The "DFRC" Method: a New Method for Structural Characterization of Lignins

F. Lu and J. Ralph

Introduction

During recent work it became clear that we could develop a new lignin characterization method. We had developed tandem reactions which were capable of breaking aryl ether bonds in lignins. This allows us now to provide a simplified alternative to the currently popular thioacidolysis method, one of the most important lignin structural tools.

The new method, based on derivatization and plant cell wall solubilization reactions via acetyl bromide followed by reductive ether cleavage, has been coined the "DFRC" Method for **D**erivatization **F**ollowed by **R**eductive **C**leavage (and reflecting the **D**airy **F**orage **R**esearch **C**enter where it was developed). The DFRC method is somewhat easier, more selective and significantly less malodorous than thioacidolysis.

The "DFRC" Method

Figure 1 shows the chemical basis of the method. In the first step, dissolution of the cell walls in an acetyl bromide/acetic acid solution provides soluble components allowing unrestricted access of the required sites to bromination and the following reductant and allowing rapid reactions without resorting to elevated temperatures. At the same time, the lignins are cleanly derivatized and some bond cleavage occurs. Thus α–ethers are selectively cleaved, all free OH's (phenolic and aliphatic) are acetylated, and benzylic positions become brominated. β–Ether units are not cleaved. These reactions on β–ether moieties are extraordinarily clean as long as conditions are not too harsh; some of the conditions recommended for the acetyl bromide method for lignin quantitation are too stringent and produce side reactions such as ring-acylation. In particular, we avoid the higher reaction temperatures (50 °C is sufficient, near 70 °C ring acylation becomes significant) and never use the perchloric acid advocated in recent versions of the acetyl bromide method. The acetyl bromide and solvents are then simply removed under vacuum or in an air stream — there is no need for product work-up.

The second step is the key to ether cleavage. A reducing source, zinc in acetic acid, rapidly cleaves the β–bromo ethers **1** that are produced in the first step. Again, this reaction is very high-yielding and clean. Tiny amounts of side-products from model compounds are detectable by GC-MS but are at such a low level that we need to purify our starting models to extraordinary lengths to detect real side products from those arising from trace impurities in our model compounds. At this point, all β–ethers and non-cyclic α–aryl ethers in lignin have been cleaved. Methoxyl groups remain intact.

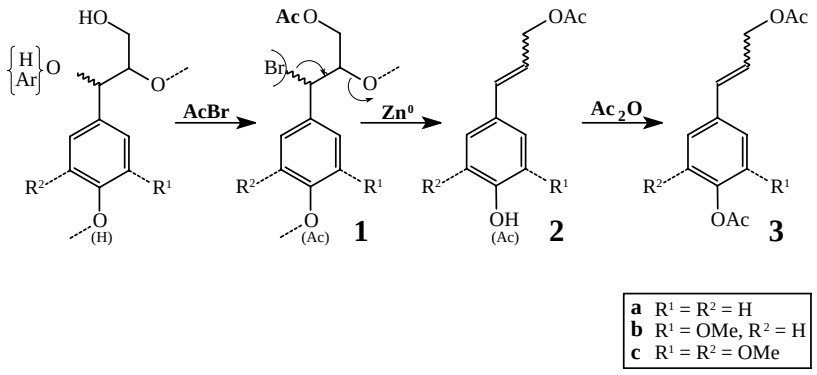

Figure 1. The reaction basis of the DFRC method. Treatment with AcBr acetylates alcohols and phenols, cleaves α-ethers, and brominates α-positions, at the same time solubilizing the whole cell wall. Two-electron reduction cleaves β-ethers. Acetylation produces the final hydroxycinnamyl acetates 3 for GC analysis.

Finally, in order to produce the simplest sample to analyze and to give quantitative data analogous to that of thioacidolysis, acetylation of phenolic hydroxyls reduces the number of distinct compounds to be quantitated. Regrettably, there are still two isomers to quantitate. In thioacidolysis, the *threo*– and *erythro*–trithioethyl

compounds result in approximately equal amounts. Here we obtain *trans*- and *cis*-hydroxycinnamyl acetates **2**, with the *trans*-isomer predominating (> 90%). When more extensive data are at hand, it might be possible to quantitate only the *trans*-isomer and calculate the total contribution from a known fixed ratio, but the *trans:cis* ratio is subtly dependent on exact reaction conditions. For crude analyses, the *cis*-isomer can simply be ignored. Attempts at removing the isomer complication, e.g. via hydrogenation, provided more complications and have now been abandoned. For now, each isomer's GC peak is largely free from contamination by other products and is readily quantitated. Figure 2 shows the results using various *p*-hydroxyphenyl/guaiacyl/syringyl model compounds; Figure 3 shows DFRC products from four lignin samples. To illustrate the cleanliness even when whole cell wall material is used, Figure 4 shows the resultant products from pine (a softwood) and bass (a hardwood).

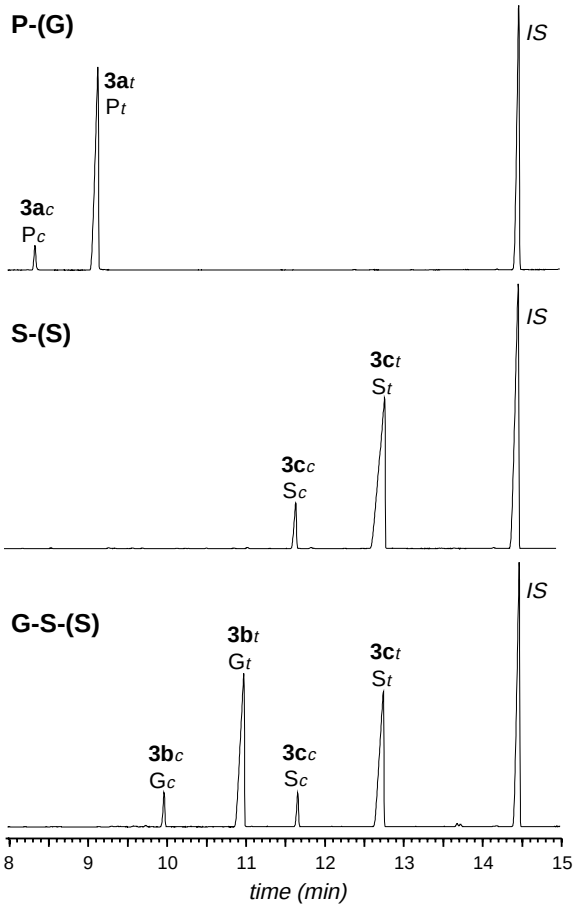

*Figure 2. GCs of DFRC products from three model compounds showing the cleanliness of the products following the three reaction steps. P, G, and S represent p-hydroxyphenyl, guaiacyl, and sinapyl units **3a-c** (Fig. 1). The final unit (in brackets) has no sidechain and is not present as such in real lignins; products from this unit appear early in the chromatogram (not shown). t = trans, c = cis .*

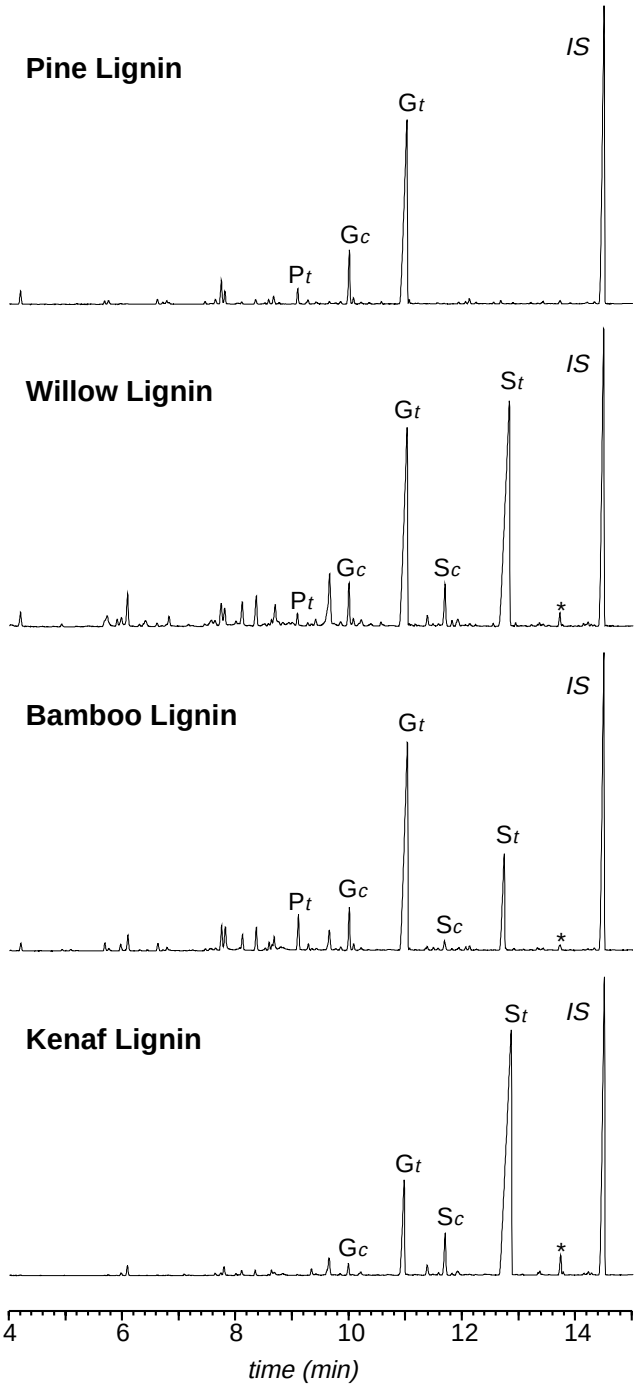

*Figure 3. GCs of DFRC products from isolated milled lignins showing the readily quantifiable P, G, and S products **3a-c** (Fig. 1). The asterisked (*) peak is from 5-hydroxyconiferyl units, detected in every S/G lignin with the DFRC method.*

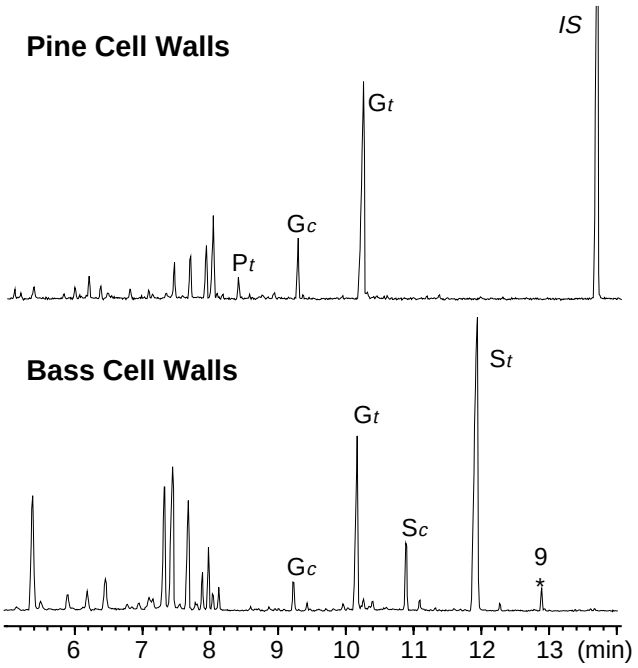

*Figure 4. Mass spec. total ion chromatograms of DFRC products from whole cell wall samples, pine (softwood) and bass (hardwood). The normal P, G and S peaks **3a-c** (Fig. 1) are shown as well as the 5-hydroxy-product. Early peaks in the chromatogram are largely polysaccharide-derived.*

Advantages of the DFRC method. In our totally biased opinions, the following advantages are seen in this method.

1. Reactions are more selective and more quantitative. As noted above, the AcBr and the Zn0 reactions are extremely clean. Reactions of model compounds through the entire process produces ~95% yields of the targeted products. Thioacidolysis yields are typically 75%. Thioacidolysis produces some chain-shortened products (aryl ethyl derivatives rather than aryl propyl) from side reactions.
2. The reactions are simpler and occur under milder conditions. Solvolytic reactions at 100+ °C under acidic conditions are not typically used in organic syntheses because they are more difficult to predict and control. The reactions described here are carried out at room temperature to 50 °C. They are sufficiently high yielding and clean to be synthetically as well as analytically useful.
3. Analyses are less sensitive to experimental conditions. Any optimization is recommended in each lab before beginning analytical determinations by thioacidolysis. Indeed in our own lab we frequently found dimeric β–ethers indicating that we are not efficiently cleaving all β–ethers. It appears that an optimization is also required when reagents are changed. The DFRC method is far less sensitive to sample and operating conditions. Although it is envisaged that some sample types may prove less amenable to AcBr reactions, the general procedure developed can be applied to a wide variety of sample types without requiring further optimization.
4. The DFRC products are stable for long periods (unlike thioacidolysis products). GC analysis can be performed and/or repeated at leisure.
5. Malodorous chemicals are not required. No discussion of thioacidolysis is complete without mention of the foul stench of the required reagent (ethane thiol). Although this can be minimized by careful work, utilizing bleach for trapping, etc., building staff are generally not supportive of thioacidolysis experiments. In some labs, it is simply not possible to run thioacidolysis because of restrictions on odor.
6. Esters are not cleaved. In thioacidolysis, esters are partially cleaved and this causes problems with grasses (for example) where *p*–coumarate esters (at the lignin γ–position) can be quite prevalent. γ–Esters remain completely intact after DFRC reactions and this can be used to tremendous advantage in identifying and characterizing esterified lignin components.
7. The scheme is amenable to targeting products and to a rather wide variety of future applications. Many are now under active investigation and will hopefully be reported in the future.
8. A lignin determination can be made at the same time as the DFRC method; the first AcBr step is similar to AcBr methods developed for lignin determination. We are currently working on integrating these methods.

Additionally the hydroxycinnamyl acetates are more diagnostic of their lignin origin than the trithioethyl derivatives or the fully saturated aryl propanes that result from the two thioacidolysis methods. For that reason, we sought to establish methods based on the hydroxycinnamyl acetates rather than aryl propanes or various unsaturated analogues that could also be targeted via modified conditions (particularly in the reduction step).

Disadvantages over thioacidolysis. The main drawbacks to the method at this point stem from its immaturity. We don't yet know what happens to a number of structural units that have already been investigated by thioacidolysis, although this number is rapidly declining. Many of these are the focus of current work.

Experimental protocol.
Acetyl Bromide Stock Solution
AcBr:acetic acid 8:92 (by volume) for model compounds and isolated lignins.
AcBr:acetic acid 20:80 (by volume) for cell wall samples.

Acetyl bromide derivatization and wall solubilization reactions. To a 10 ml round bottom flask containing about 10 mg of lignin, lignin model compound, or 20 mg cell wall sample is added 2.5 ml of AcBr stock solution. [Smaller amounts are possible for this analysis]. The mixture is gently stirred at room temperature overnight (models, lignins) or 50 °C for 3 hours (cell wall samples).

Reductive cleavage reactions. After removal of solvents by rotary evaporation at 50 °C (we think evaporation in an air stream is OK but this is still being tested), the residue is dissolved in 2.5 ml of dioxane/acetic acid/water (5:4:1, v/v/v). To the solution is added ~50 mg of zinc dust with good stirring. The mixture is stirred for 30 minutes. After addition of internal standard (tetracosane in methylene chloride, 3-5 mg for models, 300 µg for lignins, 200 µg for CW samples), the mixture is poured into methylene chloride (10 ml) and washed once with saturated ammonium chloride solution. The water phase is extracted twice with methylene chloride. The combined methylene chloride fractions are dried over anhydrous magnesium sulfate, filtered, and the solvent removed under reduced pressure. The residue is acetylated (pyridine/acetic anhydride) and the product utilized directly for NMR, GC, or GC-MS analysis.

GC quantitation. The residue from above is dissolved in methylene chloride: a) about 1.5 ml for lignin models, b) 200 µl for lignins, c) 100 µl for CW samples. A sample, 1.5 µl, is injected into a GC (Hewlett Packard 5980). [Column 0.20 mm x 30 m SPB-5 (Supelco); He carrier gas, 1 ml/min; injector 220 °C, initial column temperature 160 °C, ramped at 10 °C/min to 300 °C, hold 5 min; FID detector, 300 °C]. Response factors, determined from independently synthesized products, relative to the tetracosane internal standard and relative retention times are given in Table 1.

Conclusions
Although most of the examples given here are for woods, the method works equally well for grasses and legumes that form the basis of our core research at the Center. Applications of this method will help enormously with the characterization of forage wall components and structure. Further future extensions to this basic methodology stand poised to provide an arsenal of useful tools with which to tackle mysteries of cell wall structure and provide insights into the limitations to digestibility of its polysaccharides.

Table 1. Relative Retention Times (RRT) and Response Factors (RF) relative to tetracosane internal standard, for the DFRC-released lignin monomers.

	cis-**3a**	*trans*-**3a**	*cis*-**3b**	*trans*-**3b**	*cis*-**3c**	*trans*-**3c**
RF	1.76	1.76	1.84	1.84	2.20	2.20
RRT	0.58	0.63	0.69	0.76	0.81	0.88