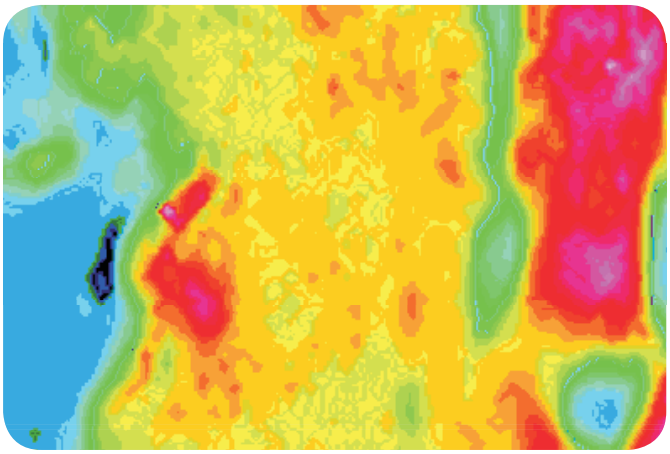

Application Note AN B403

FT-IR spectroscopy as a new imaging technique

Using IR imaging it is now possible to investigate even large sample areas with extremely high local resolution by means of IR spectroscopy. A new detection system assesses all points on the image at the same time, which means that the average measuring time is only a matter of minutes. Since a complete IR spectrum is obtained for every image point, the information content is extremely high and the technique can be used for any area of application. IR imaging has already established itself as a frequently used procedure for the investigation of different tissues and single cells in biomedical applications and the analysis of polymer mixtures in chemical applications.

The infrared spectrum (heat radiation) directly follows the longer wavelength end of the visible spectrum in the electromagnetic spectrum. In contrast to UV/VIS spectroscopy, which measures electronic transitions, infrared (IR) spectroscopy determines the vibration and rotation of molecules as absorption phenomena. Many functional groups of organic molecules show characteristic vibrations which means that they can be clearly attributed to certain absorption bands. Both the high information content and the suitability of the technique for any application in particular represent outstanding properties of the system which have been responsible for the success of IR spectroscopy as an analytical method.

Principle of the method

IR spectroscopy has been used in combination with traditional microscopy for more than a quarter of a century. On the basis of the visible image, the interesting areas of the object under scrutiny can be located and its (bio)chemical composition can then be analysed spectroscopically using this technique. Using this method, the sample does not have to be stained or chemically modified. Only the thickness of the layer has to be adjusted to the penetration depth of the IR radiation. So far, to increase local resolution, field stops were used to limit the 'field of vision' of the infrared beam to the interesting areas of the sample. For the determination of large sample areas with high spatial resolution, the sample was moved step by step past the field stop using a motorized sample support and the spectra of the different areas taken one after the other. This conventional procedure, often also termed 'mapping', was extremely time consuming, so much so that the assessment of a sample area of 0.5 x 0.5mm at a resolution of 15µm took about 10 hours. This considerably limited the use of traditional IR imaging. Especially with biological samples such as tissue slices or individual cells, it is not possible to ensure that the condition of the sample (temperature, degree of hydration etc.) remains constant throughout the entire period of determination.

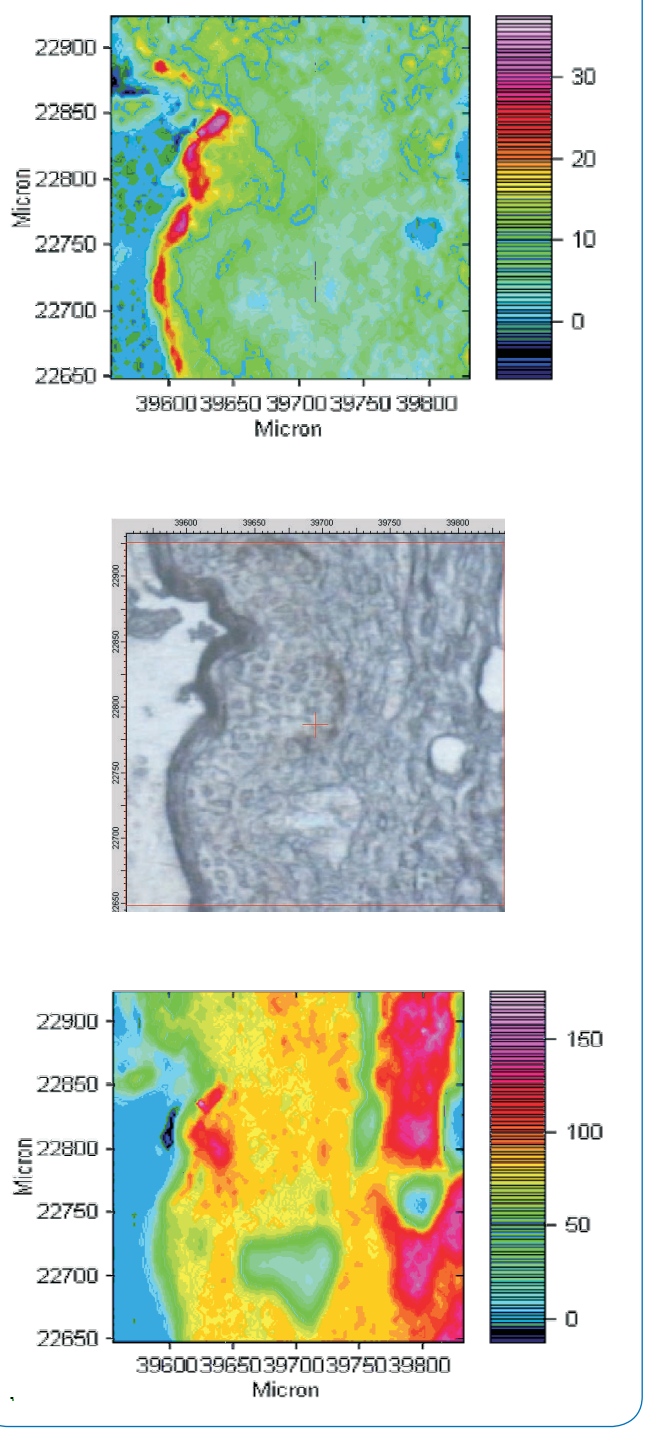

Fig. 1: IR imaging of human skin tissue. Middle panel: transverse section (250 x 250µm; 15µm), was measured using a Bruker HYPERION (8cm-1 resolution, 15x, transmission). Upper panel: integrated signal intensity in the CH stretch vibration range (3000 - 2800cm-1) = lipid distribution. Lower panel: integrated signal intensity in amide range (1720 - 1480cm- 1) = protein distribution.

In order to solve this problem, multielement detectors are used for the new IR imaging technique. The surface of these detectors consists of a square grid of detector elements. This enables large surface areas to be measured simultaneously instead of being mapped as before. Using this new detection system, it has also been possible to improve spatial resolution up to the diffraction limit, since it is no longer necessary to use field stops. The method enables simultaneous determination of areas 340 x 340 µm with a resolution of 2.7 µm. In the same way as with conventional IR microscopy, transmission, reflection and attenuated total reflection (ATR) are available as modes of measurement.

Applications

For each measurement, which takes about two minutes, a complete IR absorption spectrum is obtained for each image pixel (number = number of detector elements). In contrast to VIS and fluorescence microscopy, which result in only one data point per image point, there are generally between 300 and 600 data points per image pixel, depending on the spectral resolution specified.

These complex data obtained are selectively reduced, so that the information can be displayed in the form of two- and three-dimensional images. For example, the signal intensities of certain IR frequencies, which can clearly be assigned to a functional group, are plotted across the sample surface analysed (chemical mapping).

IR imaging has already been established for the investigation of animal and vegetable tissues. As an example, Figure 1 shows the imaging of human skin. The visible image of the area determined (250 x 250µm) of a transverse section (15µm) is shown in the middle. The upper image shows a false-colour image of the intensity determined in the CH stretch vibration range (3000 – 2800cm^{-1}), in which the distribution of the lipids in the skin area measured is recognisable. In order to illustrate the protein distribution within the tissue, the signal intensity in the amide range used to detect proteins (1720 – 1480cm^{-1}) is shown over the sample (Fig. 1, lower panel). Thus, the biochemical composition of tissues can be determined in a non-destructive manner. This technique, therefore, holds great promise particularly for the diagnosis of cancer.

Often, however, the absorption bands of several functional groups are superimposed, which hampers the correlation of spectral properties with the biochemical composition of the sample. In such cases, modern multivariate techniques can be applied for data reduction. To illustrate this, Figure 2 shows the analysis of microbeads. Such polystyrene beads are used in combinatorial chemistry for solid phase synthesis. Although the beads have a specific chemical modifica-

tion on the surface, the visual image shows no difference. The beads were taken from 4 successive steps of a synthesis reaction. The spectra of a number of representative image points in Figure 2b show that the spectral differences between different, modified beads are very small. Principal component analysis as a multivariate data processing tool was therefore applied for evaluation. This method enables a wide range of dependent variables to be transformed into a small number of independent variables, the principal components. Figure 2c shows in false colours the similarities of the original spectra to three principal components. It can easily be seen that these three principal components provide relevant spectral information, which enables differentiation between the beads. If the three images are combined into a red-green-blue (RGB) image, the different beads are seen with their own specific colours and the chemical differences become obvious.

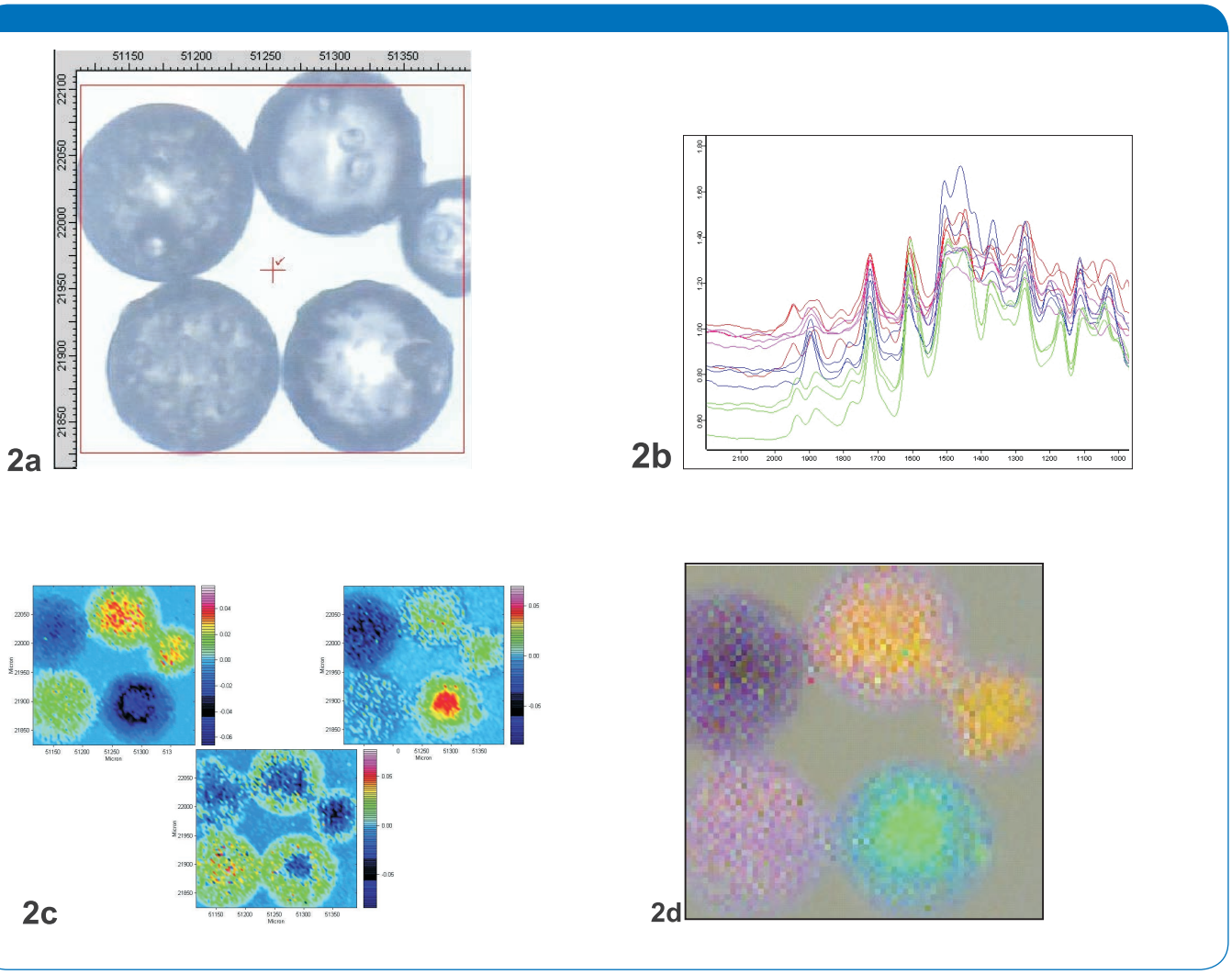

Fig. 2: IR image of polystyrene beads (8cm^{-1} resolution, 15x, transmission): Beads lock very similar in the visible image (2a). 2b: Representative IR spectra for a number of detector pixels. 2c: Similarity of the original spectra to the three relevant principal components; 2d: RGB image made up of the three relevant principal components.

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