

Isolation of Residual Kraft Lignin in High Yield and Purity

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In this paper, we report on a novel residual lignin isolation method. Initially we demonstrate that, in order to cellulolytically isolate residual lignin in high yield, the cellulosic structure of the pulp should be exposed as much as possible. Consequently, we developed a two-step method that first uses a mild enzymatic treatment to render most of the carbohydrates water-soluble and remove hemicelluloses. The solid residue obtained in this step contains about 94% of the lignin originally present in the pulp. A second mild acidolytic step is then applied (acid concentration 0.05 mol/L), which causes the quantitative cleavage of all lignin-carbohydrate linkages. Such linkages would otherwise survive an enzymatic treatment. Due to the relatively low enzyme/pulp ratio used in this step, only small amounts of proteins coexist with the lignin in the final preparation (2.3%), thus requiring no protease purification. At the same time the possibility of lignin structural changes are minimized by the low concentration of acid used throughout the developed method. The method offers a residual lignin preparation of high purity (95%) with an average yield of 70%.

La présente communication porte sur une nouvelle méthode d'isolation de la lignine résiduaire. Nous avons d'abord démontré que, pour isoler de manière cellulosique la lignine résiduaire dans les pâtes à haut rendement, la structure cellulosique de la pâte doit être exposée le plus possible. Nous avons donc développé une méthode en deux étapes qui utilise d'abord un léger traitement aux enzymes afin de rendre la plupart des hydrates de carbone hydrosolubles et d'éliminer les hémicelluloses. Le résidu solide obtenu à cette étape contient environ 94 % de la lignine initialement présente dans la pâte. Une deuxième étape acide légère est alors appliquée (teneur en acide de 0,05M) et entraîne un clivage quantitatif de tous les liens lignine-hydrates de carbone, qui survivraient autrement à un traitement aux enzymes. En raison de la proportion relativement faible enzymes-pâte à cette étape, seulement de petites quantités (2,3 %) de protéines coexistent avec la lignine dans la préparation finale, et n'exigent donc aucune purification avec des protéases. En même temps, le risque d'un changement structural de la lignine est réduit en raison de la faible concentration de l'acide employé durant toute l'analyse. Cette méthode offre une préparation de lignine résiduaire à un degré élevé de pureté (95 %) avec un rendement moyen de 70 %.

INTRODUCTION

Fundamental research aimed at improving pulping and bleaching processes almost invariably requires an understanding of the structural details of residual kraft lignin. For such endeavours it is essential that the lignin remaining on the fibre be isolated from the pulp in high yield and purity, unaltered and in the absence of contaminants. Currently, two methods are in use for the isolation of residual lignin from kraft pulp: that of enzymatic hydrolysis using cellulolytic enzymes, and that of acid hydrolysis using a solution of hydrogen chloride in dioxane/water. Despite extensive research ef-

forts, aimed at obtaining residual lignin that would completely comply with the above criteria, both of these methods have limitations that need to be addressed.

The acid hydrolysis technique [1], while it offers a residual lignin preparation of high purity, is plagued with relatively low yields [2,23] and the possibility of structural alterations induced during the acidic treatment [4].

Residual lignin isolated by enzymatic hydrolysis has been found to contain a relatively high amount of carbohydrates and proteins [5–7]. The former is believed to originate from the limited ability of enzymes to hydrolyze lignin-carbohydrate linkages [8–11], while the latter is thought to originate from the enzymes used in the hydrolysis stage. The efforts to arrive at a suitable enzymatic residual lignin preparation have been reviewed recently by Tamminen and Hortling [12]. Enzymatic residual lignin preparations have been shown to contain lignin-carbohydrate bonds [8,9] glycosidically bound to the benzyl carbons in

lignin [13]. Such lignin-carbohydrate linkages cannot be found in samples isolated by the acidolysis method. The documented acid lability of such bonds caused Wang et al. [14] to exploit it in order to purify isolated residual lignin contaminated with carbohydrates.

Since it was possible to purify lignin using a very mild acidic treatment, our efforts to develop a better residual lignin isolation method embarked from our previous account [14] and progressed to the present method. In this paper we report on a novel two-step method that initially uses a mild enzymatic treatment to render most of the carbohydrates water-soluble and remove hemicelluloses from the fibre surface [15,16]. This treatment is thought to expose the remaining lignin and lignin-carbohydrate moieties, making it possible for a second, relatively mild acidolytic step to cause the quantitative cleavage of all lignin-carbohydrate linkages. Such linkages would otherwise survive an enzymatic treatment. Using this method, the residual lignin is isolated in higher

JPPS

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yield with relatively lower protein and carbohydrate contamination demonstrating the superiority of the proposed mild two-stage approach.

EXPERIMENTAL The Two-Stage Method

Stage 1: Mild Enzymatic Hydrolysis

Never-dried (10–40 g o.d.) black spruce (*Picea mariana*) unbleached kraft pulp (kappa number 30.8) was subjected to a single enzyme treatment using cellulase (Novozym or Iogen Industrial Cellulases) with an activity of about 1300 units/mL. Under the specified reaction conditions, one unit of the enzyme reduces 1 mol of reducing end groups in carboxymethylcellulose per minute. In accord with the supplier's specifications, both enzymes contain mostly cellulase and a significant amount of hemicellulases. In general, we have discovered that the final yield of our methodology depends on the enzyme activity. Consequently, it is recommended that this be kept at a level of about 1300 units/mL. The developed procedure requires a low ratio of enzyme/pulp (360 unit/g of o.d. pulp) in order to ensure a mild enzymatic treatment causing minimum protein contamination. The enzymatic hydrolysis was carried out at pH 4.5 (acetate buffer) at a consistency of 5% in a water bath equipped with an orbital shaker set at 40°C for a period of 48 h. After the enzymatic digestion, the impure residual lignin was recovered mostly as an insoluble residue.

Stage 2: Acid Hydrolysis

The impure lignin recovered after the mild enzymatic treatment was suspended in 100 mL of a 0.05 mol/L HCl solution in dioxane–water 85:15 v/v and was refluxed (azeotrope boiling point 86°C) under nitrogen for 2 h. The resulting mixture was filtered and the lignin solution was collected. The solid residue was washed with fresh dioxane until the filtrate was clear. The lignin solution and the combined washings were then neutralized with solid sodium bicarbonate. The neutralized solution was finally precipitated in a large quantity of acidified water (pH = 2) and the precipitated lignin was isolated by centrifugation followed by freeze-drying. The lignin obtained was finally washed with dichloromethane (3 × 30 mL) to remove the existing extractives.

The Three-Stage Method

The following procedure was followed in order to validate the working hypothesis that forms the foundations of this work. The three stages are: mild acidolysis, enzymatic hydrolysis and mild acidolysis. The same kraft pulp described above was initially subjected to a mild acidolysis treatment using the procedure outlined in Stage 2. After removing the isolated lignin, the solid residue was then treated with enzyme followed by another mild acidolysis stage, using the procedure described in the two-stage approach above. All lignins were finally subjected to dichloromethane (3 × 30 mL) extractions to remove the extractives. However, this solvent was also found to remove small amounts of lignin (24%). Using pentane for the

extraction could increase the lignin yields further but the danger of fatty acid contaminants is higher.

Quantitative ³¹P NMR Spectroscopy

Quantitative ³¹P NMR spectra were obtained on a Varian 300 MHz spectrometer, following published procedures [17,18]. Pyridine/CDCl₃ (1.6/1 v/v) was used as the solvent, cyclohexanol as the internal standard, chromium acetylacetonate as the relaxation reagent and 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane as the phosphorylation reagent.

RESULTS AND DISCUSSION The Initial Three-Stage Method

The working hypothesis of our approach rests in the following description. At the end of the kraft cook, hemicelluloses and lignin are known to partly reprecipitate on the surface of cellulose microfibrils [15] possibly chemically linked to each other [9]. In order to isolate the residual lignin as quantitatively as possible, the components of the fibre wall of the pulp should be exposed. Our hypothesis was initially validated by a designed three-stage experiment composed of a mild acidolysis (acid concentration 0.05 mol/L) followed by a mild enzymatic hydrolysis and then another mild acidolysis stage (see Experimental).

If one considers the relatively low concentration of acid used in the first stage (0.05 mol/L) then the yield data of this stage seem attractive (43.6%). However, since no preliminary solvent extraction was applied on the pulp before the procedure, most of the extractives were found to accumulate in the lignin isolated during this stage. Consequently, these yield data are not representative.

After removing the lignin from the first step, the residue was treated with enzyme. During the ensuing second enzymatic hydrolysis stage, the cellular structure of the pulp and/or the lignin–carbohydrate bonds were further exposed, which made it possible for the final mild acidolytic treatment to liberate even more lignin i.e. an additional 33% of lignin was isolated during the second acidolytic stage. In total, about 66% of the lignin present on the fibre was removed. This is a significantly improved yield than any mild hydrolysis procedure has ever offered. For example, when using a higher concentration of acid (0.1 mol/L) during the conventional acidolysis procedure, no more

than 35–45% of pure lignin could be isolated. It is therefore evident that the lignin isolated from the second mild acidolytic treatment could not have been liberated from the pulp if the components of the fibre wall had not been disrupted by the enzyme stage.

The Development of the Two-Stage Method

Having defined the need to initially expose the cellulosic structure of the pulp prior to an acidolysis step, the sole use of an enzymatic stage in achieving this was then explored, thus eliminating the need for the initial acidolysis. As such, the new two-stage procedure emerged. Our two-stage approach initially aims to hydrolyze the accessible carbohydrates present on the surface of the fibres using cellulases and hemicellulases (both present in Novozym). In the presence of hemicellulase activity, the reprecipitated xylans and other hemicelluloses would also hydrolyze under the enzymatic digestion, exposing fresh cellulosic surface to the enzyme, aiding digestion.

The inability of the enzymes to further degrade the carbohydrates was rationalized on the basis that the remaining carbohydrates are largely bonded to lignin. At this point, our previous effort demonstrating the extreme lability of lignin–carbohydrate linkages [14] dictated the use of a second acidolysis step to finally free the lignin from the carbohydrates. The highly exposed cellular structure of the sample allowed for a very mild acid hydrolysis step to be developed cleaving the lignin–carbohydrate covalent bonds. At the same time, the possibility of lignin structural changes were minimized by the low concentrations of acid used.

Details of the Enzymatic Hydrolysis Stage

Gravimetric yield and lignin purity data for three different series of enzymatic hydrolysis experiments (E1, E2, E3) are shown in Table I. Experimental series E1 and E2 were carried out under otherwise identical conditions with the exception that a water bath equipped with an orbital shaker was used for series E1, while mechanical stirring was used during series E2. In relation to experiments in series E3, the conditions were similar to those of series E1, with the exception that in series E3 the amount of enzyme used was double since it was of lower activity (750 units/mL).

As implied by the data that describe experiments in series E1, a charge of Novozym

TABLE I
YIELD AND PURITY OF ENZYMATIc RESIDUES FOR THREE SERIES OF EXPERIMENTS

Series	Weight of Solid Residue from Enzymatic Hydrolysis (g)	Klason + UV Lignin in Solid Residue (%)	Lignin Yield ¹ (%)
E1	1.22	32.8	93.5 ± 2.1
E2	2.16	17.9	90.3 ± 1.7
E3	1.35	27.1	85.6 ± 0.9

All data shown are the average of duplicate experiments. 10 g of oven-dry pulp were used for each experiment.

The Klason + UV lignin content in the starting kraft pulp was 4.28%.

1. The lignin yield was calculated as (Klason + UV lignin in residue) / (Klason + UV lignin in pulp) × 100%.

TABLE II
THE YIELD AND PURITY OF LIGNINS ISOLATED AFTER THE TWO-STAGE ENZYMATIC/ACIDOLYSIS ISOLATION PROCEDURE

Series	Weight of Purified Lignin ¹ (g)	Klason + UV Lignin Content (%)	Lignin Yield (based on solid residue) ² (%)	Lignin Yield (based on pulp) ³ (%)	Protein ⁴ (%)
E1	0.316	96.8 ± 0.4	82.0	71.5 ± 1.4	2.3
E2	0.330	93.0 ± 0.7	79.4	71.7 ± 0.6	2.9
E3	0.282	94.9 ± 0.2	73.0	62.5 ± 1.8	8.8

All data shown are the average of duplicate experiments.

10 g of oven-dry pulp were used for each experiment.

1. After dichloromethane extraction to remove the extractives.

2. The lignin yield on solid residue was calculated as (Klason + UV lignin in final product)/(Klason + UV lignin in solid residue) × 100%.

3. The lignin yield based on pulp was calculated as (Klason + UV lignin in final product)/(Klason + UV lignin in pulp) × 100%.

4. The protein content calculated by multiplying the nitrogen % by 6.25.

(360 units/g of oven dry softwood pulp, over a period of 48 h) offers an enzymatic hydrolysis rate that degrades about 88% (10–1.22 g) of it, with a solid residue which amounts to 12.2% of the starting material, containing 32.8% klason lignin. About 93.5% of the residual lignin present in the original pulp was recovered after the enzymatic treatment. The mild enzymatic treatment caused the weight of the sample to be reduced by about 88% while it was heavily swollen in aqueous media; this indicated that the overall cellular structure of the material was highly exposed.

A comparison of experiments E1 and E2 shows the importance of uniform mechanical agitation. When a mechanical stirrer was used in series E2, more solid residue with lower purity was obtained, pointing to a somewhat lower efficiency of the enzymatic treatment. This lower efficiency could be due to a lower degree of aeration induced with the mechanical agitation as opposed to that offered by the orbital shaker. Despite the lower purity of the solid residue in experiments E2, the total lignin amount recovered was only slightly less than that recovered from experiments E1.

In an effort to examine the effect of the enzyme charge, experimental series E3 were carried out using double the amount of enzyme with a lower purity than in cases E1 and E2. The lignin content of the solid residue obtained from these experiments (27.1%) was lower than that obtained from the E1 series (32.8%). Despite this, however, the total amount of lignin recovered was 85.6% of that originally present on the fibre. This figure, when compared to 93.8% for the E1 series, suggests that when enzymes of lower activity are used (albeit in higher amounts) the hydrolysis efficiency is always lower. The lower lignin yields obtained from the E3 series of experiments precludes the use of these conditions for obtaining a representative sample of lignin. Since an acidolytic purification step is to follow, it was decided that the initial enzymatic hydrolysis should be optimized on the basis of yield with reasonable purity.

Acid Hydrolysis

The lignin-rich samples obtained by enzymatic hydrolysis were then subjected to mild acid hydrolysis and the resulting gravimetric

yields and purity data are shown in Table II.

The yields and purity of lignin preparations isolated in experimental series E1 and E2 (Table II) showed that an acid concentration of 0.05 mol/L, applied on the residue whose cellular structure had been exposed after an enzymatic stage, was sufficient to liberate large amounts of lignin, cleaving lignin-carbohydrate bonds and possibly hydrolyzing remaining sugars. Our data are indicative of the fact that the enzymatic and the acid hydrolysis steps play different roles during the proposed procedure.

The data of Table II further support our working hypothesis by demonstrating the critical role the enzymatic stage plays toward allowing for the release of lignin in high yield and purity. For example, when (in series E3) the enzyme activity was not adequate, the cellular structure of the pulp could not be exposed adequately. Consequently, after the second acidolytic step, the lignin yield was significantly lower (62.5%). Therefore, in order to obtain a lignin with high purity (> 95%) the extent of the first enzymatic step should go as far as series E1.

Since the proposed two-stage method uses a relatively low enzyme/pulp ratio (see series E1 & E2, Table II), the amount of protein contamination within the isolated lignins was rather low (2.3 and 2.9%, respectively). Consequently, there is no need for additional protein purification steps. Our data (Tables I, II) seem to indicate that the protein content within the final lignin preparation is proportional to the amount of enzyme used during the initial enzymatic treatment. For example, the protein contamination in series E3 (where double the amount of enzyme was used) was 8.8%, while it was only 2.3–2.9% for series E1 and E2.

Literature accounts point to the fact that a single 48 h cellulase enzymatic treatment followed by various purification procedures allows a residual lignin yield in excess of 80% [12]. These preparations are known to contain 5–10% of carbohydrates and substantial amounts of proteins [12]. In addition, the presence of lignin-carbohydrate bonds has been documented [5,19]. Furthermore, it was shown that repeated enzymatic treatments could not reduce the amount of carbohydrates further [8, 20].

In contrast, the residual lignin preparation obtained by the proposed two-step method is of considerably higher purity (93–97%, Table II) indicative of low carbohydrate and protein contamination. Furthermore, the purified lignin yields obtained via the proposed procedure were about 72%.

Yield losses were found to occur during both the enzyme hydrolysis and the acid hydrolysis steps. For example, for series E1 about 93.8% of the lignin present on the fibre was recovered after the initial enzymatic hydrolysis (Table I). However, 82% of the lignin present on the enzymatic hydrolysis residue was isolated after the mild acidolysis stage. This yield, considering the intricate nature of the lignocellulosic matrix, can be regarded as relatively high.

Compared to the conventional acidolysis method, the proposed two-stage procedure offers improved yields (by about 25–35%). In addition, the lower acid concentration used during the acidolysis stage, (0.05 m) reduces the possibility of structural modification induced on the lignin. Both of these facts contribute toward isolating a more representative sample of lignin from the starting pulp. Work aimed at further validating these statements and extending them to hardwoods is currently in progress in our laboratory.

Lignin Structural Analysis

The structure of selected lignin samples was compared using quantitative ³¹P NMR spectroscopy. More specifically, we examined a sample isolated by the standard acidolysis procedure (AL) and a sample isolated by the proposed two stage enzymatic/acidolysis method (EAL). Furthermore, in an effort to ascertain the structural differences observed between the aforementioned two samples, we subjected sample EAL to an additional acidolytic treatment using 0.1 mol/L HCl in dioxane/water (EAAL). The major hydroxyl groups present in these samples are thus depicted in Fig. 1.

The quantitative ³¹P NMR data shown in Fig. 1 indicate that all examined samples had a similar functional group distribution with the exception of sample AL which showed an obviously higher content of C5-related condensed units. These differences may be due to the stronger acidic conditions used in the acidolysis-only method (sample AL) which may have resulted in condensation reactions. An alternative hypothesis may be that the acid hydrolysis has caused the scission of certain acid-labile linkages in lignin or lignin-carbohydrate linked structures, liberating lignin that is enriched in aromatic moieties containing C5-related condensed phenolic hydroxyls.

Since the quantitative ³¹P NMR data have shown very similar amounts of condensed units for samples EAL and EAAL, this demonstrates clearly that the acidolysis stages are not responsible for the condensation. This is in agreement with the work of Jiang and Argyropoulos [2] who have shown that the acidic conditions of the traditional batch acidolysis procedure do not cause condensation in the lignin when compared to samples ob-

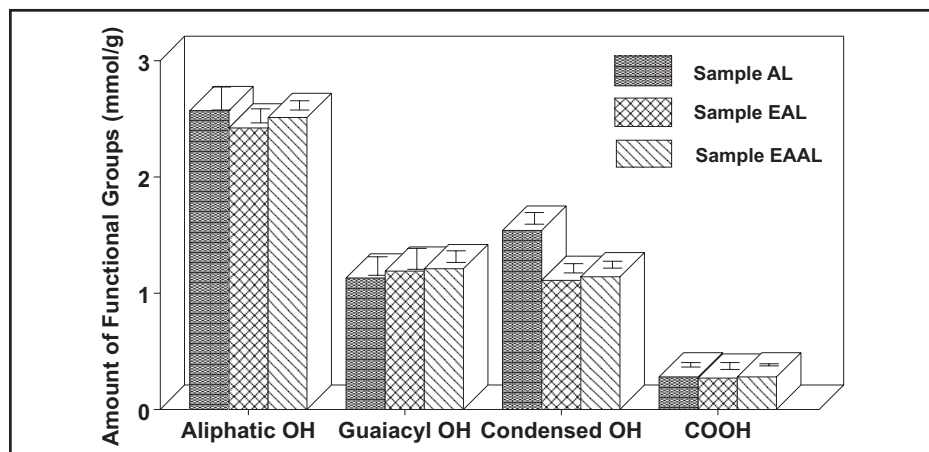


Fig. 1. The amounts (mmol/g) of the various hydroxyl groups present in softwood residual lignin isolated and treated by different methods.

TABLE III
PURITY AND YIELD DATA OF LIGNINS ISOLATED FROM
THE THREE-STAGE ISOLATION PROCEDURE

Lignin Isolated from Initial Mild Acidolysis			Lignin Isolated from Enzymatic and Acidolytic Hydrolyses			Total Purified Lignin ¹		
Weight (g)	Purity ² (%)	Yield ³ (%)	Weight (g)	Purity (%)	Yield (%)	Weight (g)	Purity (%)	Yield (%)
0.86	89.8	43.6	0.65	90.0	33.2	1.31	89.7	66.3

All data shown are the average of four experiments.
40 g of oven-dry pulp were used for each experiment.
The Klason + UV lignin content in the starting kraft pulp was 4.42%.

1. After dichloromethane extraction to remove the extractives.
2. Purity is determined by Klason and UV analyses.
3. Lignin yield was calculated as (Klason + UV lignin in product) / (Klason + UV lignin in pulp) × 100%.

tained using a flow-through reactor [2]. Despite the fact that sample EAAL was subjected twice to acidolysis conditions (a mild one at 0.05 mol/L HCl and a stronger one at 0.1 mol/L HCl), its condensed phenolic OH content was found to be less than that of the sample AL, isolated using the traditional acidolytic procedure. It is therefore, very likely that the residual kraft lignin isolated by the traditional acidolysis method is representative only of a limited fraction of the lignin present in kraft pulp. This contention is also supported by the relatively low lignin yields the procedure affords. For example, in our work the yield of the procedure offers gravimetric yields ranging from ~35–45%. Hortling and Tamminen support our conclusion by quoting a yield of 42.5% [21]. It is thus very likely that traditional acidolysis conditions afford the isolation of a particular lignin fraction enriched in C5-related condensed moieties. In this respect, the recent studies of Kleen, who used surface-sensitive analytical techniques (TOF-SIMS) to examine softwood pulp fibres, are highly relevant [3]. More specifically, these measurements showed that the residual lignin present on the outermost surface of unbleached kraft pulp fibres structurally resembles that isolated by the acidolysis method. Kleen's data, when coupled with the lower yields of the acidolysis method [21], the yield data of Tables I and III, and the structural data of Fig. 1, tend to suggest that acidolysis resid-

ual lignin is representative only of the "accessible" residual lignin present on kraft pulps. Furthermore, detailed analyses of the amounts of remaining β -O-4 ethers using DFRC/³¹P NMR [23] showed that the acidolysis lignin contained about 13% less β -O-4 ethers than either the enzymatic preparation or the two-step protocol proposed in this paper [24]. These data imply that the traditional acidolysis conditions are effectively cleaving these ethers while they remain more intact during the enzymatic or the two-step procedures.

Compared to the conventional acidolysis method, the proposed two-stage procedure offers improved yields (by about 25–35%). These field improvements seem to apply equally well to softwoods and hardwoods. However, our detailed efforts on hardwoods are to be published independently. Further efforts are currently in progress in our laboratory to ascertain whether the lignin isolated with the two-stage method is structurally more representative of the lignin in the starting pulp than that obtained by the traditional acidolysis procedure [23].

CONCLUSIONS

When kraft pulp is subjected to cellulolytic enzymatic hydrolysis or to acid hydrolysis conditions, different events occur causing the release of different residual kraft lignin fractions. It was shown that a certain lignin fraction can be released from kraft pulp

only after it has been exposed to a cellulolytic enzymatic treatment. For this reason, we propose a new isolation procedure composed of an initial mild enzymatic hydrolysis stage followed by a mild acid hydrolysis stage. This was found to be an effective way to isolate highly pure residual kraft lignin (Klason and UV soluble content >96%) at a relatively high yield (~70%). While no obvious lignin structural changes were found to occur during the mild acid hydrolysis process, the extent of the initial enzymatic stage was found to be critical to the success of the procedure.

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REFERENCE: ARGYROPOULOS, D.S., SUN, Y. and PALUŠ, E., Isolation of Residual Kraft Lignin in High Yield and Purity. *Journal of Pulp and Paper Science*, 28(2):50–54 February 2002. Paper offered as a contribution to the journal of Pulp and Paper Science. Not to be reproduced without permission from the Pulp and Paper Technical Association of Canada. Manuscript received October 13, 2000; revised manuscript approved for publication by the Review Panel May 25, 2001.

KEYWORDS: ALKALI LIGNINS, KRAFT PULPS, ISOLATION, YIELD, PURITY, PROCESSING, ENZYMATIC ACTIVITY, ACIDOLYSIS, HYDOLYSIS, NUCLEAR MAGNETIC RESONANCE.