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Determination of nitrobenzene oxidation products by GC and ^{1}H-NMR spectroscopy using 5-iodovanillin as a new internal standard

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Abstract The nitrobenzene oxidation method was modified to obtain more reproducible data and more structural information about lignin, not only by gas chromatography (GC) but also by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy for quantitative determination of the oxidation products and to simplify the procedures. The nitrobenzene oxidation mixture was directly extracted after acidification without preextraction of by-products. The direct extraction made the extractive step easy and gave reproducible data. 5-Iodovanillin was selected as a new internal standard. The reason for this selection was that 5-iodovanillin did not exist in the nitrobenzene oxidation products from any plant species and had an aldehyde group whose peak did not overlap with the other aldehyde peaks on an ^{1}H-NMR spectrum. Thus, the use of 5-iodovanillin enabled us to quantify *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde in oxidation products on the basis of ^{1}H-NMR analysis as well as GC. Furthermore, more information about the condensed structure of lignin was derived by comparing the ^{1}H-NMR and GC analyses.

Key words Lignin · 5-Iodovanillin · Modified nitrobenzene oxidation · ^{1}H-NMR analysis

Introduction

The nitrobenzene oxidation method is widely used for structural analysis of lignin.[1,2] It is comprised of the reaction steps; extraction of some by-products and products such as *p*-hydroxybenzaldehyde (2a), vanillin (3a), and syringaldehyde (4a) (Fig. 1); and instrumental analysis. The conventional method was first proposed using lignin model compounds[3] and was modified mainly after advances in analytical instruments.[4] The use of acetovanillone (5a) as an internal standard focuses on an important issue of quantitative analysis because the compound can be derived from lignin as a reaction product.[5] Alternatively, *m*-meconin has been used as an internal standard because of its absence of reaction products, but it is not commercially available. Furthermore, extraction procedures are lengthy and may cause incomplete recovery of products because of the differences in partition coefficients of products.

In this paper, we report on the improved reproducibility of the nitrobenzene oxidation method by introduction of an alternative internal standard, 5-iodovanillin (1a), which does not exist in reaction products. It is done simplifying the extraction steps and using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy together with conventional gas chromatography (GC).

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Experimental

Materials

Wood meals of eucalypt (*Eucalyptus globulus*) from Chile, spruce (*Picea abies*) from Canada, and bamboo (*Phyllostachys pubescence*) from Japan were extracted with 95% ethanol/benzene (1:2, v/v) for 6h to remove extractives using a Soxhlet apparatus and subsequently with 0.25% potassium acetate solution at 60°C for 13 days with stirring to remove pectin.[6] The resulting extracted wood meals (EWMs) were utilized in this study. The Klason lignin contents of those EWMs were determined according to the method of TAPPI Standard T 222om-83.[7]

Conventional nitrobenzene oxidation method

Samples of 50mg were reacted with 4ml of 2N NaOH solution and 0.24ml of nitrobenzene oxidation in a 10-ml

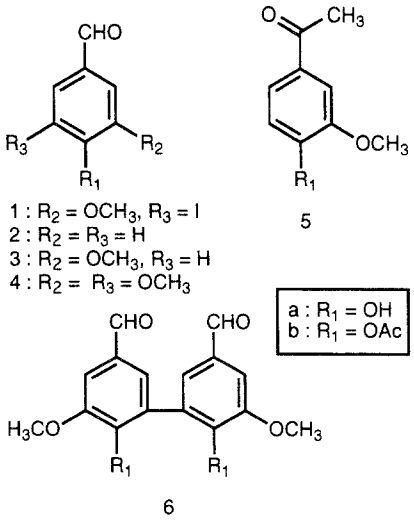

Fig. 1. Nitrobenzene oxidation products and internal standards

stainless steel vessel for 2 h, empirically determined by the products' yield at 170°C in an oil bath. The reaction mixture was cooled to room temperature by immersion in running water and then filtered with a glass filter. The residue was washed with 0.1 N NaOH (1 ml × 5). The filtrate and washings combined were extracted with diethyl ether (30 ml × 3), and the aqueous layer was acidified to pH 2–3 with 1 N HCl solution. The solution was then mixed with 0.5 ml 1,4-dioxane containing 1.5 mg acetovanillone (5a) as an internal standard. It was further extracted with diethyl ether (30 ml × 3) and dried over Na_2SO_4 and in vacuo. The products were acetylated with 2 ml of acetic anhydride/pyridine (1:1, v/v) at 50°C for 2 h. After removing the acetylating reagents by evaporation with ethanol, the acetylated products were dissolved in acetone to be analyzed by GC.[2]

Modified nitrobenzene oxidation method

The conventional method was modified as follows. Samples were reacted in a 10-ml Teflon tube at 170°C for 1 h in an autoclave (TAIATSU, TEM-D, 500 ml). The reaction mixture was cooled to about 100°C under room temperature and then soaked in running water. Before filtration, 0.5 ml 1,4-dioxane containing 2.5 mg 5-iodovanillin (1a) as an internal standard was added to the reaction mixture. NaOH filtrate and washings combined were acidified to pH 2–3 with 1 N HCl solution and extracted with ethyl acetate (30 ml × 3). The organic layer was then washed with brine and dried over Na_2SO_4 to be analyzed by GC with or without ^{1}H-NMR spectroscopy.

5-Iodovanillin

5-Iodovanillin (1a) was synthesized as follows.[8] To a stirred solution of vanillin (1.52 g, 10 mmol) in 2 N NaOH/H_2O (13.5 ml; 2:3, v/v), a solution of iodine (2.54 g; 10 mmol) and potassium iodide (2.82 g; 17 mmol) in H_2O (20 ml) was added dropwise until the red color of iodine did not disappear. The precipitated crystals were filtered and washed with water, ethanol, and *n*-hexane to yield crude crystals as slightly yellow powder. It was recrystallized three times from acetic acid (1.75 g, 63%), mp: 179.5°C (ref. 180°–181.5°C),[8] ^{1}H-NMR ($CDCl_3$): 3.97 (3H, s, —OCH_3), 7.38 (1H, d, J = 1.8 Hz,), 7.82 (1H, d, J = 1.8 Hz,), 9.77 (1H, s, —CHO).

GC analysis

Gas chromatography (Shimadzu GC18A, Kyoto, Japan) with a flame ionization detector (FID) and a fused silica capillary column (Shimadzu OV-17, 30 m × 0.25 mm i.d., coated with 0.25 μm 50% phenylmethylpolysiloxane, Kyoto, Japan) were used at column temperature 230°C, injector temperature 270°C, detector temperature 270°C, and carrier gas He (0.1 MPa). The peaks on the chromatogram were identified using GC-mass spectrometry (MS) (Shimadzu QP5000, Kyoto, Japan) with an electron impact ionization source (70 eV). As response factors for *p*-hydroxybenzaldehyde acetate (2b), vanillin acetate (3b), and syringaldehyde acetate (4b), 0.798, 0.839, and 0.828 in the case of acetovanillone as an internal standard and 1.13, 1.17, and 1.14 in the case of 5-iodovanillin were utilized by the experiment of corresponding authentic compounds. The retention times of acetylated aldehydes in the oxidation mixture were 2.045 min (2b), 2.558 min (3b), 2.939 min (5b), 3.652 min (4b) and 4.985 min (1b), respectively.

^{1}H-NMR analysis

^{1}H-NMR spectra were collected with Varian INOVA300 FT-NMR (300 MHz) spectrometer, in chloroform-*d* with tetramethylsilane (Me_4Si) as an internal standard. Chemical shifts (δ) and coupling constants (J) were given in δ values (ppm) and Hz, respectively. The oxidation monomers were quantitatively analyzed by ^{1}H-NMR spectroscopy on the basis of the aldehyde peaks area appearing at δ 9.86 ppm (1b), 9.90 ppm (4b), 9.94 ppm (3b), and 9.98 ppm (2b).

Results and discussion

The nitrobenzene oxidation method consists of three reaction steps: oxidation, extraction, and analysis of the products. In this study the oxidation and extraction steps were made simple. A new internal standard was selected to determine the nitrobenzene oxidation products by both ^{1}H-NMR spectroscopy and GC. Table 1 shows a comparison of these three steps for the conventional method and the modified method.

Oxidation step

With the conventional method, materials was heated in an oil bath at 160°–170°C for 2 h. After considering the relation

Table 1. Conventional and modified nitrobenzene oxidation methods

Parameter	Conventional method	Modified method
Oxidation conditions	160°–170°C, 2 h	170°C, 1 h
Internal standard	Acetovanillone *m*-Meconin	5-Iodovanillin
Extraction	Alkaline and acidified solutions	Acidified solution
Analysis	GC	GC and NMR

GC, gas chromatography; NMR, nuclear magnetic resonance

between the reaction time and the aldehyde yields to Klason lignin, it was found that the yields reached a maximum after 1 h of heating at 170°C with the modified method.

Internal standard

With conventional methods, acetovanillone (5a) or *m*-meconin has been used as an internal standard. Several problems remain, however, as follows.[9] Acetovanillone (5a) is comparatively unstable under basic conditions and itself exists in nitrobenzene oxidation products of lignin.[4] *m*-Meconin is not commercially available and is difficult to synthesize. Thus, acetovanillone (5a) and *m*-meconin are not suitable as internal standards for general use.

A new compound was selected as an internal standard after considering the following three points: (1) A new internal standard must give a peak well separated from those of nitrobenzene oxidation products and from by-products of nitrobenzene on the GC chromatogram. (2) All nitrobenzene oxidation products analyzed have aldehyde groups. This means that there is a good possibility of determining these aldehydes quantitatively on the basis of the peak areas of their aldehyde protons by ^{1}H-NMR spectroscopy if an internal standard with an aldehyde group is selected that does not overlap other peaks of oxidation products. (3) A new internal standard should be stable under basic conditions and also should not be given in nitrobenzene oxidation products of lignin. After considering several compounds, 5-iodovanillin (1a) was finally selected for the new internal standard (Table 1).

Extraction step

It is important that the peaks of oxidation products do not overlap other peaks of reduced by-products from nitrobenzene in the GC analysis. With the conventional method, the alkaline reaction mixture is extracted several times with organic solvents to remove the reduced products of nitrobenzene. With the ^{1}H-NMR analysis, based on the peak areas of the aldehyde peaks, it is not necessary to remove nitrobenzene-reduced products by extraction of the mixture because the reduced products of nitrobenzene do not have aldehyde groups.

The extraction process is troublesome because we must consider the partition coefficient of each compound for the quantitative determination. To minimize the influence of

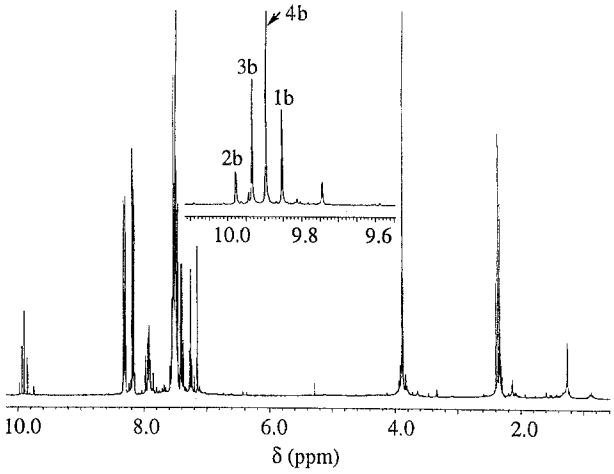

Fig. 2. The 300-MHz ^{1}H-NMR spectrum of modified nitrobenzene oxidation mixture (acetate) from bamboo (*Phyllostachys pubescence*) extracted wood meals

those partition coefficients, the process for excluding the reduced products of nitrobenzene was omitted; that is, the solution was acidified after cooling, and the reaction mixture was then immediately extracted three times with ethyl acetate (Table 1). As a result of the direct extraction, the extractive step was made easy and gave us reproducible data without any consideration of partition coefficients of the products of the extraction. Although the resulting oxidation mixture contained reduced products from nitrobenzene, the aldehyde peaks of the aldehydes (2b, 3b, 4b) and acetyl 5-iodovanillin (1b) appeared as separate peaks without any overlap with the other products from nitrobenzene on the ^{1}H-NMR spectrum after acetylation (Fig. 2). All peaks of the aldehydes and 5-iodovanillin appeared as separate peaks on the gas chromatogram as well (Fig. 3b). This shows that the oxidation products can be determined not only by ^{1}H-NMR spectroscopy but also by GC, even if nitrobenzene oxidation mixtures contain many reduced by-products from nitrobenzene.

GC and ^{1}H-NMR analyses

As a result of selecting a new internal standard, reproducible values of quantitative determinations of 2a, 3a, and 4a can be obtained by both GC and ^{1}H-NMR spectroscopy. Figure 3 shows the gas chromatograms obtained by both conventional and modified nitrobenzene oxidation methods from bamboo EWM. With the conventional method, 2b, 3b, 4b, and 5b appeare as separate peaks with slight amounts of reduced products from nitrobenzene (Fig. 3a). With the modified method, the peaks of these aldehydes (2b, 3b, 4b) also appeared as separate peaks, even though the four peaks of the reduced products of nitrobenzene existed in high yields (Fig. 3b). The acetyl 5-iodovanillin (1b) appears as a single peak that does not interfere with those of the other oxidation products; the purity of the aldehyde (1b, 2b, 3b, 4b) peaks were confirmed by GC-MS. The oxidation

products were determined on the basis of their peak areas on gas chromatograms by both the modified method and the conventional method from eucalypt, spruce, and bamboo (Table 2). Comparing the conventional method with the modified method, the yields of total amounts of 2a, 3a, and 4a to Klason lignin from three species are slightly larger with the modified method than with the conventional method.

Figure 2 shows an ^{1}H-NMR spectrum of the modified nitrobenzene oxidation products of bamboo EWM measured in $CDCl_3$. Aldehyde proton of acetyl 5-iodovanillin (1b), which appeared at δ 9.86, was well separable from the other aldehyde protons at δ 9.98 ppm (2b), δ 9.94 ppm (3b), and δ 9.90 ppm (4b). The response factors of 2b, 3b, and 4b to the internal standard (1b) should be 1.00 by ^{1}H-NMR analysis. Therefore, these oxidation products were determined on the basis of these aldehyde peaks. Figure 4 shows the ^{1}H-NMR spectra of nitrobenzene oxidation products from the EWM of spruce, bamboo, and eucalypt.[10] The peaks of these aldehydes of 1b, 2b, 3b, and 4b appeared as separate peaks in each spectrum. A small peak at δ 9.95 ppm, in addition to the peak of 3b, was found to be that of dehydrodivanillin acetate (6b).

Table 2 also shows the yields of total amounts of *p*-hydroxybenzaldehyde (2a) (H), vanillin (3a) (V), and syringaldehyde (4a) (S) to Klason lignin and the molar ratio

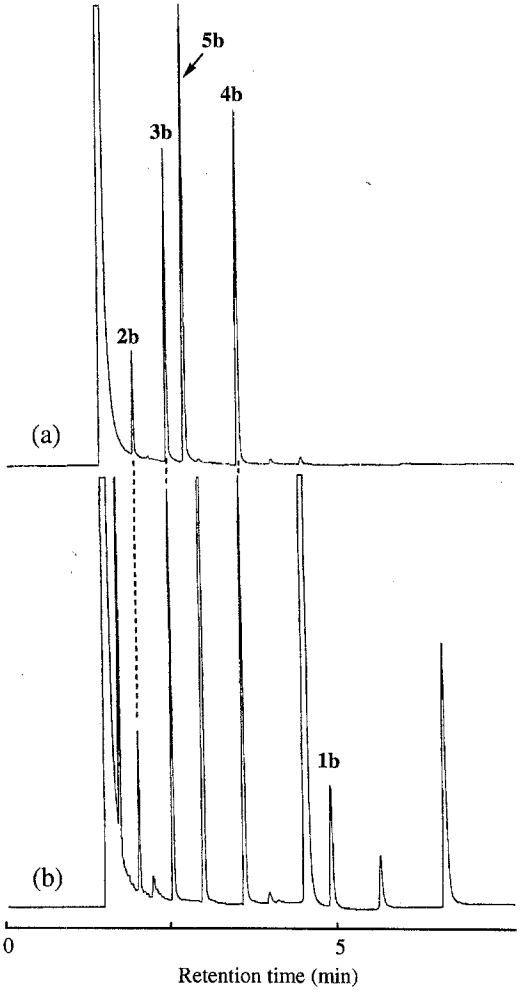

Fig. 3. Gas chromatograms of nitrobenzene oxidation mixtures from extracted wood meals of bamboo (*Phyllostachys pubescence*). Conventional method (**a**) used acetovanillone as an internal standard; modified method (**b**) used 5-iodovanillin as an internal standard. *1b*, 5-iodovanillin acetate; *2b*, *p*-hydroxybenzaldehyde acetate; *3b*, vanillin acetate; *4b*, syringaldehyde acetate; *5b*, acetovanillone acetate

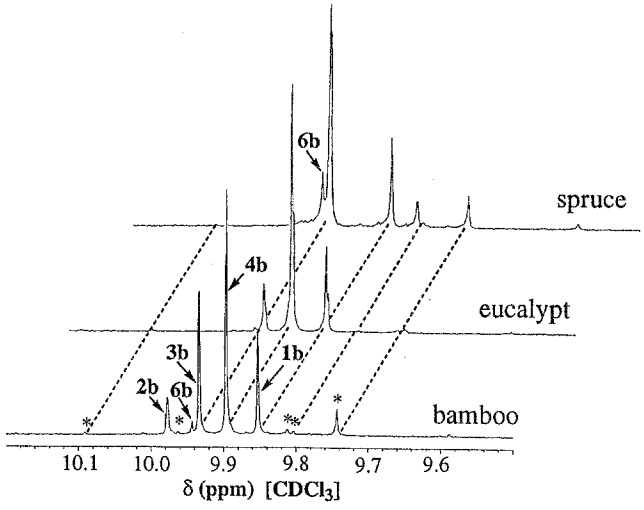

Fig. 4. The 300-MHz ^{1}H-NMR spectra of modified nitrobenzene oxidation mixture (acetate). *6b*, dehydrodivanillin acetate; *, unidentified products

Table 2. Yields of phenolic aldehydes from several plant species on nitrobenzene oxidation

Plant species[a]	Klason lignin	Conventional method GC		Modified method GC		^{1}H-NMR	
		Total aldehyde[b]	Molar ratio (H:V:S)[c]	Total aldehyde	Molar ratio (H:V:S)	Total aldehyde	Molar ratio (H:V:S)
Eucalypt (*Eucalyptus globulus*)	20.4	48.5	0:1.0:5.2	50.5	0:1.0:5.1	51.9	0:1.0:4.7
Spruce (*Picea abies*)	26.3	29.4	–	29.9	–	30.6	–
Bamboo (*Phyllostachys pubescence*)	27.5	31.9	0.4:1.0:1.4	35.5	0.3:1.0:1.4	36.7	0.3:1.0:1.4

[a] The specimens of three samples are extracted wood meals (EWMs)
[b] Total aldehyde = H + V + S / Klason lignin (yield in weight % / Klason lignin)
[c] H, *p*-hydroxybenzaldehyde (2a); V, vanillin (3a); S, syringaldehyde (4a)

of H/V/S from EWM of eucalypt, spruce, and bamboo measured by GC and ^{1}H-NMR spectroscopy using the modified method. The yields of oxidation products of three samples obtained by GC were almost the same as those seen by of ^{1}H-NMR spectroscopy. The molar ratios of H/V/S of eucalypt and bamboo measured by GC were also almost the same as those obtained by ^{1}H-NMR spectroscopy. The aldehyde yield of eucalypt by ^{1}H-NMR spectroscopy was slightly higher than that obtained by GC. This observation about the yields suggests that nitrobenzene oxidation mixtures from eucalyptus might contain some oxidation products that did not appear on the gas chromatogram but could overlap the peak of vanillin acetate (3b) on the ^{1}H-NMR spectrum. Those oxidation products may be derived from condensed structures of lignin, such as dehydrodivanillin (6a). The finding of dehydrodivanillin shows that some knowledge about condensed structures of lignin can be obtained by a comparison of GC and ^{1}H-NMR spectroscopy results. In conclusion, ^{1}H-NMR analysis was found to be an adjunctive method to GC analysis for quantitative determination of the nitrobenzene oxidation products using 5-iodovanillin as a new internal standard.

Conclusions

A modified nitrobenzene oxidation method using 5-iodovanillin as an internal standard was proposed to determine the introbenzene oxidation products by both GC and ^{1}H-NMR spectroscopy. The use of 5-iodovanillin as an internal standard made it possible to analyze nitrobenzene oxidation products by ^{1}H-NMR spectroscopy as well as by GC. The extraction step was simplified, and reproducible values were obtained. The modified nitrobenzene oxidation method was applied to eucalypt, spruce, and bamboo lignin. Regarding the total yields of aldehydes to Klason lignins from these three samples, there was little difference between GC analysis and ^{1}H-NMR analysis. There was an indication that new knowledge about condensed structures of lignin could be obtained by comparing the results of GC with those of ^{1}H-NMR spectroscopy.

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