Chapter 2

THE DFRC (DERIVATIZATION FOLLOWED BY REDUCTIVE CLEAVAGE) METHOD AND ITS APPLICATIONS FOR LIGNIN CHARACTERIZATION

Fachuang Lu* and John Ralph
Department of Biochemistry and Great Lakes Bioenergy Research Center, Wisconsin Energy Institute, University of Wisconsin-Madison, Madison, WI, US

ABSTRACT

Lignin is perhaps the most complex natural polymer present in terrestrial plants. Characterization of lignin is important and challenging because of the significance (in various fields) and complexity (compositional and structural) of lignin. The DFRC method was developed as an analytical procedure for lignin characterization that differed from the existing hydrolytic methods. It operates via selective cleavage of the most important β-O-4-linkages in lignin, producing monomers and dimers for ready analysis by GC or GC-MS. It has been used for the characterization (compositional and structural) determination of lignins, including for structural identification in the area of lignin biosynthesis, delineating biochemical processes in lignocellulosics (including in various mutant and transgenic plant lines), and in food plant research. One unique feature is that lignin γ-esters (naturally occurring in many plant species) are not cleaved during the protocol so that natural lignin acylation can be detected. A modified version of the DFRC method has even been used to detect naturally occurring acetates on lignin. Detection and identification of the unique β-β-coupled DFRC dimers bearing γ-acetates that derive from non-resinol tetrahydrofuran structures in kenaf lignin confirmed that acetylated lignins result from the participation of the pre-acetylated monolignols, primarily sinapyl γ-acetate. Furthermore, in combination with other analytical tools, DFRC analysis has provided informative data about lignin structures and the lignin biosynthetic pathway.

Keywords: Acetyl bromide, biosynthesis, lignin acylation, lignocellulosics, monolignol

* Corresponding author: E-mail: fachuanglu@wisc.edu, phone (608)-890-2552.
INTRODUCTION

The term lignin was first attributed to the residual carbon-rich substance from wood after acid treatment. Due to the complexity of lignin in nature, it may be more appropriate to use “lignins” in most situations because lignin never means a single compound but instead represents a class of aromatic biopolymers with diverse structures [1]. It has never been as easy to define lignin as for other natural polymers [2]. Lignins are mainly derived from three monomeric precursors (monolignols), namely coniferyl alcohol, sinapyl alcohol, and p-coumaryl alcohol, via an oxidase-mediated free-radical coupling process, in which the main reaction is a so-called endwise coupling of a monomer (radical) with the phenolic (radical) end of the growing polymer. Due to combinatorial coupling, a variety of linkages are formed between structural units [3]. The structural complexity of lignins comes from the irregularity in linkage ‘sequences’ between units, a variability in both composition and structure, and its association with other cell wall components such as polysaccharides or hydroxycinnamates (the latter especially in grass plants) [4], as illustrated a hypothetical lignin model in Figure 1.

As one of the major and crucial components of plant cell walls, the lignin polymer is often purported to be the second most abundant biomaterial on the earth, and constitutes ~15-25% of woody plant biomass. Lignin has very important biological functions such as providing structural rigidity to the plant body, enabling the transport of water and solutes through the vascular system, and serving as a defensive system against herbivores and pathogens [5, 6]. Although lignin is necessary to most classes of land plants, it is a problem in chemical pulping processes where wood pulps (cellulose fibers) are produced from wood chips for making paper or cellulose products, and lignin is removed as a waste or low value co-product. The pulp and paper industry produces millions of tons of such “waste” lignins annually, although it has significant value to the process, particularly in kraft or soda pulping where almost all is burned in order to recover the pulping chemicals and to provide energy [7]. With the increased interest in converting lignocellulosic biomass to fuels or chemicals, even more lignin could be available, offering potentially significant opportunities to use this most abundant renewable aromatic natural resource for production of chemicals and materials. Therefore understanding lignin structure becomes extremely important for various purposes ranging from plant breeding and genetic alteration to the ultimate use of lignocellulosics [8].

Structural analysis of lignin polymers is a crucial yet challenging task. Our knowledge of lignin structure has relied on the development of analytical methods and evolved slowly until the 1970’s when many modern methods and analytical instruments were invented [9]. Chemical degradation methods for lignin analysis had been the only way to provide important informative details about lignin structure before the non-degradative spectroscopic methods, including powerful NMR methods, were developed. Unlike other natural polymers such as proteins, polysaccharides, and nucleic acids, which contain totally degradable (enzymatically or chemically) interunit linkages, lignin contains resistant carbon-carbon and diphenyl ether bonds [10, 11]. It is impossible to completely and selectively degrade lignin polymers by methods developed so far into monomeric products due to those resistant linkages. Therefore all known degradation methods only provide insight into particular aspects of the lignin structure and a combination of methods is generally necessary to obtain more complete information [12, 13]. Although having limitations such as the low yield of measurable
degradation products, potential interference from contaminants, and side reactions, analytical degradation methods are still widely used for the analysis of the composition and structure of lignins because they have high sensitivity (require only limited amount of sample), high throughout capability, and require no expensive instrumentation. Among these widely used degradative analytical methods for lignin characterization, the DFRC (Derivatization Followed by Reductive Cleavage) method has been used for the characterization and compositional determination of lignins, including for structural identification in the area of lignin biosynthesis, delineating biochemical processes in lignocellulosics (including in various mutant and transgenic plant lines), and in food plant research.

In this chapter, the following aspects of the DFRC method will be reviewed: 1) Development of the DFRC method: chemical reactions, procedure; 2) Important features of the DFRC method; 3) Applications: compositional analysis of lignins in various plants, elucidation of biosynthetic pathways, characterization of various naturally acylated lignins, and analysis of genetically modified lignins.

Figure 1. A hypothetical lignin model showing the major types of interunit linkages and functionalities. Structures shown are only indicative and do not reflect the real frequency in any lignin.

Figure 2. The chemical transformations involved in the DFRC method, and structures of the primary lignin-derived H, G, and S DFRC monomers.
THE DFRC METHOD

Reactions Involved in the DFRC Method

The establishment of the DFRC method was based on the observation that acetyl bromide (AcBr) in acetic acid reacts with β-aryl ether lignin models producing β-bromo-ethers in very high yields [14], and the well known chemistry that β-bromo-ethers can be cleaved by two-electron reduction [15-17], in this case metallic zinc in acetic acid, to form alkenes (Figure 2). It was named the DFRC method as an acronym for the chemistry involved (Derivatization Followed by Reductive Cleavage) and the institute, the US Dairy Forage Research Center (for which DFRC is also the acronym) where the method was developed in 1997 [18].

The ability of AcBr in acetic acid to dissolve lignocellulosic materials has been recognized and used for the determination of lignin content in plant cell walls by UV absorbance spectroscopy [19-21]. However, under mild conditions the high selectivity and efficiency of AcBr reactions with β-aryl ethers of lignin were not discovered until our model studies revealed that AcBr cleanly acetylates primary alcohols and phenols, and converts the benzylic alcohols or ethers of β-aryl ether lignin model compounds to benzylic bromides [14]. We thought that the reactions between AcBr in acetic acid and β-ether lignin models could be applied to lignocellulosic samples because dissolution of lignocellulosic samples in AcBr-acetic acid should allow homogeneous reactions. Our NMR examination of the lignocellulosic samples reacted with AcBr in acetic acid confirmed that β-ether structures, the major β-O-4-linked units of lignin, react with AcBr, as the model compounds do, producing the expected β-bromo-ether derivatives with high yields [22] (Figure 3).

Another important contribution to the DFRC method came from the recognition and implementation of the reductive cleavage of β-bromo-ether derivatives of lignin by zinc in aqueous acetic acid/dioxane solvent in high efficiency. Although this kind of organic transformation is well documented in textbooks of organic chemistry, it was novel to apply this chemistry to β-bromo-ether derivatives produced from AcBr reactions with lignin, resulting in high yielding cleavage of β-ethers. Thus following two consecutive (AcBr and zinc) treatments in one pot, the β-ether lignin model compounds were broken down into monomeric units in very high yields (93-95% for all models investigated) [23]. When applied to lignin, this process cleaved β-ethers of lignin releasing monomers, dimers and higher oligomers; the monomers and dimers are suitable for analysis by GC [24, 25] (or HPLC [26]) and GC-MS [27] methods. Perhaps the most novel and interesting aspect of the DFRC method is that the primary monomeric products of this depolymerization are the lignin monomers themselves (acetylated), i.e., it is probably the only process available that can, in modest yield, convert lignin back to its component monolignols.

The DFRC Procedure

The DFRC method consists of three steps: AcBr treatment, reductive cleavage, and acetylation. A detailed description of the DFRC method was first published in 1997 [18]. Although there have been a few changes to the protocol, some users communicated with us pointing out that more specific descriptions for each steps may help to perform the method in
order to obtain more consistent results. In this section we update the DFRC protocol and make comments where appropriate.

Figure 3. $^{13}$C NMR spectra of bromo-ether model compounds A) D2 and C) T2, and bromo-derivatives from AcBr reactions with B) loblolly pine lignin and D) kenaf lignin demonstrate the high selectivity and efficiency of AcBr reaction with $\beta$-aryl ethers in lignins.
Chemicals and reagents: AcBr, 1,4-dioxane, acetic acid, acetic anhydride, pyridine, zinc dust or zinc nanopowder, were used as supplied. Analytical reagent grade solvents were used without further purification. AcBr solutions (solution A): AcBr/acetic acid, 2/8, v/v; 1,4-dioxane/acetic acid solution (solution B): 1,4-dioxane/acetic acid/water, 5/4/1, v/v/v; acetylation reagent: acetic anhydride/pyridine, 1/1, v/v; internal standard: 4,4'-ethylidenebisphenol or tetracosane (C24).

Protocol:
1. Solution A, 2.5 mL, is added to a 10 mL round bottom flask containing 5 mg of sample (lignocellulosic or lignin-containing biomass) and a magnetic stir bar.
2. After adding internal standard (the amount of internal standard should be reasonably matched to the amount of the lignin quantified [18]), the flask is sealed with a cap or stopper and heated in an oil or sand bath to 50 °C with gentle stirring for 3 h.
3. The stir bar is taken out. The solvents and reagents are removed via a rotary evaporator at 50 °C in less 10 min (to avoid decomposition of the acetylated bromo-derivatives of lignins).
4. Solution B, 2.5 mL, is added into the flask along with a stir bar. The mixture is well stirred while 50 mg zinc dust is added. Stirring is continued for 30 min.
5. The solution is completely transferred with 10 mL CH2Cl2 into a 30 mL separatory funnel and 10 mL saturated NH4Cl solution is added. The pH value of water phase is adjusted to less than 3 by adding 1 M HCl.
6. The separatory funnel is shaken for 10 s and the separated organic lower phase is collected. The water phase is extracted twice more with CH2Cl2 (2x 5 mL). The combined organic phases are dried over anhydrous MgSO4, filtered and evaporated at 40 °C under reduced pressure to dryness, usually producing an oily residue.
7. The residue is acetylated with 0.5 mL acetylation reagent in 2 mL CH2Cl2 for 40 min. Ethanol, 5 mL, is added, mixed, and evaporated under reduced pressure at 50 °C. This operation is repeated (3-5 times) until all volatile components are totally removed (no acetic acid or pyridine can be smelled).
8. (optional) The residue is loaded with 0.1 mL CH2Cl2 onto a normal-phase (silica gel) SPE column (3 mL) and eluted with 12 mL hexane/ethyl acetate (5/1, v/v). The collected solution is evaporated at 40 °C under reduced pressure to dryness.
9. The residue is dissolved in CH2Cl2 and used for GC analysis. The volume of CH2Cl2 used here can be adjusted according to the peak height/area of the lignin monomers in GC-FID or GC-TIC profiles [18].

GC conditions: column 0.20 μm x 30 m SPB-5 (Supelco); He was the carrier gas, injector 250 °C, initial column temperature 140 °C, ramped at 3 °C/min to 240 °C, hold for 1 min; then ramped at 30 °C/min to 310 °C, hold for 12 min.

The typical GC-FID chromatography is shown in Figure 4. Lignin-derived monomers (H, G, S) are quantitated by using the following equation:

\[
\text{Monomer Yields (wt) %} = 100 \times R_f \times (A_S \times W_{IS})(A_{IS} \times W_S)
\]

Where \( R_f \) is the GC response factor for individual monomer (H, G, S): 1.76, 1.85 and 2.06 for H, G and S respectively when tetracosane (C24) is used as IS; 1.43, 1.37 and 1.46 for
H, G and S respectively when 4,4'-ethylidenebisphenol is used as IS. A_S and A_IS are peak areas of monomer and IS respectively. W_S and W_IS are weight of sample used and IS added. Normally the weight% yields are converted to μmols/g lignin according to lignin content (Klason in most cases) of sample and molecular weight of the monomer: 234 for H, 264 for G, and 294 for S.

**FEATURES OF THE DFRC METHOD**

In general, most degradation methods for lignin compositional or structural analysis are based on cleavage of β-ether bonds, the major interunit linkages in lignin. The DFRC method efficiently cleaves β-ether bonds in lignin producing monomers and dimers that are amenable to GC [24] or HPLC [26] analysis. However, the DFRC method is different from other established methods such as the acidolytic methods (e.g., acidolysis, thioacidolysis) or high-temperature base method in terms of the mild conditions used and chemistries involved. Due to unique features of the DFRC method, it has provided new insights into structural features of lignin.

The cinnamyl acetates produced by DFRC degradation are characteristic products from the β-ether cleavage. For example, DFRC monomers (hydroxycinnamyl acetates, H, G and S) come from lignin units linked by one β–O–4-ether bond (for phenolic end units) or two β–O–4-ether bonds (for internal units) whereas the 4–O–β-linked cinnamyl alcohol or aldehyde end units result in other types of products [28]. Therefore the isolated 5–5 or 5–O–4 DFRC dimers from loblolly pine implicated the existence of the 5–5- or 5–O–4-structures where two β–O–4-linked units are connected via a 5–5 C–C bond or 5–O–4 di-aryl ether linkage (Figure 5). Except for thioacidolysis, no other degradation methods have this feature.

![Figure 4. Old original FID gas chromatograms of lignin-derived DFRC monomers from isolated lignins.](image-url)
The mild conditions used by the DFRC method allow some normally sensitive structural features to survive the $\beta$-ether cleavage procedure. Benzodioxane structures derived from 5-OH-coniferyl alcohol monomer found in COMT-deficient plants remain totally intact following the DFRC degradation procedure [29], for example. Thus the $\beta$–O–4-linked benzodioxane structures can be detected and determined by the DFRC method (see Application Section for detail). The fact that benzodioxane dimers and even trimers have been isolated from the DFRC products of a COMT-deficient poplar wood suggests that 5-OH-coniferyl alcohol, as a monolignol, participates in the lignification process forming lignins with the unique benzodioxane structures [29]. Isolation and identification of the novel aryl isochroman lignin trimers and related dimers from DFRC degradation products of loblolly pine wood suggested that these isochroman structures may be present in lignin and they partially survive of DFRC degradation [30]. 2D NMR examination of a isolated milled wood lignin (MWL) sample indicated that the diagnostic HMQC correlations ($\delta_C$ 41.3/\$\delta_H$ 3.60) and the complete sidechain correlations in TOCSY spectrum are consistent with those from the authenticated isolated isochroman products [31]. Although the identification of the aryl isochroman structure in the MWL can be made firmly, whether arylisochromans are present as such in native lignins is not yet clear. They could be produced during the isolation procedure and the precursor, the spirodienone, may be the in situ product, as has been proposed [32, 33]. (Figure 6)
The most important feature or advantage of the DFRC method is that the naturally occurring esters on the primary alcohol (\(\gamma\)-position) of lignin units remain totally intact following DFRC degradation. Using the DFRC method we were therefore able to detect and measure \(p\)-coumaroylated units in grass lignins confirming that \(p\)-coumarates are truly attached to the \(\gamma\)-position of lignin structural units [34]. With a modified DFRC (DFRC') method that uses propionate reagents (propionyl bromide/propionic acid/propionic anhydride) to replace all acetate analogues, the naturally occurring acetates on lignin have also been detected and determined [35]. More importantly identification, from the DFRC’ degradation products of kenaf lignin, of \(\beta-\beta\)-linked syringyl dimers bearing \(\gamma\)-acetates has provided compelling evidence that acetylation of kenaf lignin occurs not only on the \(\gamma\)-position, but through radical coupling reactions of acetylated monolignols [36, 37]. Thus, as will be discussed later in this chapter, acetylated monolignols, and acylated monolignols in general, are authentic lignin ‘monomers’ in many species.

Another feature of the DFRC method is its flexibility. With modifications by introducing appropriate reagents or chemical reactions the DFRC method can be used beyond the original scope of application. For instance, with pre-methylated lignin samples and incorporation of a propionylation step to replace the last acetylation step in the standard DFRC procedure, it was possible to detect and measure lignin units involved in phenolic end-units, \(\alpha\)-ethers, and internal \(\beta\)-ether-linked units [38], comparably with the methylation followed by thioacidolysis method [8]. Free-phenolic units could be distinguished from their etherified counterparts, however, even more simply and without the introduction of any new chemistry or new
products (other than those trivially different and readily distinguished by MS methods) to be analyzed, by using deuterated acetyl reagents and solvents (CD$_3$COOD, and (CD$_3$)$_2$O) for the DFRC method. We were therefore able to detect lignin-derived DFRC degradation products from (presumably polymeric) lignin-like substances in the red alga, *Calliarthron cheilosporioides* [27]. The DFRC method has also been used along with $^{31}$P NMR to measure $\beta$–O–4-linkage levels in various lignins [39]. Because the DFRC method does not change the stereochemistry of $\beta$-carbons on side-chains of $\beta$–5 and $\beta$–$\beta$ structures in lignin, isolation of the optically inactive $\beta$–5 and $\beta$–$\beta$ DFRC products was used to more firmly establish the racemic nature of lignin [40].

**APPLICATIONS OF THE DFRC METHOD**

**Compositional Analysis of Lignin**

As one of the major components in woody biomass, lignin is the primary obstacle for utilization of the polysaccharides in lignocellulosics. It is well known that lignin is responsible for much of the recalcitrance of biomass to enzymatic hydrolysis and affects chemical pulping efficiency. For example, lignin content greatly influences forage digestibility [41, 42], and wood containing lignin with higher ratios of syringyl/guaiacyl (S/G) units is more easily delignified and provides higher pulp yields. Therefore the structural composition of lignin is one of the important characteristics for plant breeding or selection in order to obtain woody biomass with better quality for pulp production or bioconversion to fuels and chemicals.

Many degradation methods have been used for lignin compositional analysis. The traditional and the oldest method is nitrobenzene oxidation (NBO) in alkaline solution. A detailed discussion about NBO can be found in Chapter 3 of this book. This method uses harsh conditions and is not suitable for grass materials due to interferes from hydroxycinnamates. Thioacidolysis [8] is another heavily used method for lignin compositional analysis and remains the premier method for structural studies; laboratories have become comfortable with the use of the required malodorous ethanethiol, and a recent micro-method is aiding large-scale and robotic analysis [43, 44]. The DFRC method was initially developed to provide an alternative for analysis of lignin composition and structure but thioacidolysis remains the premier method; as will be shown in subsequent sections, however, DFRC has unique attributes that makes it a particularly valuable method for some analyses.

When lignocellulosic materials are degraded by the DFRC method, the primary lignin-derived monomers released are essentially 4-acetoxycinnamyl acetate ($p$-hydroxycinnamyl peracetate, H), 4-acetoxy-3-methoxycinnamyl acetate (coniferyl peracetate, G), and 4-acetoxy-3,5-dimethoxycinnamyl acetate (sinapyl peracetate, S) (Figure 4); *trans*-isomers predominate. Listed in Table 1 are yields of lignin monomers released from DFRC degradation of several isolated lignins from softwood (loblolly pine), hardwood (willow, aspen), and non-wood (kenaf, bamboo, and bromegrass) materials. As suspected and revealed previously [11], pine (softwood) lignin is more condensed than willow or aspen (hardwood) lignins, i.e., higher yields of monomers are obtained from willow or aspen lignin than from pine lignin. Kenaf lignin has an extremely high S/G ratio and a correspondingly high $\beta$–O–4-
ether content [45], which result in very high yields of monomers from DFRC analysis. Compared to thioacidolysis, DFRC yields from kenaf and willow lignins are higher whereas DFRC yield from pine lignin is much lower. This is in accord with the results from our previous model study [23]. However, these comparison results may due to the non-optimized thioacidolysis conditions and/or to the natural esters present in these lignins that are fully retained in DFRC but may be only partially hydrolyzed in thioacidolysis [46]. Later studies revealed that the monomer yields from the DFRC method are generally lower than those from thioacidolysis but the S/G ratios are consistent with each other [38].

In addition to the major DFRC monomers derived from the lignin units connected by the dominant $\beta$–O–4-ether bonds, about 40 other minor monomers originating from other structures have been identified, as shown in Figure 7. Basically those minor peaks can be grouped into three categories (4–O-etherified end groups, $\alpha$-carbonyl units, de-methylation products) based on their origins. Regrettably, these minor units in lignin do not react selectively to produce single major products as do normal $\beta$-ether units [24]. Therefore quantitating such units is more difficult, but qualitative comparisons remain valuable.

Like other $\beta$-ether cleavage methods, the yields of released monomers are correlated to the levels of $\beta$-ether linkages in lignin. The yield of total monomers determined by the DFRC method has been used to characterize lignin preparations isolated by various isolation procedures in order to optimize isolation conditions or to establish a new isolation method [48-50]. Application of the DFRC method has shown that a significant decrease in $\beta$-aryl ether linkage content occurs in the early decay stages of wood biodegradation. The characterization by the DFRC method of the residual lignins in Pinus taeda and E. grandis biodegraded by Ceriporiopsis subvermispora suggested that an extensive depolymerization of the lignin occurred before its mineralization became significant [51]. It has been long thought that brown-rot fungi modify lignin without degrading it significantly. Using the DFRC method combined with NMR techniques, Yelle et al. demonstrated that brown rotting of spruce wood by Gloeophyllum trabeum resulted in dramatic decrease in lignin $\beta$-aryl ether content, implicating extensive degradation of lignin sidechains [52].

### Table 1. Yields of the main DFRC H, G and S monomers released from various isolated lignins, and the thioacidolysis monomer yields for comparison

<table>
<thead>
<tr>
<th>Lignin samples</th>
<th>DFRC yield$^a$ (wt%)</th>
<th>Molar yields ($\mu$mol/g lignin)</th>
<th>Monomer Distribution H/G/S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DFRC</td>
<td>Thioacidolysis</td>
<td></td>
</tr>
<tr>
<td>pine</td>
<td>17.13</td>
<td>651</td>
<td>1055$^b$</td>
</tr>
<tr>
<td>willow</td>
<td>20.58</td>
<td>732</td>
<td>597</td>
</tr>
<tr>
<td>aspen</td>
<td>19.62</td>
<td>696</td>
<td></td>
</tr>
<tr>
<td>kenaf</td>
<td>36.43</td>
<td>1258</td>
<td>967</td>
</tr>
<tr>
<td>bamboo</td>
<td>21.18</td>
<td>781</td>
<td>1086$^b$</td>
</tr>
<tr>
<td>bromegrass</td>
<td>22.04</td>
<td>800</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Yields are based on sample weights and not corrected for lignin content. $^b$ Yields are from reference [47].
Fachuang Lu and John Ralph

Figure 7. Vertically expanded FID GCs of monomeric products from pine and willow lignins, showing minor identifiable peaks whose assignments are listed at the top. cPr: cyclopropyl; P: 4-acetoxyphenyl; G: 4-acetoxy-3-methoxyphenyl; S: 4-acetoxy-3,5-dimethoxyphenyl; S': 3,4-diacetoxy-5-methoxyphenyl.

As the DFRC monomers are diagnostic of β-aryl ethers in lignin, detection of such monomers from polymeric materials (extractive-free) is an excellent indicator for the existence of lignin. With the DFRC method, we were able to detect the lignin-derived monomers from a sample of red alga Calliarthron cheilosporioides and demonstrate with a modified DFRC method using deuterated reagents that most of these lignin-derived monomers are released from etherified internal units of the lignin polymer (Figure 8) [27]. These results combined with those from immunocytochemical staining and microscopic examination provided significant evidence for the existence of lignin in the red alga, although its extremely low level leaves its biological origin and role in some contention. Cereal brans are frequently described as highly lignified based on measured Klason lignin content. However, the determination of Klason lignin does not prove that lignin is present because proteins or waxes in plant materials may contribute to acid-insoluble residues, the Klason lignin [53]. To prove the existence of true lignin, the DFRC method was applied to various cereal grain dietary fibers. To avoid the interference from low molecular weight coniferyl alcohol-ferulate crossed dimers, dietary fibers were treated with alkali followed by diethyl ether extraction. When pretreated and solvent-extracted cereal grain dietary fibers were analyzed by the DFRC, the lignin-derived monomers were detected establishing the existence of lignin in these cereal grains [54]. From the S/G ratios of the released monomers, it is suggested that lignin compositions vary among cereals. An excellent analysis of the levels of real lignin vs various other components that may analyze as lignin has been published [55].
The primary DFRC monomers detected and quantified by gas chromatography (GC) are limited to structural units connected by β-aryl ether bonds on both 4-O- and β-positions of the units or phenolic end-units connected via a β-aryl ether bond at their β-positions. However, the combination of DFRC with quantitative $^{31}$P NMR allowed a determination of all β-aryl ether linkages including those connected to condensed and non-condensed aromatic moieties in lignins (Figure 9) [39]. Guerra et al. used this binary technique to investigate the effects of the various isolation conditions on the lignin structure [50]. They found that vibratory-milling conditions cause substantial lignin depolymerization through the cleavage of uncondensed β-aryl ether linkages whereas rotary ball milling provides milder mechanical treatment producing lignin preparations with less alteration in lignin. Enzymatic mild acidolysis lignins (EMALs) from different woody species were characterized by DFRC/$^{31}$P NMR, revealing that the amounts of condensed and non-condensed β-aryl ether structures,
dibenzodioxocins, and condensed and uncondensed phenolic hydroxyl and carboxylic acid groups within lignins vary among different wood species. Specifically, *E. globulus* EMAL contains 2780 μmol/g of β-aryl ether units while EMALs from Douglas fir, white fir, and southern pine EMALs have of 1600, 1490, and 1340 μmol/g of β-aryl ether units respectively [39]. It was also found that lignin from softwood compression wood has lower β-aryl ether levels than normal wood.

Although the yields of released monomers by thioacidolysis are generally higher than those from the DFRC method, and although the total number of β-aryl ether structures in lignin determined by DFRC/31P NMR analysis has a different meaning from thioacidolysis monomer yield, it was interesting to note that the total number of β-aryl ether linkages in EMALs determined by DFRC/31P NMR was in agreement with the thioacidolysis monomer yields [56]. Recently, two MWL samples from spruce and beech wood were analyzed by Crestini et al. using an array of analytical methods including GPC, HSQC, 31P NMR, and DFRC/31P NMR to elucidate the nature and quantitative occurrence of branching units in lignin. It was found that all potential branching points (5–O–4, 5–5, and dibenzodioxocins) are phenolic terminal units implying that MWL preparations examined are linear oligomers without branches [57]; we have commented that lignin branching has likely been over-emphasized [4].

![Figure 9. A combination of the DFRC method and quantitative 31P NMR enables detailed structural information to be obtained.](image-url)
Lignin Biosynthesis Studies

Lignin has been of great interest for researchers with regard to its biological role and potential for desirable modification [58]. Lignin engineering in plants through the genetic modification of its content or composition has been a focus of research due to its impact on industry, agriculture, and the environment [59-61]. Lignin is a racemic heteropolymer produced by the combinatorial oxidative coupling of mainly three \( p \)-hydroxycinnamyl alcohols, the so-called monolignols. It is essential to fully understand or characterize genes regulating biosynthetic pathways to the formation of monolignols so that lignin content or structure can be modified in a beneficial way [62, 63]. To date, most, if not all, of the enzymes involved in the biosynthetic pathways to monolignols have been identified and characterized, with one new one, caffeoyl shikimate esterase (CSE), having just been discovered [64] and one relating to \( p \)-coumaroylation of lignins (PMT) in grasses also recently identified [65]. At least 10 enzymes are required for monolignol biosynthesis in dicots and angiosperms (a few less, the syringyl-specific enzymes, in gymnosperms): phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-(hydroxy)cinnamoyl-CoA ligase (4CL), hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (HCT), \( p \)-coumaroyl shikimate 3-hydroxylase (C3H), caffeoyl-CoA \( O \)-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), ferulic acid 5-hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid \( O \)-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD). Much work remains to better understand how these enzymes function together to determine the diversity of the lignin polymer and the coordinated regulation of their expression during development [63].

In the past, tracer experiments have played important roles in the elucidation of biosynthetic pathways to the formation of monolignols [66]. Tracer experiments using isotope-labeled precursors enable the identification of precursors and intermediates involved in certain biosynthetic pathways leading to monolignols although identification of enzymes involved is required to confirm the pathways implicated by tracer studies [67]. A rigorous study with tracers requires the recovery of readily characterized material and suitable methods for isotopic analysis. Thus most studies on lignification have preferred to degrade lignin in situ to simple and readily analyzed products. Methods used to degrade lignin have been nitrobenzene oxidation, ethanolysis, hydrogenolysis, and thioacidolysis. Nitrobenzene oxidation splits off two of the three side-chain carbons (isotope-labeling on these carbons is lost), but is easier to carry out and gives higher yields of products; Ethanolysis and hydrogenolysis have the advantage of maintaining the entire phenylpropanoid skeleton of the lignin units, but they are difficult and time-consuming to perform. Thioacidolysis produces high yielding lignin products with the entire three side-chain carbons intact, but the thioacidolysis products contain sulfur (and silica atoms after trimethylsilylation for GC-MS analysis), and the presence of natural isotopes of sulfur and silica make it very difficult to identify isotope-labeled lignin-derived monomeric products by EI-MS analysis [68]. The DFRC method therefore had a distinct advantage for characterizing isotope-labeled lignins obtained from tracer experiments because it selectively cleaves \( \beta \)-O-4-ether-linked structures in lignin to produce C6-C3 monomers, retaining the isotope-labeling (proton or carbon) in the side-chains. Identification of these isotope-labeled products becomes easier due to the fact that the DFRC monomers are composed of only C, H, and O atoms, of which each has only low levels of naturally occurring isotopes [69].
In a tracer experiment aimed at confirming a biosynthetic pathway directly from coniferyl alcohol to sinapyl alcohol, Chen et al. administered a deuterium-labeled precursor (pentadeutero $\gamma\gamma$-D$_2$, OCD$_3$ coniferyl alcohol), in which both protons at the $\gamma$-position had been replaced by deuterium, to shoots of magnolia trees. The collected newly formed xylem in which the deuterium-labeled coniferyl alcohol presumably had been incorporated into lignin was analyzed by the DFRC method. The relative labeling of G and S monomers released by the DFRC method from the collected samples was examined by GC-MS [68]. As expected it was found that pentadeuterated G monomer was detected suggesting the incorporation of deuterium-labeled coniferyl alcohol into lignin polymer. However, considerable amounts of tetradeuterated G monomers ($\gamma$-D, OCD$_3$) were also found implying that part of the administered labeled coniferyl alcohol was oxidized to coniferaldehyde, losing one $\gamma$-deuteron, and then reduced back to coniferyl alcohol before being incorporated into lignin. The most significant finding in this study was the detection of the pentadeuterated S monomers ($\gamma\gamma$-D$_2$, OCD$_3$) from the DFRC degradation products, which come from syringyl units, suggesting that the pentadeuterated coniferyl alcohol was directly converted into sinapyl alcohol, and then incorporated into lignin (Figure 10A). Therefore these results demonstrated that the monolignol biosynthetic pathway is highly diversified and that a biosynthetic pathway through coniferyl alcohol might provide an alternative mechanism for the formation of syringyl units in lignin, in addition to the pathways that involve the cinnamaldehydes, cinnamic acids and/or their CoA thioesters.

Figure 10. Isotope tracer experiments with deuterium labeled coniferyl alcohol (A) or sinapic acid (B) combined with the DFRC degradation analysis of newly formed xylem allows identification or confirmation of the biosynthetic pathway leading to the formation of sinapyl alcohol, and thus of syringyl lignin.
In order to clarify the biosynthetic pathway to sinapyl alcohol in angiosperms, Yamauchi et al. fed tetradeuterated ferulic acid-[8-D, 3-OCD₃] and heptadeuterated sinapic acid-[8-D, 3,5-di-OCD₃] to shoots of robinia (*Robinia pseudoacacia*), oleander (*Nerium indicum*), magnolia (*Magnolia kobus* DC.), and *Arabidopsis thaliana* (L.) [69]. The incorporation of the labeled precursors into lignin was monitored by the DFRC method and gas chromatography-mass spectrometry. When heptadeutero-sinapic acid was fed to robinia and oleander, which have 4-coumarate-CoA ligase (4CL) activity toward sinapic acid, syringyl units containing seven deuterium labels were apparently formed as shown by detecting the heptadeutered S monomers released by DFRC degradation of the newly formed xylem. The results of this study supported the traditionally accepted pathway that sinapic acid is converted to sinapyl alcohol through sinapoyl-CoA in robinia and oleander (Figure 10B). By contrast, when fed to magnolia and Arabidopsis, which do not have 4CL activity toward sinapic acid, the labeled sinapic acid was not incorporated into lignin suggesting that syringyl lignin biosynthesis in angiosperms operates via multiple pathways that depend on the species [70].

Hydroxycinnamyl alcohols (monolignols) are generally considered to be direct precursors of lignin, but how monolignols are transported into cell walls remains unknown. Monolignol glucosides have been conjectured to be the storage and transport forms for monolignols [71]. Feeding experiments using radiolabeled coniferin demonstrated that coniferin is efficiently incorporated into lignins. To examine the behavior of monolignol and monolignol glucosides in lignin biosynthesis, Tsuji et al. fed deuterium-labeled coniferyl alcohol and coniferin into growing *Magnolia kobus*, a representative angiosperm with an endogenous coniferin pool, and *Eucalyptus camaldulensis* that does not have one [72]. Lignins with incorporation of these labeled precursors in the newly formed xylems were comparatively analyzed by the DFRC method coupled with GC-MS. Results of this study showed that both precursors are incorporated into lignin and more labeled coniferyl alcohol was incorporated directly into guaiacyl and syringyl units than coniferin in newly formed xylem, whereas more labeled coniferin tended to be incorporated into lignin as tetradeutero units, especially in syringyl lignin in both trees. This means that coniferin is more easily (deglucosylated and) oxidized to coniferaldehyde or its glucoside that is reduced to coniferyl alcohol (incorporated into guaiacyl units) or metabolized to sinapyl alcohol (incorporated into syringyl units) (Figure 11).

In order to further investigate the behavior of glucosides of the hydroxycinnamaldehydes and hydroxycinnamyl alcohols during lignification, tracer experiments were conducted by Tsuji and Fukushima [73]. They fed the radioisotope-labeled and deuterium-labeled glucosides into two year old shoots of magnolia (*Magnolia kobus* DC) and oleander (*Nerium indicum* Mill.) for 12 h. The newly formed xylem in each was isolated and analyzed by microautoradiography or the DFRC method and GC-MS to examine the incorporation of the isotope-labeled precursors into lignin units. Results from this study showed that the aglycone from coniferaldehyde glucoside was efficiently incorporated into guaiacyl or syringyl lignin as a hydroxycinnamyl alcohol unit, i.e., the coniferaldehyde was reduced to coniferyl alcohol (incorporated into guaiacyl units) or converted to sinapyl alcohol (incorporated into syringyl units). It was also found that when coniferin was administered together with coniferaldehyde glucoside, syringyl units were rarely synthesized from coniferin via the cinnamyl alcohol stage, whereas numerous syringyl units were synthesized from coniferaldehyde glucoside. These observations suggest that the coniferaldehyde is crucial for the biosynthesis of syringyl lignin in angiosperms (Figure 11).
Characterization of Acylated Lignins

Some lignins have long been known to be naturally acylated by various acids [74]. Lignins in all grasses (both C3- and C4-grasses) are partially $p$-coumaroylated [75]; some hardwood lignins, notably in willow (Salix) and aspen (Populus), are $p$-hydroxybenzoylated [76, 77]; and acetates have been implicated in many hardwood lignins [78].

Sites of acylation (regiochemistry) become important as the position of attachment of the acyl group suggests its biochemical incorporation pathway [75]. Three pathways (Figure 12) could be responsible for acylation of lignins by acids resulting in two different regiochemistries: a) Acylation at the $\alpha$-position of the lignin side-chain implicates attack by the free acid (nucleophile) on quinone methide lignin intermediates in a purely chemical reaction; b) Acylation at the $\gamma$-position suggests that the traditional hydroxycinnamyl alcohol lignin monomers are first acylated and these acylated monolignols are then incorporated (by traditional radical-coupling mechanisms) into polymeric lignins [79-83]; (c) Acylation by activated acids (e.g., $p$-coumaroyl-CoA) occurs post lignification, resulting in esters at $\alpha$- (Lignin-I) and/or $\gamma$-positions (Lignin-III), depending on the selectivity of the (presumably required) transferase (Figure 12). However, the observation [83] that $p$-coumarates on grass lignins are on many types of units (both isomers of $\beta$-O-4-units, $\beta$-5-units, and even
cinnamyl alcohol end-groups) suggests that enzyme-assisted acylation of the lignin polymer (pathway c) is unlikely – the enzyme would have to be remarkably nonspecific.

Regiochemical determinations in lignins are complicated by acyl migration known to occur with acetates [84] and uronates [85] but not with p-coumarates [75]. p-Coumarates on grasses are exclusively at the γ-positions of lignin side-chains [83, 86]. p-Hydroxybenzoates were originally thought to be partially at the α-position in Populus [76] and bamboo [79, 81, 82], but they may also be (exclusively) at the γ-position as the small amounts of p-hydroxybenzoates released by hydrogenolysis of Populus lignins [76] and attributed to benzylic esters may easily have come from benzoates on the minor allylic cinnamyl alcohol end-groups – p-coumarates were found on such end-groups in maize lignins [83].

As mentioned above, one of the advantages over other lignin analysis methods is that esters on γ-position of side-chain in β-aryl ethers are stable through out the DFRC procedure. Therefore naturally occurring esters attached at the γ-position of the side-chain in β-aryl ether structures of lignin units can be detected and determined by the DFRC method. As illustrated in Figure 13, p-coumarate on lignins from grasses such as corn and bamboo were detected and determined by the DFRC method. Although esters on lignins can be characterized by NMR, the DFRC method is more sensitive and produces more informative data (S/G ratios of the released conjugated esters). It has been found to date that syringyl units of lignins from bamboo, corn, abaca, and curaua are acylated by p-coumarate to a much greater degree than guaiacyl units (Table 2). Interestingly, there are plants, such as wheat, where syringyl p-coumaroylation appears to be very low [87]. Although more syringyl units than guaiacyl units are likely linked by labile β-aryl ethers, the p-coumarate, acylation of sinapyl alcohol by an assumed p-coumaroyl-CoA transferase before its incorporation into lignin, seems more likely to be the main reason. Our recent studies with β-aryl ether models having γ-coumarate [34] to test the efficiency of DFRC for releasing hydroxycinnamyl p-coumarate derivatives (G-pCA and S-pCA, Figure 13) showed that the β-aryl ether linkages in models were cleaved in only about 60-65% yield, much lower than the 95% yields from normal unacylated β-aryl ether models [23]. The DFRC results may therefore have underestimated the p-coumarate levels on β-aryl ethers in lignin. However, S/G ratios of the released acylated monomers seem to objectively reflect the distributions of p-coumarates between releasable syringyl units and guaiacyl units of lignin because studies with model compounds showed that acylation did not differentially affect the release of syringyl or guaiacyl units from β-aryl ethers. In depth studies on these phenomena continue.

Although acetate groups have been recognized as being associated with lignin in kenaf [88] as well as in hardwoods [78], it has been apparently difficult to unambiguously prove their attachment to lignins, especially the regiochemistry of such acetates. Information on natural lignin acetates could have been missed easily from NMR characterization because lignins are frequently acetylated for improved NMR properties or purified using acetic acid [89, 90] when acetylation artifacts might arise. In some cases the very low abundance of acetates on lignin may also prevent NMR techniques from being able to detect acetates. The high content of acetates on lignin from kenaf (Hibiscus cannabinus L.) bast fibers was first reported by our group [45]. Up to 60% of the syringyl units are acetylated in the kenaf lignin sample investigated. In a search for an independent and highly sensitive method to determine acetates on native lignin, we thought that the DFRC could be the one of choice if appropriate modifications could be made to avoid acetylating reagents or solvents in the procedure. It was
found that a simple modified version of the method, DFRC’, in which all acetate-based reagents are replaced by their propionate analogues, allows ready detection of naturally occurring acetates on lignins [35]. Using the DFRC’ method on the kenaf lignin sample used for the previous NMR study, we were able to provide unequivocal new evidence that kenaf bast fiber lignins are indeed highly acetylated on the γ-position of side-chain in lignin units and that such acetylation is disproportionately high on syringyl units (Figure 14).

Figure 12. Three possible pathways leading to the formation of acylated lignins.
Figure 13. A reaction scheme showing how coniferyl/sinapyl dihydro-p-coumarates are released by the DFRC method from maize lignin; the partial FID gas chromatogram confirms the identity of G-pCA and S-pCA in the DFRC products from maize lignin.

Table 2. Yields of monomers and p-coumaroylated monomers from DFRC of bamboo, maize, and bromegrass lignins

<table>
<thead>
<tr>
<th>Lignin Samples</th>
<th>monomers (μmol/g)</th>
<th>p-coumarate products (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>S</td>
</tr>
<tr>
<td>Bamboo</td>
<td>504</td>
<td>222</td>
</tr>
<tr>
<td>maize</td>
<td>342</td>
<td>124</td>
</tr>
<tr>
<td>bromegrass</td>
<td>454</td>
<td>327</td>
</tr>
</tbody>
</table>

* nd, not detected; d, detected by GC-MS.

Following our first demonstration of using the DFRC’ to detect naturally occurring acetates on lignin, Del Rio et al. from Spain have conducted extensive studies on analysis of acetates on isolated lignin samples from various species of plants by using the DFRC’ method [91]. It was shown from these studies that acetylated lignin units were found in the milled wood lignins of all angiosperms investigated, including monocotyledons (sisal, palm tree, bamboo, and abaca) and eudicotyledons (beech, hornbeam, hemp, kenaf, jute, aspen, and eucalypt) but not in the gymnosperms analyzed (spruce and pine). In some plants (e.g., abaca, sisal, kenaf, or hornbeam), lignin acetylation occurred to a very high extent, exceeding 45% of the uncondensed (alkyl-aryl ether-linked) syringyl lignin units. The analyses demonstrated that the structure of the lignins from these plants is highly remarkable, being extensively acylated at the γ-carbon of the lignin side-chain (up to 80% acylation) with acetate and/or p-coumarate groups and preferentially on syringyl units. Whereas the lignins from sisal and kenaf are γ-acylated exclusively with acetate groups, the lignins from abaca and curaua are esterified with acetate and p-coumarate groups [92]. Recent reexamination of lignin acylation
in various plants has also revealed previously unreported acetylated lignin components [87, 93, 94].

Based on NMR evidence found from corn lignin it was suggested that acylation by $p$-coumarate exclusively on the $\gamma$-positions of side-chain of lignin units could result from the participation of the pre-acetylated monolignol [83]. Acetates detected in Kenaf lignin by the DFRC' method were extensively and highly selectively on $\gamma$-position of the lignin side-chain of syringyl units. These findings imply that acetates on lignin derive from pre-acetylated monolignols, especially $\gamma$-acetylated sinapyl alcohol. In order to find more concrete evidence, we examined the lignification mechanisms in plants and found that the $\beta$–$\beta$-coupling of monolignols can be significantly altered by pre-acetylation (or acylation in general) of the monolignols (Figure 15). The key concept is that, with the $\gamma$-position acetylated, $\beta$–$\beta$-coupling or cross-coupling can still presumably occur but the re-aromatization reactions following the radical coupling step can no longer be driven by the internal attack of the $\gamma$-OH on the quinone methide intermediates QM1 (Figure 15, pathway a) – the $\gamma$-acetylation prevents such a reaction to produce resinol D4. Other pathways must therefore be in effect producing other products, compounds D5 and D6 (Figure 15, pathway c or bottom part of pathway b). The important point is that the acetyl group can remain attached in non-resinol $\beta$–$\beta$-coupling products, products that could not have arisen from post-coupling acylation reactions.

Figure 14. The DFRC’ reaction scheme and GC-MS total ion chromatograms of monomers from the DFRC’ (modified DFRC with propionate reagents) procedure applied to kenaf lignin, revealing that the lignin is highly $\gamma$-acetylated, mostly on syringyl units.
The DFRC (Derivatization Followed by Reductive Cleavage) Method …

Figure 15. Dehydrodimerization of sinapyl alcohol produces syringaresinol D4, whereas sinapyl γ-esters results in tetrahydrofurans D6, and cross-coupling of sinapyl alcohol and a sinapyl γ-ester leads to tetrahydrofuran D5.

Finding the unique non-resinol syringyl structures (acetylated D5 and D6, Figure 15) in kenaf lignins established beyond reasonable doubt that acetates on kenaf lignins arise through the incorporation of pre-acetylated sinapyl alcohol, as a lignin precursor, via radical coupling mechanisms. In our preliminary report [36], the β-β-coupled dimeric DFRC’ products D8-D9 containing γ-acetate groups, which were expected to come from the non-resinol syringyl (tetrahydrofuran) structures, were tentatively identified by GC–MS. Later on, all expected diagnostic β-β-coupled DFRC’ degradation products D8-D9, D13 and D14, from those novel tetrahydrofuran structures D5 and D6 in Kenaf lignins were observed and identified by comparison of their mass spectra and GC retention times with those of synthesized authentic compounds [37] (Figure 16). More importantly 2D NMR analysis of the Kenaf lignins allowed the identification of the novel tetrahydrofuran structures, unique substructures that can be formed only from the dimerization of pre-acetylated sinapyl alcohol, in the polymer [37]. So these findings provided strong evidence that acetate groups in kenaf lignin arise from pre-acetylated sinapyl alcohol (pathway b, Figure 12). The other esters (p-hydroxybenzoate and p-coumarate found so far) on lignins are also likely to be formed in this way [95]. Therefore, there must be one or several acyltransferases responsible for acylation of monolignols in such plants. The presumed p-coumaroyl-CoA monolignol transferase has been identified recently [65] and the PMT gene has recently been shown to be the functionally active gene in Brachypodium [96].
Figure 16. Total-ion and selected-ion extraction chromatograms of TLC-fractioned DFRC’ products from Kenaf lignin showing all expected acetylated β-β-coupled products D8-D10, D13, and D14.
Genetic Modification of Lignin

Lignin engineering in plants, including trees and agricultural crops, through the modification of its content or composition, is of great interest due to its potential impact on industry, agriculture and the environment [58]. In the pulp and paper industry, lignin must be removed from the woody raw materials to obtain the desired cellulose pulp by chemical extraction processes that are polluting and energy consuming [7, 8]. In agriculture, cattle production uses large quantities of forage such as alfalfa and maize whose digestibility is improved by reduced lignin content [42, 97, 98]. Biofuel production from lignocellulosic feedstocks is also greatly affected by the lignin in the plant cell wall because it increases the cost of obtaining fermentable sugars by limiting the accessibility of cellulases for cellulose degradation [99, 100]. Driven by its significance in the economics of these industries, lignification has been one of the most intensively studied topics in plant biochemistry for more than a century. In the last decade, our growing knowledge of lignin biosynthesis, coupled with advances in plant transformation technologies, has allowed researchers to manipulate lignin content and composition in a variety of plant species using genetics and genetic engineering [101].

One of the important aspects in studies on genetic modification of lignins to produce plants with desired properties has been the characterization of lignin in the generated plants. Degradation methods (mainly thioacidolysis and the DFRC method) and NMR are frequently used for compositional analysis and structural characterization.

Brown midrib maize

Many breeding efforts for improving forage quality have focused on identifying forage crops with altered lignification. There are four naturally occurring brown midrib (bm) mutants (bm1, bm2, bm3, and bm4) known in maize (Zea mays L.). They are of interest because of their lower lignin content and more digestible cell wall structure (4, 5). Marita et al. analyzed the stalk cell walls of the four bm single mutants and a bm1-bm2 double mutant from a common genetic background (inbred A619) by the DFRC method and 2D NMR [102]. DFRC degradation products of the maize cell walls revealed the differences in their S/G ratios compared to wild type. Whereas all other maize samples had DFRC monomer S/G ratios between 0.12 and 0.16, the S/G ratio of the bm1 and bm3 mutants were 0.09 and 0.06, respectively. NMR also revealed minor differences in the S and G components of the bm1 and bm3 lignins in consistent with the DFRC results. It has been reported that the COMT activity in bm3 mutants was reduced to only about 10% of that in wild-type plants. Since COMT is responsible for converting 5-hydroxyconiferyl alcohol (5-OH-CA) to sinapyl alcohol, the strongly decreased COMT activity in the bm3 mutant would result in a reduction of S units and the concomitant accumulated 5-OH-CA could be incorporated into lignin polymer as other monolignols. The benzodioxane structure in the bm3 lignin was identified by NMR confirming the participation of 5-OH-CA in lignification in such mutants.

COMT-deficient poplar

Detailed DFRC analyses of the benzodioxane structures in lignins isolated from COMT-deficient plants have been done with a modified procedure that allow to reveal partial sequencing information for 5-OH-CA incorporation into lignin [103]. Benzodioxane
structures was first suggested to be present in lignin when the benzodioxane D15 (Figure 17) was identified in hydrogenolysis products from *Fraxinus mandshurica* var. *japonica* [104]. Later on, thioacidolysis degradation of COMT-deficient polar transgenics revealed a dimeric product that was suggested to be benzodioxane D16 by mass spectral analysis [105] (Figure 17). When COMT-deficient poplar lignins or whole plant cell wall fractions were applied to DFRC degradation, a dimeric product containing the benzodioxane structure was released and identified to be compound D17 (Figure 18). The fact that 5-hydroxyconiferyl acetate has not been found in DFRC degradation products of COMT-deficient poplar lignin implies that all of the 5-OH-CA monomer is incorporated into lignin as benzodioxane structures and these structures totally survive DFRC conditions. With model compounds it was demonstrated that the benzodioxane structures in lignin indeed remained fully intact under DFRC conditions, suggesting that DFRC can be used to detect and determine benzodioxane structures in lignins of COMT-deficient plants, and also from F5H up-regulated plants such as *Arabidopsis thaliana* [106, 107].

As seen from Figure 18, the possible analog D18 was not found, which raised a question as to whether the syringyl benzodioxane was not in this lignin, or the marker compound D18 simply could not get through the GC. With synthetic compound D18, we found that D18 could not survive the GC conditions possibly because of its thermo liability. However, the more thermally stable trimethylsilyl (TMS)-derivatized D20f and D20e (Figure 17), analogs of D18, proved to be amenable to GC quantitative analysis. So a modified DFRC procedure was developed (Figure 19) to determine the benzodioxane structures to obtain a more detailed picture of such structures in lignin polymers [103].

![Figure 17. Structures of benzodioxanes D15-D18 and D20 resulting from lignin by various degradation procedures (hydrogenolysis, thioacidolysis and the DFRC method).](image)
Following this procedure the TMS derivatives of benzodioxanes released by the DFRC degradation from COMT deficient lignins were analyzed by GC. From the results obtained, it was found that guaiacyl benzodioxanes released by DFRC were mostly (70%–86%) from the ends of lignin molecules and the released syringyl benzodioxanes were mostly (67%–77%) from internal units. One important aspect to note is that the overall yields for benzodioxanes released by DFRC are relatively low compared to the high levels found via NMR analysis of the lignins. As 5-hydroxy units could also couple with further 5-OH-CA monomers producing benzodioxane chains that are not cleavable by DFRC, trimers, tetramers, and higher oligomers of benzodioxane chains may exist in DFRC products and cannot be measured by the current GC method. However, they could be isolated through a series of liquid chromatography and thin-layer chromatography (TLC) steps. In a large-scale DFRC experiment with COMT-deficient poplar wood, the two trimeric benzodioxane products (T3G and T3S, Figure 20) have been isolated by liquid chromatography combined with thin-layer chromatography (TLC), and identified by 2D (COSY, HSQC, and HMBC) NMR spectra with comparison to synthesized and authentic model compounds [103].

Figure 18. Of the expected benzodioxane products (D17 and D18) released by the DFRC method from a COMT-deficient polar, D17 is identified by comparison of retention time and mass spectrum with the synthetic model. D18 was not detected by GC-MS because of its thermolability.
Figure 19. Modified DFRC procedure and partial GC-FIDs showing (TMS-derivatized) benzodioxane products D19 and D20 from degradation of COMT-deficient polar lignins or whole cell wall fractions by the modified DFRC procedure [103]. A) Synthesized models; B) Antisense COMT-deficient poplar lignin; C) The residue remaining after dioxane-water extraction of the lignin in B; D) Antisense COMT-deficient polar cell walls; E) Gene-silenced COMT-deficient polar lignin.
Figure 20. Chemical structures of T3G and T3S identified from DFRC degradation products of COMT-deficient poplar lignins, and HSQC NMR spectra showing the aromatic C-H correlations (top rows) and aliphatic C-H correlations (bottom rows) of synthesized models T3G and T3S (spectra B and C), and isolated DFRC degradation fractions A and B (spectra A and D).
F5H over-expressed plants

The phenylpropanoid pathway provides precursors for the biosynthesis of soluble secondary metabolites and lignin in plants. Ferulate 5-hydroxylase (F5H) is a cytochrome P450-dependent monooxygenase that catalyzes the hydroxylation of ferulic acid, coniferaldehyde and coniferyl alcohol in the pathways leading to sinapic acid and syringyl lignin biosynthesis. Therefore it was suggested that F5H is a key enzyme that determines the lignin composition. Using the DFRC Method to measure lignin composition in Arabidopsis stem cell walls of wild type, fah1, and a C4H-F5H transgenic [108], Meyer et al. reported that wild-type Arabidopsis accumulates a lignin that is dominated by guaiacyl units, and no syringyl units was detected in the fah1 mutant lignin, whereas lignin of the F5H-overexpressed transgenic appears to be derived almost solely from sinapyl alcohol monomers. These results demonstrated that modification of F5H expression may enable engineering of lignin monomer composition in important plant species.

In a later study, F5H-overexpressed transgenics from tobacco and poplar were generated to test whether high-syringyl lignins could be produced in a plant species that undergo secondary growth [109]. Again, using the DFRC method for lignin compositional analysis, they found that over-expression of F5H in tobacco with the cauliflower mosaic virus 35S promoter increased syringyl lignin units in petioles, but not in stems. By contrast, with the cinnamate 4-hydroxylase (C4H) promoter to overexpress F5H they found a significant increase in syringyl units in stem lignin. When applied to wild-type tobacco stem samples, DFRC analysis revealed 20 to 40% syringyl units in lignins. Similar levels of syringyl units were found in lignin from stems of the 35S-F5H transgenic plants and the C4H-F5H transgenic line. Consistent with the previous NBO data, DFRC analysis of the C4H-F5H lines demonstrates that the lignin in the stems of these plants is comprised of approximately 80 mol% syringyl units.

The NBO results showed that the wild type control line had 55 mol% syringyl lignin units while the C4H-F5H transgenic poplar lines had syringyl lignin units up to 85 mol%. The DFRC data were completely consistent with the NBO data, indicating that the C4H-F5H transgene leads to the deposition of lignins with high syringyl units in tree species.

In a further study to determine whether F5H overexpression leads to undesirable phenotypes, lignin quality and quantity measurements were conducted along with tests to document potential changes in wood cell wall polysaccharide content and vascular development [110]. Thioacidolysis and DFRC are complimentary methods that can effectively be used to determine lignin monomer composition. By using these two degradation methods, Huntley et al. found that lignin in 1-year-old poplar trees overexpressing F5H were significantly altered in their S/G compared to the wild type, ranging from 1.9 in the wild-type trees to a maximum of 14.2 in the transgenics. That is, the wild type lignin has a ~65 mol% syringyl units while the lignins of transgenic trees contain from 71-93.5 mol% syringyl units. More importantly these changes led to no significant difference in total lignin content and no observed phenotypic differences. Pulping experiments with such C4H-F5H-transformed trees showed significant advantages by using less chemical to produce pulp with higher yields and better quality.

By over-expressing F5H in a line of Arabidopsis mutants deficient in COMT activity, two groups generated a new types of transgenics with spectacularly altered lignin in which normal monolignols were only minor contributors and the novel 5-OH-CA monomer became major [107, 111]. DFRC analysis of the new type of lignin from the Arabidopsis mutant
demonstrated that the four COMT/F5H mutation lines had less S lignin units and showed significant decreases in G units released by DFRC compared to wild-type. This was obviously due to the enrichment of 5H units in the lignins of these plants. Further analysis by a modified DFRC procedure revealed the existence of the expected benzodioxane structures derived from 5-OH-CA incorporation. Enzymatic hydrolysis experiments with these mutants showed that significant improvement in enzymatic saccharification efficiency was obtained. Although alterations in overall growth morphology were evident in these mutants, no reduction in post-harvest biomass yields was found.

**Other mutant and transgenic plants**

The DFRC method has also been used for characterization of lignins in other mutants or genetically modified plants leading to the following results:

- Lignin deposited in the *p-coumarate 3-hydroxylase* (*C3H*) down-regulated Arabidopsis gives mainly *p*-coumaryl alcohol diacetate when subjected to DFRC degradation [112].
- The *gh2* mutant, a gold hull rice having reduced CAD activity, had a lower lignin content and gave much lower DFRC monomer yields although the monomer distributions are similar when compared to the wild-type control [113].
- The *ref3-3* Arabidopsis mutant with reduced cinnamic acid 4-hydroxylase (*C4H*) activity contains lignin with increased S/G ratios (possibly due to the reduced yields of G units) and overall lower yields of DFRC monomer products, but has similar lignin content compared to its wild-type control [114].
- A rice mutant with flexible culm (*fc1*) has reduced CAD activity and lignin with reduced H and G units (showed by the DFRC method) indicating that the *FC1* gene identified plays an important role in the biosynthesis of lignin and the control of culm strength in rice [115].

The presence of the phenylpropanoid polymer lignin in plant cell walls impedes breakdown of polysaccharides to the fermentable sugars that are used in biofuel production. Genetically modified plants with altered lignin properties hold great promise to improve biomass degradability.

**CONCLUSION**

Lignin is a complex natural polymer present in terrestrial plants. As a major and important component, lignin plays essential roles in plant development. Lignin has been and continues to be an interesting topic for academic and industrial research because of its significance in various areas including plant breeding, forest product industries, dairy forage production, biomaterials and biofuel production, etc., and its potential applications. In this context, lignin characterization (compositional and structural analysis) is crucial and informative. Due to the complexity of lignin, various analytical methods are often needed for better characterization. The DFRC method was originally developed as an alternative to thioacidolysis for lignin compositional analysis because of its potential advantages as being operationally simple and needing less smelly chemicals. In fact, the thioacidolysis method is
so useful and convenient now, due in large part to the dedicated development and applications by its chief originator, Catherine Lapierre, that is remains a premier tool for lignin structural analysis. But the DFRC has become more than just an alternative. As seen, the applications of the DFRC are beyond the original expectation and scope thanks to some of its unique features and versatility.

With the applications of the DFRC methods, our knowledge about lignin has been advanced tremendously especially in the areas of lignin biosynthesis and structure. In particular, several pathways leading to the biosynthesis of monolignols have been verified by using the DFRC method; new structures (isochromans and benzodioxanes) in lignin have been discovered and their importance detailed; the optical inactiveness of lignins has been verified; the regiochemistry of lignin acylation was identified; the pathway to lignin acylation has been proven to be through pre-acylated monolignols providing solid basis for finding the implicated monolignol acyltransferases; applications of the DFRC method helped to understand lignin structure in many mutant and transgenic plants (Arabidopsis, tobacco, rice and poplar) in which lignin biosynthetic pathway genes had been misregulated, including down-regulated \textit{COMT}, \textit{C3H}, \textit{C4H}, \textit{CAD}, and upregulated \textit{F5H} plants, and \textit{bm} maize.

It has been established that lignin modification (compositional or structural) could lead to the generation of ‘ideal’ plants with designed properties for biofuel production in order to reduce our dependence on fossil fuels. Lignin characterization (with the DFRC method and other analytical tools) will help to expedite this process. Having had many successful applications, the DFRC method is expected to continue to play an important role in areas of cellulosic biofuel production and lignin utilization.

\textbf{ACKNOWLEDGMENTS}

We thank Dr. Steven Karlen and Dr. Dharshana Pdamakshan for their interests in and constructive suggestions for improving the DFRC method. This work was funded by the DOE Great Lakes Bioenergy Research Center (DOE Office of Science BER DE-FC02-07ER64494).

\textbf{REFERENCES}


The DFRC (Derivatization Followed by Reductive Cleavage) Method ...


The DFRC (Derivatization Followed by Reductive Cleavage) Method …


