Determining the Effects of Storage on Cotton and Soybean Leaf Samples for Hyperspectral Analysis

Matthew A. Lee, *Member, IEEE,* Yanbo Huang, Haibo Yao, *Member, IEEE,* Steven J. Thomson, and Lori Mann Bruce, *Senior Member, IEEE*

***Abstract*—This paper studies the effect of storage techniques for transporting collected plant leaves from the field to the laboratory for hyperspectral analysis. The strategy of collecting leaf samples in the field for laboratory analysis is typically used when ground truthing is needed in remote sensing studies. Results indicate that the accuracy of hyperspectral measurements depends on a combination of storage technique (in a cooler or outside a cooler), time elapsed between collecting leaf samples in the field and measuring in the laboratory, and the plant species. A nonlinear model fitting method is proposed to estimate the spectrum of decaying plant leaves. This revealed that the reflectance of soybean leaves remained within the normal range for 45 min when the leaves were stored in a cooler, while soybean leaves stored outside a cooler remained within the normal range for 30 min. However, cotton leaves stored in a cooler decayed faster initially. Regardless of storage technique, results indicate that up to a maximum of 30 min can elapse between plant leaf sampling in the field and hyperspectral measurements in the laboratory. This study focused on cotton and soybean leaves, but the implication that time elapsing between sampling leaves and measuring their spectrum should be limited as much as possible can be applied to any study on other crop leaves. Results of the study also provide a guideline for crop storage limits when analyzing by laboratory hyperspectral sensing setting to improve the quality and reliability of data for precision agriculture.**

***Index Terms*—Ground truthing, hyperspectral imaging, leaf sampling, remote sensing, spectral decay.**

I. Introduction

PRECISION agriculture involves controlling agricultural inputs, such as water, fertilizer, herbicides, and pesticides, at a subfield spatial resolution to optimize agricultural profits. This means maximizing yield and minimizing production costs. This is accomplished by determining the needs of the crops at a spatial resolution that could be so finely defined that each plant has its own prescription [1].

One of the tools used in gathering information for precision agriculture is remote sensing. Remote sensing refers to gathering information about objects without physically touching them. It is often accomplished by using sensors like optical cameras or radar systems that are mounted on airborne or space borne platforms. One type of optical sensor that has been used in many applications is the hyperspectral camera. Hyperspectral sensors work by subdividing the optical part of the electromagnetic spectrum including the ultraviolet, visible, and infrared, into a large number of narrow wavelength bands. The advantage of this sensor is that it is capable of photo spectroscopy. Theoretically, hyperspectral cameras should be able to identify different types of plant stress, such as drought, nitrogen, and pest, and they should be capable of distinguishing different kinds of plants [2]. Often, hyperspectral cameras do not meet these theoretical expectations due to atmospheric interference [3]. Thus, it is often helpful to position the hyperspectral sensor closer to the crops, which reduces the amount of atmosphere the signal must pass through. At the extreme, portable hyperspectral sensors can be used on the ground to obtain hyperspectral data on the plants one at a time. These sensors are still passive, and therefore rely on solar radiation, which passes through the atmosphere on the way to the ground. Thus, the radiation interacts with the atmosphere less than the case where the sensor is airborne or spaceborne. When the atmosphere is dynamic, it may be difficult to calibrate the sensor and collect data before the atmospheric conditions change or require the use of additional equipment, and it may be advantageous to confine the hyperspectral sensor to a laboratory and collect leaves from the crop field, which are then transported to the laboratory for data measurement. The measured data can then be used to analyze the plants, or refine the analysis of a remotely sensed dataset that encompasses a larger area. The effectiveness of using a laboratory confined sensor depends on how much elapsed time is allowable between the time leaves are collected and reflectance is measured. The leaf samples change gradually after they are collected. The time that can safely elapse between collecting the leaves and measuring their reflectance depends on the rate of decay. Assuming that processing of collected leaves in the laboratory cannot be expedited, the decay rate will dictate how many leaves can be measured since the accuracy of the measurements worsen the longer the leaf remains in storage.

It is common practice to excise leaves from plants and then use them to estimate the canopy reflectance of the plant. Some researchers refrigerate leaf samples after collecting them for transport to the laboratory, but a few do not. Generally, it seems to be common practice to measure the leaf reflectance as soon as possible after collecting them. le Maire *et al.* [4] conducted a study where the chlorophyll content of deciduous tree leaves was estimated. They transported the detached leaves to laboratory and indicated that the leaves were kept cold between sampling

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M. A. Lee is with the Mississippi State University, Starkville, MS 39762 USA.

Y. Huang and S. J. Thomson are with the United States Department of Agriculture, Agricultural Research Service, Crop Production Systems Research Unit, Stoneville, MS 38776 USA (e-mail: yanbo.huang@ars.usda.gov).

H. Yao and L. M. Bruce are with the Geosystems Research Institute, Mississippi State University, Starkville, MS 38776 USA.

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and laboratory measurements. In another study by Carter and Spiering [5], the researchers also removed and refrigerated leaves from trees to transport them to the laboratory, where the hyperspectral reflectance was measured to estimate chlorophyll. Zhang *et al.* [6] also looked at the hyperspectral reflectance of tree leaves, but indicated that the leaves were stored at $0\,^{\circ}\mathrm{C}$ while being transported to the laboratory. Foster *et al.* [7] also stored samples at $0\,^{\circ}\mathrm{C}$, and their samples were switchgrass cultivars. Zhao *et al.* [8] did not refrigerate the leaves sampled from the field. Instead, the leaves were measured in the field immediately after they were removed. One of the problems with studies that collect leaves for processing in a laboratory is that spectra from sampled leaves may not characterize the spectra of the same leaves on the plant because the leaves decay during transport and storage. This can lead to erroneous conclusions because the data may not be representative of the original plant.

There have been some studies looking into the effects of excising leaves on their spectral reflectance. Foley *et al.* conducted one such study on the leaves of five different tropical rainforest trees [9]. The leaves were from the common guava (*Psidium guajava*), purple guava (*Psidium littorale*), weeping fig (*Ficus benjamina*), floss silk (*Chorisia speciosa*), and coffee (*Coffea Arabica*). In this study, the authors attempted to determine the efficacy of preserving the leaf spectra by wrapping moist gauze around the petiole of leaves and placing them in a plastic bag. Though they use a relatively small sample size (one leaf for treatment and one leaf for control for each tree species), the study showed that the leaves from each species behaved very differently after being excised. A second study was conducted by Summy *et al.* [10]. In their study, the foliage of giant reed (*Arundo donax*) was examined. Here, the authors tested many different storage combinations involving different types of bags and refrigeration. Their method involved collecting spectral radiation measurements of particular wavelength bands at 0, 24, 48, 72, and 96 h after excision, and the threshold for significant difference was determined by the lack of overlap between 95% confidence intervals on the sample means. While the method is valid and well described in the text, it is a little misleading. Assuming the variance remains approximately constant after excision, this method translates into a difference between the two sample means of about 2.717 standard deviations, which is better than a 99% confidence level. In a two class classification problem, one normally distributed feature with this description would yield an area under the receiver operating characteristic curve [11] of about 0.974, a Bhattacharyya distance [12] of about 0.929, and an overall classification accuracy of about 92% when using a maximum likelihood classifier [13]. Given that raw wavelength bands comparable to such features are rarely encountered in difficult classification problems, the confidence interval is likely too wide. Furthermore, the study conducted by Summy *et al.* does not take into account what is happening in the interval between excision and 24 h after excision, which is likely when scientists would collect spectral measurements during an experiment.

Given the fact that the behavior of leaves after excision from the plant is dependent on the species of the plant, it is critical that studies on leaves from common agricultural plants be conducted to improve research techniques in agricultural science. One such study dealing with cotton (*Gossypium hirsutum L.*) was conducted by Thomasson and Sui [14]. Their study attempted to determine if cotton leaves stored in a cooler (with ice) change within 14 h after being collected from the plant. Spectra of the five plants were measured only four times during the 14-h interval. This study assumed a linear relationship between reflectance and time. The conclusion was that the reflectance is not significantly correlated with storage time. However, due to the small number of leaves sampled, the small number of temporal measurements, and the fact that there may not be a linear relationship between spectral band reflectance values and time, it is possible that relationships were missed.

The overall goal of the study is to evaluate the efficacy of the strategy of collecting field leaf samples for analysis with a laboratory confined hyperspectral sensor. The specific objectives of the study are as follows.

1) Compare the spectral effects of storing leaf samples in a cooler and outside a cooler for two different species.
2) Evaluate accuracy of modeling the spectral decay using a linear model and a nonlinear model.

II. Materials and Methods

A. Experimental Design and Implementation

In July 2011, leaves from field grown cotton (*Gossypium hirsutum L.*) and soybean (*Glycine max (L.) Merr.*) plants were sampled at the United States Department of Agriculture, Agricultural Research Service (ARS), Stoneville, MS, USA. A total of 10 leaves of cotton and soybean were sampled for hyperspectral imaging. The cotton plants were in the seven- to eight-leaf stage, and the soybeans were in the seven- to eight-trifoliate leaf stage. For imaging one of the twin leaves for each cotton plant and the lowermost trifoliate leaves for each soybean plant were selected. Five of the cotton and soybean leaves were stored in paper bags inside a cooler, and the other five were stored in paper bags outside the cooler. The temperature inside the cooler for leaf storage stayed at about $17\,^{\circ}\mathrm{C}$ with the help of a layer of ice on the bottom of the cooler (the ice was isolated from the leaf samples with several layers of masking paper to raise the temperature above $0\,^{\circ}\mathrm{C}$ and stabilize the humidity) and the temperature of the laboratory room where the leaves outside the cooler were placed was kept at about $22\,^{\circ}\mathrm{C}$. These temperatures were monitored using thermometers placed near the leaves in storage. The leaves were imaged at 15 min after they were collected, then again at 1, 2, 3, 4, 5, 6, and 24 h after collecting them. Each time the leaves were imaged, they were placed back into their original storage method, inside or outside cooler, for subsequent imaging.

B. Leaf Image Acquisition

The image data were collected using a push broom hyperspectral imaging system [15] with an effective spectral range from 400 to 900 nm. The camera is a 14-bit PCO1600 CCD (charge-coupled device) high-resolution camera (Cooke Corporation, Romulus, MI, USA) that was integrated with an ImSpector V10E spectrograph (Spectral Imaging Ltd., Oulu, Finland) with a $30\ \mu\mathrm{m}$ entrance slit, and a 23-mm Schneider lens. The PCO1600 camera has a CCD with resolution of

1600×1200 pixels and is thermoelectrically cooled. The camera was at a range of less than 1 m, which produce images with pixels smaller than $1 \times 1\ \mathrm{mm}^2$. Image data transfer from the camera to the computer was through an IEEE 1394 "firewire" link. In order to illuminate the target area in the indoor environment, two mr16 tungsten halogen bulbs with dichroic reflectors were mounted with the sensor on an adjustable camera stand. The lamps were fitted with diffusion and color-balancing filters in order to resolve specular reflectance and to simulate natural lighting.

C. Image Processing and Data Analysis

In addition to the imagery, white reference and dark current measurements were acquired. The reflectance (R) images were computed using the formula below on each pixel in the image

$$R(\lambda) = \frac{DN_T(\lambda) - DC(\lambda)}{DN_R(\lambda) - DC(\lambda)}. \quad (1)$$

The digital numbers for the target and white reference are represented by DN_T and DN_R, respectively, and the dark current is DC.

The reflectance images of the leaves were initially segmented by thresholding the normalized difference vegetation index (NDVI) [16] computed for each pixel in the hyperspectral reflectance image. Since the background was made of felt (dark, nonvegetative fabric), this produced a rough segmentation that still mislabeled some leaf pixels as background. The segmentation was then manually cleaned up. Manual clean up involved visually comparing segmentations to the corresponding images of the leaves and correcting mislabeled pixels. If the correct label for a pixel was unclear, the pixel was labeled as background. After segmentation, the leaf pixels were averaged together to produce the reflectance spectral curve for the leaf.

The analysis started with normalizing the reflectance curves to correct the data for variations in lighting. The reflectance curves were divided by the value at 450 nm to correct for variations in light intensity during reflectance measurements [14]. After the leaf reflectances were normalized, the normalized reflectance with respect to time was fit to both the linear models

$$NR(t) = c_1 + c_2 t \quad (2)$$

and an exponential model

$$NR(t) = c_1 + c_2 e^{c_3 t} \quad (3)$$

where NR represents normalized reflectance; c_1, c_2, and c_3 are the models' coefficients; and t represents time. Exponential models of this form have been used for decades to model physical phenomenon such as nuclear and chemical decay, and the discharging of capacitors because it is the form of a solution to a first order linear differential equation [17]. Thus, it is a logical choice for modeling the decay of plant leaves in the short term. After the model parameters were chosen, both the mean squared error and an F-test [18] were computed to determine the suitability of the model for the data.

The models were used to estimate the amount of time required for each band to drift outside the normal range. Since there were no measurements of hyperspectral signatures for leaves still attached to the plant, the normal range was defined based on the values for the measurements taken very close to the time leaves were still attached to the plants. Thus the normal range was the value at 15 min plus or minus two standard deviations, which includes about 95% of all the plants assuming they take on a Gaussian distribution. When a band drifts outside the normal range, it moves into a range that is taken up by only 2.5% of the plants, and thus would be considered an outlier even if it were an accurate characterization of the leaf. The time required for bands to drift outside the normal range was estimated based on the model fit to each band by computing the intersection between the threshold values and the model function. Since the model was a monotonic function, only one threshold was crossed at a time greater than 15 min. Estimated times required to cross the threshold for all the bands were plotted with respect to wavelength. Additionally, plots were generated that show the number of bands outside the normal range with respect to time.

In order to estimate the amount of change of reflectance within a certain time, the rate of change was computed using the following formulas

$$\Delta = \frac{R_\lambda(t_2) - R_\lambda(t_1)}{t_2 - t_1} \quad (4)$$

and

$$Rate = \frac{\Delta}{R_\lambda(t_1)} \times 100. \quad (5)$$

The spectral reflectance associated with a particular wavelength is indicated by $R_\lambda(t)$. The functions and scripts used in this study were written in MATLAB (MathWorks, Inc., Natick, MA, USA).

III. Results and Discussion

Figs. 1 and 2 illustrate the mean normalized reflectance for cotton and soybean leaves. As the figures show, the spectral signatures maintain the same general shape with small variations that accumulate as time elapses. The data analysis indicates that many of these variations are significant enough to affect classification in nontrivial problems. This means leaves excised from plants may not characterize leaves still attached to the plant. It is important to consider this if the spectra measured in laboratory conditions with excised leaves are intended to be used in aiding remote sensing tasks, such as training examples for classification or pure signatures for pixel unmixing.

Fig. 3 illustrates examples of bands and model fits. The figure shows how the mean reflectance at each band drifts away from the mean at the first observation as time elapses after excision. It indicates that leaves measured quickly after excision are able to characterize the plant more accurately. In general, the data from the first 6 h conformed well to the models, but often the 24-h data would not fit any monotonic model function because the trend reverses direction at some point after 6 h. The observation that the change in spectral bands follows a different pattern after 24 h is not surprising because leaves are very complex biological systems, and contain many different substances that probably decay at different rates. It is possible the changes in the spectrum are

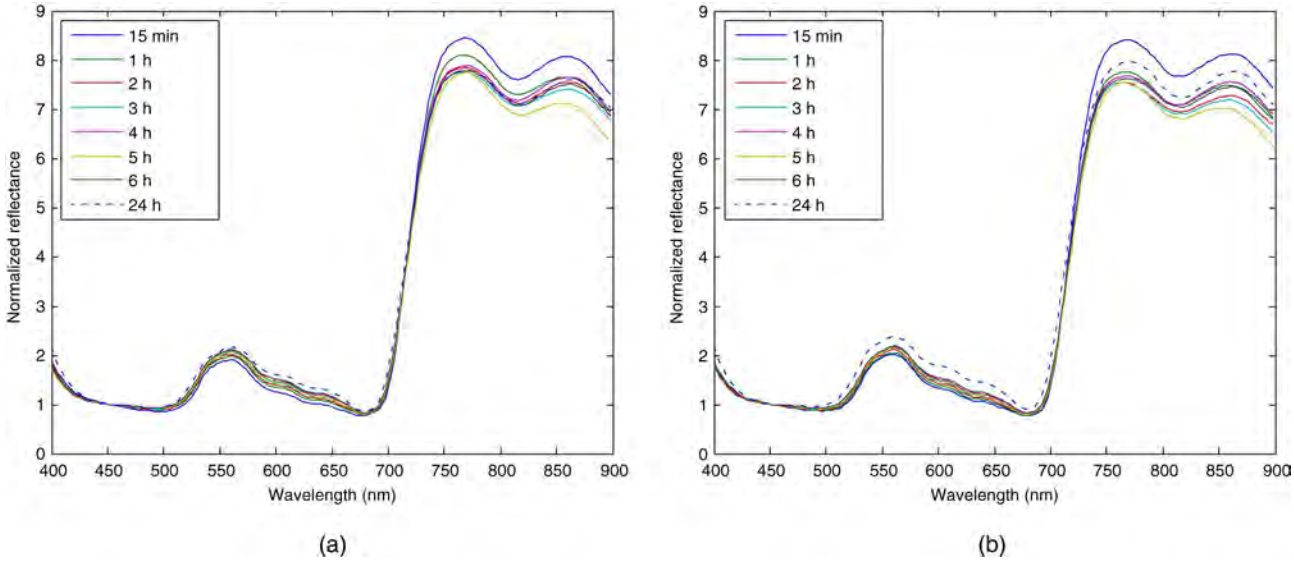

Fig. 1. Mean normalized reflectance signatures ($n = 5$) for cotton after various storage intervals. The plants in (a) were stored inside a cooler, while the plants in (b) were stored in a room outside the cooler. The signatures were normalized by dividing by the reflectance value at 450 nm.

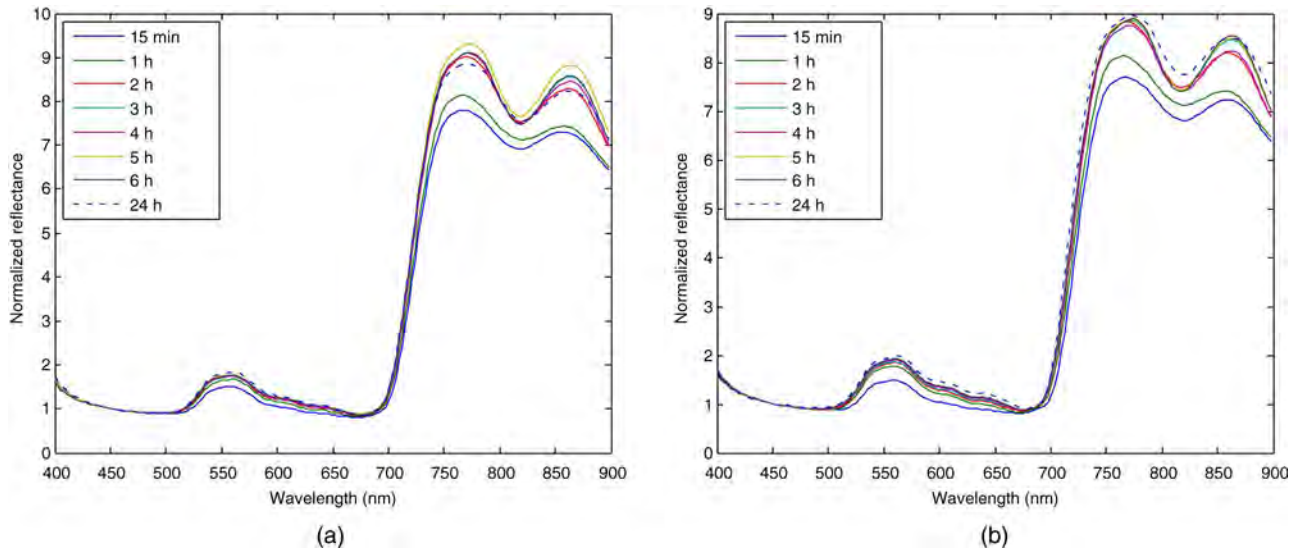

Fig. 2. Mean normalized reflectance signatures ($n = 5$) for soybeans after various storage intervals. The plants in (a) were stored inside a cooler, while the plants in (b) were stored in a room outside the cooler. The signatures were normalized by dividing by the reflectance value at 450 nm.

dominated in the first few hours by the decay of substances and structures that decay quickly, while the decay after 6 h is dominated by substances that are more persistent. In the cases where the 24-h data could not be successfully modeled by either a linear or exponential function, the 24-h data was ignored when choosing model parameters. In such cases, the model was only fitted on the data for the first 6 h. Since the exponential model had a lower squared error almost universally, the linear model was dropped from the rest of the analysis because it did not fit the data as well as the exponential model.

After the model was chosen, it was statistically justified using an F-test for each band. The results of this test are shown in Fig. 4. As shown, no bands failed at the 2.5% significance level. This means that the exponential model is accurate for all the spectral bands for both cotton and soybean leaves.

Fig. 5(a) presents the estimated time for cotton to drift outside its normal range in each wavelength band. Fig. 5(b) shows the estimated time for soybean spectral bands to drift outside the normal range. The cumulative number of bands outside the normal range for cotton and soybeans is shown in Fig. 6. As Fig. 5 shows, the cotton stored in the cooler remains more stable in the infrared portion of the spectrum, but it is less stable in the visible part of the spectrum. The cumulative plot (Fig. 6) shows that the cotton stored outside the cooler has fewer bands outside the normal range for the first hour and 30 min, and then afterward, the cotton in the cooler has fewer bands outside the normal range. There was a little effect on the stability of the reflectance caused by storage method for the soybean leaves.

The advantage of storing samples in a cooler for hyperspectral analysis depends on the kind of plant, wavelength of interest, and

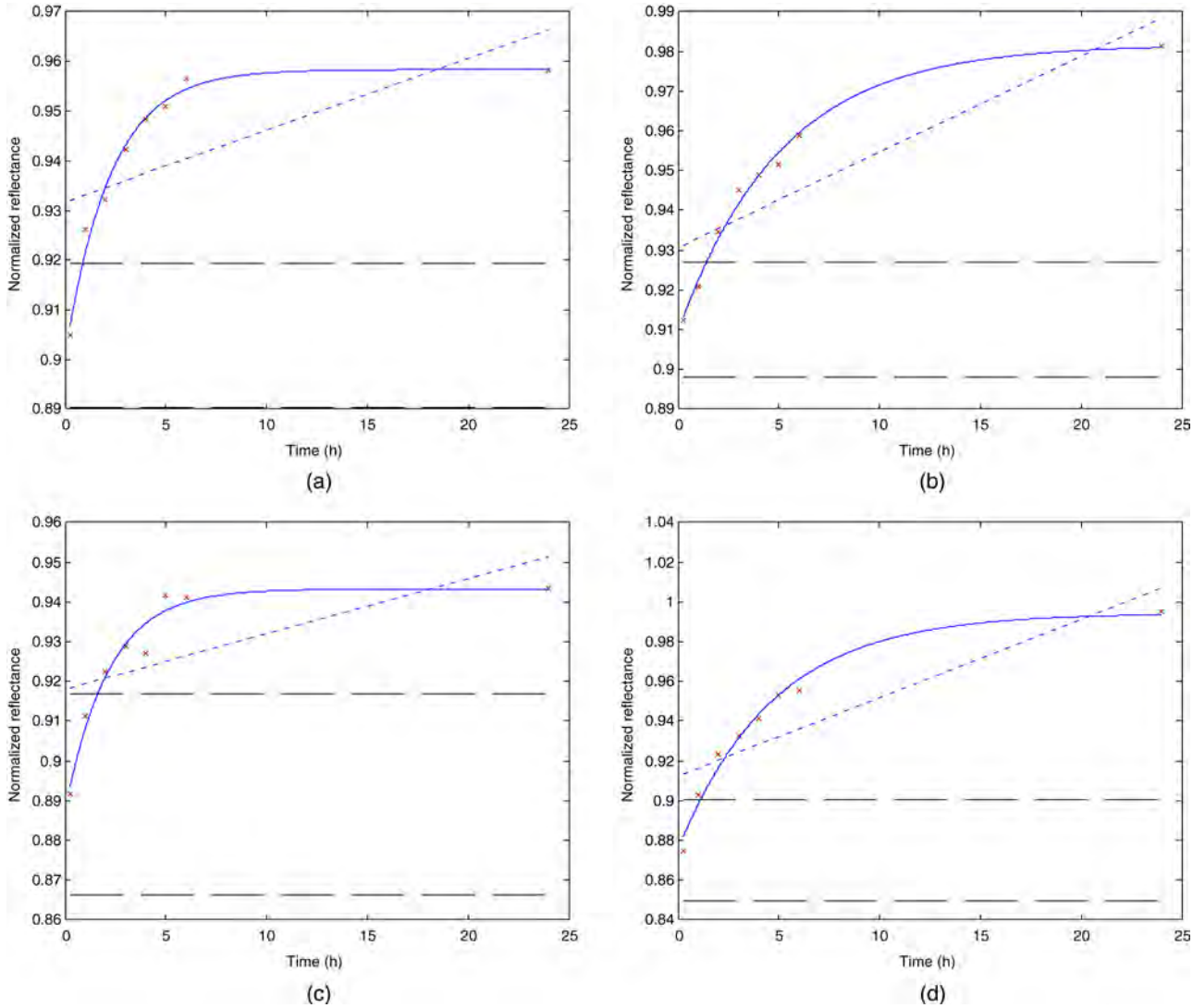

Fig. 3. Examples of reflectance band change plotted with the linear (dotted line) and exponential (solid line) models. The dashed lines represent the 2σ normal interval around the 15-min mean. (a) and (b) are cotton leaves at the 474 nm band, with (a) stored in the cooler and (b) stored in the room outside the cooler. (c) and (d) are soybean leaves at the 507 nm band, with (c) stored in the cooler and (d) stored in the room outside the cooler. Also note that both bands happen to be in regions where the normalized reflectance is close to 1.0, and other bands have values consistent with Figs. 1 and 2.

the length of storage. In the long term, a slight advantage was observed for the plants stored in a cooler, but at that point, the advantage may be meaningless because there is so much change that has already occurred. In the short term, there was no significant difference between storing soybean leaves in a cooler or outside a cooler, but for the cotton leaves, the cooler was advantageous when the storage time was greater than 1 h and 30 min, or if the near infrared range was the most important part of the spectrum for later analysis methods. For the cotton in general, it was actually better not to store the leaves in a cooler if the storage time was less than 1 h and 30 min indicating cooling not to be advantageous.

In all cases, changes were observed very soon after the experiment began. The observed change that occurred between 15 min and 1 h often remained within the normal range, but the change was consistent with the trend line established over the first 6 h, which implies that changes can be extrapolated to the interval prior to 15 min. However, it is not clear how accurate the extrapolation is, or how close to the instant the leaf is collected the extrapolation remains valid. Nevertheless, it can be expected that reflectance is changing very shortly after the leaf is removed from the plant. A second point of this study is apparent when the implication of the threshold of significance is considered. This paper considered spectral measurements to be within the normal range as long as the sample mean at the particular time were within two standard deviations of the sample mean at 15 min after excision. In a classification problem, if a feature has a normal distribution, the same standard deviation for both classes, and the means for each class separated by two standard deviations, that single feature will have an area under the receiver operating characteristic of about 0.922, a Bhattacharyya distance of about 0.500, and result in an overall classification accuracy of about 84% when a maximum likelihood classifier is used. Since it is rare to encounter raw hyperspectral bands with similar separability in difficult remote sensing problems, it is likely that even the two standard deviation threshold is too wide because the means of other classes are possibly closer than two standard deviations. Though it is naive to assume only one hyperspectral

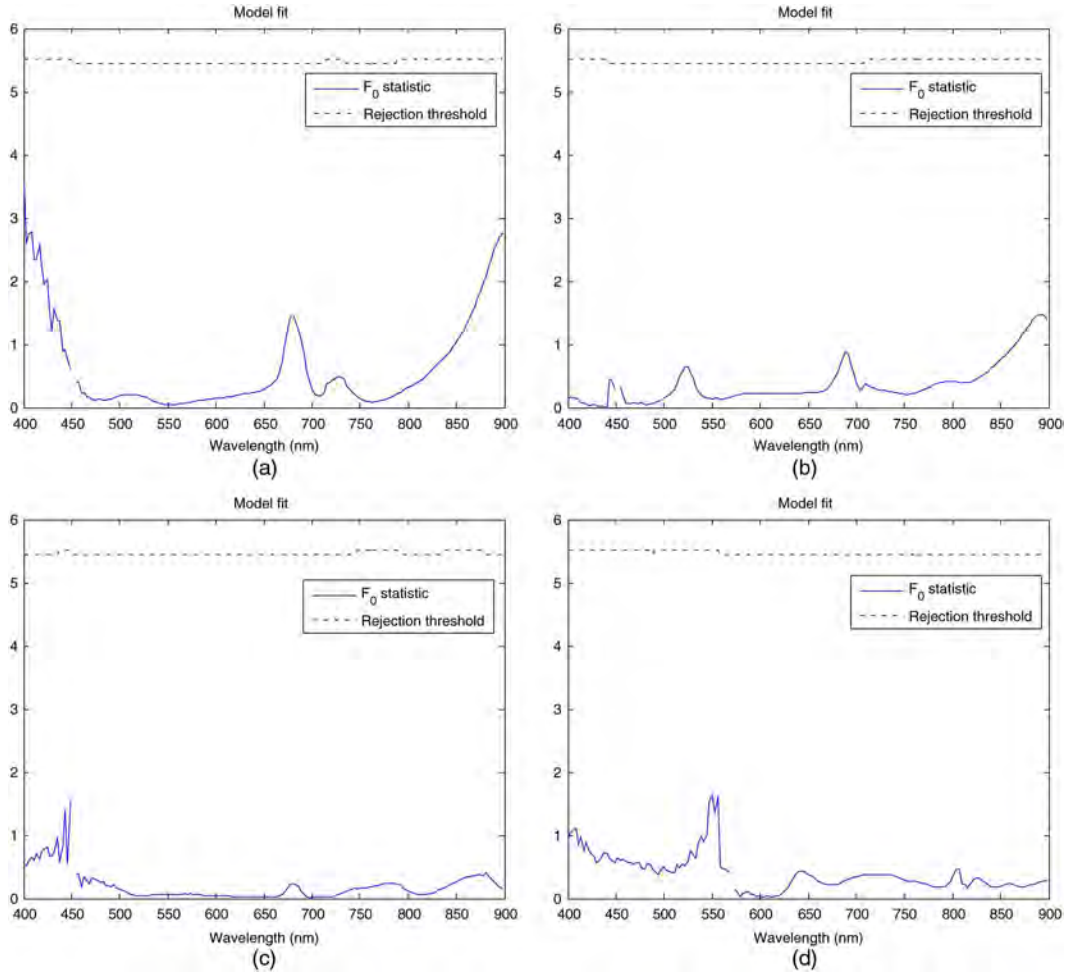

Fig. 4. Results of F-tests on model fits. (a) Test for cotton stored in a cooler. (b) Test for cotton stored outside the cooler in a room. (c) Test for soybean stored in a cooler. (d) Test for soybean stored outside the cooler in a room.

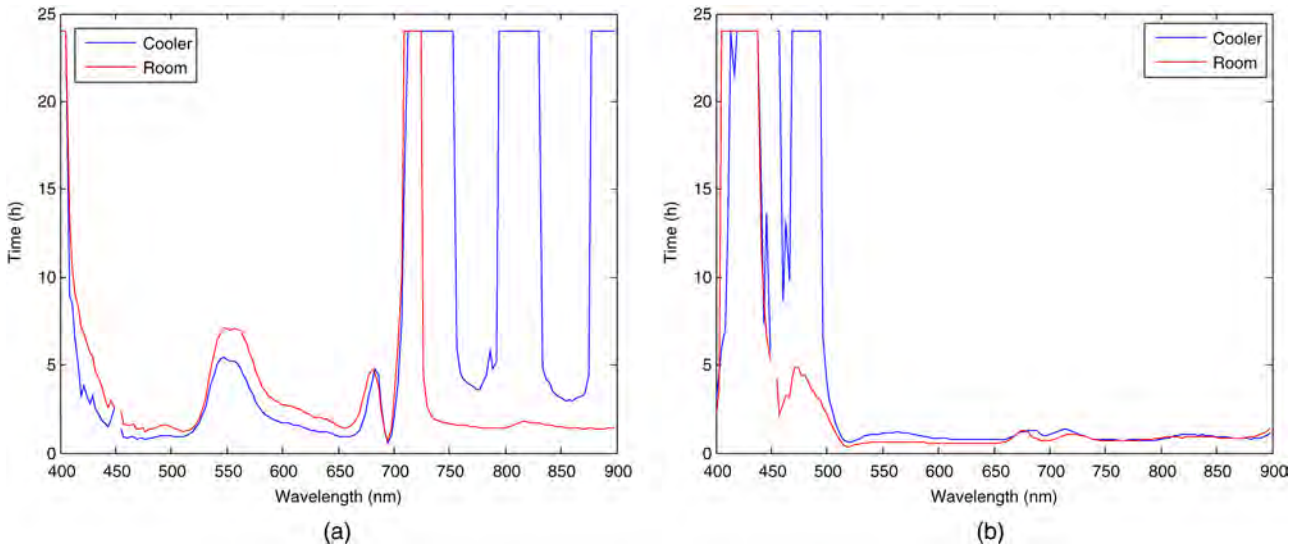

Fig. 5. Plot of time required for spectral bands to drift outside the normal range for cotton leaves (a) and soybean leaves (b). The blue and red lines represent cotton leaves stored inside a cooler and in the room, and outside the cooler, respectively.

band will be used in a study, if a classification problem is being studied where there are no hyperspectral bands with such good separability, even less time should be allowed to elapse between excision and spectral measurement if conclusions are to be extrapolated to plant canopies growing outside the laboratory. A third point about this study is that it says nothing about how the

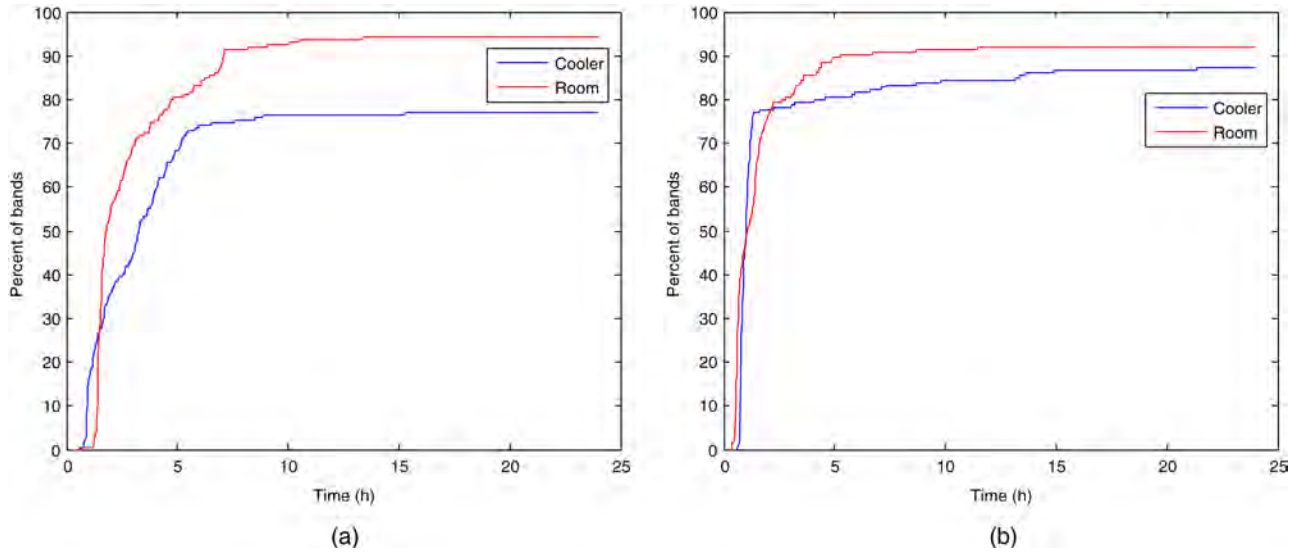

Fig. 6. Estimated percent of bands outside the normal range with respect to time for cotton leaves (a) and soybeans leaves (b). The blue and red lines represent the cotton leaves stored in a cooler and in the room, and outside the cooler, respectively.

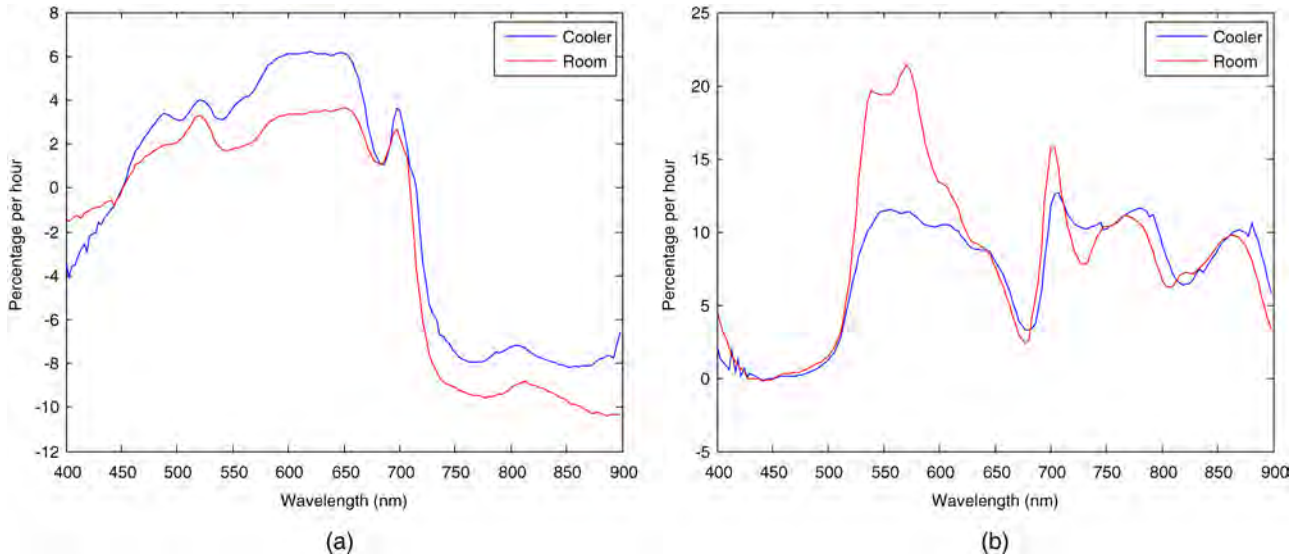

Fig. 7. Average rate of change for the first hour after collecting cotton leaves (a) and soybean leaves (b).

leaves would vary with respect to time if they were not removed, which may be as significant as the variation observed after the leaves were removed. It is clear that leaves that were removed were exposed to a different environment than the leaves that were left on the plant. Since the leaves respond to the environment they are exposed to, it is likely that changes observed from collected leaves are not representative of leaves left on the plant. If a scientist wants to design an experiment where leaves are collected from plants and sent to a laboratory where their hyprespectral signatures are measured, steps should be taken to limit the lag time between collection and measurement.

In order to estimate the amount of change in spectral reflectance within the first hour for experiment design purposes, the average rate of change was estimated for each band (Fig. 7). The rate of change is an estimate of change per hour based on the change that occurs between 15 min and 1 h. It was computed using the formulas (4) and (5). In the computation, $t_1 = 15$ min and $t_2 = 1$ h. The information can be used to roughly estimate the amount of error in the measurement if a constant rate of change over the 1-h period is assumed. One unfortunate, but not unexpected, result of this study was that the leaves of different plants (cotton and soybeans in this case) changed at different rates. This indicates that the calculation can be only applied to cotton or soybeans, and cannot be applied in general to all plants. Based on the results from these two plants, it seems to be safe to allow up to a maximum of 30 min to elapse before measuring the leaf spectrum. Waiting any longer may be risky considering that the soybean leaves showed 1.5% of the bands outside the normal range at 30 min, climbing to about 25% by 45 min. A final consideration is that measuring all the spectra from one group of plants before moving on to the next group may not be advised if the goal is to discern differences between the two groups. If the groups are completely identical, this strategy may detect differences based simply on the fact that one group was stored longer.

This analysis does not implicate any biochemical or physiological response as being responsible for the observed changes in leaf reflectance, but it is known that water content influences the near infrared portion of the spectrum and chlorophyll concentration influences the visible portion of the spectrum [19].

IV. Conclusion

The results of this study suggest that storage time has an important effect on the spectrum measured in the laboratory, and that storage technique has a lesser effect. A very important consideration is the species of plant. However, the effect caused by plant species cannot be easily predicted. It is thus best to control storage time to remain inside a range that is good for most species. It is recommended that the duration samples are in storage before analysis be limited as much as possible. If samples are in storage for 30 min or less, then it is not necessary to store leaves in a cooler. However, if the leaves are stored for several hours, then it is probably best to store the leaves in a cooler even though results suggest that storage for several hours is not advisable regardless of the storage method. For both cotton and soybeans, if the leaves are stored for 30 min or less, there should be little spectral change. The effects observed in this study were likely not observed in previous studies because linear models were used. Typically, the exponential model more closely approximated the data, and thus it was a superior model for the spectrum of sampled leaf decay.

There are many scientists conducting studies where leaves are sampled and analyzed in the lab with the expectation of providing input for remote sensing studies [4]–[7]. It is well known that atmospheric conditions influence the accuracy of any remote sensing study. This research shows that the time leaves remain in storage and transport to the laboratory confined hyperspectral sensor has an impact on the ability of the resulting laboratory measured spectra to characterize remotely sensed spectra. Thus, additional error can be introduced if samples are not measured in a timely fashion. Scientists endeavoring to use a laboratory-confined hyperspectral sensor for determining plant leaf reflectance spectra should consider the impact of the time elapsed between leaf collection and measurement.

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Matthew A. Lee (M'04) received the B.S., M.S., and Ph.D. degrees in computer engineering from Mississippi State University, Starkville, MS, USA, in 2004, 2007, and 2012, respectively.

He worked as a Post Doctorial Agricultural Research Engineer with the United States Department of Agriculture, Agricultural Research Service, Stoneville, MS, USA. In February 2014, he began working as a Post Doctorial Researcher with Electrical and Computer Engineering Department, Mississippi State University. His research interests include digital image processing and remote sensing.

Yanbo Huang received the B.E. degree in industrial automation from Beijing University of Science and Technology, Beijing, China, in 1983, and the M.E. degree in industrial automation from Chinese Academy of Mechanics and Electronics Sciences, Beijing, China, in 1986, and the Ph.D. degree in agricultural engineering from Texas A&M University, College Station, TX, USA, in 1995.

Currently, he is a Senior Research Agricultural Engineer with United Department of Agriculture (USDA), Agricultural Research Service (ARS), Stoneville, MS, USA. He is an Adjunct Professor with Texas A&M University, Mississippi State University, and Delta State University, and Guest Research Fellow with Chinese Academy of Agricultural Science. Currently, he is the Panel Manager of USDA National Institute of Food and Agriculture (NIFA) Small Business Innovation Research (SBIR) Engineering Panel. He had 4 years of research experience at Beijing Research Institute of Automation for Mechanics and Electronics Industries, Beijing, China, and 12 years of full-time research and extension experience at Texas A&M University in food processing unit operation modeling, optimization and process control, food quality quantization, and safety system simulation. In 2007, he started to work for USDA-ARS. He has authored or coauthored over 140 scientific publications, 60 of which are peer-reviewed journal articles. His research interests include aerial application (manned and unmanned), remote sensing for precision application (space-borne, airborne, and

ground truthing), soft computing and decision support for precision agriculture, spatial statistics for remote sensing data analysis, and image processing and process automation.

Dr. Huang is an Editor-in-Chief of *International Journal and Agricultural Science and Technology* (IJAST), Section Editor of *International Journal of Agricultural and Biological Engineering* (IJABE), and Associate Editor of *Transactions of the American Society of Agricultural and Biological Engineers* (ASABE).

Haibo Yao (M'04) received the Ph.D. degree in agricultural engineering from the University of Illinois at Urbana-Champaign, Urbana-Champaign, IL, USA, in 2004.

Currently, he is an Associate Research Professor with the Geosystems Research Institute, Mississippi State University, Starkville, MS, USA. He has over 60 research publications and two U.S. patents. His research interests include remote sensing and engineering solutions for agricultural, biomedical, food safety, environmental applications, GPS and GIS, algorithm development, instrumentation, as well as hyperspectral image analysis.

Steven J. Thomson received the Ph.D. degree in agricultural engineering from the University of Florida, Gainesville, FL, USA, in 1990.

Currently, he is the Lead Scientist on a research project involving spray application and efficacy, spray drift mitigation, variable rate aerial application of crop protection materials, and remote sensing. He has over 100 research publications.

Dr. Thomson is currently an Editor of the *Research Journal Transactions of the ASABE*. As Faculty Member at Virginia Tech, he received the Alpha Epsilon Award for outstanding research and extension in 1995 and was a top ten finalist out of 280 engineering faculty for a W.E. Wine Award for excellence in teaching. He has received Best Manuscript and Best Reviewer Awards, and has been invited to speak at many forums including Keynote for the First Asian Conference on Precision Agriculture in Toyohashi, Japan.

Lori Mann Bruce (S'90–M'96–SM'01) received the B.S.E. degree in electrical and computer engineering from the University of Alabama, Huntsville, AL, USA, in 1991, the Master degree from the Georgia Institute of Technology, Atlanta, GA, USA, in 1992, and the Ph.D. degree in electrical and computer engineering from the University of Alabama, in 1996.

Currently, she is a Giles Distinguished Professor of Electrical and Computer Engineering and the Associate Vice President for academic affairs and the Dean of Graduate School at Mississippi State University, Starkville, MS, USA. Prior to her current position, she has served as Associate Dean for Research and Graduate Studies in the Bagley College of Engineering, Associate Director of the Geosystems Research Institute, and the Professor of Electrical and Computer Engineering. As a Faculty Member, her research endeavors have been focused on advanced digital signal processing methodologies for exploitation of high-dimensional datasets, with particular emphasis on hyperspectral remote sensing. She has served as the Principal Investigator (PI) or coPI for more than 20 funded research grants and contracts, totaling approximately $20 million from federal agencies. As a Faculty Member, she has taught 45 sections of 17 different engineering courses and has successfully advised, as Major Professor or Thesis/Dissertation Committee Member, 75 Ph.D., and Master's students. Her research has resulted in over 130 refereed publications. Her research in hyperspectral remote sensing for agricultural and environmental applications has been presented to audiences in more than 10 countries.