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WK82642

Determination of cellulosic carbohydrate content in solid corn biomass samples

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Introduction

The US biofuel industry generates ~15 billion gallons of bioethanol annually. Most of this bioethanol is produced using 1st generation (1G) bioethanol processes that convert the starch content of corn biomass to glucose through enzymatic degradation and subsequently to ethanol via yeast fermentation. Starch (a branched polymer of α-(1,4/1,6)-D-glucose units) is the source of the majority of the glucose content present in corn. A tiny proportion of bioethanol is generated using 2nd generation (2G) bioethanol processes that convert cellulosic carbohydrates (*Cellulose*: A linear chain of β-(1-4)-D-glucose units and *Hemicellulose*: A group of heterogeneous polymers common in biomass) to glucose prior to yeast fermentation. The emergence of in-situ corn kernel fiber (CKF) (1.5G) conversion processes that allow the simultaneous conversion of starch and cellulosic content in a biomass sample presents an excellent opportunity for the sector. While starch measurement methodology has been well described in literature [1], [2], the analytical methodology required to accurately measure cellulosic content in corn biomass has not been published to date, despite the urgent requirement for same to be used in the assignment of a small but valuable percentage of total ethanol produced during these 1.5G processes as cellulosic.

Previous work – Sluiter et al (2021)

An attempt was made by the National Renewable Energy Laboratory (NREL) to address this

industry need through the seminal work of Sluiter *et al.* [3]. This study developed a method (hereafter referred to as "the NREL assay") for the measurement of cellulosic glucan in corn-based biomass that involved the use of starch degrading enzymes to afford a "cellulosic" pellet that was subsequently hydrolyzed using mineral acid to monosaccharides. Measurement of glucose by HPLC with reference to sugar recovery standards allowed calculation of the cellulosic glucan content in the original biomass sample. Excellent repeatability and spiked recoveries were demonstrated on both controls and genuine biomass samples. Advanced analytical techniques including NMR, MS and Raman spectroscopy were employed to validate the approach. The method is described in the NREL Laboratory Analytical Procedure "Determination of Cellulosic Glucan Content in Starch Containing Feedstocks" [4]. Despite the apparent utility of the method, the industry did not adopt it due to the fact that when the cellulosic glucan values obtained for biomass samples "before" and "after" conversion/fermentation (BC and AC respectively) were used to calculate cellulosic ethanol percentage using the established methodology, either extremely low or even negative results were obtained. A number of factors may contribute to this somewhat surprising outcome including a) solubilization (and subsequent loss upon filtration) of hemicellulose content facilitated by the cold caustic pretreatment performed prior to starch hydrolysis, b) as stated by Sluiter *et al*., the inability of the assay to distinguish between cellulosic content and yeast glucan that is present in AC samples and c) the fact that while commercial yeast strains have the capacity to metabolize both glucose and galactose (albeit much less favorably), only glucose, and therefore only cellulosic glucan content, is included in the analytical determination. Of these potential biases, it has now been shown that b) above is the most impactful in distorting the converted fraction calculation, as the inclusion of yeast glucan as "cellulose" in the AC sample creates an apparent increase in cellulosic content when moving from BC to AC samples that largely conceals any "true" reduction in cellulosic content that is caused by enzymatic hydrolysis through the action of endogenous or exogenous cellulases and hemicellulases.

Neogen method for the Determination of cellulosic carbohydrate content in solid corn biomass samples

The discussion that follows describes the modifications made to the NREL assay by Neogen/Megazyme, and a selection of the evidence gathered to support these modifications. The modified assay procedure, in brief, consists of a chemical pretreatment of biomass using cold caustic extraction followed by the enzymatic removal of starch in the exact same manner as performed in the NREL assay. Selective yeast glucan hydrolysis is then performed, followed by ethanolic precipitation of solubilized hemicellulosic material and acid hydrolysis of the residual carbohydrate pellet prior to measurement of D-glucose and D-galactose derived from cellulose and hemicellulose (Figure 1). Note that while glucose and galactose represent the only relevant monosaccharides metabolized by the yeasts that are currently commercially viable for use in in-situ corn kernel fiber conversion processes the bioethanol market, the inclusion of other monosaccharides in the determination of cellulosic content can obviously be accomplished in a facile manner using the same analytical HPLC method described here, if and when required, due to future developments in yeasts for the bioethanol market.

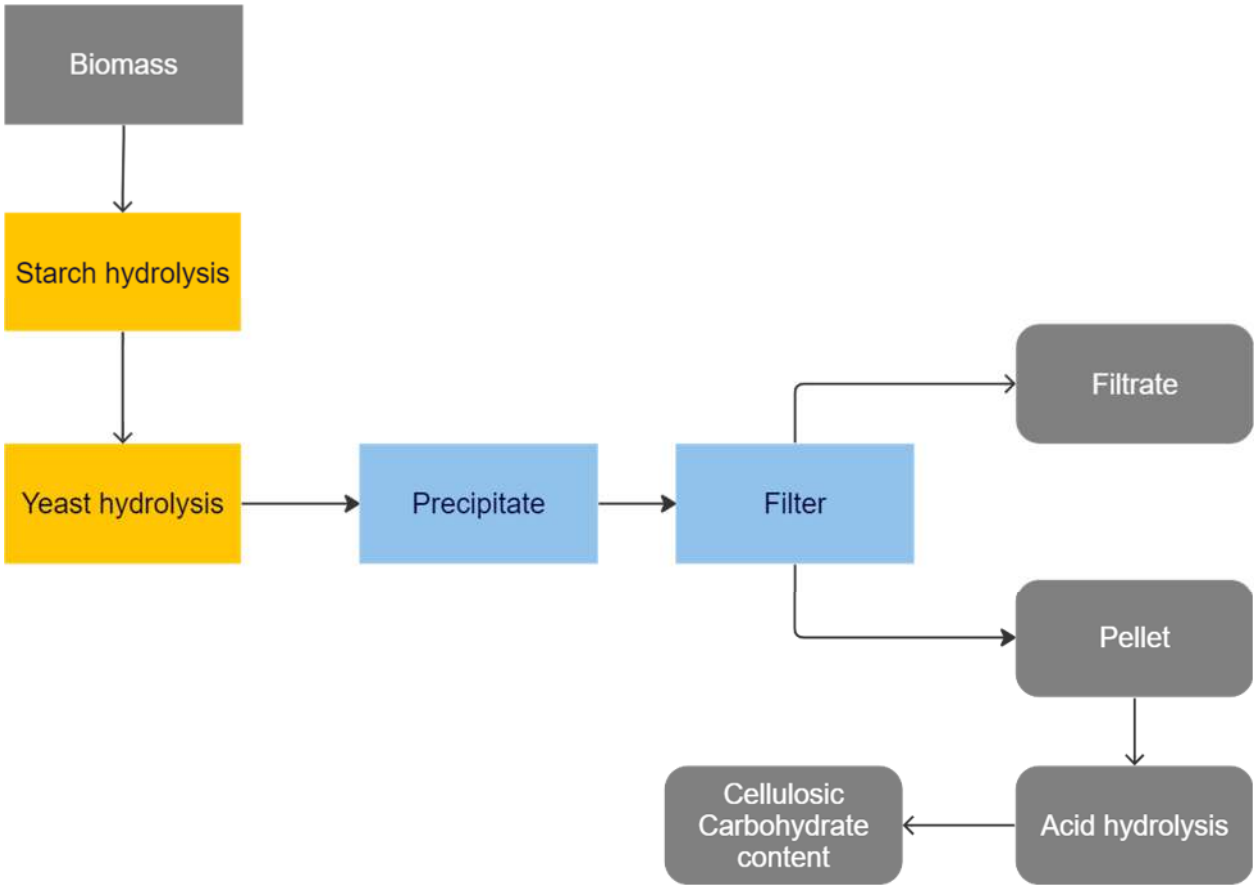

Figure 1: Schematic overview of the Neogen analytical procedure.

1. Selective hydrolysis of yeast β-glucan in AC samples

Saccharomyces cerevisiae is the most commonly employed yeast in industrial ethanol production [5] and its polysaccharide content is reported to represent 15–25 % of the yeast cell dry weight. The polysaccharides present are mainly β-glucans containing 1,3- and 1,6-linkages, followed by α-mannan, glycogen (a high molecular weight branched polysaccharide consisting of linear α-(1,4)-glucosyl chains with α-(1,6)-glucosyl branch points) and chitin (a homopolymer consisting of linear β-1,4-N-acetylglucosamine). [6].

As referenced above, Sluiter *et al*. had recognized and reported that the NREL assay could not differentiate between the various β-glucans present in biomass samples and in particular, the method did not account for β-(1,3)-and β-(1,6)-glucans found in the AC samples arising from the growth of yeast during the fermentation process.

To address this bias in the AC samples, the Neogen method employs an additional enzymatic hydrolysis step immediately following the NREL starch removal step. An enzymatic reagent (termed Yeast Degrading Cocktail or YDC) containing a suite of hydrolytic enzymes selectively degrades and facilitates removal of yeast-derived glucan in biomass samples, without the undesired hydrolysis of cellulose or hemicelluloses such as glucomannan, galactomannan, xyloglucan, arabinoxylan and 1,3:1,4-β-glucan.

Glycosyl linkage analysis is a useful tool for the characterization of complex carbohydrate structures and was employed here to demonstrate the effect of the YDC on a typical bioethanol industry fermentation yeast under conditions identical to that employed in the Neogen assay.

The analysis was performed as described by Anumula and Taylor [7]. Figure 2 clearly demonstrates the removal of the majority of glucan components present in the yeast sample.

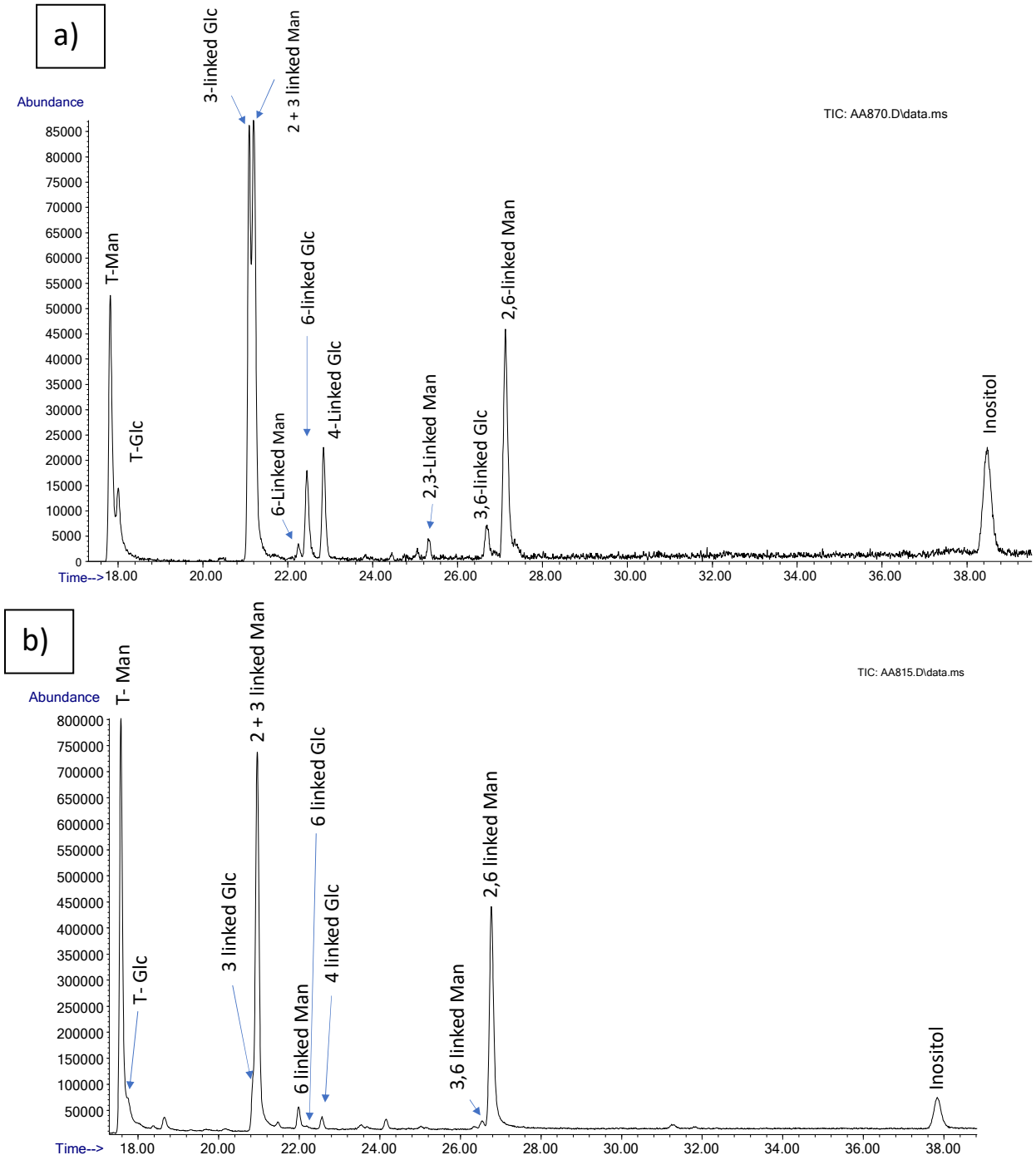

Figure 2: GC chromatograms of the permethylated alditol acetates generated from a typical bioethanol industry fermentation yeast before (a) and after (b) treatment with the YDC.

The selectivity performance of the reagent is demonstrated in Table 1. A number of structurally relevant pure polysaccharide samples (such as the cellulosic substrates, Avicel and carboxymethyl cellulose, and hemicelluloses including 1,3:1,4-β-glucan, xyloglucan, arabinoxylan, glucomannan and galactomannan) were solubilized by cold caustic extraction and incubated with YDC for a 16-hour period under the buffering, pH and temperature conditions of the Neogen assay. Resulting hydrolysis of the polysaccharides was measured by reducing sugar analysis of the hydrolysates. None of the polysaccharides tested were hydrolyzed to an extent greater than 0.1%. When applying this to a real biomass sample containing 5% of cellulose, the highest measured hydrolysis would equate to an increase in the measurement of <0.1% w/w cellulose. Two biomass BC samples were included and shown to be unaffected by the reagent, while biofuel yeast was included as a positive control, showing significant hydrolysis as expected, due to the β-glucan content present.

Sample	Sample weight (mg)	Equivalent in 200 mg Biomass (%)	Reducing sugar liberated, glucose equivalents (mg)	Hydrolysis of sample, %
Avicel (cellulose)	10	5	0	0.00
Carboxymethyl cellulose	10	5	0.009	0.09
Barley β-Glucan	10	5	0	0.00
Oat β-Glucan	10	5	0	0.00
Konjac Glucomannan	10	5	0	0.00
Carob Galactomannan	10	5	0	0.00
Wheat Flour Arabinoxylan	10	5	0.006	0.06
Tamarind Xyloglucan	10	5	0.002	0.02
Biomass Conventional BC	200	100	0	0.00
Biomass 1.5G Process BC	200	100	0	0.00
Biofuel Yeast	20	10	3.67	14.88

Table 1: Selectivity analysis of the YDC performed on a panel of pure polysaccharide samples, two BC biomass samples and yeast control sample. Incubations were carried out under Neogen assay conditions.

Further evidence of the functionality of the reagent can be found in Table 2. Firstly, the Neogen method was applied to a typical dried bioethanol industry fermentation yeast. A sample of the same yeast that had been exhaustively grown in a fermenter with glucose as feedstock was also tested to attempt to replicate the yeast form/morphology typically present in an AC sample. In both cases, removal of >80% of the yeast glucan was observed. Similarly, the effect of the YDC on real biomass BC and AC samples is shown in Table 3. The cellulosic content measured in the post-fermentation (AC) sample is reduced by approximately 1.1% due to the hydrolysis of yeast glucan present while the BC sample, which does not contain yeast in any appreciable quantity, is unaffected.

Sample Description	Treatment	Recovered carbohydrate % (w/w) n=2	%CV
Biofuel Yeast (a)	None	10.75	2.84
	+ YDC	2.04	12.72
Fermented Biofuel Yeast (b)	None	13.20	7.07
	+ YDC	2.60	7.01

Table 2: Biofuel yeast and fermented biofuel yeast samples analyzed in the Neogen assay with and without addition of Yeast Degrading Cocktail (YDC).

Sample Description	Treatment	Cellulose % (w/w) n=2	%CV
Biomass BC	None	1.86	1.59
	+ YDC	1.95	6.80
Biomass AC	None	5.73	4.42
	+ YDC	4.32	2.47

Table 3: Biomass samples (BC and AC) analyzed in the Neogen assay with and without addition of Yeast Degrading Cocktail (YDC).

2. Recovery of hemicellulosic material by ethanolic precipitation

A second source of measurement inaccuracy in the NREL assay arises from the solubilization of hemicellulose polymers by the cold caustic (sodium hydroxide) extraction utilized. Hemicellulose is a term used to describe the group of heterogeneous polymers common in biomass including 1,3:1,4-β-glucan, xyloglucans, xylans, mannans, arabinoxylan, glucuronoxylan

and glucomannans, comprising C5 and C6 sugars (namely xylose, arabinose, glucose, mannose, and galactose). Solubilization/extraction of hemicellulose in cold caustic solution has been extensively described in literature [8], [9] and its solubilization in the NREL assay results in the loss of the glucan and galactan content of these polymers to the filtrate, and an under-recovery of hemicellulose in the pellet isolated. The use of ethanolic precipitation to drive high molecular weight sugars out of solution has also been described in literature [10], and it is this technique that is utilized in the Neogen method to recover hemicellulose and ensure that it is correctly included in the cellulosic carbohydrate measurement.

Table 4 shows the resulting cellulosic carbohydrate measurement achieved using the Neogen method (without YDC) with and without precipitation applied to BC and AC samples obtained from both conventional (1G) and in-situ CKF (1.5G) processes. The degree of solubilization of hemicelluloses is sample dependent, and the additional hemicellulosic material recaptured in biomass samples was shown to vary between 0.43% and 0.91% w/w for the samples tested.

Sample	n, replicates	% CV	Minus (-) or Plus (+) Ethanolic Precipitation	Cellulosic Carbohydrate % (w/w)	Recaptured Cellulosic Carbohydrate % (w/w)
Conventional BC	2	1.5	-	1.69	0.43
	4	2.4	+	2.12	
Conventional AC	2	1.7	-	6.32	0.91
	4	0.3	+	7.23	
1.5G Process BC	2	1.3	-	1.82	0.43
	4	0.9	+	2.25	
1.5G Process AC	2	0.6	-	4.94	0.79
	4	1.1	+	5.73	

Table 4: Cellulosic carbohydrate measurement achieved using the Neogen method (without YDC) with and without precipitation.

The solubilization procedure exists within the NREL method in order to ensure the accessibility of starch prior to enzymatic treatment and removal. Alternative procedures for starch solubilization have also been reported and widely used, one such example is the dimethyl sulfoxide (DMSO) pretreatment which has been in use for more than 50 years [11]. The same samples described in Table 4 were also analyzed utilizing a soon to be published, updated DMSO extraction procedure incorporating supplemental pullulanase to ensure quantitative starch hydrolysis, which solubilizes little or no hemicellulosic material, and results were compared to those achieved using the Neogen method (Table 5). Recoveries without utilization of the ethanolic precipitation step range from 74% - 88% while recoveries when the precipitation step is included improve to between 95% - 102%.

	Recovery of cellulosic carbohydrate content (%)	
Sample	**Minus precipitation**	**Plus precipitation**
Conventional BC	79.6	95.7
Conventional AC	80.5	102.1
1.5G Process BC	88.4	95.8
1.5G Process AC	74.4	99.9

Table 5: Recovery of Cellulosic carbohydrate content achieved using the Neogen method (without YDC) with and without precipitation compared to those achieved utilizing a recently developed DMSO solubilization procedure.

3. Inclusion of galactose in monosaccharide detection step

The metabolism of galactose by *Saccharomyces cerevisiae* via the Leloir pathway has been extensively described in the literature with the GAL genes encoding this pathway being activated under conditions of high galactose and low glucose concentration [12]. S. Ostergaard *et al.*, reported that for a group of strains derived from the brewer's and distiller's strain of S. cerevisiae, the flux through the galactose utilization pathway is ~3-fold lower than the rate of glucose utilization.[13] This metabolically unfavorable pathway can become somewhat relevant in corn-based biomass conversion due to the relative ease of enzymatically driven galactose liberation from hemicellulose structures compared to that of glucose.

It has been concluded that to obtain the most accurate measurement of cellulosic content as it pertains to 1.5G bioethanol conversion processes, both glucan and galactan should be analytically quantified. Table 6 shows that the galactan content typically constitutes approximately 15% of the glucan content present in BC and AC samples. Its inclusion in the total cellulosic content measurement has, as expected given the absence of exogenous cellulases/hemicellulases, essentially no impact on the % cellulosic ethanol value obtained for

the 1G bioethanol process. The corresponding effect of its inclusion for the 1.5G process is objectively minor but not insignificant.

			Cellulosic carbohydrate content % (w/w) DWB		% Cellulosic ethanol	
Sample Description	**n**	**%CV**	**Glucan only**	**Glucan and galactan**	**Glucan only**	**Glucan and galactan**
Conventional BC	4	2.44	2.21	2.64	-0.01	0.06
Conventional AC	4	0.26	7.63	8.94		
1.5G Process BC	4	0.87	2.33	2.79	0.93	1.09
1.5G Process AC	4	1.14	6.05	7.25		

Table 6: Cellulosic carbohydrate content and % Cellulosic ethanol calculated using either glucan only or glucan plus galactan values for Conventional and 1.5G process BC and AC samples.

Overall effect of assay modifications on cellulose values and converted fraction

In order to calculate percent cellulosic ethanol a number of input values are required. Starch, Cellulose and Ash values for a conventional and 1.5G process are shown in Tables 7, 8 and 9.

A comparison between the Ash ratio and the Mass Balance ratio is supplied in Table 10. Data used to calculate the Mass Balance ratio was supplied by the biofuel producer and is not shown.

The percent cellulosic ethanol value generated using this method was calculated using Mass Balance data (Table 11) and compared to results achieved for the same samples using the NREL method and a method developed and reported by the DFO group as part of the current ASTM working group initiative (DFO method). Results for the Neogen assay are reported both as normal (including glucan and galactan content), and, in order to compare with the DFO and NREL methods on a like-for-like basis, as glucan only. The % cellulosic ethanol values derived using the DFO and NREL methods are negative in the case of conventional process and positive,

but objectively low, in the case of the 1.5G process. This can be attributed to the fact that the DFO and NREL methods do not make any correction for the bias arising from yeast glucan content in AC samples. The equivalent values derived using the Neogen assay are in line with expectations. Only trace levels of cellulosic conversion can be observed in the conventional process due perhaps to endogenous enzyme activity, while the 1.5G process facilities ~1% cellulosic ethanol production, broadly in line with most industrial samples that have been analyzed using this procedure to date (unpublished results).

% cellulosic ethanol was also calculated using the same Starch and Cellulose values but using the Ash ratio, resulting in slightly lower results for the 1.5G Process but significantly lower results for the conventional process due to a lower Ash ratio recorded for that sample set (Table 12).

	Analyst 1		***Analyst 2***		***Analyst 3***				
Sample Description	**Starch % DWB**	**%CV**	**Starch % DWB**	**%CV**	**Starch % DWB**	**%CV**	**n**	**Starch % DWB**	**%CV**
Conventional BC	67.38	*0.14*	67.18	*0.35*	67.73	*2.40*	6.00	67.43	*0.42*
Conventional AC	2.64	*2.89*	2.40	*0.29*	2.36	*0.30*	6.00	2.47	*6.13*
1.5G Process BC	66.55	*0.39*	66.93	*0.35*	67.64	*0.81*	6.00	67.04	*0.83*
1.5G Process AC	2.19	*0.64*	2.05	*0.64*	1.96	*0.02*	6.00	2.07	*5.43*

Table 7: Starch measured using the RTS-NaOH procedure (Megazyme K-TSTA 02/22, *procedure b*). Samples were analysed in duplicate by three analysts across multiple days.

	NREL Method [a]	DFO method[b]	Neogen method[c]	Neogen method[d]
Sample Description	***Cellulose % (w/w) DWB***			
Conventional BC	1.93	2.21	2.77	2.21
Conventional AC	8.09	8.30	9.29	7.41
1.5G Process BC	2.00	2.13	2.94	2.33
1.5G Process AC	6.81	7.01	7.51	5.86

Table 8: Cellulose (%, DWB) measured for Conventional and 1.5G Processes by NREL assay, DMSO solubilization method (DFO) and the Neogen assay.

[a]Data from Neogen only.

[b]Combined data from Neogen, POET, NREL, Novozymes.

[c]Combined data from Neogen, POET, NREL.

[d]Combined data from Neogen, POET, NREL, Galactan component is excluded from the cellulosic content measurement.

Sample Description	*Lab 1*	*Lab 2*	*Lab 3*	Average	St. Dev	%CV	Ash Ratio
Conventional BC	1.82	1.86	1.78	1.82	0.04	2.20	2.89
Conventional AC	5.24	5.36	5.18	5.26	0.09	1.74	
1.5G Process BC	1.88	1.80	1.72	1.80	0.08	4.36	3.29
1.5G Process AC	5.90	6.19	5.64	5.91	0.28	4.65	

Table 9: Ash values measured for Conventional and 1.5G Processes reported by each Laboratory that took part in the repeatability study

Sample Description	Ash Ratio	Mass Balance Ratio
Conventional BC	2.89	3.44
Conventional AC		
1.5G Process BC	3.29	3.53
1.5G Process AC		

Table 10: The Ash ratio for Conventional and 1.5G Processes versus the Mass Balance ratio for the same samples

	NREL Method[a]	DFO method[b]	Neogen method[c]	Neogen method[d]
Sample	***% Cellulosic ethanol***			
Conventional	-0.64	-0.30	0.11	0.08
1.5G Process	0.11	0.22	1.21	1.01

Table 11: % Cellulosic ethanol calculated using Mass Balance data for Conventional and 1.5G Processes by NREL assay, DMSO solubilization method (DFO) and the Neogen assay.

[a]Data from Neogen only

[b]Combined data from Neogen, POET, NREL, Novozymes.

[c]Combined data from Neogen, POET, NREL.

[d]Combined data from Neogen, POET, NREL - Galactan component is excluded from the cellulosic content measurement.

	NREL Method[a]	DFO method[b]	Neogen method[c]	Neogen method[d]
Sample	**% Cellulosic ethanol**			
Conventional	-1.32	-1.00	-0.67	-0.54
1.5G Process	-0.11	0.00	0.98	0.82

Table 12: % Cellulosic ethanol calculated using Ash data for Conventional and 1.5G Processes by NREL assay, DMSO solubilization method (DFO) and the Neogen assay.

[a]Data from Neogen only

[b]Combined data from Neogen, POET, NREL, Novozymes.

[c]Combined data from Neogen, POET, NREL.

[d]Combined data from Neogen, POET, NREL - Galactan component is excluded from the cellulosic content measurement.

Validation

Assay repeatability (RSD_r) and Intermediate precision (RSD_R) was determined using a series of BC and AC biomass samples, including the NIST reference materials Biomass A and Biomass B. For RSD_r (repeatability) the experiments were carried out in a single laboratory with two or three replicates per sample per run, analysed for glucose and galactose by HPLC in duplicate. For RSD_R (reproducibility), these results were compared to results achieved by two other laboratories using separate reagents and their own equipment, with two or three extractions per sample per run, analysed for glucose and galactose by HPLC in duplicate (Table 8).

	Lab	n	*% Glucan and Galactan*	RSD_r	*% Glucan and Galactan*	RSD_R
Conventional BC	1	13	2.76	2.86	2.77	1.98
	2	3	2.73	2.15		
	3	3	2.83	10.37		
Conventional AC	1	13	9.47	3.19	9.29	5.76
	2	3	9.71	0.70		
	3	3	8.69	10.13		
1.5G Process BC	1	14	2.87	4.55	2.94	2.25
	2	3	2.96	3.93		
	3	3	3.00	2.65		
1.5G Process AC	1	14	7.71	3.20	7.51	2.87
	2	3	7.55	2.65		
	3	3	7.28	4.52		
NIST Biomass A	1	14	2.64	8.01	2.50	12.64
	2	3	2.73	5.33		
	3	3	2.14	4.18		
NIST Biomass B	1	12	6.52	3.88	6.31	2.97
	2	3	6.21	1.32		
	3	3	6.18	6.67		
			Average RSD_r	4.46	**Average RSD_R**	4.74

Table 8: Repeatability (RSD_r) of the Neogen assay reported for each laboratory that took part in the study. Combined Reproducibility (RSD_R) of the Neogen assay reported for 3 laboratories.

Conclusion

This work describes a method for the measurement of cellulosic carbohydrate (glucan and galactan) in samples containing starch, cellulose, hemicellulose and yeast glucan. The method is specific for total glucan and galactan cellulosic content. When following the procedure, no starch or yeast glucan is quantified in the cellulosic measurement, and no cellulosic material is

lost into the filtrate. The precision and accuracy obtained using this method demonstrate that it can generate accurate, reproducible results.

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