# DFRC Method for Lignin Analysis. 1. New Method for β-Aryl Ether Cleavage: Lignin Model Studies

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A new method for selective and efficient cleavage of arylglycerol-β-aryl (β-O-4) ether linkages in lignins is described and applied to several lignin β-ether models. The term "DFRC" was coined for derivatization followed by reductive cleavage. Derivatization, accompanied by cell wall solubilization, is accomplished with acetyl bromide (AcBr); reductive cleavage of resulting β-bromo ethers utilizes zinc in acetic acid. Degradation monomers, 4-acetoxycinnamyl acetates, from β-ether cleavage by the DFRC method were identified by NMR, GC—MS, and comparison of GC retention times with authentic compounds. Under the conditions used in this study, the β-ether linkage of all models was cleaved in very high (>92%) yield. The DFRC method produces simpler mixtures of monomers with higher yields than alternative hydrolytic methods. Because of its relative simplicity, mild conditions, and exceptional selectivity, this method should become a powerful analytical method for lignin characterization.

**Keywords:** *Acetyl bromide; lignin; lignin model compound; β-aryl ether; thioacidolysis; β-bromo-ether; cleavage; quantitative analysis; gas chromatography; reductive elimination; acetylation; bromination*

## INTRODUCTION

Although a great deal of progress has been made in lignin chemistry, many ambiguities remain regarding lignin structure and its cross-linking with other cell wall components (Dence and Lin, 1992; Ralph and Helm, 1993). As a highly abundant, renewable raw material that is currently underutilized, lignin has attracted increasing interest in wood chemistry, plant biochemistry, and related fields (Fengel and Wegener, 1989; Chen, 1991).

Unlike other natural polymers such as proteins, polysaccharides, and nucleic acids, which have interunit linkages susceptible to enzymic and chemical hydrolyses, lignin contains resistant carbon—carbon and diphenyl ether bonds (Morohoshi, 1991; Sakakibara, 1991). It is a common practice to degrade the polymer to low molecular weight compounds in order to obtain structural information. When this strategy is applied to lignins, however, significant limitations arise such as the low yield of degradation products, interference from other contaminants, and side reactions (Lapierre, 1993).

Permanganate oxidation, alkaline—nitrobenzene oxidation, and acidolysis are traditional methods for lignin characterization, although all have shortcomings. Thioacidolysis (Lapierre, 1993; Rolando et al., 1992) was a major improvement in achieving relatively selective cleavage reactions to form diagnostic products in good yields. Recently several iodine-containing reagents have been used in attempts to degrade lignins under milder conditions; these include trimethylsilyl iodide (Makino et al., 1990; Meshitsuka et al., 1987; Shevchenko and Akim, 1995), acetyl iodide (Shevchenko and Akim, 1995), pivaloyl iodide (Fukagawa et al., 1992), and dry hydrogen iodide (Shevchenko et al., 1991; Shevchenko and Akim, 1995). Complicated mixtures of unstable degradation products formed under those conditions made it difficult to isolate, identify, and quantitate degradation products. The main degradation monomer from dry HI treatment of spruce lignin, for example, was 1,3-diiodo-1-(4-hydroxy-3-methoxyphenyl)propane which was stable for only 1—2 h (Shevchenko and Akim, 1995). Currently, thioacidolysis remains probably the most effective diagnostic method for lignin characterization (Rolando et al., 1992). However thioacidolysis is not a simple technique to perform; it requires optimization in a user's lab, and utilizes the malodorous ethane thiol. There remains a need to develop a more selective, simpler, and more powerful method for degradation of lignin to provide detailed information about lignin structure.

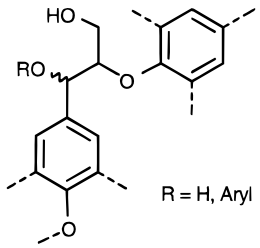


**Figure 1.** β-Ether units, major substructures in lignins.

During recent research on selective methods for cleavage of α-ether linkages in lignins, Figure 1 (R = aryl), it was found that AcBr is an ideal reagent for this purpose. AcBr treatment of α,β-diaryl ether lignin models in dioxane or acetic acid at room temperature resulted in complete formation of acetylated α-bromo derivatives along with selective α-ether cleavage/bromination (Lu and Ralph, 1996b). It is well established that β-bromo ethers or esters can be reductively cleaved by zinc dust in polar solvents (Rowlands et al., 1952; Soday and Boord, 1933; Schmitt and Boord, 1932; Kato et al., 1987), or Cr(II)en complex in dimethylformamide (DMF) (Kochi et al., 1968; Greene et al., 1987) to form alkenes. Experiments have shown that the lignin derivatives from AcBr treatment possess the β-bromo ether skeleton required for the reductive elimination (Lu

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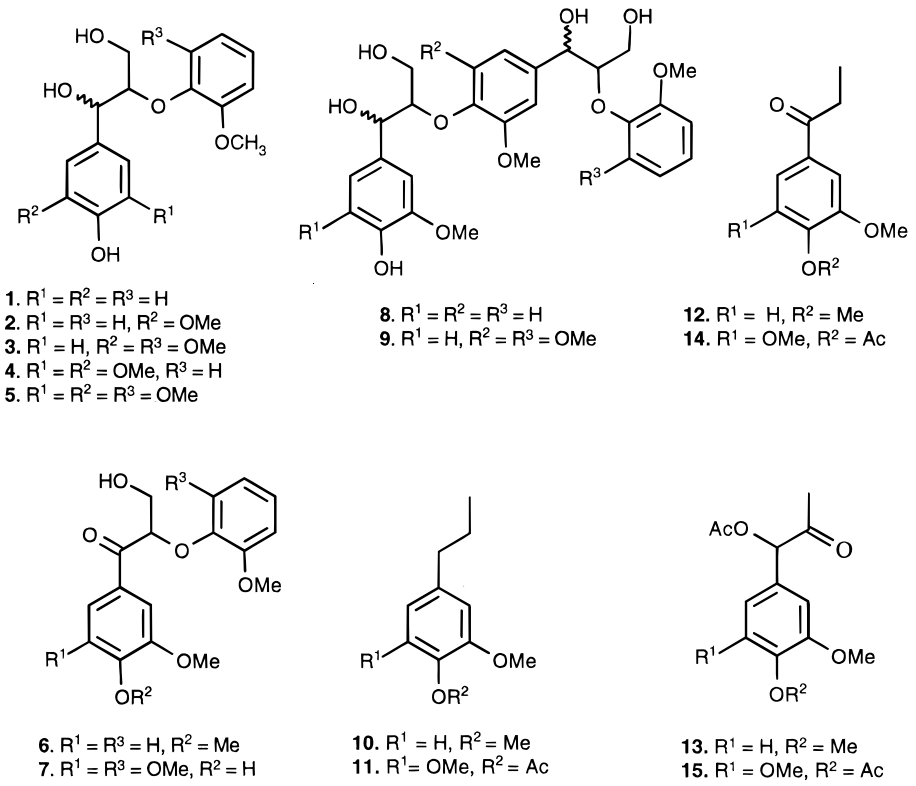


**Figure 2.** $\beta$-Ether models used for the DFRC methods, and some monomers **10**−**15** derived from dimers **6** and **7**.

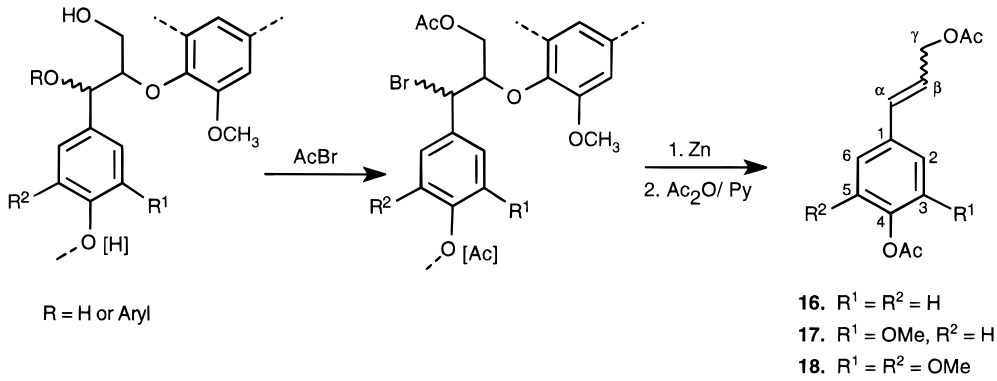


**Figure 3.** Selective ether cleavage by the DFRC method; monomers **16**−**18** derive from $\beta$-aryl ether models and lignins.

and Ralph, 1996b). We believed that AcBr treatment of lignin followed by Zn reductive cleavage would provide a pathway to selectively cleave α- and $\beta$-aryl ethers in lignin, resulting in a new method for lignin characterization (Figure 3). The new method for cleaving ethers in lignin has been given the acronym DFRC for derivatization followed by reductive cleavage (and to reflect the Dairy Forage Research Center where the method was developed). The protocol for DFRC monomer analysis has been published (Lu and Ralph, 1997a).

The first paper of this series describes a simple and efficient method (based on AcBr treatment and reductive ether cleavage) for selective cleavage of ether linkages in lignins and our evaluations using a range of representative lignin models. Because of AcBr's ability to dissolve lignin and lignin-containing plant material there is little doubt that this method can be applied to isolated lignin samples and plant materials. A following publication will report on those applications.

## EXPERIMENTAL PROCEDURES

**Materials and Reagents.** Lignin model compounds **1**−**9**, shown in Figure 2, were synthesized according to the standard methods described in literature (Ralph et al., 1986; Ralph and Helm, 1991). Hydroxycinnamyl diacetate standards **16**−**18** (*trans*-isomers, Figure 3) were prepared by acetylating the *trans*-hydroxycinnamyl alcohol parents, synthesized as previously described (Quideau and Ralph, 1992; Ludley and Ralph, 1996). NMR data for all compounds are given in the NMR Database of Lignin and Cell Wall Model Compounds (Ralph et al., 1996). Acetyl bromide, dioxane, acetic acid, and zinc dust were purchased from Aldrich Chemical Co. and used as supplied. Commercial analytical reagent grade solvents were used without further purification.

AcBr stock solution, made by mixing AcBr and acetic acid, 8:92 by volume, was stable for several weeks.

**DFRC Method.** To a 10 mL round bottom flask containing about 10 mg of lignin model, 2.5 mL of AcBr stock solution was added. The mixture was kept at room temperature and stirred gently overnight. After removal of solvent by rotary evaporation at below 50 °C, the residue was dissolved in 2.5 mL of dioxane/acetic acid/water (5:4:1, v/v/v) solution. Zinc dust (50 mg) was added to the well-stirred solution. Stirring was continued for 30 min. After addition of internal standard (3 mg of teracosane in methylene chloride) the mixture was quantitatively transferred into a separatory funnel with $CH_2Cl_2$ (10 mL) and saturated $NH_4Cl$ (10 mL). The pH of the aqueous phase was adjusted to less than 3 by adding 3% HCl,

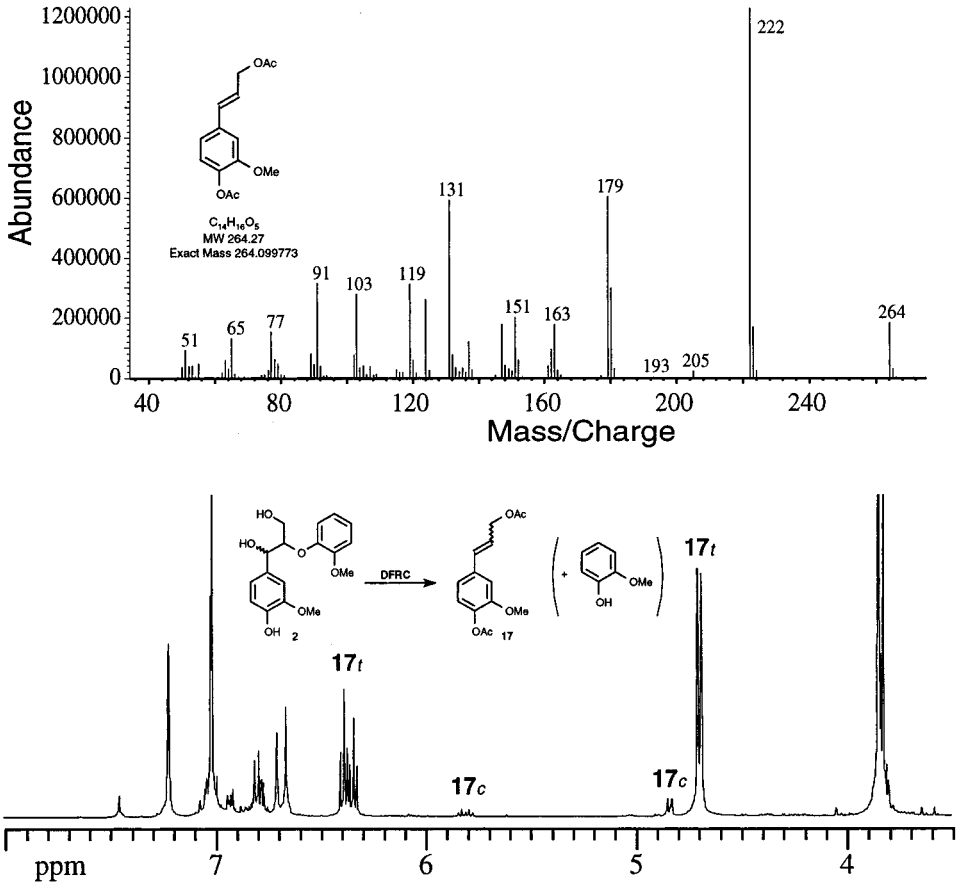


**Figure 4.** Mass spectrum of product **17** (coniferyl diacetate) and partial proton NMR spectrum of crude products (**17***t* and **17***c*) from dimer **2** by the DFRC method. *c* = *cis*, *t* = *trans*.

**Table 1. Mass Spectral and GC (RRT and RF) Data of Degradation Monomers 10−18**

| peak | RRT | RF | *m*/*z* (rel intensity) |
|---|---|---|---|
| 10 | 0.31 | | 180(15), 151(100), 135(8), 107(30), 91(24), 77(42) |
| 11 | 0.52 | | 238(4), 196(56), 167(100), 151(5), 137(7), 122(9), 77(12) |
| 12 | 0.48 | | 194(11), 165(100), 137(15), 122(22), 107(18), 92(22), 79(83) |
| 13 | 0.60 | | 252(5), 209(8), 167(100), 139(99), 124(25), 95(20), 77(41) |
| 14 | 0.66 | | 252(2), 210(36), 181(100), 167(5), 151(12) |
| 15 | 0.78 | | 310(2), 268(9), 225(15), 183(100), 155(11), 123(15), 95(9) |
| 16*c* | 0.58 | 1.76 | 234(19), 192(100), 149(85), 133(54), 121(44), 107(28), 94(29), 77(24) |
| 16*t* | 0.63 | 1.76 | |
| 17*c* | 0.69 | 1.85 | 264(11), 222(100), 179(37), 163(9), 151(12), 131(27), 119(15), 91(14) |
| 17*t* | 0.76 | 1.85 | |
| 18*c* | 0.81 | 2.06 | 294(8), 252(100), 209(24), 193(10), 181(8), 161(17), 149(13), 133(6) |
| 18*t* | 0.88 | 2.06 | |

the solution was vigorously mixed, and the organic layer was separated. The water phase was then extracted 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. The residue was acetylated in 1.5 mL dichloromethane containing 0.2 mL acetic anhydride and 0.2 mL pyridine for 40 min. All volatile components were removed completely by coevaporation with ethanol under reduced pressure. The residue was used for NMR, GC, and GC−MS characterization.

**GC Determination and GC−MS.** The degraded monomers from models were dissolved in 1.5 mL of methylene chloride and 1 μL of this solution was used for GC analysis. The degraded monomers from lignins or models were quantitatively determined by GLC (Hewlett Packard 5980, Atlanta, GA): column 0.20 mm × 30 m SPB-5 (Supelco, Bellefonte, PA); 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; flame ionization detector (FID), 300 °C. The amounts of individual monomers **16**−**18** were determined using response factors (RFs) derived from pure monomer standards using tetracosane as internal standard. Relative retention times (RRTs) and GC response factors relative to the tetracosane internal standard are given in Table 1. Electron ionization (EI)−MS (70 eV) data were collected on a Hewlett Packard HP5970 mass selective detector attached to the same GC. The column and flow conditions differed slightly between the GC−FID and GC−MS setups; thus, absolute retention times differ between the two.

## RESULTS AND DISCUSSION

Because the most frequent interunit linkages in lignin are arylglycerol-β-aryl ethers (Figure 1) (Adler, 1977), the cleavage of β-aryl ethers has been studied extensively (Makino et al., 1990; Meshitsuka et al., 1987; Fukagawa et al., 1992; Shevchenko et al., 1991; Lapierre et al., 1985; Lapierre et al., 1983). If these β-O-4 ether linkages were to be cleaved selectively and completely, the characterization of the degradation monomers (along with dimers and even trimers) would provide valuable structural information regarding the initial lignin. It has long been one of the most important research targets for lignin chemists to find a mild, selective, and efficient method for β-O-4 ether cleavage (Lundquist,

1992; Nimz, 1969, 1974; Lapierre et al., 1991). Such cleavage is a key requirement either for an efficient degradation of polymeric lignins during chemical pulping or for analysis of various linkages present in lignins.

For simplicity many studies on lignin reactions begin with model compounds. Interpretation of the chemical behavior of lignin, especially in degradation reactions, is largely based on results obtained from studies of appropriate model compounds under analogous conditions. It is essential that any proposed reactions on representative models yield desired products with high yields. If this requirement is not met, it is unreasonable to expect that the significantly more complex plant matrix will react efficiently. To determine the efficiency of the AcBr/Zn mediated ether cleavage method, $\beta$-O-4 aryl ether model compounds shown in Figure 2 were studied.

**Identification of Degradation Monomers.** As shown in Figure 3, the DFRC degradation method requires two key steps: (1) bromination and acetylation with AcBr, and (2) reductive cleavage with Zn dust. Final acetylation is used to acetylate free phenolic groups that are produced following the reduction step and reduce the number of compounds to be quantitated. The reaction of lignin or lignin models with AcBr in acetic acid results in complete dissolution of lignin and formation of acetylated lignin α-bromo-derivatives which possess the $\beta$-bromo ether skeleton. In the next step the bromo ethers are cleaved by reductive elimination with Zn dust in aqueous dioxane/acetic acid solution, forming a pair of 4-hydroxycinnamyl acetate isomers. Thus $\beta$-ether dimer **2** was cleaved through the DFRC procedure resulting in 4-acetoxy-3-methoxycinnamyl acetates **17**, which were identified by NMR and GC−MS (Figure 4). Monomers derived from DFRC degradation of various models representing $\beta$-ether structures in softwood and hardwood lignins are essentially 4-acetoxycinnamyl acetate **16**, 4-acetoxy-3-methoxycinnamyl acetate **17**, and 4-acetoxy-3,5-dimethoxycinnamyl acetate **18**. Two trimeric models, **8** and **9**, which more accurately model internal $\beta$-ether units than do dimeric models, were subjected to DFRC, forming monomer **17** (from trimer **8**) and monomers **17** and **18** (from trimer **9**) in 93, 97, and 93% yields, respectively (Figure 5 and Table 2).

The NMR and mass spectral data of degradation monomers derived from $\beta$-ether models by the DFRC method are listed in Table 1. As shown in Figures 5 and 6, GC chromatograms of the monomers obtained from the DFRC method are clean and simple. Only one pair of monomers is formed from the corresponding uncondensed lignin structural unit.

A further hydrogenation step to reduce the number of degradation monomer isomers and make quantitation easier was considered (Lu and Ralph, 1996a). However competing hydrogenolysis is unavoidable. For example, hydrogenation of 4-acetoxycinnamyl acetate **18** was always accompanied by the hydrogenolysis byproduct propylphenyl acetate **15**, which is also derived from other lignin substructures. Thus hydrogenation does not actually help quantitative analysis and makes the procedure less diagnostic and more time consuming. Distinct advantages of the current DFRC protocol are that degradation monomers have a $C_6-C_3$ skeleton representing the phenylpropanoid characteristic of lignin, and that ester groups on $\gamma$-positions of lignin side chains remain fully intact (Lu and Ralph, 1997b,c). To our knowledge, this method is the first one possessing such features that will be exploited in future work.

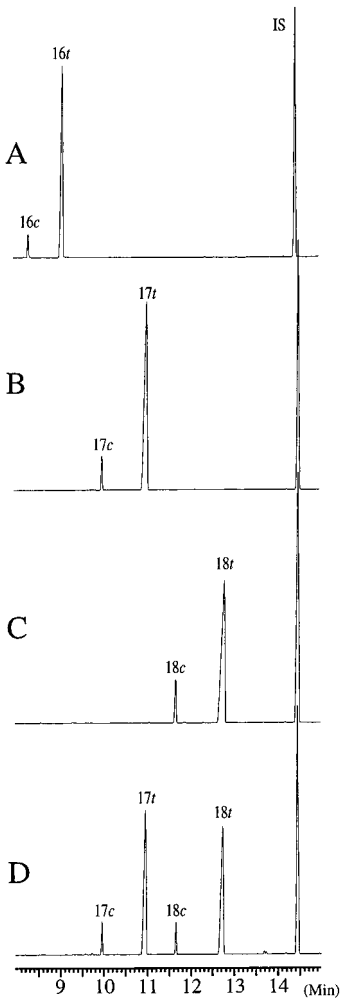


**Figure 5.** GC−FID chromatograms of DFRC monomers from (A) dimer **1**, (B) dimer **2**, (C) dimer **5**, (D) trimer **8**. *c* = *cis*, *t* = *trans*.

The primary degradation products of $\beta$-O-4 ether models possessing α-carbonyl groups are propiophenones **12** and **14** and 1-acetoxy-1-phenylacetones **13** and **15**, although the mechanisms involved are not understood at present. Those products are well separated in GC chromatograms from monomers derived from normal $\beta$-O-4 ethers. Some minor compounds, **10** and **11**, are also formed as shown in Figure 6. Although reactions are not as clean as for arylglycerol-$\beta$-aryl ether structures, it is possible to estimate α-carbonyl groups present in lignin units connected through $\beta$-O-4 ether bonds by using an extended reduction step in the DFRC method.

**Quantitative Determination of the Degradation Monomers.** We have shown (Lu and Ralph, 1996b) with lignin models that AcBr in acetic acid at room temperature is highly selective in its reactions with lignin, i.e., α-hydroxyls and α-ethers become α-bromo derivatives and $\gamma$-hydroxyls are acetylated rapidly, and phenolic hydroxyls are acetylated more slowly. Brominated acetylated derivatives of lignin models (and lignins) are formed in almost quantitative yields. The only detectable monomers from Zn reductive cleavage steps on $\beta$-ether units are pairs of 4-acetoxycinnamyl acetates, confirmed by NMR and GC−MS (see Figures 4 and 5). The relative simplicity in the resulting monomer composition makes quantitative analysis easy

**Table 2. Comparison of Monomer Yields from Thioacidolysis, Acidolysis, and DFRC Methods**

| | **1** (PG) | **2** (GG) | **3** (GS) | **4** (SG) | **5** (SS) | **8** (GGG) | **9** (GSS) G% | **9** (GSS) S% |
|---|---|---|---|---|---|---|---|---|
| DFRC method | 93.2 | 94.5 | 95.0 | 93.5 | 96.4 | 92.6 | 97.0 | 93.4 |
| thioacidolysis | | 70/75 | | 75 | 52−57 | | | |
| acidolysis | | 69 | | | 32 | | | |

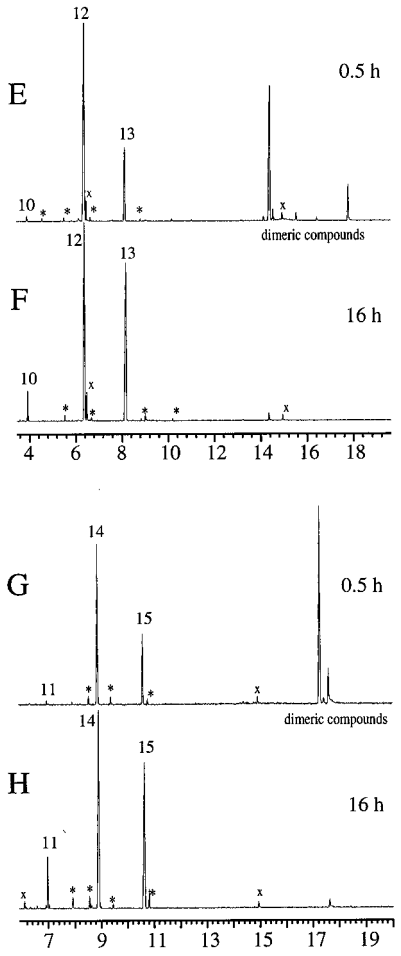


**Figure 6.** GC−MS total ion chromatograms of DFRC products from α-carbonyl dimers **6** (E, F) and **7** (G, H) using two Zn treatment times. x = Impurities, * = currently unidentified products.

and accurate. Reproducibility of the DFRC method is about 3−5%, comparable to other analytical techniques.

Monomer yields of AcBr/Zn degradation of models are 92−97% (Table 2), much higher than those of acidolysis or thioacidolysis (Lapierre et al., 1985; Grabber et al., 1996). Extensive nonspecific derivatization of lignin and numerous side reactions are generally difficult to avoid in lignin degradation reactions carried out at high temperatures with reactive acids or Lewis acids. It is believed that the frequency of condensation reactions is lower for model compounds than is the case in real lignin. Even for thioacidolysis, the mildest and most selective method to date, the best monomer yield from model compounds is far from quantitative (Lapierre et al., 1985; Grabber et al., 1996). It is unlikely that, when applied to real lignins, thioacidolysis can completely cleave all desired linkages and recover desired monomers quantitatively. Thioacidolysis is also sensitive to experimental conditions. For example, we recently found significant quantities of $\beta$-O-4 ether dimers in our thioacidolysis runs when the experimental conditions were not optimized (Ralph and Grabber, 1996). Forthcoming publications will examine how the DFRC method compares with respect to sensitivity to sample type, temperature, duration of the steps involved, and sample moisture content.

The high yield of degradation monomers from $\beta$-ether models gives the DFRC method the potential to quantitate uncondensed lignin structural units and be further used in studies on lignin cross-linking chemistry in cell walls. All reactions involved in the DFRC method are run at room temperature (up to 50 °C for lignins and cell wall samples) and released products are reasonably stable, so secondary condensation reactions can presumably be avoided. This was reflected by high monomer yields obtained for lignin models subjected to the DFRC method (Table 2). No apparent differences were found between monomer yields from *p*-hydoxyphenyl, guaiacyl, and syringyl models. Therefore, all units connected by $\beta$-O-4 ethers in lignin can be cleaved equally efficiently and the monomer ratio between *p*-hydroxyphenyl, guaiacyl, and syringyl derivatives is diagnostic of the proportions of those units releasable as monomers.

As for model compounds **6** and **7** possessing α-carbonyl groups, $\beta$-ether cleavage by the standard DFRC procedure is less efficient than in normal $\beta$-ether models **1**−**5** and **8** and **9**, although up to 60% of their ether linkages were cleaved as shown by GC chromatograms (Figure 6). Because models **6** and **7** have phenacyl ether skeletons which can be cleaved almost quantitatively by Zn in acetic acid (Hendrickson and Kandall, 1970), the ether linkages in models **6** and **7** could be eventually cleaved as long as the Zn treatment times are sufficiently long. This idea proved to be correct when models **6** and **7** were subjected to the DFRC method with overnight (16 h) Zn treatment. The starting dimeric compound almost completely disappeared in GC−MS total ion chromatograms of the corresponding degradation products (Figure 6). The main products recovered were **12** or **13** and **14** or **15** plus small amounts of **10** or **11**. Therefore, caution is required when this method is applied to lignins with high α-carbonyl contents. Reducing carbonyls to hydroxyl groups by sodium borohydride (or sodium borodeuteride to allow mass spectrometric distinction) before the DFRC procedure, or prolonging the zinc treatment may be worthy modifications to apply when quantitation of α-carbonyl groups becomes a crucial consideration.

## CONCLUSION

From this study it has been demonstrated that $\beta$-ether linkages in lignin models were efficiently cleaved through derivatization with AcBr followed by reductive cleavage, now referred to as the DFRC method. This method can be applied to isolated lignin and lignins in situ as will be shown in forthcoming publications. Its simplicity and cleanliness, and the use of relatively innocuous reagents, may provide a suitable alternative to the widely used thioacidolysis procedure for some analyses.

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