Potential Applications of Infrared and Raman Spectromicroscopy for Agricultural Biomass

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ABSTRACT

The low bulk density agricultural biomass should be processed and densified making it suitable for biorefineries. However, many agricultural biomass (lignocellulosic) especially those from straw and stover results in poorly formed pellets or compacts that are more often dusty, difficult to handle and costly to manufacture. The binding characteristics of biomass can be enhanced by modifying the structure of lignocellulose matrix (cellulose-hemicellulose-lignin) by different pre-processing and pre-treatment methods. However, it is not well understood as to how various pre-processing and pre-treatment methods affect the lignocellulosic matrix at the molecular level. Therefore, it is essential to determine chemical composition of agricultural biomass and the distribution of lignin relative to cellulose and hemicellulose before and after application of various treatment methods and after densification process. In this paper, the structural characteristics of lignocellulosic plant biomass and applications of Infrared (IR) and Raman spectromicroscopy methods are reviewed. The IR and Raman methods have good potential to determine the structural characteristics and distribution of chemical components in lignocellulosic biomass. Both methods have their own advantages and drawbacks, and should be used as complementary techniques.

Keywords: Infrared, Raman, Lignocellulose, Cellulose, Hemicellulose, Lignin, Spectromicroscopy, Densification, Biomass, Vibrational and Rotational Spectroscopy

1. INTRODUCTION

Agricultural biomass residues have the potential for the sustainable production of bio-fuels and to offset greenhouse gas emissions (Campbell *et al.,* 2002; Sokhansanj *et al.,* 2006). The straw and agricultural residues existing in the waste streams from commercial crop processing plants have little inherent value and have traditionally constituted a disposal problem. In fact, these residues

represent an abundant, inexpensive and readily available source of renewable lignocellulosic biomass (Liu *et al.,* 2005). New methodologies need to be developed to process the biomass making it suitable feedstock for bio-fuel production. In addition, some of the barriers to the economic use of agricultural crop residue have uncertainty in its availability, variablility in quality, cost of collection, problems in transportation and storage (Bowyer and Stockmann, 2001; Sokhansanj *et al.,* 2006).

Biomass must be processed and handled in an efficient manner in order to reduce industry's operational cost as well as to meet the requirement of raw material for biofuel production. Biomass has low bulk density, making it difficult and expensive to store and transport in its native loose form. The bulk density of dried alfalfa straw is as low as 40 kg/m^3. The bulk density of pelleted forage can be as high as 1250 kg/m^3 (Adapa *et al.,* 2002). When densified, many agricultural biomass, especially those from straw and stover, result in a poorly formed pellets or compacts that are more often dusty, difficult to handle and costly to manufacture. This is a result of lack of complete understanding on the binding characteristics of the components that make up biomass (Sokhansanj *et al.,* 2005).

The binding characteristics of lignocellulosic biomass can be enhanced by modifying the structure of cellulose-hemicellulose-lignin matrix by application of pre-processing and pre-treatment methods (Sokhansanj *et al.,* 2005). However, it is not well understood on how various pre-processing and pre-treatment methods affect the lignocellulosic matrix at the molecular level. Most studies focused on quantification of chemical composition of biomass such as total energy content, crude protein and carbohydrates (structural and non-structural) using traditional chemical or proximate analysis methods (Adapa *et al.,* 2004). The proximate analysis of lignocellulosic material relies on the separation of the component of interest from the complex matrix that makes up the biomass. As a result, information on the spatial origin and distribution of the component of interest is lost and the object of the analysis is destroyed (Budevska, 2002; Yu *et al.,* 2007). In addition, applications of pre-processing methods such as size reduction or increasing porosity, and pre-treatment techniques such as steam explosion and pulse electric methods on agricultural biomass have demonstrated an improvement in pellet (compact) quality, that can be attributed to the changes in the lignocellulosic components and distribution (Ade-Omowaye *et al.,* 2001; Bagby, 1982; Bhazal *et al.,* 2003; Focher *et al.,* 1998).

Structural characteristics and chemical compounds distribution of agricultural biomass at microscopic level before and after pre-treatment, and after densification can be studied using Infrared (IR) and Raman spectromicroscopy. This could reveal structural and chemical changes that occur when particular combinations of treatment variables (temperature, pressure, hold time, moisture content, etc.) are applied to produce an optimized and high quality pelletized/densified product development. According to the knowledge of the authors, no such studies have been conducted to determine the structural characteristics and chemical components distribution in agricultural biomass using IR and Raman methods enabling it suitable for biorefineries. Therefore, the objectives of this study are:

- to review the structural and chemical characteristics of agriculture-based lignocellulosic biomass;
- to review the concepts and application of IR and Raman spectromicroscopy methods for biomass research; and

- to explore basic concepts, and evaluate the strengths and drawbacks of spectromicroscopy as applied to lignocellulosic material.

2. LIGNOCELLULOSIC MATERIAL

Lignocellulosic material refers to plant biomass that is composed of cellulose, hemicellulose, and lignin (Lin and Tanaka, 2006). The major combustible component of non-food energy crops is cellulose, followed by lignin. Non-food energy crops are more energy efficient than edible energy crops that have a large starch component (Holt-Gimenez, 2007).

2.1 Cellulose

Cellulose is an organic polysaccharide (fig. 1) consisting of a linear chain of several hundred to over nine thousand β(1→4) linked D-glucose $(C_6H_{10}O_5)_n$ units (Crawford, 1981; Updegraff, 1969). Cellulose, a fibrous, tough, water-insoluble substance, is found in the cell walls of plants, particularly in the stalks, stems, trunks and all the woody portions of the plant body (Nelson and Cox, 2005). Cellulose comprises 40-60 % of the dry weight of plant material (the cellulose content of cotton is 90 % and that of wood is 50 %) (Encyclopædia Britannica 2008; USDE 2006). The multiple hydroxyl groups on the glucose residues from one chain form hydrogen bonds with oxygen molecules on another chain, holding the chains firmly together side-by-side and forming *microfibrils* with high tensile strength (fig. 2). This strength is important in cell walls, where they are meshed into a carbohydrate *matrix*, conferring rigidity to plant cells (Murphy and McCarthy, 2005).

Zandersons and co-workers (2004) and Shaw (2008) reported that binding of wood material during hot pressing or densification is mainly dependent on the transition of cellulose into the amorphous state. According to Hon (1989), due to the semi-crystalline structure, hydrogen bonded cellulose cannot be dissolved easily in conventional solvents, and it cannot be melted before it burns; hence, cellulose itself is not a suitable adhesive. This can be overcome by breaking the hydrogen bonds, thus making the cellulose molecule more flexible (Hon 1989). Cellulose requires a temperature of 320 °C and pressure of 25 MPa to become amorphous in water (Deguchi *et al.* 2006).

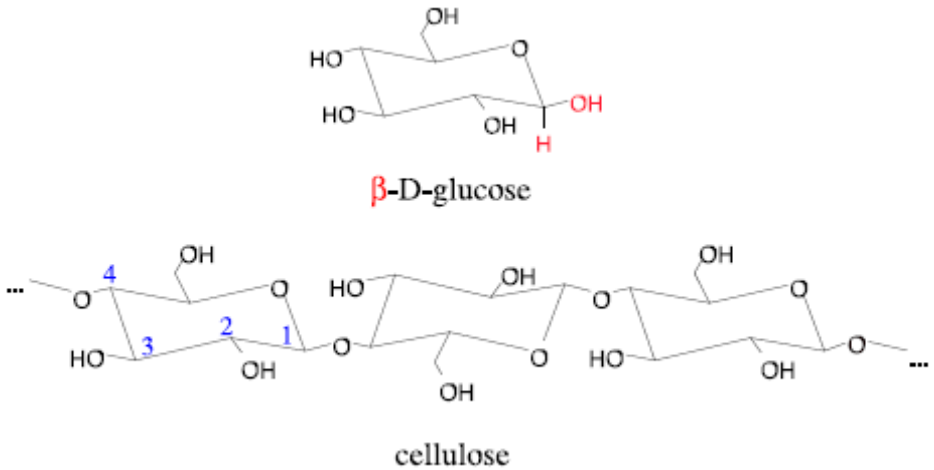

Figure 1: The glucose unit and the cellulose chain (Vainio, 2007).

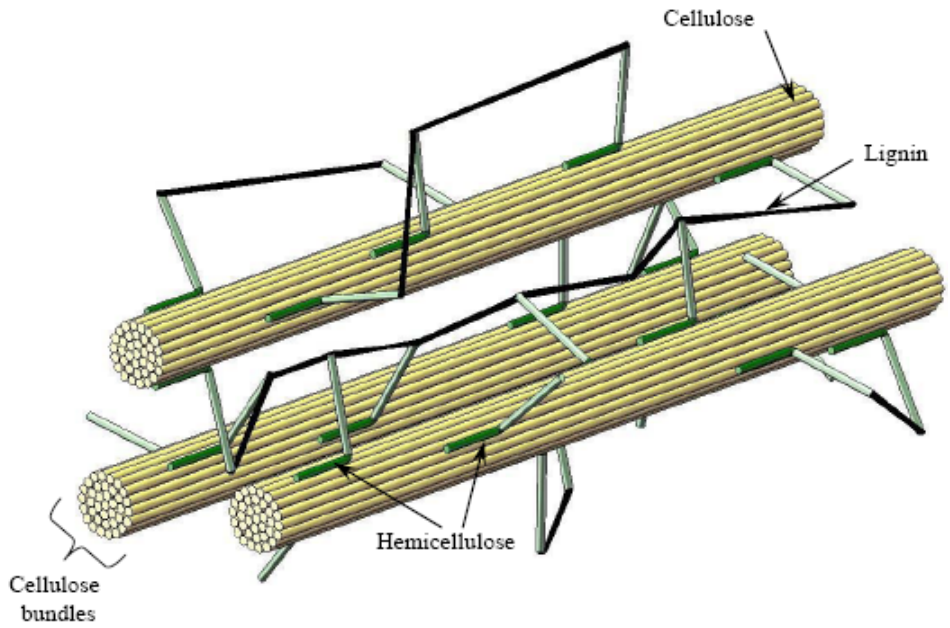

Figure 2: Location and arrangement of cellulose microfibrils in plant cell walls (Murphy and McCarthy, 2005; Shaw, 2008).

2.2 Hemicellulose

Hemicellulose is made of several heteropolymers (matrix polysaccharides) present in almost all plant cell walls along with cellulose (fig. 2). While cellulose is crystalline, strong, and resistant to hydrolysis; hemicellulose has a random, amorphous structure with less strength. Hemicellulose is a polysaccharide related to cellulose and comprises 20-40 % of the biomass of most plants. In contrast to cellulose, hemicellulose is derived from several sugars in addition to glucose, including especially xylose but also mannose, galactose, rhamnose and arabinose (Shambe and Kennedy, 1985). Branching in hemicellulose produces an amorphous structure that is more easily hydrolyzed than cellulose (Shaw, 2008). Also, hemicellulose can be dissolved in strong alkali solutions. Hemicellulose provides structural integrity to the cell. Some researchers believe that natural bonding may occur due to the adhesive properties of degraded hemicellulose (Bhattacharya *et al.,* 1989).

2.3 Lignin

Lignin is a complex chemical compound most commonly derived from wood and is an integral part of the cell walls of plants (Lebo *et al.,* 2001; Zandersons *et al.,* 2004). The compound has several unusual properties as a biopolymer, not the least its heterogeneity in lacking a defined primary structure. Lignin fills the spaces in the cell wall between cellulose and hemicellulose (fig. 2). It is covalently linked to hemicellulose and thereby crosslinks different plant polysaccharides, conferring mechanical strength to the cell wall and consequently to the whole plant structure (Chabannes *et al.,* 2001).

There are three monomers of lignin, methoxylated to various degrees: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (fig. 3) (Freudenberg and Nash, 1968). These are incorporated into lignin in the form of the phenylpropanoids *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), respectively (Boerjan *et al.,* 2003). Gymnosperms have a lignin that consists almost entirely of G with small quantities of H. The dicotyledonic angiosperms is more often a mixture of G and S (with very little H), and monocotyledonic lignin is a mixture of all three (Boerjan *et al.,* 2003). Many grasses have mostly G, while some plants have mainly S. All lignin contain small amounts of incomplete or modified monolignols, and other monomers are prominent in non-woody plants (Ralph *et al.,* 2001).

Lignin acts as a binder for the cellulose fibres (fig. 2). Van Dam and co-workers (2004) have reported that lignin can be used as an intrinsic resin in binderless board production due to the fact that when lignin melts (temperatures above 140 °C), it exhibits thermosetting properties. Lignin is the component that permits adhesion in the wood structure, and is a rigidifying and bulking agent (Anglès *et al.,* 2001). Lehtikangas (2001) stated that 8-15 % water in pellets will reduce the softening temperature of lignin to 100-135 °C by plasticizing the molecular chains. The adhesive properties of thermally softened lignin are thought to contribute considerably to the strength characteristics of briquettes made of lignocellulosic materials (Granada *et al.,* 2002; Shaw, 2008).

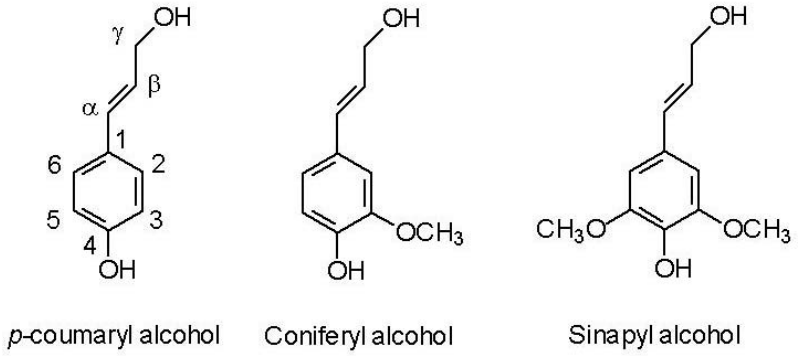

Figure 3: The three common monolignols (Freudenberg and Nash, 1968).

It is apparent that the application of various pre-processing and pre-treatment methods to enhance the availability and distribution of lignin is critical. Various traditional and proximate analysis methods are available to determine the chemical composition of lignocellulosic biomass. However, there is a lack of literature and understanding to determine chemical compounds distribution of agricultural biomass at microscopic level before and after pre-treatment, and after densification using IR and Raman spectromicroscopy. Therefore, the following sections will explore the basic concepts of IR and Raman spectromicroscopy as applied to lignocellulosic biomass.

3. VIBRATIONAL AND ROTATIONAL SPECTROSCOPY

Spectroscopy may be defined as the study of the quantized interaction of electromagnetic radiations with matter. The electromagnetic radiations are produced by the oscillation of electric charge on an atom in a molecule (Yadav, 2005). Electromagnetic radiation is characterized by its wavelength λ (the length of one wave, cm), its frequency ν (the number of vibrations per unit time, Hz), and its wavenumber $\bar{\nu}$ (the number of waves per unit length). The wavenumber expressed in cm^{-1} is the number of waves in a 1 cm-long wavetrain (Colthup *et al.,* 1990). The wavenumber $\bar{\nu}$, in waves per centimetre (cm^{-1}), is related to the other parameters by (eq. 1)

$$\bar{\nu} = \frac{\nu}{\left(c/n\right)} = \frac{1}{\lambda} \tag{1}$$

where:
c = velocity of light in vacuum (2.998×10^{10} cm/s), and
(c/n) = velocity of light in a medium whose refractive index is n, in which the wavenumber is measured.

It is important to understand the distribution of energy possessed by a molecule at any given moment, defined as the sum of the contributing energy terms (eq. 2):

$$E_{total} = E_{electronic} + E_{vibrational} + E_{rotational} + E_{translational} \tag{2}$$

In the above equation, the translational energy relates to the displacement of molecules in space as a function of the normal thermal motions of matter. The rotational energy, which gives rise to its own form of spectroscopy, is observed as the tumbling motion of a molecule, which is the result of the absorption of energy within the microwave region. The vibrational energy component is a higher energy term and corresponds to the absorption of energy by a molecule as the component atoms vibrate about the mean centre of their chemical bonds. The electronic component is linked to the energy transitions of electrons as they are distributed throughout the molecule, either localized within specific bonds, or delocalized over structures, such as an aromatic ring (Coates, 2000). Energy E in eV for a single photon is given by equation (3):

$$E = h\nu = \frac{hc}{\lambda} \tag{3}$$

where: h = Planck's constant (6.6256×10^{-34} Js**)**

3.1 Electromagnetic Radiation and Spectrum

Electromagnetic spectrum covers a very wide range of electromagnetic radiations. The arrangement of all types of electromagnetic radiations in order of their wavelengths or frequencies is shown in Figure 4.

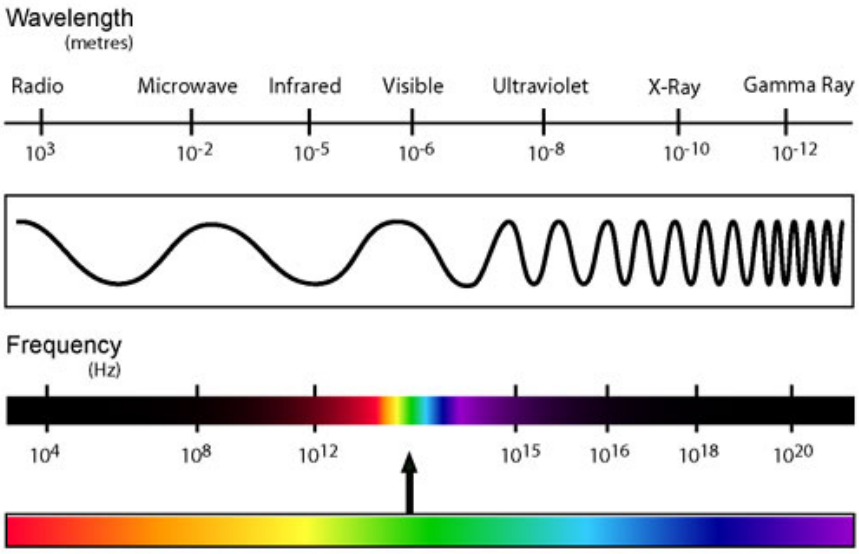

Figure 4: Electromagnetic spectrum (Colour Therapy Healing, 2008).

3.2 Absorption and Emission Spectra

When electromagnetic radiation is passed through an organic compound, it may be absorbed to induce electronic, vibrational and rotational transitions in the molecules. The energy required for each of these transitions is quantized. Thus, only the radiation supplying the required quantum (photon) of energy is absorbed and the remaining portion of the incident radiations is transmitted. Generally, a spectrometer records an absorption spectrum as a plot of the intensity of absorbed or transmitted radiations versus their wavelengths or frequencies and is called absorption spectra (fig. 5) (Schienmann, 1970; Williams and Fleming, 1966; Yadav, 2005).

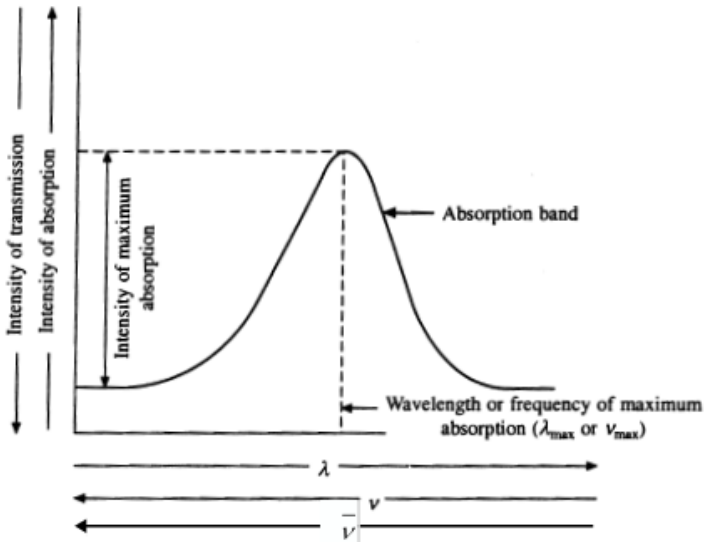

Figure 5: Schematic of absorption spectra (Yadav, 2005).

4. INFRARED SPECTROMICROSCOPY

With the introduction of the IR spectromicroscopy, it is possible to measure spectra of objects as well as structures on the micrometer scale. Analysis of biological tissues became an important area of interest due to the possibility to perform chemical analysis on a very small scale and link it to morphology (Budevska, 2002).

IR spectroscopy deals with the recording of the absorption of radiations in the infrared region of the electromagnetic spectrum. The position of a given infrared absorption is expressed in terms of wavenumber $\bar{\nu}$ (cm^{-1}) as it is directly proportional to energy. The ordinary infrared region 4000-667 cm^{-1} is of greatest practical use to organic chemists. The region 12,500-4000 cm^{-1} is called the near infrared and the region 667-50 cm^{-1} as the far infrared region. The absorption of infrared radiation by a molecule occurs due to quantized vibrational and rotational energy changes when it is subjected to infrared irradiation (Colthup *et al.,* 1990; Yadav, 2005).

When a molecule absorbs IR radiation below 100 cm^{-1}, the absorbed radiation causes transitions in its rotational energy levels. Since these energy levels are quantized, a molecular rotational spectrum consists of discrete lines. When a molecule absorbs IR radiation in the range 100-10,000 cm^{-1}, the absorbed radiation causes transitions in its vibrational energy levels. These energy levels are also quantized, but vibrational spectra appear as bands rather than discrete lines. The rotational energy levels of a molecule are far less than that between its vibrational energy levels. Thus, a single transition in vibrational energy levels is accompanied by a large number of transitions in rotational energy levels and so the vibrational spectra appear as vibrational-rotational bands instead of discrete lines (Colthup *et al.,* 1990; Yadav, 2005).

Infrared radiation is absorbed when the oscillating dipole moment, due to a molecular vibration, interacts with the oscillating electric field of the infrared beam. This interaction occurs and hence, an absorption band appears only when a molecular vibration produces a change in the dipole moment of the molecule. Otherwise, the vibration is said to be infrared inactive and will show no absorption band in the infrared spectrum. Usually, the larger the change in dipole moment, the higher is the intensity of absorption. It is not necessary for a molecule to have a permanent dipole moment for IR absorption (Yadav, 2005).

4.1 Calculation of Vibrational Frequencies

The characteristic frequencies of particular combination of atoms within a molecule could be explained by the basic model of the simple harmonic oscillator and its modification to account for anharmonicity (Coates, 2000). Because of the importance in the study of molecular vibrations, the classical vibrational frequency for a diatomic molecule will be derived using a model represented by two masses m_1 and m_2 connected by a mass-less spring (Colthup *et al.,* 1990) (fig. 6).

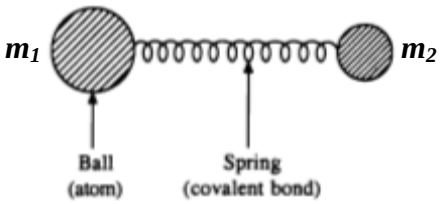

Figure 6: Representation of atoms using a simple harmonic oscillator model (Yadav, 2005).

Hooke's law could be used to express the fundamental vibrational frequency of a molecular ensemble (eq. 4) (Coates, 2000).

$$\nu = \frac{1}{2\pi c}\sqrt{\frac{f}{\mu}} \tag{4}$$

where:

$\mu = \frac{m_1 m_2}{m_1 + m_2}$ is the reduced mass, m_1 and m_2 are the masses (g) of the atoms linked to the particular bond,

f = force constant of the bond in dyn/cm,

This simple equation provides a link between the strength (or springiness) of the covalent bond between two atoms (or molecular fragments), the mass of the interacting atoms and the frequency of vibration. This simple model does not account for repulsion and attraction of the electron cloud at the extremes of the vibration, and does not accommodate the concept of bond dissociation at high levels of absorbed energy (Coates, 2000).

4.2 Fundamental Molecular Vibrations

The IR spectra of polyatomic molecules may exhibit more than one vibrational absorption band. The number of these bands corresponds to the number of fundamental vibrations in the molecule which can be calculated from the degrees of freedom of the molecule. The degrees of freedom of a molecule are equal to the total degrees of freedom of its individual atoms. Each atom has three degrees of freedom corresponding to the three Cartesian coordinates (x, y and z) necessary to describe its position relative to other atoms in the molecule. Therefore, a molecule having n atoms will have $3n$ degrees of freedom. In case of a non-linear molecule, three of the degrees of freedom describe rotation and three describe translation. Thus, the remaining $(3n - 3 - 3) = 3n - 6$ degrees of freedom are its vibrational degrees of freedom or fundamental vibrations (Coates, 2000; Colthup *et al.,* 1990; Yadav, 2005).

In case of a linear molecule, only two degrees of freedom describe rotation (because rotation around its axis of linearity does not change the positions of the atom) and three describe translation. Thus, the remaining $(3n - 2 - 3) = 3n - 5$ degrees of freedom are vibrational degrees of freedom or fundamental vibrations (Coates, 2000; Colthup *et al.,* 1990; Yadav, 2005).

The two types (modes) of fundamental molecular vibrations known are: a) stretching; and b) bending vibrations (deformations) (Coates, 2008; Colthup *et al.,* 1990; Yadav, 2005).

4.2.1 Stretching Vibrations

In stretching vibrations, the distance between two atoms increases or decreases, but the atoms remain in the same bond axis. Stretching vibrations are of two types (Colthup *et al.,* 1990; Yadav, 2005):

a) Symmetrical stretching - in this mode of vibration, the movement of atoms with respect to the common (or central) atom is simultaneously in the same direction along the same bond axis (fig. 7(a)); and
b) Asymmetrical stretching - in this vibration, one atom approaches the common atom while the other departs from it (fig. 7(b)).

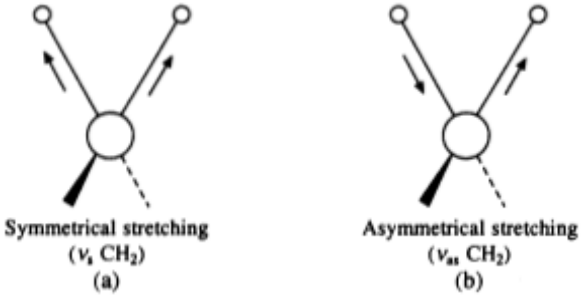

Figure 7: Stretching vibrations of CH_2 group ($\nu\,CH_2$) (Yadav, 2005).

In practice, various other deformation motions (angular changes), such as bending and twisting about certain centers within a molecule, also have impact, and contribute to the overall absorption spectrum. By rationalizing the effort needed to move the atoms relative to each other, one can appreciate that it takes less energy to bend a bond than to stretch it. Consequently, the stretching absorptions of a vibrating chemical bond occur at higher frequencies (wavenumbers) than the corresponding bending or bond deformation vibrations, with the understanding that energy and frequency are proportionally related. In addition, it takes slightly more energy to excite a molecule to an asymmetric than a symmetric vibration (Coates, 2000).

4.2.2 Bending Vibrations (Deformations)

In bending vibrations, the positions of the atoms change with respect to their original bond axes. Bending vibrations are of four types (Colthup *et al.,* 1990; Yadav, 2005):

a) Scissoring - in this mode of vibration, the movement of atoms is in the opposite direction with change in their bond axes as well as in the bond angle they form with the central atom (fig. 8(a));
b) Rocking - in this vibration, the movement of atoms takes place in the same direction with change in their bond axes (fig. 8(b)). Both scissoring and rocking are in-plane bendings;

c) Wagging - in this vibration, two atoms simultaneously move above and below the plane with respect to the common atom (fig. 8(c)); and
d) Twisting - in this mode of vibration, one of the atom moves up and the other moves down the plane with respect to the common atom (fig. 8(d)).

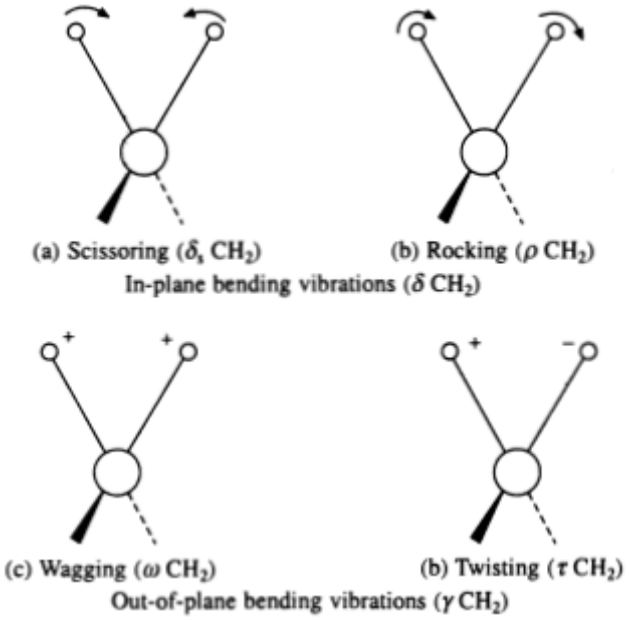

Figure 8: Bending vibrations (deformations) of CH_2 group (+ and – signs indicate movements perpendicular to the plane of the paper) (Yadav, 2005).

4.3 Fingerprint Region

It is not possible for any two different compounds to have exactly the same IR spectrum. Therefore, the IR spectrum of a compound is called its fingerprint. The region below 1500 cm^{-1} is called fingerprint region because every compound has unique absorption pattern in this region. The fingerprint region contains many absorption bands caused by bending vibrations as well as absorption bands caused by stretching vibrations. Since the number of bending vibrations in a molecule is much greater than its stretching vibrations, the fingerprint region is rich in absorption bands and shoulders. Thus, the superimposability of IR bands of the spectra of any two different compounds becomes impossible in this region. However, similar compounds may show very similar spectra above 1500 cm^{-1} (Coates, 2000).

5. RAMAN SPECTROMICROSCOPY

Infrared and Raman spectroscopy are closely related as both originate from the transition in vibrational and rotational energy levels of the molecule on absorption of radiations. The intensity of IR absorption depends on the change in dipole moment of the bond, whereas Raman intensity depends on the change in polarizability of the bond accompanying the excitation. Thus, an electrically symmetrical bond (i.e. having no dipole moment) does not absorb in IR region (i.e. the transition is forbidden) but it does absorb in Raman scattering (i.e. the transition is allowed).

In other words, an electrically symmetrical bond is Raman active but IR inactive. However, an electrically unsymmetrical bond may be IR active and Raman inactive or both IR and Raman active (Anderson, 1973; Colthup *et al.,* 1990).

IR and Raman spectroscopy are complementary techniques. For example, studies on bond angles, bond lengths and other structural confirmations require Raman data in addition to IR analysis (Yadav, 2005).

According to Raman effect, when a beam of strong radiation of a definite frequency is passed through a transparent substance (gas, liquid or solid), the radiation scattered at right angles has not only the original frequency but also some other frequencies which are generally lower and occasionally higher than that of the incident radiation. This is known as Raman scattering or *Raman effect*. The spectral lines resulting from lower frequencies than that of the incident radiation are called *Stokes lines* and those from higher frequencies are called *anti-Stokes lines*. The spectral lines whose frequencies have been modified in *Raman effect* are called Raman lines. Thus, Stokes and anti-Stokes lines are *Raman lines*. The Raman spectra are a manifestation of *Raman effect*, which is accompanied by transitions in vibrational and rotational energy levels of the molecule. Similar to IR spectra, the position of spectral lines (or bands) in Raman spectra are also reported in wavenumbers (cm^{-1}) (Anderson 1973; Colthup *et al.* 1964).

The incident light is associated with energy $h\nu_i$, a part of which is used for causing transitions from lower to higher vibrational and rotational energy levels, so the scattered radiation has a lower energy content $h\nu_s$ and thus a new line (Raman line) appears in the spectrum. Raman also discovered (Yadav, 2005) that the frequency difference $\Delta\nu$ between the incident frequency ν_i and any scattered frequency ν_s is constant and characteristic of the substance exposed to radiation and is completely independent from the frequency of the incident radiation ν_i. $\Delta\nu$ is known as *Raman frequency shift* or *Raman shift* and is given by the Equation (5) (Anderson, 1973; Yadav, 2005).

$$\Delta\nu = \nu_i - \nu_s \tag{5}$$

where $\Delta\nu$ is positive for Stokes line and negative for anti-Stokes lines. Although Raman shifts $\Delta\nu$ correspond to IR absorption or emission, IR and Raman spectra of a substance are not always identical.

6. APPLICATIONS OF VIBRATIONAL SPECTROMICROSCOPY FOR AGRICULTURAL BIOMASS

The ability to produce qualitative and quantitative analytical data for samples with minimum or no sample preparation has made the vibrational spectroscopic approach the preferred one in many cases where speed and high throughput are of great importance. The ability to fingerprint the chemical composition in a spatially-resolved manner will, most likely, prove to be unique and most valuable, especially in the characterization of agricultural materials. Applications developed for agricultural purposes span a wide range including satellite and aerial remote sensing,

macroscopic imaging for quality control and in vivo measurements, and microscopic applications aimed at increasing the fundamental understanding of plant physiology on the cellular level (Budevska, 2002).

IR and Raman spectroscopy are complementary techniques that provide information on molecular structure. By combining spectroscopy with microscopy, molecular information can be obtained with great spatial resolution (at the micrometer scale) at the microscopic level (Thygesen *et al.,* 2003).

6.1 Applications of IR Spectromicroscopy

Among all the properties of an organic compound, no single property gives as much information about the compound's structure as its IR spectrum. Thus, IR spectroscopy is the most widely used method for the compositional determination of organic compounds. The basic reason why IR spectra are of such value to organic chemists is that molecular vibrations depend on interatomic distances, bond angles and bond strengths, rather than on bulk properties of the compound. Thus, these vibrational frequencies provide a molecular fingerprint which enables the identification of the compound either in the pure state or in mixtures (Yadav, 2005).

Synchrotron based IR spectromicroscopy has better signal to noise ratio and spatial resolution compared to globar sources. The technique is used to identify molecular constituents in biological samples from their vibrational spectra in the mid-IR region (4000-667 cm^{-1}), and is capable of exploring the molecular chemistry (structural make-up) within microstructures of biological tissues at a cellular level without destruction of internal structures at a high spatial resolution (3-10 μm) (Marinkovic *et al.,* 2002; Yu *et al.,* 2007).

A drawback of globar-source IR spectromicroscopy is the diffraction effects which result in reduced aperture size that limit the field of view to a small region of interest. At the same time, less light overall results in a decrease in the signal-to-noise ratio. For this kind of study on a plant tissue's molecular structural-chemical features for which spectral data need to be collected at the diffraction limit (a few micrometers) in each spatial dimension, only advanced synchrotron and free-electron lasers can be used. Furthermore, the brightness of conventional bench-top IR source is lower by two to three orders of magnitude (Raab and Martin, 2001).

The IR spectromicroscopy have been used to study chemical composition and distribution of various lignocellulosic biomasses as applied to food, feed, biocomposite, textile, and paper and pulp industries. The summary of IR study on various lignocellulosic materials is listed in Table 1. Yu *et al.* (2007) characterized the molecular chemistry of the internal structure of wheat and reported both its structural chemical make-up and nutrient component matrix by analyzing the intensity and spatial distribution of molecular functional groups within the intact seed using advanced synchrotron-based IR spectromicroscopy. A spectral examination of wheat tissue by Yu *et al.* (2007) provided a unique absorption band of lignin at 1510 cm^{-1} in the mid-IR region of the electromagnetic spectrum. This is considered to be indicative of the aromatic character of the lignin. An aromatic compound gives two major bands at 1600 and 1510 cm^{-1}, resulting from quadrant and semicircle ring stretching, respectively (Colthup *et al.,* 1990). Yu *et al.* (2007)

observed strong bands for both structural and non-structural carbohydrates, particularly in the 1100-1025 cm^{-1} region. A presence of moderate intensity bands at 1420, 1370 and 1335 cm^{-1} are characteristics of structural carbohydrates. A peak at 1246 cm^{-1} is used to indicate the presence of structural carbohydrate such as cellulose (Wetzel *et al.,* 1998; Wetzel, 2001).

Himmelsbach *et al.* (1998) performed experiments to determine the distribution of chemical components in two varieties of flax stems. They have observed bands at 1595 and 1510 cm^{-1} located in the core, cuticle and epidermal tissue and assigned it to aromatic compounds (lignin). Cellulose was monitored by the band at 1335 cm^{-1} and acetylated hemicellulosic materials were monitored at 1250 cm^{-1}.

Stewart *et al.* (1995) used IR and Raman spectroscopy techniques to investigate the changes in the composition and structure of oak wood and barley straw that had been subjected to chemical and biochemical treatments. Both spectral techniques have been shown to be useful in the analysis of plant and cell walls. The use of both techniques in isolation provided general structural and compositional information of oak wood and barley after biochemical treatments (Table 1).

Wilson *et al.* (2000) performed IR spectromicroscopy analysis of the onion cell and determined the orientation of macromolecules in single cell wall. The IR spectrum of onion was dominated by absorption bands of cellulose and pectin, while minor constituents such as protein, ferulic acid, lignin and hemicelluloses were also detected (Table 1).

Yu (2005) used synchrotron-based IR spectromicroscopy to determine molecular chemistry of various feeds (including grain corn and oat hull tissues) to reveal their ultra-structural and chemical composition (Table 1). The molecular chemical information can be linked to structural and nutritional information. Such information can also be used for biological structural study.

Table 1: Summary of FT-IR spectra obtained for various lignocellulosic materials.

Material	Wavenumber (cm^{-1})	Researcher(s)
Lignin		
Wheat Seed Tissue	1600 – quadrant ring stretching – aromatic lignin 1510 – semicircle ring stretching – aromatic lignin	Colthup *et al.* 1990; Yu *et al.* 2007
Wheat Straw	1595 – very strong aromatic ring stretch; aromatic C-O stretching 1510 – very strong aromatic ring stretch; aromatic C-O stretching	Revol 1982; Stewart *et al.* 1995
Steam Exploded Wheat Straw	1513 & 1433 – aromatic C=C stretch 1473 & 1380 – C-H symmetric and asymmetric deformation 1327 – C-C and C-O skeletal stretch	Sun *et al.* 2005
Flax Stems	1595 – phenylpropanoid polymer 1510 – phenylpropanoid polymer	Himmelsbach *et al.* 1998
Corn Kernel	1514 semicircle stretching – para-substituted	Lin-Vein *et al.* 1991;

	benzene rings	Budevska 2002
Barley Straw	1595 – aromatic skeletal vibrations plus C=O stretch 1510 –aromatic ring stretch	Lin and Dence 1992; Stewart *et al.* 1995
Grain Corn Tissue	1510 – aromatic lignin characteristics	Yu 2005
Oat Hull Tissue	1510 – aromatic lignin characteristics	Yu 2005
Cellulose		
Wheat Seed Tissue	1420 Weak C-O stretching 1370 Weak C-O stretching 1335 Weak C-O stretching 1246 Strong C-O stretching	Yu *et al.* 2007; Wetzel *et al.* 1998
Wheat Straw	1162, 1130, 1098 900 – anti-symmetric out-of-plane ring stretch of amorphous cellulose; C-O stretching 1500-1300 – C-H bending	Michell 1990; Stewart et al. 1995; Yu *et al.* 2007
Flax Stems	1335	Himmelsbach *et al.* 1998
Barley Straw	1130 – ? (reason not reported) 1098 – weak absorbance 900 – anti-symmetric out-of-plane ring stretch of amorphous cellulose; C-O stretching	Stewart *et al.* 1995
Onion	1430 – CH_2 in-plane bending vibrations 1336 – C-H ring in-plane bending vibrations 1162 – C-O-C ring vibrational stretching 1125/1110 – C-O and C-C ring vibrational stretching 1060 – C-O stretching and O-C-H in-plane bending vibrations 1035 – C-O, C=C and C-C-O vibrational stretching 985 – OCH_3	Schulz and Baranska 2007; Wilson *et al.* 2000
Grain Corn Tissue	1246 – cellulosic compounds	Yu 2005
Oat Hull Tissue	1246 – cellulosic compounds	Yu 2005
Hemicellulose		
Wheat Straw	1735 – ? (reason not reported)	Chen *et al.* 1997; Gastaldi *et al.* 1998
Flax Stems	1250 – Acetylated Hemicellulose	Himmelsbach *et al.* 1998
Onion	815 – ? (reason not reported)	Schulz and Baranska 2007; Wilson *et al.* 2000

Note: The references of Researchers provided in Table 1 – Column 3 are for both assignment and observed wavenumbers

6.2 Applications of Raman Spectromicroscopy

Although cellulose and lignocellulosic materials have been studied using conventional Raman spectroscopy, availability of the Raman instrumentation has made studying these materials more convenient (Agarwal, 1999). Many Raman studies of such materials have been published including studies of quantitative nature (Agarwal *et al.,* 2003; Ona *et al.,* 1998; Sun *et al.,* 1997).

For *in situ* structural analysis of lignocellulosic materials, which are heterogeneous composites of cellulose, lignin and hemicellulose and whose microstructures are composed of morphologically distinct regions, Raman spectroscopy is a good technique. Capability to analyze microscopic regions is another important tool which makes Raman microscopy suitable (Turrell and Corsett, 1996). Further considering that presence of water in a sample is not a problem (unlike IR) and information on the orientation of macromolecular components can be obtained. Raman spectroscopy has capabilities that are not provided by any other method (Agarwal, 2008).

The use of Raman spectromicroscopy mapping has been explored for studies of flax stem (Himmelsbach *et al.,* 1999). The results were in agreement with the one obtained with IR microscopic mapping (Himmelsbach *et al.,* 1998).

Assignment of bands/wavenumbers in the Raman spectra of lignocellulosics is an important topic of research. Although some information is already available (Agarwal *et al.,* 1997; Takei *et al.,* 1995), research in this area needs to be accelerated considering that more and more lignin-containing materials are being studied using Raman spectroscopy, which is primarily responsible for the laser induced fluorescence. For interpreting the Raman spectrum of a multi-component material like lignocellulose, not only the contribution of each component needs to be identified but the latter needs to be assigned to component-specific structural units and/or functional groups (Agarwal, 2008).

In this context, note that Raman features of cellulose have already been assigned (Wiley and Atalla, 1987). Moreover, hemicellulose spectral assignments are expected to be very similar to that of cellulose (Agarwal and Ralph, 1997). Therefore, it is primarily lignin for which bands need to be assigned. Assignment for softwood-cellulose Raman bands is given in Table 2 (Agarwal, 1997).

Table 2. Assignment of bands in the FT-Raman spectrum of softwood-cellulose (Agarwal, 1997)

Wavenumber (cm^{-1})	**Assignment**[a]
330 sh[b]	Heavy atom bending
351 w	Some heavy atom stretching
380 m	Some heavy atom stretching
406 vw	? (reason not reported)
435 m	Some heavy atom stretching
458 m	Some heavy atom stretching
492 w	? (reason not reported)
520 m	Some heavy atom stretching
899 m	HCC and HCO bending at C6[c]

971 vw	Heavy atom (CC and CO) stretching
1000 vw	Heavy atom (CC and CO) stretching
1037 sh	Heavy atom (CC and CO) stretching
1063 sh	Heavy atom (CC and CO) stretching
1073 sh	Heavy atom (CC and CO) stretching
1095 s	Heavy atom (CC and CO) stretching
1123 s	Heavy atom (CC and CO) stretching
1149 sh	Heavy atom (CC and CO) stretching plus HCC and HCO bending
1298 sh	HCC and HCO bending
1338 m	HCC and HCO bending
1377 m	HCC, HCO and HOC bending
1456 m	HCH and HOC bending
2740 vw	? (reason not reported)
2848 sh	CH and CH_2 stretching[d]
2895 vs	CH and CH_2 stretching

[a]Assignment based on reference (Wiley and Atalla 1987)
[b]Note: vs is very strong; s is strong; m is medium; w is weak; vw is very weak; sh is shoulder. Band intensities are relative to other peaks in the spectrum.
[c]In reference (Wiley and Atalla 1987) the band is at 913 cm^{-1}
[d]In reference (Wiley and Atalla 1987) the band is at 2868 cm^{-1}

Further considering that lignin and hemicellulose molecular structures are somewhat different in different lignocellulosic materials (e.g., in softwood, hardwood and grasses), it is even more important that the goal of band assignment for each class of differing lignocellulosics be accomplished (Agarwal, 2008). When assignments of bands are available, one can evaluate how structural differences and similarities of lignin and carbohydrate polymers are reflected in their individual Raman spectra. For black spruce (softwood) lignin, Raman bands have been assigned (Table 3) (Agarwal *et al.,* 1997).

Table 3. Assignment of bands in the FT-Raman spectrum of softwood lignin (Agarwal, 2008)

Wavenumber (cm^{-1})	**Assignment[a]**
357 w[b]	Skeletal deformation of aromatic rings, substituent groups and side chains
384 w	Skeletal deformation of aromatic rings, substituent groups and side chains
463 vw	Skeletal deformation of aromatic rings, substituent groups and side chains
491 vw	Skeletal deformation of aromatic rings, substituent groups and side chains
537 vw	Skeletal deformation of aromatic rings, substituent groups and side chains
555 vw	Skeletal deformation of aromatic rings, substituent groups and side chains
591 vw	Skeletal deformation of aromatic rings, substituent groups and side chains
634 vw	Skeletal deformation of aromatic rings, substituent groups and side chains
731 w	Skeletal deformation of aromatic rings, substituent groups and side chains
787 w	Skeletal deformation of aromatic rings, substituent groups and side chains
900 vw	Skeletal deformation of aromatic rings, substituent groups and side chains
926 vw	CCH wag
969 vw	CCH and -HC=CH- deformation
1033 w	C-O of aryl-O-CH_3 and aryl-OH
1102 w	Out of phase C-C-O stretch of phenol
1134 m	A mode of coniferaldehyde

1191 w	A phenol mode
1216 vw	aryl-O of aryl-OH and aryl-O-CH_3; guaiacyl ring (with C=O group) mode
1271 m	aryl-O of aryl-OH and aryl-O-CH_3; guaiacyl ring (with C=O group) mode
1297 sh	aryl-O of aryl-OH and aryl-O-CH_3; C=C stretch of coniferyl alcohol
1333 m	Aliphatic O-H bend
1363 sh	C-H bend in R_3C-H
1393 sh	Phenolic O-H bend
1428 w	O-CH_3 deformation; CH_2 scissoring; guaiacyl ring vibration
1454 m	O-CH_3 deformation; CH_2 scissoring; guaiacyl ring vibration
1508 vw	Aryl ring stretching, asymmetric
1602 vs	Aryl ring stretching, symmetric
1620 h	Ring conjugated C=C stretch of coniferaldehyde
1658 s	Ring conjugated C=C stretch of coniferyl alcohol; C=O stretch of coniferaldehyde
2843 m	C-H stretch in OCH_3, symmetric
2886 sh	C-H stretch in R_3C-H
2938 m	C-H stretch in OCH_3, asymmetric
3007 sh	C-H stretch in OCH_3, asymmetric
3065 m	Aromatic C-H stretch

[a]Assignment taken from reference Agarwal *et al.* 1997

[b]Note: vs is very strong; s is strong; m is medium; w is weak; vw is very weak; sh is shoulder. Band intensities are relative to other peaks in the spectrum.

Samples of flax (*Linum usitatissimum L.*) stem and its anatomical parts were studied by near-infrared Raman (NIR-Raman) spectroscopy to determine if the major chemical components of each could be detected by this method (Himmelsbach and Akin, 1998). The bands for cellulose were primarily observed in the fibres and hemicellulose polysaccharides were observed to be prevalent in bast tissue and fibres (Table 4).

Table 4. Regions assigned to cellulose and hemicellulose present in flax fibres (Himmelsbach and Akin, 1998; Jahn *et al.,* 2002).

Wavenumber (cm^{-1})	**Assignment**
Cellulose	
3000-2800	C-H stretch region; OH/CH deformation; CH/CH_2 wag region
1500-1200	C-O stretch
1200-950	Ring mode region
1120 and 1098	Symmetrical C-O-C and asymmetrical C-O-C vibrational stretching
950-700	Side-group deformation region for COH, CCH and OCH
910-890	HCC and HCO bending at the C6
Hemicellulose	
890	HCC and HCO bending at C6
515-470	HCC and HCO bending at C6
2895	Very strong C-H stretch

7. ADVANTAGES AND DISADVANTAGES OF IR AND RAMAN SPECTROMICROSCOPY

Although Raman shifts fall in the IR region of the electromagnetic spectrum, Raman spectra are quite different from IR spectra (Colthup *et al.,* 1990; Himmelsbach and Akin, 1998; Pistorius, 1995; Schenzel and Fischer, 2001; Schulz and Baranska, 2007; Thygesen *et al.,* 2003; West, 1996; Williams and Manson, 1990; Yadav, 2005). Table 5 summarizes the advantages and disadvantages of IR and Raman spectra deduced from literature review performed in previous sections. Both techniques have been proved beneficial in obtaining chemical compound information and their spatial distribution in lignocellusic biomass, when applied in coordination.

Table 5. Advantages and disadvantages of IR and Raman spectroscopy

Infrared Spectra	**Raman Spectra**
These originate from absorption of radiation by vibrating and rotating molecules	These originate from scattering of radiation by vibrating and rotating molecules
IR spectroscopy detects vibrations during which the electrical dipole moment changes	Raman spectroscopy is based on the detection of vibrations during which the electrical polarisability changes
For liquid samples, generally, dilute solutions are preferred	Raman lines are weak in intensity, hence concentrated solutions are preferred to get enough intensity
Wet samples can not be used for spectroscopic study because the O-H stretching vibration is very strong in IR and will give false spectra	Wet or dry sample can be used for spectroscopic study because the O-H stretching vibration is very weak in Raman since O-H bonds are weakly polarized
Optical systems of IR spectrometer are made of NaCl, NaBr, KCl, KBr, etc.	Optical systems of Raman spectrometer are made of glass or quartz
Fluorescent/Photochemical reactions do not take place	Sometimes fluorescent/photochemical reactions take place in the frequency regions of Raman lines and so create difficulties
IR has high signal-to-noise ratio resulting in shorter sampling times	Signal-to-noise ratio is much lower, and if the sample fluoresces, measurements may even be impossible. Therefore, long sampling times and/or repeated samplings are desired
The IR spectromicroscopy has spatial resolution of $\geq 10\ \mu m^2$ (Thygesen *et al.* 2003) and does not offer confocality i.e. it is not possible to focus on different planes below the sample	Raman spectromicroscopy has the potential of a better spatial resolution ($\geq 1\ \mu m^2$) due to lower wavelengths used and is possible to focus on different planes below the sample

surface	surface
Method is accurate as well as very sensitive	Method is accurate but not very sensitive
These are recorded by using a beam of radiation having a large number of frequencies in the IR region	These are recorded by using a beam of monochromatic radiation
Homonuclear diatomic molecules are IR inactive	Homonuclear diatomic molecules are Raman active
IR spectroscopy has negligible concerns of heating the sample	Raman spectroscopy has the problem of heating the sample due to heat generated by the laser. Therefore, short sampling times are recommended to avoid any alteration to the sample
Studies by the IR spectra do not require a high degree of purity	Pure substances are required for studies by Raman spectra

8. SUMMARY

The IR and Raman spectromicroscopic methods have the potential to determine the structural characteristics and chemical compound distribution in agricultural (lignocellulosic) biomass enabling it suitable for biorefineries. However, both these methods have their own advantages and drawbacks, and should be used as complementary techniques. By combining spectroscopy with microscopy molecular information can be obtained with great spatial resolution at the microscopic level.

The literature review of lignocellulosic biomass have indicated that IR and Raman spectromicroscopy could be used successfully to study the chemical structure and spatial distribution of cellulose, hemicellulose and lignin in various agricultural biomasses as applied to food, feed, biocomposite, textile, and paper and pulp industries. However, no published research has been found that could address the need to determine the structural characteristics and chemical components distribution in agricultural biomass using IR and Raman spectroscopic methods enabling it suitable for biorefineries. There is a need to initiate studies that could develop information on the spatial origin and distribution of the components of interest when subjected to various pre-processing and pre-treatment methods at microscopic level as opposed to quantification of chemical composition of biomass by relying on the separation of the components of interest.

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