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STRUCTURAL INVESTIGATION OF MISTLETOE PLANTS FROM VARIOUS HOSTS EXHIBITING DIVERSE LIGNIN PHENOTYPES

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The aim of the study was to identify the characteristics of the mistletoe lignin, through the qualitative evaluation of the chemical and biochemical modifications. The analysis of lignins by FTIR spectroscopy was accomplished by evaluating the intensity of the absorption bands, determined by the wide number of functional groups present in the structure of these aromatic compounds. The 1700–900 cm^{-1} domain represents an area in which absorption bands corresponding to the guaiacil and siringil structural units with different substitutions meet. The intensity of the absorption peak is different for the three studied samples. All the investigated samples present a wide central band around the value of 2930 cm^{-1}, assigned to a methylenic spread vibration $\nu(CH_2)$, and a second central band at 2873 cm^{-1}, assigned to a methylic spread vibration $\nu(CH_3)$. In the case of these samples, there can be observed that the spread of the absorption peak is higher for the sample harvested from the birch, decreasing in intensity for the samples harvested from fir and ash, respectively, which can be a proof for including –OH groups at the level of the aromatic rings.

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1. Introduction

The biomass includes any type of regenerable organic material, comprising the terrestrial plants (agricultural crops for foods, trees and crops designed for the production of energy, industrial plants, and feeds) as well as ensemble of wastes and organic residues from agriculture, pisciculture, forestry, municipal wastes and other wastes. The chemical composition of the biomass differs a lot depending on the species, but there can be said that the plants contain (15-30% dry matter) lignin ($C_{40}H_{44}O_6$) and carbohydrates (sugars, glucides). The carbohydrate fraction is composed of many glucidic molecules linked together in long chains or polymers. The two representative categories of carbohydrates are (40-45%) cellulose ($C_6H_{10}O5$) and (20-35%) hemicellulose. The lignin fraction is composed of different molecules from those of the sugars. The long cellulose polymers are used by nature to build fibers that makes the plant rigid [1]. The lignin fraction acts as a ligand which holds the cellulose fibers together. In the past years, advanced technologies have been developed for the conversion of biomass into fuels or for efficient burning. Of course, not all the biomass resources may be used for energy purposes. Biomass represents, at the same time, an important source of food, timber, paper and several valuable chemicals. For this reason, its use for energy purposes must be integrated with other important applications. The use of biomass for energy purposes may bring significant social and economic benefits both for the rural and for the urban areas. The present lack of access to convenient sources limits the quality of life for millions of people all around the world, especially for those in the rural areas and in the developing countries [2]. Lignins are macromolecular substances that accompany cellulose in different organs and tissues of the plants. They are substances very widely spread in the plant regnum, having the second place after cellulose. They are found in the wood of different plant species in proportion of 21-30% dry matter. The linen and

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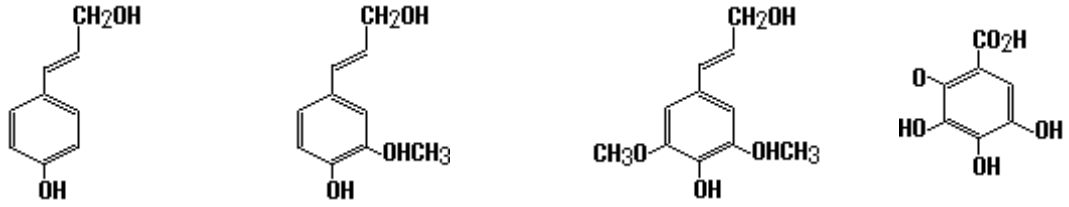
hemp fibers are poor in lignin, and those of jute contain 19% lignin [3]. Lignins are tertiary substances, composed of C, H and O, of aromatic nature [4]. Characteristic to lignin is the presence of methoxyl and hydroxyl groups in the molecule. The main constituent substances for lignin are the aromatic alcohols derived from phenylpropane: coniferilic alcohol, hydroxyconiferilic alcohol and sinapinic alcohol (figure 1 and 2) [5]. Lignins are formed by the condensation of these alcohols. The molecular weight of lignins varies between 700-100.000 Da. They are amorphous substances, dark coloured, insoluble in water, diluted in acids and bases. They are soluble in strong concentrated acids and concentrated bases [6]. Lignins are intertwined with the cellulose fibers through physical and chemical (etheric) bonds.



p-coumaric alcohol ferulic alcohol syringyl alcohol gallic acid

Fig. 1. Monolignols

They confer, to the cellulose fibers, a higher strenght towards the action of water because they reduce their absorbing power, thus decreasing the elasticity. Lignin can be separated from cellulose by dissolving the cellulose in adequate reactants, either by dissolving in alcaline concentrated hydroxids and subsequent precipitation out of the solution [7]. Lignins may be recognized by treating them with a solution of fluoroglucine in clorhidric acid, with the appearance of the red color [8].

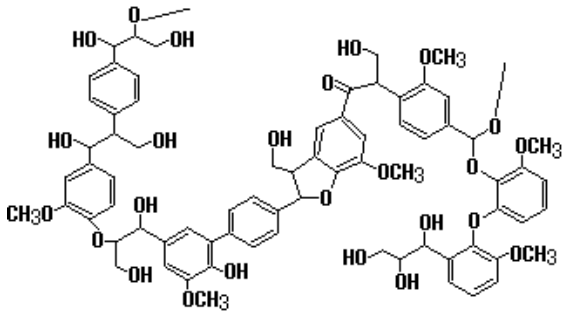


Fig. 2. The major building blocks of lignin (suggests a possible fragment of a lignin co-polymer based on these three monomers)

Because of its complex structure [9], only several types of microorganisms are capable of degrading lignin [10, 11]. Lignins have an important role in forming high stability humus, because it resists the action of chemical agents and are hardly attacked by microorganisms. They are used for the obtaining of synthetic plastic materials, smoke black and as replacement for plant tannins. The study of metabolic processes for the polyphenolic and ligninic products has created the possibility of using them in different scientific areas [12]. There can be said that these products, used in judicious quantities, influence in a positive way the development of plants, take part in soil bioremediation, in the sense of fertilizing it or removing different pollutants. Lignin is one of the main aromatic components that are characteristic to superior plants. Worldwide, this results from the production of cellulose or from the technologies of plant biomass hydrolysis [13] and may be considered raw material with high potential for exploitation [14], as a follow-up of its origin from regenerable resources and due to a low price [15]. Starting from this premise, that lignins from different plants are not identical (they differ by the number and the type of the structural units), in this study there were compared, through IR spectroscopy, mistletoe lignin samples harvested from different hosts. The aim of the study was the evaluation of the changes suffered by the aromatic polymer (lignin) under the action of extracellular enzymatic systems using IR spectroscopy for the lignin isolated from the mistletoe of birch, fir and ash.

2. Materials and Methods

Vegetal samples. The samples were collected from forestry located in western Romania. The plant material used for the determinations, in the laboratory stage, was represented by the stems of mistletoe grown on birch, fir and ash, with a relative humidity of 15%, harvested in November 2011. After the pre-treatment of auto-hydrolysis there are formed two phases: the solid phase (cellulose and lignin) and the liquid phase (monosaccharides, oligosaccharides, degradation compounds) [16-18]. These two phases were analysed and quantified for each component.

Characteristics of mistletoe wood - determination of lignin content. The procedure was referred from TAPPI Standard T 222 os-74. 1 g of air dried extractive free mistletoe was weighed out precisely in weighing bottle and transferred in a 50 mL beaker. 10 mL of 72% H_2SO_4 were added meticulously with a pipetted and the mixture was stirred with a small glass rod (which is left in beaker). The mixture was left quantitatively with a wash bottle (water) to a 500 mL round-bottle flask and diluted until the volume is 300 mL (with water). While the solution was refluxing (boiled under reflex 3 h), a crucible was oven dried for 1 h at 110°C, then allowed to cooled in a desiccator (15 min and precisely weighed. When the refluxing was completed, the insoluble lignin was recovered by filtration through the crucible after allowing the lignin to settle to facilitate filtration. The lignin free was washed from act with 250 mL of hot distilled water. The crucible containing the lignin was dried at 110°C for 1 h, cooled in a desiccator (15 min) and weighed. Lignin content was reported as reported as percentage by weight of the dried sample [19].

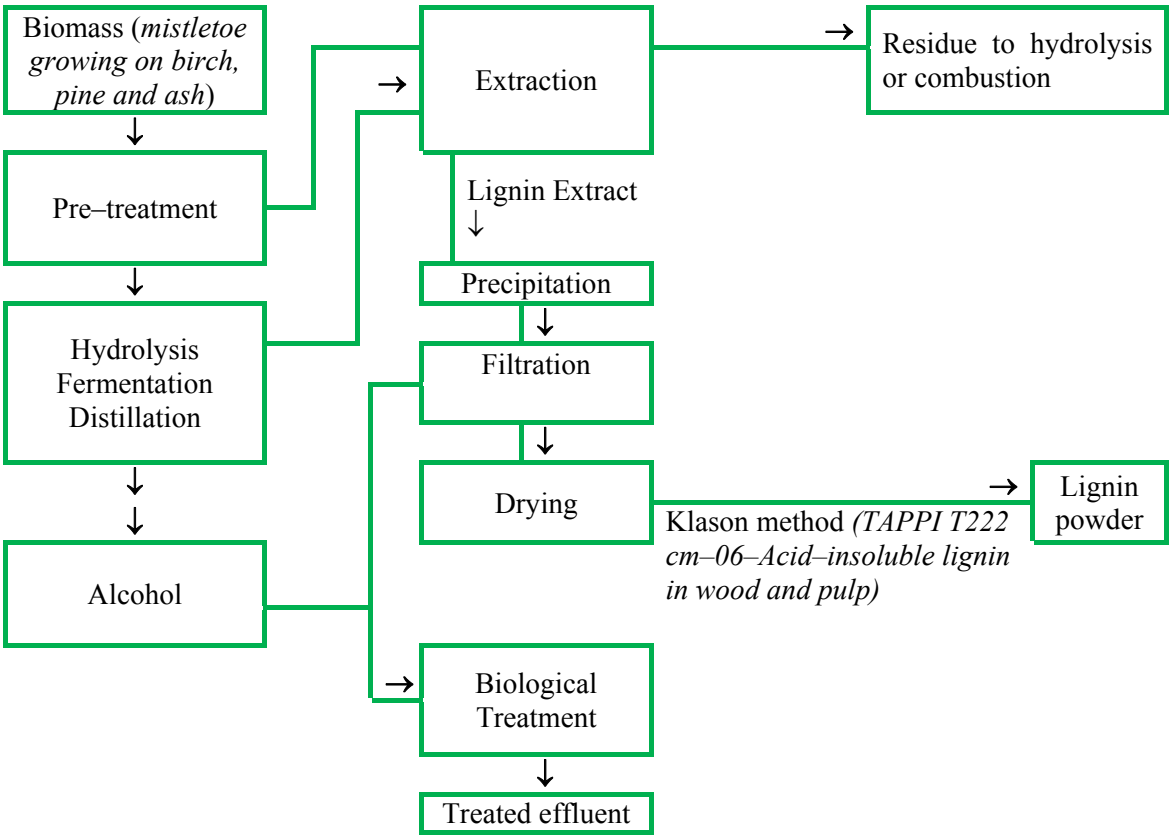


Figure 3. Schematic experimental procedure used to determine the stages of mistletoe lignin increased on birch, fir and ash.

Methods of analysis. The used methods are standardized methods by the *Technical Association of the Pulp and Paper Industry* (TAPPI) and classical methods widely used in the literature. Because these methods are described in standard collections or in the cited literature, they are only informally presented:

- *Cellulose:* cellulose; α-cellulose have been determined according of standard methods (TAPPI T203 cm–09–Alpha–, beta–, and gamma–cellulose in pulp); holocellulose; pentosans –(TAPPI T 223 cm–01–*Pentosans in wood and pulp*); lignin – Klason method (TAPPI T222 om–06–*Acid–insoluble lignin in wood and pulp*);
- *Extraction substances:* extraction with alcohol-benzene mixture (TAPPI T204 cm–07–*Solvent extractives of wood and pulp*, TAPPI T264 cm–07–*Preparation of wood for chemical analysis*); extraction with alcohol (TAPPI T204 cm–07–*Solvent extractives of wood and pulp*, T264 cm–07–*Preparation of wood for chemical analysis*); extraction with cold water (TAPPI T207 cm–08–*Water solubility of wood and pulp*); extraction with warm water (TAPPI T207 cm–08–*Water solubility of wood and pulp*); extraction with 1% solution of NaOH (TAPPI T212 cm–07–*One percent–sodium hydroxide solubility of wood and pulp*);
- *Ash* (TAPPI T211 cm–07–*Ash in wood, pulp, paper, and paperboard: Combustion at 525°C*);
- *Humidity* (TAPPI T210 cm–03–*Weighing, sampling and testing pulp for moisture*; TAPPI T258 cm–06–*Basic density and moisture content of pulpwood*).

The lignin content (L) in the cellulose obtained after the alkaline boiling was determined by the Kappa Index following the equation: L(%)=0.15×Kappa, where: *Kappa* represents the Kappa Index of cellulose [20, 21].

The caloric power of the biomass is closely related to the lignin content. Thus, the superior caloric power for a dry sample that has a lack of ash may be calculated with the equation: Qs = 88.9 (LC) + 16821.8; [kJ/kg], where (LC) represents the lignin content correlated to the dry and ash free state, %.

The superior caloric power of the biofuels can be calculated depending on the fixed carbon content, Cf (%) with the formula: Qs = 196 Cf + 14119; [kJ/kg],

In the literature there were developed formulas for the estimation of the caloric power for the fuels from different lingo-cellulosic materials based on their chemical analysis. For the solid bio-fuels there can be used the modified Dulong's formula, as dependent on the carbon content, C (%), hydrogen, H (%), oxygen O (%) and nitrogen, N (%): Qs = 33500 C + 142300 H–15400 O–14500 N; [kJ/kg],

The following formula can be also found:

$Qsanh$=349.1$Canh$+1178.3$Hanh$+100.5$Sanh$–15.1$Nanh$–103.4$Oanh$–21.1$Aanh$; [kJ/kg],

where $Canh$, $Hanh$, $Sanh$, $Nanh$, $Oanh$, $Aanh$ represent the contents (in percentage) of carbon, hydrogen, sulphur, nitrogen, oxygen and ash, respectively, reported to the anhydrous state [22, 23].

Characterization of lignins by FTIR spectroscopy. FTIR spectroscopy can be used to monitor the functional modifications that occur in the structure of the lignin macromolecule in accordance to the initial samples. The spectra have been recorded in KBr probe (tablet), in a ratio of 1:200. For spectral characterization ((Fourier Transform Infrared spectrometry), samples were prepared as follows: compounds obtained after heat treatment were mixed with potassium bromide powder. Samples were previously dried for 24h, mass ratio of 0.04:1. After advanced grinding in agate mortar, appropriate pressure was applied (0.3 GPa, normal atmosphere); as a result, pellets with thickness of 0.5–0.75 mm and 13 mm in diameter were obtained. Pellets were analysed with a *JASCO 660 PLUS* spectrophotometer in optical area 4000 cm^{-1}–400 cm^{-1}. The work parameters were: resolution 4 cm^{-1} and scan number 20. The FTIR spectra were processed with the help of a specialized program from the *SpectraManager* series.

3. Results

In accordance with the Mullen and collaborators 2010 [24] deploying the bio-char co-product, which contains most of the nutrient minerals from the biomass, as well as a substantial quantity of carbon, to the land can increase soil quality, sequester carbon, and attenuate environmental problems associated with elimination of wood residues from fields and wood and wood waste combusted directly for energy.

Using the methods mentioned in the *Materials and Methods* section, there were obtained the following values for the chemical composition for the mistletoe wood grown on birch, fir and ash, in comparison with the wood composition reported in the literature (expressed as mean value of the measurement ± standard deviation).

Table 1. The chemical composition of the mistletoe wood grown on birch, fir and ash
Proximate analysis (wt%, dry basis) and higher heating values *(HHV in* kJ/kg*, dry basis)*

Samples of mistletoe	References	Birch	Pine	Ash tree
Holocellulose *[(mg/kg dry)]*	69.9±0.7	69.03	66.02	58.01
Cellulose *[(mg/kg dry)]*	46.0±0.7	42.02	44.04	39.02
Hemicellulose *[(mg/kg dry)]*	23.9±0.6	27.03	21.01	18.02
Lignin *[(mg/kg dry)]*	28.4±0.3	28.01	27.03	28.02
Extractibile *[(mg/kg dry)]*	2.3±1.1	2.5	2.4	2.8
Ash *[(mg/kg dry)]*	0.3±0.1	0.2	0.2	0.2
Heating values *[(HHV in* kJ/kg*, dry basis)]*	14.4–22.6	18.9	19.4	20.7

Lignin content in the three samples is approximately the same and has values comparable with the values found in the literature [25]. Lignocellulosic biomass: the alternative to fill the gap. Lignin Potential Mass Yield on biomass: 15 – 30%Equivalent to 60 – 100 + % of ethanol yield. Lignin Energy Content: ~ 28 kJ/kg Equivalent to 93% of energy content of ethanol.

Spectral characterization of the lignin products. The FTIR spectra are recorded as analytical instrument for the qualitative evaluation of the chemical and biochemical changes, occurred subsequent to lignin biosynthesis, according to the type of host.

In comparison to the conventional chemical analyses, this technique requires small sample quantities and a short analysis time. The lignin from birch mistletoe (S1), from fir mistletoe (S2) and the one from ash mistletoe, chosen for testing, were analysed from the spectral point of view, using the FTIR spectroscopy. From the spectral analysis there can be observed that in the 3000–2846 cm^{-1} area there are found vibrations characteristic to the C-H aromatic bonds, aliphatic bonds and phenolic hydroxyl groups. Lignin from ash mistletoe (S3) presents higher peak intensity in the 1710–1600 cm^{-1} domain, which is attributed to the etheric bonds and to the carbonyl groups linked to the aromatic nucleus. The 1700–900 cm^{-1} domain represents a characteristic area for the absorption corresponding to the guaiacyl and siringyl structural units with different substitution. The absorption band present at 3400 cm^{-1}, is attributed to the phenolic hydroxyl groups and to the aromatic structures. In the case of these samples, it can be observed that the width of the absorption peak is higher for the sample harvested from birch (S1), decreasing in intensity for the sample harvested from fir (S2) and ash (S3), respectively, which may be a proof for the introduction of –OH groups at the level of the aromatic nucleus. The 2873–2934 cm^{-1} domain represents an area in which there are found absorptions corresponding to the vibrations of methoxy groups linked to the aromatic nucleus, methyl-alifatic groups and vibrations of the –C-O- un-conjugated bonds specific to the ketonic, carbonylic and eteric groups.

4. Discussion

For the lignin sample of ash mistletoe there can be observed the well delimited peaks in this interval (2873–2934 cm^{-1}) [26-28]. For the lignin samples, this domain is affected, by decreasing the intensity of the peaks, sometimes observing their disappearance [29, 30]. These observations may be correlated with the demethoxylation of the aromatic rings from the structure of lignin by the characteristic enzymatic systems, or by the attack on the –C-O bond. At 1708 cm^{-1} there are recorded absorptions characteristic to the valence vibrations of the carbonyl and carboxyl groups. For the recovered lignin samples there can be observed changes of the intenstities of these peaks, modifications that may be determined by the oxidation reactions of the enzymatic systems [31]. The 1500–1590 cm^{-1} domain corresponds to the vibrations of the –C=C– and –C–C– bonds

of the aromatic nucleus. There can be observed modifications of the intensity of peaks in this domain in comparison to the ash mistletoe sample. These modifications occur probably due to the attack of the enzymatic systems on the aromatic rings and due to the type of the host, taking into consideration the data in the literature [32, 33], according to which, the synthesized enzymes, would attack the aromatic nucleus in the structure of lignin, previously hydroxylated. Therefore, the functional groups on surface were studied by FT-IR. The band assignments are shown in Table 2.

Table 2. Infrared band assignments for the cellulosic fibers

Position / cm^{-1}	**Assignment**
~3335	ν(OH) free
~2900	ν(C-H)
~2850	ν(CH_2) symmetrical stretching
~1735	ν(C=O) ester
~1635	adsorbed water
~1595	ν(C=C) aromatic in–plane
~1505	ν(C=C) aromatic in–plane
~1475	δ(CH_2) scissoring
~1455	δ(C–H); δ(C–OH) 1 & 2 alcohol
~1420	δ(C–H)
~1365	δ(C–H)
~1335	δ(CH_2) wagging
~1315	δ(C–H)
~1280	δ(CH_2) twisting
~1235	δ(C–OH) out-of-plane
~1200	δ(C–OH); δ(C–CH)
~1155	ν(C–C) ring breathing, asymmetric
~1105	ν(C-O-C) glycosidic
~1050	ν(C-OH) 2 alcohol
~1025	ν(C-OH) 1 alcohol
~1005	ρ(-CH-)
~985	ρ(–CH–)
~895	ν(C–O–C) in plane, symmetric

In the 1330–1370 cm^{-1} absorption domain there are vibrations of the siryngyl and guaiacyl units present in the structure of lignin in different ratios, depending on the plant species, on the modifications brought in its structure and on biosynthesis. The changes at this level occur probably because of the transformations of the products at the level of the aromatic rings under the action of the enzymes. The presence of the absorption bands in the 1300–900 cm^{-1} domain are considered to be specific to the C-O bonds in the secondary alcohols, to the ester and ether bonds, to the C-C bonds and to their deformation [34, 35]. The intensity of the absorption is different, for the three studied samples, which occurred subsequent to lignin biosynthesis according to the type of host. The chemical composition of the mistletoe wood grown on birch, fir and ash, determined through standard methods is approximately the same, and has comparable values with the ones found in the literature [36]. In the analysis of the spectra, there were followed the modifications at the level of the hydroxyl, carbonyl, carboxyl and methyl groups, and even at the level of the bezenic, guaiacilic and siringilic structures. Thus, in the hardwood lignins, the sinapinic alcohol prevails, and in those of conifers, the coniferilic alcohol prevails [24].

At the level of these bonds, different changes occur, thus, the absorption bands decrease or increase in intensity, sometimes even disappearing. All these modifications are characteristic for a series of reactions like: oxidation reactions, dehydrogenation reactions and possibly condensation

reactions between the structural units in the lignin macromolecule. The IR spectrum for the lignin of fir mistletoe (S2), presents specific bands for the phenolic –OH groups and for the aliphatic structures in the 3410–3460 cm^{-1} spectral domain, bands that have a high intensity, in comparison with the spectra of lignin from ash mistletoe, where the absorption peak is missing. As it can be observed, the enzymatic systems synthesized by the lignin of the fir mistletoe (S2), modify the structure of lignin at the level of different groups and bonds. The peaks in the 3411–3391 cm^{-1} domain, characteristic to the vibration of the phenolic hydroxyl groups and to the aliphatic structures, suffer modifications regarding the intensity of the absorption signals. The signals in the 2873–2899 cm^{-1} domain are responsible for the vibrations of the un-conjugated –C-O bonds, specific to the ketone, carbonyl and ether groups, and in the case of the other samples, they disappear. This is done probably, due to the enzymatic systems that cause the breaking of the –C-O bonds in the structure of lignin.

The domain in the interval 1590–1740 cm^{-1}, which is responsible for the vibrations of the –C=O bonds, suffer modifications especially by intensifying the peaks that are found around the value of 1600 cm^{-1}, characteristic to the vibration of the conjugated –C=O bonds at the level of the ketone and carbonyl groups. The signal responsible for the vibration of the bonds of the aromatic nucleus (1505–1590 cm^{-1}), is well defined and presents a high intensity, whereas the peaks specific for the samples of lignin separated from the lignin of ash mistletoe (S3), present signals of low intensity. In the 1270–1275 cm^{-1} domain, there are vibrations of the phenol or ether bonds, where the intensity of the absorption signals is modified in the case of the samples of lignin separated from the ash mistletoe (S3), in comparison with the sample of fir mistletoe (S2).

Therewith, the signals present in the 1126–1127 cm^{-1} absorption domain, assigned to the vibrations of the –C-H bond of the aromatic ring, characteristic to the siryngic units, or assigned to the vibrations specific to the elongation of the –C-O bond present at the level of the primary alcohols, and the one at 1035 cm^{-1}, characteristic to the deformation of the –C-O bonds, present at the level of primary alcohols and of the –C-H bonds in the aromatic ring, suffer significant modifications, by lowering their intensities. This decrease can be explained by the fact that at the level of these functional groups present in the structure of lignin, oxidations can occur, which lead to the breaking of the chemical bonds.

5. Conclusions

It can be stated that, subsequent to the spectral analysis of the birch, fir and ash lignin samples (S1, S2, S3), there were underlined significant modifications at the level of the functional groups in the structure of lignin determined by the type of host. Other spectral signals, common both to the lignin isolated from the birch, fir and ash mistletoe, were identified in the 842 ÷ 1329 cm^{-1} spectral domain as follows:

- At 1329 cm^{-1}, corresponding to the shear vibrations $\delta (CH_2)$ and $\delta (CH_3)$;
- la 1263 cm^{-1}, corresponding to the deformation vibrations $\delta (CH_2)$ and $\delta (CH_3)$;
- la 1200 cm^{-1}, corresponding to the deformation vibrations δ (CCH) and δ (C–O);
- la 918 cm^{-1}, corresponding to the vibrations $\rho (CH_2)$ and $\rho (CH_3)$;
- As well as around the 842 cm^{-1} value, corresponding to the isolated vibrations ν (CC).

If we focus on the spectral differences between the birch, fir and ash lignin samples (S1, S2, S3), we can observe around the 1617 cm^{-1} band, corresponding to the elongation vibrations ν (C=C) aromatic, the ash lignin samples have a median intensity signal, in comparison with the samples of fir lignin, which only have a low intensity signal. The FTIR spectra can be considered significant in order to emphasize the functionality modifications, in the majority of the oxidation reactions which determine the erosion of the lignin structures in order to supply a carbon source for microorganisms.

A useful means for the comparison of the biomass is based on the *O: C* and *H:C* ratios, known as the Van Krevlen diagram. The more the ratios are smaller, the bigger is the energy content of the studied matter.

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