Plant Biotechnology 27, 305–310 (2010)

Original Paper

Microscale alkaline nitrobenzene oxidation method for high-throughput determination of lignin aromatic components

Masaomi Yamamura[1], Takefumi Hattori[1], Shiro Suzuki[2], Daisuke Shibata[3], Toshiaki Umezawa[1,2,*]

[1] Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan; [2] Institute of Sustainability Science, Kyoto University, Uji, Kyoto 611-0011, Japan; [3] Kazusa DNA Research Institute, Kisarazu, Chiba 292-0818, Japan

* E-mail: tumezawa@rish.kyoto-u.ac.jp Tel: +81-774-38-3625 Fax: +81-774-38-3682

Received December 17, 2009; accepted March 31, 2010 (Edited by M. Yamazaki)

Abstract The nitrobenzene oxidation method is widely used for structural analysis of lignin. However, the conventional nitrobenzene oxidation method has several drawbacks including the requirement of a sizeable amount of sample material and the rather slow completion of the reaction process. In this paper, we describe a microscale nitrobenzene oxidation method using deuterium-labeled vanillin and syringaldehyde as internal standards. Using this method, we show that the microscale nitrobenzene oxidation method realized high-throughput determination of lignin components using a small amount of sample, i.e. 10 mg per reaction, and high reproducibility. Due to the small sample sizes, the oxidation reaction and extraction steps of a number of samples can be completed in parallel, thus enabling us to process more than 40 samples per day.

Key words: High-throughput, lignin, nitrobenzene oxidation.

Lignin is a major component of the secondary cell wall of vascular plants, and fills the spaces between cell walls and cell wall polysaccharides. Lignin confers mechanical strength and imperviousness to the cell wall, allowing plants to grow upward (Boerjan et al. 2003; Vanholme et al. 2008). Therefore, the ability to synthesize lignin has been essential in the evolutionary adaptation of plants from an aquatic environment to land (Boerjan et al. 2003).

Lignin has several properties that present obstacles to chemical pulping, forage digestion and the conversion of plant cell wall polysaccharides into biofuels. To improve these processes the characterization of lignin is essential.

Lignins are generally classified into three major groups: guaiacyl (4-hydroxy-3-methoxyphenyl), syringyl (3,5-dimethoxy-4-hydroxyphenyl) and *p*-hydroxyphenyl lignins. Gymnosperm (softwood) lignin is mainly composed of guaiacyl lignin. In contrast, angiosperm (hardwood) lignin consists of both guaiacyl and syringyl lignins. Grass (gramineae) lignins are also classified as guaiacyl-syringyl lignins. However, unlike other angiosperm lignins, grass lignins additionally contain small, yet significant amounts of *p*-hydroxyphenyl lignin (Sarkanen and Hergert 1971). In addition, the syringyl/guaiacyl (S/G) ratio varies between plant species. Plants with higher S/G values are more easily delignified in kraft pulping (Chiang and Funaoka 1990). Consequently, the aromatic composition of lignin is one of the essential criteria used to characterize lignins.

Alkaline nitrobenzene oxidation was introduced as a diagnostic device of lignin in 1939 by Feudenberg and co-workers, being an adaptation of a long known technique for the conversion of isoeugenol to vanillin (2) (Chang and Allan 1971; Chen 1992). When applied to lignin-containing materials, the major oxidation products are phenolic aldehydes such as *p*-hydroxybenzaldehyde (1), vanillin (2) and syringaldehyde (3) (Figure 1). These products are derived from oxidative degradation of the corresponding 4-hydroxyphenylpropane units and their ethers, that is, the corresponding 4-*O*-alkylated (α-*O*-4 and β-*O*-4) lignin substructures. The molar ratio of these aldehydes provides information on the relative amounts of the uncondensed *p*-hydroxyphenyl, guaiacyl and syringyl units that constitute the original lignin. Thus, the syringyl to guaiacyl (S/G) ratio has long been identified as a significant parameter for wood lignin characterization (Chang and Allan 1971). The optimal reaction conditions for nitrobenzene oxidation of lignins were elucidated by Leopold (1950). In general, nitrobenzene oxidation of lignins has been conducted in laboratories according to modifications of the original procedure developed by Leopold (1950) (Chen 1992).

This article can be found at http://www.jspcmb.jp/

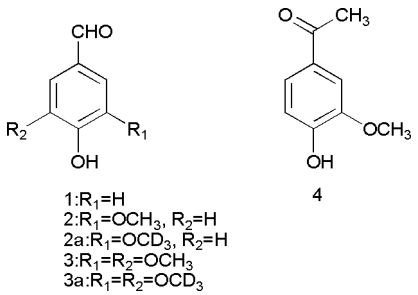

Figure 1. Nitrobenzene oxidation products and internal standards.

In the conventional method for nitrobenzene oxidation of lignin (Chen 1992), samples are reacted in stainless steel vessels at 170°C in an oil bath. The vessels are sealed tightly with a screw cap fitted with a Teflon gasket. However, this process of sealing the vessel is problematic and often fails, leading to reactant leakage. Katahira and Nakatsubo (2001) reported an improved method that uses a Teflon tube as a reaction vessel which is heated and pressurized in an autoclave. This approach eliminated the possibility of reactant leakage. In addition, 5-iodovanillin was used as an internal standard, which enables the analysis of the products by ^{1}H-NMR without the removal of unreacted nitrobenzene and products therein (Katahira and Nakatsubo 2001).

Recently, we have proposed a modification of the method by Katahira and Nakatsubo (2001), which enables the complete reaction to be completed in a standard working day, i.e. 8 hours (Umezawa et al. 2007).

During the last two decades, there has been intense interest in the metabolic engineering of lignin biosynthesis to develop plant materials which are suitable for chemical pulping, forage digestion and biofuel production, as well as the elucidation of the functions of the genes involved in lignin biosynthesis (Vanholme et al. 2008). In these research and development activities, a large number of transgenic plant lines have been produced. Such transgenic plant lines often have subtle changes in lignins which require rapid and precise characterization. However, the conventional nitrobenzene oxidation methods are inefficient for accurate high-throughput characterization, because such approaches are time-consuming and data variations are non-negligible. Herein, we report a microscale, high-throughput and highly accurate nitrobenzene oxidation method using deuterium-labeled internal standards.

Materials and methods

Plant materials

Acacia mangium heartwood meal was a gift from Koshii Wood Industry Co., Ltd. The wood meal was freeze dried and further powdered with a TissueLyser (Qiagen Co., Ltd.) for 2.5 min at 26 Hz and room temperature. The wood meal obtained was extracted under reflux with a 99.8% ethanol/toluene (2:1, v/v) solution for 6 h and subsequently with methanol for 2 h using a Soxlet apparatus. The extracted wood meal was subsequently dried under high-vacuum.

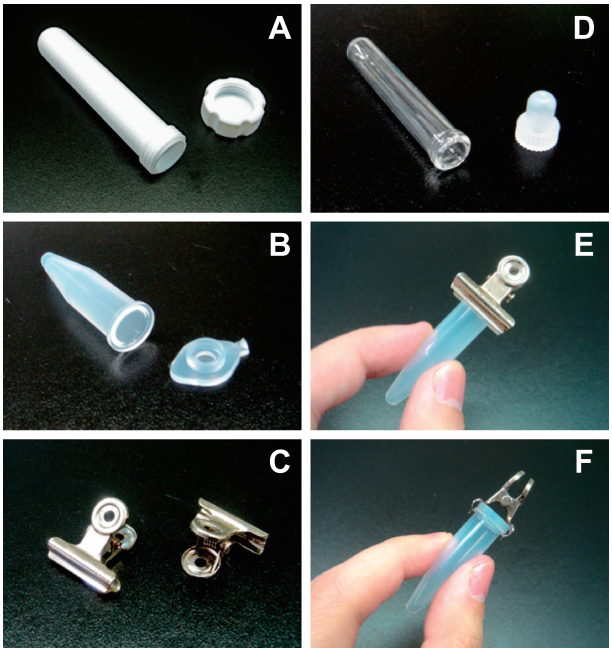

Figure 2. Tubes used for nitrobenzene oxidation and its work up. (A) 18 ml Teflon tube. (B) 1.5 ml Teflon microcentrifuge tube. (C) Medama clip. (D) 1 ml grass microtube. E and F, Illustrates how a medama clip clamps the lid of the microcentrifuge tube.

Chemicals

Vanillin-d_3 (2a) and syringaldehyde-d_6 (3a) were synthesized previously (Sakakibara et al. 2007). All other reagents were obtained from Nacalai Tesque Co., Ltd. or Wako Pure Chemicals Co., Ltd., unless otherwise noted.

Instrumentation

Gas chromatography-mass spectrometry (GC-MS) was carried out with a Shimadzu QP-5050A GC-MS system (Shimadzu Co., Ltd.) [electron impact mode (70 eV); column, Shimadzu Hicap CBP-10-M25-0.25 column (25 m×0.22 mm); carrier gas, helium; injection temperature, 240°C; oven temperature 40°C at $t=0$ to 2 min, then to 230°C at 40°C min^{-1}]. The samples for GC-MS were dissolved in 8 µl of *N*,*O*-Bis(trimethylsilyl)acetamide (BSA) and left standing at 60°C for 45 min; then an aliquot of the BSA solution was subjected to GC-MS analysis.

Conventional nitrobenzene oxidation method

A slight modification (Umezawa et al. 2007) of the method by Katahira and Nakatsubo (2001) was employed. Briefly, 50 mg of dried sample was suspended in 4 ml of 2 N NaOH and 0.24 ml of nitrobenzene in a 18 ml Teflon tube (Sogo Rikagaku Glass Works Co., Ltd.; 18 ml round-bottomed Teflon tube, Catalog number 355-04, Figure 2A) and heated in an autoclave (Taiatsu Techno Co., Ltd.; Metal Reactor TEM-D1000M) at 170°C and 0.8 MPa for 1 h. The autoclave reactor was sub-

sequently cooled down to ~80°C and the Teflon tube was taken out of the reactor and cooled under running water. One hundred µl of acetovanillone (4) in 1,4-dioxane (10 mg ml^{-1}) was added to the reaction mixture as an internal standard. Next, the reaction mixture was filtered and the filtrate was extracted with ethyl acetate (30 ml×3) to remove unreacted nitrobenzene and products derived there of. The alkaline water layer was acidified to pH 2–3 with a 1 N HCl solution and extracted with ethyl acetate (30 ml×3). The organic layer was washed with brine and dried over anhydrous Na_2SO_4 and the solvent was removed by evaporation. The sample obtained was analyzed by GC-MS after trimethylsilylation. Vanillin and syringaldehyde were quantified using a calibration curve for either vanillin or syringaldehyde {$Y=2.769X+0.019$, 0.52–6.5 nmol range, vanillin; $Y=24.188X-0.022$, 0.44–44 nmol range, syringaldehyde. X=vanillin or syringaldehyde (nmol), Y=ion current ratio of vanillin or syringaldehyde/acetovanillone (1.2 nmol)}.

Microscale nitrobenzene oxidation method

Ten mg of dried sample was placed into a 1.5 ml Teflon microcentrifuge tube (Flonchemical Co., Ltd.; 1.5 ml PFA sampling cup, Catalog number 1322-02, Figure 2B). Five-hundred µl of 2 M NaOH and 30 µl of nitrobenzene were added to the tube and the resulting suspension was mixed by vortexing. The lid of the tube was clamped using a stainless-clip (KOKUYO Co., Ltd.; Medama Clip, Catalog number Kuri-17B, Figure 2C) to prevent the lid from opening as shown in Figure 2E and F. The reaction mixture in the tube was heated and pressurized at 170°C and 0.8 MPa for 1 h in the autoclave (Taiatsu Techno Co., Ltd.; Metal Reactor TEM-D1000M). The autoclave reactor was cooled to about 100°C at room temperature. The reaction tube was removed from the reaction vessel and left standing on ice for several minutes. Subsequently, 10 µl of 1,4-dioxane containing 100 µg acetovanillone as an internal standard was added to the reaction mixture and this solution was mixed by vortexing and centrifuged (2000×g). Two hundred and fifty µl of the supernatant of the total reaction mixture was transferred to a 1 ml glass microtube (Figure 2D) {Maruemu Co., Ltd.; 1 ml microtube (No. 1), Catalog number 0407-02}, and extracted with ethyl acetate (200 µl×4). The aqueous layer was then transferred to a new 1 ml glass microtube, acidified to pH 2–3 by the addition of 60 µl of 6 N HCl and extracted with ethyl acetate (200 µl×3). Here, the 1 ml grass microtube was placed in a 2 ml polypropylene microcentrifuge tube without a lid. The glass tube inside the 2 ml microcentrifuge tube was centrifuged at 2000×g to ensure the complete separation of the ethyl acetate and water layers. The combined organic solutions were washed with a saturated aqueous NaCl solution and dried over anhydrous Na_2SO_4. Subsequently, 10 µl of the organic solution obtained was evaporated under high-vacuum and subjected to GC-MS analysis after trimethylsilylation.

In separate experiments, the nitrobenzene oxidation reactions were carried out as described above with the following modifications. First, the mixture of deuterium-labeled compounds, vanillin-d_3 (2a) and syringaldehyde-d_6 (3a), were used as internal standards instead of acetovanillone. Second, a 1.5 ml polypropylene microcentrifuge tube was used rather than the 1 ml glass microtube. Following the completion of the reaction, 20 µl of a mixture of deuterium-labeled internal standards, vanillin-d_3 (10 mg ml^{-1}) and syringaldehide-d_6 (10 mg ml^{-1}), were added to the reaction mixture. This solution was mixed thoroughly and centrifuged. Two hundred and fifty µl of the supernatant of the total reaction mixture was transferred to a new 1.5 ml polypropylene microcentrifuge tube and extracted with ethyl acetate (200 µl×4). The aqueous layer was then transferred to a new 1.5 ml polypropylene microcentrifuge tube, acidified to pH 2–3 by the addition of 60 µl of 6 N HCl, and extracted with ethyl acetate (200 µl×3). The combined organic solutions were washed with a saturated aqueous NaCl solution, dried over anhydrous Na_2SO_4 and a portion of this solution was evaporated under high-vacuum. The reaction products were analyzed by GC-MS after trimethylsilylation {$Y=1.030X-0.046$, 1–18 nmol range, vanillin; $Y=1.012X+0.0673$, 1–18 nmol range, syringaldehyde. X=vanillin or syringaldehyde (nmol), Y=ion current ratio of vanillin/vanillin-d_3 (1 nmol) or syringaldehyde/syringaldehyde-d_6 (1 nmol)}.

Results and discussion

Historically, the nitrobenzene oxidation method was established for the analysis of lignin present in the secondary xylem of woody plants. The conventional method requires rather large amounts of samples (ca. 200 mg for triplicate analysis) and a standard working day (8 hours) to complete the reaction and work up. Although such a protocol is time consuming and requires large amounts of material, the technique has been successfully applied to analyse the lignin content of woody plants. This is because this approach provides key information about the lignin character, including the aromatic composition, and because in general it is easy to obtain large amounts of wood samples, and the number of samples to be analyzed is usually small.

However, recent research and development activities of lignin metabolic engineering necessitates an accurate high-throughput method to analyze lignin content, because these activities handle a large number of transgenic lines which often show only slight modifications of lignin character when compared with control plants.

In this context, we have recently established a high-throughput method for lignin determination as thioglycolic acid lignin from rice straw using disposable plastic microtubes, a small ball-mill homogenizer and a UV microplate reader (Suzuki et al. 2009).

In the present study, we have established a microscale, high-throughput method for aromatic unit characterization of lignins using a nitrobenzene oxidation method. We have confirmed the formation of the nitrobenzene oxidation products, vanillin and syringaldehyde, from the *Acacia mangium* heartwood using the present microscale method. The products were identified by comparison of their retention times and mass spectra (trimethylsilyl [TMS] ethers) with those of unlabelled authentic samples (Figure 3A, B). As shown in Figure 3A, the retention times of nitrobenzene oxidation product 1 and 2 (7.42

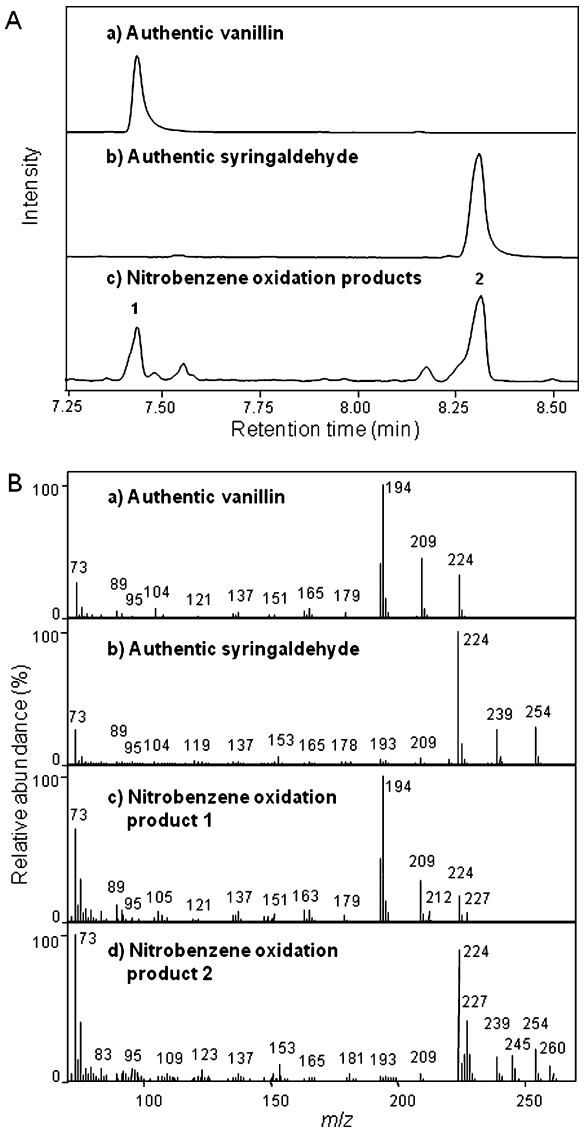

Figure 3. GC-MS analysis of TMS ethers of reaction products obtained by microscale nitrobenzene oxidation. (A) Total ion chromatograms of TMS ethers of phenolic aldehydes. a, Authentic vanillin; b, Authentic syringaldehyde; c, Nitrobenzene oxidation products. (B) Mass spectra of TMS ethers of phenolic aldehydes. a, Authentic vanillin; b, Authentic syringaldehyde; c, Nitrobenzene oxidation product 1; d, Nitrobenzene oxidation product 2. Note that vanillin-d_3 ($[M]^+ + 3 = m/z$ 227) and syringaldehyde-d_6 ($[M]^+ + 6 = m/z$ 260) were added as internal standards in nitrobenzene oxidation products (A-c, B-c, B-d).

and 8.29 min, Figure 3A-c) were identical to those of authentic vanillin (Figure 3A-a) and syringaldehyde (Figure 3A-b) compounds, respectively. In addition, the mass spectra of nitrobenzene oxidation product 1 and 2 (Figure 3B-c, B-d) were also identical to those of authentic vanillin (Figure 3B-a) and syringaldehyde (Figure 3B-b), respectively. The mass spectra of the reaction products contain deuterium labeled internal standards which gave additional ion peaks of *m/z* 227 ($[M]^+ + 3$) and 212 (vanillin-d_3) in Figure 3B-c, and those of *m/z* 260 ($[M]^+ + 6$), 245 and 227 (syringaldehyde-d_6) in Figure 3B-d. These results unequivocally show that vanillin and syringaldehyde were formed as the oxidation products by microscale nitrobenzene oxidation.

Having confirmed that the chemical reaction in the microscale method proceeds effectively, we next compared the microscale and conventional methods using *Acacia mangium* heartwood as the test sample. Here, we focused on the time required to collect the data and the reproducibility (Table 1).

The microscale method requires 5–10 mg of sample and a 1.5 ml Teflon microcentrifuge tube as a reaction container. All the handling of the oxidation reaction was carried out using 1.5 ml polypropylene microcentrifuge tubes and micropipetters, which are commonly used in biochemical and molecular biology laboratories. The use of microcentrifuge tubes and micropipetting saves significantly on sample amounts and operation time; both sample amounts and operation time required for the reaction and work-up are one tenth of the conventional method. In the conventional nitrobenzene oxidation method (Katahira and Nakatsubo 2001; Umezawa et al. 2007), we previously had to spend a considerable amount of time in the work-up to the reaction, because each sample was extracted one-by-one using a separatory funnel. On average, only four samples could be handled within a standard working day (8 h). In contrast, the present microscale nitrobenzene oxidation method can simultaneously deal with many samples, since the extraction process of the microscale method is performed using disposable 1.5 ml polypropylene microcentrifuge tubes, a vortex and a mini centrifuge. Up to twenty samples can be handled in a single autoclaving cycle and the subsequent preparation stage. This process only takes a few hours. Hence, the whole process can be repeated twice or, at most, three times a day. In combination, the throughput is approximately ten-fold greater when compared with the conventional method.

In addition, the microscale method using deuterium-labeled internal standards shows higher quantitative accuracy. As shown in Table 2, standard deviations of the S/G ratio determined by the conventional method were in the range of 0.2. Conversely, the deviations by the microscale method using deuterium-labeled internal standards were 0.01, clearly indicating that the microscale method shows much higher accuracy. This is ascribed at least in part to the use of the internal deuterium-labeled standards, because the reproducibility of the microscale method which used acetovanillone as an internal standard was lower than the microscale method using the deuterium-labeled internal standards. We carried out statistical analysis by Student's *t*-test on the condition that the standard deviation by the microscale method using deuterium-labeled internal standard are always 0.01. This statistical analysis showed

Table 1. A comparison of the conventional and microscale nitrobenzene oxidation methods

	Katahira and Nakatsubo's method (Katahira and Nakatsubo 2001; Umezawa et al. 2007)	Microscale method (The present study)
Oxidative reaction		
Vessel	18 ml Teflon tube	1.5 ml Teflon microcentrifuge tube
Amount of sample	50–100 mg	5–10 mg
Device and conditions	Autoclave (170°C, 1 h)	Autoclave (170°C, 1 h)
Internal standard	5-Iodovanillin, acetovanillone	Acetovanillone, or vanillin-d_3 and syringaldehyde-d_6
Work up	Extraction with ethylacetate three times using a separatory funnel	Extraction with ethylacetate three times using a 1.5 ml polypropylene microcentrifuge tube (or a 1.0 ml glass tube), vortex and mini-centrifuge
	Solvent (about 30 ml) evaporation with a rotary evaporator	Solvent (about 0.5 ml) evaporation with a diaphragm pump or centrifugal concentrator
Throughput	4 samples/8 h	40–60 samples/8 h

Table 2. Syringyl/guaiacyl ratio of *Acacia mangium* lignin.

Method	Conventional	Microscale	Microscale
Internal standard	Acetovanillone	Acetovanillone	Vanillin-d_3 and syringaldehyde-d_6
S/G ratio	0.61 (± 0.20)*	0.67 (± 0.08)*	0.64 (± 0.01)*

*Number of repetitions was 20.

this microscale method using deuterium-labeled internal standard can sufficiently distinguish difference of S/G ratio ($P<0.03$, $n=20$) when the difference of S/G ratio is more than 0.01. This result suggested that the present method can determine the slight modification of lignin structure between transgenic plant lines.

Although the stable isotope dilution technique requires isotope labeled internal standards that are generally not commercially available, this technique has several important advantages in relation to improvement in reproducibility and accuracy (Baillie 1981; Sakakibara et al. 2007). First, the loss of the target compounds during extraction and derivatization can be ignored due to the presence of the labeled internal standards. Second, incomplete chromatographic separation can be overcome by mass separation. Third, ion suppression in mass spectrometry due to a significant reduction of the ion intensity of the target compound by the coexisting compounds can be neglected. Together, these features most likely contribute to the observed improvement in the reproducibility of the microscale nitrobenzene oxidation method over the conventional method.

The microscale method can also be perfo rmed with acetovanillone as an internal standard, instead of the deuterium labeled standards. The use of acetovanillone still has the advantage of high-throughput compared with the conventional method. It should be noted that the use of acetovanillone in the microscale method requires a 1.0 ml glass tube for the work-up process rather than the 1.5 ml polypropylene microcentrifuge tube. This might be due to undesirable adsorption of acetovanillone on to the polypropylene microcentrifuge tubes. On the other hand, the use of stable isotope labeled internal standards can ignore the possible adsorption of vanillin and syringaldehyde, because both unlabeled and labeled compounds are adsorbed to the same extent, and the ratio of the unlabeled products and the labeled internal standards remains unchanged.

In conclusion, we have demonstrated that the microscale nitrobenzene oxidation method using deuterium-labeled internal standards provides excellent reproducibility and high-throughput.

Acknowledgements

This research was partly supported by the grant "Research and development of genetic and metabolic information of energy producing plants for breeding programs" from the New Energy and Industrial Technology Development Organization (NEDO), Japan. A part of this research was conducted by Forest Biomass Analytical System, Research Institute for Sustainable Humanosphere, Kyoto University.

References

Baillie TA (1981) The use of stable isotopes in pharmacological research. *Pharmacological Rev* 33: 81–132

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. *Annu Rev Plant Biol* 54: 519–546

Chang H-M, Allan GG (1971) Oxidation. In: Sarkanen KV, Ludwig CH (eds) *Lignins*. Wiley-Interscience, New York, pp 433–485

Chen C-L (1992) Nitrobenzen and cupric oxide oxidations. In: Lin SY, Dence CW (eds) *Methods in Lignin Chemistry*. Springer, Berlin, pp 301–321

Chiang VL, Funaoka M (1990) The difference between guaiacyl and guaiacyl-syringyl lignins in their responses to kraft delignification. *Holzforschung* 44: 309–313

Katahira R, Nakatsubo F (2001) Determination of nitrobenzene oxidation products by GC and ^{1}H-NMR spectroscopy using 5-iodovanillin as a new internal standard. *J Wood Sci* 47: 378–382

Leopold B (1950) Aromatic keto- and hydroxyl-polyethers as lignin models. III. *Acta Chem Scand* 4: 1523–1537

Sakakibara N, Nakatsubo T, Suzuki S, Shibata D, Shimada M, Umezawa T (2007) Metabolic analysis of the cinnamate/monolignol pathway in *Carthamus tinctorius* seeds by a stable-isotope-dilution method. *Org Biomol Chem* 5: 802–815

Sarkanen KV, Hergert HL (1971) Classification and distribution. In: Sarkanen KV, Ludwig CH (eds) *Lignins*. Wiley-Interscience, New York, pp 43–94

Suzuki S, Suzuki Y, Yamamoto N, Hattori T, Sakamoto M, Umezawa T (2009) High-throughput determination of thioglycolic acid lignin from rice. *Plant Biotechnol* 26: 337–340

Umezawa T, Wada S, Yamamura M, Sakakibara N, Nakatsubo T, Suzuki S, Hattori T, Koda M (2007) Protocols for lignin analysis for Forest Biomass Analytical System of RISH, Kyoto University. *Res Sustainable Humanosphere* (*Seizonken Kenkyu*) 3: 73–75 (in Japanese)

Vanholme R, Morreel K, Ralph J, Boerjan W (2008) Lignin engineering. *Curr Opin Plant Biol* 11: 278–285