ATR FT-IR Imaging of Human Hair Cross-Section

Introduction

Human hair is typically 50-100 µ in diameter, previous transmission and ATR studies of its cross section[1,2] reveal three major identifiable regions. The core, or medulla, typically 5-10 µ in diameter, is surrounded by the cortex, making up the bulk of the hair and finally an outer layer or cuticle, typically less than 5 µ in thickness. Biochemical compositional studies may be performed by examining the IR spectra in the –C-H stretching region (ca. 3000 cm^{-1}), and amide (I) and (II) regions like (ca. 1700-1400 cm^{-1}). Using single point IR transmission microspectroscopy however, neglecting sample and instrumental effects, spatial resolution is ultimately limited by the wavelength of the IR radiation ca. 6-11 µ in the fingerprint region. Furthermore, to obtain relatively pure transmission spectra of the core area, very small (< 10 µ) apertures are required. Signal-to-noise and alignment problems can become a significant issue, and consequently, this study has been subject to IR microspectroscopy using a synchrotron as the radiation source[3]. However, this is clearly a highly specialized option.

ATR FT-IR imaging using a germanium crystal offers the opportunity to achieve higher spatial resolution at a given wavelength than using standard transmission techniques[2,4,5]. An ATR imaging device has been developed for the PerkinElmer® Spotlight™ Imaging System and used to collect very high quality ATR images of human hair cross-sections. It is demonstrated that high quality IR spectra can be obtained from sectioned samples in relatively short measurement times and presents new measurement opportunities in biomedical research. In particular, in situations where it is needed to discern relatively subtle spectral differences, the system is capable of high signal-to-noise performance as well as delivering improved spatial resolution. For hair sections, the quality of data can be favorably compared with transmission data obtained with more elaborate systems with a synchrotron source, and since the two methods differ in the actual amount of sample probed, they could be considered as

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precisely.

complementary. In addition, when used in combination with principal components analysis (PCA), the technique allows for rapid survey image acquisition to significantly reduce overall experiment times.

Experimental

Sample preparation

Freshly cut human hair strands were clamped in sample fixation clips and embedded in a polyester resin (available from Struers Ltd., UK) and left to cure for 24 hours. The resin block (ca. 25 mm diameter, 8 mm deep) was then sectioned and polished using a succession of abrasive sheets ranging from 30 µ to 1 µ grit size. Finally, the surface was cleaned using a lens tissue.

FT-IR Imaging

Images were collected using a PerkinElmer Spotlight mid-IR Imaging System equipped with a standard mid-IR source. The system uses a linear MCT detector array combined with a moving sample stage/ATR crystal to generate images. The ATR accessory uses a germanium crystal which provides a useful sample contact area defined by a circle of approximately 500 µ diameter. For example, images of 400 x 400 µ, or other rectilinear areas of various aspect ratios which can be fitted into such a circle can be readily obtained. Image pixel size was set to 1.56 x 1.56 µ. A nominal spectral resolution of either 4, 8 or 16 cm^{-1} was used for the images.

Actual image sizes varied from 100 x 100 µ to 300 x 150 µ. Number of sample scans per pixel ranged from 1 to 16, with typical image acquisition times ranging from ca. 1.5 minutes to ca. 60 minutes. Image data processing included simple peak ratio and principal components methods provided by the standard Spotlight Software, and some advanced processing including sample masking operations performed using the PerkinElmer Hypersoft™ Software Version 3.0.

Results and discussion

A visible image of the polished surface of the embedded sample is shown in Figure 1a. The medulla (central core) sections can be seen in most samples – not all, as this section is often discontinuous or even absent from some samples.

The visible image was collected with the resin block in place on the ATR accessory. The mechanical arrangement allows the user to locate the region of interest (ROI) using the visible image and lower the ATR crystal onto the ROI without moving the sample.

The survey ATR image corresponding to this visible image is shown in Figure 1b. The gross features in the IR image are highlighted using the 'Show Structure' command available in the Spotlight (Version 1.4) Software. This performs baseline correction and principal components analysis on the spectral data and overlays the resulting principal component score images. There is good overall correspondence between the visible and IR images at a high level. For example, the medulla is clearly absent in two of the hair sections.

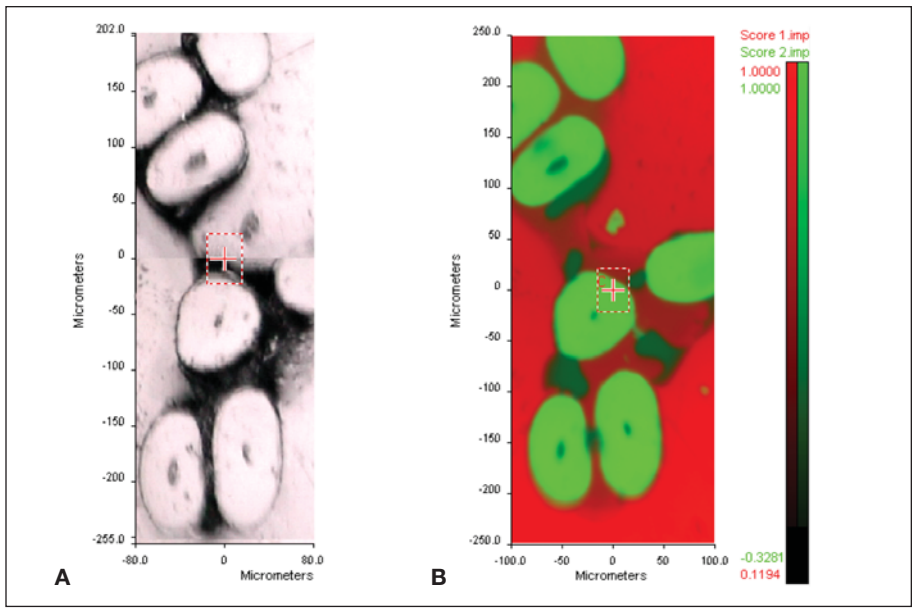

Figure 1. Visible and infrared survey images of embedded hair samples (A) visible and (B) infrared survey images.

A smaller region of interest (200 x 150 µ) was scanned using 16 scans per pixel to reveal the intra-sample structure. Figure 2a shows the simple integrated absorbance image after simple baseline offset correction.

The three primary areas of the sections are clearly seen. Typical spectra extracted from the medulla and cortex regions are shown in Figure 2b. The major difference between these two regions is an overall lower intensity of the medulla spectra, which is consistent with the suggestion of a region of more loosely packed cells. Closer investigation of the spectra reveals a difference in relative intensities of the –C-H bands around 2900 cm^{-1} compared with the intensities of the amide (I) and (II) in the region at ca. 1700-1500 cm^{-1}. Even simple plotting of the ratios of the integrated areas of the amide (I) and (II) shows some structure within the individual samples, Figure 3. It is well known from the detailed band assignments of the amide group vibrations that the observed band contour is a superposition of a number of distinct bands due to –C=O, -N-H, and –C-N and these are conformationally sensitive. The amide (I) band envelope has been used to address the secondary structure relative composition[1] and while a detailed analysis of this envelope is not performed here, it is believed the spectra are of sufficient quality to allow this. Indeed a variation in the lineshape of the amide (I) is observed and this is possibly due to variations in relative a-helix, b-sheet and random coil structures.

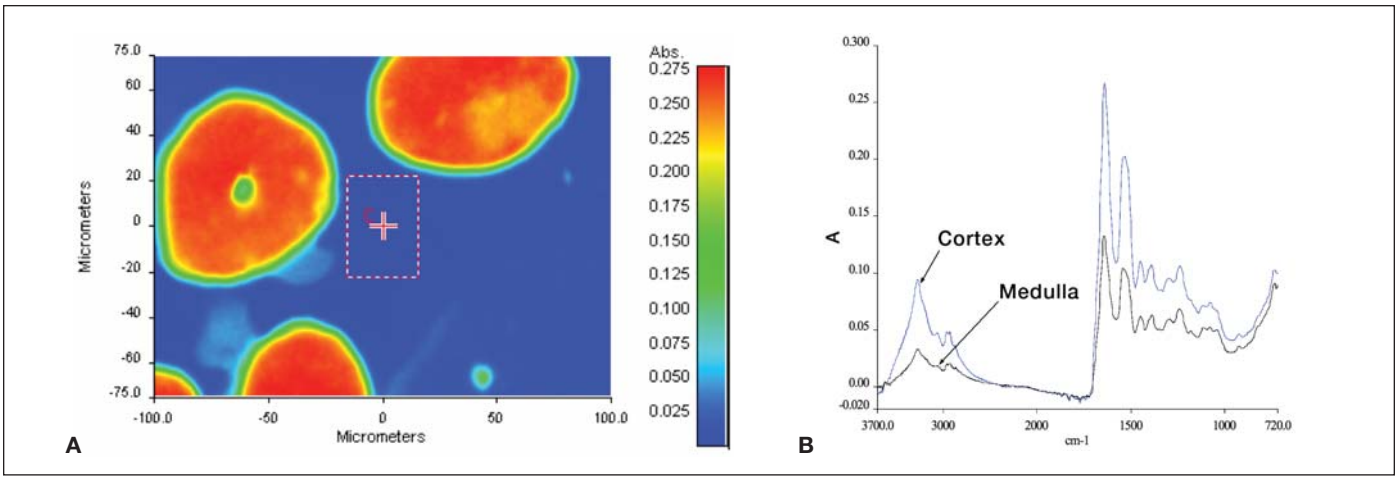

Figure 2. (A) Integrated absorbance peak image and (B) typical raw image spectra from medulla and cortex regions.

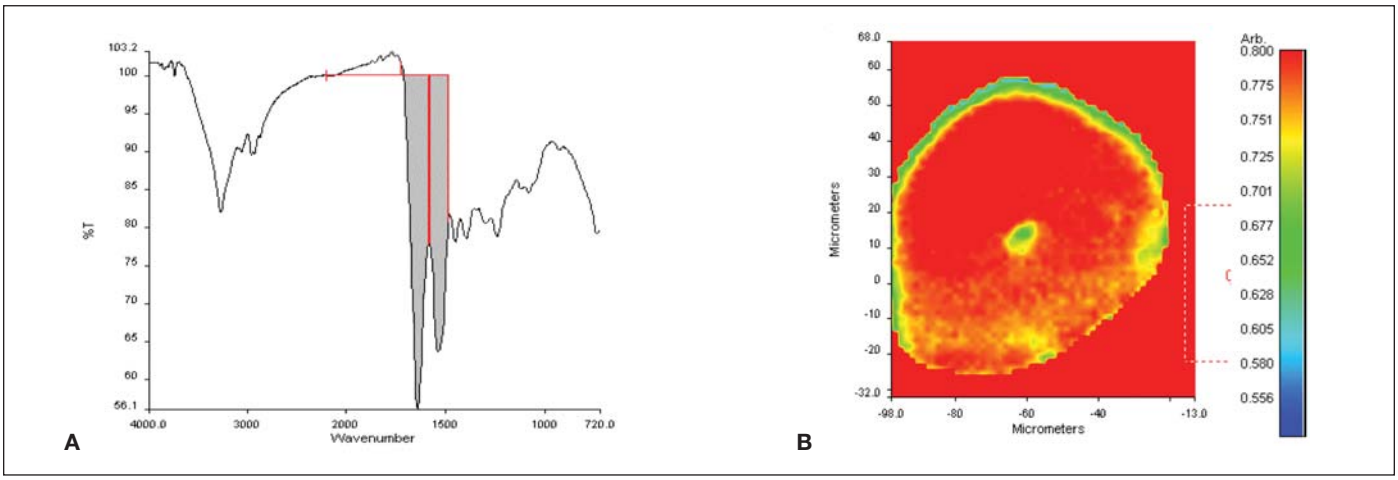

Figure 3. (A) Amide (I) and (II) envelopes and (B) peak ratio image.

From the relatively coarse differences between spectra, the major regions can be differentiated and even measured. Figure 4 shows a profile of the amide (I) envelope intensity as a function of distance along a line drawn through the center of the medulla, allowing an estimate of the core diameter of ca. 7 µ.

Figure 5 shows spectra extracted from the image along a line through the outer edge, or cuticle. Significant spectral differences are observed in a similar manner to those seen between the medulla and cortex regions. In this instance, the outer layer forms a distinct edge as evidenced from the spectra extracted ca. 5 µ away from the cell edge. No significant absorptions from the embedding material (an epoxy resin) are seen. This somewhat fortuitous result suggests the final polishing is perhaps abrading the polymer in preference to the hair sample, since this effect was not restricted to this point but was apparent around the whole periphery of the section in this sample.

Potential of Principal Components Analysis (PCA) with ATR Imaging

Two particular potential benefits of using PCA are highlighted (a) the ability to assist with generation of survey images to locate finer detail with the minimal number of scans and (b) the ability to assist with interpretation of relatively subtle spectral differences in image data.

Role in generation of survey images

Strictly speaking, ATR is potentially a 'destructive' IR sampling technique since the ATR crystal must come into physical contact with the sample in order to generate an ATR spectrum. It is clearly desirable to perform this operation only once, using a freshly prepared sample and perfectly clean ATR crystal. Using this ATR device, it is possible to generate an image 400 x 400 or 160000 μ^2 composed of 1.56 µ square pixels. A typical hair medulla may be say 25-30 μ^2 in area. From a practical perspective, it is advantageous to survey the larger

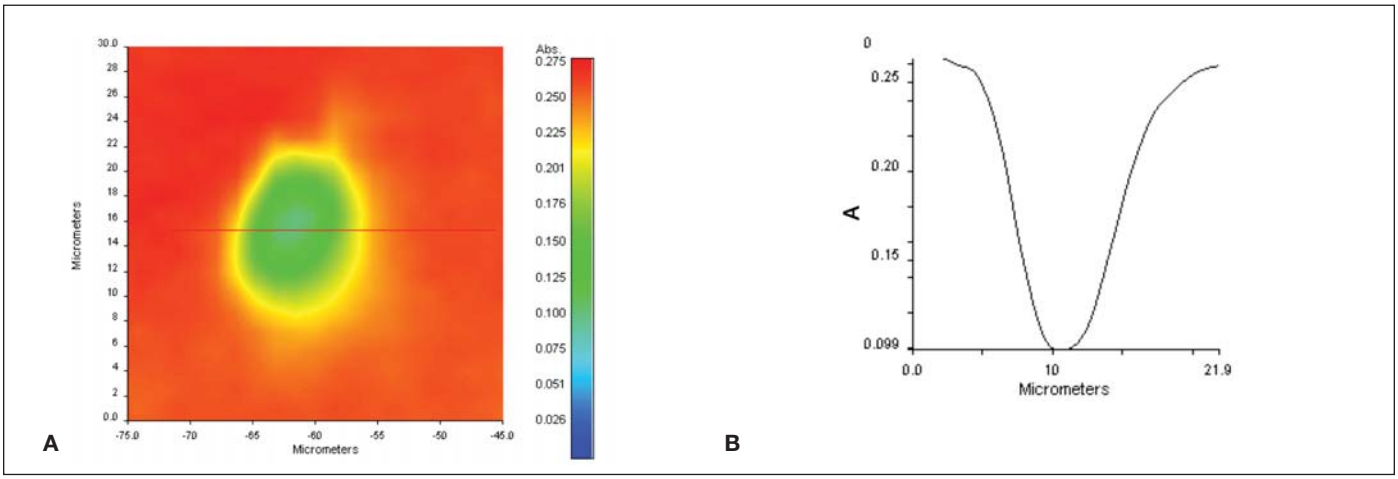

Figure 4. (A) Central section of image around medulla region and (B) peak intensity profile through medulla.

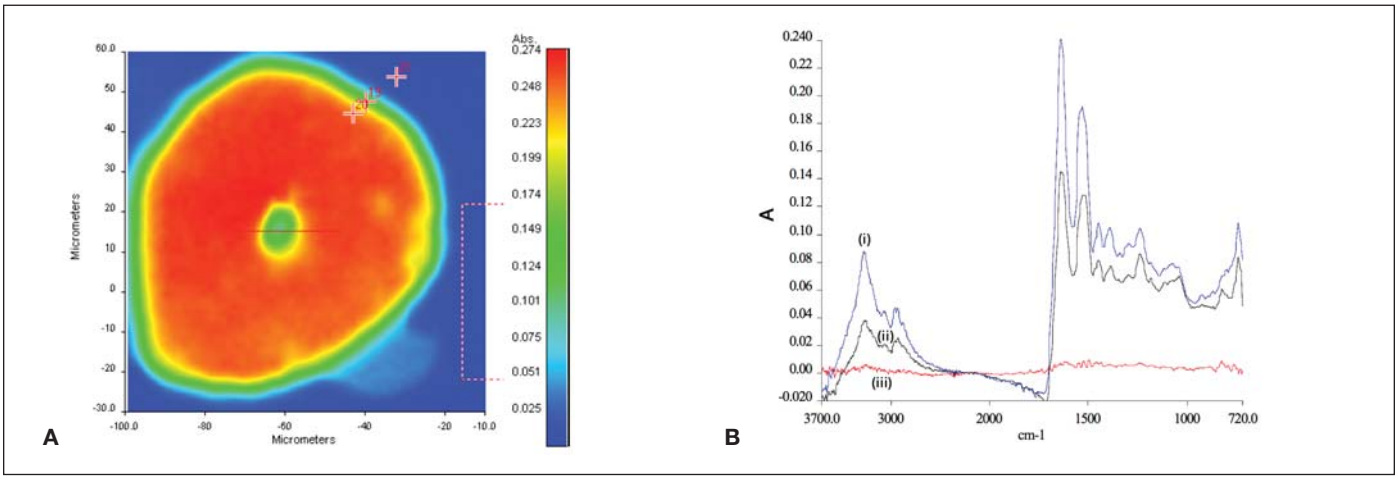

Figure 5. (A) Imaging outer hair layer, (B) Spectra from cortex (i), cuticle (ii) and beyond edge (iii).

area of the sample first using a minimal number of scans to enable a preliminary check, for example, adequate sample contact, lack of contamination etc. at the region of interest before collecting the higher fidelity image using co-added scans. For example, a 400 x 400 µ image could take 211 minutes using 16 scans/pixel for a given scanner velocity. Scanning with 1 scan/pixel takes around 12.5 minutes. Using principal components analysis takes advantage of the existence of tens of thousands of spectra in the image to effect noise reduction[5]. Used with care, to ensure useful information is not relegated to lower, discarded principal components, the technique can be highly useful in enhancing spatial contrast to help locate the ROI. Figure 6 shows a reconstructed score image of a 200 x 200 µ area collected using 1 scan/pixel (ca. 3 minutes scan time).

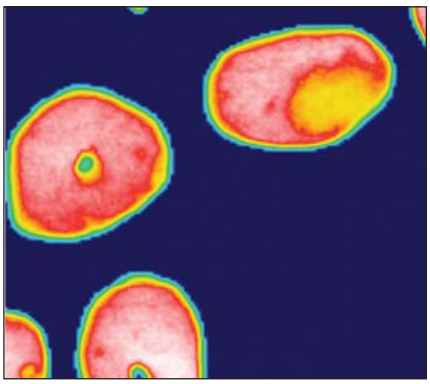

Figure 6. Principal components score image from 1/scan/pixel image.

The image is clearly sharpened with respect to the main features and the reconstructed spectra are of high quality. A combination of 1 scan/pixel and PCA takes ca. 4 minutes and provides confidence that the sample is well positioned and in contact prior to the higher signal-to-noise (S/N) run taking > 1 hour.

Assistance with structure elucidation of ATR images

Due to the suspected subtle spectral variation within the hair structure, the central region around the medulla was subject to PCA over a relatively narrow wavenumber range (3100-2700 cm^{-1}). The results are shown in Figure 7. Using 2nd derivative (no smoothing) data pre-treatment, the first two principal components clearly demonstrate the –CH2 plus -CH3 features and –CH2 respectively. The score images on these first two principal components is remarkable: the first score image again clearly distinguishes the medulla region and is expected. The second score image however highlights an increased relative contribution of –CH2 in certain regions. A similar observation was made from the analysis of high quality synchrotron images of hair sections[1]. This could, for example, be due to an increased abundance of lipids with longer aliphatic chains. A detailed analysis of this finer structure is beyond the scope of this note, but it is worth noting that further PCA over an area extending through the medulla, cortex and cuticle revealed principal components with similar structure

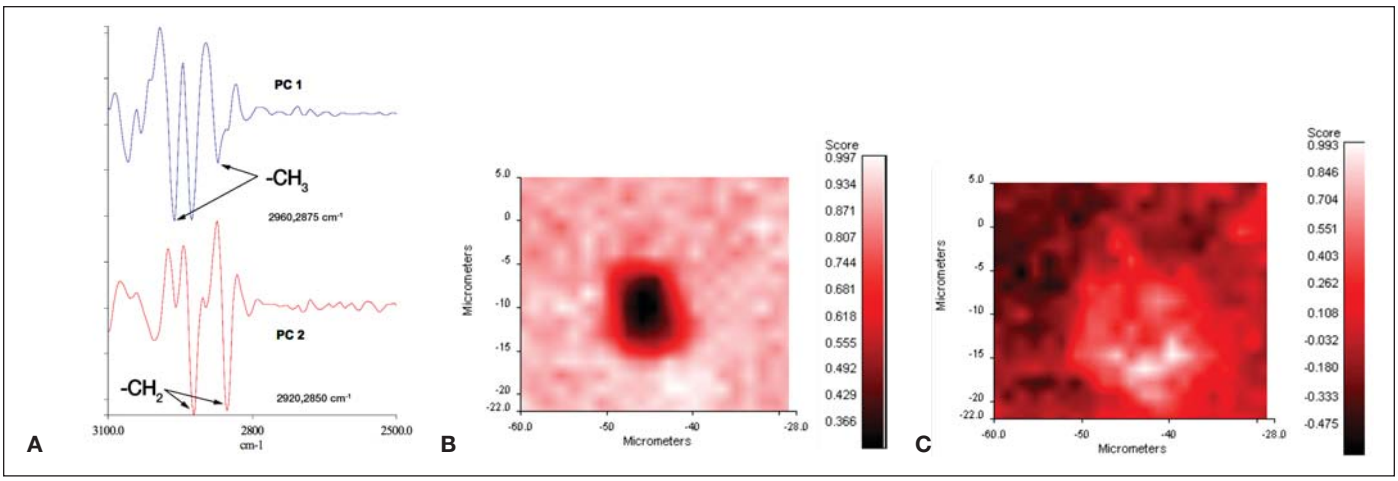

Figure 7. Principal components analysis (A) first and second principal components (2nd derivative, A, peaks extend downwards). (B) image of first principal component. (C) image of second principal component.

i.e. highlighting –CH2 rich areas in the medulla region and cuticle regions [Figure 8]. It is believed that the lipids are arranged in an organized state[7] and such organized structure has been seen in the skin stratum corneum, by X-ray diffraction and IR spectroscopy. In IR, this lipid organization is believed to be manifested by a slight downward shift of symmetric –CH2 modes towards the outer layer in the hair. This is consistent with the observations reported here.

While the technique is believed to offer invaluable help in enhancing image contrast where subtle effects are found, it must be used with caution to avoid misinterpretation of results. ATR is essentially a surface technique, and using a germanium crystal, the depth of penetration into the sample is believed to be of the order of 1 micron or less. Extra caution must be taken to guard against surface contamination since relative spectral contributions are likely to be high, compared with, for example, MIR transmission and certainly NIR imaging by diffuse reflectance. Occasionally this benefits the user, because the contaminant is so strikingly obvious it can be easily isolated and masked out of the PCA analysis using the spatial masking feature in the Hyperview Software. Consequently, whenever structure is observed following a PCA, it is **always** advisable to go back and examine the raw (or

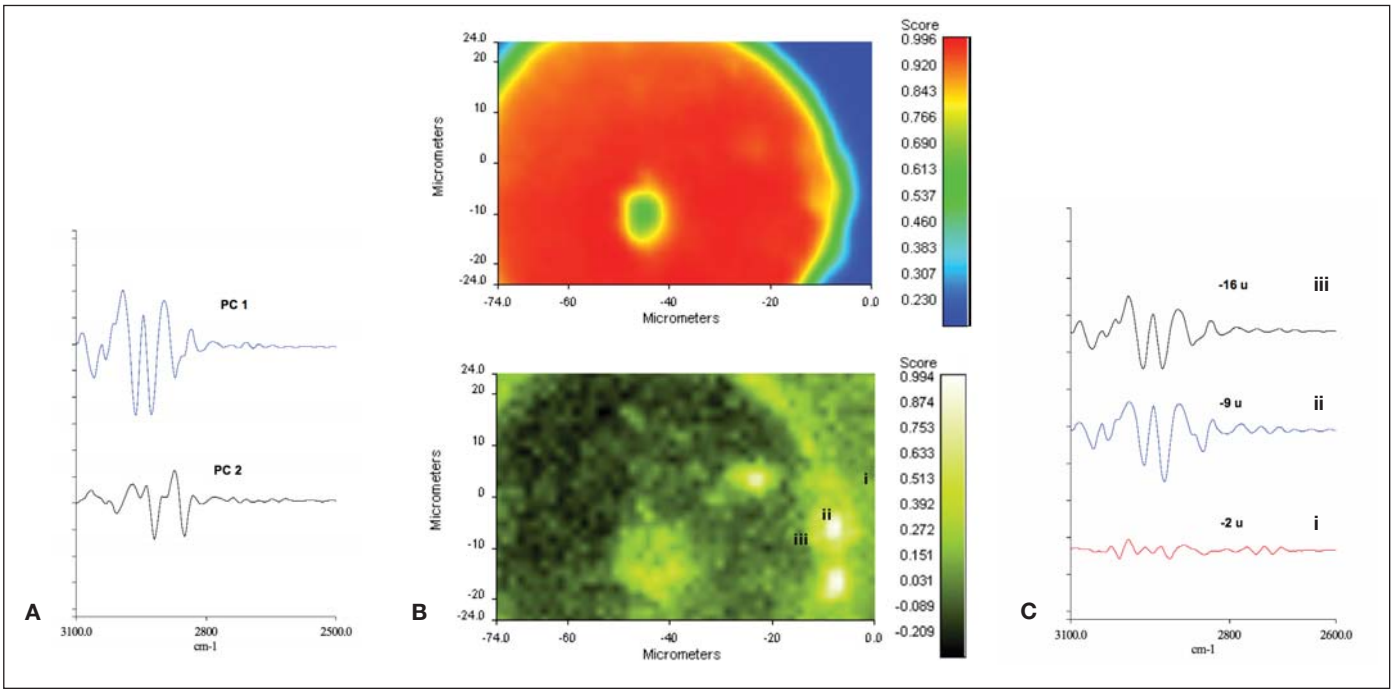

Figure 8. First two principal components (A), score images (B), and spectra from (C) i, ii, iii showing enhanced –CH2 in the cuticle region.

pre-PCA) data for confirmation. Figure 9 illustrates the high spots associated with the enhanced –CH2 intensity (ca. 2850 cm^{-1}, 2920 cm^{-1}) regions and the corresponding full range raw spectrum at a high spot overlaid with the spectrum from the mounting resin. The resin contains distinct bands at 825 cm^{-1} and 1032 cm^{-1} for example, which do not overlap with the hair spectra. Consequently, they would provide useful markers for possible resin contamination. The absence of these bands at the high spots in the medulla and cuticle regions help rule out the possibility of resin contamination.

Conclusion

Human hair sections provide excellent samples for demonstrating the potential of mid-IR ATR imaging. Measurements of cross-sections of human hair samples using the Spotlight System demonstrate the ability to deliver high signal-to-noise spectra of hair samples with improved spatial resolution and good signal-to-noise compared with standard transmission microspectroscopy. The flexibility of the PerkinElmer Spotlight Imaging System using a linear detector array can be further exploited using principal components analysis, enabling high quality survey images to be generated often using a single scan per pixel. Another potential use of PCA is the elucidation of more subtle spectral features in images. Provided the technique is used with caution and with an understanding of the inherent attributes of ATR imaging, subtle information can be discerned in ATR images using relatively short measurement times.

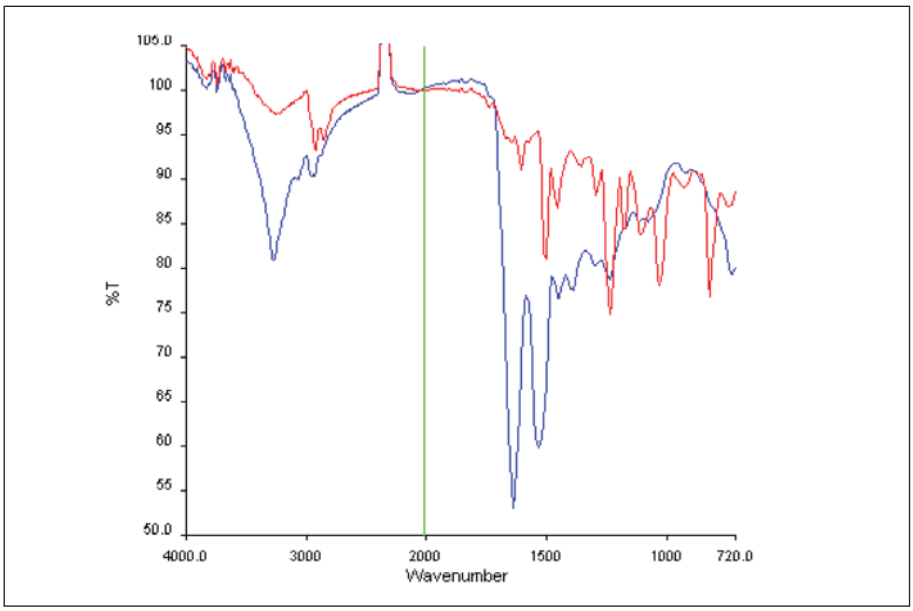

Figure 9. Spectra from region of enhanced relative CH2 intensity (blue) overlaid with mounting resin spectrum (red).

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PerkinElmer Life and Analytical Sciences
710 Bridgeport Avenue
Shelton, CT 06484-4794 USA
Phone: (800) 762-4000 or
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