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Rapid, Microscale, Acetyl Bromide-Based Method for High-Throughput Determination of Lignin Content in *Arabidopsis thaliana*

XUE FENG CHANG,[†] RICHARD CHANDRA,[†] THOMAS BERLETH,[§] AND RODGER P. BEATSON*[,#]

Department of Wood Science, The University of British Columbia, 2900-2424 Main Mall, Vancouver, BC, Canada V6T 1Z4, Department of Cell and Systems Biology, University of Toronto, 25 Willcocks Street, Toronto, Ontario, Canada M5S 3B2, Chair of Advanced Papermaking, British Columbia Institute of Technology, 3700 Willingdon Avenue, Burnaby, BC, Canada V5G 3H2

The acetyl bromide method has been modified to enable the rapid microscale determination of lignin content in *Arabidopsis* with the goal of determining the genes that control lignin in plants. Modifications include reduction in sample size, use of a microball mill, adoption of a modified rapid method of extraction, use of an ice-bath to stabilize solutions and reduction in the volume of solutions. The microscale method was shown to be rapid, accurate and precise with values in agreement with those determined by the full-scale acetyl bromide method. The extinction coefficient for *Arabidopsis* lignin, dissolved using acetyl bromide, was determined to be 23.35 g^{-1} L cm^{-1} at 280 nm. This value is independent of the *Arabidopsis* accession, environmental growth conditions and is insensitive to lignin structure. The newly developed method can be used to determine lignin content in the inflorescence stems of *Arabidopsis* for mapping of lignin-related genes.

KEYWORDS: Lignin determination; *Arabidopsis*; acetyl bromide; microscale; high-throughput; extinction coefficient; extraction

INTRODUCTION

Lignin is a major chemical component of plants and the second most abundant natural polymer after cellulose (*1*). It is found mainly in the thickened secondary cell wall, strengthening stem and vascular tissue, allowing upward growth and permitting water and minerals to be conducted through the xylem under negative pressure (*2*). Lignin is a complex aromatic polymer mainly comprised of three phenylpropanoid units, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (*3*). Although lignin provides a renewable source of phenolic polymers, its presence in plants is often detrimental for other applications. High lignin content limits the digestibility of forage crops by cattle (*4*), inhibits the enzyme hydrolysis process in the biofuel industry (*5*) and adversely affects chemical pulping and bleaching processes (*6, 7*). These concerns and interests of agriculture and industry have stimulated the study of genes governing lignin content in plants.

Arabidopsis thaliana provides a model system for the study of the genes governing lignin content in angiosperms. Full genome sequence comparison, as well as expression data and genetic studies have demonstrated high conservation of major signaling and biosynthesis pathways across the angiosperms, which include all major crop plants and hardwood trees (*8–10*). Signaling and biosynthesis pathways are amenable to genetic dissection in *Arabidopsis*, because the short growth cycle, small size, and small genome, facilitate growth and analysis of large numbers of genetic variants at low costs (*11, 12*). There is abundant genetic and phenotypic variation among naturally occurring populations of *Arabidopsis*. This natural diversity has been used for the mapping of Quantitative Trait Loci (QTL) and, based on this, for the cloning of relevant genes in processes such as flowering time, fruit size and root growth (*13, 14*). Also QTLs affecting lignification and cell wall digestibility have been mapped in *Arabidopsis* using established recombinant inbred lines (*15*). Such results can potentially be extrapolated to crop plants (*16*) and trees and thus may be used to custom design plants for specific end-uses.

The task of identifying genes that determine lignin content in *Arabidopsis* illustrates the need for a small-scale high-throughput protocol for lignin quantification. First, to reveal natural variation, lignin content has to be determined across a large number of *Arabidopsis* local varieties, called accessions. Then, once accessions with divergent lignin content have been identified, new mapping populations can be established from a cross of selected accessions. To provide sufficient genetic

* Address correspondence to this author at Chair of Advanced Papermaking, British Columbia Institute of Technology, 3700 Willingdon Ave., Burnaby, BC, Canada V5G 3H2 [e-mail Rodger_Beatson@bcit.ca; telephone (604) 432-8951; fax (604) 432-9572].

[†] The University of British Columbia.

[§] University of Toronto.

[#] British Columbia Institute of Technology.

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resolution for mapping and cloning of lignin related genes, it is necessary to determine lignin content in around 500 individual F2 plants (segregants) of the mapping population (*17*). A rapid method of lignin content determination in *Arabidopsis* that can handle a large number of samples at a microscale is essential for the success of this approach.

Many methods have been developed to quantitatively determine the amount of lignin in plants. The principle types of approaches are based on weighing or spectroscopy (*18*). Gravimetric methods such as digestion with 72% sulfuric acid prior to weighing are time-consuming and require a relatively large sample size and a high precision balance to ensure accuracy. When applied to herbaceous samples, the 72% sulfuric acid method may result in an overestimation of lignin due to coprecipitation of proteinaceous substances and the presence of inorganic ash (*18*). Spectrophotometric methods including infrared spectroscopy (FTIR), near-infrared spectroscopy (NIR), and the acetyl bromide digestion combined with ultraviolet spectroscopy (UV) only require small amounts of sample due to their high sensitivity. One challenge in using spectrophotometric methods for the determination of lignin content is the definition of an appropriate calibration standard (*19*). This challenge arises from the heterogeneity of lignin composition and chemical structure (*19, 20*).

Prior investigations have indicated that variation in lignin composition and structure may not have a large influence on the UV absorbance of lignin solubilized using the acetyl bromide method. Iiyama and Wallis (*21*) found that acetyl bromide treatment leads to a very similar extinction coefficient at 280 nm for lignin obtained from different species. Thus, we hypothesized that it may also be possible to use a single extinction coefficient, at 280 nm, derived from lignin isolated from multiple *Arabidopsis* accessions, to estimate the total lignin content in various lines of *Arabidopsis* when using an acetyl bromide-based method.

Since the acetyl bromide method was first introduced for the determination of lignin content in wood samples by Johnson et al. in 1961 (*22*), many modifications to the original procedure have been made to suit different purposes. For example, Morrison (*23*) adapted the acetyl bromide method for nonwoody samples because it was found that the high protein content in such samples does not interfere with the determination of lignin content. Morrison (*23*) also modified the pretreatment of grass samples allowing for a more rapid determination of lignin. Iiyama and Wallis (*24*) scaled down the volume of the digested solution from 200 to 50 mL and found that the precision of the method was not affected. Rodrigues et al. (*25*) froze the digested samples in the freezer at −10 °C to stabilize solutions, enabling the preparation of several reaction batches before the absorbance measurements. These developments, to the conventional acetyl bromide method, indicate the potential for further modification to the procedure to enable determination of lignin content in a large number of small samples of *Arabidopsis* with high accuracy and precision.

In this paper, modifications of the conventional acetyl bromide method to enable rapid microscale determination of lignin content in *Arabidopsis* are proposed. The extinction coefficients of lignins isolated from different *Arabidopsis* accessions, using the newly developed microscale method, are reported.

MATERIALS AND METHODS

Plant Materials. Seeds of *A. thaliana* accessions were purchased from the *Arabidopsis* Biological Resource Center (ABRC) and TAIR (The *Arabidopsis* Information Resource). The accessions were chosen from lines collected from different continents. Plants were grown in Terra-Lite Redi-earth (W. R. Grace & Co.) soil under various conditions. The growth conditions for Columbia-0 (Col-0) were controlled using a growth chamber with 16-h-light/8-h-dark cycles at 21 °C and fluorescent lamps at a light intensity of 100−150 μmols^{-1} m^{-2}. Accessions other than Col-0 were grown in separate batches in the greenhouse of the University of British Columbia. Each accession was harvested once it reached full maturity and all the siliques had turned brown. Harvested plants were conditioned at 50% relative humidity (RH) and 23 °C for 48 h prior to grinding the inflorescence stems.

Grinding. The inflorescence stems of *Arabidopsis* accessions were ground to pass a 40-mesh screen using a Wiley mill or ground to 80-mesh using a microball mill to meet the requirements of the subsequent extraction method. Prior to extraction, the ground samples were dried in a vacuum oven at 40 °C for 48 h and conditioned in a vacuumed desiccator over phosphorus pentoxide (P_2O_5) overnight.

Extractions Methods. *Four-Stage Soxhlet Extraction.* The method of Fukushima and Hatfield (*26*) was followed. Approximately 2.5 g of dried 40-mesh sample was sequentially extracted with water, 95% ethanol, chloroform and acetone using a Soxhlet apparatus. Extraction with each solvent was for 8 h at a reflux rate of approximately 8 cycles/h. Specified samples were also extracted for 24 h with each solvent.

Single-Stage Soxhlet Extraction. Approximately 2.5 g of dried 40-mesh sample was extracted with 95% ethanol/benzene (1:2, v/v) for 24 or 48 h. The rate of solvent reflux was approximately 8 cycles/h.

Rapid Extraction by Washing. The procedure was based on Morrison's quick extraction method (*23*) with reduction in sample particle size and weight. A fine filtration membrane was also used. Approximately 0.1 g of dried 80-mesh sample was put in a test tube, soaked with 15 mL of distilled water, heated in a water bath at 65 °C for 30 min with occasional shaking, and then filtered hot through a dry and preweighed 0.45 μm nylon membrane using a Millipore filter. The residual was first washed with 20 × 2 mL of deionized water, then sequentially with 20 × 1 mL of each of ethanol, acetone, and diethyl ether. The residual was then transferred with the nylon membrane to a preweighed aluminum pan by brushing.

The extracted samples were dried in a vacuum oven at 40 °C for 48 h and conditioned in a vacuum desiccator over P_2O_5 overnight for further analysis. The total amount of extractives from each sample was determined by the difference in sample weight before and after the extractions. Where specified, extractives removed by each solvent were determined by the weight of residuals obtained from evaporating the solvent with a rotary evaporator at 30−35 °C.

Preparation of Samples with Various Lignin Contents. Lignin content in Col-0 was increased by cellulase treatment and decreased by addition of holocellulose. The holocellulose was prepared using the method of Yokoyama et al. (*27*) except that the material used for the isolation of holocellulose was extracted Col-0 inflorescence stems rather than wood wafers. For cellulase treatment, 1 g of ground and dried *Arabidopsis* stems extracted by the four-stage Soxhlet extraction method was incubated for 3 h in 50 mL of 50 mM acetic buffer (pH 4.8) containing 7 IU of Novozyme 188 and 3.5 filter paper units of Celluclast 1.5L (Novozyme, Franklinton, NC) at 45 °C. After hydrolysis, the residual was centrifuged, washed three times by resuspension in 600 mL distilled water and shaken by sonication at 40 KHz for 60 min in a TP 680 DH ultrasonic water bath (Elma Hans Schmidbauer Gmbh & Co., Singen, Germany).

Preparation of Samples with the Same Lignin Content. Col-0 plants grown under the same conditions (16 h light, 150 μmol^{-1} m^{-2}, 21 °C) were separated into three different groups. The inflorescence stems within each group were combined, ground and extracted by the rapid extraction method as above.

Lignin Isolation for Extinction Coefficient Determination. Lignin was isolated using dioxane acidified with HCl by the method of Fukushima and Hatfield (*26*) except that refluxing of the solutions under N_2 was for 60 min rather than 30 min.

Lignin Content Determination by the Acetyl Bromide Method. *Conventional Method.* The method of Iiyama and Wallis (*24*) was followed, except that the samples for the analysis were extracted using the four-stage Soxhlet extraction rather than a two-stage extraction with 80%

ethanol and chloroform, and the extracted samples were ground to 80-mesh in the microball mill prior to drying over P_2O_5 in a vacuum desiccator. The dried samples (5 ± 1 mg), weighed to the nearest 0.01 mg, were added to a 10 mL glass tube with 2.5 mL of 25% acetyl bromide in acetic acid. The tubes were tightly sealed with Teflon lined caps and put in a 70 °C water bath for 30 min with shaking at 10 min intervals. After cooling the tubes to room temperature, the samples were transferred to 50 mL volumetric flasks containing 2.5 mL 2 M NaOH and 12 mL acetic acid. The tubes were rinsed with acetic acid to complete the transfer. Then 0.5 mL 7.5 M hydroxylamine hydrochloride was added to the volumetric flasks which were then made up to 50 mL with acetic acid. The absorbance of the solutions was read at 280 nm using a Varian Cary 50 spectrophotometer. A blank was included to correct for background absorbance by the reagents.

Microscale Method Using Cuvettes. The sample size for grinding was reduced from 2.0 to 0.1 g prior to extraction using the rapid extraction method. Dried 80-mesh samples (5 ± 1 mg) of the ground and extracted *Arabidopsis* stems were weighed to the nearest 0.01 mg, and then digested with 1.000 mL of 25% acetyl bromide in acetic acid as above. After the digestion, the tubes were cooled and stored in an ice bath for a period of between 5 and 120 min. During this period, 5.000 mL of acetic acid was added to each tube, followed by mixing using a Vortex apparatus. Then 300 μL of the mixture was transferred to a quartz cuvette, followed by 400 μL of 1.5 M NaOH and 300 μL of 0.5 M hydroxylamine hydrochloride. The contents were mixed then diluted with 1.500 mL acetic acid. The absorbance of the solution was measured against a blank as above.

Microscale Method Using Microplates. The digestion and cooling of the samples were the same as used in the microscale method using cuvettes. The digested samples were removed from the ice bath and 5.000 mL of acetic acid were added to each sample. After mixing, 30 μL of the mixed content, in triplicate, were transferred to the wells of a 96-well quartz microplate. The transfer of the samples to the microplate was completed within 5 min before sequentially adding 40 μL of 1.5 M NaOH, 30 μL of 0.5 M hydroxylamine hydrochloride and 150 μL acetic acid to each well using a ten-channel multiple pipet. The absorbance of the solutions in the wells at 280 nm was measured using a Perkin-Elmer Wallac 1420 microplate reader. A blank was included to correct for background absorbance by the reagents.

Lignin Content Determination Using the 72% Sulfuric Acid Method. The modified Klason method of Huntley et al. (*28*) was used to determine lignin content in *Arabidopsis* stems except that the Klason lignin was corrected for crude protein contamination. The hydrolysates were saved for acid-soluble lignin determination and neutral sugars and uronosyls analysis. Acid-soluble lignin was determined by absorption spectroscopy at 205 nm using the Varian Cary 50 spectrophotometer (*18*).

Neutral Sugars and Uronosyls Analysis. Neutral sugars and uronosyls contents in *Arabidopsis* inflorescence stem samples were determined using the hydrolysates from the 72% sulfuric acid method. Hydrolysis with 72% sulfuric acid was also used for the determination of neutal sugars and uronosyls in isolated dioxane lignins. Because of reduced sample availabilty and low carbohydrate content, weights of the isolated lignins used in the hydrolysis were one tenth of those used in *Arabidopsis* stem analysis and the amounts of reagents applied were reduced by 100 fold.

Neutral sugar concentrations in the hydrolysates were determined by high-performance liquid chromatography (HPLC) (Dionex DX-500, Dionex, Sunnyvale, CA) equipped with an ion exchange PA1 column, an ED40 electrochemical detector, and an AS3500 autosampler. The hydrolysates were filtered through 0.45 μm filters (Millipore, Bedford, MA) before loading the samples on HPLC. A volume of 20 μL was injected onto the column equilibrated with 250 mM NaOH and eluted with deionized water at a flow rate of 1.0 mL min^{-1}, followed by postcolumn addition of 200 mM NaOH at a flow rate of 0.5 mL min^{-1}. Each sample was determined in duplicate.

Uronosyls concentrations in the hydrolysates were determinated using the phenyl phenol method (*29*).

Crude Protein Analysis. Crude protein content in dioxane isolated and Klason lignins was estimated as N% × 6.25. Percent of nitrogen in the samples was determined using Perkin-Elmer Series II CHNS/O 2400 analyzer (Norwalk, CT) according to the manufacturer's instructions.

Ash Determination. Ash determination was performed according to TAPPI test method T211 om-93.

Determination of Extinction Coefficients of Isolated Lignins. Ten milligrams of isolated lignin (after correction for contamination from carbohydrates and proteins as above) were dissolved in 5.0 mL of dioxane. Aliquots of 0.3, 0.4, 0.5, 0.6, and 0.7 mL of the solution were freeze-dried in 10 mL test tubes prior to digestion with acetyl bromide solution (*26*). The absorbance values at 280 nm were measured by the microscale acetyl bromide method using cuvettes as above. The extinction coefficient of the isolated lignin was obtained from the slope of the standard curve of absorbance value versus lignin concentration. The standard curve for each lignin was developed in duplicate.

Lignin Phenylpropane Unit Determination. The phenylpropane unit composition (*p*-hydroxyphenyl:guaiacyl:syringyl) of isolated lignin was determined by thioacidolysis (*30*) using 5 mg (after correction for contamination by carbohydrates and proteins) of isolated lignin. Tetracosane (2 mL of 0.25 mg mL^{-1} in CH_2Cl_2) was used as the internal standard. The silylation reaction proceeded for a minimum of 4 h. GC-MS analysis was conducted on a HP 6890 series GC system fitted with a HP 5973 mass selective detector and a HP 7683 series injector. The GC was equipped with a 30 m × 0.25 mm DB-5 column (J&W Scientific). The GC conditions were set as: injection volume 2.0 μL, inlet temperature 250 °C, helium carrier gas flow at 1.0 mL/min, injector spit ratio of 10:1. The initial oven temperature was set to 130 °C, held for 3 min, then ramped at a rate of 3 °C min^{-1} to 260 °C and held for 5 min. For the MS detector, electron ionization potential was set at 70 eV with the source at 100 °C. Ions were scanned across the range of 50 - 450 mu (mass unit).

Statistical Analysis. Analysis of variance (ANOVA) was conducted to compare the specific absorbance value of preparations with varying lignin content and the same lignin content using the SAS software package (SAS Institute Inc., Cary, NC). Paired *t* test was performed to compare the absorbance value of samples before and after scaling down the volume of solutions in the acetyl bromide method. Data in tables and figures indicate means of measurements (±SD). The measurements are duplicated unless specified. Error bars in all graphs refer to 95% significant confidence intervals.

RESULTS AND DISCUSSION

Modification of the Acetyl Bromide Method. Here we report a newly developed microscale assay for lignin content that adapts the conventional acetyl bromide method so that it can be used as a high throughput method. The applications of the method include the use of the natural variation of lignin content in *Arabidopsis* accessions to identify the genetic loci controlling lignin content and analysis of lignin metabolomics. The following critical steps were incorporated into the acetyl-bromide based microscale lignin assay. The sample size used for grinding was reduced from approximately 2 to 0.1 g by adoption of a microball mill. The extraction process was scaled down and sped up by extracting small amounts of samples having a small particle size in a Millipore filter apparatus. The process of sample treatment was accelerated by scaling down the volume of solutions thus facilitating the use of micropipettes or multichannel pipettes for solution transfer. The digested samples treated by acetyl bromide solution were stored and stabilized in ice bath, allowing high sample throughput to be handled.

Grinding of Arabidopsis Samples Using a Microball Mill. The acetyl bromide method requires that samples are ground to pass an 80-mesh screen to ensure a complete digestion of samples in 25% acetyl bromide in glacial acetic acid after heating at 70 °C for 30 min (*22*). Although the digestion of

Table 1. Comparison of Extractive Content Determined by Different Extraction Methods Using Inflorescence Stems of Col-0 (Results Expressed as a Percentage of the Weight of the Original, Oven-Dried Sample)

		extractives by four-stage Soxhlet extraction		extractives by single-stage Soxhlet extraction	
solvent	extractives using rapid extraction by washing	8 h/stage	24 h/stage	24 h	48 h
water	20.13 ± 0.80	17.65 ± 0.37	18.11 ± 0.45	NA	NA
ethanol	1.46 ± 0.08	1.68 ± 0.05	1.71 ± 0.06	NA	NA
chloroform	NA	0.72 ± 0.02	0.76 ± 0.03	NA	NA
acetone	1.80 ± 0.05	1.05 ± 0.06	1.34 ± 0.08	NA	NA
ethyl ether	0.45 ± 0.01	NA	NA	NA	NA
ethanol/benzene (1:2)	NA	NA	NA	5.73 ± 0.27	6.51 ± 0.30
total	23.84 ± 0.94	21.10 ± 0.50	21.92 ± 0.62	5.73 ± 0.28	6.51 ± 0.31

coarsely ground samples can be promoted by adding perchloric acid to the digestion solution (*31*), later work has shown that perchloric acid might cause interference with lignin content determination due to the degradation of hemicelluloses to products that absorb at 280 nm (*32*). Rather than adding perchloric acid, the best approach is to grind the samples until they are fine enough to ensure complete digestion. Samples are usually ground using a micro Wiley mill. However, this is not feasible with samples less than 0.1 g as will be encountered in lignin content determination in *Arabidopsis*. An alternative better suited to small samples turned out to be grinding with a microball mill.

Using a microball mill, 0.1 g of *Arabidopsis* stem sample could be ground to 80-mesh in two minutes. The digestion of a ground stem sample from *Arabidopsis* accession Columbia-0 (Col-0) in 25% acetyl bromide solution at 70 °C indicated that the sample was dissolved in approximately 14 min with the absorbance rising rapidly during this period. At 30 min, the rate of change in absorbance was small, which was consistent with the results of Morrison (*23*). A sediment was found after centrifuging the digested solution. A similar sediment was also found with grass samples by Morrison, who ascertained that it consisted almost entirely of proteins and thus did not cause errors in the lignin content determination (*23*).

Adaptation of the Rapid Extraction Method. Extractives may interfere with lignin determination by the acetyl bromide method (*23*). To apply this method for the rapid determination of lignin content in a large number of *Arabidopsis* samples, a simple and efficient extraction method needs to be developed.

According to Morrison (*23*), grass samples heated with distilled water, filtered and washed thoroughly with water, ethanol and acetone and diethyl ether underwent the same degree of extraction as the same samples extracted consecutively with solvents such as water, ethanol, chloroform and acetone in a Soxhlet apparatus. By adapting Morrison's extraction method, it proved possible to increase the efficiency of the extraction process and facilitate the handling of small amounts (mg quantities) of samples. It was found that approximately 25 *Arabidopsis* samples could be extracted per day once Morrison's extraction method was modified for use with small samples having a small particle size. In contrast, the normal extraction method applying a Soxhlet apparatus requires 32 h for one cycle of extraction using water, ethanol, acetone and diethyl ether.

To determine how effective the modified rapid extraction method was for *Arabidopsis*, the amount of extractives removed from ground samples of Col-0 stem by each solvent during rapid extraction (using 80-mesh sample), conventional four-stage Soxhlet extraction (using 40-mesh sample) and the single-stage Soxhlet extraction method (using 40-mesh sample), were compared (**Table 1**).

The results in **Table 1** demonstrate that extraction efficiency was mainly dependent on the extraction method and was not improved significantly by increasing extraction time. The rapid extraction method removed more of the nonvolatile extractives than the other methods. This is probably a result of the use of small amounts of finely ground sample for the rapid extraction. The total amount of extractives obtained through the four-stage Soxhlet extraction was close to that obtained in the rapid extraction. Both these methods removed around 3.5 times more extractives than the single-stage Soxhlet extraction method.

The reason for the differences in extraction efficiency among the different extraction methods can be deduced from the changes in the chemical macrocomponent before and after extraction (*33*). **Table 2** shows that the four-stage Soxhlet extraction removed approximately 88.5% of the ash, 13.4% of total carbohydrates (neutral sugars plus uronosyls) and 48.7% of proteins from the original sample while the single-stage Soxhlet extraction method only removed around 18.0% of the ash, 8.0% total carbohydrates and 21.5% of proteins. Clearly, the properties of solvents used for extraction affect the efficiency of extraction. The four-stage Soxhlet extraction can efficiently remove inorganic ash, proteins, carbohydrates and other extractives with small molecule weight because water and other solvents with a wide range of polarities are used sequentially for extraction. For a water extraction combined with an ethanol/benzene extraction, the extraction efficiency may be expected to approach that of the four-stage Soxhlet extraction, as can be inferred from Morrison's study (*23*).

Table 2 also shows that the rapid extraction removed around 1.5% more of the carbohydrates and 0.5% more of ash from the original samples than the four-stage Soxhlet extraction. The rapid extraction method removed a further 0.3% of extractives beyond that accounted for by carbohydrates, ash and proteins. These results indicate that the rapid extraction removed the extractives slightly more efficiently than the four-stage Soxhlet extraction.

The extractions affect the measurements of Klason lignin and acid soluble lignin in *Arabidopsis* using the sulfuric acid method. After correction for protein contamination, the values of Klason lignin for the extracted samples were 2.5−3.2% lower than those in the original sample (**Table 2**). The values for acid soluble lignin in the extracted samples were 0.8−1.7% lower than those in the original sample (**Table 2**). The total of Klason lignin and acid soluble lignin in the sample extracted by the four-stage Soxhlet was 0.96% lower than that obtained by single-stage Soxhlet extraction (**Table 2**). The differences in the results may be due to the interferences present in original nonextracted sample or may be because extractions with hot water or ethanol removed a portion of lignin in the original samples.

The rapid extraction method uses mild extraction conditions with 65 °C being the highest extraction temperature. At this temperature removal of polymeric lignin is unlikely. The difference in lignin contents is most probably due to interferences from extractives in the original sample. The similarity in

Table 2. Components of Col-0 Inflorescence Stems As Determined by the Analysis of the Samples with No Extraction, Single-Stage Soxhlet Extraction, Four-Stage Soxhlet Extraction, and Rapid Extraction by Washing (Results Expressed as a Percentage of the Weight of the Original, Oven-Dried Sample)

component	original	single-stage Soxhlet extracted[a]	four-stage Soxhlet extracted[b]	rapid extraction by washing
total glycans[c]	55.77 ± 1.14	51.73 ± 0.74	48.64 ± 0.23	47.21 ± 0.07
fucan	0.18	0.16	0.12	0.12
arabinan	1.36	1.23	0.78	0.9
rhamnan	1.39	0.93	0.65	0.7
galactan	1.83	1.65	1.18	1.22
glucan	35.84	33.44	31.98	30.63
xylan	13.24	12.44	12.28	11.96
mannan	1.92	1.87	1.65	1.68
uronic acids[c]	5.68 ± 0.18	4.82 ± 0.19	4.58 ± 0.21	4.53 ± 0.18
ash	13.35 ± 0.12	10.94 ± 0.11	1.54 ± 0.03	1.05 ± 0.03
crude protein	6.88 ± 0.06	5.40 ± 0.11	3.53 ± 0.05	3.52 ± 0.14
Klason lignin[d]	17.51 ± 0.16	15.03 ± 0.19	14.35 ± 0.12	14.20 ± 0.14
acid soluble lignin	2.96 ± 0.12	1.57 ± 0.02	1.29 ± 0.06	1.24 ± 0.06
extractives	NA	6.12 ± 0.28	21.52 ± 0.56	23.84 ± 0.70
total	102.15	95.61	95.45	95.59

[a] Combined sample from 24 and 48 h extractions. [b] Combine sample from 8 and 24 h extraction stages. [c] The contents of polysaccharides were converted from pentose and hexoses/uronic acids by multiplying the factors of 0.88 and 0.90, respectively. [d] Values after correction for crude protein in the Klason lignin residue.

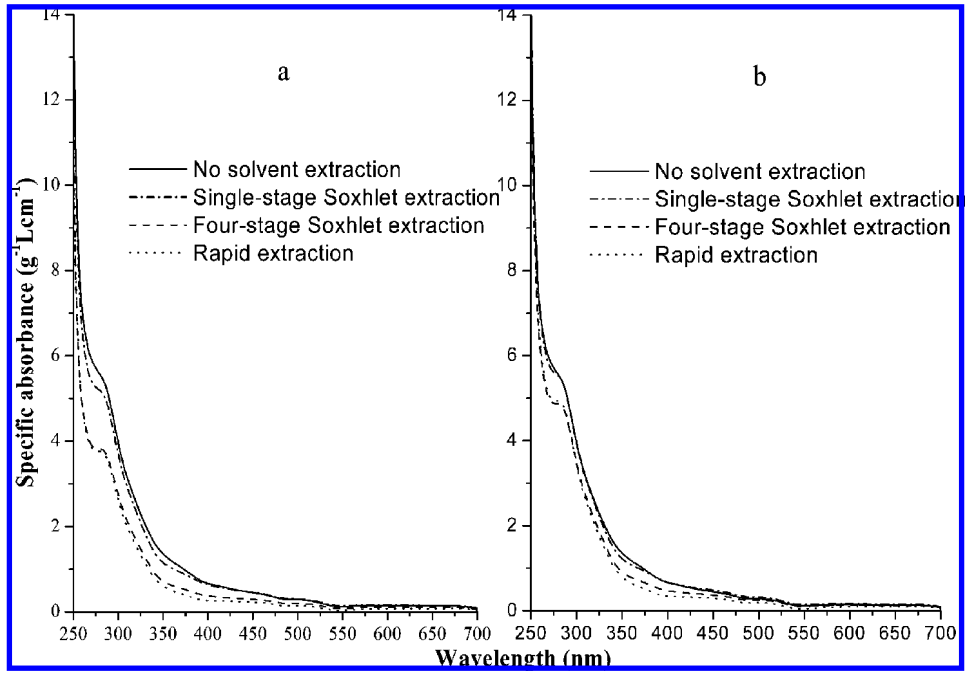

Figure 1. Spectra of the original, single-stage Soxhlet extracted, four-stage Soxhlet extracted, and rapidly extracted samples of Col-0 inflorescence stems treated with acetyl bromide solution: (**a**) values based on the weight of original, oven-dried sample; (**b**) values based on the weight of extracted, oven-dried sample.

lignin content of samples obtained through the rapid extraction and four-stage Soxhlet extraction indicates that these two extraction methods have similar extraction efficiency and lead to a more accurate lignin content measurement by the sulfuric acid method than obtained through single-stage Soxhlet extraction or without extraction.

The extraction methods also affect the spectra of samples treated by acetyl bromide solution and thus can lead to errors in lignin content determination using the acetyl bromide method. **Figure 1** shows that, in the range of 250 to 700 nm, the samples after four-stage Soxhlet extraction had lower specific absorbance values than the samples with no solvent extraction or after a single-stage Soxhlet extraction. The rapid extraction had similar effects on the specific absorbance values as the four-stage Soxhlet extraction. The differences in specific absorbance values among the differently extracted samples mainly occurred in the ranges of 350−400 nm and 260−290 nm. The higher specific absorbance values between 350 and 400 nm are probably caused by the presence of pigments such as chlorophyll and carotenoids that were not removed during the extraction process. The higher specific absorbances caused by such pigments could extend to the 280 nm region used for lignin determination.

In the 260−290 nm region, the specific absorbance values obtained using four-stage Soxhlet extraction and rapid extraction were lower than those obtained without solvent extraction or when using a single-stage Soxhlet extraction. Statistical analysis showed that, the specific absorbance value at 280 nm after the four-stage Soxhlet extraction was significantly different from that obtained without solvent extraction or when using a single-stage Soxhlet extraction ($P < 0.001$), but was not significantly different from that obtained after the rapid extraction process ($P = 0.211$). The lower specific absorbance values of the samples after four-stage Soxhlet extraction and rapid extraction in the 260−290 nm region indicate a minimum interference from

Table 3. Specific Absorbance Values of Inflorescence Stems of Various *Arabidopsis* Accessions Obtained on Scaling Down of the Volume of Solutions Used in the Acetyl Bromide Method (Values Are Averages of Three Measurements and Based on the Weight of the Original, Oven-Dried Sample)

	specific absorbance at 280 nm (g^{-1} L cm^{-1})		
accession	50 mL scale[a]	2.5 mL scale[b]	0.25 mL scale[b]
Columbia-0 (Col-0)	3.73 ± 0.11	3.75 ± 0.08	3.72 ± 0.13
Catania (Ct-1)	3.11 ± 0.15	3.24 ± 0.05	3.14 ± 0.08
Kendalville	2.60 ± 0.07	2.63 ± 0.06	2.50 ± 0.10
Lezoux (Lz-0)	3.28 ± 0.02	3.26 ± 0.04	3.20 ± 0.09
Nossen (No)	3.19 ± 0.05	3.22 ± 0.09	3.15 ± 0.13
Shakdara (Sha)	3.72 ± 0.09	3.66 ± 0.05	3.71 ± 0.08
mixed[c]	2.99 ± 0.07	3.09 ± 0.05	3.05 ± 0.08

[a] Using 80 mesh and four-stage Soxhlet extracted samples. [b] Using 80 mesh and rapidly extracted samples. [c] A mixture of Estland (Est-1), Br (Br-0), Merzhausen (Mz-0), Borky (Bor-4), and Gückingen (Gü-0).

'pseudolignin'. Thus, the four-stage Soxhlet extraction and the rapid extraction provide more representative values of the specific absorbance of lignin at 280 nm.

Scaling down the Volume of Solutions. Scaling down the volume of the digested solutions will accelerate the process of sample treatment through the use of micropipettes or multichannel pipettes for solution transfer. In the conventional acetyl bromide method defined by Iiyama and Wallis (*24*), the final sample volume is 50 mL. In the work reported here, the analysis was first scaled down to 2.5 mL and further scaled down to 0.25 mL allowing for use of microplates for the measurement of UV absorbance.

The two scaled-down methods (with rapid extraction) were compared to the conventional method (with four-stage Soxhlet extraction) by testing the specific absorbance values of digested 80-mesh samples of different *Arabidopsis* accessions (**Table 3**). Paired *t* test of the results show that the specific absorbance values obtained with the conventional 50 mL scale are not significantly different from those obtained with 2.5 mL scale using cuvettes ($P = 0.258$) and 0.25 mL scale using microplates ($P = 0.385$). Thus, the scaling down does not affect the accuracy and precision of the acetyl bromide method.

Stability of the Solutions. In order to use the acetyl bromide method to efficiently analyze many samples at the same time the spectral properties of the digested samples must be stable over the period of time required for processing prior to measurement. It proved possible to do this by cooling the digested samples in an ice bath (*22, 25*). Samples of Col-0 stems digested with acetyl bromide in acetic acid were placed in an ice bath to verify if cooling the samples could effectively stabilize the absorbance. The absorbance of the samples remained constant during two hours in an ice-bath. In contrast, the digested solution at room temperature showed a slow decrease in absorbance at a rate of 1.58% over 20 min. The use of an ice bath enabled digested samples to be stored prior to UV absorbance measurements thus allowing the acetyl bromide method to be used for high sample throughput.

Furthermore, the stability of the acetyl bromide solutions of digested Col-0 stems at room temperature increased after the solutions were finally diluted with acetic acid, as was discussed by Johnson et al. (*22*). The absorbance of solutions after final dilution decreased by less than 0.6% in 20 min. The results are similar to those of Rodrigues et al. (*25*) who showed that the absorbance of the diluted acetyl bromide solution decreased at a low rate of 0.008 in 20 min.

The effects of time lapse among samples on the absorbance values are limited for the microscale method incorporating the above four modifications. In the microscale method using cuvettes, there is no time lapse between transfer of the solution from ice bath and the UV absorbance measurement during high throughput determinations of lignin content. With the microscale method using microplates, the time lapse between transfer of the solution from ice bath and UV absorbance measurement can be limited to seven minutes. The absorbance measurement error caused by the total seven minutes of time lapse among the samples would be less than 0.5%.

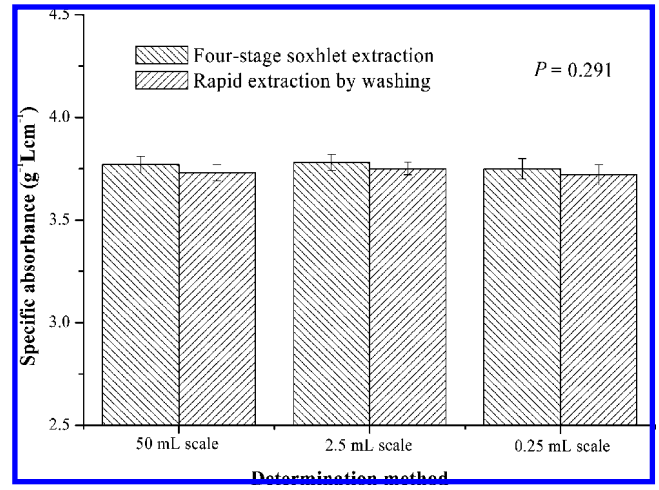

Figure 2. Comparison of specific absorbance values using different extraction procedures and scales in the acetyl bromide method. Values are averages of 30 measurements.

Validation of the Microscale Methods. The results have shown that, considered independently, neither the changes in extraction nor scaling-down affected the results from the acetyl bromide lignin determination. To test if these two modifications combined have an effect on the absorbance measurements at 280 nm, the specific absorbance values of a homogenized Col-0 stem sample were determined using different combinations of the two extraction procedures (four-stage Soxhlet extraction and rapid extraction) and three scales in the acetyl bromide method (conventional 50 mL scale, 2.5 mL scale using cuvettes and 0.25 mL scale using microplates) as shown in **Figure 2**. The results show that the measured values were not significantly different ($P = 0.291$), again confirming that the modifications do not change the accuracy of the conventional method.

To further confirm the accuracy and precision of the microscale methods, individual plants of Col-0 grown at the same condition (16 h light, 150 μmol^{-1} m^{-2}, 21 °C) were collected and combined into group A, B, and C. The specific absorbance values of samples from the three groups were determined using both of the microscale acetyl bromide methods. The test results demonstrate that the measured values were not significantly different for the microscale methods using either the cuvettes ($P = 0.238$) or microplates ($P = 0.128$) (**Figure 3**). The relative standard deviation of the measurements by the microscale method using cuvettes was approximately 2.8% while for the microscale method using microplates the relative standard deviation was 3.4%. The latter value was little higher than the former value probably because the absorbance measurement using microplates is not only affected by the lignin concentration changes due to volume variation of reagents added to the digested acetyl bromide solution, but is also affected by light path length variation caused by solution volume changes in the cell of the microplate. Another possible reason for more variation in the measurements using microplates is the time effects on the absorbance of the solution when a high throughput

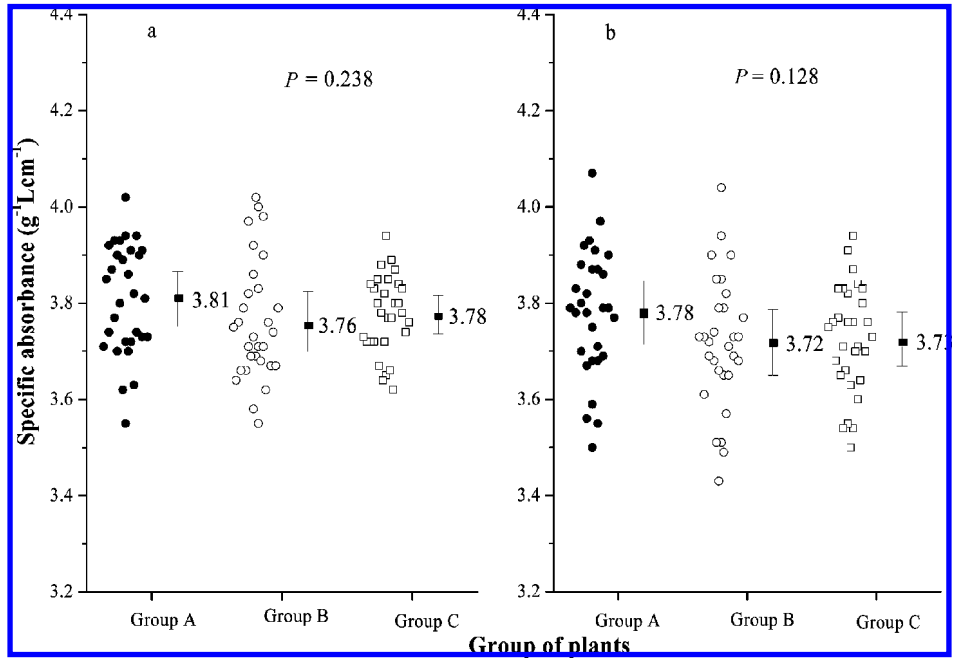

Figure 3. Accuracy and precision test of the microscale acetyl bromide methods using the inflorescence stems of three groups of Col-0 grown under the same conditions (16 h light, 150 μmol^{-1} m^{-2}, 21 °C): (**a**) by the microscale method using cuvettes; (**b**) by the microscale method using microplates. Values are based on the weight of original, oven-dried samples.

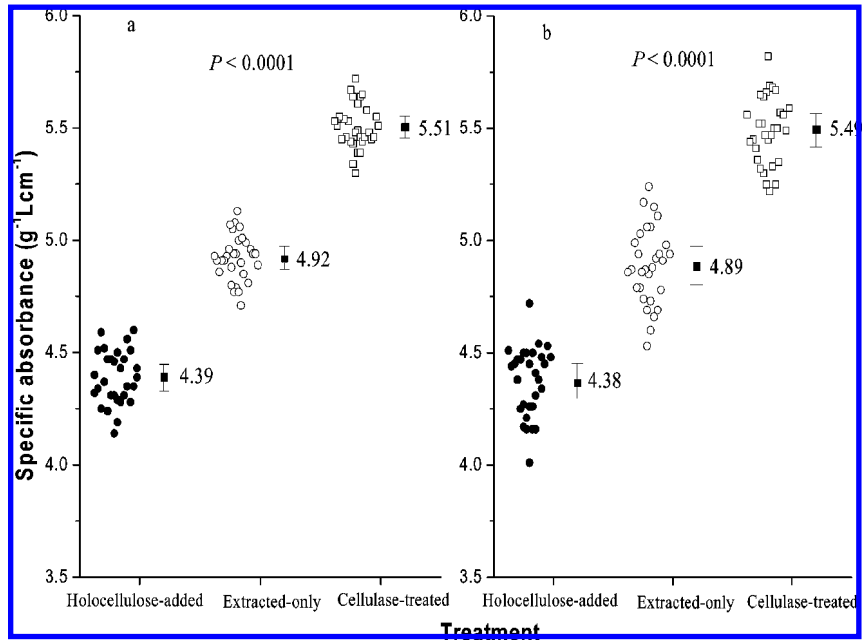

Figure 4. Accuracy and precision test of the microscale acetyl bromide methods using extracted-only, holocellulose-added, and cellulase-treated samples of inflorescence stems of Col-0: (**a**) by the microscale method using cuvettes; (**b**) by the microscale method using microplates. Values are based on the weight of treated, oven-dried samples.

of samples is being handled, as was discussed above. However, the precision of both microscale methods is sufficient for the screening program of lignin content in *Arabidopsis* accessions. In conclusion, the microscale methods using cuvettes and microplates can identify samples with the same lignin concentration accurately and precisely.

The microscale methods can identify samples with the same lignin concentration but it is also important to distinguish between samples with different lignin concentrations. To verify if the microscale methods are capable doing this, one portion of an extracted sample of Col-0 inflorescence stems was treated with cellulase to increase lignin concentration while another portion was mixed with holocellulose, isolated from Col-0, to decrease lignin concentration. The specific absorbance values of the holocellulose-added, extracted-only, and cellullase-treated samples were determined respectively by the microscale acetyl bromide method using either cuvettes or microplates. **Figure 4** shows that the specific absorbance values of the three differently treated samples were significantly different by the method using cuvettes ($P < 0.0001$) and by the method using microplates ($P < 0.0001$). The relative standard deviation of the measurements obtained by the microscale method using cuvettes was approximately 2.2% while that of the measurements obtained by the microscale method using microplates was 3.3%.

Determination of the Extinction Coefficients of Lignins Isolated from Different Accessions of *Arabidopsis*. The

Table 4. Impurities in Isolated Lignin from Inflorescence Stems of *Arabidopsis* Accessions (Results Expressed as a Percentage of the Weight of the Isolated Lignin)

accession	total neutral sugars	uronic acids	crude protein	total
Columbia-0 (Col-0)	2.54 ± 0.08	0.86 ± 0.04	3.69 ± 0.18	7.09 ± 0.30
Bensheim (Be)	2.62 ± 0.01	1.08 ± 0.05	4.28 ± 0.02	7.98 ± 0.08
Catania (Ct-1)	1.30 ± 0.01	0.49 ± 0.03	4.72 ± 0.02	6.51 ± 0.06
Hannover/Stroehen (Hs-0)	3.44 ± 0.04	0.96 ± 0.05	3.12 ± 0.12	7.52 ± 0.21
Kendalville	1.73 ± 0.01	0.76 ± 0.04	4.38 ± 0.20	6.87 ± 0.07
Lezoux (Lz-0)	2.42 ± 0.07	0.67 ± 0.03	5.34 ± 0.04	8.43 ± 0.14
Nossen (No)	1.62 ± 0.07	0.58 ± 0.03	4.16 ± 0.13	6.36 ± 0.23
Shakdara (Sha)	1.26 ± 0.05	0.38 ± 0.01	5.34 ± 0.04	6.98 ± 0.10
Wietze (Wt-5)	1.78 ± 0.01	0.59 ± 0.01	2.62 ± 0.01	4.99 ± 0.03
Zdarec (Zdr-6)	2.91 ± 0.01	0.89 ± 0.04	3.03 ± 0.01	6.83 ± 0.06
mixed[a]	1.16 ± 0.01	0.41 ± 0.02	4.75 ± 0.12	6.32 ± 0.15
mean	2.07	0.70	4.13	6.70

[a] A mixture of Estland (Est-1), Br (Br-0), Merzhausen (Mz-0), Borky (Bor-4), and Gückingen (Gü-0).

Table 5. Lignin Phenylpropane Unit Ratios, Determined by Thioacidolysis, and Extinction Coefficients at 280 nm of Lignins Isolated from Inflorescence Stems of *Arabidopsis* Accessions by the Acetyl Bromide Method

		lignin phenylpropane unit ratio			
accession	extinction coefficient (g^{-1} L cm^{-1})	hydroxyphenyl	guaiacyl	syringyl	total phenylpropane yield[a] (μmol g^{-1})
Columbia-0 (Col-0)	23.29	1.9	58.2	39.9	1104.7
Bensheim (Be)	23.60	1.8	56.3	41.9	924.5
Catania (Ct-1)	22.96	1.2	53.8	45.0	1068.6
Hannover/Stroehen (Hs-0)	23.60	3.4	56.7	39.9	627.3
Kendalville	23.23	1.4	49.5	49.1	1165.2
Lezoux (Lz-0)	23.32	1.7	53.4	44.9	965.0
Nossen (No)	23.20	1.2	49.9	48.9	1206.8
Shakdara (Sha)	23.53	1.4	55.1	43.5	1000.1
Wietze (Wt-5)	23.48	1.5	59.5	39.1	965.2
Zdarec (Zdr-6)	23.33	2.5	52.8	44.7	801.1
mixed[b]	23.33	1.0	53.3	45.7	1238.3

[a] The total phenylpropane yield is the quantity of lignin phenylpropane units recovered by thioacidolysis on the basis of the starting lignin content. [b] Sample is a mixture of Estland (Est-1), Br (Br-0), Merzhausen (Mz-0), Borky (Bor-4), and Gückingen (Gü-0).

microscale acetyl bromide method has been shown to be accurate and precise, with no significant differences in the determined values of lignin content compared to the conventional method. The modifications to the acetyl bromide method enable small samples to be easily and efficiently handled. All of this has been proven using the same *Arabidopsis* accession. However, to use the microscale acetyl bromide method for the determination of lignin content in different *Arabidopsis* accessions requires there to be a single common extinction coefficient. Variation of lignin structure among different samples could lead to variation in extinction coefficients, resulting in error in the subsequent calculation of lignin content. To determine the extinction coefficients of lignins in *Arabidopsis* accessions by the microscale acetyl bromide method, lignins were isolated from a set of *Arabidopsis* accessions grown under various growing conditions, using the procedure proposed by Fukushima and Hatfield who developed lignin extinction coefficients for alfalfa and bromegrass (*26*). The set of *Arabidopsis* accessions were chosen according to observed phenotypic variation and genetic distance. For example, Shakdara (Sha) is a Central-Asian accession, which is genetically distant from European accessions (*34*), and has a medium size of rosettes and leaves. On the other hand, Hannover/Stroehen (Hs-0) is a German accession with large rosettes and leaves. In this way, the plants used for lignin isolation were representative of the population of *Arabidopsis* accessions and contained the effects of environmental variation on the plants.

To determine the extinction coefficient values of the isolated lignins, impurities in the lignins including neutral sugars, uronic acids and proteins were determined in order to correct the lignin weight. The major sugar in the lignin isolated from *Arabidopsis* accessions was xylose. The total carbohydrates, neutral sugars plus uronic acids, were in the range of 1.57% to 4.40% and proteins ranged from 2.62 to 5.34% (**Table 4**). These results are similar to the results for lignins isolated from other nonwoody samples as reported by Fukushima and Hatfield (*26*).

Based on the impurity-corrected weight of lignin, the extinction coefficient values of each isolated lignin were determined and found to be in the range of 22.96 and 23.60 g^{-1} L cm^{-1} (**Table 5**). The mean and confidence interval for the extinction coefficient is 23.35 ± 0.13 g^{-1} L $cm^{-1}$and variability is less than 1%. The results are consistent with the work of Johnson et al. (*22*) who found the extinction coefficients of acetyl bromide derived lignins from various softwoods and hardwoods ranged from 23.3 to 23.6 g^{-1} L cm^{-1}.

Although the extinction coefficient values of the isolated lignins from *Arabidopsis* accessions were very close, the structures of the lignins were different. Thioacidolysis analysis of the isolated lignins showed that the syringyl content of the lignins varied from 39.1 to 49.1% (**Table 5**). Furthermore, the total monomer yield of the isolated lignins ranged 627.3 to 1238.3 μmol g^{-1}, which also indicates variation in the lignin structures since the yield of thioacidolysis mainly depends on the cleavage of β-aryl ether linkages in lignins (*30*). These results demonstrate that the extinction coefficient values using the acetyl bromide method are insensitive to the lignin structure differences caused by changes in plant growth conditions and genetic variation in *Arabidopsis*. Thus, the average extinction coefficient value can be applied to lignin content determination of inflorescence stems of different *Arabidopsis* accessions and

their segregants harvested at the mature stage. In short, the microscale acetyl bromide method can be used for determination of lignin in inflorescence stems of *Arabidopsis*, for mapping of lignin-related genes. The microscale acetyl bromide method should be also suitable for general metabolomic analyses of lignin in *Arabidopsis*, as the extinction coefficient value is not likely to be significantly affected by the lignin structure variation that occurs at different growth stages (*35*).

ABBREVIATIONS USED

ANOVA, analysis of variance; Col-0, Columbia-0; FTIR, Fourier transform infrared spectroscopy; G, guaiacyl; GC-MS, gas chromatography-mass spectrometry; H, *p*-hydroxyphenyl; Hs-0, Hannover/Stroehen; HPLC, high-performance liquid chromatography; NA, not available; NIR, near-infrared spectroscopy; QTL, quantitative trait loci; RH, relative humidity; S, syringyl; Sha, Shakdara; UV, ultraviolet light.

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