

A THREE-STAGE KLASON METHOD FOR MORE ACCURATE DETERMINATIONS OF HARDWOOD LIGNIN CONTENT

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A three-stage Klason, or 72 wt% sulfuric acid, method is described for hardwoods. As compared to the traditional two-stage method, the primary hydrolysis (PH) stage was modified to improve mixing of the wood meal into the 72% H₂SO₄. The PH stage was followed by two 1 h secondary hydrolysis (SH) stages. The 72% H₂SO₄ slurry was first diluted to 40 wt% H₂SO₄ and heated at 80 °C then diluted to 3 wt% H₂SO₄ and refluxed. When equivalent PH stages were used, convincing evidence was obtained showing that the hydrolytic intensity in the cleavage of lignin-carbohydrate complexes (LCC) was much higher for the modified SH stages, as compared to the traditional refluxing of a 3% H₂SO₄ slurry for 4 h. The accuracy in lignin content determination should be equal or superior for the three-stage method, which takes approximately 2 h less time. Results are presented for 4 hardwoods from 3 different genera.

Keywords: hardwoods, lignin content, sulfuric acid hydrolysis, acid insoluble lignin, acid soluble lignin

INTRODUCTION

Research on the sulfuric acid method for lignin content determination began in the early 1900's. By 1923, Peter Klason realized that optimization of the primary hydrolysis (PH) stage with concentrated H₂SO₄ was critical.¹ He concluded that 61-68 wt% H₂SO₄ at a low temperature was close to optimum and used his method to obtain an acid insoluble lignin (AIL) content of 28% for European spruce wood. AIL values in the range of 28% for European spruce is now widely accepted.² The 72% sulfuric acid method was later published by Ritter *et al.* in 1932.³ This method is still the most commonly used method for lignin content determination of woody species^{4,5} and modifications to the conditions used by Ritter *et al.*³ have been minor. The AIL by the method of Ritter *et al.*³ is now called Klason lignin. Extensive data were presented on the effect of PH treatment time in 72% H₂SO₄ at different temperatures.³ The hardwood used was sugar maple (*Acer saccharum*) and the consistency of the reaction slurry was ~1.0 g of air-dried wood meal in 12.5 ml of 72% H₂SO₄. After PH the slurry was diluted to 3 wt% H₂SO₄

and boiled under reflux for 4 h in a secondary hydrolysis (SH) stage.³

Ritter *et al.* observed no significant difference in Klason or acid insoluble lignin (KL or AIL) yield, when the PH stage was performed for 1-3 h at 20 °C.³ For a 2 h treatment, Ritter *et al.* observed only a slight increase in KL or AIL in going from 20 °C to 25 °C or 30 °C (21.6% vs. 21.8% vs. 22.0%). The standard sulfuric acid or Klason method now calls for ~1.0 g of wood meal (oven-dried or OD basis) in 15 ml of 72% H₂SO₄ with 2 h of hydrolysis at 20 °C in the PH stage. This is followed by dilution to 3% H₂SO₄ and reflux at ~100 °C for 4 h.^{4,5} This method is referred to as the traditional method in this publication, since there have not been any significant changes to it since 1967.^{4,5} In the laboratory of the present authors, the PH standard is 1.0 g of air-dried wood meal (of known moisture content) in 15 ml of 72% H₂SO₄ for 2 h at 25 °C and with a mixing protocol that will be described later.⁶

Whether or not the standard or traditional method consistently cleaves all carbohydrates

from the KL or AIL has been an issue of on-going concern. An example would be the 1984 results of Matsumoto *et al.*⁷ Those authors used 1.0 g of wood meal in 20 ml of 72% H₂SO₄ and ran their PH stage for 4 h at 20 °C. Their PH stage was followed by dilution of the slurry to 3% H₂SO₄ and refluxing for 2 h in a SH stage. Matsumoto *et al.* found that the KL or AIL from a softwood contained 0.75% neutral sugars (% on KL or AIL), while the value was 0.95% for a hardwood KL or AIL.⁷ Furthermore, they commented that the actual sugar contents on the KL or AIL were likely to be higher than the values above.⁷

While performing research on carbohydrate compositional analyses of hardwoods, Bose *et al.* obtained data indicating the possibility of a significant retention of carbohydrates on the AIL if mixing in the PH stage was inadequate.⁶ The PH stage used in that research⁶ will be described shortly and was very similar to that of the standard Klason method.^{4,5} Most quantification of carbohydrate monomers from biomass use a hydrolysis protocol close to the one first reported in 1954 by Saeman *et al.*⁸ That method calls for ~0.5 g or biomass meal to be PH treated in 3 ml of 72% H₂SO₄ for 1 h at 30 °C, the slurry is then diluted to 4% H₂SO₄ and autoclaved for an additional hour at 121 °C.⁸⁻¹¹ Bose *et al.* tried to improve on xylose yield from the method of Saeman *et al.*,⁸ by increasing the acid hydrolytic intensity in a modified two stage hydrolysis.⁶ Their idea was to perform the SH stage in 40% H₂SO₄ at 80 °C instead of 4% H₂SO₄ at 121 °C. The thinking of Bose *et al.* was aided by the fact that they had access to a method and equipment for sugar monomer analysis by ¹H NMR.⁶ A high H₂SO₄ concentration is helpful to the ¹H NMR method, because it shifts the H₂O peak out of the carbohydrate region in the NMR spectrum.¹² Saeman *et al.* used only 3 ml of 72% H₂SO₄ in PH because of the large dilution factor in going from 72% to 4% H₂SO₄ in the SH stage.⁸ Since their dilution would be from 72% to only 40% H₂SO₄, Bose *et al.* could afford to use 0.5 g of wood meal in 16 ml of 72% H₂SO₄ for 2 h at 25 °C in the PH stage.⁶ Due to both stages being performed at <100 °C, this method could handle more samples simultaneously.⁶ After the PH stage, those authors simply added 21 ml of H₂O to the PH slurry, sealed the centrifuge tube, shook it vigorously and placed it in an 80 °C water bath. During their initial data collection with 0.5 g of wood meal in 16 ml of 72% H₂SO₄, nine samples were hydrolyzed simultaneously.⁶ However, in their

first attempt with 1.0 g of a poplar wood meal in 16 ml of 72% H₂SO₄, no adjustment was made for the increased time requirement that would be needed for the mixing of 1.0 g instead of 0.5 g of wood meal in 16 ml of 72% H₂SO₄. The true glucan and xylan content of the sample was 51.0% and 17.5%, respectively and the summative analysis was 99.7%.⁶ However, in their initial trial only one of nine samples gave glucan and xylan yields >49% and >17%, respectively. Those authors later isolated the problem to inadequate mixing in the PH stage.⁶ When 1.0 g of wood meal is being used for either lignin or sugar monomers quantification, nine runs at a time is not recommended.

The results of Bose *et al.*⁶ would suggest that if mixing is inadequate, incomplete hydrolysis of lignin-carbohydrate (L-C) linkages, is possible when 1.0 g of wood meal is hydrolyzed in 15 ml of 72% H₂SO₄ in the PH stage of the Klason method. There is no standard related to adequate mixing in the PH stage of the traditional Klason method.^{4,5} The objective of this research was to investigate a three stage hydrolytic procedure for KL or AIL to see if a more extensive cleavage of L-C linkages could be obtained. After the PH stage, the sample would be diluted to 40% H₂SO₄ and hydrolyzed at 80 °C for 1 h (SH-1) then further diluted to 3% H₂SO₄ and refluxed for 1 h at ~100 °C (SH-2).

The research program was greatly influenced by KL or AIL values reported for extractive-free sugar maple, a hardwood species commonly used by the present research group.¹³⁻¹⁶ Ritter *et al.* obtained seven close to optimum KL or AIL values in the range of 21.5% to 22.0% for this species.³ Later, a value of 22% was reported by the US Forest Service.¹⁷ The present research group has analyzed many samples of this species by the standard or traditional method, but always with good mixing in the PH stage. At least five samples were harvested from different sites in Central New York and one sample from Central Pennsylvania. The KL or AIL values all fell in a narrow range of 21.5% to 22.5%. However, others have reported KL or AIL values >24% on an extractive-free basis for North American grown sugar maple.^{18,19} As previously discussed, the hydrolytic procedures for KL or AIL and the generation of sugar monomers are fairly similar. Kaar *et al.*^{10,11} used the Saeman method⁸ to analyze seven hardwoods and three softwoods. Their summative analyses varied from 98.3% to 99.6% for the ten samples. One interesting aspect

of the research of Kaar *et al.* was that they estimated their AIL by filtering the slurry after one hour at 121 °C SH hydrolysis.^{10,11} On the other hand, the modified method of Bose *et al.*⁶ for sugar monomers along with the traditional Klason + acid soluble lignin (ASL) methods were used to determine the contents of lignin and carbohydrates in three temperate hardwoods,⁶ a Brazilian eucalyptus,²⁰ three Mediterranean eucalypti,²¹ a tropical hardwood,²² two sugar cane bagasse samples²⁰ and a bamboo sample.²⁰ The summative analyses varied from 98.5-101.5% for all the samples.^{6,20-22} Coincidentally, sugar maple was the only overlapping species between the research of Kaar *et al.*^{10,11} and the Bose *et al.* method. Kaar *et al.* obtained lignin and xylan contents of 28.9% (Klason + ASL) and 16.1% on an extractive-free basis, respectively.¹¹ In contrast, Bose *et al.* obtained corresponding values of 25.0% and 18.6%, respectively.⁶ It appeared to the present group of researchers that SH stages in 3% H₂SO₄ for 4 h at ~100 °C^{4,5} or in 4% H₂SO₄ for 1 h at 121 °C⁸⁻¹¹ may not always offer sufficient cleavage of L-C linkages in hardwoods, thus resulting in an artificially high value for lignin content and an artificially low value for xylan content. This was the primary motivation for research on SH in 40% H₂SO₄ at 80 °C. This SH condition was previously shown to be effective at cleaving almost all of the L-C linkages in both hardwoods and non-woods.^{6,20-22}

EXPERIMENTAL

Wood meal samples

Starting with debarked wood chips, wood meals (30 mesh) were prepared from paper birch (*Betula papyrifera* Marsh), sugar maple, aspen (*Populus tremuloides*) and a poplar identified as W 79. The birch, maple and aspen were harvested in Central New York, while W 79 was harvested from a commercial plantation in Wallula, WA and is described elsewhere.²³ All of the wood meals were soxhlet extracted by a 2:1 (v/v) mixture of toluene/ethanol.

Traditional and modified Klason determinations

The PH stage was performed with 1.00 g of air-dried wood meal in 15 ml of 72% H₂SO₄ in a 50 ml polypropylene centrifuge tube with a maximum useable temperature of 121 °C. The reaction temperature was 25 °C and the wood meal was thoroughly mixed into the 72% H₂SO₄ at the beginning. A glass rod was used and it was not removed from the centrifuge tube until the end of the PH stage. The use of the centrifuge tube with a conical bottom allowed for the sample to be converted to a liquid with only a small amount of fine particles after

only ~10 minutes. The slurry was mixed after 15 minutes of reaction and every 15 minutes thereafter. This PH protocol was used for both methods.

In the traditional method, there were no changes to the standard 4 h SH stage.^{4,5} In the three-stage method, 19.6 g of water was added to the PH slurry that started with 15 ml of 72% H₂SO₄ (density of 1.63 g/cm³). The centrifuge tube now containing a 40 wt% H₂SO₄ solution was vigorously shaken before being heated in an 80 °C thermostat controlled water bath for 60 minutes (SH-1). The sample was shaken every 15 min. That solution was then diluted to 3 wt% H₂SO₄ and refluxed for 1 h (SH-2). A few experiments were performed where the PH stage was shortened to 15 minutes and with frequent mixing (every 1-2 min). This was followed by the conventional 4 h SH or SH-1 + SH-2 treatment from above.

Acid soluble lignin (ASL) determination

The lignin remaining soluble in the 3% H₂SO₄ solution after filtration of the KL or AIL was estimated by the UV spectroscopic method described in Tappi Method UM 250.²⁴ There was no change to the traditional filtration process.^{4,5} A standard dilution factor of 20 and lignin absorptivity of 110 l/g.cm at 203 nm were used.

Elemental composition of Klason lignins

The analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee, USA.

RESULTS AND DISCUSSION

Preliminary research with W 79

The research was initiated with poplar W 79 with a reported total lignin content (Klason + ASL) of 27.3%.²³ In order to get some idea of the importance of the PH stage on the cleavage of lignin-carbohydrate complexes (LCC), the PH time in the traditional method was shortened to 15 min. Very high duplicate values of both AIL and ASL were obtained for W 79 (Table 1). The duplicate values were 32.7% and 35.3% for AIL and 3.7% and 3.6% for ASL. These AIL values appeared unexpectedly high because both wood meal samples were completely liquefied 5-10 min into the PH stage. It was assumed that a 4 h reflux in 3 wt% H₂SO₄ would have cleaved most of the LCC linkages. The next experiment was to confirm that poplar W 79 did indeed have a lignin content of only ~27% when a 120 min PH was used. This was confirmed and duplicate total lignin values of 27.0% and 26.7% were obtained by the traditional method (Table 1). The KL or AIL values were 24.0% and 24.1% for PH = 120 min. The experiment with PH = 15 min was repeated a few days later and the duplicate values

were 32.8% and 34.2% for AIL and 4.3% and 4.0% for ASL. The four AIL data points for PH = 15 min gave an average of 33.8%. This value clearly indicated that significant amounts of non-lignin components were attached to the AIL and almost all of these components had to be of carbohydrate origin. The difference in AIL values

between PH = 15 min and 120 min was 9.7% on wood (33.8%-24.1%). Kaar *et al.* analyzed all seven of their hardwood species for protein and ash content and the total of the two was <1.0% on wood in all cases. Therefore, approximately 9.0% on wood of the non-lignin component attached to the AIL had to be of carbohydrate origin.

Table 1
Lignin content of extractive-free W 79 poplar by traditional and modified methods

PH time (min)	T or M ¹	Klason lignin ²	ASL ²	Total ²
15	T	32.7 (35.3) ³	3.7 (3.6) ³	36.4 (38.9) ³
15	T ⁴	32.8 (34.2)	4.3 (4.0)	37.1 (38.2)
120	T	24.1 (24.0)	2.9 (2.7)	27.0 (26.7)
15	M	27.2 (26.5)	2.8 (2.6)	30.0 (29.1)
15	M ⁴	28.3 (28.0)	2.6 (2.3)	30.9 (30.3)
120	M	24.7 (24.2)	2.4 (2.5)	27.1 (26.7)

¹Traditional or modified; ²wt% based on wood meal (OD basis); ³Duplicate result in parentheses; ⁴Performed at a later date compared to run above

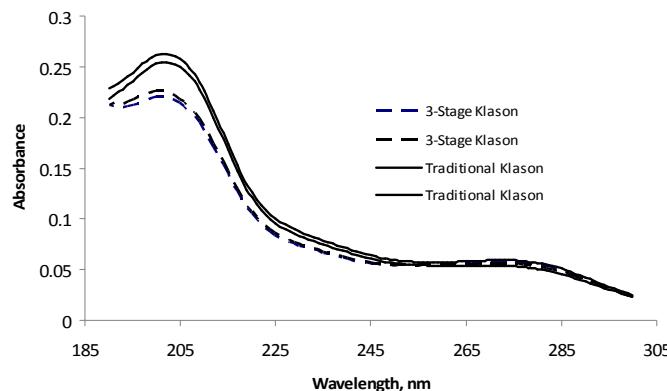


Figure 1: UV spectra of filtrate from traditional and modified Klason determinations performed on W 79 poplar (duplicates for each method)

The modified or three stage method (PH = 120 min) was then attempted and duplicate total lignin contents of 27.1% and 26.7% were obtained. These values were nearly identical to the two stage method. However, the KL or AIL values were slightly higher for the modified method, while the ASL values were slightly lower. This may be due to a higher rate of lignin-lignin condensation²⁵ in the three stage method and particularly in SH-1 that utilized 40% H₂SO₄ at 80 °C. The duplicate values of KL or AIL were 24.7% and 24.2% for the modified method, as compared to 24.1% and 24.0% for the traditional method. The corresponding numbers for ASL were 2.4% and 2.5% for the modified method and 2.9% and 2.7% for the traditional method. The

UV spectra for the four diluted Klason filtrate (duplicates for both traditional and modified methods) are shown in Figure 1. The trend of slightly higher KL or AIL and lower ASL for the modified method was consistently observed for all four wood species (W 79, sugar maple, birch and aspen).

Determinations using the modified method and PH = 15 minutes were then performed. The duplicate values for AIL were 27.2% and 26.5%. The experiments were repeated at a later date and duplicate AIL values of 28.3% and 28.0% were obtained. The average AIL value for the four runs was 27.5%, which is much lower than the 33.8% value obtained when PH = 15 min was used in the

traditional method. The PH = 15 min results prove that the hydrolytic intensity was higher when the two SH stages of the modified method were compared to the 4 h SH stage of the traditional method. While the traditional method gave an AIL with 9.7% on wood of non-lignin components (33.8%-24.1%), the value was only 3.4% for the modified method (27.5%-24.1%). Furthermore, when PH = 120 min plus the present 40 wt% H₂SO₄ SH-1 treatment were used to produce carbohydrate monomers, not much furfural or hydroxymethylfurfural was generated.⁶ If these compounds were to be generated at significant yields from xylose and glucose, respectively, they could possibly condense with lignin²⁶ and result in an artificially high KL or AIL content.

Based on the earlier results showing almost complete hydrolysis of carbohydrates from lignin and their depolymerization to monomers,^{6,20-22} it is highly likely that the SH-2 hydrolysis in the modified method is not needed for accurate determinations of lignin content. However, the standard method is based on filtration of KL or AIL from a 3 wt% H₂SO₄ slurry after a reflux treatment. Filtering the KL or AIL from a 40 wt% H₂SO₄ slurry would introduce an additional variable to be investigated. Also, the safety risk

associated with filtering a 40 wt% H₂SO₄ slurry is likely to be more significant than filtering a 3 wt% H₂SO₄ slurry. Since SH-1 was a straightforward 1 h hydrolysis, it was concluded that an additional 1 h reflux was preferable to filtering a 40 wt% H₂SO₄ slurry. Even with this additional SH-2 treatment, the modified method is still ~2 h shorter than the traditional method.

Other wood species

The traditional and modified methods (PH = 120 min in both) were then compared using sugar maple. The results (KL + ASL) for the modified method were 22.4% + 2.5% and 22.5% + 2.5% (Table 2). For the traditional method, one sample gave 22.0% + 3.1%, but the other sample was lost due to experimental error. The traditional method was repeated at a later date and duplicate results of 22.0% + 2.9% and 22.3% + 2.8% were obtained (Table 2). Therefore, total lignin contents of 24.9% and 25.0% were obtained from the modified method, while values of 25.1%, 24.9%, and 25.1% were obtained by the traditional method. It should be noted that for the traditional method, KL or AIL values in the range of 22.0%-22.3% were obtained (Table 2) and this is very similar to the 21.5%-22.0% range reported by Ritter *et al.*³

Table 2
Lignin content of extractive-free maple, birch and aspen by traditional and modified methods

PH time (min)	T or M ¹	Klason lignin ²	ASL ²	Total ²
Sugar maple				
120	T	22.0	3.1	25.1
120	T	22.0 (22.3) ^{3,4}	2.9 (2.8)	24.9 (25.1)
120	M	22.4 (22.5)	2.5 (2.6)	24.9 (25.1)
Paper birch				
120	T	18.5 (18.7)	3.7 (3.7)	22.2 (22.4)
120	M	19.2 (18.9))	3.3 (3.3)	22.5 (22.2)
Aspen				
120	T	18.0 (18.3)	2.3 (2.3)	20.3 (20.6)
120	M	18.5 (18.3)	1.9 (1.9)	20.4 (20.2)

¹Traditional or modified; ²wt% based on wood meal (OD basis); ³Duplicate results in parentheses; ⁴Performed at a later date compared to run above

Table 3
Comparison of elemental composition of KL or AIL between modified and traditional method

Wood sample	%C	%H	%O	%S
Poplar W79	60.5 (60.6) ¹	5.6 (5.7)	31.7 (31.9)	<0.2 (<0.2)
Sugar maple	58.7 (59.0)	5.5 (5.6)	33.2 (33.1)	<0.2 (<0.2)
Paper birch	60.4 (60.4)	5.5 (5.7)	32.0 (32.2)	<0.2 (<0.2)

¹Traditional KL or AIL followed by modified KL or AIL in parentheses

The traditional method was then compared to the modified method for paper birch and aspen. The duplicates for birch by the traditional method averaged a total lignin of 22.3%, while the average for the modified method was 22.4% (Table 2). The corresponding values for the aspen were 20.5% by the traditional method and 20.3% by the modified method (Table 2).

Preliminary results regarding the acid soluble lignin (ASL)

The modified method gave consistently lower ASL values (~0.4% on wood) than the conventional method. These lower values appear reasonable in light of the fact that the modified method gave a slightly higher KL or AIL. However, some preliminary research was performed to get an indication as to whether or not the ASL from the modified method could have had a significantly different absorptivity value, as compared to the ASL from the traditional method. If there were to be a significant difference in ASL, then one would expect some significant differences in the KL or AIL as well. When elemental analyses were performed on KL or AIL from three of the wood species, there were no significant differences in C, H, O, or S content between the traditional and modified methods (Table 3).

Interestingly, the ranges of C, H, and O content in Table 3, i.e. 58.7%-60.6% for C, 5.5%-5.7% for H and 31.7%-33.2% for O are very similar to values reported by Bjorkman and Person²⁷ for milled wood lignin (MWL) from three other hardwoods. Those values were 58.8%-60.4% for C, 6.1%-6.5% for H and 33.0%-34.0% for O. The decrease in H and O contents for the KL or AIL, as compared to MWL, is due in part (most likely) to the loss of OH groups from α -carbon atoms in the lignin side chain, as a result of acid-catalyzed lignin condensation (C-C bond formation) reactions during H_2SO_4 treatments.²⁵ However, the losses in H and O were not major and KL or AIL may be less condensed than previously thought.²⁸

CONCLUSION

The Klason, or 72 wt% sulfuric acid, method for the determination of lignin content is very accurate and reproducible if performed by experienced analysts. However, data in the literature suggest that the KL or AIL can contain significant amounts of carbohydrate residues if mixing is inadequate in the PH stage. When the

effectiveness of the PH stage was deliberately lowered by its treatment time, being decreased from 120 min to 15 min, it was observed that the SH-1 + SH-2 treatments of the modified method afforded an AIL value of 27.5% for a poplar, while the corresponding value for the single 4 h SH stage of the traditional method was 33.8%. In each case, the poplar sample was analyzed in duplicate on separate days, thus each average above was for 4 different runs. The minimum AIL value (PH = 120 min) for the poplar was 24.1%. The 33.8% and 27.5% AIL values for the traditional and modified methods when PH = 15 min was used clearly indicated that significant amounts of non-lignin components were attached to the AIL (9.7% for the traditional method and 3.4% for the modified method). Kaar *et al.* analyzed all seven of their hardwood species for protein and ash content and the total of the two was <1.0% on wood in all cases. Therefore, almost all of these non-lignin components had to be of carbohydrate origin. The difference between 33.8% AIL for the traditional method and 27.5% for the modified method is a clear demonstration that the hydrolytic intensity in LCC cleavage was much higher for the modified method.

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