DEVELOPMENT AND APPLICATION OF A RAPID MICRO-SCALE METHOD OF LIGNIN CONTENT DETERMINATION IN *ARABIDOPSIS THALIANA* ACCESSIONS

by

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ABSTRACT

Lignin is a major chemical component of plants and the second most abundant natural polymer after cellulose. The concerns and interests of agriculture and industry have stimulated the study of genes governing lignin content in plants in an effort to adapt plants to human purposes. *Arabidopsis thaliana* provides a convenient model for the study of the genes governing lignin content because of its short growth cycle, small plant size, and small completely sequenced genome. In order to identify the genes controlling lignin content in *Arabidopsis* accessions using Quantitative Trait Locus (QTL) analysis, a rapid micro-scale method of lignin determination is required.

The acetyl bromide method has been modified to enable the rapid micro-scale determination of lignin content in *Arabidopsis*. Modifications included the use of a micro-ball mill, adoption of a modified rapid method of extraction, use of an ice-bath to stabilize solutions and reduction in solution volumes. The modified method was shown to be accurate and precise with values in agreement with those determined by the conventional method. The extinction coefficient for *Arabidopsis* lignin, dissolved using acetyl bromide, was determined to be 23.35 $g^{-1}Lcm^{-1}$. This value is independent of the *Arabidopsis* accession, environmental growth conditions and is insensitive to syringyl/guaiacyl ratio. The modified acetyl bromide method was shown to be well correlated with the 72% sulfuric acid method once the latter had been corrected for protein contamination and acid-soluble lignin content ($R^2 = 0.988$, $P < 0.0001$).

As determined by the newly developed acetyl bromide method and confirmed by the sulfuric acid method, lignin content in *Arabidopsis* was found to be a divergent property. Lignin content in *Arabidopsis* was found to be weekly correlated with growth rate among *Arabidopsis*

accessions (R^2 = 0.48, P = 0.011). Lignin content was also found to be correlated with plant height among *Arabidopsis* accessions (R^2 = 0.491, P < 0.0001).

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
AcBr	acetyl bromide
ANOVA	analysis of variance
°C	degrees Celsius
cm	centimeter
F5H	ferulate 5-hydroxylase
FPU	filter paper units
FTIR	Fourier transform infrared spectroscopy
g	gram
G	guaiacyl
GC-MS	gas chromatography-mass spectrometry
h	hour
H	p-hydroxyphenyl
HPLC	high performance liquid chromatography
IU	international units
KHz	kilohertz
L	liter
m	meter
M	molar

mg	milligram
min	minute
mL	milliliter
mm	millimeter
mu	mass unit
m/z	mass to charge ratio
NIR	near-infrared spectroscopy
nm	nanometer
QTL	Quantitative Trait Locus
RH	relative humidity
RILs	recombinant inbred lines
S	syringyl
µg	microgram
µL	microliter
µm	micrometer
µmol	micromolar
UV	ultraviolet light
v/v	volume by volume

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1 INTRODUCTION

1.1 Importance of lignin study

Lignin, which is the second most abundant biopolymer on Earth (Battle et al., 2000), represents a significant carbon sink in the biosphere. Lignin has played a major role in the competitive survival of vascular plants. Its deposition in the cell walls of xylem confers hydrophobicity and mechanical strength providing resistance to the negative pressure generated by transpiration. In addition, lignin reduces the susceptibility of cell walls to attack by pathogens (Monties, 1989). The defense response of lignification to environmental cues such as wounding and pathogen attack is vital for plant survival (Lange, Lapierre, & Sandermann, 1995; Lawton & Lamb, 1987; Vance, Kirk, & Sherwood, 1980).

On the other hand, lignin has far-reaching impacts on agriculture and industry. The digestibility of forage crops by cattle is negatively correlated with the lignin content (Moore & Jung, 2001). The enzyme hydrolysis process in the biofuel industry is inhibited by the presence of lignin (Koullas, Christakopoulos, Kekos, Macris, & Koukios, 1992; Mansfield, Mooney, & Saddler, 1999). Kraft pulping and bleaching processes are adversely affected by the lignin which needs to be removed from the wood using large amounts of energy and chemicals (Axegard, Jacobson, Ljunggren, & Nilvebrant, 1992; Chiang, Puumala, Takeuchi, & Eckert, 1988).

As a result, the study of the lignification process in plants is now receiving increased attention in an effort to adapt plants to human purposes. With the development of

molecular techniques, it is now possible to manipulate the lignification process in plant by altering enzymes within the lignin biosynthesis pathway or cloning genes associated with lignification. The modification of plants to reduce lignin content would provide livestock more readily digestible forage and improve biofuel and pulp production by reducing the requirements for energy and chemicals (Baucher, Monties, Van Montagu, & Boerjan, 1998). Meanwhile, altering lignin quantity through genetic modification should not lead to pleiotropic effects incompatible with the normal growth and development of plants. The applied objectives require a thorough knowledge of lignification of plants, a topic that has been studied for more than a century, yet many fundamental questions still remain (Whetten, MacKay, & Sederoff, 1998). The limitation of the present knowledge of lignification are mainly due to the complexity of lignin structures and heterogeneity of lignin in plants (Boudet, Lapierre, & Grimapettenati, 1995; Joseleau & Ruel, 1997).

1.2 Lignin structure

1.2.1 Lignin components

The complexity of lignin structure is exemplified by variations in monomeric composition. Lignin is a complex aromatic polymer derived from three main hydroxycinnamyl alcohol precursors or monolignols, which differ in the extent of methoxylation (Higuchi, 1985). These alcohols, 4-hydroxycinnamyl alcohol, coniferyl alcohol, and sinapyl alcohol, give rise to p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units respectively when incorporated into the lignin polymer (Figure 1).

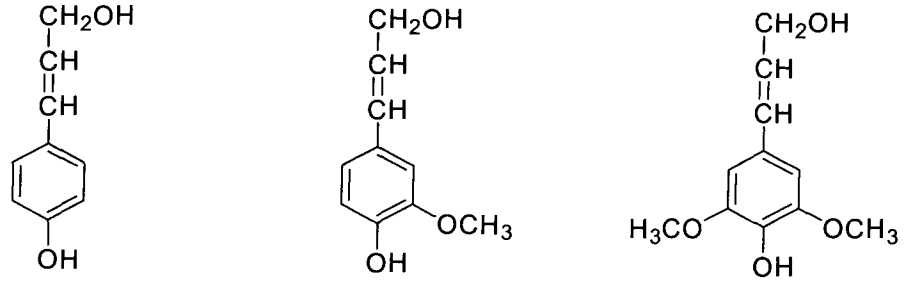

Figure 1: Chemical structure of the hydroxycinnamyl alcohol precursors of lignin. Source: Adapted from (Rogers & Campbell, 2004).

In addition to the three monolignols, small amounts of other monomers are also present in the lignin polymer in many plants. These monomers apparently arise from incomplete monolignol biosynthesis and other biosynthesis side reactions (Ralph et al., 2001). For example, p-hydroxybenzoates are found in the lignin of poplar, palms and willows, p-coumarates are detected in the lignin of all grasses, and acetates appeared in kenaf lignin (Ralph et al., 2001). The acetylated components, which are referred as non-core lignin compounds in the broader definition of lignin, derive from acylated monolignols (Boerjan, Ralph, & Baucher, 2003).

1.2.2 Inter-unit linkages of lignin

The complexity of lignin structures is also reflected in the variation of inter-unit linkages. In lignin, the alcohol precursors are interconnected by several types of ether and carbon-carbon linkages formed through dehydrogenative polymerization involving random coupling of radicals. In the polymerization process, the alcohol precursors are oxidized by peroxidases or laccases to form reactive phenoxy radicals which further are coupled. Figure 2 shows the four main resonance forms of coniferyl alcohol radicals obtained from

oxidation of the coniferyl alcohol by peroxidase (Glasser, 1980). The coupling reaction of the radicals produces a variety of inter-unit bonds. The most frequent inter-unit linkages are β-O-4 ether linkages (Sakakibara, 1980) (Figure 3). The other prominent linkages are β-5, β-1, β-β, 5-5 (C-C linkages), and 4-O-5 (ether) linkages. The linkages of α-O-4 (ether) can be formed as phenyl coumaran or dibenzodioxocin structures (Figure 3). The phenyl coumaran structures are formed by intramolecular ring closure between the phenolic groups of a β-5-linked lignin unit to a quinone methide (Sjöström & Alén, 1999). The dibenzodioxocin structures are formed by reaction of the phenolic groups of 5-5-linked units to an adjacent quinone methide (Ralph et al., 2004). The relative abundance of the different linkages depends largely on the composition of the different units in the lignin polymer (Boerjan et al., 2003). Softwood lignins, which are composed predominantly of G units, contain more C-C linkages (β-5 and 5–5) than hardwood lignins incorporating S units, because of the greater availability of the C5 position for coupling (Table 1) (Boerjan et al., 2003). Indeed, approximately 25% of C–C linkages are found at the C5 position in softwoods compared with only 10% in hardwoods (Monties, 1989).

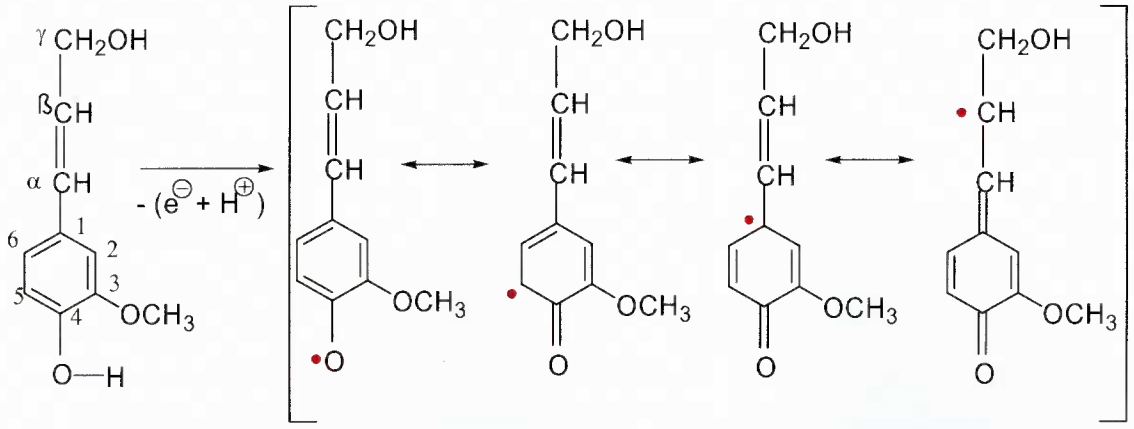

Figure 2: Main resonance forms of coniferyl alcohol radicals formed from oxidation of the conferyl alcohol by peroxidase.
Source: Adapted from (Glasser, 1980).

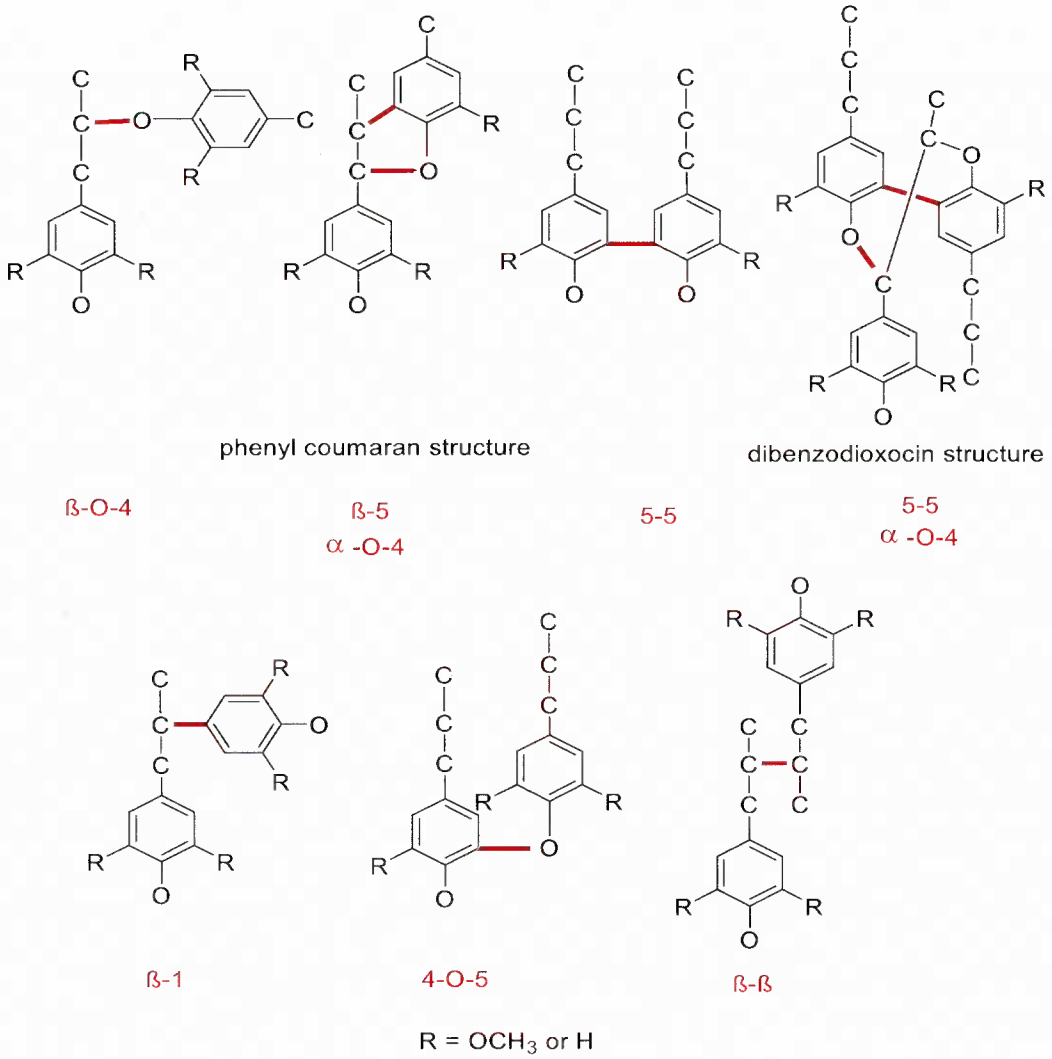

Figure 3: The most common intermonomeric linkages in lignin.

Source: Adaped from (Baucher et al., 1998; Ralph et al., 2004).

Table 1: Relative Frequencies of Predominant Inter-unit Linkages in Lignins.

Source: Adapted from (Sjöström & Alén, 1999).

Type of linkage	Percentage of total linkages	
	Softwood	Hardwood
β-O-4	50	60
α-O-4	2-8	7
β-5	9-12	6
5-5	10-11	5
4-O-5	4	7
β-1	7	7
β-β	2	3

1.2.3 Linkages between lignin and other polymers

Linkages exist not only between the units of lignin but also the between lignin and other cell wall polymers such as polysaccharides and proteins. The types of linkages between lignin and proteins are not clear (Iiyama, Lam, & Stone, 1994). The cross-links between polysaccharides and lignin have been proposed and are shown in Figure 4 (Iiyama et al., 1994). The linkages can be direct ester types bonds between uronic acids, such as the 4-O-methylglucuronic acid units present in xylan, and hydroxyl groups (α and γ) of the side chain of the monolignols (Figure 4a) (Iiyama et al., 1994; Sjöström & Alén, 1999). The linkages between polysaccharides and lignin can also be direct ether type bonds (Figure 4b). The bonding groups of polysaccharides can be the arabinose unit (HO-2 or HO-3) in xylans, or the galactose unit (HO-2) in galactoglucomannans. The bonding point in lignin is most likely the α-position (Sjöström & Alén, 1999). The linkages to the α-position likely result from reaction of alcohol or acid with quinone methide intermediates produced through reaction of peroxidase with monomeric lignin units (Iiyama et al., 1994).

The ester-ether bridges between polysaccharides and lignins (Figure 4f) can be formed by the non-core lignin component such as ferulic acid and p-coumaric acid, which are esterified or etherified to polysacchrides (Figure 4c) and monolignols (Figure 4d and 4e). (Iiyama et al., 1994). The mechanism of the formation of ester-ether bridges to lignin was suggested to be addition of hydroxycinnamoyl residues on polysaccharides to quinone methides giving benzylaryl ethers (α-ethers) (Scalbert, Monties, Rolando, & Sierraescudero, 1986). The derivatives of ferulic acid (dehydrodiferulic acid) can form diester linkage between polysaccharides (Figure 4g) and may also be etherfied to

monolignols (Figure 4h) (Lam, Iiyama, & Stone, 1992). The formation of the ether bonds could be due to etherification of lignin monomers either through quinone methide intermediates or incorporation through radical copolymerization (Ralph, Helm, Quideau, & Hatfield, 1992).

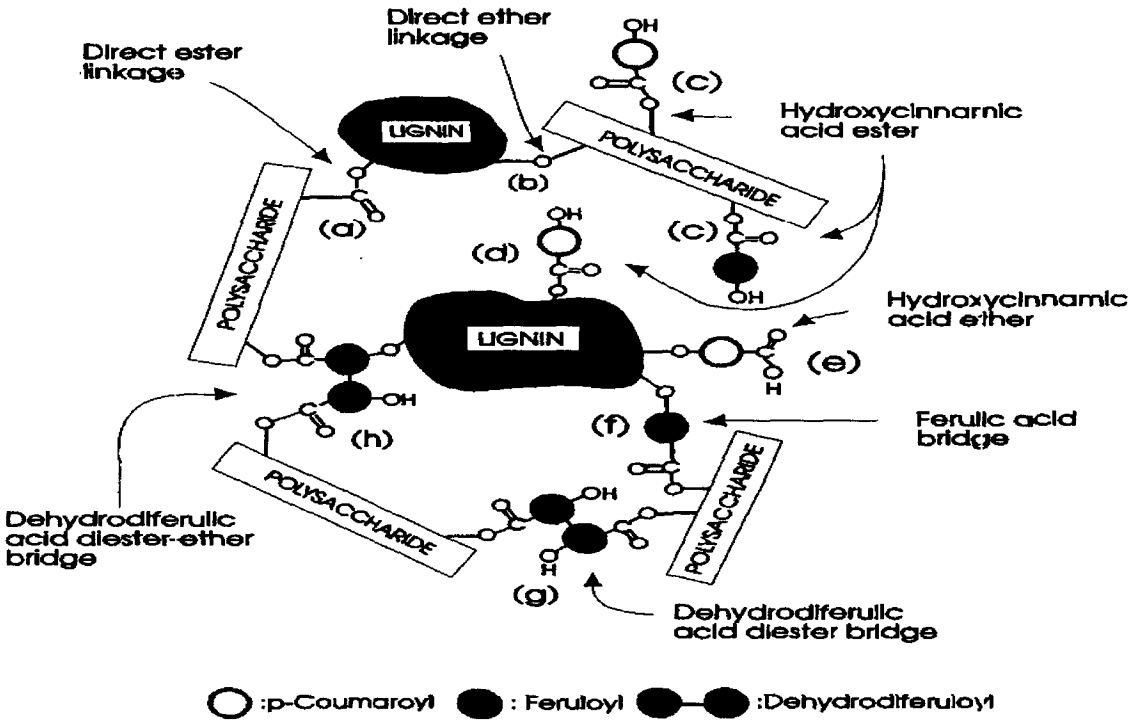

Figure 4: Possible covalent cross-links between polysaccharides and lignin in cell walls.
Source: Taken from (Iiyama et al., 1994). The material is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

1.3 Heterogeneity of lignin

In addition to the complexity of lignin structures led by the diversity of lignin composition and linkages, the heterogeneity of lignin composition and content in various regions of plants represents another level of complexity in the study of lignification.

Lignins found in the primary wall (P), secondary wall (S1, S2, and S3), middle lamella

(ML), and cell corners are all different (Figure 5) (Saka & Goring, 1985; Terashima & Fukushima, 1988). In the cell wall of pine, a higher amount of H units of lignin are present in the cell corner and the middle lamella, more G units are deposited in the second wall, and S units, which are minor components of gymnosperm, are deposited in the S3 layer of the secondary wall (Terashima & Fukushima, 1988). Furthermore, lignin content in the different subcellular regions varies. In the tracheids of gymnosperms, the secondary wall has the highest amount of lignin due to a large proportion of the total tissue volumes, while the middle lamella and cell corners have a higher lignin concentration than the secondary wall (Baucher et al., 1998).

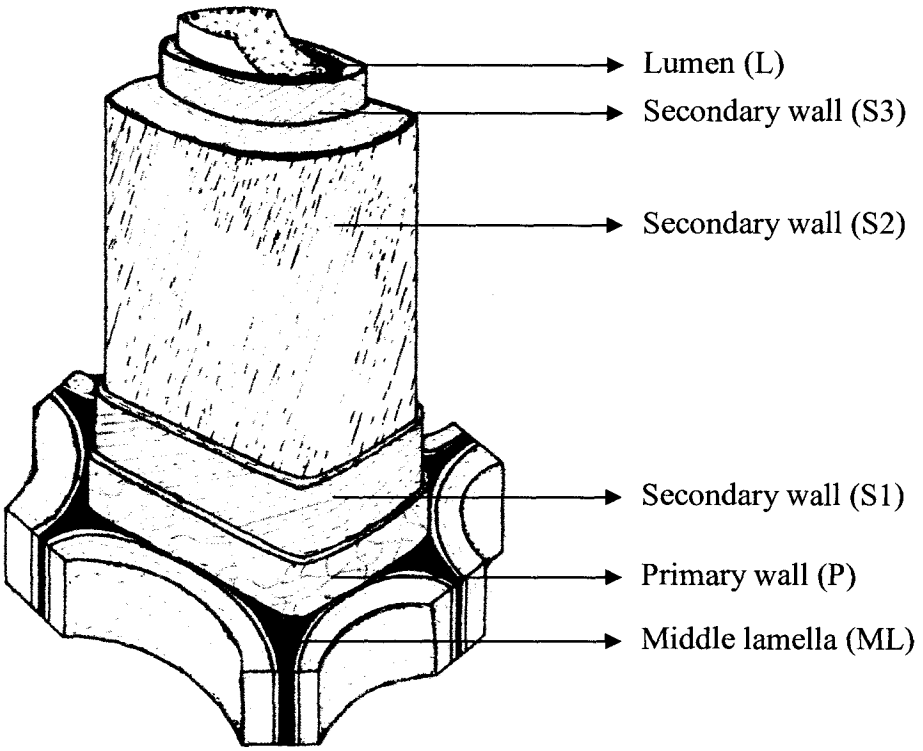

Figure 5: Cell wall organization.
Source: Adapted from (Smook, 2002).

Lignin content and composition vary with different cell types of a single tissue. In the secondary wall of angiosperm, lignin in fibers is generally enriched in S units while

lignin in vessels is enriched in G units. Lignin concentration is generally higher in secondary walls of vessels than in those of fibers. The proportion of total lignin content is larger in latewood than in early wood (Baucher et al., 1998).

Lignin also varies among taxa. Lignin in gymnosperms is mainly composed of G units whereas angiosperm lignin consists of G and S units with the ratio varying from 4:1 to 1:2 (Sjöström & Alén, 1999).

Lignin amount and composition in plants are also influenced by environmental conditions. For example, in normal gymnosperm wood tissues, lignin is mainly composed of G units and the lignin content is about 30%. On the other hand, the lignin in gymnosperm compression wood, which is formed in response to a non-vertical orientation of the stem caused by winds, snow or slope, contains a higher amount of H units that have multiple conjugating sites to form a highly condensed lignin. The lignin content in compression wood can be as high as 40% (Timell, 1986).

1.4 Lignification study with *Arabidopsis*

The complexity of lignin structure and its variation with cell wall layer, cell wall type, species, and environmental conditions create challenges in the study of lignification in plants. Although the basic outlines of lignin biosynthesis have been known for about thirty years, many uncertainties still exist about how lignin monomers are synthesized, how they are transported to the cell wall, and how they are polymerized into lignins (Whetten et al., 1998). In fact, the monolignol biosynthetic pathway has been redrawn many times and remains a matter of debate (Humphreys & Chapple, 2002). Advances in our fundamental understanding of lignin biosynthesis have been limited as research has

being conducted with a wide range of species, resulting in a large body of knowledge which is difficult to interpret. A more systematic approach is needed. The emergence of *Arabidopsis thaliana* as a model organism in biology has made a dramatic shift toward using *Arabidopsis* as the main plant for systematic lignification studies (Anterola & Lewis, 2002; Meinke, Cherry, Dean, Rounsley, & Koornneef, 1998).

1.4.1 Characteristics of *Arabidopsis thaliana*

Arabidopsis thaliana, which is a dicotyledon angiosperm in the family mustard, has become the organism of choice for a wide range of studies in plant science because of the advantages of a large genetic variation among naturally occurring populations, small completely sequenced genome, selfing nature of the plant, short growth cycle, and small plant size (Meinke et al., 1998).

The large genetic variation among the naturally occurring population of *Arabidopsis* is a reflection of the adaptation of *Arabidopsis* to a wide geographical distribution covering a diversity of environments. Many *Arabidopsis* accessions, which are different wild lines distributed in various geographical regions, have been collected in Europe, Asia, Africa and North America; from northern Scandinavia at 68° to the Cape Verde Islands at 16°; from sea level in the Netherlands to the high western Himalayan (Alonso-Blanco & Koornneef, 2000).

The genome of *Arabidopsis* is small. The entire genome, which contains 150 megabases and is organized into five chromosomes, was sequenced in 2000. Furthermore, the selfing nature of the plant reduces the chance of cross-contamination during generating progeny plants, which assists genetic experiments.

As an annual plant, *Arabidopsis* has a rapid life cycle. The entire cycle of germination, formation of plant rosette, bolting of the main stem, flowering and maturation of the first seeds takes about six weeks. Bolting occurs about three weeks after planting, resulting in an inflorescence stem (Meinke et al., 1998).

Arabidopsis has a small size. The leaves, which form a rosette with a diameter in the range of 2-10 cm at the base of the plant, are 1.5-5 cm long and 2-10 mm broad. The inflorescence stems, which are produced from the rosettes during the transition from vegetative to reproductive growth, are typically 30-50 cm long. The flowers are 2 mm long while the fruits known as siliques are 5-20 mm long, containing 20-30 seeds (Meinke et al., 1998). The small size of *Arabidopsis* allows it to grow in a variety of settings including growth rooms under florescent lights in the laboratory, greenhouses or growth chambers (Meinke et al., 1998).

1.4.2 Significance of *Arabidopsis thaliana* research

The advantages of *Arabidopsis* for plant study may have been difficult to foresee before the 1980s. Before the advent of gene cloning, several plants including maize, tomato, pea and barley remained the major organisms for genetic study because of the perceived linkage between plant breeding and genetics. After the establishment of transformation protocols in the 1980s and the demonstration that *Arabidopsis* had a small genome for detailed molecular analysis, *Arabidopsis* started to become widely used in plant research (Meinke et al., 1998). Many plant biologists experienced in the analysis of other organisms were attracted to *Arabidopsis* as a promising organism for basic research in plant physiology, biochemistry, and development. The completion of the *Arabidopsis*

genome sequence in 2000 stimulated additional enthusiasm for the use *Arabidopsis* as a model to solve practical problems in plant research. The genome sequence information helps researchers identify every essential gene of *Arabidopsis* and decipher the regulatory networking embodying biological functions.

The advances with *Arabidopsis* can be applied to other high plants. *Arabidopsis* evolved, within the last 150 million years, from a common ancestor with 250,000 other species of angiosperms. This relative recent evolution has resulted in a high degree of similarity between *Arabidopsis* and other angiosperms in developmental, metabolic, biochemical, and environmental aspects of biology. For example, it has been demonstrated that tomato and *Arabidopsis* have common genome sequences controlling fruit development and *Arabidopsis* genomes can be exploited for tomato research. Rice and *Arabidopsis* have been found to have the same mechanism governing flowing time (Hayama & Coupland, 2003). Thus, the research of *Arabidopsis* can produce a deep understanding and knowledge of other plant species.

In fact, research on *Arabidopsis* has given new insights into plant physiology and biochemistry that is shared with other organisms (Alonso-Blanco & Koornneef, 2000). For example, the genetic analysis of *Arabidopsis* mutants has revealed a protein complex that controls a complex signal transduction network that was later found throughout the eukaryotes (Alonso-Blanco & Koornneef, 2000).

1.4.3 Effectiveness of lignification research with *Arabidopsis*

As a model organism, *Arabidopsis* can be used in studies of lignin biosynthesis of plants. It has been found that the development pattern of lignification in *Arabidopsis* is similar to

other angiosperms (Barriere et al., 2005; Dharmawardhana, Ellis, & Carlson, 1992; Goujon, Sibout, Eudes, MacKay, & Joulanin, 2003).

1.4.3.1 Lignified tissues in *Arabidopsis* inflorescence stems

In the inflorescence stems of *Arabidopsis*, there are typically eight discrete vascular bundles that are separated by cells of the interfascicular regions. Xylem tissues in the vascular bundles and the cells from the interfascicular regions are characterized by strong lignification, which can be visualized as a red/magenta color on staining with phloroglucinol-HCl (Figure 6).

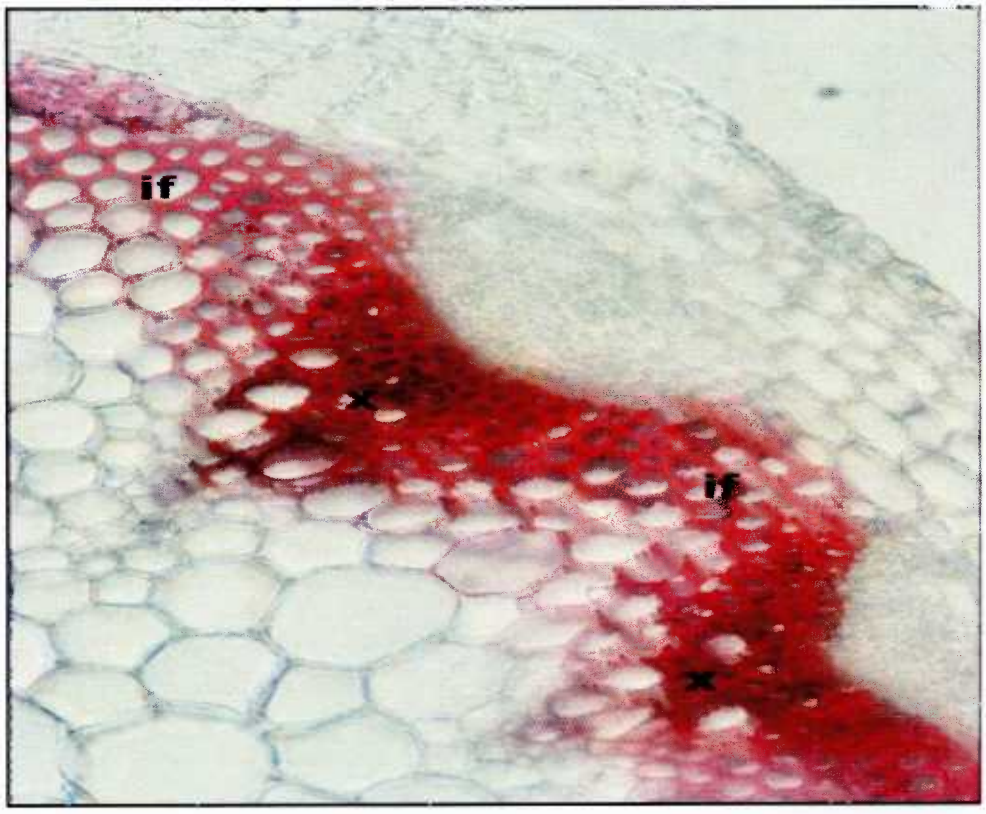

Figure 6: Lignin deposition in a cross section of an *Arabidopsis* inflorescence stem. Lignins appear to be red/magenta in both xylem cells (x) and interfascicular cells (IF) stained with phloroglucinol-HCl.
Source: Taken from (Rogers & Campbell, 2004). The material is reprinted with permission of New Phytologist, copyright 2004.

Xylem tissues in the vascular bundles first arise from the cells of the procambium during the primary growth stage. The primary xylem tissue in the vascular bundles consists of the tracheary elements and xylem parenchyma cells (Figure 7) (Turner & Sieburth, 2003). In common with most flowering plants, the tracheary elements, which differentiate from procambial cells, undergo the significant elongation, secondary wall thickening, lignification and programmed cell death (Turner & Sieburth, 2003). The xylem parenchyma cells, which do not undergo cell death, are characterized by short length and uniform lignified secondary cell wall. The development process of the primary xylem tissue involves the formation of the early protoxylem and later metaxylem (Figure 7) (Turner & Sieburth, 2003). The protoxylem elements differentiate toward the outside of the vascular bundle in the top of inflorescence stems. The metaxylem elements differentiate toward the inside of the vascular bundle in the top of inflorescence stems (Turner & Sieburth, 2003).

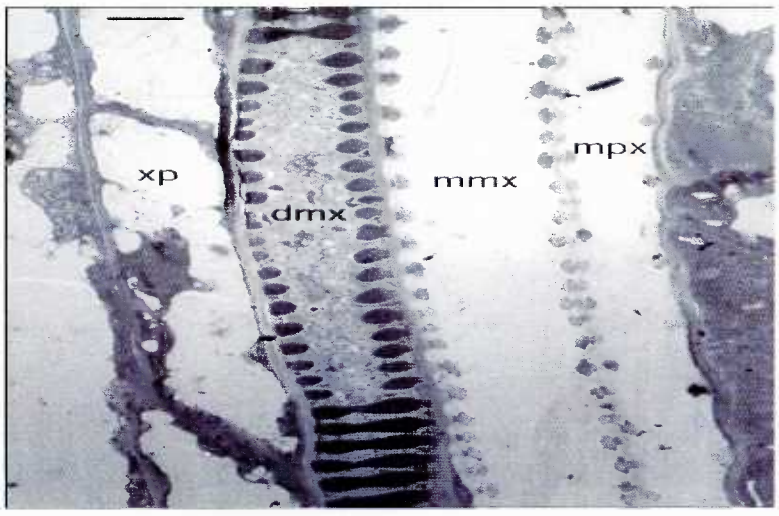

Figure 7: Xylem cells in a vascular bundle from an *Arabidopsis* inflorescence stem in the primary growth stage. Mature protoxylem (mpx), mature metaxylem (mmx), developing metaxylem (dmx) and xylem parenchyma (xp) are indicated.
Source: Taken from (Turner & Sieburth, 2003). The material is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

Concurrent with the development of metaxylem in the vascular bundles, the interfascicular fibers are forming from the fiber precursor cells, interfascicular parenchyma cells, in the interfascicular regions (Ye, Freshour, Hahn, Burk, & Zhong, 2002). The fiber precursor cells undergo significantly greater elongation than neighboring cells such as endodermis probably because the tapered end of the fiber precursor cells assist in their penetration between the walls of cells (Ye et al., 2002). When the elongation of the fiber precursor cells stops, thick secondary walls with distinct layers start being synthesized. The secondary walls of the mature interfascicular fibers are impregnated with lignin (Ye et al., 2002).

Following the development of primary xylem and interfascicular fibers, secondary xylem tissues may also be produced, in both vascular bundles and the interfascicular regions, along the entire inflorescence stems in the secondary growth stage (Ye et al., 2002). In both of the vascular bundles and the interfascicular regions, the produced secondary xylem consists of lignified vessel elements with pitted secondary wall and lignified xylary fibers with tapered ends (Ye et al., 2002). The amount of produced secondary xylem depends on the activity of cambial and interfascicular cambial cells which can be stimulated by growing plants for a prolonged period under a long-night photoperiod or by cutting the developing inflorescences (Barriere et al., 2005; Levyadun, 1994; Ye et al., 2002).

In short, the mature inflorescence stems of *Arabidopsis* contain lignified vascular bundles joined by a lignified arc. In the vascular bundles, there are sequentially developed cells of protoxylem, xylem parenchyma, metaxylem, secondary xylem. The lignified arc linking the vascular bundles consists of interfascicular fibers and secondary xylem cells.

These cells in the tissues contain a large amount of lignin. The development pattern of lignified tissues in *Arabidopsis* appears to be typical of other higher plants such as maize (Barriere et al., 2004; Barriere et al., 2005). As a result, *Arabidopsis* has been widely used as a model system in the study of cell wall syntheses including lignification (Barriere et al., 2005; Reiter, 1998).

1.4.3.2 Lignin in *Arabidopsis* inflorescence stems

Besides the typical development pattern of lignified tissues in *Arabidopsis* inflorescence stems, the monomer composition of the lignin in the tissues was also found to be common to that in many herbaceous angiosperms with a predominant amount of guaiacyl (G) and syringyl (S) units (Dharmawardhana et al., 1992). Lignin content in dry inflorescence stems of *Arabidopsis* is around 12-18%, depending upon the growth condition and accessions (Goujon et al., 2003).

In conclusion, the typical development pattern of lignified tissues and the nature the lignin formation indicate that *Arabidopsis* can serve as a convenient model to explore the regulatory mechanisms of lignin biosynthesis. The results of lignification research with *Arabidopsis* can potentially be extrapolated to other high plants.

1.4.4 Advances in lignification research with *Arabidopsis*

Research with *Arabidopsis* has been giving new insights into many aspects of lignification (Goujon et al., 2003). For example, the analysis of *Arabidopsis* ferulate-5-hydroxylase (F5H), an enzyme involved in lignin biosynthesis, provided the evidence for the now revised model of phenylpropanoid pathway (Humphreys & Chapple, 2002). The

analysis of the transcript abundance of *Arabidopsis* genes revealed that lignin biosynthesis appeared to be regulated in a circadian fashion (Harmer et al., 2000). This work led to the proposal that lignin biosynthesis pathway is coordinately controlled with cell elongation (Rogers & Campbell, 2004).

The advance in lignification research with *Arabidopsis* has been largely based on the classic genetics analysis of the mutants with altered expression of a particular gene affecting lignification (Alonso-Blanco & Koornneef, 2000; Rogers & Campbell, 2004). Using this method, the mutants affecting lignin biosynthesis pathway can be isolated to dissect the lignin biosynthesis process and to identify and analyze the genes underlying the altered expression pattern of lignification. Many mutants of *Arabidopsis* in lignin biosynthesis processes have been identified and analyzed (Goujon et al., 2003). For example, mutant, *irx4*, was found to have 50% less lignin than wild types and the further analysis indicated that the reduction of lignin content was due to a cinnamoyl-CoA reductase (CCR) gene (Jones, Ennos, & Turner, 2001). The identification of genes with altered expression of lignification in the mutants and their associated functions have contributed to a molecular understanding of analogous processes in a wide range of plant species.

Since lignification is not under control of a single but rather multiple genes, the analysis of such mutants may not provide all the genetic backgrounds about lignification (Alonso-Blanco & Koornneef, 2000; Goujon et al., 2003; Loudet, Chaillou, Camilleri, Bouchez, & Daniel-Vedele, 2002). For example, the genotype of the wild lines used for analysis will affect the identification of genes governing lignification. If the wild lines carry a functionally null or weak allele, the genes affecting the lignification might not be

detected (Alonso-Blanco & Koornneef, 2000).

A possible alternative to lignification research based on mutants is to exploit the wealthy natural variation among *Arabidopsis* accessions by the Quantitative Trait Locus (QTL) analysis (Alonso-Blanco & Koornneef, 2000; Loudet et al., 2002). By the QTL analysis, the loci that affect a divergent trait of *Arabidopsis* can be identified and characterized. Indeed, the *Arabidopsis* traits such as disease resistance, tolerance to stresses and light responses have already been mapped down to the molecular level by the QTL analysis (Alonso-Blanco & Koornneef, 2000). Using *Arabidopsis* accessions, it is possible to first determine if lignin is a divergent property and later determine underlying genes governing lignin content. Further molecular isolation and characterization of the genes governing lignin content can yield additional information about lignin biosynthesis and obtain novel insight into regulatory aspects of ligninfication. At the same time, the *Arabidopsis* accessions can be used to assess if lignin content varies independently with plant growth rates and height among natural accessions. This will help to evaluate whether decreasing lignin content by generating transgenic plants alters the yield of carbohydrates such as cellulose in the plants.

1.5 Determination of lignin content in *Arabidopsis* accessions

In order to determine if lignin is a divergent property and if lignin content varies independently with plant growth rates and height among *Arabidopsis* accessions, a rapid method of lignin determination in *Arabidopsis* accessions that can handle a large number of samples at a micro-scale is required.

1.5.1 Current methods available for lignin content determination

Many methods have been developed to quantitatively determine the amount of lignin in plant samples. The principle types of approach are based on weighing, oxidant consumption or spectroscopy (Dence, 1992). The methods based on weighing involve an isolation of lignin residuals for gravimetric analysis through hydrolysis and solubilization of carbohydrates in the samples. The hydrolysis of the carbohydrates is catalyzed by a variety of strong mineral acids. The most widely used gravimetric method is the Klason or 72% sulfuric acid procedure, which employs 72% sulfuric acid to promote carbohydrate hydrolysis (Dence, 1992). The methods based on oxidant consumption involve measurement of the amount of oxidant consumed by lignin per unit weight of sample. The most common procedure in this category is the Kappa number method which employs potassium permanganate as the oxidant (Dence, 1992). The spectroscopic methods for the determination of lignin content include staining combined with visible spectroscopy, infrared spectroscopy (FTIR), near-infrared spectroscopy (NIR), ultra-violet microscopy and the acetyl bromide digestion combined with ultra-violet spectroscopy.

Many of the procedures first developed for wood, pulp and paper have been adapted for herbaceous samples (Hatfield & Fukushima, 2005). However, none of the methods currently available would be considered a standard unambiguous method suitable to measure lignin in all samples (Hatfield & Fukushima, 2005). As a result of this, it is necessary to consider the efficacy and limitations of the currently available methods for determining lignin content.

1.5.1.1 The 72% sulfuric acid (Klason) method

The 72% sulfuric acid procedure was originally developed in the early 1900s by Klason and is now applied to determination of lignin in pulp, wood and herbaceous samples. This method involves the partial digestion of the plant material with 72% sulfuric acid (H_2SO_4). In the process of digestion, long chains of polysaccharides are broken down by acid-catalyzed hydrolysis into smaller chains or into simple carbohydrates which are soluble in water (Figure 8). Moreover, condensation reactions of lignin occur. In this process, carbonium ions which are generated from the lignin units react with weakly nucleophilic 1- and 6- (or 5-) positions of other lignin units (Sjöström & Alén, 1999), forming carbon-carbon bonds and leaving an insoluble (Klason lignin) residue to be determined by gravimetric measurement (Figure 9).

This 72% sulfuric acid method also releases small amounts of acid-soluble lignin, which must be independently determined and added to the acid-insoluble lignin to obtain a more accurate value for the total lignin content. In the determination of acid-soluble lignin, the absorbance value of the filtrate from the acid-insoluble lignin, at a wavelength (205 nm), is measured and converted to lignin content through application of Beer's law. The extinction coefficient used to calculate the lignin content may vary among different samples and it would be desirable to determine this for each sample individually (Dence, 1992). Practically, an average extinction coefficient reported in the literature (110 $g^{-1}Lcm^{-1}$) is commonly used to roughly calculate the soluble lignin content (Dence, 1992). In some species of angiosperm and grasses, the acid-soluble lignin content can be higher than 5% (Lai & Sarkanen, 1971).

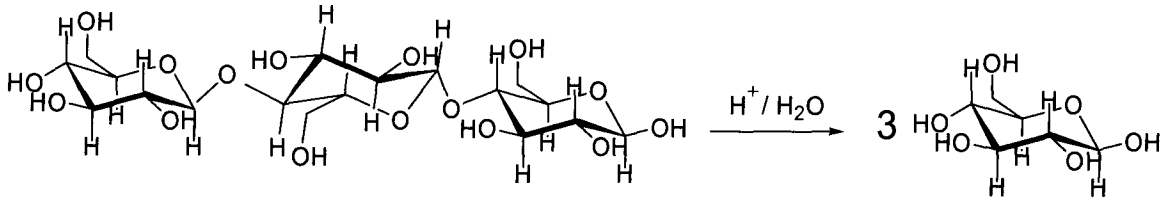

Figure 8: The hydrolysis of carbohydrates.
Source: Adapted from (Sjöström & Alén, 1999).

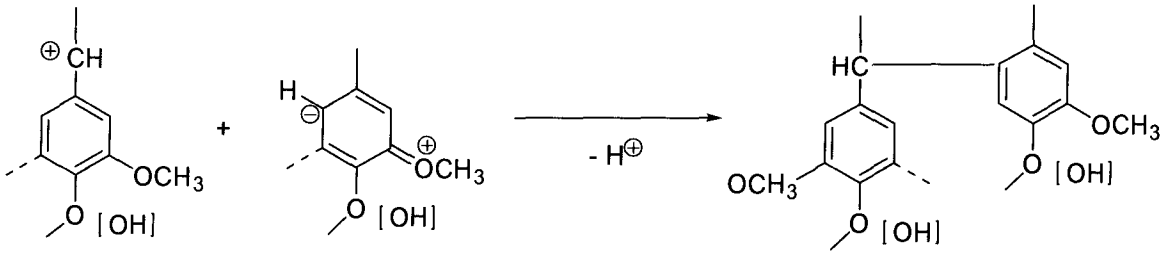

Figure 9: The condensation reaction of lignin
Source: Adapted from (Sjöström & Alén, 1999).

The 72% sulfuric acid method requires a relatively large amount of sample in order to provide enough lignin (approximately 20 mg) for an accurate weighing (Dence, 1992). The samples should be ground to pass a 40 mesh (0.4mm) screen to ensure complete carbohydrate hydrolysis during the acid digestion. The ground samples should be thoroughly extracted with solvents in a Soxhlet apparatus before digestion to remove extractives which would otherwise interfere with the determination. The solvents commonly used for extraction are ethanol-benzene (1:2, v/v) for wood samples and a combination of solvents such as ethanol-benzene (1:2, v/v), 95% ethanol and acetone, which are applied successively, for herbaceous samples (Dence, 1992). When applied to herbaceous samples, the 72% sulfuric acid method may require additional procedures to handle potential over-estimation of lignin due to the co-precipitation of proteinaceous substances and the presence of non-lignin inorganic ash constituents (Dence, 1992).

1.5.1.2 The Kappa number method

The Kappa number method is based on the oxidative consumption of lignin with acidic permanganate. Because the oxidative consumption of lignin is affected by the size of the sample and the amount of permanganate applied, discontinuities in the oxidation-lignin relationship will occur if the sample size or applied permanganate volume is changed. To avoid this problem, Tasman and Berzins (1957) proposed to adjust the sample to ensure that approximately half of the applied permanganate (i.e. 50 mL of 0.10 N $KMnO_4$) is consumed . If the actual volume of permanganate consumed by the sample is more or less than 50% of the applied amount, correction factors are applied. The corrected amount of permanganate consumed per unit of weight of sample is calculated and expressed as "Kappa number". Kappa number has a linear relationship with lignin content below 10% (Dence, 1992). The repeatability of the Kappa number determination is high for the samples with low lignin content (below 10%) but decreases with increasing lignin content (Dence, 1992). Therefore, this method is used exclusively in the analysis of unbleached pulps.

The Kappa number method is rapid, simple and useful for routine quality control in pulp and paper mills. In pulp and paper mills, Kappa number is used as an indicator of the degree of delignification during pulping and chemical requirement for bleaching. The Kappa number determination has been adopted as a standard procedure by many pulp and paper organizations (Dence, 1992).

1.5.1.3 The staining method combined with visible spectroscopy

The staining method is based on the color reactions of specific structural elements in lignified tissue with certain organic and inorganic reagents. More than 150 reagents are proposed for use in these color reactions (Nakano & Meshitsuka, 1992). The most commonly used reagent for detection and quantitative determination of lignin content is phloroglucinol-hydrochloric acid (Nakano & Meshitsuka, 1992).

When phloroglucinol-hydrochloric acid is applied dropwise to a lignified tissue slice or meal, a reddish violet appears and the color intensity can be measured at 550 nm by visible spectroscopy. It has been proposed that the color formation is due to the reaction between the coniferaldehyde units in lignin with phloroglucinol-hydrochloric acid (Figure 10) (Nakano & Meshitsuka, 1992). Thus, the determination based on this reaction is an evaluation of the relative amount of coniferaldehyde units in different samples. Practically, this method is often used to qualitatively indicate and compare the extent of lignification in plants. The sample preparation for staining should include a thorough extraction of samples first with ethanol-benzene (1:2, v/v) and then with water in a Soxhlet apparatus to remove extractives that otherwise interfere with the test.

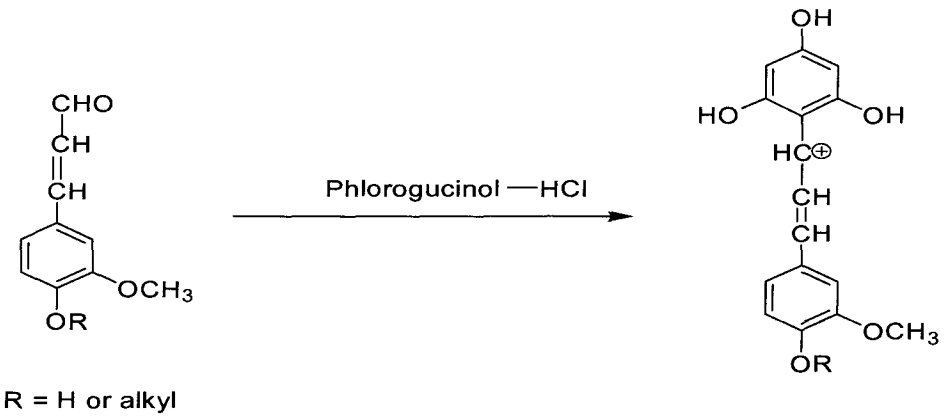

Figure 10: Color reaction between the coniferaldehyde units in lignin with phloroglucinol-hydrochloric acid
Source: Adapted from (Nakano & Meshitsuka, 1992)

1.5.1.4 Infrared spectroscopy (FTIR)

In Fourier transform infrared spectroscopy (FTIR), an interference infrared wave produced in an interferometer interacts with the sample. The modification of the interference light beam by the interaction with the sample is recorded and energy modes of bond types such as C-O, C=O, C-H stretching and vibrational energies are measured (Hatfield & Fukushima, 2005). In the FTIR spectra, the O-H, C-H, and C=O stretching modes above 1600 cm^{-1} and the aromatic skeletal vibration around 1510 cm^{-1} are unequivocally assigned because they are "pure" bands without the overlap of peak intensities (Dence, 1992). On the other hand, the interpretation of the bands below 1430 cm^{-1} and of the skeletal vibration band at 1600 cm^{-1} is difficult because these bands are complex with contributions from various stretching and/or vibration modes (Dence, 1992).

Although there are FTIR bands that can be relatively specific to lignin, the intensities of these bands may vary with the ratio of H, G, and S units of lignin in samples. For example, the bands in the wavenumber range 1505-1510 cm^{-1}, which are conventionally used as reference bands for lignin, have higher intensities for G units than for H units in lignin. As a result, it is difficult to use FTIR to determine the total lignin content in different types of samples due to difficulties of defining an appropriate standard for spectral calibration (Hatfield & Fukushima, 2005).

However, FTIR can be used to compare the structure and composition of lignin in different types of samples because the spectra characteristics of FTIR spectra are affected by the lignin structure. For instance, lignin in samples can be classified into G, GS (with

four subgroups), and HGS types based on the ratio of the intensities of the 1509 cm^{-1}/ 1426 cm^{-1} (Faix, 1992).

Sample preparation in FTIR is relatively easy compared to sample preparation in the wet chemistry methods, such as the 72% sulfuric acid method (Hatfield & Fukushima, 2005). Samples for FTIR measurement are typically extracted with ethanol-benzene (1:2, v/v) in a Soxhlet apparatus then finely ground. The ground samples are embedded in an alkali halide such as potassium bromide for transmission mode measurements or made into a powder for diffusion reflectance mode measurements to obtain the FTIR spectra (Faix, 1992). Thus, FTIR is rapid and nondestructive. Another advantage of FTIR is that it has a high signal-to-noise ratio and requires a small amount of sample due to the high sensitivity (Faix, 1992).

1.5.1.5 Near infrared spectroscopy (NIRS)

Near infrared spectroscopy (NIRS) uses the range of electromagnetic spectrum between the visible and infrared wavelength. NIRS shows minor but significant differences between the spectra of different lignin samples. These differences are useful for quantitative analysis and can be accentuated by derivative conversion of the spectral measurement (Faix, 1992). The converted spectral measurements are not directly proportional to the concentration of lignin because Beer's law does not hold. Instead, a calibration model that correlates the spectral measurement with corresponding lignin concentration, measured by wet chemistry, must be established to determine lignin content (Hatfield & Fukushima, 2005). To establish the calibration model, statistical

techniques including multiple linear regression (MLR), principal component regression (PCR) and the partial least squares (PLS) are commonly used (Faix, 1992).

The established calibration model should represent the full scope of variation in the samples for analyses (Poke, Wright, & Raymond, 2004). Thus, the calibration model in NIRS is specific and only applicable for determination of lignin content in same types of samples as those included in the model development. Moreover, the calibration model may be affected by the precision of the wet chemistry method used for the lignin assay and may change with the applied range of lignin content in the calibration sets. This will result in predicted values of lignin content that vary with the nature of the calibration model (Hatfield & Fukushima, 2005; Sykes et al., 2005; Yamada et al., 2006).

NIRS involves the same 'simple' sample preparation as stated in FTIR above. The diffusion reflectance technique requires relatively large amount of sample (several grams) to ensure the accuracy of results due to the large variation caused by the small penetration depth (1-4 mm) of the beam and heterogeneity of samples (Faix, 1992; Yamada et al., 2006; Yeh, Chang, & Kadla, 2004). The transmission technique requires less then 100 mg of sample because samples are fully penetrated and more tolerant to the heterogeneity than when using the reflectance technique (Yamada et al., 2006; Yeh et al., 2004).

1.5.1.6 Ultra-violet microscopy

Ultra-violet (UV) microscopy has been developed into a quantitative method for determination of the lignin distribution in particular morphological regions of a plant tissue (Nakano & Meshitsuka, 1992). The UV absorbance of lignin in the sample produces an image that can be taken as a photomicrograph for densitometric analysis.

The densitometric analysis provides a quantitative measure of lignin concentration according to the Beer's law:

$$A = abc,$$

where A = absorbance, a = extinction coefficient ($g^{-1}Lcm^{-1}$), b = section thickness or length of light path through the samples (cm), c = lignin concentration (gL^{-1}).

If the UV extinction coefficient of lignin in the sample remains constant, the lignin concentration is proportional to UV absorbance recorded on the photomicrograph and measured by densitometric analysis. However, p-hydroxylphenyl, guaiacyl and syringyl lignin unit have different absorbtivities at the same wavelength. Fergus and Goring (1970) found the absorption maximum for guaiacyl and syringyl lignin were at 280 nm and 270 nm respectively. Thus, if lignin composition in the area under study varies, the extinction coefficient of lignin is not constant and can not be used to calculate and compare lignin concentration within the samples.

Since gymnosperms lignin is mostly composed of guaiacyl units, a constant value of extinction coefficient can be assumed for various samples (Fergus & Goring, 1970). On the other hand, angiosperm lignin has variable ratios of guaiacyl and syringyl lignin units and thus use of the UV microscopy is limited.

Small wood chips are employed in the ultra-violet (UV) microscopy measurement. The wood chips are typically extracted sequentially with n-hexane at room temperature, alcohol-benzene (1:2, v/v) in a Soxhlet apparatus and acetone-water (1:1, v/v) at room temperature to remove 'pseudolignin' before the quantitative analysis of lignin (Nakano & Meshitsuka, 1992).

1.5.1.7 The acetyl bromide method

In the acetyl bromide method, the samples are solubilized in a solution of 25% acetyl bromide in glacial acetic acid, resulting in the formation of acetyl derivatives of unsubstituted OH groups within the lignin polymer and bromide replacement of α-carbon OH groups (Figure 11) (Hatfield & Fukushima, 2005). The absorbance of the solution at 280 nm, which is specific for aromatic compounds, is measured. Lignin concentration in the solution can be calculated from the absorbance value according to the Beer's law. The extinction coefficient used to convert absorbance value to lignin concentration seems to be constant for a wide range of samples. Johnson et al. (1961) determined the extinction coefficients of lignins from a range of softwood and hardwood lignins solubilized using the acetyl bromide method and found the extinction coefficients were similar and ranged from 23.3 to 23.6 $g^{-1}Lcm^{-1}$ at 280 nm.

The acetyl bromide method provides precise absorbance values for the determination of lignin content, and does not require a correction for acid-soluble lignin (Dence, 1992).

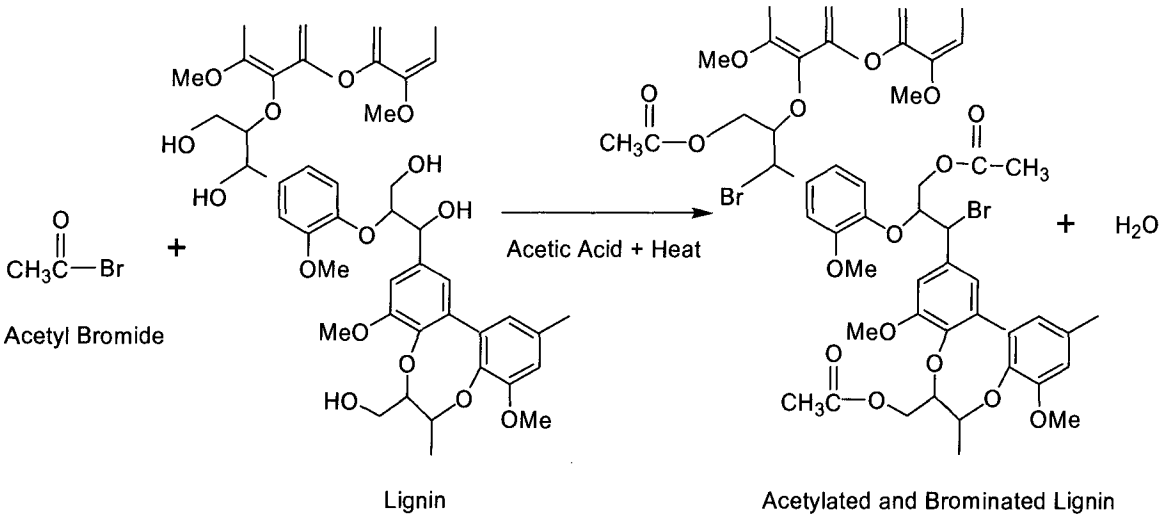

Figure 11: Reaction of acetyl bromide with lignin
Source: Adapted from (Hatfield & Fukushima, 2005).

The acetyl bromide method has been applied to wood, pulp, paper and herbaceous samples. Johnson et al. first introduced the acetyl bromide method for the determination of lignin content in wood samples. Morton (1967) modified the acetyl bromide method for the analysis of unbleached pulp and uncoated paper samples. Morrison (1972) further adapted the acetyl bromide method for non-woody samples because it was found that proteins in such samples did not dissolve in the acetyl bromide solution and that did not give rise to absorbance at 280 nm. When using the acetyl bromide method, it should be remembered that non-core lignin components will be included in the lignin content measurement due to the overlaps of the absorbance peaks between the three lignin units (H, G, and S units) and the non-core lignin components such as p-coumaric acids (Dence, 1992). The acetyl bromide method is applicable to samples where only small amounts (mg size) are available. Lignin in the acetyl bromide solution can be detected at ppm level with an absorbance reading between 0.2 and 0.7 (Dence, 1992).

Using the acetyl bromide method, the samples have to be ground finely (80 mesh) to ensure a complete digestion. Although the digestion of coarsely ground samples can be promoted by adding perchloric acid to the digestion solution (Iiyama & Wallis, 1988), later work has shown that that perchloric acid might cause interference with the lignin content determination due to the degradation of hemicelluloses to products that absorb at 280 nm (Hatfield, Grabber, Ralph, & Brei, 1999). The ground samples should be thoroughly extracted with solvents (Morrison, 1972). Johnson (1961) extracted wood samples with ethanol-benzene (1:2, v/v) in a Soxhlet apparatus; Morrison (1972) heated grass samples with distilled water in at 60 to 65 ^{o}C, and then filtered and washed the samples through a No. 52 filter paper with water, ethanol and acetone and diethyl ether;

Fukushima and Hatfield (2001) extracted both wood and non-wood samples with water, ethanol, chloroform, and acetone sequentially in a Soxhlet apparatus.

1.5.2 Requirements for the determination of lignin content in *Arabidopsis* accessions

The above methods have different features and are suitable for the determination of lignin content in different scenarios. To obtain an appropriate method for the determination of lignin content in *Arabidopsis* accessions, it is essential to define the requirements for the determination method:

- Large number of samples: To determine if lignin content is a divergent property, the determination of lignin content will have to be conducted on a large number of *Arabidopsis* accessions. Once lignin content is proven as a divergent trait, the method may further be used for QTL analysis of the recombinant inbred lines (RILs), which are generated by crossing the accessions with extreme high and low lignin content, and (RILs) progeny to locate the loci of genes governing lignin content. In short, the analysis will be based on the determination of lignin in a large number of samples in order to reach statistically significant results (Loudet et al., 2002).
- Various types of samples: The samples for the determination of lignin content will come from different *Arabidopsis* lines.
- Small amount of samples available: The determination of lignin content will have to be conducted using small amounts of samples. The weight of *Arabidopsis* inflorescence stem is less than 0.1 grams, while the analysis for

QTL analysis will be based on the determination of lignin content in individual plants.

- Non-woody samples: *Arabidopsis* is a non-wood angiosperm. Protein content and inorganic ash in *Arabidopsis* will be higher than wood samples.

1.5.3 Challenges for the determination of lignin content in *Arabidopsis* accessions

The sample features and requirements of the project represent challenges in obtaining an appropriate method for the determination of lignin content. Lignin structures, which affect lignin content determinations by many methods, may vary with *Arabidopsis* accessions due to the heterogeneity of the deposition of lignin monomers and inter-unit linkages among the different types of samples. The possible high amount of protein content and inorganic ash may interfere with lignin content determination. The high throughput of samples requires the determination method to be efficient while the low amount of samples available requires the determination to be performed at a micro scale.

1.5.4 Selection of methods for the determination of lignin content in *Arabidopsis* accessions

Among the currently available methods for the determination of lignin content, the 72% sulfuric acid method is time-consuming and not suitable for a high throughput of samples. Using this method, each set of determinations requires 3-4 hours, and the numbers of samples that can be performed in one set are limited by the availability of filtration stations. Determinations by this method will be more tedious if extra procedures such as

pepsin pretreatment of samples or protein and/or ash content determination in the acid-insoluble residue are incorporated to avoid or correct for the possible over-estimation caused by the protein content and inorganic ash. Moreover, determination by this method may be not accurate if the amount of sample available is less than 0.1 gram.

The Kappa number method is rapid and simple but is used exclusively in the analysis of unbleached pulps because the repeatability of the method decreases with increasing lignin content.

The method of staining combined with visible spectroscopy is commonly used as a qualitative method for indication of the extent of lignification in plants and is not sufficiently quantitative for our purposes.

FTIR, NIR, and ultra-violet microscopy require small amounts of sample due to the high sensitivity of these spectrophotometric methods. However, one challenge in using these methods for the determination of lignin content among *Arabidopsis* accessions is definition of an appropriate standard (Hatfield & Fukushima, 2005). The lignin composition and chemical structure variation among *Arabidopsis* accessions can lead to different spectral characteristics of lignin.

The acetyl bromide method may be an appropriate method for determination of lignin content in *Arabidopsis* accessions. The acetyl bromide method is appropriate for small sample sizes (mg level) and does not require a correction for acid-soluble lignin and non-lignin inorganic ash constituents. Determination of lignin by the acetyl bromide method would not be interfered with by proteins (Dence, 1992). Furthermore, prior investigations have indicated that the lignin composition and structure variations may not have a large influence on the UV absorbance of lignin solubized using the acetyl bromide method.

Iiyama and Wallis (1989) found that acetyl bromide treatment leads to very similar extinction coefficients at 280 nm for lignin obtained from different species. Thus, it may be possible to use a single extinction coefficient, at 280 nm, derived from lignin isolated from multiple *Arabidopsis* samples to estimate the total lignin content in various lines of *Arabidopsis*.

1.5.5 Adaptation of acetyl bromide method for determination of lignin content in *Arabidopsis* accessions

Besides having a possible well defined lignin standard and low detection limits, the acetyl bromide method can be potentially modified to be a high throughput rapid method suitable for the determination of lignin in a large number of *Arabidopsis* samples. In fact, since the acetyl bromide method was first introduced for the determination of lignin content in wood samples by Johnson et al. in 1961, many modifications to the original procedure have been made to suit different purposes.

For example, Morrison (1972) modified the pretreatment of grass samples to speed up the determination. According to Morrison, 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 water and other solvents such methanol, chloroform, acetone and ethanol-benzene in a Soxhlet apparatus (Morrison, 1972).

Iiyama and Wallis (1990) found that the precision of the method was not affected when they scaled down the volume of the digested solution from 200 mL to 50 mL.

Rodrigues et al. (1999) froze the digested samples in the freezer at –10 ^{0}C to stabilize solutions enabling the preparation of several reaction batches before the UV absorbance measurement.

These developments made over years to adapt the conventional acetyl bromide method indicate that 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.

1.6 Correlation between the acetyl bromide method and the 72% sulfuric acid method

The modified acetyl bromide method can be compared to 72% sulfuric acid method, which is widely accepted for the determination of lignin content in woody and non-woody samples. Fukushima and Hatfield (2001) found reasonable correlation between the acetyl bromide method and 72% sulfuric acid method when measuring the acid insoluble lignin (Klason lignin). The ratio between the values determined by the two methods among different plant samples ranged from 0.80 to 1.20. The variation of the ratio was probably due to protein contamination in the acid-insoluble residue and release of acid-soluble lignin which was not measured in this case. If these factors with the 72% sulfuric acid method can be eliminated, a higher correlation between the two methods might be expected.

1.7 Correlation between lignin content and growth rate of *Arabidopsis*

Using an altered acetyl bromide test, it may be possible to study the differences in lignin content that could exist among the *Arabidopsis* accessions. The determined values of lignin content in *Arabidopsis* accessions can be used to examine if lignin content is correlated with plant properties such as growth rate and height. The information gained through the lignin measurements could be used to support subsequent projects focused on genetic modification of plants.

1.8 Hypotheses

The hypotheses of the project are:

- The acetyl bromide method can be adapted to a rapid micro-scale method for the determination of lignin content in various accessions of *Arabidopsis* without significant effects on the accuracy and precision of the method.
- The adapted acetyl bromide method has a good correlation with 72% sulfuric acid method.
- Natural variation of lignin content among various accessions of *Arabidopsis* exists.
- Lignin content is correlated with plant growth rate and height among *Arabidopsis* accessions.

1.9 Objectives

The research objectives of the project are:

- Development of a high-throughput method for lignin determination in *Arabidopsis.*
- Determination of the lignin content in various accessions of *Arabidopsis.*
- Examination of the lignin content variation with plant growth rate and height among *Arabidopsis* accessions.

2 MATERIALS AND METHODS

2.1 Plant materials

Seeds of *Arabidopsis thaliana* accessions were purchased from the *Arabidopsis* Biological Resource Center (ABRC) and TAIR (The *Arabidopsis* Information Resource). The seeds of *Arabidopsis* accessions were soaked in Falcon tubes with water, vernalized for 7 days at 4 °C in the dark and then sown onto Terra-Lite Redi-earth (WR Grace & Co) soil in 72-well culture plates. The accessions were grown under various growth conditions depending on the following purposes of the experiments.

2.1.1 Plants for lignin isolation and the development of the lignin content determination methods

The *Arabidopsis* accessions used for lignin isolation and the development of the lignin content determination methods were chosen from the lines collected from different continents and grown either in the greenhouse or a growth chamber. The growth environment for Col-0 was controlled under 16-h-light/8-h-dark cycles at 21 °C, with fluorescent lamps at a light intensity of 100-150 $\mu mols^{-1}$ m^{-2} in a growth chamber. The accessions other than Col-0 were grown in separate batches in the greenhouse of the University of British Colombia. For each accession, around 500 individual plants were planted at the same time to yield enough biomass for the experiments.

2.1.2 Plants for examination of lignin content variation among accessions and their corresponding growth rates

The sixty-five accessions of *Arabidopsis* used to examine if natural variation of lignin content exists among accessions were grown together and randomly distributed in a 72-well culture plate. For each accession, there were six individual plants grown in different culture plates in a growth chamber controlled under 16-h-light/8-h-dark cycles at 21 °C, with fluorescent lamps at a light intensity of 100-150 $\mu mols^{-1} m^{-2}$ in a growth chamber to ensure a uniform environmental condition. When the first flower of the plants opened, the growth rates of the plants were measured according to the measurement method below.

All of the plants were harvested when they reached full maturity with all the siliques turning brown. Harvested plants were conditioned at 50% RH and 23 °C for 48 hours prior to grinding the inflorescence stems to make a pool of the each accession grown under the same conditions.

2.2 Grinding

The collected inflorescence stems of each *Arabidopsis* accession were ground to pass a 40-mesh screen using a Wiley mill or ground to 80-mesh using a micro ball mill depending on the extraction methods. The ground samples were dried in a vacuum oven at 40 °C for 48 h and conditioned in a vacuumed desiccator over P_2O_5 overnight.

2.3 Extractions

Ground and dried samples were extracted using the following extraction methods.

2.3.1 Four-stage Soxhlet extraction

The method of Fukushima and Hatfield (2001) was followed. Approximately 2.5 g of dried 40-mesh samples were sequentially extracted with water, 95% ethanol, chloroform and acetone using a Soxhlet apparatus. The rate of solvent refluxing through the samples was adjusted to allow for approximately 8 cycles/h. Each sample was extracted for 8 h for each solvent. Specified samples were also extracted for 24 h using each solvent for comparison of extraction efficiencies.

2.3.2 Single-stage Soxhlet extraction

Approximately 2.5 g of dried 40-mesh samples were extracted with 95% ethanol/benzene (1:2, v/v) for 24 h and 48 h for comparison of extraction efficiency. The rate of solvent refluxing through the samples was adjusted to allow for approximately 8 cycles/h.

2.3.3 Rapid extraction by washing

This procedure was based on Morrison's quick extraction method (1972) but with reductions in sample particle size and weight. A fine filtration membrane was also used. Approximately 0.1 g of dried 80-mesh samples 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 pre-weighed 0.45 μm nylon membrane using a Millipore filter. The residual was washed with 2 mL x 20 deionized water, 1mL x 20

ethanol, acetone, and diethyl ether respectively. The residual was then transferred with the nylon membrane to a pre-weighed 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 removed extractives in each sample was determined by the difference of 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.

2.4 Lignin isolation

Lignin was isolated from the extracted samples of *Arabidopsis* accessions using dioxane acidified with HCl by the method of Fukushima and Hatfield (2001) except that the refluxing of solutions under N_2 was for 60 minutes rather than 30 minutes. Briefly, 5 g of extracted and dry inflorescence stems of *Arabidopsis* accessions were put into a 250-mL round bottom flask. After adding 100 mL of acidic dioxane (90 mL dioxane + 10 mL 2 M HCl solution) to the flask, the solution was flushed with N_2 gas for 30 seconds and then refluxed under N_2 for 60 minutes. After cooling, the solution was filtered through a glass fiber filter and collected in an Erlenmeyer flask. The residue on the filter was washed with 20 mL of 96% dioxane and the wash was combined with the filtrate in the Erlenmeyer flask. The solution in the Erlenmeyer flask was neutralized with four grams of sodium bicarbonate and then filtered through a 0.45 μm nylon membrane using a Millipore filter. The filtered solution was concentrated to 10-15 mL in a 250-mL round

bottom flask using a rotary evaporator at 40 °C. The concentrated solution was added dropwise to 200 mL of rapidly stirring distilled water in four 50-mL centrifuge bottles. The residue in the round bottom flask was washed with 2.0 mL of 96% dioxane solution and also added dropwise to the stirring distilled water. While stirring, 2.0 g of anhydrous sodium sulfate was added to the centrifuge bottles to help to flocculate the lignin. The centrifuge bottles were then centrifuged to obtain lignin pellet by removing the supernatant. The lignin pellet in the centrifuge bottles was partially dried in forced air oven at 60 °C for 15 min. The lignin pellet was dissolved in 4-5 mL of dioxane. The dissolved lignin solution was filtered through a 0.45 μm nylon membrane and added dropwise to 200 mL of stirring anhydrous diethyl ether in four 50-mL centrifuge bottles. The lignin pellet formed in the bottles was obtained by centrifuging (9000*g*, 15min, 0 °C) and removing the supernatant. The step of dissolving the lignin pellet in dioxane and precipitating in ether was repeated once before the lignin pellet was stirred with 60 mL of petroleum ether using a spatula. The finally purified lignin was obtained by allowing the residue to settle and removing the supernatant. The purified isolated lignin was freeze-dried for 48 h and stored in a vacuum desiccator over P_2O_5.

2.5 Holocellulose and cellulose preparation

Holocellulose and cellulose were isolated from extracted samples of Col-0 inflorescence stems. The methods of Yokoyama et al. (2002) were followed except that the material used for the isolation of holocellulose and cellulose was *Arabidopsis* inflorescence stems rather than wood wafers.

2.5.1 Holocellulose isolation from Col-0 inflorescence stems

Approximately 100 mg of dried 40-mesh Col-0 inflorescence stems was put in a 10-mL round-bottom flask with a glass stopper. The flask was then heated in a water bath at 90 °C. To the heated flask, 0.5 mL of a solution of 200 mg of 80% sodium chlorite dissolved in 2 mL of deionized water and 0.2 mL of acetic acid were added to start the reaction. At 30-min intervals, 0.5 mL of the sodium chlorite solution was added to the reaction, for a total of 2 mL. After four additions of the sodium chlorite solution, the mixture was cooled in a cold water bath and filtered through a coarse sintered glass filter. The residue of filtration was thoroughly washed with 3 x 50 mL of deionized water before drying in an oven at 105 °C. The dry residue was weighed to calculate the yield of holocellulose.

2.5.2 Cellulose isolation from Col-0 inflorescence stems

Approximately 50 mg of the isolated holocellulose from Col-0 inflorescence stems was put in a 10-mL beaker and left at room temperature to allow moisture equilibration. After 30 min of equilibration, 4 mL of 17.5% sodium hydroxide was added to the beaker and left at room temperature for another 30 min. Four milliliters of deionized water was then added to the beaker. The mixture in the beaker was stirred for one minute, left at room temperature for an additional 29 min and then filtered through a coarse sintered glass filter. The residue of filtration was washed with 3 x 30 mL deionized water, then soaked in 1.0 M acetic acid solution for 5 min. The neutralized α-cellulose was then thoroughly washed with 3 x 30 mL deionized water again before being dried in an oven at 105 °C. The dry residue was weighed to calculate the yield of the cellulose.

2.6 Cellulase treatment of Col-0 inflorescence stems

The Col-0 inflorescence stem samples prepared by the four-stage extraction were used for cellulase treatment. One gram of the dried samples was incubated for 3 h in 50 mL of 50 mM acetic buffer (pH 4.8) containing Celluclast 1.5L (3.5 filter paper units) and Novozyme 188 (7 IU) at 45 °C. After hydrolysis, the residual was centrifuged, washed three times by re-suspension 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)

2.7 Lignin analysis by the acetyl bromide method

2.7.1 Conventional method

The method of Iiyama and Wallis (1990) 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 micro ball mill. The dried sample (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 of 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 at 280 nm,

which was limited to the range of 0.2 to 0.8 by adjusting the amount of assay samples, was read using a Varian Cary 50 spectrophotometer. A blank was included to correct for background absorbance by the reagents.

2.7.2 Modified method using cuvettes

Dried 80-mesh samples (5 ± 1 mg) extracted using the rapid extraction method 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 between 5 min and 120 min. During this period, 5.000 mL of acetic acid was added to the tubes and mixed with 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, ranged from 0.2 to 0.8, was measured against a blank as above.

2.7.3 Modified method using microplates

The digestion and cooling of the samples were the same as used in the modified method using cuvettes. The digested samples were removed from the ice bath and 5.000 ml of acetic acid were added. After mixing, 30 μl of the mixed content in triplicate were transferred to the wells of a 96-wells 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 pipette. The absorbance of the solutions in the wells at 280 nm,

ranged from 0.2 to 0.5, was measured by a Perkin Elmer Wallac 1420 microplate reader. A blank was included to correct for background absorbance by the reagents.

2.8 Lignin analysis by the 72% sulfuric acid method

2.8.1 The 72% sulfuric acid method without pepsin pretreatment

The modified Klason method of Huntley et al. (2003) was followed except that the samples for the analysis were extracted using the four-stage Soxhlet extraction rather than a two-stage extraction using ethanol-benzene and 95% ethanol. Briefly, 0.2 g of extracted and dried samples (40-mesh) was reacted with 3 mL of 72% H_2SO_4 at 20 °C for 2 h with mixing every 10 min. The mixture was then diluted with 112 mL of deionized water and autoclaved at 121 °C for 1 h in a serum bottle. The mixture was filtered through a medium coarseness sintered glass filter and the acid-insoluble lignin was determined gravimetrically. Acid-soluble lignin was determined by absorption spectroscopy at 205 nm with the Varian Cary 50 spectrophotometer using an extinction coefficient of 110 $g^{-1}Lcm^{-1}$ (Dence, 1992).

2.8.2 The 72% sulfuric acid method with pepsin pretreatment

The method adopted by the Association of Official Agriculture Chemists was followed except that the samples for the analysis were extracted using the four-stage Soxhlet extraction rather than a single-stage Soxhlet extraction with ethanol-benzene and washing with ethanol and ether (Dence, 1992). Approximately 0.8 g of dried and extracted samples (40-mesh) was put in a 250-mL Erlenmeyer flask, and 40 mL of 1% pepsin in 0.1 N hydrochloric acid was added. The mixture was incubated in a water bath at 40 °C

overnight. Thirty milliliters of deionized hot water was added and the mixture was filtered. After washing the residue with additional 30 mL of deionized hot water, the residue was transferred to a 250-mL round bottom flask with 5% sulfuric acid and the volume of the mixture was made up to 150 mL with the acid. The mixture was refluxed for 1 h and then filtered. The residual was washed with 3 x 30 mL of hot water, 2 x 20 mL of ethanol and 2 x 15 mL of ether. The residual was then transferred to a beaker by tapping and brushing. Twenty milliliters of 72% H_2SO_4 was added to the beaker and the mixture was kept at 20 °C for 2 h with occasional mixing. One hundred and twenty five milliliters of water were added to the beaker and residual was filtered and washed with 20 mL of hot water. The residual was transferred to a 250-mL round bottom flask and refluxed with 150 mL of 3% H_2SO_4 for 2 h. The mixture was filtered through a medium coarseness sintered glass filter and the acid-insoluble lignin was determined gravimetrically. Acid-soluble lignin was determined by absorption spectroscopy at 205 nm with the Varian Cary 50 spectrophotometer using an extinction coefficient of 110 $g^{-1}Lcm^{-1}$.

2.9 Neutral sugars and uronosyls analysis

Neutral sugars and uronosyls in *Arabidopsis* inflorescence stem samples and isolated lignins were determined using the hydrolysates from the 72% sulfuric acid method (without pepsin treatment). For the determination in isolated lignins, because of the small sample sizes and low concentration of carbohydrates, the two step hydrolyses were carried out in a GC vial with a Teflon lined cap using a tenth amount of samples and a hundredth of reagents.

2.9.1 Neutral sugars analysis

Neutral sugar concentration in the hydrolysates were determined by high-performance liquid chromatography (HPLC) (Dionex DX-500, Dionex, CA) equipped with an ion exchange PA1 column, a pulsed amperometric detector with a gold electrode, and an AS50 auto-injector. The hydrolysates were filtered through 0.45 mm 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 de-ionized water at a flow rate of 1.0 mL min^{-1}, followed by post-column addition of 200 mM NaOH at a flow rate of 0.5 mL min^{-1}. Each sample was determined in duplicate.

2.9.2 Uronosyls analysis

Uronosyls concentrations in the hydrolysates were determinated using the phenyl phenol method (Blumenkr & Asboehan, 1973). Hydrolysates (0.2 mL) containing 0.5 to 20 μg of uronic acids was transferred to a tube. One point two milliliters of 0.0125 M solution of tetraborate in concentrated sulfuric acid was added to the tube and kept in an ice bath. The tube was then shaken with a Vortex mixer and put in a water bath at 100 °C for 5 min. The mixture was cooled in the ice bath again and 20 μL of 0.15% solution of meta-hydroxydiphenyl in 0.5% NaOH was added. The tube was shaken and the absorbance at 520 nm of the solution was measured with the Varian Cary 50 spectrophotometer within 2 min. A blank was incorporated each sample to account for the absorbance caused by carbohydrates and sulfuric acid/tetraborate. The blank sample was added with 20 μL 0.5% NaOH instead of the meta-hydroxydiphenyl solution. The absorbance of the blank

was subtracted from the total absorbance of the sample. Glucuronic acid was used as a standard for calibration in the determination of uronic acids.

2.10 Crude protein analysis

Crude protein was estimated as N% x 6.25. Percent of nitrogen in the samples was determined using Perkin Elmer Series II CHNS/O 2400 analyzer (Norwalk, CT, USA) according to the manufacturer's instructions.

2.11 Ash determination

2.11.1 Total ash content

The ash determination was performed according to a TAPPI test method T 211 cm-86. Briefly, around 5 g of dried samples weighed to the nearest 0.1 mg was put in a pre-heated and pre-weighed crucible then transferred to a muffle furnace at a temperature less than 100 °C. The temperature of the furnace was gradually raised to 575 ± 25 °C and held for around 3 h until all the black particles in the crucible disappeared. The crucible with the sample was then cooled in a dessicator and weighed to nearest 0.1 mg to determine the total ash content in samples.

2.11.2 Insoluble ash content

The insoluble ash content determination was performed according to TAPPI test method T 244 om-88 except with that the ash samples were solubilzed in a 50-mL beaker instead of a platinum dish. Ash residue from the total ash determination was transferred to a 50-

mL beaker by brushing. The first portion and second portion of 5 mL 6 M HCl were added to the beaker and evaporated slowly to dryness sequentially. After the third portion of 6 M HCl had been added, the sample was heated and diluted with 20 mL of water. The diluted sample was filtered through an ash-free filter paper and thoroughly washed with deionized water. The sample with the filter paper was put in a pre-heated and pre-weighed crucible and transferred to a muffle furnace as the method for the determination of total ash content.

2.12 Determination of extinction coefficients of isolated lignins

Ten milligrams of isolated lignin (after correction for contamination from carbohydrates and proteins) was 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 (Fukushima & Hatfield, 2001). The absorbance values of the dried aliquots of lignin were measured by the modified acetyl bromide method using cuvettes as above. The extinction coefficient of an 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.

2.13 Monolignol quantification

The monolignol ratio of isolated lignin was determined by thioacidolysis (1992) 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 x 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 MS detector, electron ionization potential was 70 eV with a source at 100 °C, and ions were scanned across the range of 50-450 mu (mass unit).

2.14 Growth rates measurement

The growth rates of the plants in the growth chamber were measured by marking two points one centimeter and two centimeters down from the top of the stem respectively when the first flower of the plants opened. The diameters of the stems at the lower of the marks were also measured with a digital caliper when the two points were marked. After five days, the distance from the top of the plants to the first mark, the distance from the first mark to the second mark and the diameters of the stem at the lower mark were measured with the digital caliper and the average daily growth rates of the corresponding sections over five days were calculated respectively. The growth rates of each *Arabidopsis* accession were the average values measured on six individual plants.

2.15 Statistical analysis

Analysis of variance (ANOVA) was conducted to compare the absorbance value of samples with different treatments or from different groups using the SAS software package (SAS institute Inc., Cary, NC). Canonical correlation analysis was conducted to determine the correlation between growth rates and lignin content of plants among *Arabidopsis* accessions 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.

3 RESULTS AND DISCUSSION

3.1 Modification of the acetyl bromide method

In order to use the natural variation of lignin content in *Arabidopsis* accessions to identify the genetic loci controlling lignin content, a rapid micro-scale method of lignin determination is required. This necessitated several modifications to the standard acetyl bromide method of lignin analysis. The grinding of the samples was scaled down using a micro ball mill; the extraction process was scaled down and sped up by making changes to Morrison's rapid extraction method (Morrison, 1972); the volumes of solutions used in the conventional acetyl bromide method were scaled down; and an ice bath was used to stabilize the digested samples treated by acetyl bromide solution.

3.1.1 Grinding *Arabidopsis* samples with a micro ball 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 ^{o}C for 30 min (Johnson et al., 1961). Although the digestion of coarsely ground samples can be promoted by adding perchloric acid to the digestion solution (Iiyama & Wallis, 1988), later work has shown that that perchloric acid might cause interference with the lignin content determination due to the degradation of hemicelluloses to products that absorb at 280 nm (Hatfield et al., 1999). 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 grams as will be encountered in lignin content determination in *Arabidopsis*. A possible alternative, better suited to small samples, could be grinding with a micro ball mill.

Using a micro ball mill, 0.1 g of *Arabidopsis* stem sample could be ground to 80-mesh in two minutes. The digestion process of a ground sample of Col-0 in 25% acetyl bromide solution at 70 °C indicated that the sample was dissolved in approximately 10 min with the specific absorbance (absorbance per unit of light path length for 1 g of assay sample in 1 L of solution, $g^{-1}Lcm^{-1}$) rising rapidly during this period (Figure 12). At 30 min, the rate of change in specific absorbance was small, which was consistent with the results of Morrison (1972). Sediment was found after centrifuging the digested solution. A similar sediment was also found in grass samples processed by Morrison, who ascertained that it consisted almost entirely of proteins and thus did not cause errors in the lignin content determination (Morrison, 1972).

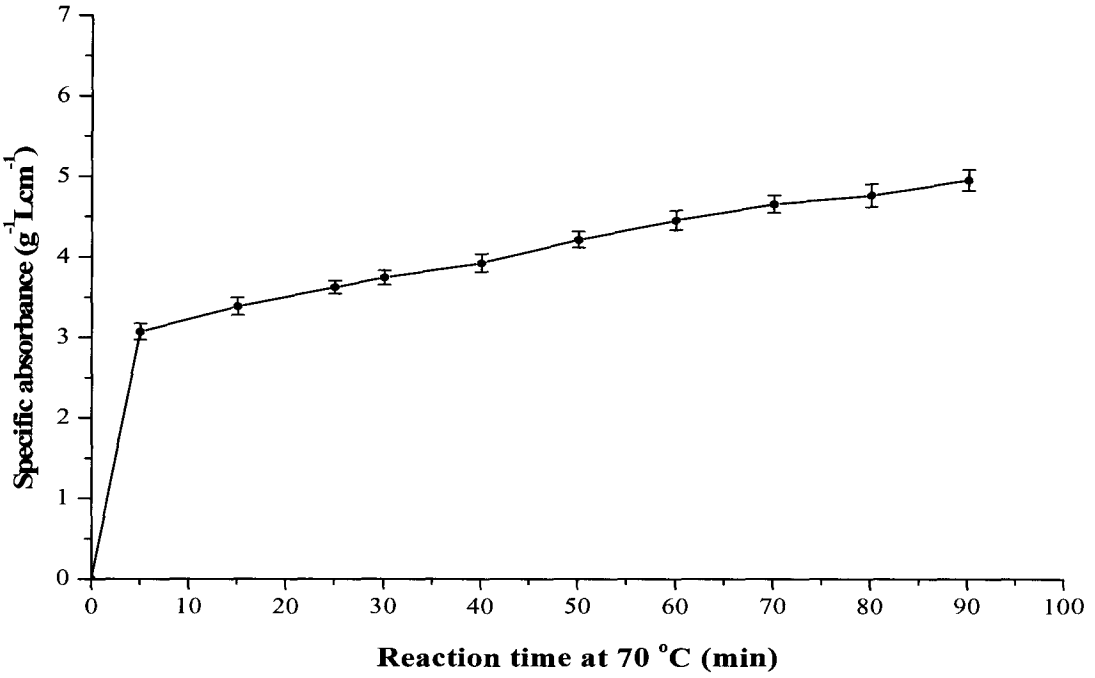

Figure 12: Specific absorbance changes of the ground sample of Col-0 with the digestion time at 70 °C. Values were an average of three measurements and were based on the weight of original, oven-dried sample.

3.1.2 Adaptation of the rapid extraction method

Extractives interfere with lignin determination by the acetyl bromide method (Morrison, 1972). 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 (1972), 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 may be 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 hours 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), normally used for extraction of woody samples, were compared (Table 2).

Table 2: Comparison of Extractive Content Determined by Different Extraction Methods Using Inflorescence Stems of Col-0 (Results Expressed as a Percentage of the Weight of Original, Oven-Dried Sample).

	Extractives using rapid extraction by washing	Extractives by four-stage Soxhlet extraction		Extractives by single-stage Soxhlet extraction	
Solvent		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

The results in Table 2 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 fine and small amounts of samples for the

extraction. The 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 amongst the different extraction methods can be deduced from the changes in the chemical macrocomponent before and after extraction. Table 3 shows that the four-stage Soxhlet extraction removed approximately 88.5% of total ash, 13.4% of total carbohydrates and 48.7% of proteins from the original sample while the single-stage Soxhlet extraction method only removed around 10% of ash content, 8% 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 were used sequentially for extraction. Analysis of holocellulose and cellulose content in the original, single-stage Soxhlet extracted and four-stage Soxhlet extracted samples confirmed the removal of hemicellulose during solvent extraction (Table 4). Compared with the original sample, hemicellulose content in the four-stage Soxhlet extracted samples decreased 18% while that in the single-stage Soxhlet extracted sample decreased 10%. Cellulose content in the three different samples was similar, consistent with its high crystallinity and low solubility.

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 (1972).

Table 3: Components of Col-0 Inflorescence Stems as Determined by the Analysis of the Samples with No Extraction, Single-stage Soxhlet Extraction and Four-stage Soxhlet Extraction (Results Expressed as a Percentage of the Weight of Original, Oven-dried Sample).

Component	Original	Single-stage Soxhlet extracted [a]	Four-stage Soxhlet extracted [b]
Total glycans [c]	55.77 ± 1.14	51.73 ± 0.74	48.64 ± 0.23
Fucan	0.18	0.16	0.12
Arabinan	1.36	1.23	0.78
Rhamnan	1.39	0.93	0.65
Galactan	1.83	1.65	1.18
Glucan	35.84	33.44	31.98
Xylan	13.24	12.44	12.28
Mannan	1.92	1.87	1.65
Uronic acids [c]	5.68 ± 0.18	4.82 ± 0.19	4.58 ± 0.21
Ash	13.35 ± 0.12	10.94 ± 0.11	1.54 ± 0.03
Proteins	6.88 ± 0.06	5.40 ± 0.11	3.53 ± 0.05
Klason lignin [d]	17.51 ± 0.16	15.03 ± 0.19	14.35 ± 0.12
Acid-soluble lignin	2.96 ± 0.12	1.57 ± 0.02	1.29 ± 0.06
Extractives	NA	6.12 ± 0.28	21.52 ± 0.56
Total	102.15	95. 60	95.45

[a] The sample was a combination of preparations of 24 h and 48 h extractions. [b] The sample was a combination of preparations of 8 h and 24 h extractions each stage. [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 with correction of crude proteins in the Klason lignin residue.

Table 4: Determination of Cellulose and Hemicellulose in the Samples of Col-0 Inflorescence Stems with No Extraction, Four-stage Soxhlet Extraction, and Single-stage Soxhlet Extraction (Results Expressed as a Percentage of the Weight of Original, Oven-dried Sample).

Component	Original	Single-stage Soxhlet extracted [a]	Four-stage Soxhlet extracted [b]
Cellulose	33.34 ± 0.63	32.42 ± 0.93	31.97 ± 0.80
Hemicellulose	34.87 ± 1.23	31.21 ± 2.13	28.63 ± 1.71
Holocellulose	68.21 ± 0.60	63.63 ± 1.20	60.60 ± 0.91

[a] The sample was a combination of preparations of 24 h and 48 h extractions. [b] The sample was a combination of preparations of 8 h and 24 h extractions each stage

Table 5 shows that the rapid extraction removed around 1.5% more carbohydrates, 0.5% more ash content and 0.1% more protein 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 various extractives slightly more efficiently than the four-stage Soxhlet extraction.

Table 5: Components of Col-0 Inflorescence Stems as Determined by the Analysis of the Samples with Four-stage Soxhlet Extraction and Rapid Extraction by Washing (Results Expressed as a Percentage of the Weight of Original, Oven-dried Sample).

Component	Four-stage Soxhlet extracted [a]	Rapid extraction by washing
Total glycans [b]	48.64 ± 0.23	47.21 ± 0.07
Fucan	0.12	0.12
Arabinan	0.78	0.9
Rhamnan	0.65	0.7
Galactan	1.18	1.22
Glucan	31.98	30.63
Xylan	12.28	11.96
Mannan	1.65	1.68
Uronic acids [b]	4.58 ± 0.21	4.53 ± 0.18
Ash	1.54 ± 0.03	1.05 ± 0.03
Proteins	3.53 ± 0.05	3.52 ± 0.14
Klason lignin [c]	14.35 ± 0.12	14.20 ± 0.14
Acid-soluble lignin	1.29 ± 0.06	1.24 ± 0.06
Extractives	21.52 ± 0.56	23.84 ± 0.70
Total	95.45	95.59

[a] The ample was a combination of preparations of 8 h and 24 h extractions each stage. [b] The contents of polysaccharides were converted from pentose and hexoses/uronic acids by multiplying the factors of 0.88 and 0.90 respectively. [c] Values with correction of crude proteins in the Klason lignin residue.

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 (Tables 3 and 5). The values for acid soluble lignin in the extracted samples were 0.8-1.7% lower than those in the original sample (Tables 3 and 5). 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 3). The differences in the results may be due to the interferences present in original non-extracted 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 lignin removal is unlikely. Thus, the different lignin content is most probably due to interferences from extractives in the original sample. The similarity in 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 truer lignin content measurement by sulfuric acid method than obtained through single-stage Soxhlet extraction and non-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 spectroscopic methods. Figure 13 shows that, in the range of 250 nm to 700 nm, the samples after four-stage Soxhlet extraction had lower specific absorbance values than the samples with no solvent extraction or 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 amongst the differently extracted samples mainly occurred at the ranges of 350-400 nm and 260-290 nm. The higher specific absorbance values of samples with no solvent extraction and single-stage Soxhlet

extraction at 350-400 nm is probably caused by the presence of pigments such as chlorophyll and carotenoids that were not removed during the extraction process. The higher specific absorbance values at 350-400 nm caused by such pigments likely extend to the 280 nm region used for lignin determination. The higher specific absorbance values of samples with no solvent extraction and single-stage Soxhlet extraction at 260-290 nm may be caused by the interference of small molecular weight phenolic compounds such as flavonoids. The lower specific absorbance values of the samples with four-stage Soxhlet extraction at 260-400 nm indicate a minimum interference from 'pseudolignin' in the sample. In fact, the spectra of sample treated by four-stage Soxhlet extraction had a similar shape to the spectrum of lignin isolated from *Arabidopsis* Col-0 after acetyl bromide treatment (Figure 14).

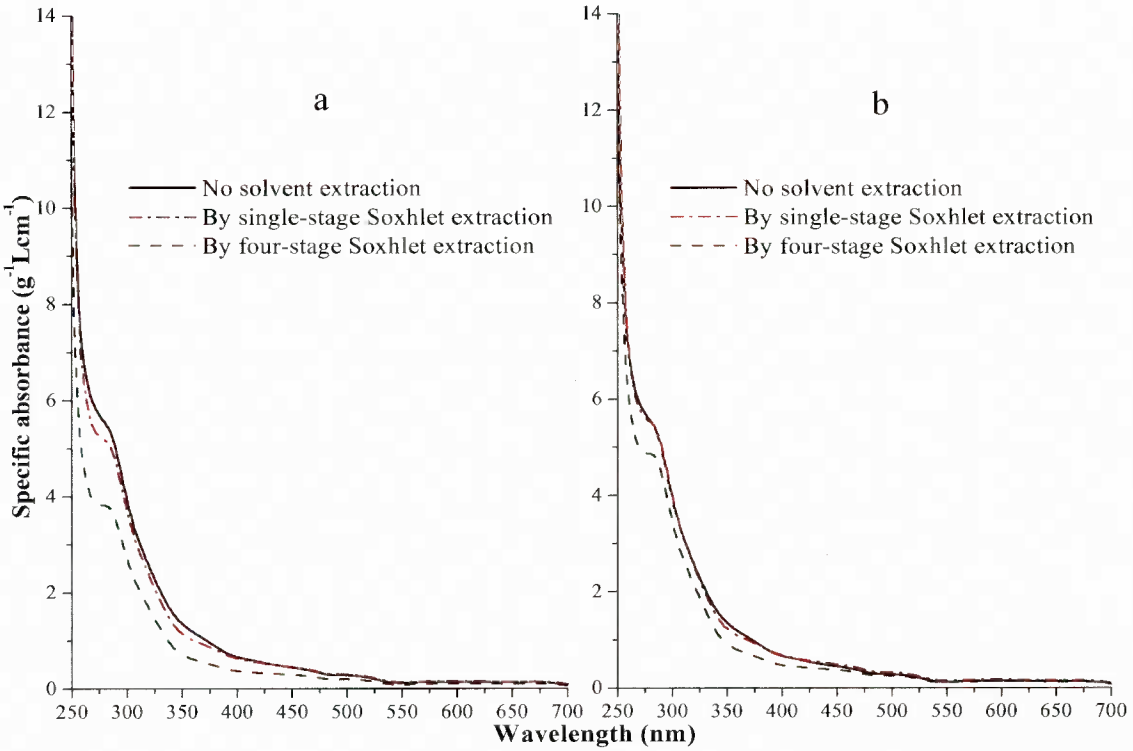

Figure 13: Spectra of the original, single-stage Soxhlet extracted and four-stage Soxhlet extracted samples of Col-0 inflorescence stems treated by acetyl bromide solution. Values were an average of three measurements. (a) Values based on the weight of original, oven-dried sample. (b) Values based on the weight of extracted, oven-dried sample.

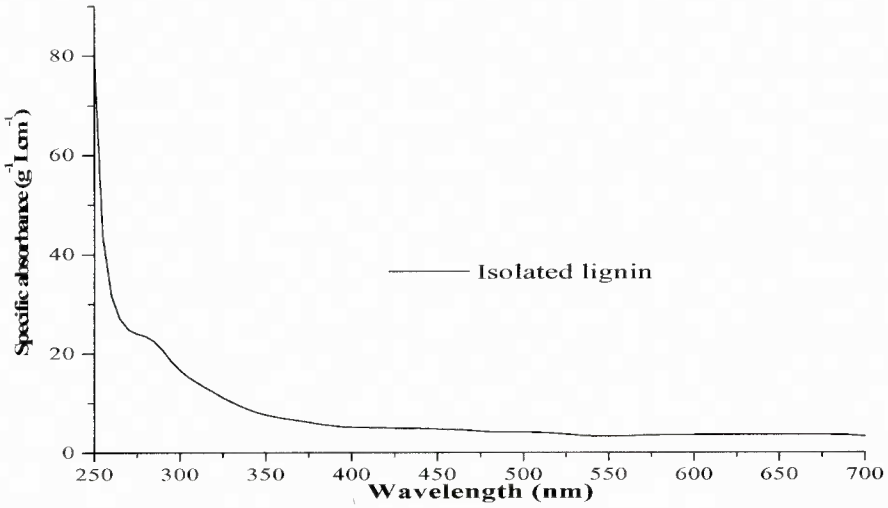

Figure 14: Spectrum of isolated lignin from Col-0 inflorescence stems treated by acetyl bromide solution. Values were an average of three measurements and were based on the weight of lignin with correction for impurities.

Figure 15 demonstrates that the rapid extraction had similar effects to the four-stage Soxhlet extraction on the specific absorbance values, in the 250 nm to 700 nm range, of samples in acetyl bromide solution.

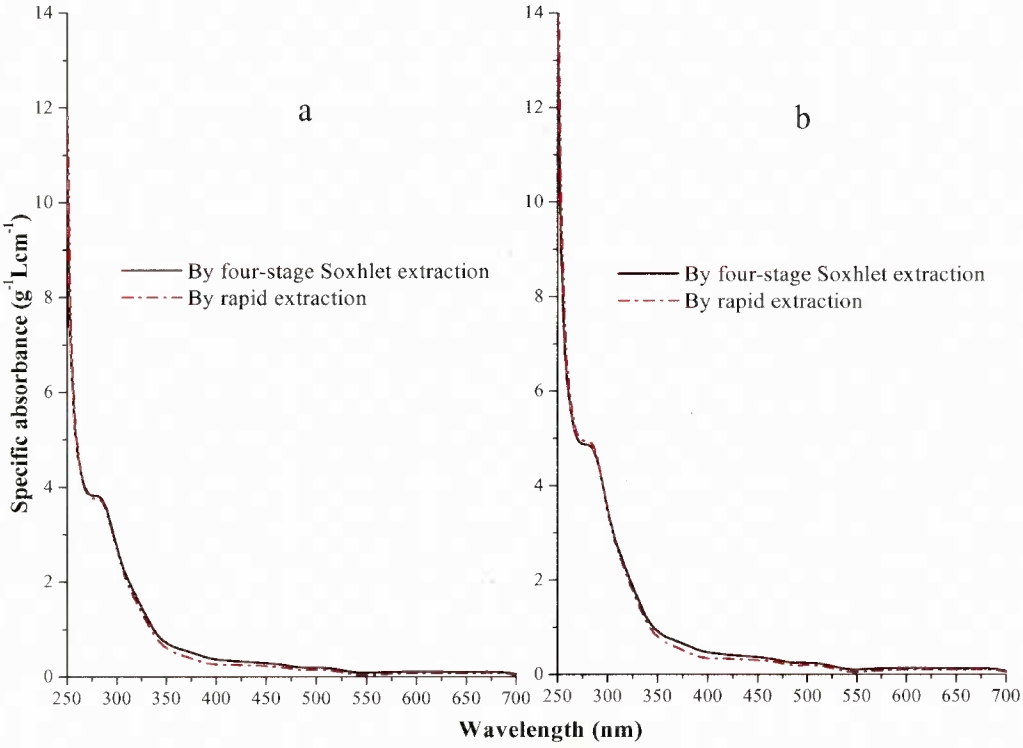

Figure 15: Spectra of the four-stage Soxhlet extracted and rapidly extracted samples of Col-0 inflorescence stems treated by acetyl bromide solution. Values were an average of three measurements. (a) Values based on the weight of original, oven-dried sample. (b) Values based on the weight of extracted, oven-dried sample.

The specific absorbance values at 280 nm of the samples pretreated by different extractions methods are compared in Figure 16. The specific absorbance of sample pretreated by the four-stage Soxhlet extraction was significantly lower than that of samples with no solvent extraction or a single-stage Soxhlet extraction ($p < 0.0001$) but it was not significantly different from that of sample treated by the rapid extraction process

(P = 0.211) . For the same reason as the determination of lignin content using the sulfuric acid method, the lower measured values of specific absorbance at 280 nm of the samples extracted by the four-stage Soxhlet extraction and the rapid extraction are more representative of the true absorbance value of lignin in the samples.

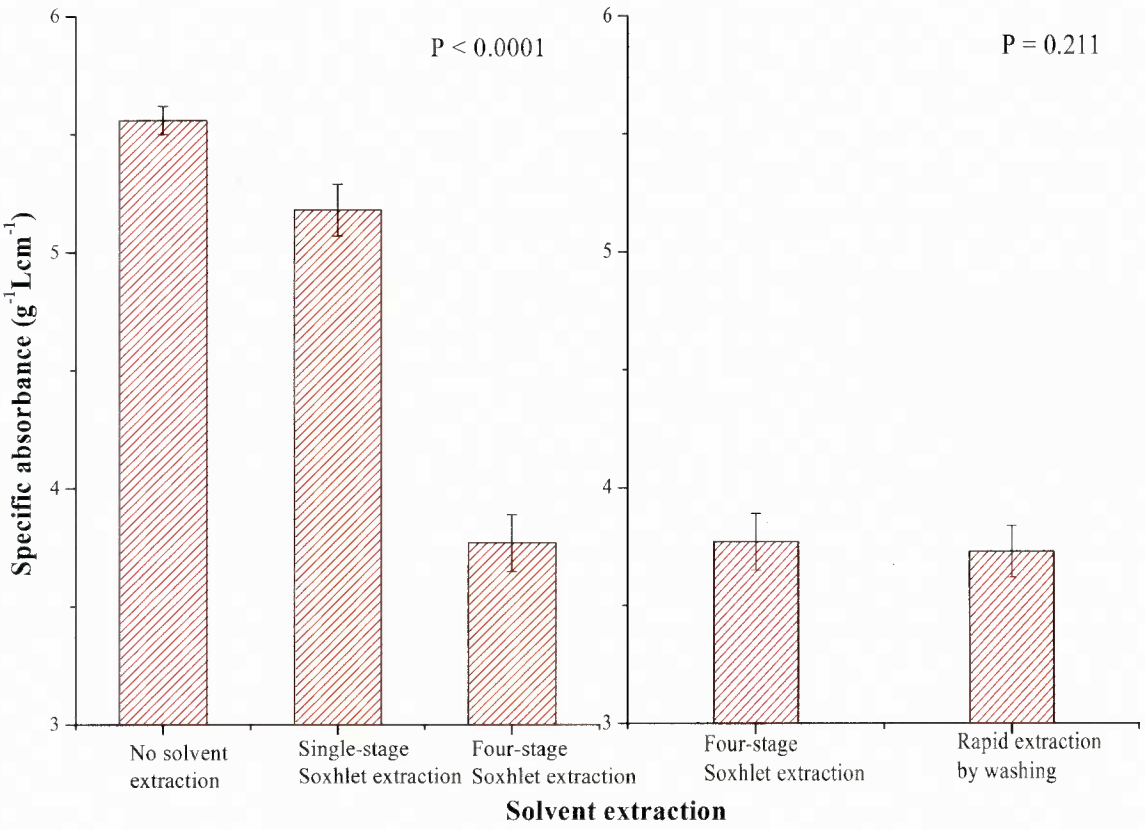

Figure 16: The effect of solvent extractions on the absorption values of inflorescence stems of Col-0 at 280 nm using the acetyl bromide method (values were an average of three measurements and were based on the weight of original, oven-dried sample).

Extraction of samples of other accessions of *Arabidopsis* grown under various conditions also shows that the rapid extraction has similar extraction efficiency to the four-stage Soxhlet extraction as can be seen from the good correspondence between the amounts of

extractives derived by the two methods (Table 6). Therefore, the rapid extraction can remove the 'pseudolignin' which interferes lignin content determination using acetyl bromide method as efficiently as can the four-stage Soxhlet extraction.

Table 6: Extractive Content from Inflorescence Stems of Different *Arabidopsis* Accessions Determined by Four-stage Soxhlet Extraction and Rapid Extraction by Washing (Results Expressed as a Percentage of Weight of Oven-dried Sample).

Accession	Extractive content	
	Four-stage Soxhlet extraction	Rapid extraction by washing
Col-0	21.52 ± 0.49	22.84 ± 0.70
Be	32.52 ± 0.65	33.80 ± 0.57
Ct-1	29.40 ± 0.58	31.14 ± 0.31
Hs-0	21.75 ± 0.44	23.19 ± 0.23
Kendalville	33.12 ± 0.76	33.30 ± 0.53
Lz-0	30.70 ± 0.31	29.96 ± 0.20
No	28.00 ± 0.45	28.28 ± 0.80
Sakhdara	29.00 ± 0.54	29.10 ± 0.40
Wt-5	21.55 ± 0.33	23.08 ± 0.28
Zdr-6	24.14 ± 0.36	25.79 ± 0.30
Mixed [a]	24.73 ± 0.21	25.06 ± 0.15

[a] A mixture of Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

3.1.3 Scaling down the volume of solutions

Scaling down the volume of the digested solution will accelerate the process of sample treatment through the use of micro-pipettes or multi-channel pipettes for solution transfer. In the conventional acetyl bromide method defined by Iiyama and Wallis (1990), the final sample volume is 50 mL. In the work reported here, the test 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.

Determining right dosages of reagents to be added to the digested acetyl bromide solution is an important step in scaling down the conventional method. The blank acetyl bromide digestion solution has strong absorption at 280 nm because of the presence of polybromide anion Br_x^- (Iiyama & Wallis, 1988; Johnson et al., 1961). Addition of sodium hydroxide and hydroxylamine can eliminate the absorbance contributed by polybromide anion Br_x^-, so that absorbance at 280 nm can be used to determine lignin concentration. The elimination of the effects of polybromide anion Br_x^- is shown from the spectral changes in the blank digestion solution before and after adding sodium hydroxide and hydroxylamine (Figure 17).

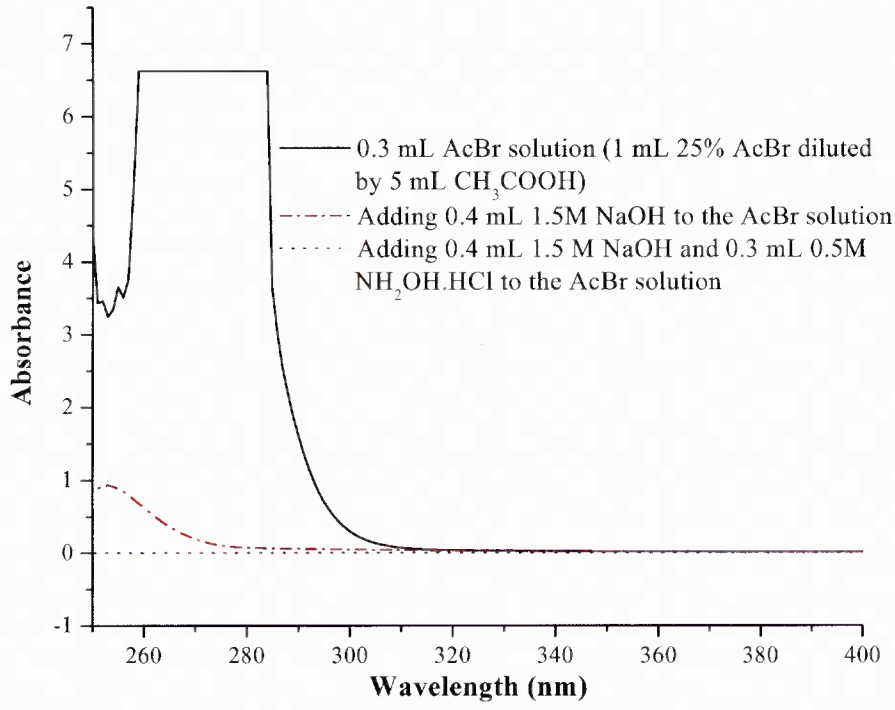

Figure 17: The UV absorbance change in the blank on addition of NaOH and $NH_2OH{\cdot}HCl$ by the modified method (measurement against CH_3COOH).

After obtaining the right proportions of reagents that needed to be added to the acetyl bromide solution, the method was scaled down to 2.5 mL and further scaled down to 0.25

mL using a cuvette and a microplate for UV absorbance measurement respectively. 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 7). Paired t-test of the results shows that the specific absorbance values obtained using the conventional method are not significantly different from those by the modified method using cuvettes and modified method using microplates (Table 8). Thus, the scaling down did not affect the accuracy and precision of the acetyl bromide method.

Table 7: Comparison of Lignin Content Determinations by the Conventional and Modified Acetyl Bromide Methods Using Inflorescence Stems of Various *Arabidopsis* Accessions (Values Based on the Weight of the Original, Oven-dried Sample).

Accession	Specific absorbance at 280 nm ($g^{-1}Lcm^{-1}$)		
	Conventional method [a]	Modified method using cuvettes [b]	Modified method using microplates [b]
Col-0	3.73 ± 0.11	3.75 ± 0.08	3.72 ± 0.13
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
Lz-0	3.28 ± 0.02	3.26 ± 0.04	3.20 ± 0.09
No	3.19 ± 0.05	3.22 ± 0.09	3.15 ± 0.13
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 Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

Table 8: Significance Test of the Effect of Scaling-down the Volume of Solutions on the Lignin Content Determination by the Acetyl Bromide Method.

Pair test	Degree of freedom	Mean of difference	Test statistics (t)	P value
Conventional method versus modified method using cuvettes	6	0.03	1.302	0.258
Conventional method versus modified method using microplates	6	- 0.02	- 0.995	0.385

3.1.4 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 may be possible to do this by cooling the digested samples in an ice bath (Johnson et al., 1961; Rodrigues et al., 1999). Samples of Col-0 stem 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 specific absorbance of the samples did not change with time (R^2 = 0.314, P = 0.321) during two hours in an ice-bath (Figure 18). In contrast, the digested solution at room temperature showed a trend of decreasing specific absorbance with time (R^2 = 0.967, P = 0.00161) at a rate of 1.58% over 20 min (Figure 19). 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.

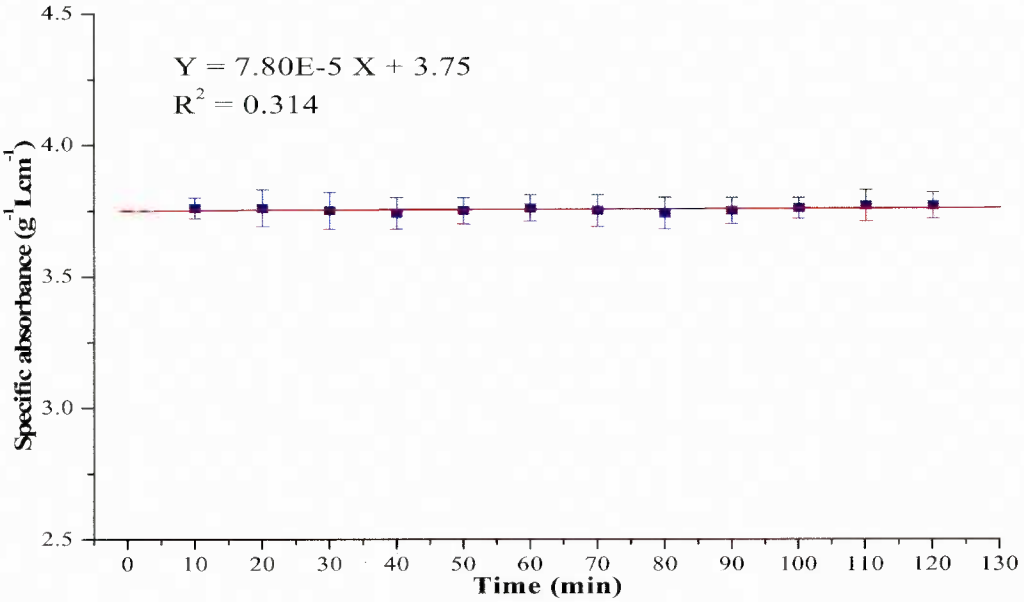

Figure 18: The effect of cooling solution in milled ice on the optical stability at 280 nm of solutions using Col-0 inflorescence stems by the acetyl bromide method.

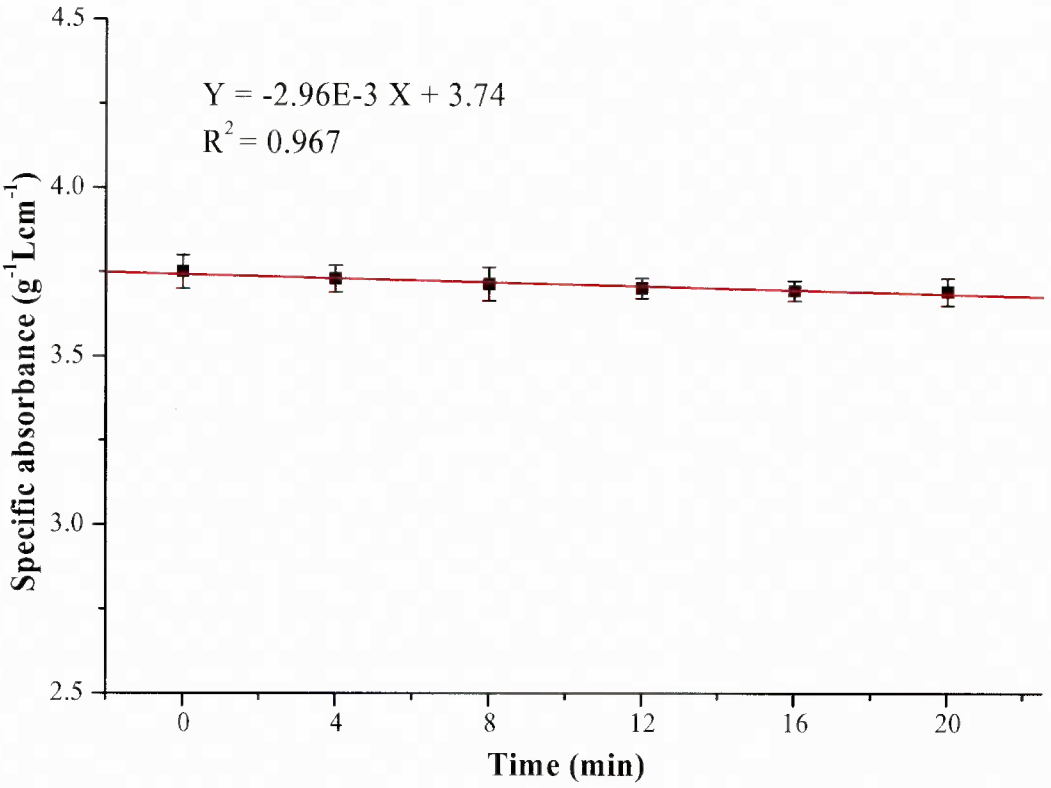

Figure 19: The optical stability of a lignin solution digested by acetyl bromide at room temperature, 280 nm and before adding NaOH.

Furthermore, the stability of solutions at room temperature increased after the solutions were finally diluted with acetic acid, as was discussed by Johnson et al. (1961). The specific absorbance of solutions after final dilution decreased with time ($R^2 = 0.930$, $P < 0.0001$) at less than 0.6% in 20 min (Figure 20). The results are similar to those of Rodrigues et al. (1999) who showed that the absorbance of the diluted acetyl bromide solution decreased at a low rate of 0.008 in 20 min.

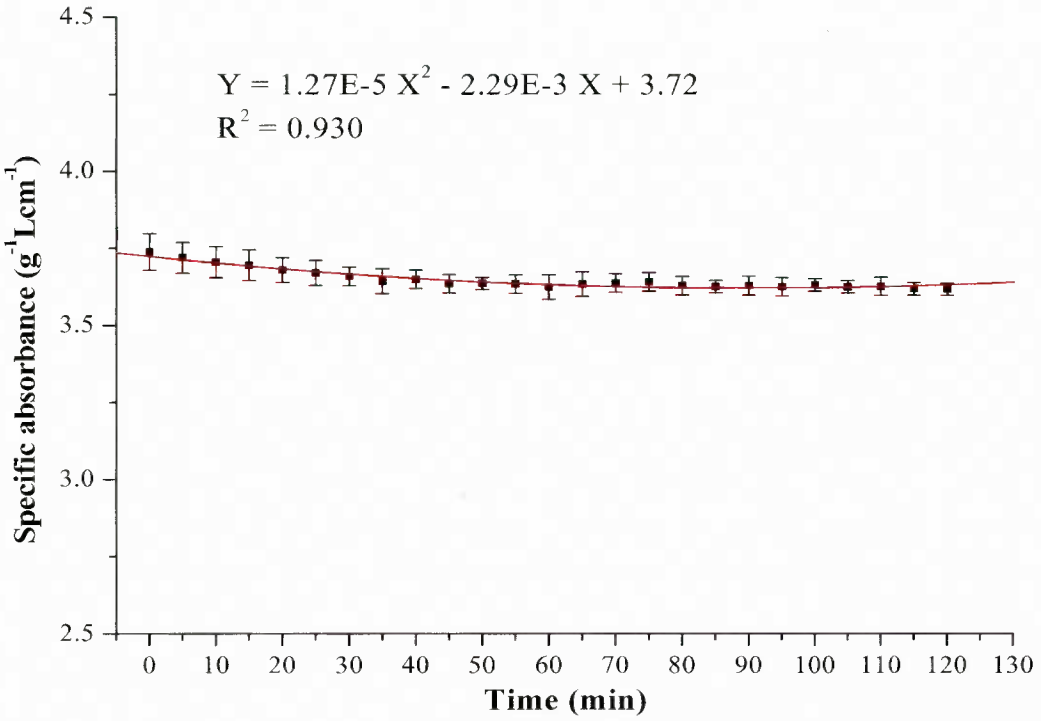

Figure 20: The optical stability of a lignin solution digested by acetyl bromide at room temperature, 280 nm and after final dilution with acetic acid.

In the modified method using cuvettes, there is no time lapse during transfer of the solution from ice bath and the UV absorbance measurement during high throughput determinations of lignin content. With the modified method using microplates, the time elapse during transfer of the solution from ice bath and final dilution of digested samples can be limited to five minutes. The time elapse during final dilution of digested samples and UV absorbance measurement is less than two minutes. The absorbance measurement error caused by total time elapse would be not more than 0.5%.

3.1.5 Validation of the modified methods

In the process of changing the extraction method and scaling-down the acetyl bromide method, the test results have shown no significant difference between the conventional

method and the adapted method for either of the modifications. To test if the 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 the methods with different combination of two extraction methods (four-stage Soxhlet extraction and rapid extraction) and three scales of acetyl bromide method (conventional scale, 2.5 mL scale using cuvettes and 0.25 mL scale using microplates (Figure 21). The results showed that the measured values were not significantly different (P = 0.291). Thus, the modification of extraction method and scaling down of the acetyl bromide method did not change the accuracy of the conventional method.

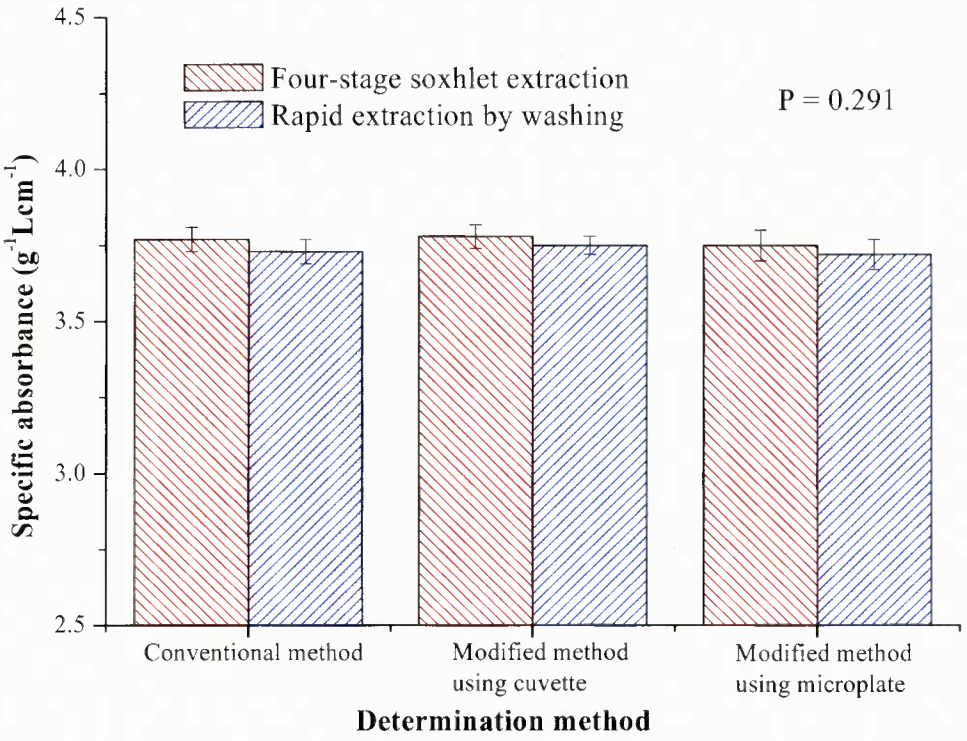

Figure 21: Comparison of measured specific absorbance values using different extractions and scales of the acetyl bromide method. Values were an average of three measurements.

To further confirm the accuracy and precision of the modified methods, individual plants of Col-0 grown at the same condition (16 h light, 100-150 $\mu mols^{-1} m^{-2}$, 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 the both of the modified acetyl bromide methods. The test results demonstrated that the measured values were not significantly different both by the modified method using either cuvettes ($P = 0.238$) or microplates ($P = 0.128$) (Figure 22). The relative standard deviation of the measurements by the modified method using cuvettes was approximately 2.8% while for the modified method using microplates the 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 cells 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 of samples is being handled, as was discussed above. However, the precision of the both modified methods is sufficient for the screening program of lignin content in *Arabidopsis* accessions. In conclusion, the modified methods using cuvettes and microplates can identify samples with the same lignin concentration accurately and precisely.

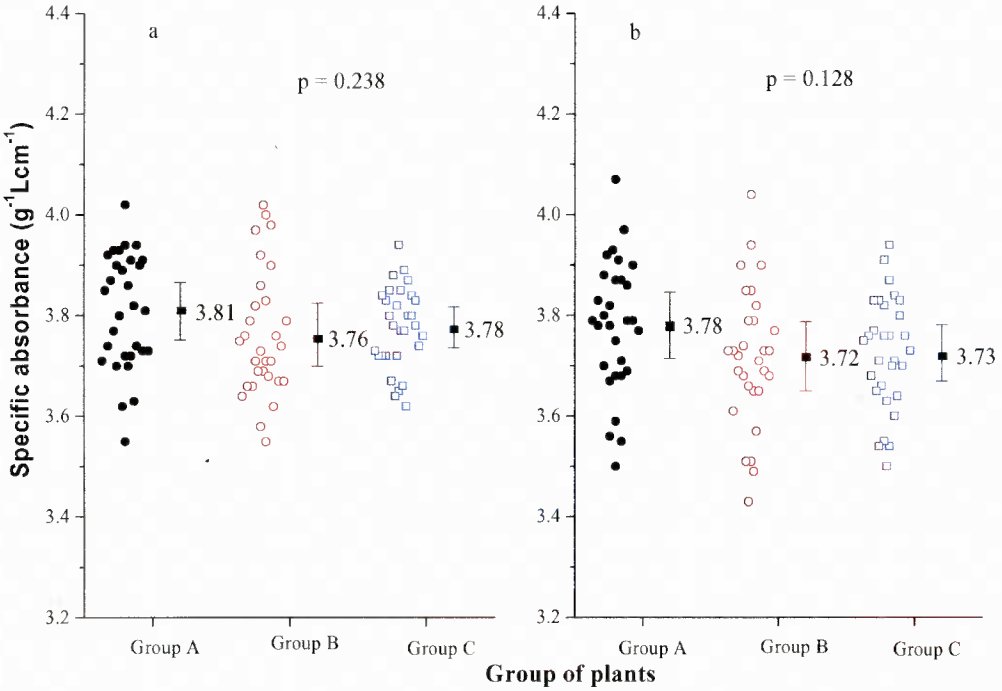

Figure 22: Accuracy and precision test of the modified acetyl bromide methods using the inflorescence stems of three groups of Col-0 grown under the same condition (16 h light, 100-150 µmols^{-1} m^{-2}, 21°C). (a) By the modified method using cuvettes. (b) By the modified method using microplates. (Values based on the weight of original, oven-dried samples).

The modified 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 modified 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 of the sample was mixed with holocellulose isolated from the same sample to decrease lignin concentration. The specific absorbance values of the holocellulose-added, extracted-only, and cellullase-treated samples were determined respectively by the modified acetyl bromide method using cuvettes and microplates. Figure 23 shows that the measured specific absorbance values of the three differently

treated samples were significantly different by the modified method using cuvettes ($P < 0.0001$) and using microplates ($P < 0.0001$). The relative standard deviation of the measurement by the modified method using cuvettes was approximately 2.2% while that by the modified method using microplates was 3.3%.

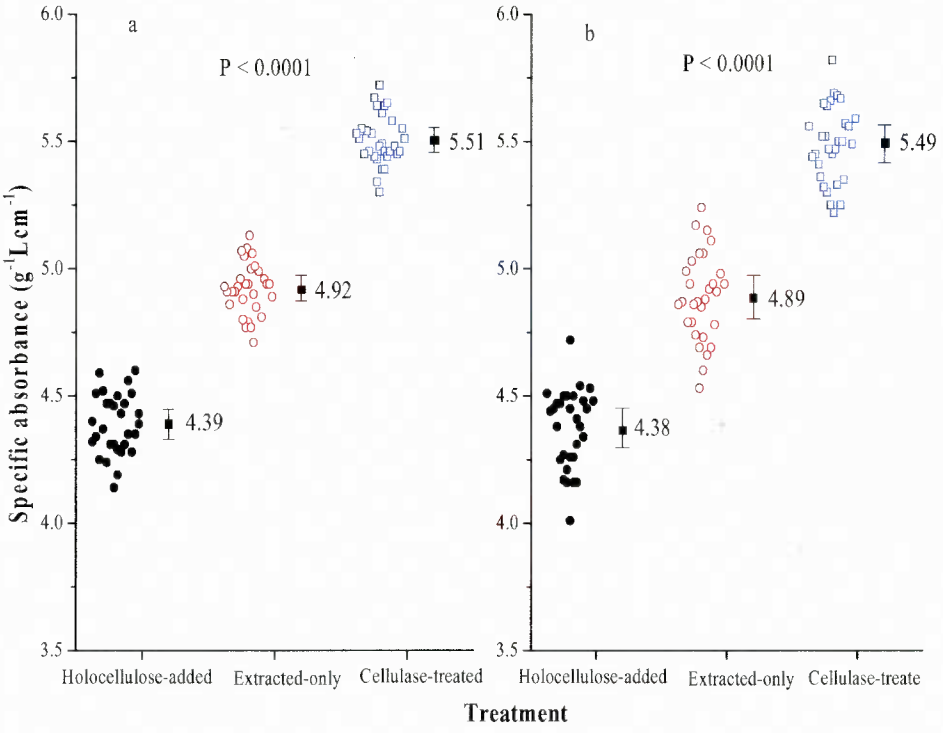

Figure 23: Accuracy and precision test of the modified acetyl bromide methods using extracted-only, holocellulose added and cellulase-treated samples of inflorescence stems of Col-0. (a) By the modified method using cuvettes. (b) By the modified method using microplates. (Values based on the weight of treated, oven-dried samples).

3.2 Extinction coefficient determination of the isolated lignins from different accessions of *Arabidopsis*

The modified acetyl bromide method was shown to be accurate and precise, with no significant differences in the determined values of lignin content compared to the conventional method. The modifications of the acetyl bromide method enable small samples to be easily and efficiently handled. All this has been proven using one accession, Col-0. However, to use the modified acetyl bromide method for the determination of lignin content of different of *Arabidopsis* accessions requires there to be a single common extinction coefficient for the lignin amongst all the accessions.

3.2.1 Isolation of lignins from *Arabidopsis* accessions

A set of *Arabidopsis* accessions were grown under various growing conditions and the lignins isolated using the procedure proposed by Fukushima and Hatfield who developed lignin extinction coefficients for alfalfa and bromegrass (Fukushima & Hatfield, 2001). The set of *Arabidopsis* accessions were chosen according to observed phenotypic variation and genetic distance. For example, Sakhdara is a Central-Asian accession, which is genetically distant from European accessions (Loudet et al., 2002), and has medium size of rosettes and leaves. On the other hand, 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. Table 9 shows the *Arabidopsis* accessions used for lignin isolation and their yields of the isolation.

Table 9: Yield of Isolated Lignin from Inflorescence Stems of *Arabidopsis* Accessions (Results Expressed as a Percentage of the Weight of Total Lignin Content [a] Determined by the Sulfuric Acid Method).

Accession	Yield of isolated lignin (%)
Col-0	25.32
Be	18.33
Ct-1	22.63
Hs-0	21.28
Kendalville	16.55
Lz-0	25.44
No	18.54
Sakhdara	19.22
Wt-5	18.40
Zdr-6	20.62
Mixed [b]	20.63
Mean	20.63

[a] Including acid soluble lignin and Klason lignin with correction of crude proteins. [b] A mixture of Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

3.2.2 Impurities in the isolated lignins

To determine the extinction coefficients of the isolated lignins, impurities in the lignins including neutral sugars, uronic acids and proteins were determined to correct the lignin weight (Table 10). The amount of each individual sugar in the isolated lignin is listed in Table 11.The results show that the major sugar in the lignin isolated from the *Arabidopsis* accessions was xylose, the total carbohydrates were in the range of 1.57% to 4.40% and proteins ranged from 2.62 to 5.34%. These results are similar to the results for lignins isolated from other non-woody samples as reported by Fukushima and Hatfield (2001).

Table 10: 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	Proteins	Total
Col-0	2.54 ± 0.08	0.86 ± 0.04	3.69 ± 0.18	7.09 ± 0.30
Be	2.62 ± 0.01	1.08 ± 0.05	4.28 ± 0.02	7.98 ± 0.08
Ct-1	1.30 ± 0.01	0.49 ± 0.03	4.72 ± 0.02	6.51 ± 0.06
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
Lz-0	2.42 ± 0.07	0.67 ± 0.03	5.34 ± 0.04	8.43 ± 0.14
No	1.62 ± 0.07	0.58 ± 0.03	4.16 ± 0.13	6.36 ± 0.23
Sakhdara	1.26 ± 0.05	0.38 ± 0.01	5.34 ± 0.04	6.98 ± 0.10
Wt-5	1.78 ± 0.01	0.59 ± 0.01	2.62 ± 0.01	4.99 ± 0.03
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 Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

Table 11: Individual Neutral Sugars in Isolated Lignin from Inflorescence Stems of *Arabidopsis* Accessions (Results Expressed as a Percentage of the Weight of the Isolated Lignin).

Accession	Fucose	Arabinose	Rhamnose	Galactose	Glucose	Xylose	Mannose
Col-0	0.03	0.14	0.12	0.16	0.16	1.88	0.05
Be	0.05	0.12	0.22	0.19	0.12	1.84	0.08
Ct-1	0.02	0.03	0.07	0.08	0.07	0.99	0.04
Hs-0	0.04	0.22	0.29	0.27	0.12	2.40	0.10
Kendalville	0.02	0.07	0.13	0.11	0.06	1.30	0.04
Lz-0	0.05	0.16	0.14	0.19	0.11	1.70	0.07
No	0.02	0.07	0.08	0.10	0.06	1.26	0.03
Sakhdara	0.02	0.03	0.05	0.08	0.07	0.96	0.05
Wt-5	0.01	0.09	0.09	0.11	0.07	1.33	0.08
Zdr-6	0.04	0.19	0.28	0.23	0.11	1.96	0.10
Mixed [a]	0.01	0.05	0.06	0.08	0.05	0.87	0.04
Mean	0.03	0.11	0.14	0.15	0.09	1.50	0.06

[a] A mixture of Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

3.2.3 Extinction coefficient determination of isolated lignins

Based on the impurity-corrected weight of lignin, the extinction coefficients of each isolated lignin standard were determined and were found to be in the range of 22.96 and 23.60 $g^{-1}Lcm^{-1}$ (Table 12). The mean and confidence interval for the extinction coefficient is 23.35 ± 0.13 $g^{-1}Lcm^{-1}$and variability is less than 1%. The results are consistent with the work of Johnson et al. (1961) who found the extinction coefficients of acetyl bromide derived lignins from various softwoods and hardwoods to range from 23.3 to 23.6 $g^{-1}Lcm^{-1}$.

Table 12: Determination of Monolignol Ratios by Thioacidolysis and Extinction Coefficients at 280 nm by the Acetyl Bromide Method for Isolated Lignins from Inflorescence Stems of *Arabidopsis* Accessions.

Accession	Extinction coefficient [a]	Lignin monolignol ratio [a]			Total monolignol
	($g^{-1}Lcm^{-1}$)	hydroxyphenyl	guaiacyl	syringyl	yield [b] (μmol g-1)
Col-0	23.29	1.9	58.2	39.9	1104.7
Be	23.60	1.8	56.3	41.9	924.5
Ct-1	22.96	1.2	53.8	45.0	1068.6
Hs-0	23.60	3.4	56.7	39.9	627.3
Kendalville	23.23	1.4	49.5	49.1	1165.2
Lz-0	23.32	1.7	53.4	44.9	965.0
No	23.20	1.2	49.9	48.9	1206.8
Sakhdara	23.53	1.4	55.1	43.5	1000.1
Wt-5	23.48	1.5	59.5	39.1	965.2
Zdr-6	23.33	2.5	52.8	44.7	801.1
Mixed [c]	23.33	1.0	53.3	45.7	1238.3

[a] Data are means of two measurements. [b] The total monlignol yield was the quantity of monolignols recovered by thioacidolysis based on the starting lignin content. [c] Sample was a mixture of Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

3.2.4 Monolignol ratios of the isolated lignins

To determine if the small variation of extinction coefficient values of the isolated lignins from *Arabidopsis* accessions was due to similar chemical structures of the lignins,

thioacidolysis analysis was performed to determine the monolignol ratios on the lignins. Figure 24 shows an example of a GC chromatogram of the thioacidolysis products of an isolated lignin from *Arabidopsis* inflorescence stems. Figure 25, 26, 27 and 28 show an example of mass spectrum of the GC peaks with retention time at 36.00, 37.79, 40.14 and 42.51 min respectively for the identification of the GC peaks. The GC peaks were identified to be the thioacidolysis products of tetracosane (the internal standard), hydroxyphenyl lignin, guaiacyl lignin and syringyl lignin with the corresponding most abundant ion having m/z value of 57, 239, 269, and 299 respectively on the mass spectrum, as stated by Rolando et al. (1992).

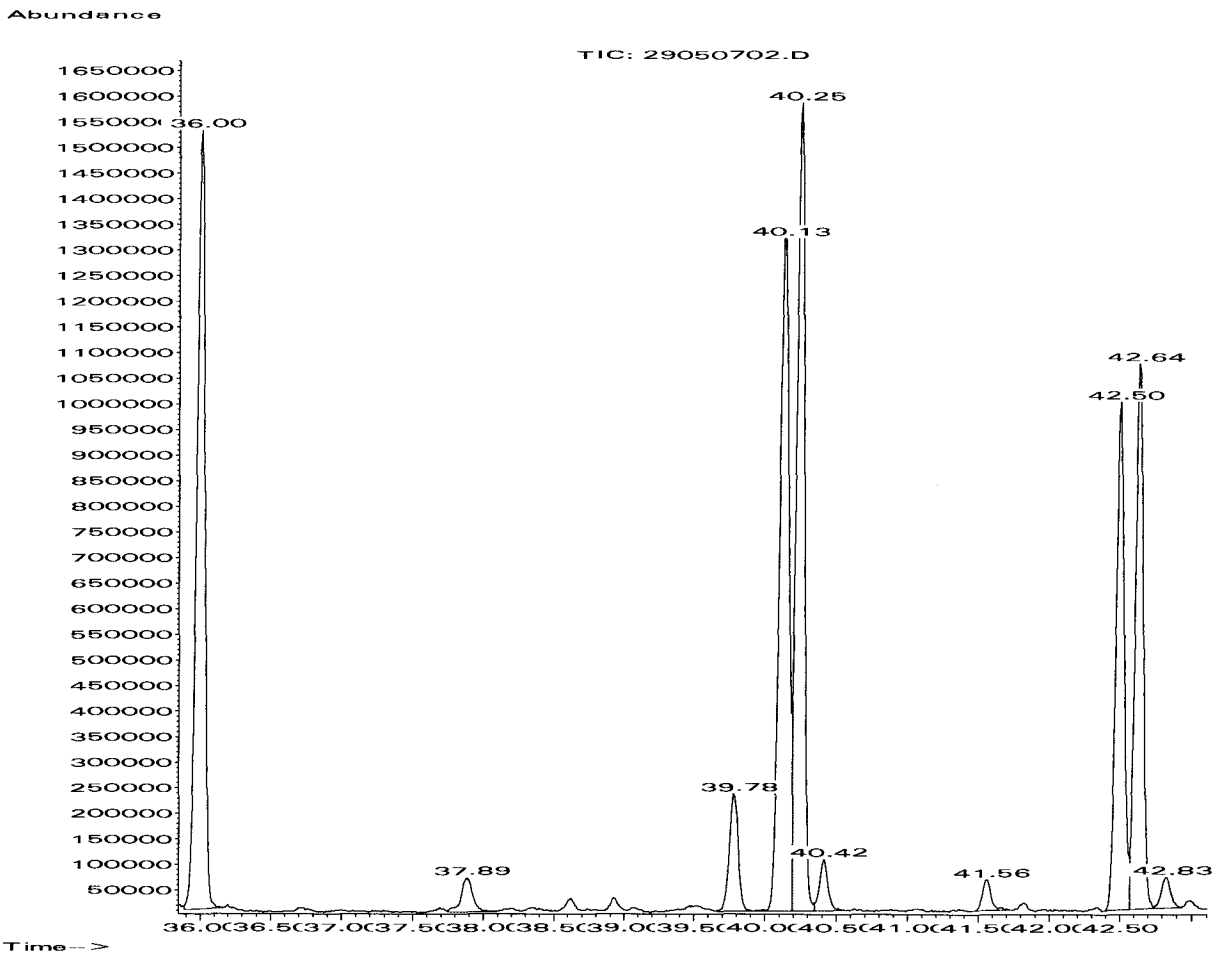

Figure 24: Gas chromatogram of thioacidolysis products of an isolated lignin from *Arabidopsis* inflorescence stems.

Measurement of the peak areas for the isolated lignins shows that that the syringyl content of lignins varied from 39.9 to 49.9% (Table 12). Furthermore, the total monolignol yield of the isolated lignin ranged from 627.3 to 1238.3 μmol g^{-1}, which may also indicate the variation of the lignin structures since the yield of thioacidolysis mainly depends on the cleavage of β-aryl ether linkages in lignins (Table 12) (Rolando et al., 1992). These results demonstrate that the extinction coefficients using the acetyl bromide method is 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 the isogenized progenies of all segregants in a mapping population (RILs), harvested at mature stage.

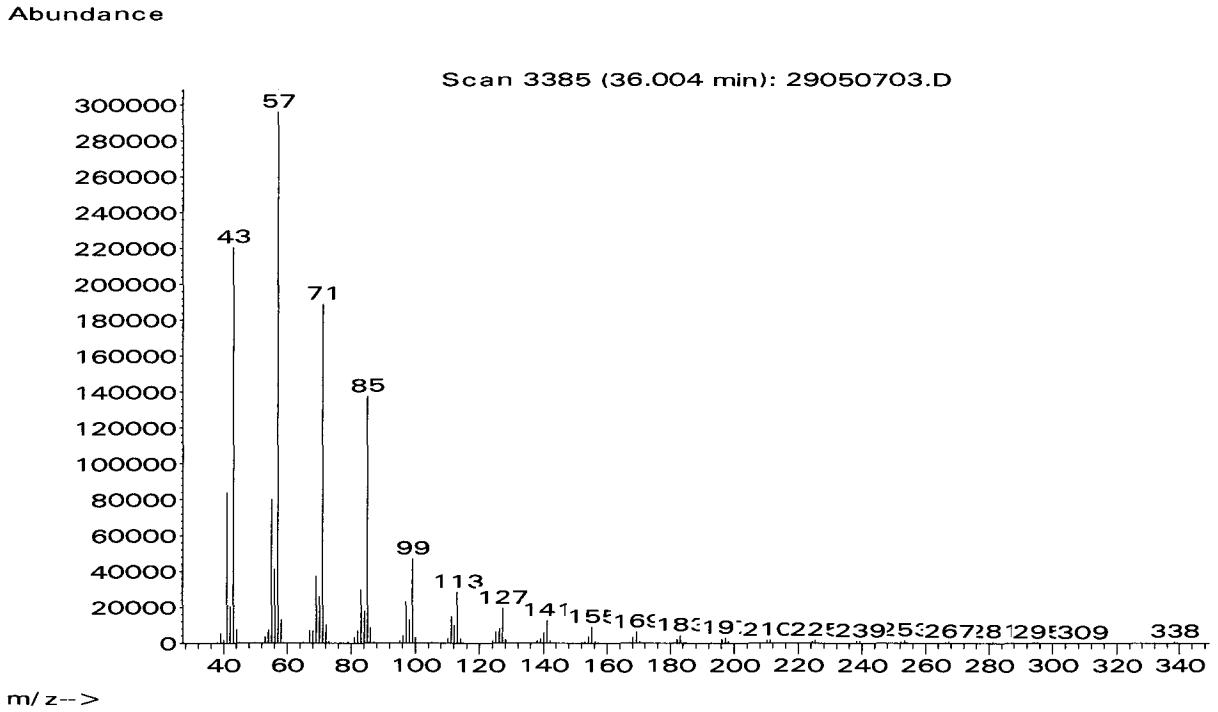

Figure 25: The mass spectrum of trimethylsilylated tetracosane thioacidolysis derivative with GC retention time at 36.00 min.

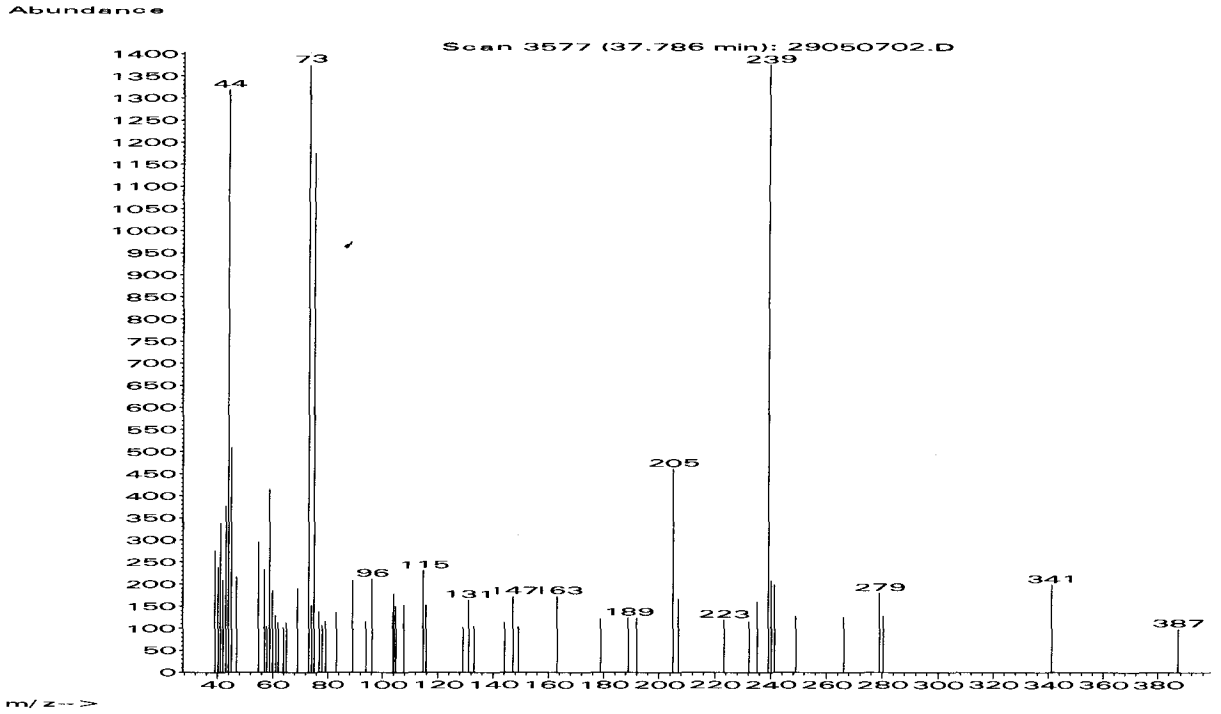

Figure 26: The mass spectrum of trimethylsilylated hydroxyphenyl lignin thioacidolysis derivative with GC retention time at 37.79 min.

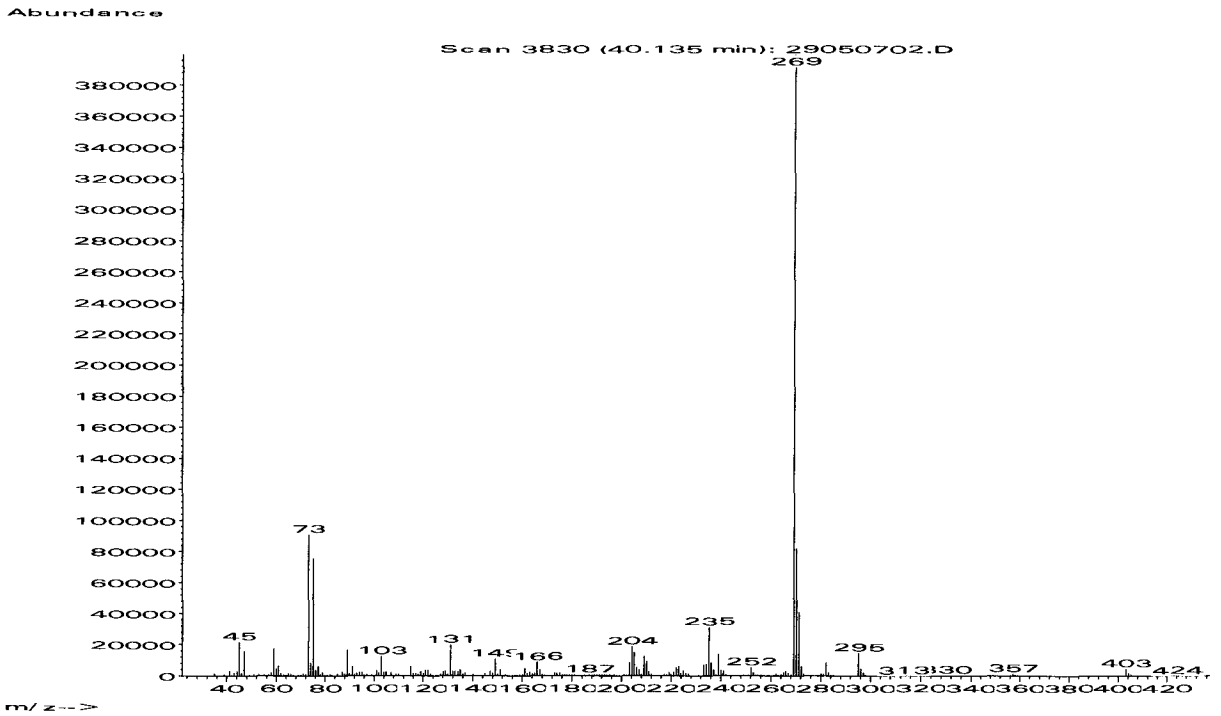

Figure 27: The mass spectrum of trimethylsilylated guaiacyl lignin thioacidolysis derivative with GC retention time at 40.14 min.

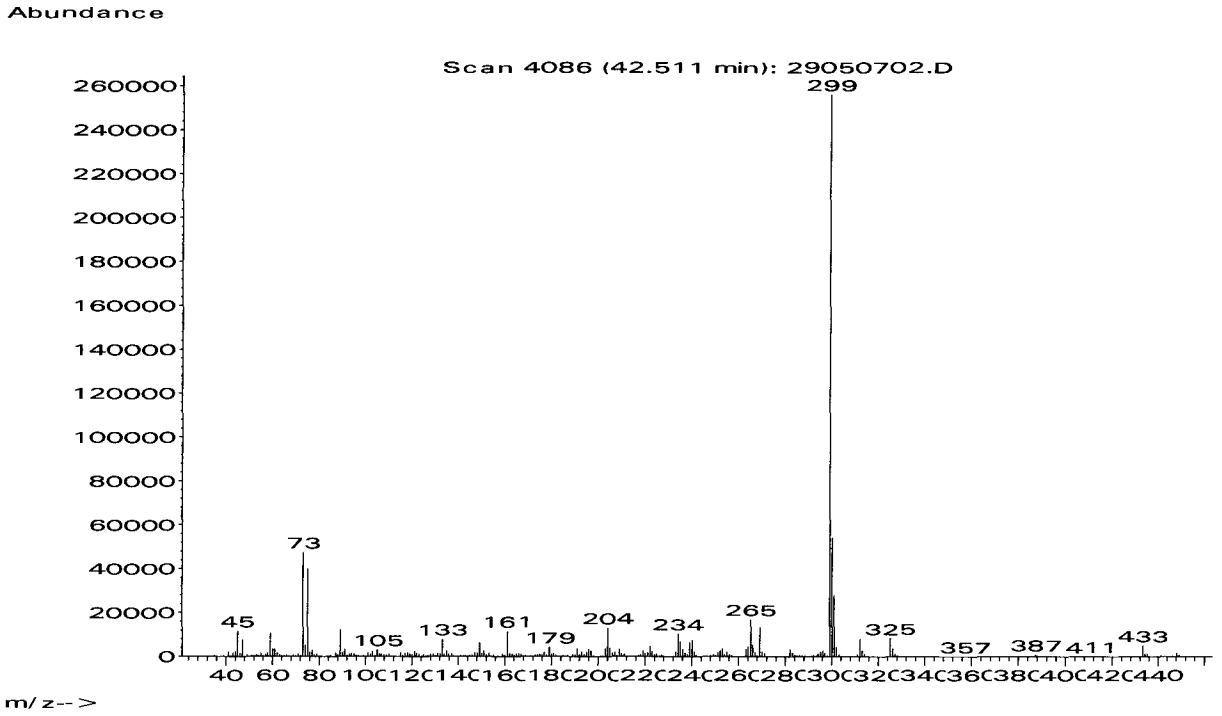

Figure 28: The mass spectrum of trimethylsilylated syringyl lignin thioacidolysis derivative with GC retention time at 42.51 min.

3.3 Comparison of lignin content determination by the acetyl bromide method and sulfuric acid method

The modified acetyl bromide method for lignin content determination of *Arabidopsis* accessions can be compared to other methods of lignin content determination. Since the acetyl bromide method is based on relating spectral response of lignins to lignin concentration, the methods used for comparison would be preferred to be non spectrophotometric methods. The sulfuric acid method is a good candidate for comparison since it is a widely accepted method in which lignin is isolated and determined gravimetrically. The speed of the sulfuric acid method is not critical for the

comparison purpose, but it is important to investigate uncertainties associated with the determination of lignin content in *Arabidopsis* using the sulfuric acid method.

3.3.1 Factors affecting the determination of lignin content in *Arabidopsis* using the sulfuric acid method

The sulfuric acid method for lignin content determination is standardized for wood and pulp. When applied to non-woody samples, the sulfuric acid method may result in an over-estimation of lignin due to the co-precipitation of proteinaceous substances and the presence of non-lignin inorganic ash constituents (Dence, 1992). Furthermore, the sulfuric acid method releases small amounts of acid-soluble lignin, which must be independently determined and added to the acid-insoluble lignin content to obtain a more accurate value for the total lignin content. In some species of angiosperms and grasses, the acid-soluble lignin content can be higher than 5% (Lai & Sarkanen, 1971). Therefore, when using the sulfuric acid method for determination of lignin content in *Arabidopsis,* the effects of inorganic ash, proteins and acid-soluble lignin on the determination should be analyzed.

3.3.1.1 Ash content

Ash determination in *Arabidopsis* accessions (Table 13) shows that total ash percentage in *Arabidopsis* is high with mean and confidence interval of 11.20% ± 2.0% while insoluble ash is low with mean and confidence interval of 0.37% ± 0.11%. Since only insoluble ash, which is mainly silica, can precipitate with lignin residual in the

determination of lignin content using the sulfuric acid method, the potential interference caused by ash content will be negligible.

Table 13: Total Ash and Insoluble Ash Content in Inflorescence Stems of Different Accessions of *Arabidopsis* (Results Expressed as a Percentage of the Weight of Original, Oven-dried Sample).

Accession	Total ash	Insoluble ash
Col-0	13.35 ± 0.15	0.191 ± 0.010
Be	15.90 ± 0.11	0.200 ± 0.010
Ct-1	12.73 ± 0.19	0.510 ± 0.010
Hs-0	7.05 ± 0.11	0.172 ± 0.005
Kendalville	9.05 ± 0.11	0.212 ± 0.006
Lz-0	11.60 ± 0.21	0.313 ± 0.008
No	9.80 ± 0.10	0.351 ± 0.012
Sakhdara	14.80 ± 0.12	0.430 ± 0.011
Wt-5	6.90 ± 0.07	0.451 ± 0.015
Zdr-6	9.49 ± 0.29	0.730 ± 0.021
Mixed [a]	12.5 ± 0.29	0.462 ± 0.013

[a] A mixture of Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

3.3.1.2 Protein content

Nitrogen analysis demonstrated that crude proteins in the original samples of *Arabidopsis* accessions were in the range between 6.88% and 18.81% (Table 14). Extraction of the samples decreased the amount of crude proteins but did not thoroughly remove them from the samples. The determination of crude proteins in *Arabidopsis* accessions (Table 14) shows that the mean and confidence interval of crude proteins content in the extracted samples of *Arabidopsis* accessions is 7.28% ± 1.44% (Table 14). Since the magnitude of the error caused by protein in the lignin determination using the sulfuric acid method is probably high for the samples having protein content more than 2.5% (Dence, 1992), the

determination of lignin content in *Arabidopsis* accessions using the sulfuric acid method should include procedures for avoiding or correcting for protein interference.

Table 14: Crude Protein Content in the Extracted [a] and Original Inflorescence Stems of *Arabidopsis* Accessions (Results Expressed as a Percentage of the Weight of Original and Extracted Sample Respectively).

	Crude proteins	
Accession	In the original samples	In the extracted samples
Col-0 [b]	6.88 ± 0.06	4.50 ± 0.06
Kendalville [c]	18.81 ± 0.50	7.41 ± 0.04
Ct-1 [c]	13.75 ± 0.25	7.12 ± 0.40
Sakhdara [c]	11.38 ± 0.50	6.19 ± 0.06
Lz-0 [c]	14.31 ± 0.12	9.12 ± 0.12
No [c]	14.75 ± 0.12	8.50 ± 0.31
Mixed[c d]	16.67 ± 0.25	8.12 ± 0.12

[a] Extracted by the four-stage Soxhlet extraction method. [b] A combination of preparations of 8 h and 24 h extractions each stage. [c] A preparation of 8 h extractions each stage. [d] A mixture of Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

To avoid the interference of proteins in lignin determination using the sulfuric acid method, the samples can be pretreated by pepsin to remove proteins from extracted samples. Table 15 shows the comparison of measured lignin content values in *Arabidopsis* accessions by the sulfuric acid method with and without pepsin treatment. The results demonstrated that the measured acid-insoluble lignin values using the sulfuric acid method with pepsin treatment were approximately 3%-4% lower than those without pepsin treatment for the same accession of *Arabidopsis*.

Table 15: Lignin Content in Inflorescence Stems of *Arabidopsis* Accessions Determined by the Sulfuric Acid Method with and without Pepsin Pretreatment (Results Expressed as a Percentage of the Weight of Original, Oven-dried Sample).

Accession	Method without pepsin pretreatment		Method with pepsin pretreatment	
	Acid-insoluble (Klason) lignin	Acid soluble lignin	Acid-insoluble lignin	Acid soluble lignin
Col-0	15.34 ± 0.13	1.29 ± 0.09	12.56 ± 0.32	0.12 ± 0.01
Kendalville	11.97 ± 0.13	1.47 ± 0.08	8.80 ± 0.03	0.10 ± 0.01
Ct-1	14.33 ± 0.28	1.64 ± 0.09	10.22 ± 0.01	0.11 ± 0.01
Sakhdara	15.9 ± 0.14	1.50 ± 0.04	11.81±0.20	0.11 ± 0.01
Lz-0	14.41 ± 0.21	1.80 ± 0.07	10.88 ± 0.21	0.17 ± 0.01
No	14.04 ± 0.22	1.92 ± 0.03	10.8 ± 0.20	0.19 ± 0.03
Mixed [a]	14.45 ± 0.16	1.50 ± 0.04	10.31 ± 0.29	0.31 ± 0.01

[a] A mixture of Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

The difference of the measured lignin for the same samples may be due to the complete remove of proteins from samples using the sulfuric acid method with pepsin treatment and a high amount of proteins precipitated with lignin residuals using the sulfuric acid method without pepsin treatment. To verify this, crude proteins in the lignin residuals were estimated by multiplying the nitrogen content by the factor 6.25 (Table 16). The results in Table 16 indicates that pepsin pretreatment did not completely eliminate proteins from samples although the amount of proteins precipitated with lignin residuals using the method with pepsin treatment is approximately half of those using the method without pepsin treatment. Thus, both the sulfuric acid method with and without pepsin treatment require protein correction for lignin determination.

Table 16: Crude Proteins in the residual of Acid-insoluble Lignin Obtained by the Sulfuric Acid method with and without Pepsin Pretreatment Using Inflorescence Stems of *Arabidopsis* Accessions (Results Expressed as a Percentage of the Weight of the Acid-insoluble Residual).

Accession	Crude proteins in acid-insoluble residual obtained by H_2SO_4 method	
	Without pepsin pretreatment	With pepsin pretreatment
Col-0	6.47 ± 0.12	4.97 ± 0.12
Kendalville	14.06 ± 0.31	8.04 ± 0.24
Ct-1	11.87 ± 0.50	6.80 ± 0.20
Sakhdara	12.19 ± 0.31	6.94 ± 0.06
Lz-0	15.81 ± 0.37	6.80 ± 0.31
No	16.12 ± 0.12	6. 90 ± 0.35
Mixed [a]	15.43 ± 0.37	6.71 ± 0.17
Mean	13.14	6.74

[a] A mixture of Est-1, Br-0, Mz-0, Bor-4, and Gu-0.

3.3.1.3 Acid-soluble lignin

Measured acid soluble lignin using sulfuric acid methods without pepsin treatment was approximately 1.5%, which was around ten times higher than that using the sulfuric acid method with pepsin treatment (Table 15). This may indicate that large amount of lignin goes into solution during the pepsin pretreatment.

Since the pepsin pretreatment may lead to underestimation of acid soluble lignin and could not completely remove proteins from the samples, the determination of lignin content in *Arabidopsis* accession using sulfuric acid methods may not need the pepsin pretreatment. It is probably better to correct the acid insoluble lignin with co-condensed proteins by estimating protein as N% x 6.25 and then add the acid soluble lignin to obtain the total amount of lignin in *Arabidopsis* samples.

3.3.2 Correlation between lignin content determination by the modified acetyl bromide method and by the sulfuric acid method

Proper handling of the factors affecting the determination of lignin content in *Arabidopsis* using the sulfuric acid method allows the sulfuric acid method to be used for comparison with the modified acetyl bromide method. The comparison was done on holocellulose-added, extracted-only, and cellulase-treated samples of inflorescence stems of Col-0 and samples of inflorescence stems of different *Arabidopsis* accession respectively to examine the correlation between the two methods. The comparison was under the assumption that the determined values of a blank sample by the two methods are all zero. Figure 29 shows a good agreement ($R^2 = 0.998$, $P < 0.0001$)) among the holocellulose-added, extracted-only, and cellulase-treated samples analyzed by the two methods. Figure 30 demonstrates a high correlation ($R^2 = 0.988$, $P < 0.0001$) between the determined values of lignin content by the sulfuric acid method and the modified acetyl bromide method on different accessions of *Arabidopsis*. The results are consistent with the work done by Fukushima and Hatfield (2001), who found a good correlation between the acetyl bromide method and Klason method and a ratio between the measured values by the two methods on four-stage Soxhlet extracted samples of alfalfa, bromegrass, pine and corn stalk ranging from 0.81 to 1.20. Fukushima and Hatfield (2001) also found that the ratio between the measured values by the two methods could be closer to 1.0 after correction of protein contamination and acid insoluble lignin, as is the case in our measurements with *Arabidopsis*.

The excellence of the correlation is somewhat surprising as other factors such as guiaicyl/syringyl (S/G) ratio and the presence of p-coumaric acid and ferulic acid linked

by ester bonds to lignin would be expected to have divergent impacts on the two lignin determination methods.

Using the acetyl bromide method, p-coumaric and ferulic ester groups will be solublized and absorb at 280 nm. The extinction coefficients of acetyl bromide treated p-coumaric acid and ferulic acid have been shown to be close to those of lignin units (Iiyama & Wallis, 1989). In fact, the extinction coefficient at 280 nm using the acetyl bromide method has been verified to be independent of the types of sample of *Arabidopsis* as discussed above.

Using the sulfuric acid method, p-coumaric and ferulic ester groups either precipitate with lignin residual or are partially hydrolyzed into solution (Lai & Sarkanen, 1971). Since the acid-soluble lignin is measured and included in the total lignin content in *Arabidopsis* samples using sulfuric acid method, the determination includes p-coumaric acid and ferulic acid. The extinction coefficients used to calculate the acid-soluble lignin may vary with S/G ratio of lignin and the amount of p-coumaric acid and ferulic acid among different samples and it would be desirable to determine this for each sample individually (Dence, 1992). Practically, an average extinction coefficient reported in the literature (110 $g^{-1}Lcm^{-1}$) is commonly used to roughly calculate the soluble lignin content (Dence, 1992). However, the uncertainty of the extinction coefficient is not likely to lead to large error of the determination of acid-soluble lignin, which includes hydrolyzed p-coumaric and ferulic ester groups, due to its relatively small amount compared to the total lignin content (Goldschmid, 1971).

Thus, the contribution of p-coumaric acid and ferulic acid in both instances will be included. The uncertainty of the extinction coefficient caused by S/G ratio of lignin and

the presence of p-coumaric acid and ferulic acid using the sulfuric acid method is unlikely to significantly affect the correlation between the measured values by the two methods. The sulfuric acid method can be used for cross-checking the lignin content determination in *Arabidopsis* accessions by the modified acetyl bromide method.

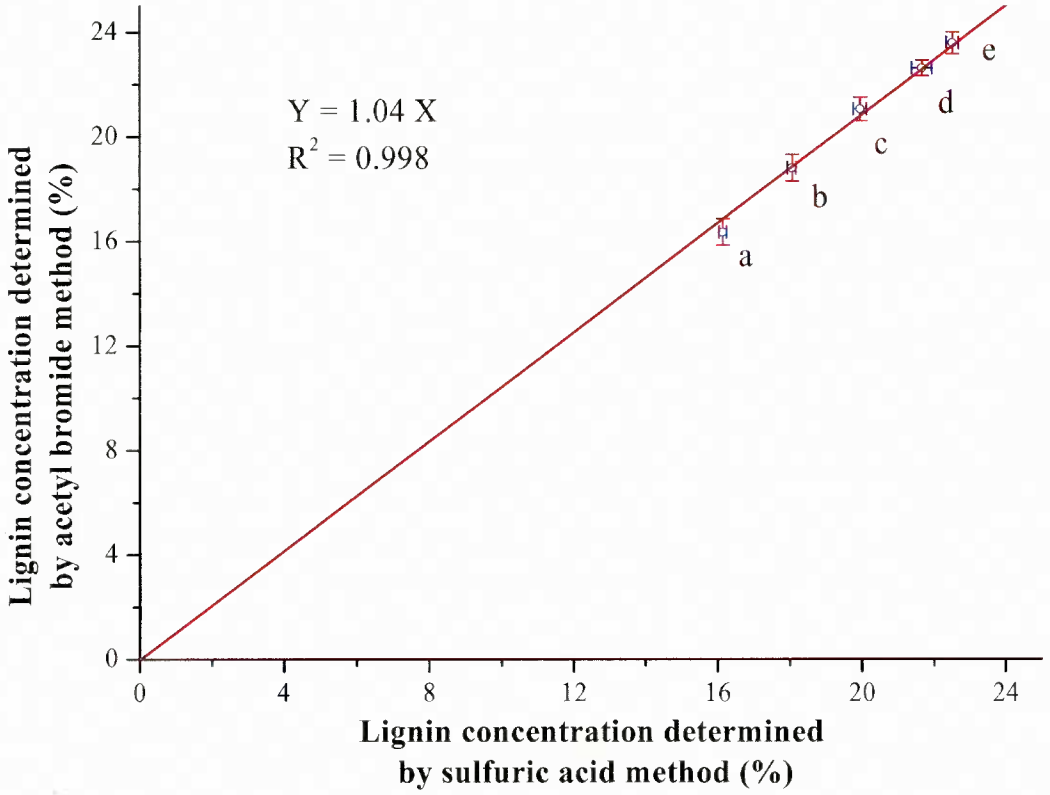

Figure 29: Correlation between the determinations of lignin content by the acetyl bromide method (using the modified method with cuvettes) and the sulfuric acid method (including acid-soluble and acid-insoluble with crude proteins correction) using holocellulose-added (a and b), extracted-only (c), and cellulase-treated (d and e) samples of inflorescence stems of Col-O.

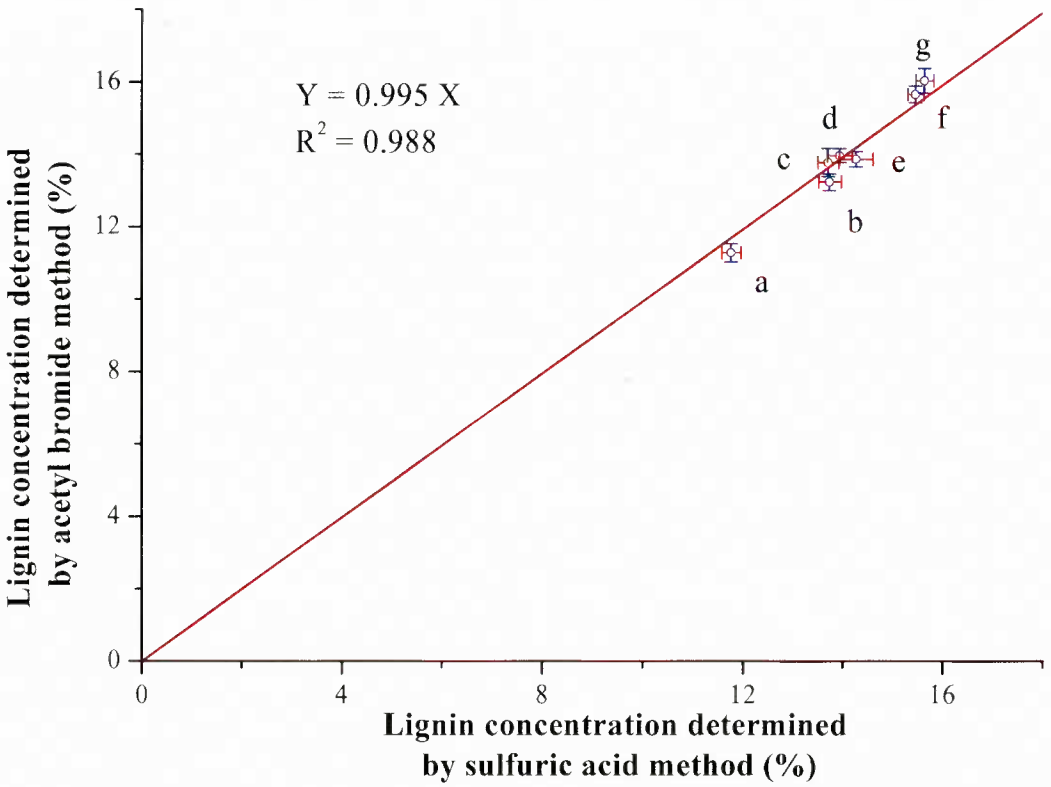

Figure 30: Correlation between the determinations of lignin content by the acetyl bromide method (using the modified method with cuvettes) and sulfuric acid method (including acid-soluble and acid-insoluble with crude proteins correction) using inflorescence stems of different *Arabidopsis* accessions grown at various conditions. (a). Kendalville. (b). No. (c). Mixed (d). Lz-0. (e). Ct-1. (f). Sakhdara. (g). Col-0. Values based on the weight of original, oven-dried sample.

3.4 Application of the modified acetyl bromide method

The established high-throughput acetyl bromide method and the standard value of extinction coefficient at 280 nm for lignin in *Arabidopsis*, 23.35 $g^{-1}Lcm^{-1}$, can now be used to determine lignin content of various accessions of *Arabidopsis*. The sulfuric acid method after correction for co-condensed proteins and the acid soluble lignin can be used for confirmation of lignin content determination in the *Arabidopsis* accessions.

3.4.1 Sampling method for determination of lignin content of *Arabidopsis* accessions

To compare lignin content in various accessions of *Arabidopsis*, the sampling method should be well defined. It was found that the stain in cross sections obtained from various regions of an inflorescence stem of *Arabidopsis* increases basipetally using phloroglucinol-HCl, as xylem vessels and inter-fascicular fibres become progressively more differentiated (Rogers & Campbell, 2004). It is possible that different sections of *Arabidopsis* inflorescence stems have different lignin concentrations. To verify this, inflorescence stems of Col-0 plants grown at controlled condition (16 h light, 100-150 $\mu mols^{-1}m^{-2}$, 21 °C) were divided into top, middle and bottom sections according to their heights. The top, middle and bottom sections of the inflorescence stems were combined respectively to make three pools. The measured values of lignin content in the three sections by the modified acetyl bromide method were significantly different (Figure 31). Thus, whole inflorescence stems of *Arabidopsis* should be sampled for the determination of lignin content in *Arabidopsis* accessions.

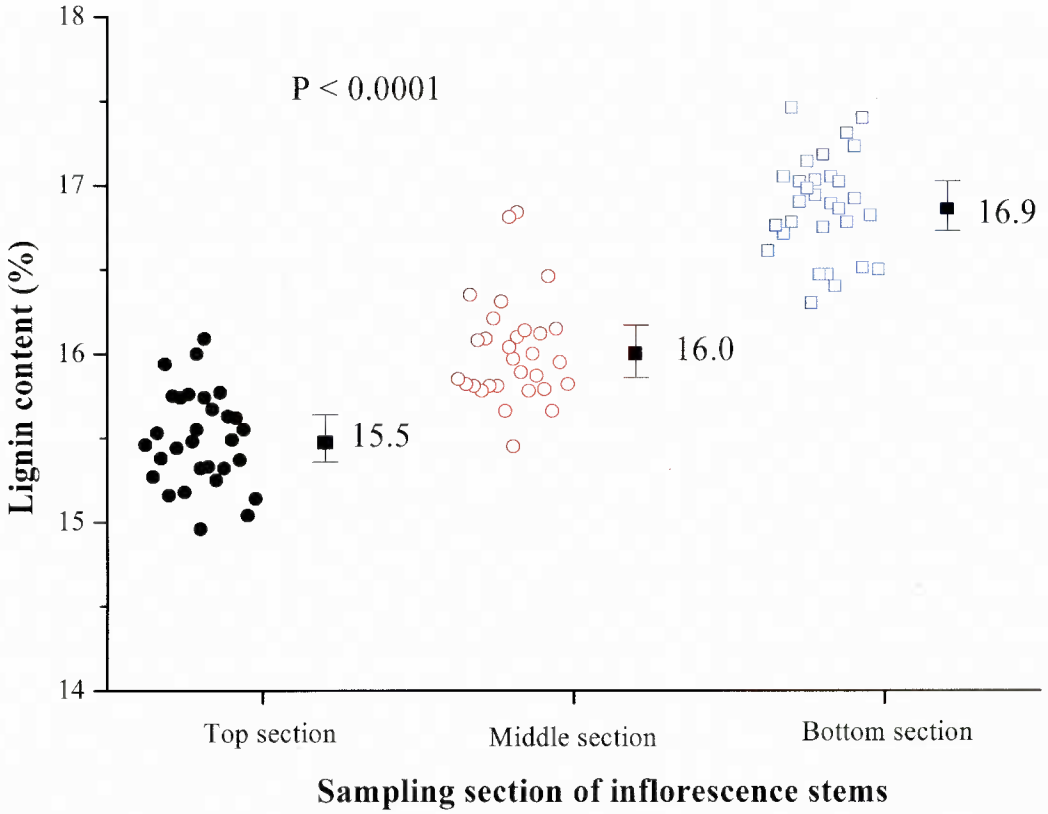

Figure 31: Comparison of lignin content in different sections of inflorescence stems of Col-0 by the modified acetyl bromide method (Results expressed as a percentage of the weight of original, oven-dried sample).

3.4.2 Determination of lignin content in *Arabidopsis* accessions

The well defined sampling method provides a basis for the comparison of lignin content in *Arabidopsis* accessions. Sixty-five accessions of *Arabidopsis* were planted under the same growing conditions in a growth chamber (16 h light, 150 $\mu mols^{-1} m^{-2}$, 21 ^{o}C). The plants were planted in random locations throughout the temperature controlled growth chamber. For each accession, the stems of approximately six individual plants were collected and the whole plant stems were ground, mixed and used for the determination of lignin. Therefore, the pooled samples of each accession are representative of the

population of the accession and the phenotype variations among the different accessions grown under the same condition would be due to genetic variations.

Table 17 shows the extraction yields and lignin content in samples of the sixty-five accessions of *Arabidopsis* using the modified acetyl bromide method.

Table 17: Determination of Lignin Content in Inflorescence Stems of 65 *Arabidopsis* Accessions Grown in a Controlled Environment in a Growth Chamber with 16 h daylight, 150 $\mu mols^{-1}$ m^{-2}, 21 °C. (Results Expressed as a Percentage of the Weight of Oven-dried sample).

Accession	Extraction yield	Lignin content	
		Based on extracted sample	Based on orignial sample
Ren-1	73.38 ± 0.56	17.8 ± 0.1	13.0 ± 0.2
Bay-0	73.41 ± 0.61	18.0 ± 0.2	13.2 ± 0.2
Nok-3	72.10 ± 0.83	18.7 ± 0.3	13.5 ± 0.4
Kin-0	79.30 ± 0.54	17.0 ± 0.2	13.5 ± 0.2
Bor-4	76.04 ± 0.34	17.8 ± 0.1	13.6 ± 0.1
Knox-18	71.78 ± 0.34	19.2 ± 0.1	13.8 ± 0.1
Sorbo	76.54 ± 0.16	18.9 ± 0.4	14.5 ± 0.4
Van-0	75.64 ± 0.61	19.9 ± 0.4	15.1 ± 0.4
Col-1	77.16 ± 0.76	19.7 ± 0.4	15.2 ± 0.5
Uod-7	76.66 ± 0.02	20.0 ± 0.1	15.4 ± 0.1
En-1	82.98 ± 0.71	18.8 ± 0.1	15.6 ± 0.2
Pro-0	79.53 ± 1.20	19.7 ± 0.3	15.6 ± 0.5
Bch-3	80.57 ± 0.51	19.4 ± 0.1	15.7 ± 0.2
Ws-0	73.72 ± 0.62	21.4 ± 0.1	15.7 ± 0.2
Berkeley	77.79 ± 0.60	20.2 ± 0.4	15.7 ± 0.4
Shakdara	78.12 ± 0.34	20.2 ± 0.1	15.8 ± 0.2
Mr-0	80.00 ± 0.71	19.7 ± 0.2	15.8 ± 0.3
Col-PRL	75.55 ± 0.33	21.0 ± 0.4	15.8 ± 0.4
Zdr-1	80.81 ± 0.15	19.7 ± 0.1	15.9 ± 0.1
Nd	75.92 ± 0.53	21.0 ± 0.2	16.0 ± 0.2
Lp2-6	77.31 ± 0.52	20.7 ± 0.2	16.0 ± 0.3
Col-0	76.10 ± 0.58	21.1 ± 0.3	16.1 ± 0.3
Uod-1	79.33 ± 0.28	20.8 ± 0.1	16.5 ± 0.1
Rennes-11	83.18 ± 0.15	20.0 ± 0.5	16.7 ± 0.4
Bu-6	79.09 ± 0.40	21.2 ± 0.3	16.7 ± 0.4
Lz-0	77.28 ± 0.81	21.7 ± 0.3	16.8 ± 0.4
Kondara	78.45 ± 0.68	21.4 ± 0.1	16.8 ± 0.2
Tsu-1	79.14 ± 0.44	21.2 ± 0.4	16.8 ± 0.4
Ull2-5	78.41 ± 0.04	21.4 ± 0.3	16.8 ± 0.3
Cape Verde	78.72 ± 0.35	21.5 ± 0.4	17.0 ± 0.4
Kendalville	78.38 ± 0.33	21.7 ± 0.3	17.0 ± 0.3
Kz-7	78.49 ± 0.56	21.7 ± 0.5	17.1 ± 0.5

Table 17 (continued)

Accession	Extraction yield	Lignin content	
		Based on extracted sample	Based on orignial sample
Hr-8	78.81 ± 0.01	21.8 ± 0.5	17.1 ± 0.4
Ct-1	82.48 ± 0.92	20.8 ± 0.2	17.2 ± 0.4
Lc-0	81.21 ± 0.64	21.9 ± 0.2	17.2 ± 0.3
Mrk-0	79.06 ± 0.30	21.7 ± 0.3	17.2 ± 0.3
S96	81.39 ± 0.49	21.2 ± 0.3	17.2 ± 0.3
Br-0	82.89 ± 0.79	20.8 ± 0.1	17.2 ± 0.2
Fei-0	83.90 ± 0.01	20.6 ± 0.1	17.3 ± 0.1
Gu-0	81.80 ± 0.78	21.2 ± 0.2	17.3 ± 0.3
Durh-1	79.85 ± 0.08	21.7 ± 0.1	17.3 ± 0.1
RLD1	80.97 ± 0.41	21.4 ± 0.1	17.3 ± 0.2
Bor-1	83.24 ± 0.59	20.9 ± 0.1	17.4 ± 0.1
Ga-0	78.40 ± 0.10	22.3 ± 0.1	17.5 ± 0.1
Zdr-6	80.17 ± 0.02	22.0 ± 0.1	17.6 ± 0.1
Ws	81.44 ± 0.08	21.8 ± 0.5	17.8 ± 0.4
Dijon-G	83.22 ± 0.08	21.4 ± 0.1	17.8 ± 0.1
Mt-0	80.74 ± 0.46	22.2 ± 0.3	17.9 ± 0.3
HR-10	85.17 ± 0.69	21.0 ± 0.2	17.9 ± 0.3
An-1	82.55 ± 0.79	21.8 ± 0.3	18.0 ± 0.4
Kn-0	81.78 ± 0.05	22.1 ± 0.2	18.1 ± 0.2
Be	78.99 ± 0.08	22.9 ± 0.1	18.1 ± 0.1
Bs-1	81.16 ± 0.74	22.3 ± 0.1	18.1 ± 0.2
Gr-3	80.17 ± 0.33	22.8 ± 0.1	18.3 ± 0.1
Aua/Rhön	80.77 ± 0.26	23.0 ± 0.1	18.6 ± 0.1
Hr-5	80.97 ± 0.81	23.0 ± 0.1	18.7 ± 0.2
Kz9	82.47 ± 0.55	22.7 ± 0.2	18.7 ± 0.3
No	83.65 ± 1.21	22.4 ± 0.4	18.7 ± 0.6
Ra-0	81.90 ± 0.50	22.9 ± 0.1	18.7 ± 0.2
Oy-0	80.10 ± 0.44	23.4 ± 0.1	18.8 ± 0.2
Wt-5	80.96 ± 0.15	23.2 ± 0.2	18.8 ± 0.2
Mühlen	81.85 ± 0.30	23.0 ± 0.1	18.8 ± 0.2
Kz-1	78.58 ± 0.44	24.0 ± 0.3	18.8 ± 0.4
Est	82.33 ± 0.24	22.9 ± 0.2	18.8 ± 0.2
Gre-0	82.82 ± 0.92	23.1 ± 0.1	19.1 ± 0.2
Ler-0	83.01 ± 0.51	24.1 ± 0.1	20.0 ± 0.2

The results demonstrate that natural variation of lignin content exists among *Arabidopsis* accessions. In the sixty-five *Arabidopsis* accessions, lignin content based on the weight of original sample ranged from 13.0% to 20.0% while lignin content based on the weight of extracted sample varied from 17.0% to 24.1%. The ogive shows that based on the weight

of original sample, approximately 10% of the *Arabidopsis* accessions have lignin content less than 15% while 10% of accessions have lignin content more than 18.5% (Figure 32).

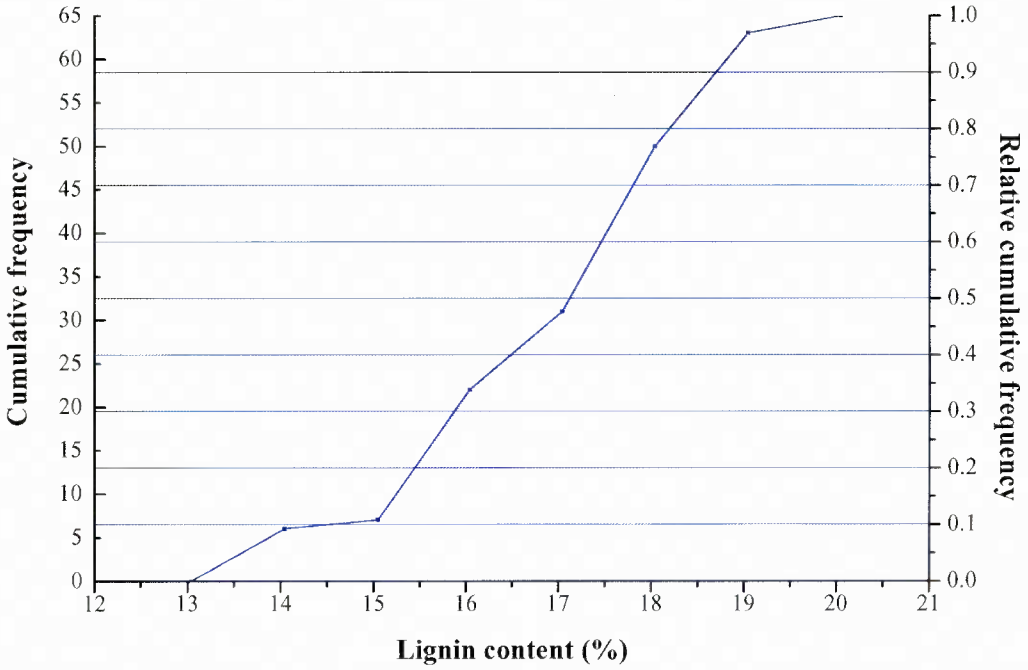

Figure 32: The cumulative frequency ogive and the relative cumulative frequency ogive for lignin content in inflorescence stems of 65 *Arabidopsis* accession determined by the modified acetyl bromide method (Lignin concentration expressed as a percentage of the weight of the original, oven-dried sample).

3.4.3 Spiking isolated lignin to cellulose

In the process of screening program for lignin content in *Arabidopsis* accessions using the modified acetyl bromide method, isolated lignin was spiked into isolated cellulose and the spiked lignin concentration was compared with the measured lignin concentration in the spiked samples for quality control. Figure 33 demonstrates a good correlation between the two concentration values and the ratio of the two values was close to 1.0. Thus, the accuracy of the measurements in the screening program was confirmed.

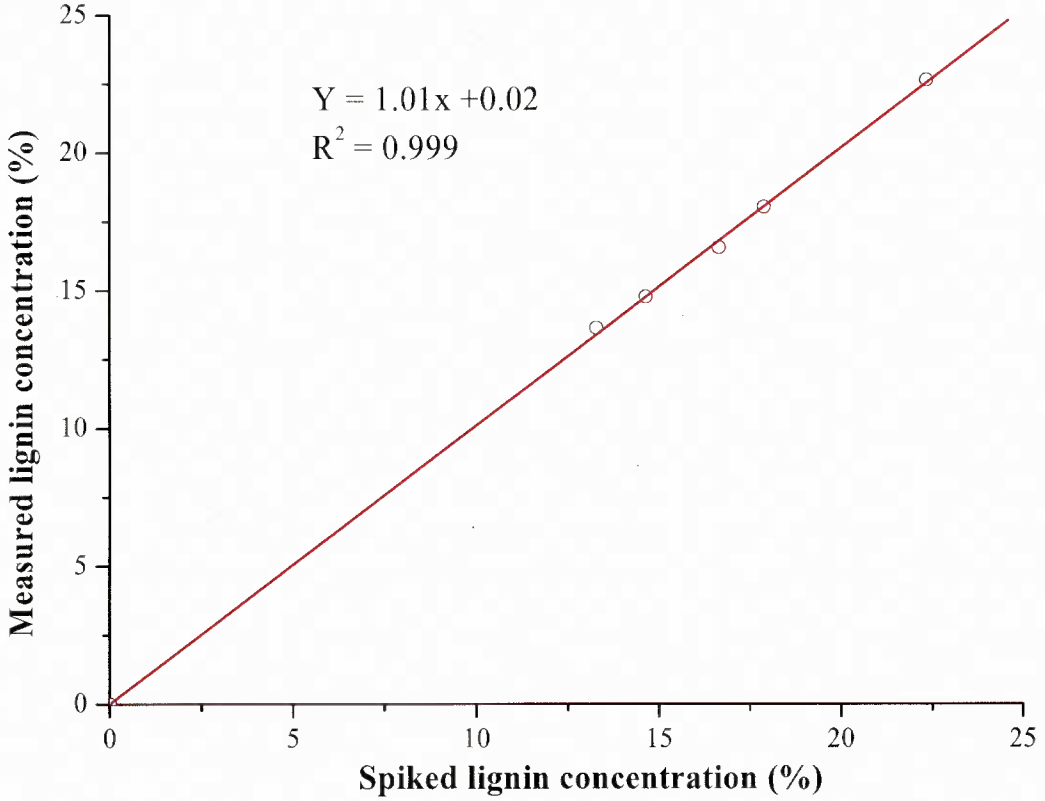

Figure 33: Quality control using isolated lignin spiked to isolated cellulose from inflorescence stems of Col-0 (Results expressed as lignin concentrations in final solution for absorbance measurement)

3.4.4 Cross-checking lignin content in *Arabidopsis* accessions by the sulfuric acid method

Lignin content in the twelve *Arabidopsis* accessions in the screening program using the modified acetyl bromide method was cross-checked by the sulfuric acid method, which had shown a good correlation with the acetyl bromide method. Figure 34 demonstrates that the measured lignin content values in twelve *Arabidopsis* accessions were either

extreme high or extreme low by both of the acetyl bromide method and sulfuric acid method.

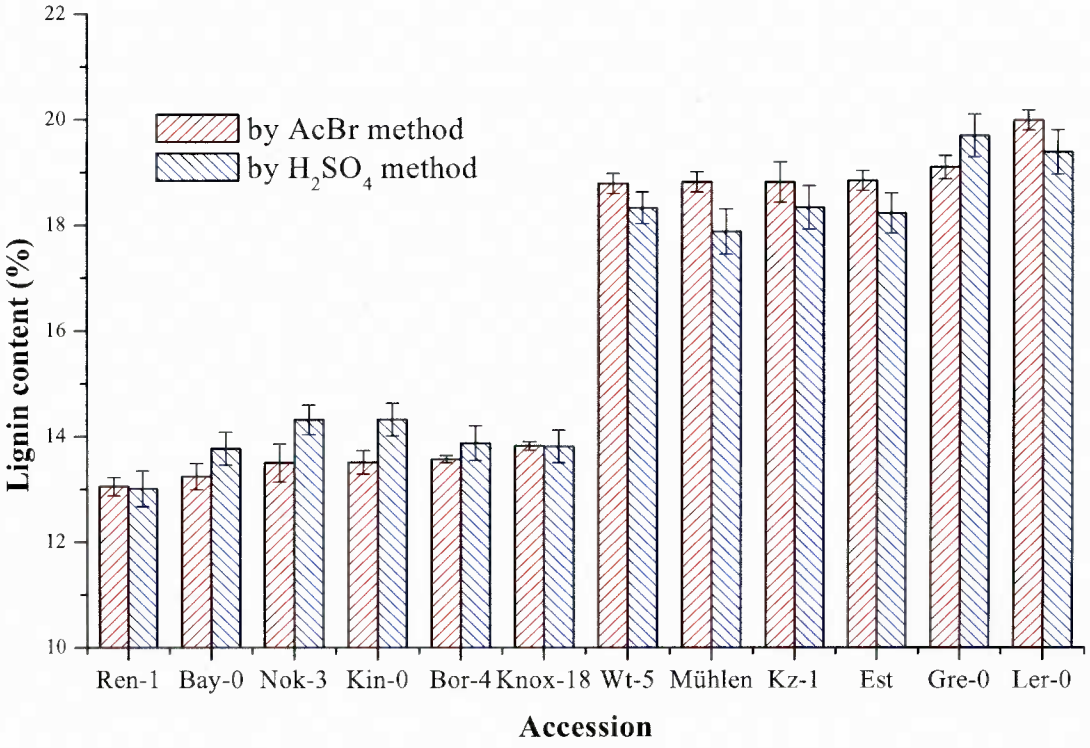

Figure 34: Cross-check of lignin content in inflorescence stems of 12 extreme *Arabidopsis* accessions determined by acetyl bromide method (using the modified method with cuvettes) with the sulfuric acid method (including acid-soluble and acid-insoluble with crude proteins correction). Results were obtained from triplicate measurements and expressed as a percentage of the weight of original, oven-dried sample.

In short, lignin content determination in *Arabidopsis* accessions using the modified acetyl bromide and the sulfuric acid method shows the lignin content is divergent property of *Arabidopsis*. The accessions of *Arabidopsis* with extreme amount of lignin content could be used for subsequent projects focused on genetic modification of *Arabidopsis*.

3.4.5 Correlation between lignin content and growth rate among *Arabidopsis* accessions

The results of lignin content determination in *Arabidopsis* accessions could be used to examine if lignin content has a correlation with growth rates among *Arabidopsis* accessions.

Kirst et al. (2004) found evidence that the same genomic regions regulate both growth rate and lignin content in an interspecific backcross of Eucalytus. It may be possible to find a similar relationship between lignin content and growth rates using the inflorescence stems of *Arabidopsis* accessions.

3.4.5.1 Measurement of growth rate of *Arabidopsis* accessions

To measure and compare the growth rates of various *Arabidopsis* accessions, the position of the measurement on the inflorescence stem and the maturity of the plants have to be well defined because the pattern of growth rate may vary with cell development stages and the plant growth stages. Imoto et al. (2005) found that sections along the *Arabidopsis* inflorescence stem have a decreasing elongation rate as one moves from the apex to the basal mature region. Moreover, the elongation rate of the each section decreases with the maturity of the plants.

For these reasons, the measurement of growth rate of *Arabidopsis* accessions was begun by marking two points on the *Arabidopsis* inflorescence stems at one centimeter and two centimeters down from the apical meristem when the first flower of the plants opened (Figure 35). This ensures that the collection of growth rate data for all the plants begins at

the same plant development stage, growth stage 6.00, with the first flower of the plant easily serving as a plant developmental landmark (Boyes et al., 2001). This readily determined growth stage of 6.00 can accommodate the comparison of growth rate among different *Arabidopsis* accessions. It has previously been shown that the inflorescence stems of *Arabidopsis* have a maximum elongation rate between growth stages 6.00 and 6.50 (Boyes et al., 2001).

The position for the growth rate measurement of the inflorescence stems of *Arabidopsis* accessions was defined by distance from the apical meristem. The longitudinal growth rate of stem was measured through the elongation rates of the upper section between the plant apex and the first mark, and the lower section between the first mark and the second mark on the inflorescence stems. The rate of increase in stem width was monitored at the second mark on the inflorescence stems (Figure 35).

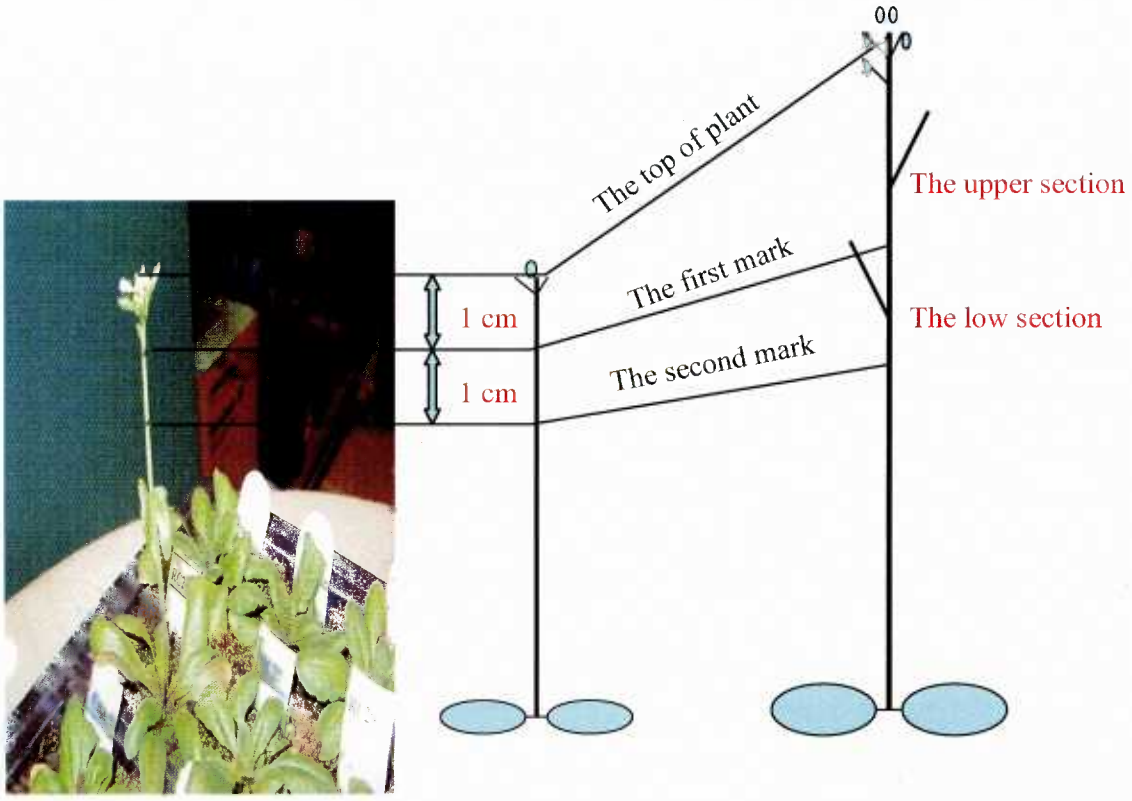

Figure 35: Diagram showing the measurement method for the growth rate of *Arabidopsis* accessions

Using the same individual plants as were used for lignin content determination, the elongation rates of the upper and lower sections, and the width growth rate of the stems at the second mark, were determined in different *Arabidopsis* accessions over a five day period. The growth rates of each of the *Arabidopsis* accessions are reported as the averages of six individual plants (Table 18).

Table 18: Measurement of the growth rate of *Arabidopsis* accessions Grown in a Controlled Environment in a Growth Chamber with 16 h daylight, 150 $\mu mols^{-1} m^{-2}$, 21 °C.

Accession	Elongation rate of the upper section (cm/day)	Elongation rate of the lower section (cm/day)	Width growth rate at the second mark (cm/day)
Gu-0	1.730 ± 0.105	0.274 ± 0.027	0.053 ± 0.004
Ler-0	1.739 ± 0.177	0.277 ± 0.021	0.073 ± 0.006
Wt-5	1.746 ± 0.118	0.133 ± 0.007	0.042 ± 0.003
Zdr-6	1.822 ± 0.082	0.174 ± 0.009	0.044 ± 0.003
An-1	1.908 ± 0.199	0.160 ± 0.007	0.020 ± 0.002
HR-10	1.954 ± 0.154	0.282 ± 0.029	0.059 ± 0.008
Pro-0	1.958 ± 0.180	0.237 ± 0.020	0.044 ± 0.006
Aua/Rhön	2.038 ± 0.121	0.338 ± 0.020	0.063 ± 0.004
Bu-6	2.069 ± 0.158	0.273 ± 0.016	0.036 ± 0.003
Zdr-1	2.072 ± 0.030	0.193 ± 0.020	0.049 ± 0.005
S96	2.086 ± 0.030	0.277 ± 0.051	0.041 ± 0.002
Durh-1	2.133 ± 0.024	0.368 ± 0.042	0.050 ± 0.006
Mr-0	2.141 ± 0.061	0.354 ± 0.031	0.044 ± 0.003
Ra-0	2.155 ± 0.223	0.297 ± 0.006	0.036 ± 0.003
Kz-7	2.162 ± 0.303	0.311 ± 0.039	0.052 ± 0.005
Bs-1	2.182 ± 0.064	0.324 ± 0.033	0.043 ± 0.005
Ren-1	2.243 ± 0.016	0.155 ± 0.031	0.046 ± 0.004
Uod-1	2.255 ± 0.082	0.308 ± 0.030	0.076 ± 0.008
Gre-0	2.280 ± 0.098	0.283 ± 0.009	0.057 ± 0.006
Hr-5	2.283 ± 0.374	0.370 ± 0.013	0.059 ± 0.003
Col-0	2.291 ± 0.271	0.307 ± 0.029	0.039 ± 0.001
Sakhdara	2.295 ± 0.155	0.345 ± 0.031	0.071 ± 0.006
Fei-0	2.318 ± 0.227	0.354 ± 0.034	0.036 ± 0.002
Nd	2.326 ± 0.170	0.317 ± 0.033	0.043 ± 0.002
Mrk-0	2.356 ± 0.123	0.301 ± 0.018	0.092 ± 0.005
Rennes-11	2.359 ± 0.120	0.361 ± 0.023	0.057 ± 0.005
Lc-0	2.372 ± 0.134	0.294 ± 0.031	0.058 ± 0.004
Dijon-G	2.392 ± 0.146	0.449 ± 0.037	0.065 ± 0.006
Cape Verde	2.412 ± 0.142	0.393 ± 0.040	0.063 ± 0.005
T8	2.413 ± 0.079	0.403 ± 0.048	0.055 ± 0.005
Mühlen	2.413 ± 0.024	0.325 ± 0.019	0.054 ± 0.006
Mt-0	2.423 ± 0.135	0.480 ± 0.007	0.050 ± 0.005
No	2.450 ± 0.122	0.347 ± 0.006	0.074 ± 0.004
Col-PRL	2.455 ± 0.012	0.300 ± 0.024	0.053 ± 0.004
Sorbo	2.459 ± 0.111	0.306 ± 0.014	0.053 ± 0.002
Bor-1	2.512 ± 0.136	0.335 ± 0.036	0.046 ± 0.004
Kz9	2.563 ± 0.249	0.446 ± 0.004	0.060 ± 0.005

Table 18 (continued)

Accession	Elongation rate of the upper section (cm/day)	Elongation rate of the lower section (cm/day)	Width growth rate at the second mark (cm/day)
Kendalville	2.581 ± 0.141	0.424 ± 0.022	0.094 ± 0.004
Kondara	2.582 ± 0.112	0.198 ± 0.013	0.062 ± 0.003
Kin-0	2.586 ± 0.111	0.317 ± 0.032	0.039 ± 0.003
Kz-1	2.599 ± 0.293	0.469 ± 0.051	0.042 ± 0.003
Lz-0	2.671 ± 0.127	0.313 ± 0.026	0.070 ± 0.004
Est	2.692 ± 0.108	0.370 ± 0.026	0.084 ± 0.007
Ct-1	2.787 ± 0.128	0.421 ± 0.031	0.068 ± 0.004
Ws	2.847 ± 0.120	0.409 ± 0.038	0.055 ± 0.005
En-1	2.860 ± 0.127	0.380 ± 0.034	0.053 ± 0.005
RLD1	2.874 ± 0.170	0.496 ± 0.045	0.058 ± 0.004

The elongation rate of the upper section (Table 18) may reflect the speed of both cell division and cell elongation while the elongation rate of the lower section will only indicate the rate of cell elongation. Longitudinally, cell division occurs in the apical meristem and cell elongation occurs just below the apical meristem until the cell reaches maturity. Consequently, the cells along the inflorescence stems from the apical meristem to the basal mature region have a gradual order of cell development, with the upper sections comprising shorter cells than the lower sections (Imoto et al., 2005).

The gradient of cell length in the upper section and lower section along the inflorescence stem is present not only in the epidermal cells, on which the marks and measurements were performed, but also in the inner tissues (Imoto et al., 2005). Therefore, the measurements of elongation rates of the upper section and lower section reflect the longitudinal rates of cell development in the inner cells such as lignified xylem cells and interfascicular fibers (Imoto et al., 2005).

Similarly, the gradient of cell widths along the inflorescence stem corresponds in both epidermal cells and inner cells (Imoto et al., 2005). As such, the measured width growth

rate in Table 18 will reflect the cell width change of inner cells such as in xylem cells and interfascicular fibers at the second mark on the inflorescence stems.

It is unlikely that width development at the second mark on the inflorescence stems occurrs by cell division in the cambium and interfascicular cambium because only primary growth can be expected in the *Arabidopsis* inflorescence stem under the conditions used in this project. Previous studies show that the secondary growth of cells in the vascular bundles and lignified arc of *Arabidopsis* only occurs to a significant level when plants are grown under a long-night photoperiod and/or very low density or when the developing inflorescences are cut (Altamura et al., 2001; Barriere et al., 2005; Levyadun, 1994; Levyadun, 1994; Ye et al., 2002). The plants of *Arabidopsis* accessions in this project were grown under long-day photoperiod, with the density of 72 plants per Petri dish and without cutting of inflorescences. Thus, the changes of width at the second mark on the inflorescence stem probably mainly indicate the rate of secondary wall thickening of cells such as protoxylem, xylem parenchyma, metaxylem, and interfascicular fibers, all of which are accompanied by deposition of lignin.

In short, the results of elongation rate of the upper section may reflect the speed of both cell division and cell elongation of the early development stage of outer and inner cells. The elongation rate of the lower section may indicate the rate of cell elongation of the outer and inner cells of the inflorescences stem section. The width growth rate at the second mark may reflect the rate of secondary cell wall thickening of cells such as protoxylem, xylem parenchyma, metaxylem, and interfascicular fibers.

3.4.5.2 Correlation between lignin content and growth rate among *Arabidopsis* accessions

The elongation rate of the upper section, elongation rate of the lower section, and the width growth rate at the second mark on the inflorescence stems of forty-seven *Arabidopsis* accessions were used to determine whether lignin content of the whole stem is correlated with growth rate. Canonical correlation analysis reveals that growth rate in terms of all of the above three growth parameters has a week correlation with lignin content in *Arabidopsis* accessions (R^2 = 0.48, P = 0.011). The original variable of the elongation rate of the upper section is negatively related with its canonical variate (R^2 = 0.34). The original variables of the elongation rate of the lower section and the width growth rate at the second mark are positively related to their respective canonical variates (R^2 = 0.43 and R^2 = 0.37 respectively). This shows that growth rate and the lignin content of *Arabidopsis* are not thoroughly independent and thus the three growth parameters relate with lignin content in different ways.

A simple correlation between two sets of variables also shows that the elongation rate of the lower section is positively related to lignin content in *Arabidopsis* accessions (R^2 = 0.205) (Figure 36). This result indicates that accessions with high cell elongation rate have high lignin content in the inflorescence stems. A high lignin content would provide mechanical rigidity to strengthen the inflorescence stems of *Arabidopsis*, aiding upward growth of the plants.

Since the inflorescence stems of *Arabidopsis* comprise different cell types, the pattern of cell elongation is not simple. For example, the interfascicular fibers may undergo dramatic intrusive growth with the aid of their tapered ends and they are five to eight

times longer than their neighboring parenchyma cells (Ye et al., 2002). The elongation rates of different cells are not the same. However, because of the correlated growth of the epidermal cells and the cells of inner tissues along the inflorescence stems (Imoto et al., 2005), the measured elongation rate of the lower section could be an indication of the elongation rate of all cells and thus may reflect the positive correlation with lignin content in the *Arabidopsis* accessions.

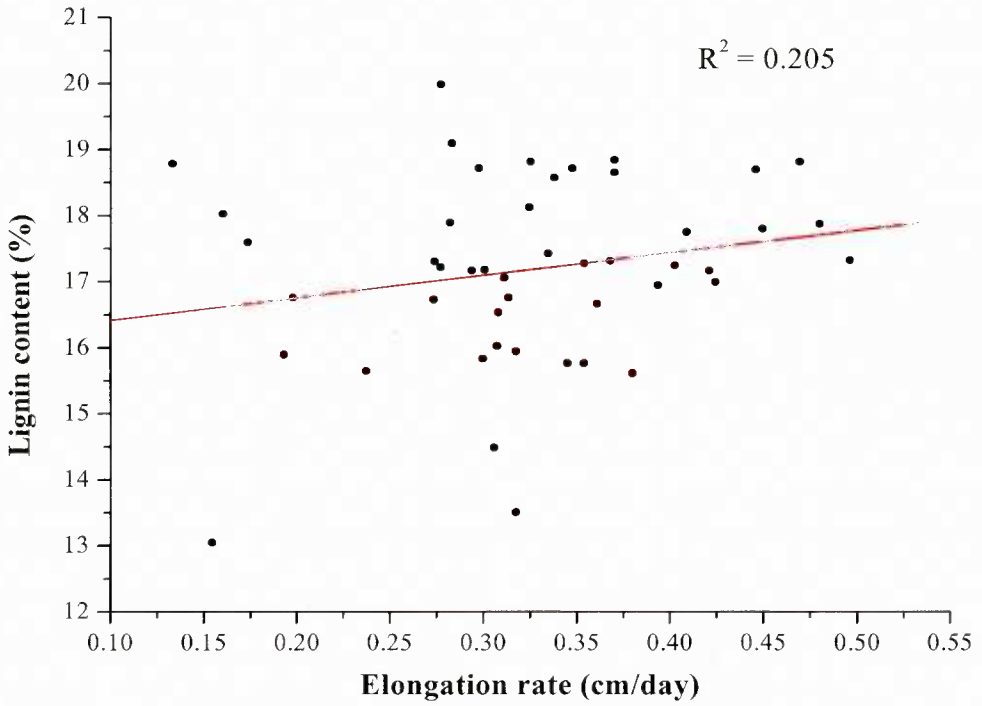

Figure 36: Correlation between lignin content and the rate of cell elongation of the lower section of *Arabidopsis* accessions

Consistent with the canonical correlation analysis, a simple correlation between two sets of variables shows that the elongation rate of the upper section is negatively related to lignin content in *Arabidopsis* accessions ($R^2 = 0.163$) (Figure 37). This indicates that *Arabidopsis* accessions with high rates of cell division have low lignin content in the inflorescence stems. The measured values of the elongation rate of the upper section

reflect the rates of both cell division and cell elongation in the section. Whereas, the elongation rate of cells has shown a positive correlation with lignin content. It is possible that the rate of cell division could have a strong negative correlation with lignin content, resulting in a net negative correlation between the elongation rate of the upper section and lignin content. This observation is consistent with the work by Kirst et al. (2004) who demonstrated a correlation between a high rate of lignin biosynthesis and reduced cell division in the radial direction of hybrid populations of Eucalytus. The negative correlation between cell division and lignin content could be due to the competition for carbon flow between lignin biosynthesis and cell division during growth of plants, as discussed by Kirst et al. (2004).

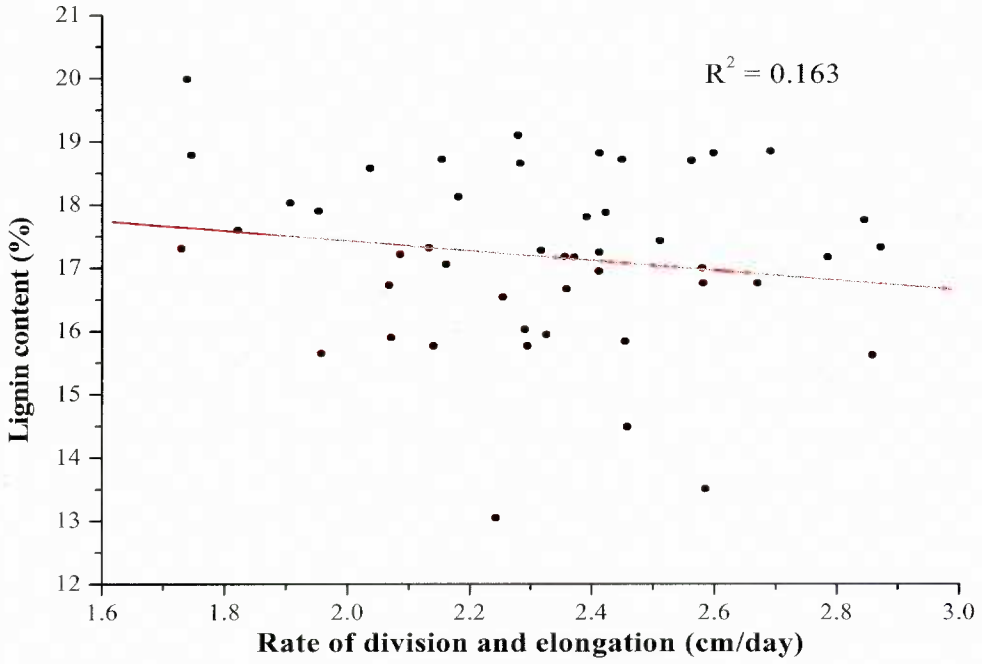

Figure 37: Correlation between lignin content and the rate of cell division and elongation of the upper section of *Arabidopsis* accessions

Moreover, a simple correlation between two set of variables shows that the width growth rate at the second mark is positively related to lignin content in *Arabidopsis* accessions (R^2 = 0.18) (Figure 38). This may show that *Arabidopsis* accessions with high rates of secondary wall thickening of cells have high lignin content in the inflorescence stems. Since lignin is deposited chiefly in the thickened secondary wall, the accessions with high proportion of secondary wall in the inflorescence stems would have more lignin. The accessions with high rates of secondary wall thickening probably would have high amounts of secondary wall and thus high lignin content.

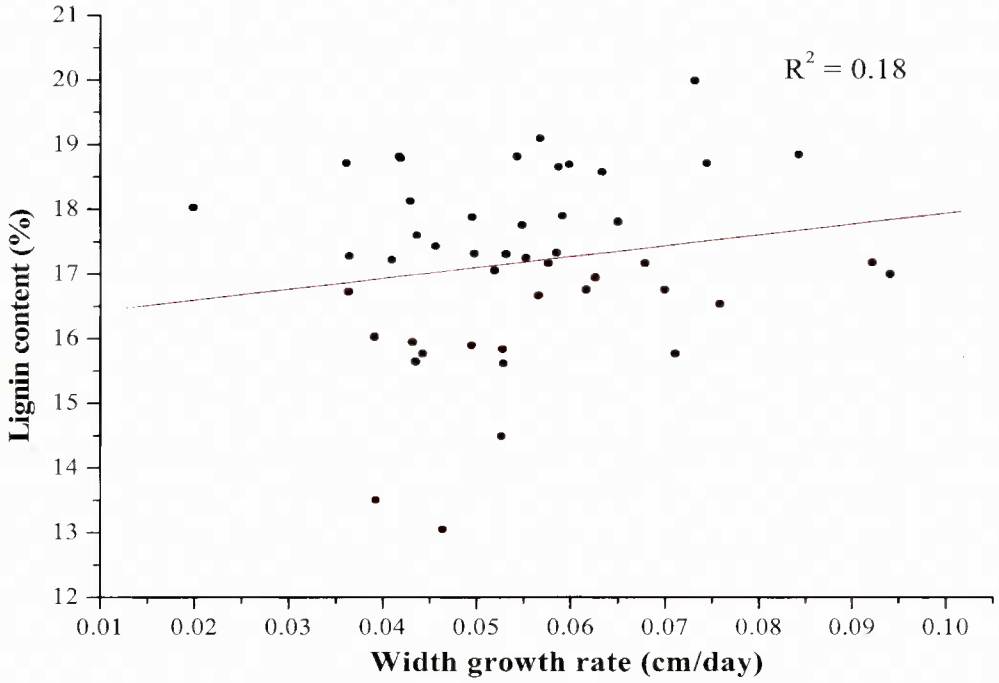

Figure 38: Correlation between lignin content and the width growth rate at the second mark on the inflorescence stems of *Arabidopsis* accessions

In conclusion, growth rate and lignin content of *Arabidopsis* are weakly correlated. The elongation rate of the lower section is positively related to lignin content in *Arabidopsis* accessions, whereas, the elongation rate of the upper section is negatively related to lignin

content. The width growth rate at the second mark is positively related to lignin content in *Arabidopsis* accessions. Therefore, *Arabidopsis* accessions with high cell elongation rate are likely to have high lignin content the inflorescence stems. The accessions with high rate of cell division tend to have low lignin content. The accessions with high rates of secondary wall thickening of cells have high lignin content.

3.4.6 Correlation between lignin content and plant height among *Arabidopsis* accessions

The results of lignin content determination in *Arabidopsis* accessions could be also used to examine if lignin content has a correlation with plant height among *Arabidopsis* accessions. Figure 39 shows a correlation exists between lignin content and plant height among *Arabidopsis* accessions ($R^2 = 0.491$, $P < 0.0001$). The results indicate that tall *Arabidopsis* accessions are likely to have high lignin content in the inflorescence stems. Tall plants have long xylem paths through which water is pulled from the soil to leaves by evaporation. Since a long xylem path will cause high hydraulic resistance, high negative pressure in stems is required so that the water can flux up the plant under tension according to the cohesion tension theory. The mechanical strength of stems required to endure the high negative pressure can be provided by lignin; therefore, high lignin content in the stems of tall *Arabidopsis* accessions allows water to be conducted through the xylem without collapse of the tissue. Moreover, high lignin content in the stems of tall *Arabidopsis* accessions would provide mechanical support for the inflorescence stems of *Arabidopsis*.

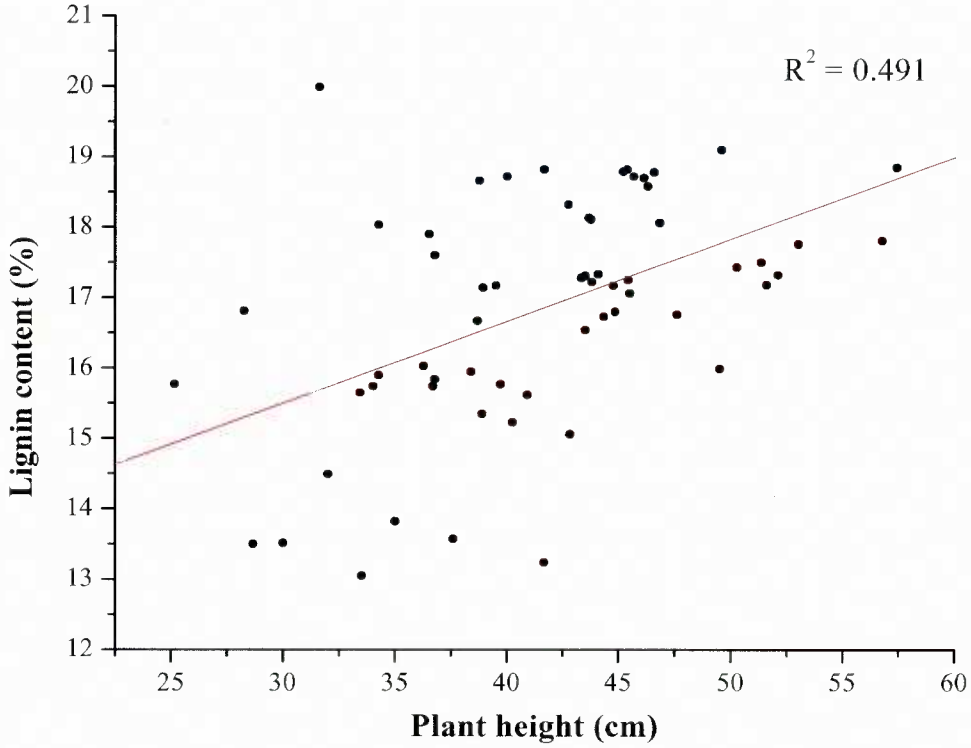

Figure 39: Correlation between lignin content and plant height among *Arabidopsis* accessions

4 CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

The acetyl bromide method was modified to enable the rapid micro-scale determination of lignin in *Arabidopsis* accessions with the goal of ascertaining the genes that control lignin content in plants. The modifications included the use of a micro-ball mill, adoption of a modified rapid method of extraction, use of an ice-bath to stabilize solutions and reduction in solution volumes.

Using a micro ball mill, 0.1 g of *Arabidopsis* stem sample could be ground to 80-mesh in two minutes. The ground stem could be completely digested in 25% acetyl bromide in glacial acetic acid after heating at 70 °C for 30 min.

The rapid extraction method, adapted from Morrison's method (1972), had high extraction efficiency and facilitated the handling of small amounts (mg quantities) of samples. Using the modified rapid method of extraction, approximately 25 *Arabidopsis* samples could be extracted per day. In contrast, the normal extraction method applying a Soxhlet apparatus (four-stage Soxhlet extraction) requires 32 hours for one cycle of extraction using water, ethanol, acetone and diethyl ether. The rapid extraction removed various extractives slightly more efficiently than the four-stage Soxhlet extraction, probably due to the use of finer and smaller amounts of sample with the former extraction. The similarly thorough extractions by the rapid and the four-stage Soxhlet extraction methods led to truer lignin content determination than when a single-stage

ethanol/benzene Soxhlet extraction was used. This was true for lignin determinations by both the sulfuric acid method and acetyl bromide method.

Scaling down the volume of the digested solution accelerated the process of sample treatment through the use of micro-pipettes or multi-channel pipettes for solution transfer. The volume of the samples for the UV absorbance measurement was first reduced from 50 mL to 2.5 mL, and then further scaled-down to 0.25 mL, allowing for use of microplates. The scaling-down did not affect the accuracy and precision of the acetyl bromide method.

Cooling the digested samples in an ice bath stabilized the spectral properties of the samples prior to UV measurement, allowing many samples to be handled at the same time. The error in absorbance caused by total time elapse during transfer of the solution from ice bath and the UV absorbance measurement would be less than 0.5%.

The modified methods using cuvettes (2.5 mL scale) and microplates (0.25 mL scale) were shown to be accurate and precise with values in agreement with those determined by the conventional acetyl bromide method. The modified methods can not only identify samples with the same lignin concentration but also distinguish between samples with different lignin concentrations. The relative standard deviation of the measurements by the modified method using cuvettes was approximately 2.5% while for the modified method using microplates the standard deviation was around 3.4%.

The extinction coefficient for *Arabidopsis* lignins dissolved using acetyl bromide was determined to be 23.35 ± 0.13 $g^{-1}Lcm^{-1}$. This value is independent of the *Arabidopsis* accession, environmental growth conditions and is insensitive to syringyl/guaiacyl ratio.

Using the single extinction coefficient, 23.35 ± 0.13 $g^{-1}Lcm^{-1}$, for lignin determination in a wide range of *Arabidopsis* samples, the acetyl bromide method was shown to be well correlated to the 72% sulfuric acid method after correction for protein contamination and acid-soluble lignin (R^2 = 0.988, P < 0.0001). The ratio between the measured values of lignin content in *Arabidopsis* by the two methods was close to 1.0. The high correlation between the measured values by the two methods indicates that rapid micro-scale acetyl bromide method is a valid way of determining lignin content of *Arabidopsis* accessions.

As determined by the modified acetyl bromide method and confirmed by the sulfuric acid method, lignin content in *Arabidopsis* has been shown to be a divergent property. Natural variation of lignin content exists among Arabidopsis accessions with approximately 10% of the accessions having less than 15.0% of lignin and 10% of accessions having more than 18.5% of lignin in the whole inflorescence stem.

Lignin content determined by the modified acetyl bromide method was found to be weekly correlated with growth rate among *Arabidopsis* accessions (R^2 = 0.48, P = 0.011); therefore, growth rate and lignin content of *Arabidopsis* are not thoroughly independent.

Lignin content determined by the modified acetyl bromide method was also found to be positively correlated with plant height among *Arabidopsis* accessions (R^2 = 0.491, P < 0.0001).

4.2 Future work

This study has addressed the determination method of lignin content in *Arabidopsis* accessions harvested at mature stage. The determination of lignin content in *Arabidopsis* accessions was performed using plants grown under one controlled condition to provide a

basis for the genetic study of lignin in *Arabidopsis*. In terms of a wider application of the method in genetic studies, the following future work would be helpful.

4.2.1 Extinction coefficient of lignin of *Arabidopsis* harvested at various growth stages

In this study, a single common extinction coefficient for *Arabidopsis* lignins, dissolved using acetyl bromide, has been obtained using the lignins isolated from plants representative of the whole population of *Arabidopsis* accessions and grown under different environmental variations. The extinction coefficient can be applied, with confidence, to lignin content determination of inflorescence stems of different *Arabidopsis* accessions and their RIL progenies grown under various conditions and harvested at mature stage. However, it is uncertain whether the average extinction coefficient can be used for the determination of lignin content in *Arabidopsis* lines at different growth stages, especially as lignin structure is known to vary with developmental stage (Kim et al., 2004). Thus, it is important to test if a single extinction coefficient can be used for lignin in *Arabidopsis* samples collected at different growth stages.

4.2.2 Stability of the lignin content divergence under different growth conditions

In this study, lignin content has been shown to be a divergent property among *Arabidopsis* accessions. However, for the genetic studies it is also important that this divergence in lignin content is independent of growth conditions. Therefore, the

accessions with extreme values of measured lignin content should be grown under various growth conditions and then analyzed for lignin content.

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