# Derivatization Followed by Reductive Cleavage (DFRC Method), a New Method for Lignin Analysis: Protocol for Analysis of DFRC Monomers

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A new method for selective and efficient cleavage of arylglycerol−$\beta$-aryl ($\beta$-O-4) ether linkages in lignins is introduced. The acronym "DFRC" relates to the reactions involved, **d**erivatization **f**ollowed by **r**eductive **c**leavage. Derivatization, accompanied by cell wall solubilization, is accomplished with acetyl bromide in acetic acid; reductive cleavage of resulting $\beta$-bromo ethers utilizes zinc in an acidic medium. Following acetylation, degradation monomers (4-acetoxycinnamyl acetates) are quantified by GC, providing data analogous to those from analytical thioacidolysis.

**Keywords:** *Acetyl bromide; lignin; $\beta$-aryl ether; thioacidolysis; reductive elimination*

## INTRODUCTION

It is a common practice to degrade lignins to low molecular weight compounds in order to obtain structural information. At this time, thioacidolysis is probably the most diagnostic method for lignin characterization (Rolando *et al.*, 1992; Lapierre, 1993). However it is not a simple technique to perform and has certain drawbacks such as requiring a malodorous reagent, side chain degradation, potential incomplete cleavage (Ralph and Grabber, 1996), and the need for optimization in each laboratory.

Here we provide the protocol for a robust new method that efficiently cleaves α- and $\beta$-aryl ethers in lignins releasing analyzable monomers for quantification (Lu and Ralph, 1996a; Ralph *et al.*, 1996; Lu and Ralph, 1997). The method has been given the acronym "DFRC" to describe the reactions involved (**d**erivatization **f**ollowed by **r**eductive **c**leavage) and to reflect the Dairy Forage Research Center where it was developed. It provides data analogous to those from the basic thioacidolysis method. Full papers describing the reactions, detailing yields from model compounds and real-world samples, describing products from lignins' minor components, and illustrating further applications will follow. This note provides details for laboratories anxious to begin using the method for their own analyses.

## PROTOCOL

**Materials and Reagents.** Acetyl bromide (AcBr), dioxane, acetic acid, and zinc dust (<10 $\mu$m) were purchased from Aldrich Chemical Co. and used as supplied. Commercial analytical reagent grade solvents were used without further purification. Cell wall samples were from plant materials ground through a Wiley mill with a 0.5 mm screen (a 1 mm screen also provides a satisfactory particle size) and solvent-extracted. Removal of major extractives with 80% ethanol using the Uppsala method (Theander, 1991; Theander and Westerlund, 1993) is adequate. AcBr stock solution: AcBr: acetic acid, 8:92 or 20:80 by volume; stable for several weeks. Acidic reduction medium: dioxane/acetic acid/water (5:4:1, v/v/v); stable for several months. Acetylation reagent: 1:1 pyridine:acetic anhydride. Internal standard for GC quantification: tetracosane.

Table 1 summarizes the conditions for various sample types and the GC parameters required for quantification. A standard sample is available from the authors to establish response factors in users' laboratories.

**Detailed Experimental Method.** Consult Table 1 for amounts, volumes, reaction times, etc., for each of the sample types.

*(1) AcBr Step.* To a 10 mL round bottom flask containing approximately the amounts given in Table 1 of lignin model, lignin, or cell wall sample was added the AcBr stock solution. The mixture was gently stirred at either room temperature or 50 °C for times given in Table 1. Finally, the solvent was completely removed by rotary evaporation below 50 °C. (Blowing down under a stream of air appears to be satisfactory.)

*(2) Reductive Cleavage Step.* The above residue was dissolved in the acidic reduction solvent. Zinc dust (50 mg) was added to the well-stirred solution. Stirring was continued for 30 min. The mixture was quantitatively transferred into a separatory funnel with $CH_2Cl_2$ (10 mL) and saturated $NH_4Cl$ (10 mL) and internal standard (tetracosane in methylene chloride) added. The pH of the aqueous phase was adjusted to less than 3 by adding 3% HCl, the mixture vigorously mixed, and the organic layer separated. The water phase was extracted twice more with $CH_2Cl_2$ (2 × 5 mL). The combined $CH_2Cl_2$ fractions were dried over $MgSO_4$, and the filtrate was evaporated under reduced pressure.

*(3) Acetylation Step.* The residue was acetylated for 40 min in 1.5 mL of dichloromethane containing 0.2 mL of acetic anhydride and 0.2 mL of pyridine. All volatile components were removed completely by coevaporation with ethanol under reduced pressure. The residue was used for GC quantification. Final products are quite stable if dried down and kept out of the light; some *cis*−*trans* isomerization can occur.

*(4) GC Quantification.* The degraded products were dissolved in methylene chloride, and 1−2 $\mu$L of this solution was used for GC analysis. In our case, the degraded monomers were quantitatively determined by GLC (Hewlett Packard 5980): column, 0.20 mm × 30 m SPB-5 (Supelco); He carrier gas, 1 mL/min; 30:1 split ratio; injector 220 °C, FID detector, 300 °C, temperature program as in Table 1. The amounts of individual monomers, *p*-acetoxycinnamyl acetate, coniferyl diacetate, and sinapyl diacetate (P, G, and S), were determined using response factors (RFs) derived from pure monomer standards using tetracosane as internal standard. Relative

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**Table 1. Summary of Reaction Conditions for the DFRC Method on Various Substrates, and GC Parameters for Each of the Monomers Analyzed**

| description | models | lignins | cell walls |
|---|---|---|---|
| wt of material (mg) | 5 | 5–10 | 20[a] |
| (1) AcBr Step | | | |
| AcBr reagent | 8:92 | 8:92 | 20:80 |
| volume (mL) | 2.5 | 2.5 | 3 |
| temperature (°C) | RT | RT/50 | 50 |
| time (h) | 4 | 16/2 | 3 |
| (2) Reductive Cleavage Step | | | |
| acidic solvent (mL) | 2.5 | 2.5 | 3 |
| zinc dust (mg) | 50 | 50 | 50 |
| time (h) | 0.5 | 0.5 | 0.5 |
| internal standard (tetracosane, mg) | 2.5 | 0.3 | 0.2[b] |
| (3) Acetylation Step | | | |
| acetylation solvent ($CH_2Cl_2$, mL) | 1.5 | 1.5 | 1.5 |
| pyridine (mL) | 0.2 | 0.2 | 0.2 |
| acetic anhydride (mL) | 0.2 | 0.2 | 0.2 |
| acetylation time (min) | 40 | 40 | 40 |
| (4) GC Quantification | | | |
| volume with $CH_2Cl_2$ (mL) | 2 | 0.25 | 0.2 |
| volume injected ($\mu$L) | 1.5 | 1.5 | 2 |
| initial temp (°C) (hold time, min) | 150 (1) | 150 (1) | 140 (1) |
| intermed temp (°C) (ramp rate, °C/min; hold time, min) | – | – | 240 (3, 1) |
| final temp (°C) (ramp rate, °C/min) | 310 (10) | 310 (10) | 310 (30) |
| final temp (°C) (hold time, min) | 310 (17) | 310 (17) | 310 (12) |
| total run time (min) | 34 | 34 | 50 |

GC Relative Retention Times and Response Factors for Monomers

| | P*c* | P*t* | G*c* | G*t* | S*c* | S*t* |
|---|---|---|---|---|---|---|
| relative retention time | 0.45 | 0.51 | 0.59 | 0.68 | 0.74 | 0.84 |
| response factor | 1.76 | 1.76 | 1.85 | 1.85 | 2.06 | 2.06 |

[a] The amount of sample required depends on the amount of lignin in the sample and the cleavable $\beta$-ether frequency; 20 mg is a comfortable level for most cell wall samples. The volume of AcBr solution should be scaled proportionally if this amount is increased. It is possible to use lesser quantities for the three sample types listed here. [b] As with any such analysis, the amount of standard should be reasonably matched to the amount of the compounds being quantified. Using the conditions described in the rest of the table, the following may help in selecting the appropriate level for your analysis: basswood (hardwood), 0.3 mg; pine (softwood), 0.2 mg; alfalfa (legume), 0.05 mg; corn (grass, but perhaps one of the worst!), 0.05 mg. Note: For lignins, using the longer GC temperature program that is used for cell walls may improve resolution and separate out minor peaks. GC injector temperature, 220 °C; detector, 300 °C.

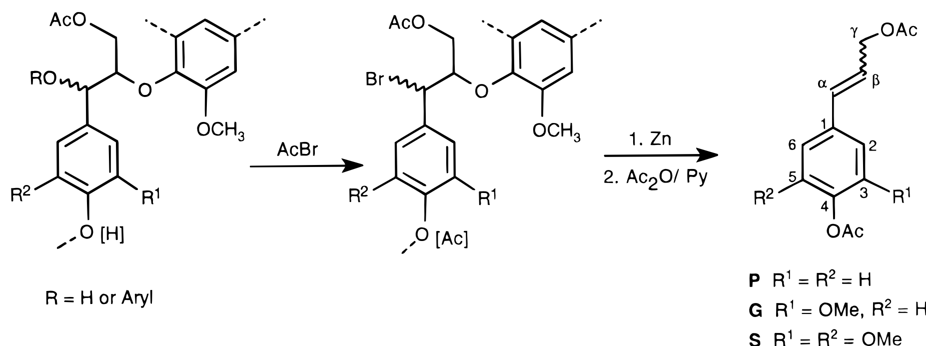


**Figure 1.** Ether cleavage in lignins by the DFRC method produces quantifiable 4-acetoxycinnamyl acetate (P), coniferyl diacetate (G), and sinapyl diacetate (S) monomers from *p*-hydroxyphenyl, guaiacyl, and syringyl units.

retention times and GC response factors relative to the tetracosane internal standard are also given in Table 1.

## RESULTS AND DISCUSSION

The basic steps of the procedure are illustrated in Figure 1. Derivatization, accompanied by cell wall solubilization, is accomplished with AcBr in a procedure similar to those used for the AcBr-based lignin determinations (Johnson *et al.*, 1961; Martin, 1967; Morrison, 1972; Bagby *et al.*, 1973; Iiyama and Wallis, 1988; Dence, 1992). We are careful to use lower temperatures to avoid side reactions (Lu and Ralph, 1996b) and do not use added perchloric acid. Optimization studies show that the lignin becomes fully solubilized from 0.5 or 1 mm Wiley-milled woods in *ca.* 3 h. Reductive cleavage of resulting $\beta$-bromo ethers utilizes zinc in an acidic medium. Following acetylation, 4-acetoxycinnamyl acetate monomers P, G, and S (Figure 1) are quantified by GC, providing data analogous to those from analytical thioacidolysis. It should be noted that yields of monomers from $\beta$-ether model compounds are ~95%, significantly higher than for thioacidolysis. This is due to the cleaner reactions over the two crucial steps of the DFRC method than in the high-temperature thioacidolysis reaction. Molar monomer yields from isolated lignins are also higher in our comparisons.

GC FID chromatograms are shown in Figure 2 for a representative softwood (loblolly pine) and hardwood (basswood). Major peaks arise from guaiacyl and syringyl units involved in ether linkages. The total yields

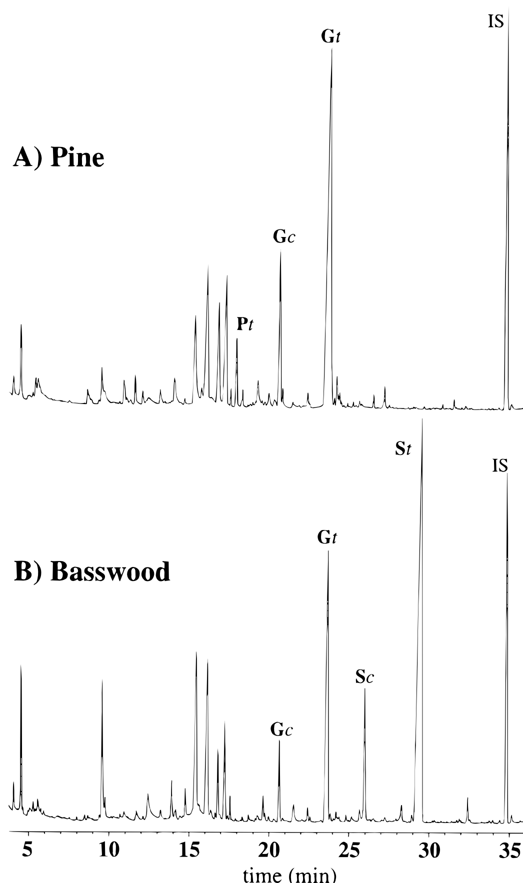


**Figure 2.** GC−FID chromatograms of DFRC monomers from extractive-free wood samples: (A) loblolly pine (*Pinus taeda*, a softwood) and (B) basswood (*Tilia americana*, a hardwood). P, G, and S are the acetoxycinnamyl acetates defined in Figure 1. In the pine product, the *cis*-P peak is obscured by carbohydrate products but can be identified from selected ion chromatograms in mass spectrometry. IS, internal standard (tetracosane); *t*, *trans*; *c*, *cis*.

of the three monomers were ∼640 and ∼1440 μmol/g of Klason lignin in these particular pine and basswood samples. Smaller amounts of *p*-hydroxyphenyl units are also apparent in the pine. Early peaks arise predominantly from degraded polysaccharides. Although their interference is minimal in this protocol, we are exploring the use of solid-phase extraction to clean up samples. Full details of the methods, the chemistry involved, and comparisons with thioacidolysis will appear in forthcoming manuscripts.

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