JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Imaging Molecular Chemistry of Pioneer Corn

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Synchrotron Fourier transform infrared (FTIR) microspectroscopy as a rapid, direct, and nondestructive analytical technique can explore molecular chemical features of the microstructure of biological samples. The objective of this study was to use synchrotron FTIR microspectroscopy to image the molecular chemistry of corn (cv. Pioneer 39P78) to reveal spatial intensity and distribution of chemical functional groups in corn tissue. This experiment was performed at the U2B station of the National Synchrotron Light Source in Brookhaven National Laboratory (NSLS-BNL, Upton, NY). The Pioneer corn tissue was imaged from the pericarp, seed coat, aleurone, and endosperm under peaks at 1736 (carbonyl C═O ester), 1510 (aromatic compound), 1650 (amide I), 1550 (amide II), 1246 (cellulosic material), 1160 (CHO), 1150 (CHO), 1080 (CHO), 929 (CHO), 860 (CHO), 3350 (OH and NH stretching), 2929 (CH_2 stretching band), and 2885 cm^{-1} (CH_3 stretching band). The results showed that with synchrotron FTIR microspectroscopy, the images of the molecular chemistry of Pioneer corn could be generated. Such information on the microstructural−chemical features of grain corn can also be used for corn breeding programs for selecting superior varieties of corn for targeted food and feed purposes and for prediction of corn quality and nutritive value for humans and animals.

KEYWORDS: Synchrotron; FTIR spectroscopic characteristics; corn; molecular chemistry; imaging

INTRODUCTION

Grain corn is commonly grown in the world for human and animal diets (*1*, *2*). The selection of corn varieties has been one of several approaches undertaken to improve the nutritive value and nutrient utilization of corn. Most studies have focused on total chemical composition of seeds including total protein and carbohydrates (structural and nonstructural) components using traditional "wet" chemical analysis.

As we know, traditional analytical chemistry has several disadvantages: (1) the analyses rely heavily on the use of harsh chemicals and derivatization, therefore altering the native plant structures and possibly generating artifacts; (2) the analyses also require reasonable amounts of plant material (gram or milligram levels), which usually means that they are carried out on composite samples of tissues (*3*); (3) the analyses look for a specific component through homogenization of the tissue and separation of the component of interest from the complex matrix. As a result, information about the spatial origin and distribution of the component of interest is lost and the object of the analysis is destroyed (*4*).

Infrared (IR) spectroscopy is able to identify molecular constituents in biological samples from their vibration spectra in the mid-IR region (*5*). Synchrotron-based Fourier transform infrared (FTIR) microspectroscopy is a rapid, direct, nondestructive, and noninvasive chemical analytical technique. It can detect molecular chemical features of biological samples at high spatial resolutions without destroying the inherent structure of the tissue (*5*−*13*). The chemical information (qualitative and quantitative analytical results) can be linked to structural information (*4*). Compared to other traditional technologies in plant analysis, the synchrotron FTIR microspectroscopy has several advantages and has the potential to be expanded to the nutritive evaluation field. The advantages of synchrotron-based FTIR microspectroscopy have been detailed in Wetzel et al. (*6*, *14*).

No study, to our knowledge, has been carried out to explore the structural−chemical features (bonding and functional group characteristics) of corn seeds tissue within cellular dimensions. These features are associated with corn quality and with nutritive value in human and animal diets. The objective of this study was to use synchrotron FTIR microspectroscopy to explore the molecular chemical features of the microstructure of Pioneer corn. Such information would aid grain corn breeding programs for selecting superior varieties of corn for targeted food and feed purposes and for prediction of corn quality and nutritive value for humans and animals.

MATERIALS AND METHODS

Pioneer Corn Tissue. Grain corn (cv. Pinoneer 39P78) at harvest maturity was obtained from Henry Penner (Morden, MB), arranged by Prairie Feed Resource Centre (Canada) (Director, Vern Racz). Harvest maturity is often defined as that grain moisture content when harvest can occur with minimal kernel damage and mechanical harvest loss.

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10.1021/jf049291b CCC: $27.50
Published on Web 11/05/2004

Synchrotron FTIR Slide Preparation. The Pioneer corn was frozen at −20 °C and then was cut into thin cross sections (~6 μm thick) using a microtome at The Western College of Veterinary Medicine, University of Saskatchewan. The unstained cross sections were mounted onto Low-e IR microscope slides (Kevley Technologies, Chesterland, OH) for synchrotron FTIR microspectroscopy in a reflectance mode.

Photomicrographs of Cross Sections of Pioneer Corn Tissues. Photomicrographs of the cross-section of the cereal grain tissues were taken by a microscope with a digital camera from slides (objective ×10). The magnification of photomicrographs was 10 × 10.

Synchrotron Light Source and Synchrotron FTIR Microspectroscopy. The experiment was carried out on the U2B beamline at Albert Einstein Synchrotron Bioscience Center in the National Synchrotron Light Source at Brookhaven National Laboratory (NSLS-BNL, Upton, NY). The beamline is equipped with a FTIR spectrometer (Nicolet Magna 860) with a KBr beam splitter and a mercury cadmium telluride (MCT-A) detector coupled with an IR microscope (Nic-Plan, Nicolet Instruments, Madison, WI). The bench was configured to use collimated synchrotron light (beam energy 800 MeV) through an external input of the spectrometer. The modulated light was passed through the Nic-Plan IR microscope to perform reflection microscopy. The IR spectra were collected in the mid-IR range 4000−800 cm^{-1} at a resolution of 4 cm^{-1} with 64 scans co-added and an aperture setting of ca. 10 μm × 10 μm. The reasons for the chosen aperture size of 10 μm × 10 μm were as follows: (1) The size was within the cellular dimension. (2) The 10 × 10 μm aperture size was the best for getting good signal-to-noise ratio spectrum mapping of Pioneer corn tissue. To minimize IR absorption by CO_2 and water vapor in ambient air, the optics were purged using dry N_2. A background spectroscopic image file was collected from an area free of sample. The mapping steps were equal to aperture size. Stage control and data collection and processing were performed using OMNIC 6.0 (Thermo-Nicolet, Madison, WI). Scanned visible images were obtained using a charge-coupled device (CCD) camera linked to the infrared images (objective × 32).

In the microspectroscopic area mapping, the spatial information was obtained by translating the tissue along the *x*- and *y*-axes and positioning different parts of the designated tissues in the synchrotron IR beam of the microspectroscope at the U2B station. The motorized computer-control stage was programmed to trace the designated areas in the tissues. After the spectra of all parts of interest had been measured, the spectral information was related to the visible images. As a result, spectral data sets were formed with the *xy* surface, corresponding to the scanned area of the sample, and *z* direction, which contained the spectral information. Functional group images (such as amide I, aromatic compound, carbonyl ester) were generated by plotting the intensity of synchrotron IR absorption bands as a function of the *xy* position (*4*). Different coverage of the sample with measurements could also be achieved by varying the step size and the dimensions of the image mask (aperture size). The big advantage of using the synchrotron beam with FTIR spectroscopy to map the tissues is that diffraction limits and spatial resolution are achievable (*6*, *14*).

Data Analysis and Chemical Imaging. The spectral data of the Pioneer corn tissues were collected, corrected for the background spectrum, and analyzed using OMNIC software 6.0 (Thermo-Nicolet). A baseline correction was applied to generate the final spectra. The synchrotron FTIR bands were expressed as log(1/*R*), because these units in reflection mode correspond to absorbance in a regular transmission method. The data can be displayed either as a series of spectroscopic images collected at individual wavelengths or as a collection of infrared spectra obtained at each pixel position in the image.

The molecular chemistry of functional groups from the pericarp to endosperm was imaged under peaks at 1736, 1510, 1650, 1246, 1160, 1150, 1080, 929, 860, 3350, 2929, and 2885 cm^{-1} using the OMNIC software 6.0 (Thermo-Nicolet) (*4*, *6*, *8*, *15*, *16*). False color maps were used (colors representing band intensities), which were derived from the area under particular spectral features.

RESULTS AND DISCUSSION

Photomicrograph of Pioneer Corn Tissues. The chemical microstructure of cereal grains has been of interest in our laboratory in regard to nutritive value and nutrient utilization. **Figure 1** illustrates the intrinsic structure of Pioneer tissue [6 μm thickness cross section; the magnification of the photomicrograph is 10 × 10 (figure is reproduced here at ~80% of original size)] from the pericarp at the outside of the seed, to the seed coat, through to the aleurone layer and the part of endosperm. The detailed chemistry of each layer of grain seeds has been discussed in previous publications (*6*, *14*, *16*). The pericarp forms the tough outer covering of the seed kernel and provides protection for the interior components. The seed coat is found between the pericarp and the aleurone layer. The aleurone cells play an important role during seedling development. The endosperm consists of cells filled mainly with starch granules. However, the photomicrographs of the tissue are not informative with respect to molecular chemical features of the inherent structures at spatial resolution.

Molecular Chemistry Mapping To Reveal Structural Features. Mid-IR spectroscopy (4000−600 cm^{-1}) measures the contribution from vibrations of particular organic and inorganic functional groups within molecules (*5*, *10*, *16*). A drawback of a Globar-sourced FTIR microspectroscopy is resulting diffraction effects if the aperture is decreased to limit the field of view to a small region of interest. At the same time, less light overall reaches the detectors, and hence the signal-to-noise ratio decreases (*9*, *10*). For this kind of study of cereal grain tissue on structural−chemical features within cellular dimensions, to collect spectral data at the diffraction limit or a few micrometers in each spatial dimension, only synchrotrons and free electron lasers can be used. The brightness of conventional benchtop IR sources is simple to lower by 2−3 orders of magnitude (*9*, *10*). On the basis of the advantages offered by synchrotron FTIR microspectroscopy, the molecular chemistry of Pioneer corn was imaged.

Figure 2 represents color maps of functional groups of a cross section of the Pioneer corn tissue and single-pixel spectra from a sample area of 10 × 10 μm. It shows visible and chemical images in false-color representation of chemical component intensities and spectra at various pixels. Using synchrotron FTIR microspectroscopy, the distribution and relative concentration of the chemical functional groups associated with the corn tissue inherent structure were mapped. The infrared images were taken from the region of the visible image outlined by the rectangle area. The size of the rectangle area in the visible image was 25 μm × 8 μm.

The molecular chemistry of functional groups from the pericarp, seed coat, aleurone, and part of the endosperm was imaged under peaks at 1736 (carbonyl C═O), 1510 (aromatic character of lignin), 1650 (amide I C═O), 1550 (amide II N−H and C−N), 1246 (cellulosic), 1160 (CHO), 1150 (CHO), 1080 (CHO), 929 (CHO), 860 (CHO), 3350 (NH and OH stretching), 2929 (CH_2 stretching vibrations), and 2885 cm^{-1} (CH_3 stretching vibrations) (**Figure 2**). The functional group band assignments (for cereal grain) were according to publications (*6*, *14*−*16*).

The dominant absorption features in the lipid spectrum are found in the region 2800−3000 cm^{-1} and assigned, by analogy with the IR spectra of alkanes, predominantly to asymmetric and symmetric stretching vibrations of CH_3 (ca. 2956 and 2874 cm^{-1}) and CH_2 (ca. 2922 and 2852 cm^{-1}) groups of the acyl chains (*7*, *17*). As expected given the greater number of CH_2 groups, the corresponding peak intensities are 10−20 times greater than that of CH_3. The frequency of the CH_2 stretching absorptions provides a useful probe of lipid bilayer order, low frequencies being associated with a higher degree of conformational order (*17*). In addition, the strong band at ~1736 cm^{-1} arises from the stretching vibration of the ester C═O groups in the lipid (*5*−*7*). **Figure 2a** is a chemical image under area at

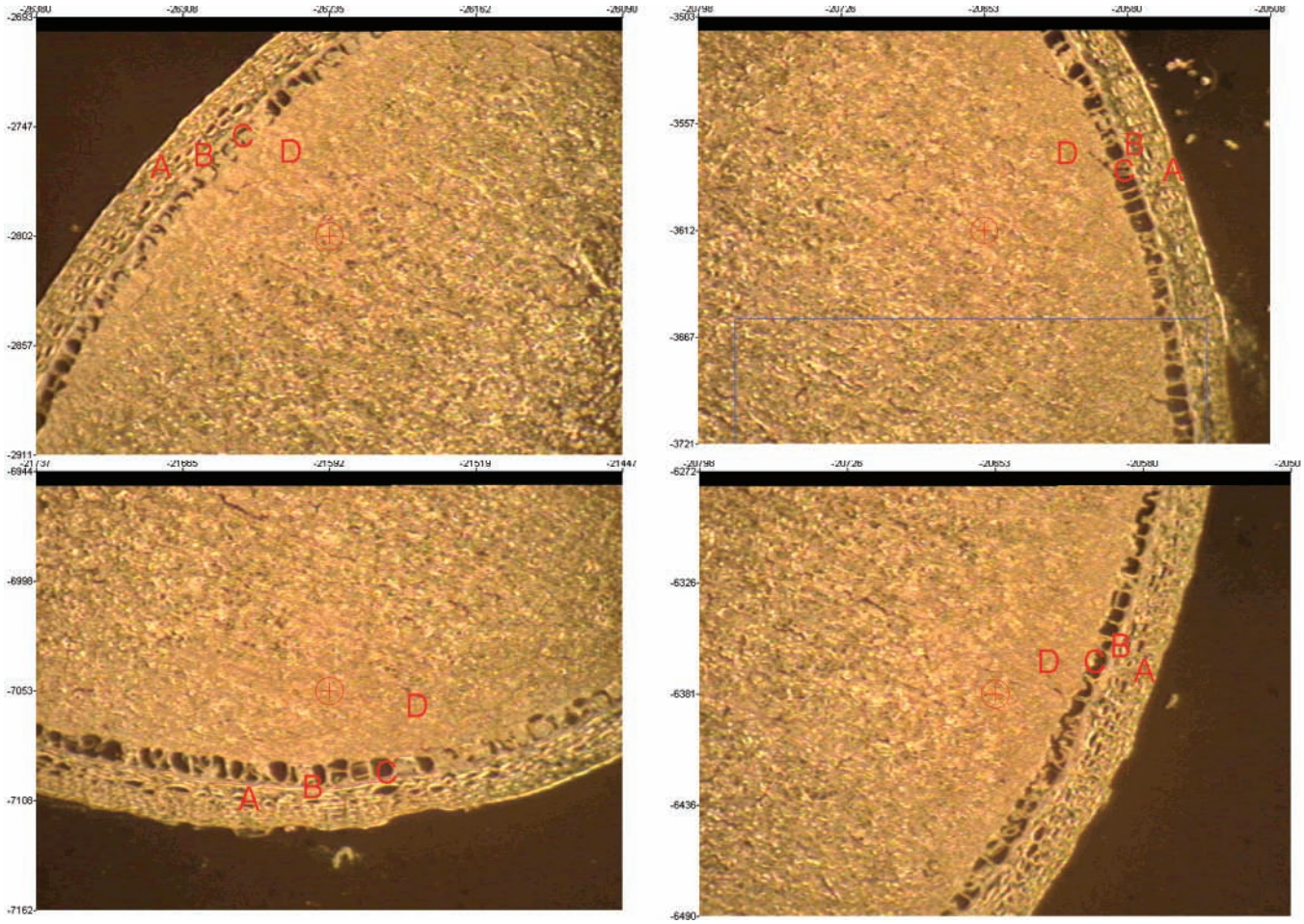


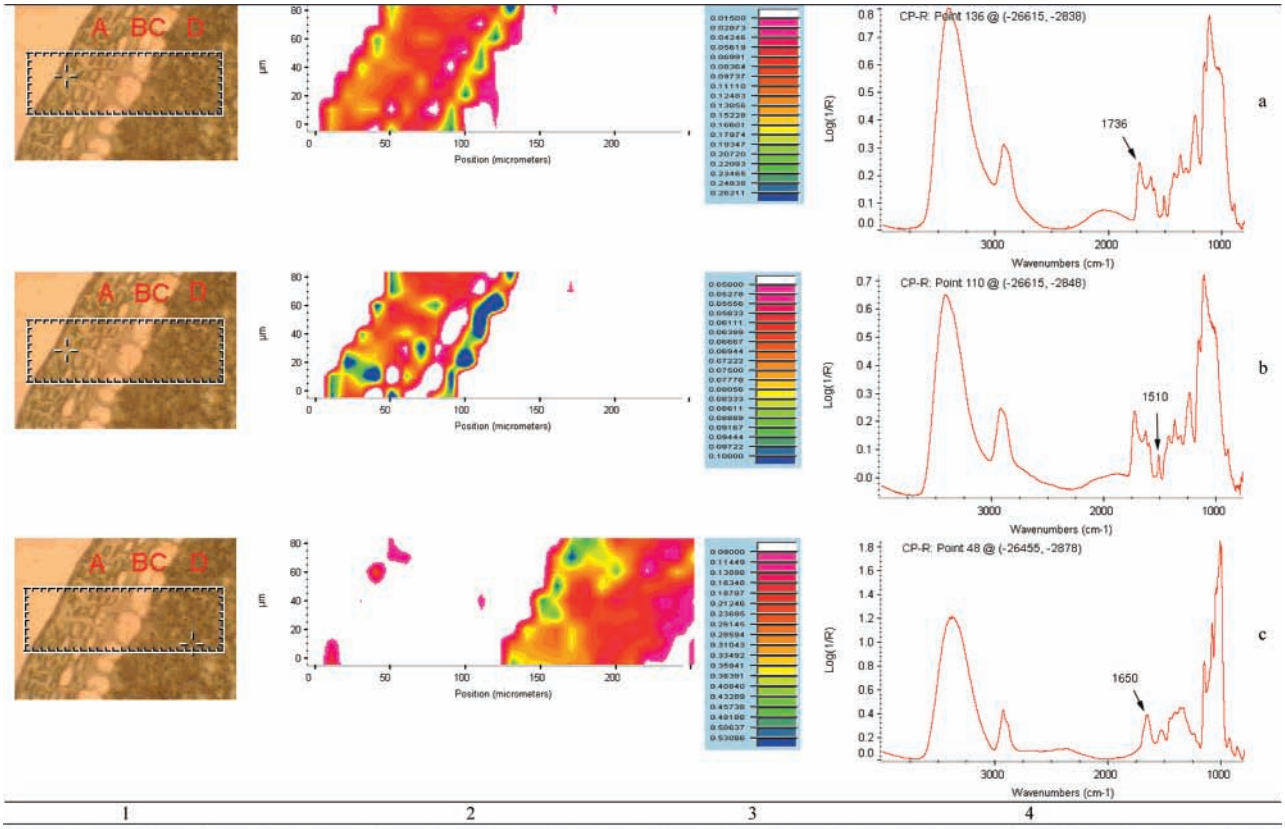
Figure 1. Photomicrograph of cross-section of Pioneer corn tissue (6 μm) showing the intrinsic structure: pericarp (outside of seed) (**A**), seed coat (**B**), aleurone (**C**), and endosperm (**D**). [Magnification: 10 × 10 (figure is reproduced here at ~80% of original size).]

~1736 cm^{-1} showing the lipid distribution. The band at ~1736 cm^{-1} is due to the carbonyl group (C═O) stretching vibration in the ester linkage in the pericarp. The endosperm areas contain little lipid. The spectra on the right in each figure correspond to the pixel in the cross-hair and were selected to represent the value of the integrated peak. The represented spectra show a wide variety of spectral characteristics, which manifest the chemical composition in different morphological parts of the seed. In plant leaves, the 1736 cm^{-1} feature may also arise from methylesterified pectins (*11*), not just lipid in cereal grain (*6*).

The unique absorption band of lignin is found at 1510 cm^{-1} in the mid-IR region. This is considered to be indicative of the aromatic character of the lignin. An aromatic compound gives two major bands at ca. 1600 and 1500 cm^{-1}, referred to as quadrant and semicircle ring stretch, respectively (*18*). These are well exemplified in the lignin spectrum that shows bands at 1595 and 1510 cm^{-1}. The first of these bands possibly interferes with the other bands. The second of these bands, the peak at 1510 cm^{-1}, shows no significant interference with any other bands and thus is an excellent diagnostic criterion for aromatics. **Figure 2b** is a chemical image under area at ~1510 cm^{-1}, showing the spatial lignin distribution and concentration of the area under peaks centered at 1510 cm^{-1}. This infrared data image was taken at the area of the visible image outlined by the rectangle. The 1510 cm^{-1} band (*6*, *15*, *18*) corresponds to the stretch associated with para-substituted benzene rings and can be associated with aromatic species present only in the pericarp of the Pioneer corn.

The protein IR spectrum has two primary features, the amide I (~1600−1700 cm^{-1}) and amide II (~1500−1560 cm^{-1}) bands, which arise from specific stretching and bending vibrations of the protein backbone. The amide I band arises predominantly from the C═O stretching vibration of the amide C═O group. The frequency of the amide I band is particularly sensitive to protein secondary structure (*5*, *7*, *14*) and can be used to predict protein secondary structure. Wetzel et al. (*14*) reported the comparison of the protein secondary structure in terms of α-helix and β-sheet among six varieties of hard wheat and four varieties of soft wheat. In this paper, total protein spatial distribution and intensity was imaged. However, by using a function of the secondary derivative of spectrum in some software, the protein secondary α-helix and β-sheet components could also be imaged, separately. The amide II (predominantly an N−H bending vibration coupled to C−N stretching) is also used to assess protein conformation. However, as it arises from complex vibrations involving multiple functional groups, it is less useful for protein structure prediction than the amide I (*17*). **Figure 2c** is a chemical image under area at ~1650 cm^{-1}, showing the area under the 1650 cm^{-1} peak attributed to protein absorption (amide I). The amide I (1650 cm^{-1}) and amide II (1550 cm^{-1}) are characteristics of C═O and N−H bonds in the protein backbone and are indicators of the area of the sample where protein is present. **Figure 2c** shows that the pericarp region has no protein associated with it and that protein concentration is high in the subaleurone and endosperm layers.

The major absorptions from carbohydrates are found in the 1180−950 cm^{-1} region of the spectrum and are attributed to C−O stretching vibrations. When studying cereal grain materials, we normally look for structural carbohydrates such as cellulose or hemicellulose (*6*, *16*). A peak area of the ~1420


A BC D
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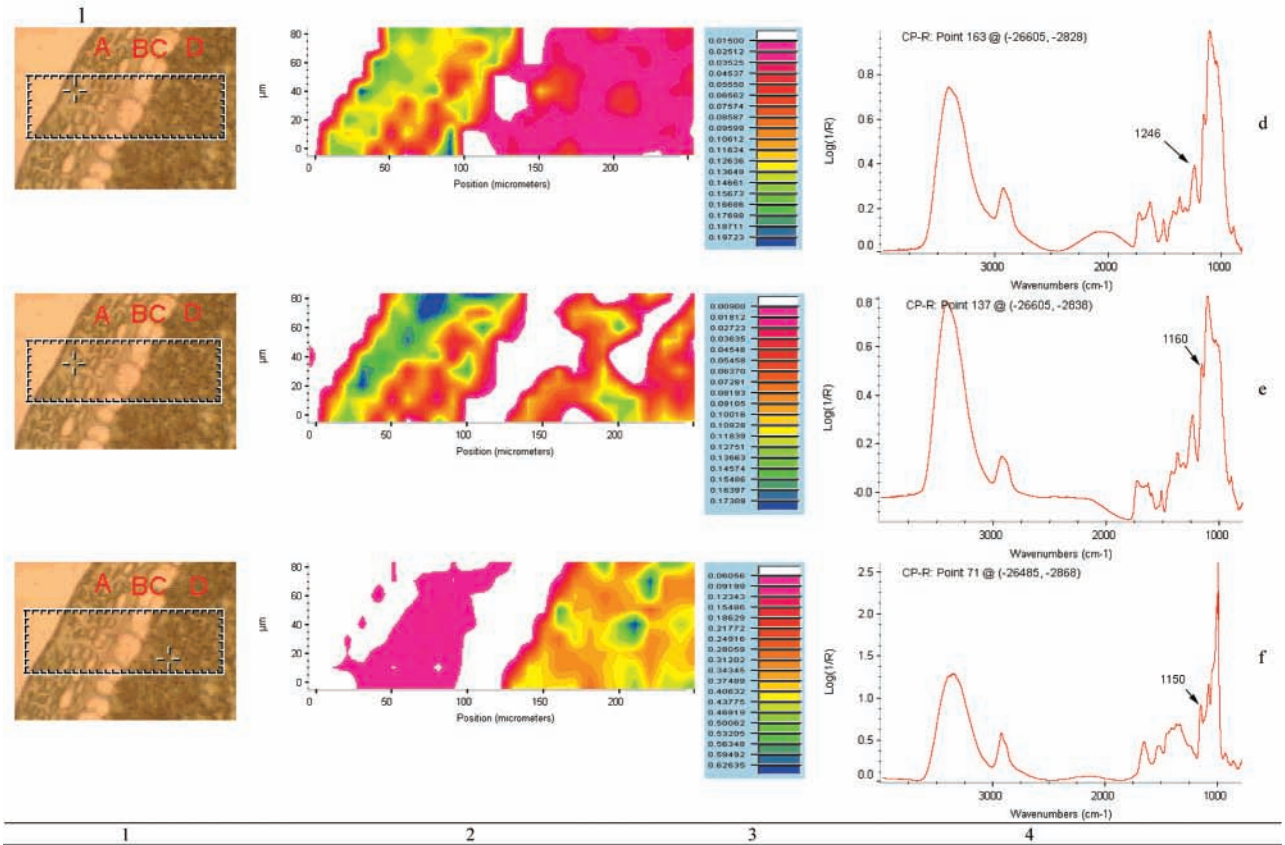
1736
a
CP-R: Point 110 @ (-26615, -2848)
1510
b
CP-R: Point 48 @ (-26455, -2878)
1650
c
Wavenumbers (cm-1)
Log(1/R)
1
2
3
4


1
A BC D
Position (micrometers)
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1246
d
CP-R: Point 137 @ (-26605, -2838)
1160
e
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1150
f
Wavenumbers (cm-1)
Log(1/R)
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2
3
4

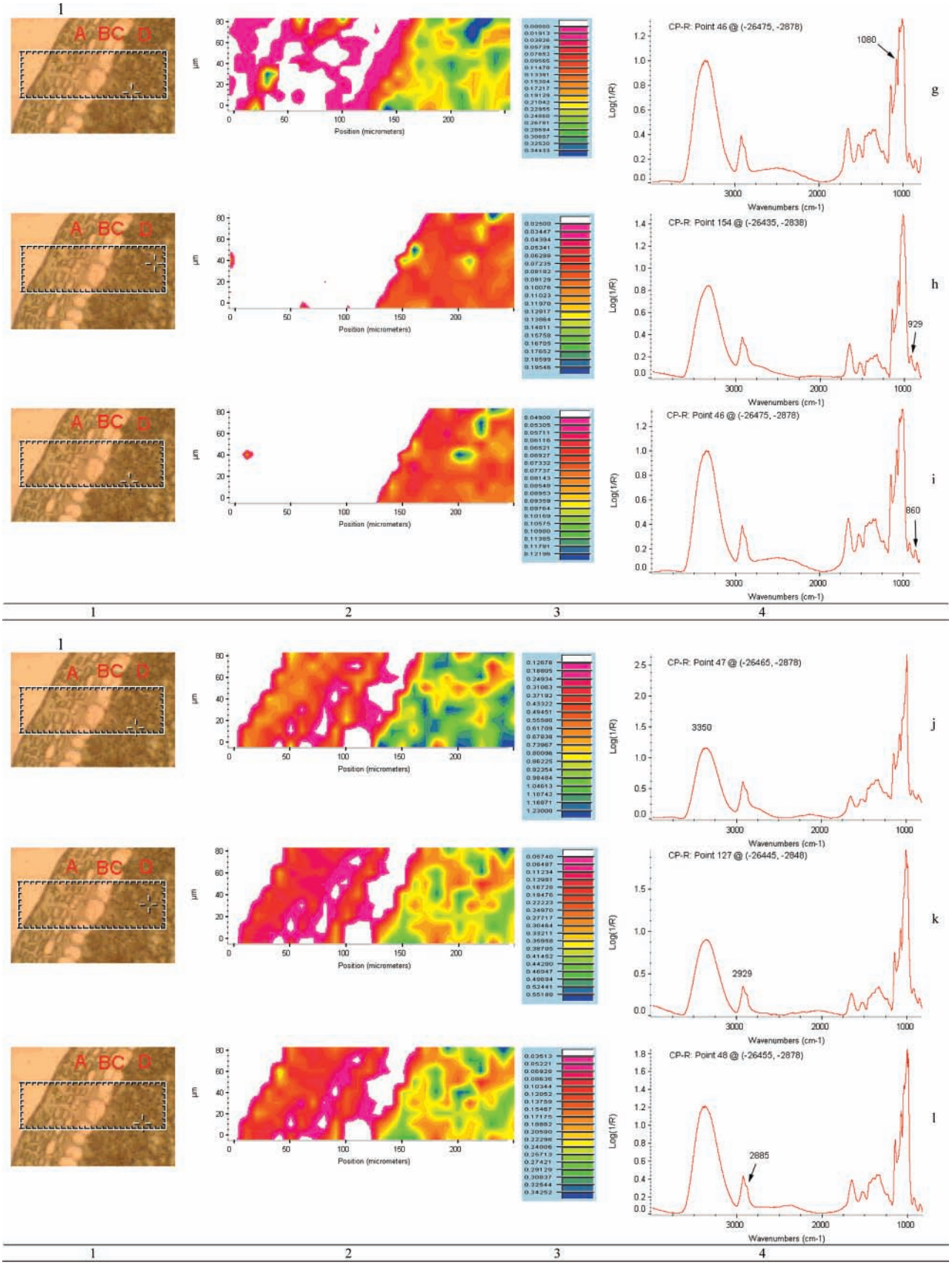
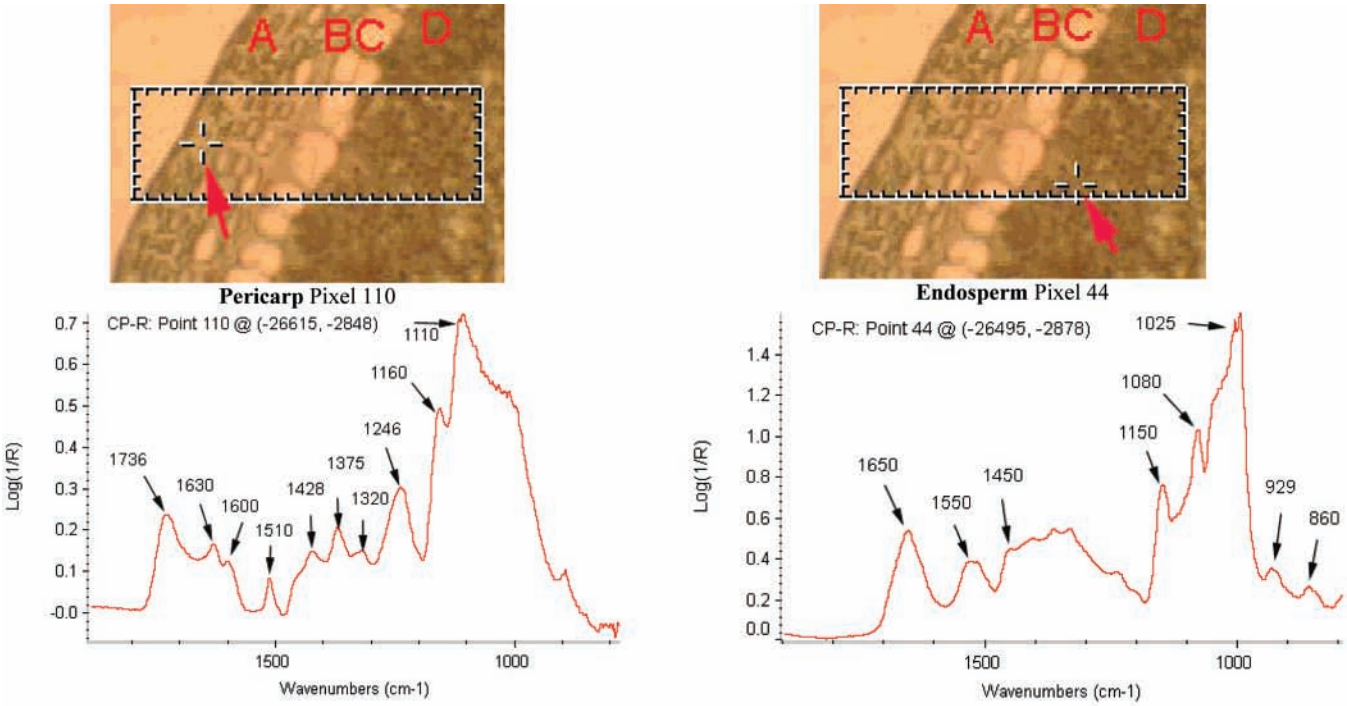


Figure 2. Functional group images of the Pioneer corn tissue from the pericarp (**A**), seed coat (**B**), aleurone (**C**), and endosperm (**D**): 1, visible image; 2, chemical image; 3, chemical intensity; 4, spectra corresponding to the pixel at the cross-hair in the visible image. The rectangle area size in the visible images is 25 μm × 8 μm: (**a**) area under 1736 cm^{-1} peak (carbonyl C═O); (**b**) area under 1510 cm^{-1} peak (aromatic compound); (**c**) area under 1650 cm^{-1} peak (amide I); (**d**) area under 1246 cm^{-1} peak (cellulosic materials); (**e**) area under 1160 cm^{-1} peak (CHO); (**f**) area under 1150 cm^{-1} peak (CHO); (**g**) area under 1080 cm^{-1} peak (CHO); (**h**) area under 929 cm^{-1} peak (CHO); (**i**) area under 860 cm^{-1} peak (CHO); (**j**) area under 3350 cm^{-1} peak (OH and NH); (**k**) area under 2929 cm^{-1} peak (CH_2); (**l**) area under 2885 cm^{-1} peak (CH_3).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1110
1160
1246
1375
1428
1320
1736
1630
1600
1510
Log(1/R)
Wavenumbers (cm-1)
A BC D
Endosperm Pixel 44
CP-R: Point 44 @ (-26495, -2878)
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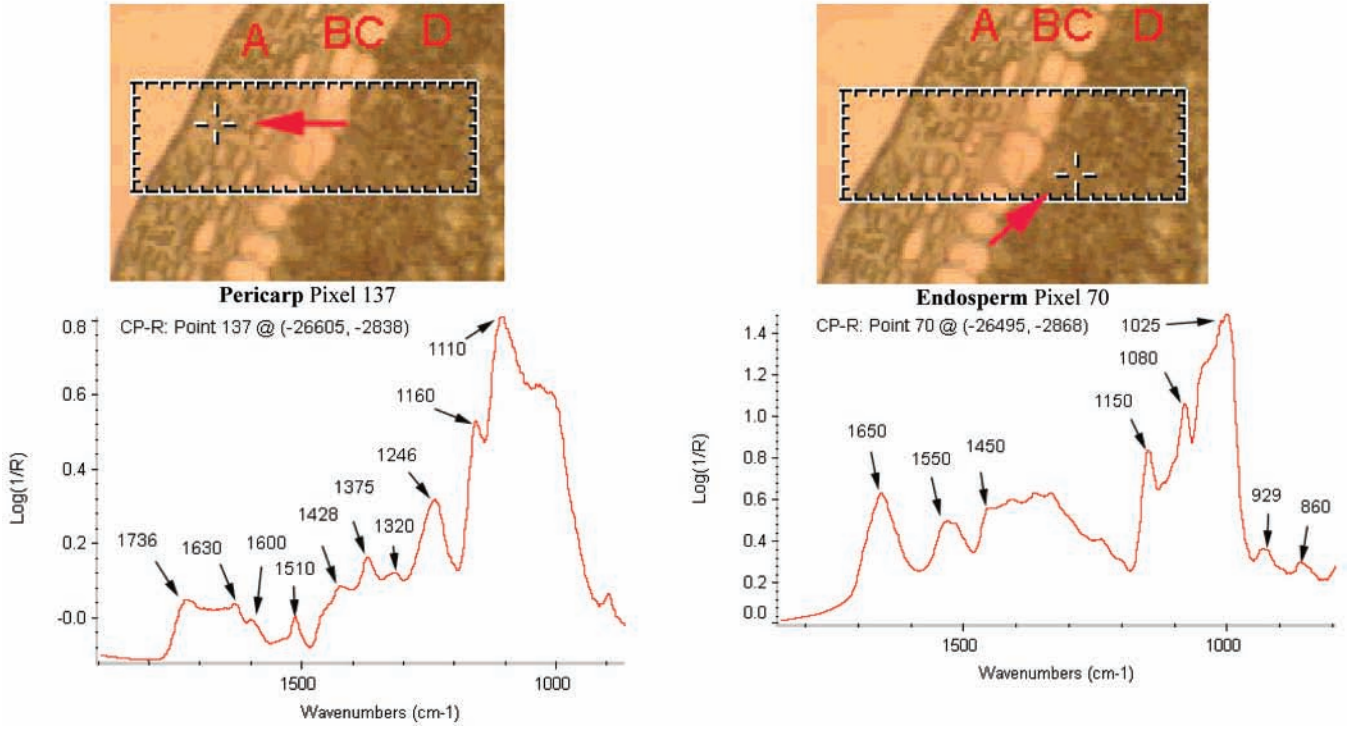
1080
1150
1650
1550
1450
929
860
Log(1/R)
Wavenumbers (cm-1)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1428
1320
1736
1630
1600
1510
Log(1/R)
Wavenumbers (cm-1)
A BC D
Endosperm Pixel 70
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Wavenumbers (cm-1)

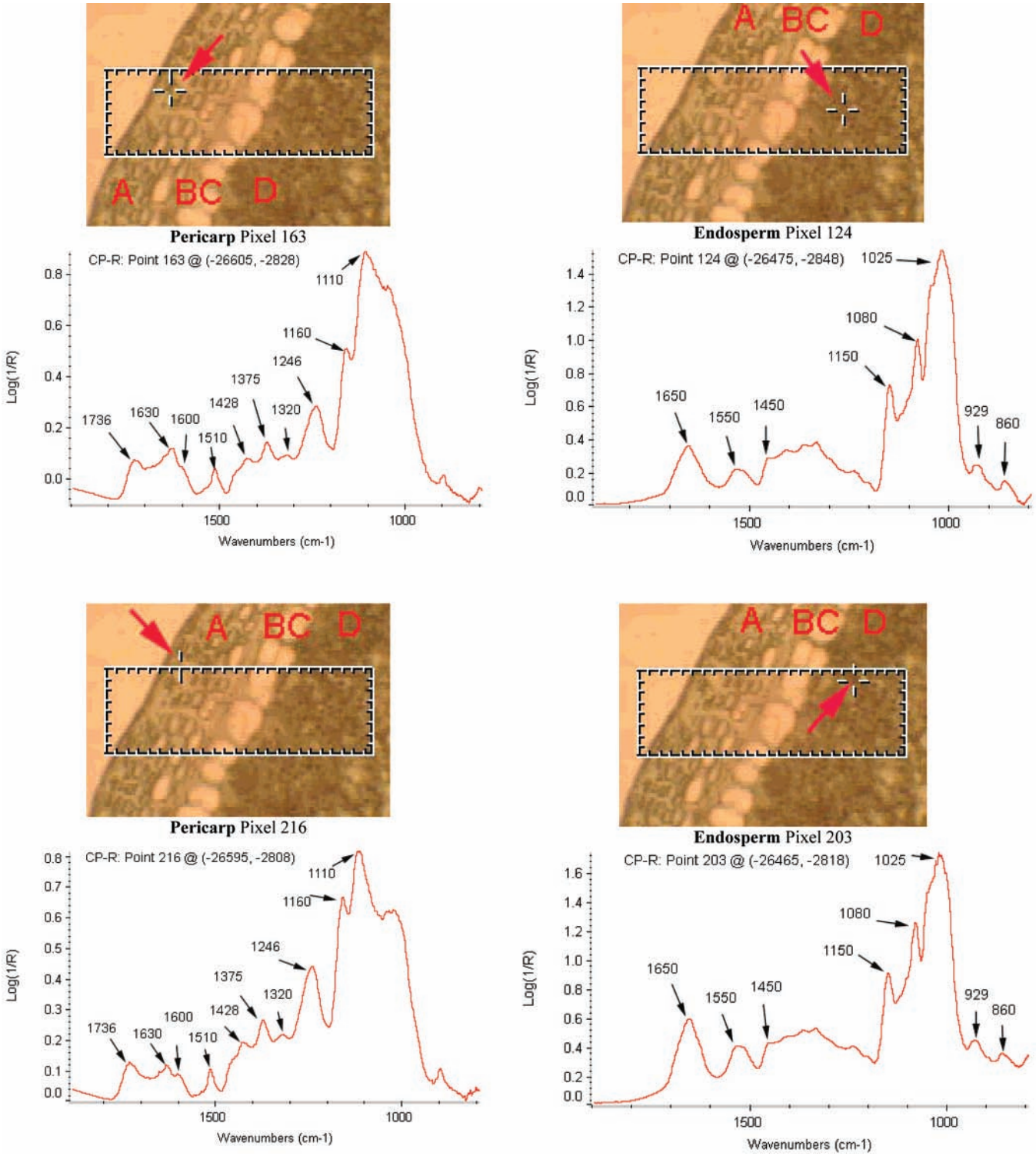

Figure 3. Spectra of pericarp and endosperm of Pioneer corn tissues selected from corresponding area from the visible images, showing that similar morphological parts exhibit similar spectral characteristics and chemical composition (**A**, pericarp; **B**, seed coat; **C**, aleurone; **D**, endosperm). The rectangle area size in the visible image is 25 μm × 8 μm.

cm^{-1} band is to look for irregular depositions of this particular type of carbohydrate—β-glucan (*6*). A peak area of the 1025 cm^{-1} band in cereal grains (*6*) is used to indicate nonstructural carbohydrate such as starch. Starch is an $\alpha(1, 4)$ glucose polymer, often branched, one of the principal energy storage forms for plant growth. A peak area of the 1246 cm^{-1} band is used to indicate cellulosic material in cereal cell walls (*6*). Cellulose is a $\beta(1, 4)$ glucose polymer, laid down in long parallel strands. **Figure 2d** is a chemical image under area at ~1246 cm^{-1}, showing the spatial cellulosic material distribution and concentration and that pericarp contains high cellulosic materials. Panels **e**−**i** of **Figure 2** show different carbohydrate spatial distributions. The spectra for carbohydrate assignments are very complicated and are not fully understood (*9*−*11*).

Figure 3 presents spectra of the pericarp and endosperm layers, showing that similar morphological parts exhibit similar spectral characteristics, functional groups, and biological components. All pixels in the pericarp region show spectral peaks at 1736, 1630, 1600, 1510, 1428, 1735, 1320, 1246, 1160, and

1110 cm^{-1}. All pixels in the endosperm region show spectral peaks at 1650, 1550, 1450, 1160, 1080, and 1025 cm^{-1}. This indicated that pericarp contains higher concentrations of lignin (1510 cm^{-1}), cellulose (1246 cm^{-1}), and lipid (1738 cm^{-1}) materials and little concentration of protein (1650 cm^{-1} for amide I band and 1550 cm^{-1} for amide II band). Contrary to that, the endosperm region has practically no lignin (1510 cm^{-1}) and higher protein (1650 and 1550 cm^{-1}) and starch (1025 cm^{-1}).

It should be mentioned that the functional group mapping in this study was done only from the pericarp, seed coat, and aleurone to part of the endosperm area of the Pioneer corn. The purpose of this study was to show how to use synchrotron-based FTIR microspectroscopy to map functional groups to reveal biological components' spatial distribution and intensity. If people want to know the specific functional group distribution and intensity, they need to map a whole corn cross section. This is especially true for the endosperm. For example, the starch granules are much more tightly packed in the horny starch area than in the floury starch area. Therefore, it is risky to extrapolate the data obtained from one specific area to the whole endosperm. Also, starch granule sizes are different, so suitable aperture size settings need to be adjusted.

Conclusions and Implications. The above work clearly shows that imaging molecular chemistry (chemical and functional group distribution) across cereal grain tissue at spatial resolution can be accomplished using synchrotron FTIR microspectroscopy. This molecular chemical information can be linked to structural and nutritional information. In contrast to traditional "wet" chemical methods, which during processing for analysis often result in the destruction of the intrinsic microstructures of corn tissues, the synchrotron FTIR technique leaves the corn sample intact and, as a result, allows one to relate metabolic function to the anatomical structure of corn. The implications from this study are that the spectroscopic profile differences in different varieties compared with a normal variety could be determined by synchrotron-based FTIR microspectroscopy. With respect to human and animal nutrition, it is possible to relate digestive function and nutrient utilization to the specific chemical makeup of intrinsic structures of grain corn. This technique is able to provide some information relating to the quantity, composition, structure, and distribution of chemical constituents and functional groups in grain corn. Such information on the microstructural−chemical features of grain corn can also be used in corn breeding programs for selecting superior varieties of corn for targeted food and feed purposes and for prediction of corn quality and nutritive value for humans and animals.

ACKNOWLEDGMENT

We are grateful to Drs. N. S. Marinkovic and L. M. Miller (NSLS-BNL, Upton, NY) for helpful discussion and data collection at U2B station in NSLS and Vern J. Racz (Director, Prairie Feed Resource Centre) for supplying grain corn samples.

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Received for review May 4, 2004. Revised manuscript received September 13, 2004. Accepted September 17, 2004. This research has been supported by grants from Saskatchewan Agricultural Development Fund (ADF). The National Synchrotron Light Source in Brookhaven National Laboratory (NSLS-BNL, Upton, NY) is supported by the U.S. Department of Energy contract DE-AC02-98CH10886.

JF049291B