0.7 +/- 0.01
Yestimune	79.1 +/- 0.6	68.2 +/- 1.2	10.8 +/- 0.1
Immiglucan	72.9 +/- 2.0	60.9 +/- 1.3	16.1 +/- 0.3

Table 2: *Re-detection of different yeast β-glucan preparations added to two different bread types at different addition levels. Values are per 100 mg dry weight and represent the mean +/- st.dev. of 4 different measurements from two independent extractions.*

Type of test food	Type of yeast β-glucan preparation	Measured amount of 1,3-β-G or yeast β-glucan preparation (mg /100 mg)	Added amount of yeast β-glucan preparation (mg /100 mg)	Actual added amount of 1,3-β-G (mg /100 mg)	Recovery of added amount (%)
Using the calibration curve for dispersible Wellmune corrected for actual 1,3-β-G content					
bread	dispersible Wellmune	0.298 +/-0.028	0.5	0.3315	89.0 +/- 8.2
bread	dispersible Wellmune	0.135 +/- 0.020	0.2	0.1326	100.9 +/- 15.2
crisp bread	dispersible Wellmune	0.837 +/- 0.001	1.4	0.936	89.26 +/- 0.2
crisp bread	dispersible Wellmune	0.304 +/- 0.018	0.5	0.3315	90.6 +/- 5.5
crisp bread	dispersible Wellmune	0.139 +/- 0.002	0.2	0.1326	103.7 +/- 1.8
bread	NBG	0.305 +/- 0.004	0.5	0.3095	95.1 +/- 1.3
bread	NBG	0.156 +/- 0.002	0.2	0.1238	121.5 +/- 1.8
crisp bread	NBG	0.366 +/- 0.005	0.5	0.3095	114.3 +/- 1.5
crisp bread	NBG	0.165 +/- 0.006	0.2	0.1238	128.6 +/- 4.5
bread	Yestimun	0.504 +/- 0.022	0.5	0.3955	147.8 +/- 6.3
bread	Yestimun	0.225 +/- 0.002	0.2	0.1582	165.1 +/- 1.5
crisp bread	Yestimun	0.520 +/- 0.017	0.5	0.3955	152.5 +/- 4.9
crisp bread	Yestimun	0.223 +/- 0.007	0.2	0.1582	163.7 +/- 5.1
Using individual calibration curves without correction of actual 1,3-β-G content					
bread	Immiglucan	0.475 +/- 0.011	0.5		95.1 +/- 2.3
bread	Immiglucan	0.208 +/- 0.005	0.2		104.1 +/- 2.9
crisp bread	Immiglucan	0.479 +/- 0.012	0.5		95.7 +/- 2.5
crisp bread	Immiglucan	0.204 +/- 0.008	0.2		102.2 +/- 4.0
bread	soluble Wellmune	0.802 +/- 0.022	1		80.2 +/- 2.2
bread	soluble Wellmune	0.420 +/- 0.025	0.5		83.9 +/- 4.9
crisp bread	soluble Wellmune	0.637 +/- 0.046	1		63.7 +/- 4.6
crisp bread	soluble Wellmune	0.363 +/- 0.016	0.5		72.6 +/- 3.2
Amounts of soluble Wellmune corrected for average spike recovery of 83.5%					

bread	soluble Wellmune	0.960 +/- 0.026	1	96.0 +/- 2.6
bread	soluble Wellmune	0.502 +/- 0.029	0.5	100.5 +/- 5.9
crisp bread	soluble Wellmune	0.763 +/- 0.055	1	76.3 +/- 5.5
crisp bread	soluble Wellmune	0.434 +/- 0.019	0.5	86.9 +/- 3.9

Table 3: *Background fluorescence of dry yeast and control breads formulated without addition of yeast β-glucan preparations. The corresponding hypothetical 1,3-β-G contents of the samples were calculated using the calibration curve for dispersible Wellmune.*

Material tested	**Blank corrected fluorescence at 500 nm**	**Hypothetical 1,3-β-G content (mg /100 mg)**
bread	6630 +/- 879	0.029 +/- 0.007
crisp bread	4547 +/- 913	0.016 +/- 0.006
dry yeast	1423 +/- 129	-0.005