Research Journal of Pharmacognosy (RJP) 4(2), 2017: 1-13
Received: Oct 2016
Accepted: Jan 2017

Original article

Characterization of the lignin polymer in Brassicaceae family

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Abstract

Background and objectives: Residues of medicinal plants after extraction and weeds are suitable candidates for bioethanol production. Significant barriers exist to make the conversion of lignocellulosic feedstock to biofuel cost effective and environmentally friendly; one of which is the lignin polymer. Brassicaceae family is one of the potential targets for biofuel production. The structural characteristics of lignin from *Hirschfeldia incana, Sisymbrium altissimum* and *Cardaria draba* were studied in comparison to that of *Brassica napus.* **Methods:** Lignin deposition was observed by phloroglucinol and Mäule staining. The total lignin content was determined by Klason method. Maximum UV absorbance and FT-IR spectra were compared. Ratio of syringyl to guaiacyl lignin (S/G ratio) as a metric of lignin digestibility was determined by DFRC followed by GC-MS analysis. ^{1}H-NMR spectra of the total lignin was compared with other spectroscopic methods. **Results:** Staining of the stem cross sections of *C. draba* showed higher G units in contrast to the higher S units in *S. altissimum* which was in agreement with ^{1}H-NMR analysis. Total lignin content for *H. incana*, *C. draba* and *S. altissimum* was 27.10%, 23.8% and 24.5%, respectively. The specific maximum UV absorbance appeared between 230-260 nm. FT-IR analysis confirmed the presence of more aromatic structures in the seed maturation stage than the flowering stage. S/G ratio was 0.26, 0.10 and 0.22 for *H. incana*, *C. draba* and *S. altissimum*, respectively. **Conclusion:** Except *Cardaria draba* with the predominance of G subunits in lignin polymer, *Hirschfeldia incana* and *Sisymbrium altissimum* are suitable candidates for bioethanol production.

Keywords: Brassicaceae, *Cardaria draba, Hirschfeldia incana*, lignin, *Sisymbrium altissimum*

Introduction

Growing demand for energy, concerns about greenhouse gas emission and energy security of fossil fuels, encourage scientists to investigate renewable and sustainable biofuel resources [1]. Lignocellulosic biomass from agricultural wastes is of considerable interest as the second generation feedstock for bioethanol production. Although various potential biomass feedstocks are available to meet the criteria of biofuel targets, several obstacles remain to making this process beneficial [2]. One of the mechanical hurdles affect the feasibility in processing cellulosic ethanol is the complex lignin matrix in which crystalline cellulose polymer is embedded. Lignin adsorbs hydrolytic enzymes, and acts as a major barrier for these enzymes to access

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cellulose, and negatively affects cellulosic ethanol production [3]. The interaction between cell wall lignin and polysaccharides should be reduced by a physical, chemical, biological or combined pretreatment [4]. Reduction of processing cost is a key challenge to commercializing cellulosic fuel production. Pretreatment is one of the most costly steps in biomass conversion [5]. Therefore, optimal characteristics are essential for the selection of bioenergy crops.

Lignin as a secondary metabolite is one of the most abundant natural polymers (accounts for about 30% of organic carbon in the biosphere) and the building block of secondary cell walls in plants [1]. This aromatic polymer provides strength for vascular tissues and allows water conductance under negative pressure [6]. Differing in the degree of methoxylation, *p*-coumaryl, coniferyl and sinapyl alcohol (the end products of the monolignol pathway) result in the formation of H, G and S lignin, respectively (figure 1) [7]. While gymnosperms (softwoods) are rich in G lignin, dicotyledonous angiosperms (hardwoods) are classified as G-S groups and herbaceous plants (monocots) contain H-G-S lignin [8]. Using laccases/peroxidases, phenolic radicals are formed from monolignols, which then cross couple with the growing polymer. The phenoxy radicals on the polymer form linkages on their 4-*O*- or 5- positions. Since the 5-position is occupied by a methoxy group in S units, ß-5 coupling is only observed in G units. ß-*O*-4 linkages are considered as non-condensed while C-C linkages are known as condensed which results in the higher degradation resistance of G rich polymers in comparison to high S content lignins.

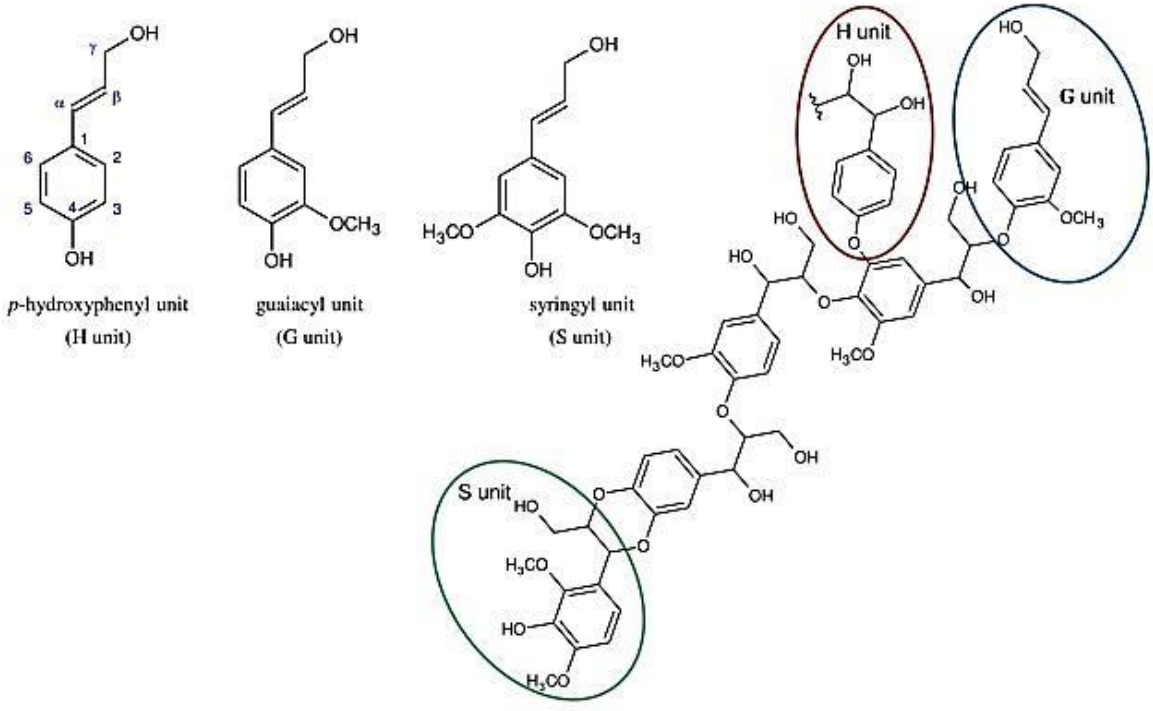

Figure 1. Lignin polymer and *p*- coumaryl, coniferyl and sinapyl alcohol, the final products of the monolignol pathway which result in the formation of *p*-hydroxy phenyl (H), guaiacyl (G), and syringyl (S) lignin units, respectively

ß-*O*-4 linkages are observed in monolignol couplings both to a G or S unit [7]. This results in the higher frequency of ß-ether linkages (50-80% of linkages in hardwoods and 45-50% of linkages in softwoods) which are easily cleaved chemically by treatments like thioacidolysis or derivatization followed by reductive cleavage (DFRC) [9]. Since the DFRC analysis is performed under milder experimental conditions with tolerable odor and cleaner ion peaks, this method is currently preferred to the conventional thioacidolysis. The measurement of S to G moieties is an indicator of the type and number of crosslinks in lignin polymer and S/G ratio is a metric of lignin digestibility which leads to the identification of biomass species with reduced recalcitrance [10]. However, when the S/G ratio is higher than a moderate value, no further improvement is observed in lignin deconstruction [10].

Considerable variation in lignin composition exists in natural populations [10]. Screening for biomass resource with low lignin content or lignin composition with higher digestibility is one of the strategies to investigate suitable feedstocks. *Brassica napus* L. -a salt tolerant crop- is widely adopted in many areas under variable temperatures. It is a short rotation crop and grows at high density [12]. *B. napus* is globally one of the largest seed producers as the edible oil. In spite of superior combustion properties of the biodiesel produced from canola oil, a huge concern about consequences on the food security exists [13]. On the other hand, an enormous amount of canola stem and stover is potentially available for fuel production [14]. The cultivation area of canola in Iran exceeds 100,000 ha, with 2035 kgh^{-1} and 1580 kgh^{-1} of product in Chaharmahal-va-Bakhtiari and Golestan, the western and northern provinces respectively [15]. With annual production of 500,000 tons of canola straw in Iran, a potential candidate for bioethanol production is available [16]. Competitive suppression of crop productivity by weeds is a major challenge. Weeds of Brassicaceae family reduce the productivity of canola plants and reduce the oil quality [17]. Available herbicides do not have selectivity against mustard weeds in comparison to mustard crops [18]. There is also the possibility of inter-specific crossing of pollen movement from herbicide tolerant canola cultivars to mustard weeds [19]. *Hirschfeldia incana* L. Lagr.-Foss sometimes called Mediterranean mustard and *Sisymbrium altissimum* L. (tall mustard) are perennial plants of Brassicaceae family which have been determined as an injurious weed worldwide and in canola farms as well. These noxious weeds can be considered as a potential cheap feedstock for the production of lignocellulosic ethanol. Residues of medicinal plants are also potential candidates for biofuel production after the extraction of the active components. *Cardaria draba* L. Deav. (hoary cress) the other allelopathic wild type member of Brassicaceae is native to Western Asia. *C. draba* has been introduced as a medicinal plant containing glucosinolates, flavonoids, alkaloids and saponins with tonic, diuretic and antimicrobial properties [20-22].

Although several studies on the seed coat lignin content of *B. napus* and its manipulation have been conducted [23,24], an intensive study on the stover of the other species of Brassicaceae family has not been performed. In this study, we have determined lignin characteristics of *H. incana*, *S. altissimum* and *C. draba* stover by localization of lignin monomers using phloroglucinol and Mäule staining. A quantitative determination of the S and G lignin was performed by GC-MS after a DFRC procedure to measure S/G ratio. In addition to Klason and UV spectroscopic analysis, FT-IR and NMR characteristics were measured to find possible correlations between various methods. Although, harvesting weeds at the flowering stage helps to decrease the threat to canola crops, lignin properties in the seed bearing stage have been analyzed to observe the effect of the developmental stage on the lignin accumulation.

Experimental

Chemicals

Phloroglucinol, ethanol (analysis grade), HCl 37%, potassium permanganate ($KMnO_4$), ammonia solution (32%), acetylbromide, acetic

acid, pyridine and 4,4'-(propane-2,2-diyl) diphenol were purchased from Merck Millipore (USA). Glutaraldehyde, 1,4-dioxane (99.8%), Zinc dust (98%) and dimethyl sulfoxide-*d6* (DMSO-*d6*) were from Sigma-Aldrich (USA). H_2SO_4 (96%) was from Daejung Metals & Chemicals Co Ltd., (South Korea).

Plant material

Wild type *S. altissimum* and *C. draba* plants were harvested from Roknabad, Shiraz, (4900 feet altitude) in March 2014. *B. napus* and *H. incana* were harvested from canola fields in Gachsaran (Iran) where the elevation is 720 m from sea level. Samples were gathered in the flowering stage and seed maturation stage in March and May 2014, respectively. Plant samples were identified by the plant systematic specialist and retained at the Herbarium of the School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran. Herbarium specimens of *S. altissimum*, *C. draba*, *H. incana* and *B. napus* were deposited under DBSH-102, DBSH-103, DBSH-110 and DBSH-111 voucher numbers, respectively. Fresh plant material was used for staining procedures. The stems were then dried in air at room temperature and milled for further analysis.

Histochemical staining

For phloroglucinol staining, hand sections of inflorescence stems were incubated in 16% ethanol (v/v), 10% HCl (v/v), and 0.2% phloroglucinol for 5 min, and then photographed with a SONY camera (Japan) attached to a CETI light microscope (UK). For Mäule staining, hand sections of inflorescence stems were fixed in 1.25% glutaraldehyde, rinsed with H_2O, and treated for 10 min with 0.5% $KMnO_4$. Sections were rinsed twice with water, treated with HCl 10% (5 min), washed and mounted in concentrated NH_4OH for examination under light microscope [25].

Cell wall preparation and Klason lignin analysis

Milled stems were washed repeatedly with 80% ethanol at 80 °C in 15-30 min intervals until the supernatant became clear. Dried cell walls were used for Klason analysis. Klason lignin preparation was performed according to Weng *et al*. [26]. Briefly, 3 mL of 72% H_2SO_4 was added per 100 mg cell wall and incubated at 30 °C for 30 min. The sulfuric acid of samples was diluted to 4% and autoclaved at 120 °C for 1 h. The residue was filtered, dried and weighed.

DFRC analysis

The DFRC lignin analysis was performed according to Lu and Ralph [27]. Briefly, cell walls were dissolved in acetylbromide/acetic acid solution, containing 4,4'-(propane-2,2-diyl) diphenol as internal standard. The reaction products were dried down using nitrogen gas, dissolved in dioxin/acetic acid/water (5/4/1, v/v/v), reacted with zinc dust and purified with C-18 SPE column (SUPELCO, USA). After acetylation with pyridine/acetic anhydride (2/3,v/v), the lignin derivatives were analyzed by an Agilent 7890A (USA) gas chromatograph attached to a 7000 triplequad mass detector with a 0.25 mm × 30 m polysiloxan (DBI-MS) column. The GC program was as follows: initial column temperature was 35 °C, hold 3 min; ramp 1 was 25 °C/min to 180 °C, hold 1 min; ramp 2 was 1 °C/min to 235 °C, ramp 3 was 35 °C/min to 300 °C, hold for 10 min.

FT-IR, UV and ^{1}H-NMR analysis

The FT-IR spectra of Klason lignin samples were recorded on a PG Instrument (UK) FT-IR spectrophotometer. KBr pellets were prepared using a lignin concentration of 16% (w/w). Recorded spectra were the average of 128 scans between 400-4000 cm^{-1}. Five mg Klason lignin was dissolved in 10 mL dioxane (99.8%) and stirred for 15 min. The UV spectra were recorded using a Bruker (USA) UV/Vis spectrophotometer and the UV absorbance was measured between 190-600 nm. ^{1}H-NMR spectra were recorded by dissolving 20 mg of lignin in DMSO-*d6* at 300 MHz on a Bruker AVANCE III Ascend™ -300 NMR instrument (USA). The standard Bruker Topspin™ NMR software was used to process data. Chemical structures were drawn by ACD/ChemSketch software version 14.0.1.

Results and Discussion

To evaluate the presence of normal lignin deposition, and changes in lignin characteristics during the maturation process, phloroglucinol and Mäule staining were performed on the stem cross section of plants. Phloroglucinol which defines lignin associated hydroxycinnamaldehyde end groups results in red staining. Compared with the flowering stage, *B. napus* and *H. incana* plants displayed much stronger phloroglucinol-HCl staining at the seed maturation stage (figure 2A-C-E-G). This observation was consistent with the increased total lignin during maturation observed by Klason lignin analysis (table 1). The phloroglucinol staining in the seed maturation stage was more highly colored in *H. incana* (figure 2G) than in *B. napus* (figure 2C) suggesting a higher aldehyde composition. Stem cross sections showed higher G units in *C. draba* (figure 2I) in comparison to higher S units in *S. altissimum* (figure 2K). S and G moieties showed red and brown color in Mäule staining, respectively. As shown in figure 2 the vascular bundles were brown, sclerified parenchyma stained red which indicated the presence of syringyl residue in the secondary cell wall of all plants. Higher intensity Mäule staining in the seed maturation stage *vs* flowering stage of *B. napus* and *H. incana* was consistent with the increased amount of S and G units during maturation confirmed by the DFRC method. Higher S/G ratio has been observed in *S. altissimum* (figure 2L) in comparison to *H. incana* and *C. draba*. Cell wall staining suggested *S. altissimum* and *H. incana*, in their flowering stage, were suitable candidates for digestion. The total amount of lignin content was determined by Klason lignin analysis. Hemicelluloses and cellulose were hydrolyzed and solubilized by sulfuric acid. After removing non lignin components, insoluble lignin provided a black amorphous powder. Klason lignin in hardwood and softwoods is 22-30% and 26-28.8%, respectively [28]. Interestingly, similar total lignin content has been observed for all plants in the flowering stage (table 1). As lignification proceeds during maturation, the total lignin content increases (table 1). Although total lignin in the flowering stage of our plant species was in the normal range, one should consider that even after several solvent extractions which remove low molecular weight compounds; phenolic acids, ashes and proteins can influence Klason lignin and result in the overestimation of the total lignin amount [29].

Derivatization followed by reductive cleavage (DFRC) efficiently cleaves arylglycerol-ß-aryl (ß-O-4) ether linkages in the lignin polymer [30]. While arylether linkages predominate in the lignin polymer, some major linkages like C-C are not cleaved by DFRC [7].

Table 1. Lignin content of cell walls from Klason analysis

Sample	Klason lignin content (% dry weight ± SD) (n=3)
Hirschfeldia incana (flowering stage)	27.10 ± 1.67
Hirschfeldia incana (seed maturation stage)	35.33 ± 2.00
Cardaria draba (flowering stage)	23.8 ± 2.93
Sisymbrium altissimum (flowering stage)	24.5 ± 2.02

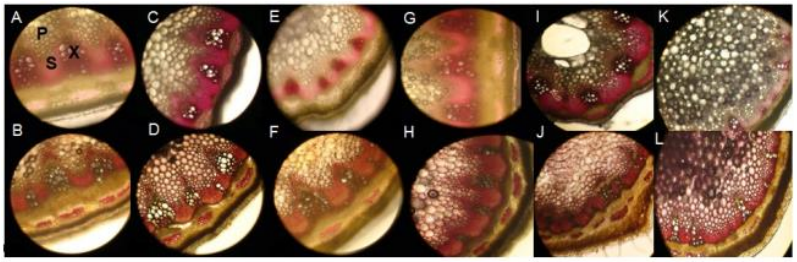

Figure 2. Phloroglucinol-HCl (top) and Mäule (bottom) histochemical staining of the stem cross section; A & B) *Brassica napus* flowering stage; C & D) *Brassica napus* seed maturation stage; E & F) *Hirschfeldia incana* flowering stage; G & H) *Hirschfeldia incana* seed maturation stage; I & J) *Cardaria draba* flowering stage; K & L) *Sisymbrium altissimum* flowering stage; P: pith, S: sclerified paranchyma; X: xylem

The DFRC method would be able to cleave more than half of the linkages in the polymer. Degradation products of arylether inter-unit linkages by the DFRC analysis are G_{cis} and G_{trans} at m/z = 264 as well as S_{cis} and S_{trans} at m/z = 294 (figure 3). H unit is very low or absent in hardwood lignins and was not detectable in our analysis. The content of G and S monomers has been summarized in table 2. The total G content was higher than S unit in all the species. High amount of G monomers determined by DFRC indicated the high arylether type lignin content in these plants.

Since lignins in different sources vary in the degree of crosslinking [31], they noticeably differ in the ratio of the DFRC products. Lignins are sometimes classified as G rich (G/S ratio > 3), S rich (S/G ratio > 3) and balanced (0.3< G/S ratio < 3) [28]. According to DFRC analysis *H. incana*, *C. draba* and *S. altissimum* can be considered as G rich type lignin.G lignin provides water proof structure of xylem in angiosperms while S lignin is responsible for the mechanical strength [32]. The guaiacyl rich structure of these plants indicated that water conductance is superior to mechanical strength. S/G ratio is considered as an important factor in biomass digestibility [8]. This ratio was about 0.2-0.3 for *H. incana* and *S. altissimum* (table 2) which is comparable with the ratio (0.4) observed for *Arabidopsis thaliana* (Brassicacea) [33].

This ratio was 1.3-2 in *Cynara cardunculus*, 1.37, 2.48 and 3.35 for magnolia, birch and beach, respectively [34, 35]. S/G ratio measurement for 800 greenhouse poplars has been reported between 0.5-1.5 [36]. However, one should note that contraindicatory results would be observed on the dependence of digestibility and S/G ratio. While Davison *et al.* [37] argued that a decrease in S/G ratio in poplar improves degradation, Huntley *et al*. [38] has shown that high S/G ratio in poplar decreases recalcitrance. Investigations on the production of ethanol from *B. napus* lignocellulose and efforts on the digestion of canola fibers by ruminal bacteria have shown the necessity of genetic engineering in favor of easy digestible monomers [24,39,40]. The cell wall traits by altering lignin composition have been studied intensively [41]. Studying *Arabidopsis* mutants in which carbon flux was directed toward synthesis of 90 mol% S units resulted in a much higher glucose yield than G rich or wild type samples [42]. Thus, maximizing reactive C-O bonds in interunit linkages yields to more favorable lignin quality for the pretreatment process of Brassicaceae.

To investigate differences in the structure of lignin samples and evaluate the role of growth stage on the lignin formation, infrared spectra of Klason lignins were recorded as depicted in figure 4.

Table 2. Syringyl to guaiacyl ratio measured by DFRC analysis

Sample name	G unit (µmol/gKlason)	S unit (µmol/gKlason)	S/G ratio
Hirschfeldia incana (flowering stage)	4.84	1.28	0.26
Hirschfeldia incana (seed maturation stage)	8.16	1.34	0.16
Cardaria draba (flowering stage)	6.38	0.67	0.10
Sisymbrium altissimum (flowering stage)	3.23	0.72	0.22

The broad band at 3341 and 3348 cm^{-1} in the seed maturation stage corresponds to the OH stretching vibration (figure 4B & 4D). Similar band at 3375 cm^{-1} has been detected for *C. draba* (figure 4E). C-H stretching vibration of methyl or methylene groups appeared at 2923 cm^{-1}. C-H stretching vibration of methoxy groups was near 2860 cm^{-1}. The intensive vibration of lignin which corresponded to the aromatic ring appeared between 1400-1600 cm^{-1} with a higher intensity in the seed maturation stage in comparison to the flowering stage. The peak near 1715 cm^{-1} was assigned to the carbonyl group of coniferaldehyde or syringaldehyde. The peak at 1615 cm^{-1} which had a higher intensity at seed maturation stage of *B. napus* and *H. incana* was assigned to the ring conjugated C=C bond or β-1, β-β, 5-5, or β-5 inter monomeric lignin linkages. The aromatic skeletal vibration appeared at 1504 cm^{-1}. The absence of a peak at 1363 cm^{-1} indicates that all 4-OH groups were etherified. The presence of a peak at 1286 cm^{-1} in the seed

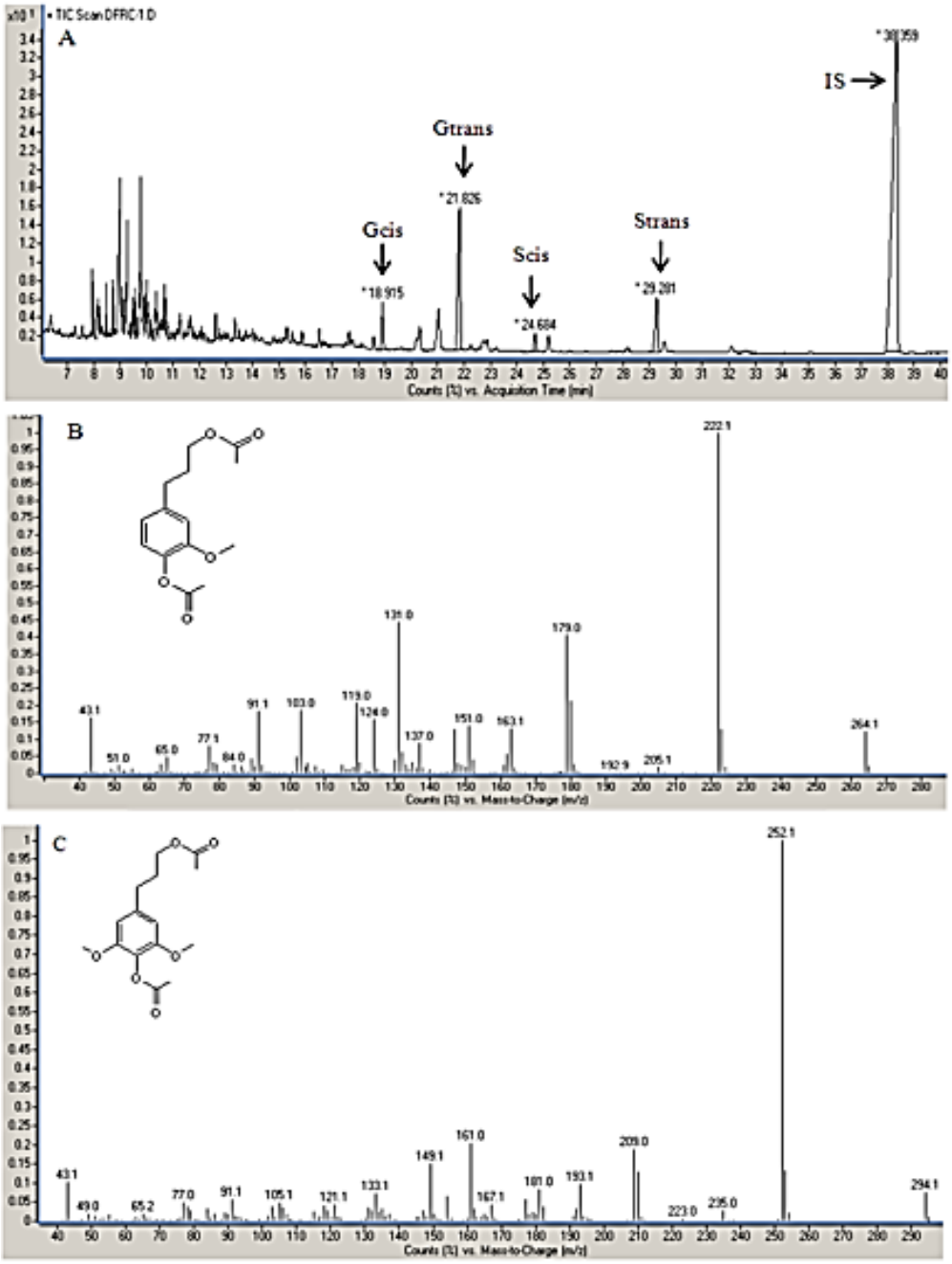

Figure 3. GC-MS analysis of lignin monomer composition; A) DFRC- GC chromatogram of *Hirschfeldia incana* in seed maturation stage. G and S are guiaiacyl and syringyl lignin derived hydroxycinnamyl alcohol peracetates. IS: internal standard; B & C) MS confirmation of the identity of peracetatemonolignols derived from DFRC-GC analysis of G and S units, respectively

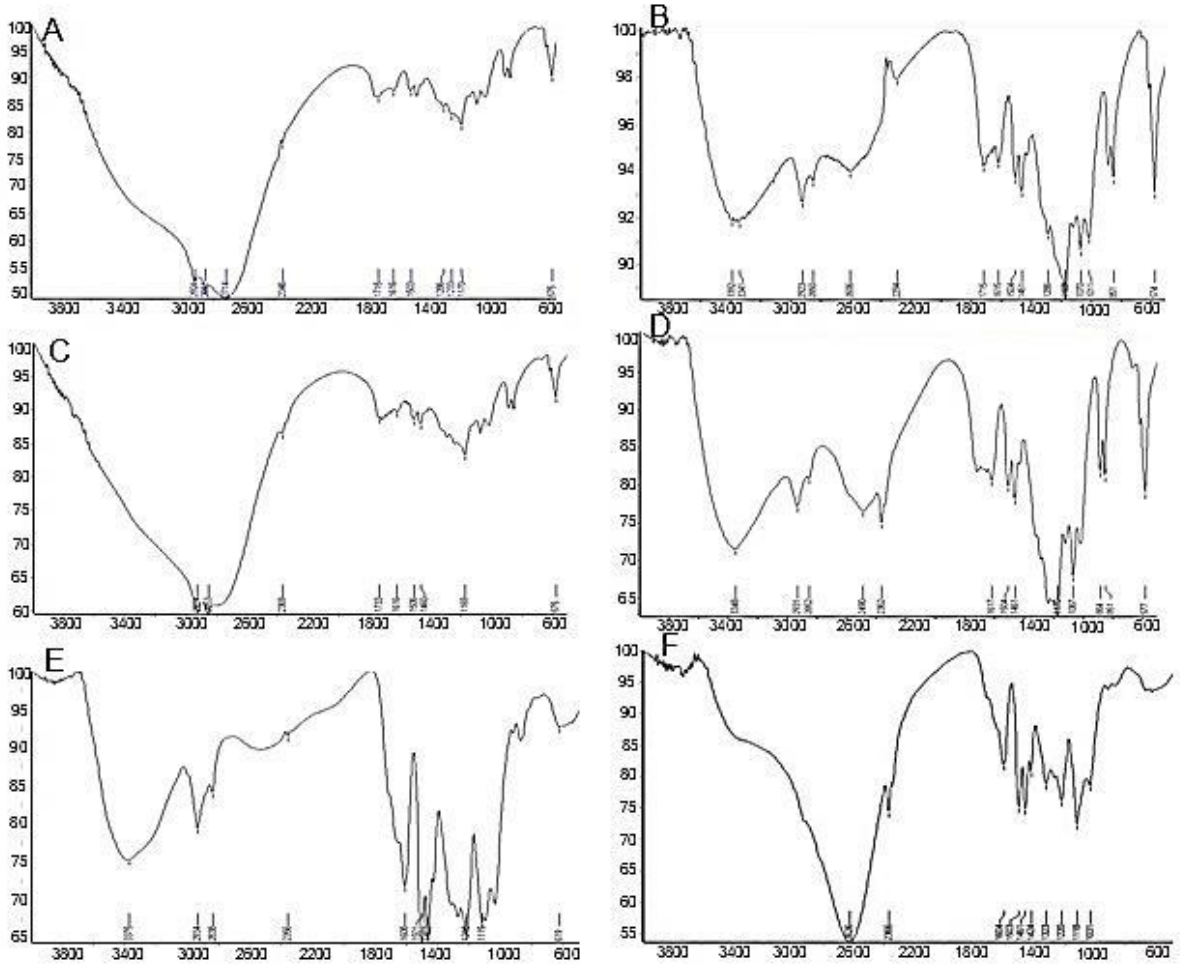

Figure 4. The FT-IR spectra of Klason lignin samples; A) *Brassica napus* flowering stage; B) *Brassica napus* seed maturation stage; C) *Hirschfeldia incana* flowering stage; D) *Hirschfeldia incana* seed maturation stage; E) *Cardaria draba* flowering stage; F) *Sisymbrium altissimum* flowering stage

maturation stage of *B. napus* and its absence in other species demonstrated the greater amount of 4-*O*-5 inter monomeric linkages in canola. The peak at 1170 cm^{-1} is related to the alkyl substituted ether (O-CH_3 or O-CH_2 stretch) and the peak at 1070 cm^{-1} corresponded to the cyclic ether large ring stretching. The intensity of the latter peak was higher in the seed maturation stage, which is indicative of higher cyclic ether linkage. The peak near 851 cm^{-1} was attributed to the vinyl ether. The total FT-IR pattern of the flowering stage of *B. napus*, *H. incana* and *S. altissimum* were similar while *C. draba* had a FT-IR pattern like the seed maturation stage which was in agreement with staining and GC-MS analysis. Dioxan solutions of Klason lignins were subjected to the UV absorption measurement at 190-600 nm. *C. draba* and *S. altissimum* lignins showed the maximum UV absorption at 233 nm and 244 nm, respectively. The maximum absorption of both *H. incana* and *B. napus* appeared at 260 nm. Characteristic maximum UV absorption of lignin spectrum is expected at 200-230 and 260-280 nm [43]. The lower wavelength corresponds to the aromatic structure of lignin while the large cyclic ether ring absorbances appear at higher wavelengths [44]. UV absorption maxima in the seed maturation stage of *H. incana*

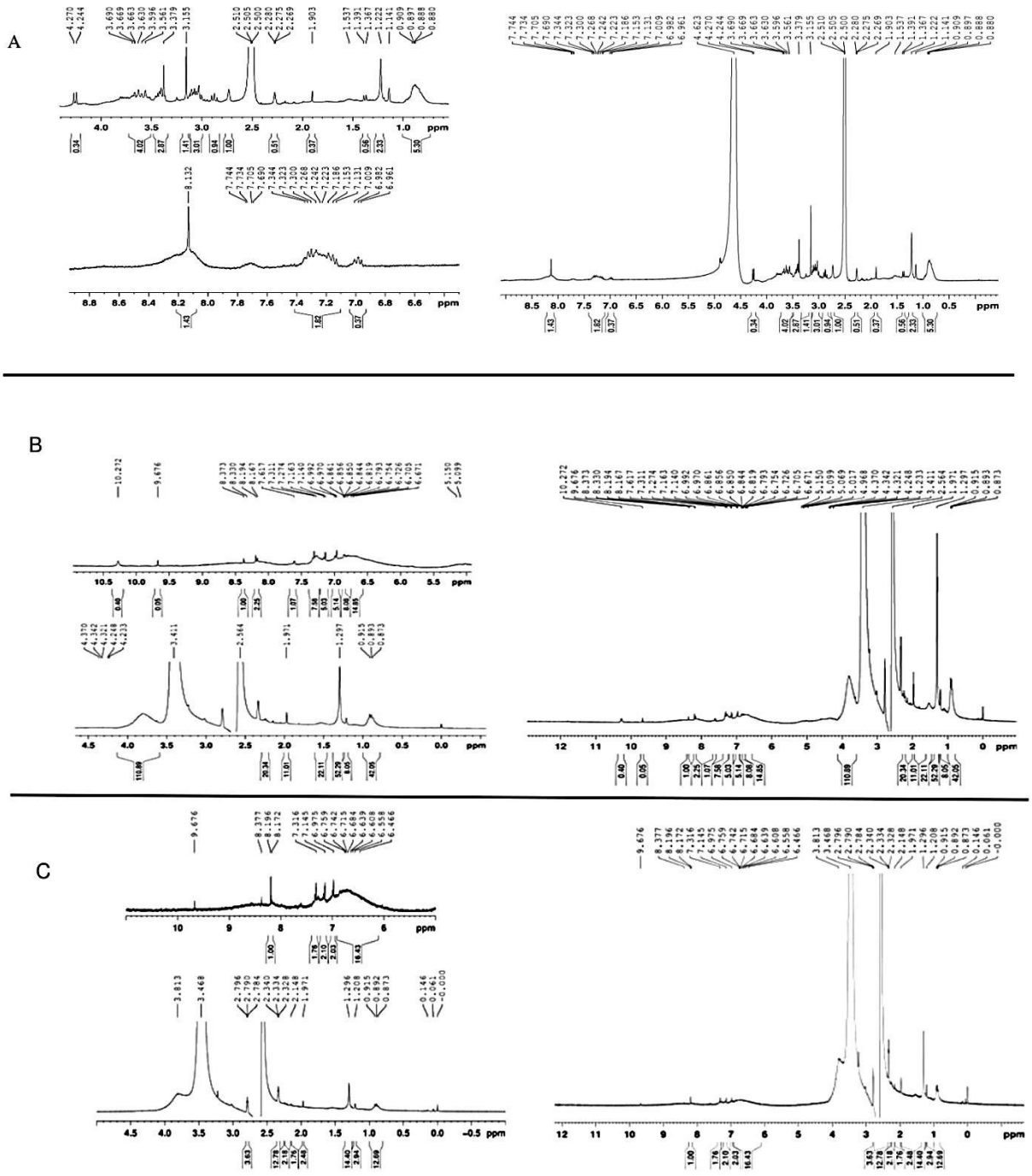

Figure 5. [1]H-NMR spectra of lignin in the flowering stage; A) *Hirschfeldia incana*; B) *Cardaria draba*; C) *Sisymbrium altissimum*

and *B. napus* had higher intensity than in the flowering stage, which is in good agreement with the higher lignin content in the seed maturation stage than the flowering stage confirmed by staining and Klason analyses. To evaluate if independent analysis of lignin with ^{1}H-NMR spectra provided similar interpretations for screening the suitability of lignocellulosic feedstocks for bioethanol production ^{1}H-NMR analysis was conducted on the total lignin (figure 5A-C). Signals between 0.8-1.3 were related to protons in aliphatic groups. Protons of methyl/methylene groups adjacent to carbonyl groups or double bonds appeared between 1.9-2.3 ppm. Residual protons in DMSO-*d6* resulted in a strong solvent peak at 2.5 ppm (with carbon satellite peaks at 2.28 and 2.73 ppm). An intense signal at 3.4 ppm was assigned to polysaccharides which emphasized the necessity for polysaccharide hydrolyses during treatment and prior to analysis. Signals between 3.8-4 ppm were associated with β-O-4 and β-β' moieties. Peaks at 5.3 ppm were assigned to phenylcoumarans. Phenylcoumaran moieties might be very low or absent in these polymers since no peak at 5.3 ppm has been detected. Aromatic protons of the S subunit appeared at 6.25-6.80 ppm. Peaks at 6.80-7.25 have been attributed to the G subunits. Integration of such peaks showed that *C. draba* and *H. incana* were rich in G lignin. *S. altissimum* showed higher syringyl units. However, one must consider the misinterpretation of weak signals as well as overlaps in ^{1}H-NMR spectra. Although this observation was in agreement with former analysis, NMR instruments with higher magnetic field and 2D-NMR techniques are suggested for screening the suitability of lignocellulosic feedstocks.

Usage of the residues of medicinal plants and weeds as nonfood, nonfeed stocks provides a cost effective resource of lignocellulosic material for ethanol production. Histochemical, GC-MS and FT-IR analyses showed that *H. incana*, *S. altissimum* and *C. draba* have the G-S type lignin contents. Total Klason lignin content in the flowering stage for the studied species fell within the normal range in our analyses. So, we propose that harvesting at early stages is appropriate for biofuel targets. Although the total lignin was appropriate, the influence of syringyl to guaiacyl monomer ratio should also be taken into account. S rich lignin is more linear and shows a lower degree of polymerization which results in lower resistance under thermal and chemical treatments, and needs less input energy for digestion during industrial biofuel production. The low S/G ratio obtained in this study by DFRC analysis suggests that the composition of lignin in the candidate feedstocks can be manipulated by genetic engineering for higher S content which results in higher S/G ratio and easier digestion of lignin during industrial treatments.

Acknowledgments

This work was financially supported by Shiraz University of Medical Sciences, Shiraz, Iran under grant numbers 93-01-05-8278 and 94-01-36-9385. This report was parts of the thesis of Navid Mehrabi and Nafiseh Babazadeh.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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