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Chapter 15

Microprobing Structural Architecture Using Mid-Infrared Vibrational Molecular Spectroscopy

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Abstract

The biofunctions of biopolymers are closely related to their microstructures in the complex plant-based tissue in biological systems. In this chapter, molecular spectroscopy is introduced as an approach to microprobe the structural architecture of plant-based seed tissues. Some recent progresses are made using molecular spectroscopy techniques. The working principles of the techniques, along with the methods of molecular spectral analyses and applications in feed architecture research are described.

Keywords: molecular spectroscopy, chemical functional groups, biopolymers, plant-based feed and food, bio-functions

1. Molecular spectroscopy techniques

According to wavelengths from short to long, the electromagnetic spectrum includes gamma rays, hard X-rays, soft X-rays, ultraviolet, visible light, near infrared, mid-infrared, far infrared, microwaves, and radio waves. Gamma rays have the highest frequency and strongest penetration ability. They are produced by the most energetic objects in the universe, or by nuclear explosions, lightning, and radioactive decay on the earth. X-rays are widely used in medical and science areas, while soft X-rays are also used to analyze the characterization of different layers of plant tissues [1, 2]. Near infrared (NIR), as well as mid-infrared, are effective tools in feed analysis and quality assessment. The mid-IR spectral region (ca. 4000–400 cm^{-1}) is a domain of interest to many scientific areas because many molecules have strong characteristic vibrational transitions, especially in the wave number range of ca. 1800–800 cm^{-1}, which is also called the "fingerprint region" [3–5].

As we know, plants are made up of molecules and the internal molecular energy consists of the electronic, translational, rotational, and vibrational energies. Under normal conditions, the functional groups in organic molecules vibrate independently and only interact weakly with each other. However, the interference from outside, such as electromagnetic radiation, could trigger the nonequilibrium phase and cause energy transitions between the rotational and vibrational energies, which can induce the net change in the electric dipole moment and the absorption of the IR. As the ratio of absorption and transmission IR differs between molecules, nearly every molecular species gives a unique IR absorption spectrum. Hence, IR spectrometry could be used to identify molecular functional groups [5].

2. ATR-FTIR molecular spectroscopy techniques

2.1. Working principles

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) is a globar-sourced FTIR spectroscopy, which could be used to identify the molecular constituents in a wide range of samples in areas such as physics, chemistry, and biology. ATR-FTIR is mainly the combination of the globar light source and a microscope and based on the attenuation effect of light [5, 6].

The core part of a FTIR spectrometer is the Michelson interferometer (**Figure 1**, adapted from [9]).

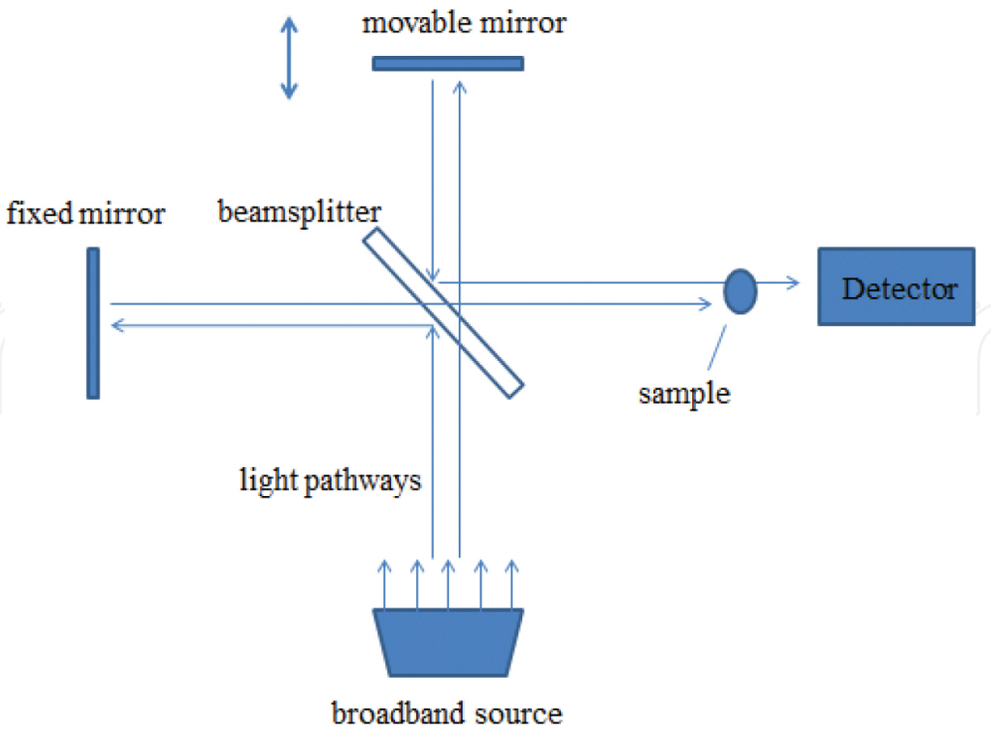

Figure 1. Schematic diagram of a FTIR spectrometer (adapted with permission from McCluskey [9]).

The collimated light from the broadband source travels through the beamsplitter and is split into two beams. One beam travels through the splitter and is reflected by a movable mirror, and the other beam travels to a fixed mirror and is reflected back. A portion of the light finally reaches the sample that may be placed in a liquid-helium cryostat with IR-transparent windows made of ZnSe, KBr, or polypropylene. In an ATR-FTIR instrument, a crystal made of material such as zinc selenide (ZnSe), germanium (Ge), and thallium-iodide is placed under the samples and the incident beam entering at an angle larger than the critical angle, the total reflection could be achieved and only the part of energy that is absorbed by the sample is lost during the process [7, 8]. The part of light that passes through the sample is sensed by the detector, which could be a photoconducting detector such as Ge:Cu placed right behind the sample or a mercury-cadmium-telluride (MCT) mounted in the outside [9]. In this way, all spectral elements are measured simultaneously on the detector and the time consumption (Fellgett advantage or multiplex advantage) depends primarily on the movement of the movable mirror, which could be very short [8, 10].

This technology has a high spectral resolution, a broad measure range and short measure time [9]. At the same time, with no slits to attenuate the infrared light, FTIR has a higher throughput of radiation compared to conventional IR methods (Jacquinot advantage) [10]. Another advantage of ATR-FTIR is it only requires simple sample preparation by finely grinding them and depositing a thin layer on the infrared transparent windows [6].

Nevertheless, the technique also has its shortcomings. Due to the limited brightness, when ATR-FTIR is used to analyze a small region of interest, the decreased aperture would result in the diffraction effects and reduce the signal-to-noise ratio [5]. It is reported that as the plant cell size is normally between 5 and 30 μm, the globar sourced FTIR is not able to obtain a good signal-to-noise ratio within this dimension [5].

2.2. Application of ATR-FTIR techniques in feed research

The ATR-FTIR technique has been proven to be effective in mineral samples in oil shale [11] and biological samples such as cytology and tissue sections, live cells or biofluids [12], and plant samples including transgenic alfalfa [13], hulless barley [14, 15], corn forage [16], DDGS [17, 18], coproducts from bio-oil processing [19–22], heat processing impact [23, 24].

The lipid region, protein region, and carbohydrate region are the three main regions in a spectrum of feed material being analyzed (**Figure 2**). Taking cereal grains as an example, the assessed items included infrared intensity of protein amide I (ca. 1725–1578 cm^{-1}), amide II (ca. 1578–1482 cm^{-1}), amide I peak height (ca. ~1647 cm^{-1}), amide II peak height (ca. ~1537 cm^{-1}), α-helix height (ca. ~1653 cm^{-1}), β-sheet (ca. ~1632 cm^{-1}), lipid (ca. 1798–1709 cm^{-1}) and its peak height (ca. ~1744 cm^{-1}), cellulosic compounds (ca. 1291–1184 cm^{-1}) and its peak height (ca. ~1238 cm^{-1}), total carbohydrates (CHO; ca. 1191–944 cm^{-1}), three major CHO peaks: first peak (ca. 1191–1132 cm^{-1}) and its peak height (ca. ~1150 cm^{-1}), second peak (ca. 1132–1066 cm^{-1}) and its peak height (ca. 67–1078 cm^{-1}), third peak (ca. 1066–944 cm^{-1}) and its peak height (ca. ~1012 cm^{-1}) [25].

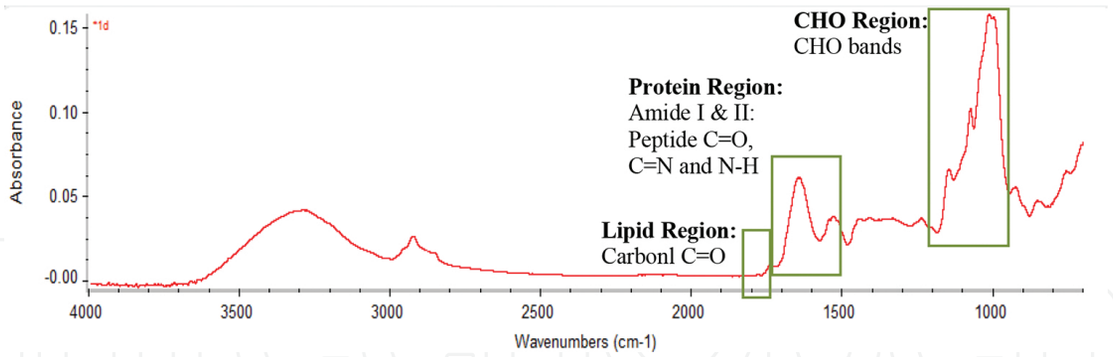

Figure 2. Spectra and fingerprint region and chemical functional groups in plant-based feeds and food.

3. Synchrotron-based molecular spectroscopy techniques (SR-IMS)

3.1. Working principles and advantages

The biggest advantage of SR-IMS is it could preserve the information about the spatial distribution of the objects when detecting the inner structures. This is achieved by the use of synchrotron infrared light source. The nondivergent, intense and extremely fine beamline is created by a giant particle accelerator that turns electrons into light, which is 100–1000 times brighter than the globar source [5]. Therefore, high spatial resolution and signal to noise spectra can be collected at a faster speed [5].

3.2. Novel applications of SR-IMS techniques in feed research

The SR-IMS technology was first applied to animal feed research in 1999 [26]. Since then it has been utilized on several feeds, including transgenic alfalfa [27, 28], hulless barley [5, 15, 29], canola seeds [5, 30], corn seed [28], flaxseeds [28, 30], sorghum seeds [31], wheat [30, 32], wheat DDGS [28], and corn DDGS [28]. In spite of all these applications, this research is still in its infancy.

4. Spectra analysis

Functional groups such as amide I and amide II bonds have certain percentages of C═O, C─N, and N─H stretching vibrations, the wave numbers (cm^{-1}) at which they are absorbed and generally fixed, but they also slightly shift depending on the samples [5]. Some typical IR absorption bands include: amide I (centered at about 1650 cm^{-1}, includes about 80% C═O stretching, 10% C─N stretching and 10% N─H bending), amide II (centered at about 1550 cm^{-1}, includes about 60% N─H bending and 40% C─N stretching), lipid carbonyl C═O (peaks at about 1738 cm^{-1}), and cellulose (at about 1100 cm^{-1}) [33]. Among them, amides I and II are the most dominant vibrational bands of the protein backbone and amide I, due to its high

C═O stretching composition, is the most sensitive and highly related to secondary structural elements of proteins [34].

4.1. Univariate analysis

Using univariate analysis, it is possible to discover quantitative differences in the spectra information, such as the component areas, peak heights, and ratios among different components. Univariate analysis gives very straightforward results in terms of what changes occurred on the mathematical parameters characterizing the spectrum, such as the band intensities, integrated intensities, band frequencies and the band intensity ratios. In addition, this method makes it possible to connect the spectra information to the biological meaning on a mathematical basis [35].

4.2. Multivariate analysis

Multivariate analysis is capable of analyzing multiple variables at same time. Principal component analysis (PCA) and hierarchical cluster analysis (CLA or HCHA) are two of the commonly used methods.

The PCA transforms the original set of variables based on the correlations among them, into a set of independent linear combinations called principal components (PCs), which contain most of the information in the original variables and empirically summarizes their correlations [32, 36]. The first few PCs usually account for more than 95% of the total variation among the variables [27].

The CLA is another data reduction method that calculates a distance matrix, searches for the two most similar objects, and displays the results as dendrograms [32, 37]. In the hierarchical approach, the object or objects are gathered as a group step by step, being nested to the previous groups. Thus, the number of clusters reduces sequentially as the clusters' sizes grow and end up with only one [37].

5. Application

5.1. Application 1: structural responses of functional groups in cereal grains to heat processing methods

The research [25] in processing-induced molecular structure study showed that the sensitivity and responses of functional groups can be detected by both ATR-FTIR and SR-IMS techniques, and different functional groups in cereal grain tissues respond differently to the heating methods, although not all heat-induced structural changes detected by the two mid-IR techniques are highly related to the nutrient availability of cereal grains in ruminants.

Due to the difference in sample-preparation and sampling areas, the results found by the two mid-IR techniques were also different. Similar to the conventional studies, the grains were ground and well-mixed before using the ATR-FTIR technique. The results found in the

conventional studies indicated that moist heating had greater impact on nutrient availabilities compared with dry heating. In accordance with such results, ATR-FTIR method also detected stronger influence on spectral peak areas, heights, and ratios (**Figure 3**). These alterations, especially the changes on protein secondary structure, were highly related with the nutrient availability in cereal grains.

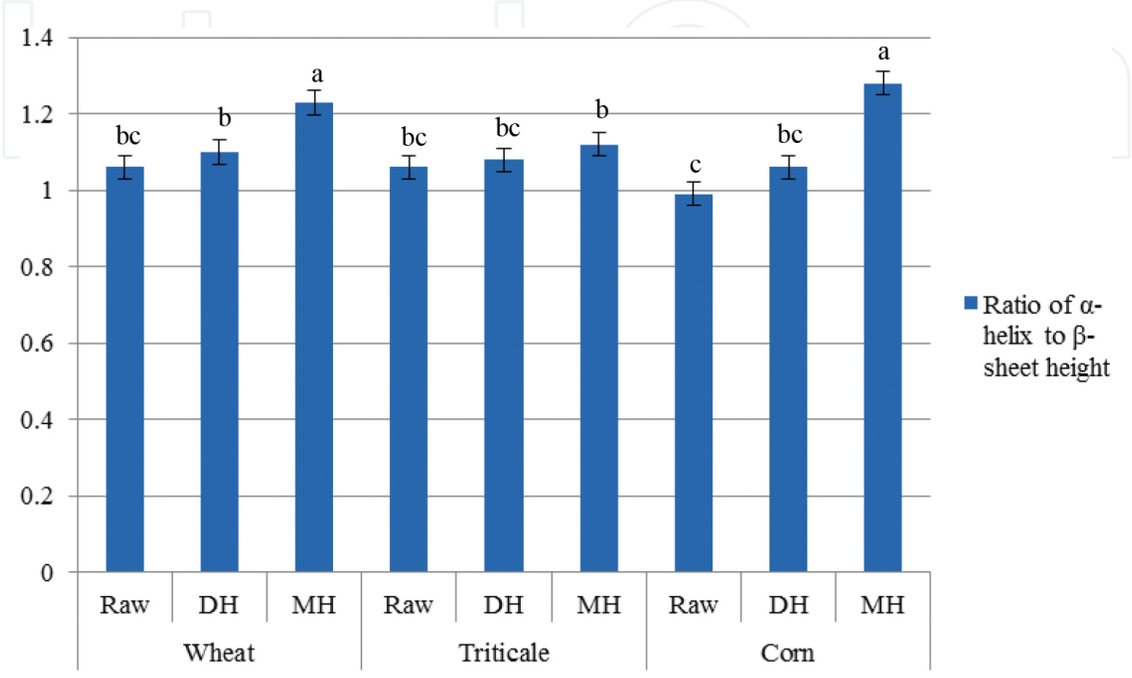

Figure 3. Ratios of modeled α-helix to modeled β-sheet height affected by processing methods detected by ATR-FTIR technique.

The grain seeds were cross-sectioned into thin (6 μm) sections and spectra were collected from the endosperm area. The results discovered by the SR-IMS technique indicated that dry heating also played a big role in changing the secondary structures and functional groups of the grains. As the peak areas and peak heights represent combined information of nutrient amount and molecular structure, with the nutrient contents affected by moist heating, it is very likely that the molecular structures in the endosperm were also changed. Unfortunately, such change was not specified in this part of study. In comparison with results found by the ATR-FTIR technique, less and weaker correlation was discovered between the heat-induced structural changes and the nutrient availability in the endosperm area of cereal grains in ruminants by the SR-IMS technique.

5.2. Application 2: microwave irradiation-induced changes in protein molecular structures of barley grains: relationship with changes in protein chemical profile, protein subfractions, and digestion in dairy cows

These studies [38, 39] aimed to evaluate microwave irradiation (MIR)-induced changes in crude protein (CP) subfraction profiles; ruminal CP degradation characteristics and intestinal digestibility of rumen undegraded protein (RUP); and protein molecular structures in barley

(*Hordeum vulgare*) grains. Samples from hulled and hulless cultivars of barley, harvested in two consecutive years from four replicate plots, were evaluated. The samples were either kept as raw or irradiated in a microwave for 3 min (MIR3) or 5 min (MIR5). Compared with raw grains, MIR5 decreased the contents rapidly degradable CP subfraction (45.2–6.4% CP) and the ruminal degradation rate (8.16–3.53%/h) of potentially degradable subfraction. As a consequence the effective ruminal degradability of CP decreased (55.7–34.1% CP), and RUP supply (43.3–65.9% CP) to the postruminal tract increased. The MIR decreased the spectral intensities of amide 1, amide II, α-helix and β-sheet, and increased their ratios. The changes in protein spectral intensities were strongly correlated with the changes in CP subfractions and digestive kinetics. These results show that MIR for a short period (5 min) with a lower energy input can improve the nutritive value and utilization of CP in barely grains.

6. Summary, implications, and future research areas

6.1. Summary

As different ratios of IR could be absorbed in different molecules when mid-IR is applied, the functional groups have their unique spectra, especially in the "fingerprint region." There have been many applications of ATR-FTIR and SR-IMS on animal feed researches in recent years. The results show that these two advanced mid-IR approaches can effectively detect the microstructural changes in some plant tissues. With the help of the statistical analysis, quantitative differences lie between different spectra could be discovered.

6.2. Implications

Functional groups in different type of plants could have different sensibility and react differently to external changes. Feed processing methods could change the inner structure of the plant tissues, such change can probably be detected by mid-IR techniques such as SR-IMS and ATR-FTIR. Combined with conventional animal nutrition studies, the link between structural changes in spectral areas such as amide, CHO, and cellulosic compounds and nutrient availability of the plant could be found.

6.3. Further research areas

Our further research plans include using ATR-FTIR technique to detect the sensitivity and responses of various chemical functional groups in different types of feed materials to different types of feed processing methods, and building up models using the spectral parameters to estimate the nutrient utilization and availability in ruminants. We would also expand the sampling areas when using the SR-IMS technique and combine methods such as Mid-IR microspectroscopic mapping to better understand the inner structural changes in the plant tissues.

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