# A Proton Nuclear Magnetic Resonance Method for the Quantitative Analysis on a Dry Weight Basis of (1→3)-β-D-Glucans in a Complex, Solvent-Wet Matrix

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Health benefits of the polysaccharide (1→3)-β-D-glucan, reported to induce immunobiological, hypocholesterolemic, and hypoglycemic effects in humans and animals, have made the isolation, characterization, and assay of a viable glucan product critical. A new analytical method, based on internal standard proton NMR analysis, for the assay of solvent-wet samples containing (1→3)-β-D-glucan is presented. The method enables glucan identification, provides a solvent-free assay, and improves upon the previous multistep extraction and lyophilization procedure by reducing the 1−2 day analysis time to 1−2 h. NMR offers a rapid method for quantifying the glucan in commercial samples, such as nutraceuticals, as well as industrial samples enabling better evaluation of the efficacy of these carbohydrates in health-related applications.

**Keywords:** *Carbohydrate; nutraceutical; nutritional supplements*

## INTRODUCTION

The polysaccharide (1→3)-β-D-glucan (Figure 1) has been reported to induce immunobiological (*1*, *2*), hypocholesterolemic (*3*, *4*), and hypoglycemic (*5*−*7*) effects in humans and animals. Naturally occurring (1→3)-β-D-glucan polymers can be isolated from bacterial and fungal cell walls (*8*−*10*), and a mixed linkage (1→3,1→4)-β-glucan polymer can be isolated from plant and cereal grain extracts (*7*, *11*). The pure (1→3)-β-D linked polymers are associated with immunobiological activity (*2*), whereas the mixed linkage polymers have been reported to exert hypocholesterolemic and/or hypoglycemic activity (*5*−*7*). There is a growing awareness of the potential health benefits of glucans, which has increased their importance in the nutraceutical industry. This enhanced awareness has made their isolation, characterization, and assay critical to the industrial production of a viable glucan.

A major hindrance to the introduction of glucans into appropriate health-related applications, where very large quantities of glucan are required, is the availability of a fast assay methodology. Isolated glucan product of known purity is critical for optimum evaluation of the efficacy of these products in the nutraceutical industry.

Current characterization methodologies for (1→3)-β-D-glucan quality are very time-consuming and labor intensive. Modifications to the method of Di Luzio et al. (*7*) for the recovery of particulate glucan from *Saccharomyces cerevisiae* yeast have been reported (*11*, *12*) but do not result in a significant reduction in time and labor. The Williams et al. (*12*) process results in a ∼2% yield of glucan on a solvent-free basis. These methods also require removal of solvent residues for an accurate determination of the glucan content on a dry-weight basis.

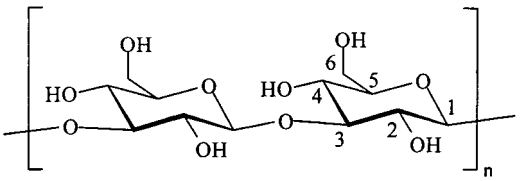


**Figure 1.** Structure of (1→3)-β-D-glucan.

Other methods of quantifying (1→3)-β-D-glucan have been reported. For example, Izawa (*13*) developed a flow injection method based on gel filtration using calcofluor, a fluorescent compound that specifically binds to the β-glucan. Wakshull et al. (*14*) quantified (1→3)-β-D-glucan using a glucan-binding agent, such as a glycosphingolipid.

Solution-state proton nuclear magnetic resonance spectroscopy (NMR) has not been applied to the nondestructive, quantitative analysis of glucans, such as (1→3)-β-D-glucan. However, quantitative NMR has found applicability for the analysis of simple sugars in plant cell wall extracts (*15*), in fruits (*16*−*18*) and vegetables (*19*), in aqueous extracts (*20*, *21*), and in root crops (*22*, *23*). In these analyses, the simple sugars are quantified in a wet sample, not on a dry-weight basis.

The purpose of this study was to develop an improved methodology for assaying the glucan content in a solvent-wet complex matrix, such as a microbial cell wall extract, plant and cereal grain cell wall extracts, microbial fermentations, and dietary fibers. This methodology, based on the application of internal standard quantitative NMR (*24*), allows for the characterization of the constituents in a complex matrix on a solvent-free basis without an extensive extraction, isolation, and drying protocol. In addition to quantification and spe-

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ciation of the glucan polysaccharides, other matrix components, such as other polysaccharides, lipids, and residual solvents, can be analyzed. This NMR method reduces the time requirements from 1−2 days to a few hours per sample and provides a direct measure of the glucan content on a dry-weight basis, even in the presence of one or more identifiable solvents, such as water, acetic acid, or ethanol.

## MATERIALS AND METHODS

**Sources of Glucan-Containing Samples.** Glucan from yeast sample 1 was isolated according to the procedure of Mueller et al. (*9*). Glucan from yeast sample 2 was isolated according to the procedure of Williams et al. (*12*). Nutrex 370 inactive yeast was extracted at Eastman Chemical Co. according to a modification of a previously described method (*12*).

**NMR Spectroscopy.** Spectra were collected at 80 °C with a JEOL model DELTA-400 NMR spectrometer using 5-mm NMR tubes. In those cases when the water resonance chemical shift was too close to the chemical shift of the aromatic protons of the internal standard, dimethyl terephthalate, a few drops of trifluoroacetic acid-*d* (99.8% deuterated or better from Cambridge Isotope Laboratories; TFA-*d*) were added to the solution to shift the water resonance downfield (*25*). Chemical shift referencing was accomplished relative to the residual proton resonance from perdeuterated dimethyl sulfoxide (DMSO-$d_6$) at 2.50 ppm. Proton chemical shift assignments for (1→3)-$\beta$-D-glucan are reported elsewhere (*8*).

**Sample Preparation.** Fifteen milligrams of the internal standard, dimethyl terephthalate, and 1 mL of DMSO-$d_6$ were added to a 3-dram vial. A blank sample was measured with each set of glucan-containing samples. Analyte samples were obtained by weighing 15.0−30.0 mg of a solvent-wet glucan-containing sample, wt of glucan, and 15.0 mg of dimethyl terephthalate into a 3-dram vial and adding 1 mL of DMSO-$d_6$. For high-purity glucans, 15.0 mg is sufficient, whereas extracts containing lower levels of glucans may need up to 30.0 mg of sample. The sample and blank vials were capped and placed on a hot plate at 80 °C with stirring using a magnetic stirring bar. If insoluble components were present, the sample was transferred to the NMR tube with filtration.

**NMR Data Collection.** NMR spectra were collected at 80 °C, with 64 scans, 15 s recycle delay, 32768 complex data points, and a 15 ppm spectral window centered at 5.0 ppm. The spectra were processed with exponential apodization. Elevated temperature was used to reduce solution viscosity and improve resonance line widths and the experiment sensitivity.

**Calculations.** The concentration of water as HOD in the blank sample, mmol of HOD (blank), was determined using

$$\text{mmol of HOD (blank)} = \frac{4W_{DMT}I_{HOD}}{194.14I_{DMT}} \quad (1)$$

where $W_{DMT}$ = the weight of dimethyl terephthalate in mg, $I_{HOD}$ = the integral area of the HOD proton resonance, 194.14 = the molecular weight of the dimethyl terephthalate, and $I_{DMT}$ = the integral area of the singlet resonance at 8.05 ppm from the four aromatic protons of dimethyl terephthalate. HOD arises as the predominant species from the rapid H/D exchange equilibrium between the TFA-*d* deuteron and $H_2O$ in the blank on the NMR time scale.

The concentration of water as HOD in the glucan-containing sample, mmol of HOD (sample), was determined using eq 1. Rapid H/D exchange equilibrium between TFA-*d* and the hydroxyl protons of the glucan, and other carbohydrates present, also contributes to the HOD resonance intensity.

The concentration of glucan in the sample, mmol of glucan, was determined using eq 1 after $I_{HOD}$ was replaced by $I_{glucan}$, where $I_{glucan}$ is the integral area of the glucan anhydroglucose repeat unit (AGRU) anomeric proton, H1, resonance at 4.52 ppm.

The quantities of mmol of HOD (blank) and mmol of HOD (sample) were converted to $wtH_2O$ (blank) and $wtH_2O$ (sample), the weight of water in the blank and in the glucan-containing samples, respectively, using

$$wtH_2O = (\text{mmol of HOD} \times 18.02)/2 \quad (2)$$

where 18.02 is the molecular weight of water and 2 is the number of protons in $H_2O$.

The weight contributed to the water resonance from the three exchangeable AGRU hydroxyl protons, wt3OH(glucan), was determined using

$$\text{wt3OH (glucan)} = (3 \times \text{mmol of glucan} \times 18.02)/2 \quad (3)$$

The weight of water, $wtH_2O$, in the water-wet glucan extract was determined using

$$wtH_2O = wtH_2O\ (\text{sample}) - wtH_2O\ (\text{blank}) - \text{wt3OH (glucan)} \quad (4)$$

The dry weight of the glucan-containing extract, dry wt of glucan, was determined using

$$\text{dry wt of glucan} = \text{wt of glucan} - wtH_2O \quad (5)$$

The weight percent glucan, wt % of glucan, in the glucan extract on a dry-weight basis was determined using

$$\text{wt \% of glucan} = \frac{400 \times 162.15 \times W_{DMT}I_{glucan}}{194.19 \times \text{dry wt of glucan} \times I_{DMT}} \quad (6)$$

where 162.15 = the formula weight of the glucan AGRU and 400 represents the product of 4 for the aromatic protons of dimethyl terephthalate and 100 for the conversion to percent.

## RESULTS AND DISCUSSION

The methodology for determination of the glucan content on a dry-weight basis in a partially dried sample containing residual water is demonstrated. All identifiable sources of exchangeable protons are incorporated in the quantitative analysis for water. Also, other non-hydroxyl-containing solvents are considered in these calculations. The weights of these solvents, including water, are subtracted from the solvent-wet sample weight to give a calculated dry weight for the glucan-containing matrix.

Duplicate analyses of yeast sample 1 by the NMR method agreed to within 0.9 wt % with an average glucan content of 97.4 wt % on a dry-weight basis. The remaining material was unidentified as non-lipid-containing material (*9*). A typical NMR spectrum is shown in Figure 2A. By the standard total glucose assay after hydrolysis, the glucan content of yeast sample 1 was >98 wt %, in agreement with the NMR result. The NMR method also provided improved precision of 0.6 wt % compared to the multistep procedure that exhibited a precision of 2−3 wt % (*9*). Residual water is a significant portion of the sample. From the NMR analysis, it can be concluded that 33 wt % of the solvent-wet glucan sample resulted from water left from the purification procedure and/or from readsorption on standing. Glucans are known to be hygroscopic (*9*). By this example, the validity of the NMR method for the determination of glucan content on a dry-weight basis in a water-wet sample is clearly demonstrated.

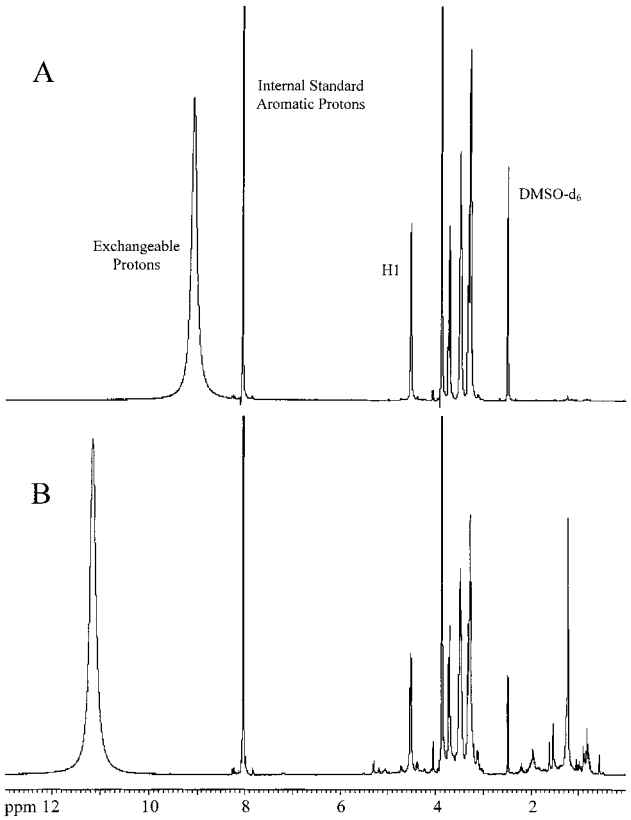


**Figure 2.** Proton NMR spectra of a highly purified (1→3)-*β*-D-glucan (A) and a crude isolation of (1→3)-*β*-D-glucan from Nutrex 370 inactive yeast (B) dissolved in DMSO-$d_6$ containing internal standard, (dimethyl terephthalate), TFA-*d*, and residual water. The exchangeable proton resonance contains protons from water, TFA-*d*, and the glucan.

Even though yeast sample 2 is less pure than yeast sample 1, quantification of the glucan content by the NMR method gives excellent agreement compared to the multistep approach. The NMR method indicates a glucan content of 89.26 wt %, whereas the total glucose analysis after hydrolysis indicates >90% purity. A higher level of lipid impurities (*9*) in yeast sample 2 relative to yeast sample 1 is also confirmed by these results.

A typical NMR spectrum of the Nutrex 370 inactive yeast extract is shown in Figure 2B. The presence of the desired extracted glucan, (1→3)-*β*-D-glucan, is clearly evident along with several other components, including lipids and other carbohydrates. Analysis of the extract, provided as a water-wet solid, required only 1−2 h. The extract was determined to contain 46.22 wt % glucan on a dry-weight basis.

The method developed provides an assay for glucan content on a solvent-free basis without requiring time-consuming sample purification and drying while enabling a straightforward identification of the desired glucan. In addition, the NMR method offers a means of quickly quantifying and identifying the glucan in commercial samples, such as nutraceuticals, as well as industrial samples.

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