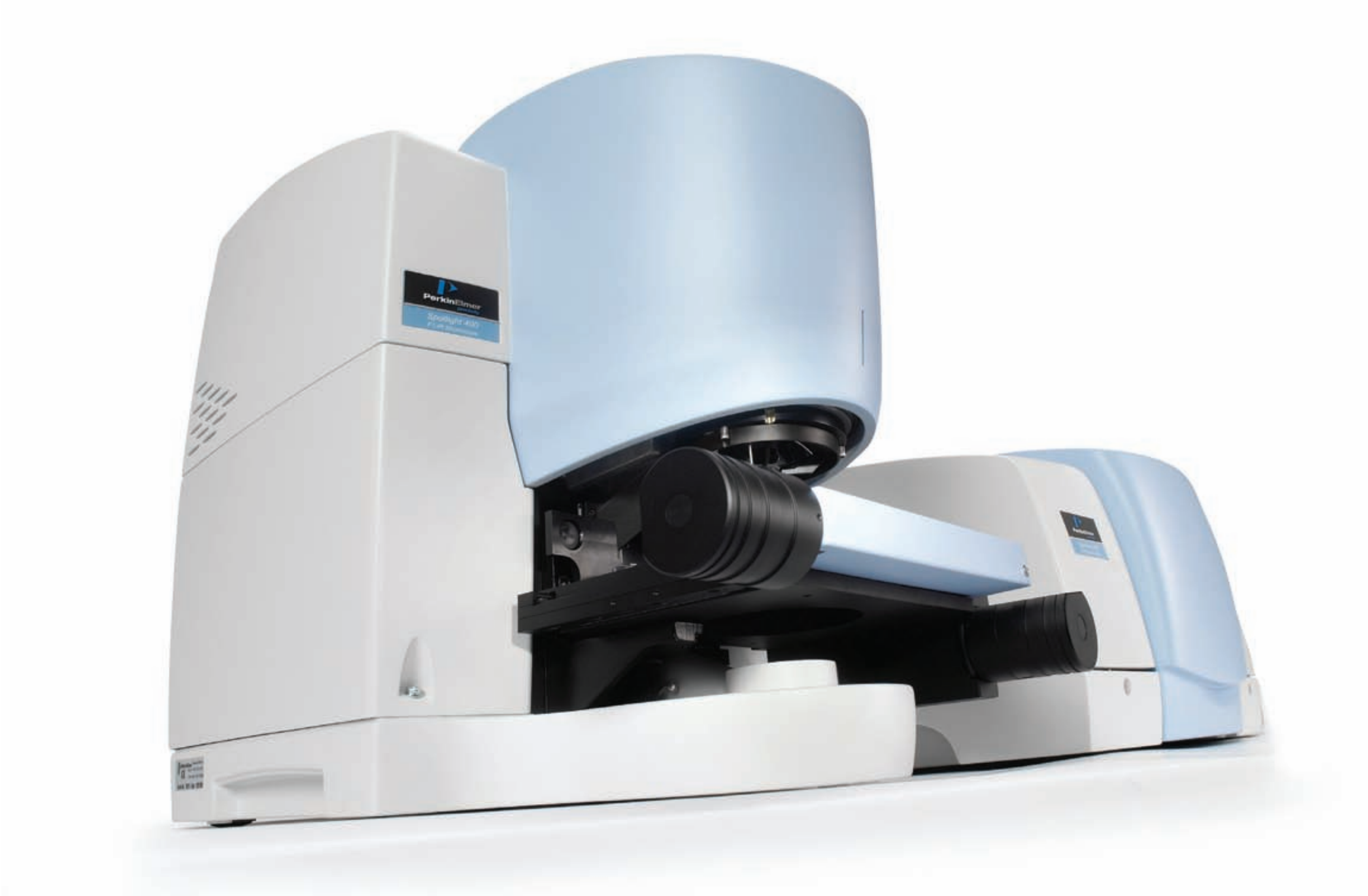
CASE STUDY

Infrared Spectroscopy




# Infrared Microscopy Provides More Accurate Kidney Stone Diagnosis

A recent study has demonstrated that the accuracy of kidney stone diagnosis can be substantially improved by switching from conventional optical microscopy and contrast staining to infrared (IR) microspectroscopy. There are over a dozen different types of kidney stone, and diagnosing the type is important because the most effective treatment methods vary for each. Infrared spectroscopy and x-ray diffraction have been successfully used on isolated kidney stones because of their ability to yield highly accurate information regarding mineral composition. However, the problem with the methods used in the past is that they analyzed macroscopic-sized samples of kidney stones. Mineral inclusions in the tissue trigger the onset of larger stone formation and it is these inclusions that must be analyzed in the early stages of the disease. Thin sectioning and contrast staining procedures combined with optical microscopy are currently used in most tissue biopsies to determine the type of mineral inclusion. With more than 20 steps required in this style of sample preparation, the accuracy of results depends on the skill level of the histologist and the experience of the pathologist.

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Researchers at the Molecular Microspectroscopy Laboratory led by Andrè J. Sommer of the Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio, and Andy Evan of the Department of Anatomy and Cell Biology, Indiana School of Medicine, Indiana-Purdue University, Indianapolis, Indiana, are pioneering a new method that uses infrared absorption microspectroscopy to identify the type of mineral inclusion present, substantially increasing the diagnostic accuracy. “Parallel studies have shown that 40% of current-day pathological studies incorrectly identify the type of mineral inclusion,” Sommer said. “By providing more accurate diagnoses using IR microspectroscopy we are able to deliver superior treatments while reducing medical costs.”

## Previous kidney stone diagnostic methods

Approximately 5% of the population will suffer from kidney stones at some point in their life. About 70% of renal stones are composed of calcium oxalate with small amounts of calcium hydroxyapatite; 10% to 12% contain uric acid; 10% to 20% contain magnesium ammonium phosphate; 5% contain more than 50% hydroxyapatite or calcium monohydrogen phosphate; and less than 1% are composed of cystine. These and other types of stones each have their own treatment regimens. Determination of mineral inclusion composition combined with patient history and laboratory investigation is fundamental in establishing the cause and likelihood of recurrence of renal stones, as well as an appropriate patient treatment.

For over 160 years, disease detection in biopsied tissue has relied on the painstaking preparation of thin sections followed by contrast staining to visibly signal the presence or absence of diseases[1]. Mineral inclusions associated with kidney disease are identified by the visual inspection of crystal morphology using an optical light microscope. Often, selective staining protocols are employed to further enhance the contrast between different mineral types seen under the light microscope. This method involves complicated sample preparation procedures and relies heavily on the experience and skills of both the histologist and pathologist to achieve an accurate diagnosis. The difficulty of determining the type of kidney stone is particularly great in the very early stages of the disease, when it is most treatable. Misdiagnosis of the type of stone is not life-threatening to the patient but may waste time and money in indicating a treatment that later needs to be abandoned because the patient was unresponsive.

## Advent of IR microscopy

IR spectroscopy has gained wide acceptance in the analytical community both as a qualitative and a quantitative research tool, because it provides direct molecular identification of the materials being studied. Infrared spectra are unique molecular fingerprints, which provide bonding information about compounds with activity in the infrared region.

The integration of a microscope with the infrared spectrometer allows the infrared beam to be focused on microscopic mineral inclusions at specific areas of a larger sample. This approach is termed infrared (IR) microspectroscopy and allows the unique molecular fingerprint of the tiny mineral inclusions to be obtained. This technique has previously been employed on tissue sections with positive results, but never on minerals embedded in tissue. In addition, previous studies used tissue samples that were stained and held between two infrared-transparent windows, while the new method employed at Miami University offers the option of using unstained samples that are merely mounted onto a reflective glass substrate.

## New diagnostic method

“Our laboratory is interested in determining the pathogenesis of the most common form of kidney stones: calcium oxalate,” Evan said. “In order to determine the mechanisms responsible for the formation of these stones, we have to be able to accurately characterize the composition of very small mineral inclusions. In our collaborative effort, we have developed a new diagnostic method that uses infrared microspectroscopy along with low energy reflective slide substrates to accurately determine the composition of small mineral inclusions. A major innovation in this new protocol is that tissue sections

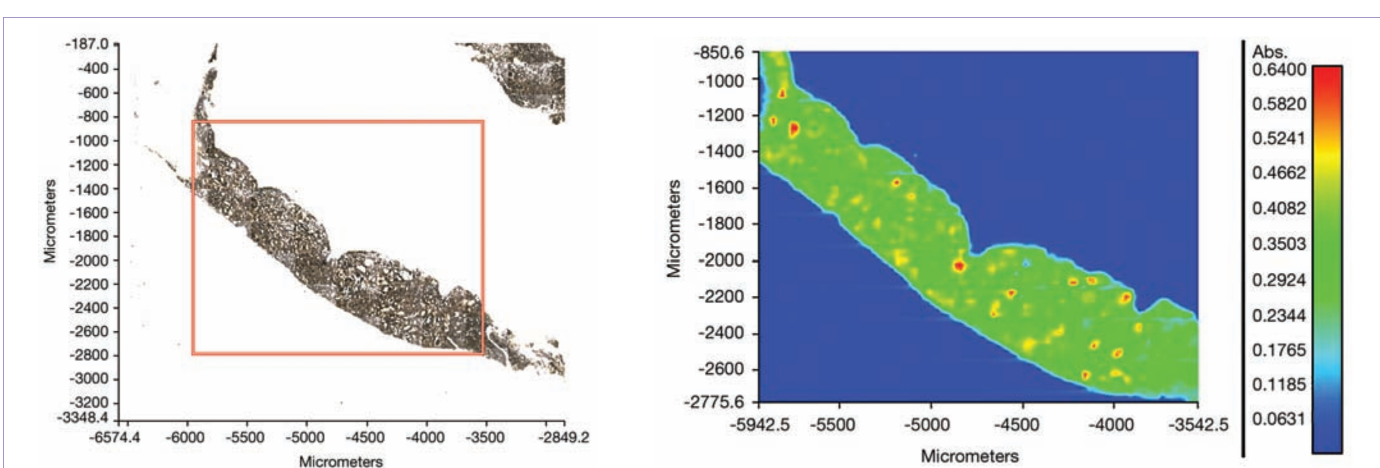


*Figure 1.* Visible and infrared image of thin section mounted on low-E glass.

are prepared in the traditional manner, but mounted on a reflective slide that is used for optical as well as infrared analysis of the tissue section."

All samples were analyzed with a PerkinElmer® Spotlight™ 300 IR microscope equipped with an array detector for the rapid acquisition of molecular images and a single point detector for the acquisition of high signal to noise spectra with a lower wavenumber cut-off of 580 $cm^{-1}$. Both detectors are based on mercury cadmium telluride technology. The minimum sample size that can be analyzed with either detector is approximately 2 times the wavelength of light being measured, or approximately 6 micrometers. Both reflectance/absorption (R/A) and attenuated total reflection (ATR) analysis of tissue and mineral inclusions can be performed on the low-E substrate, yielding qualitative, unbiased and accurate information about the mineralized inclusions and tissue.

"Low-E" stands for "low emissivity", which means that the substrate is highly reflective (and not diffusely scattering) to heat, i.e., IR radiation. Low-E glass is used in many buildings where the uncoated glass side faces the outdoors and a coated side (making it low-E) faces the inside. As such, light and heat from the sun can enter through the glass window, but heat isn't allowed to exit, reflected by the low-E coating on the inside.

## Advantages of PerkinElmer instrumentation

"We began using the PerkinElmer AutoIMAGE™ microscope because of its high quality optics and high signal-to-noise ratio," Sommer said. "More recently we have switched to the PerkinElmer Spotlight 300, which offers the same optical advantages plus major improvements in detector technology." Spotlight's patented detector incorporates an array detector and single point detector on the same substrate in the same Dewar. The array detector provides high quality images of large areas rapidly while the single point detector is useful for the toughest of spatial resolution problems and for the ultimate in sensitivity, easily outperforming top-of-the-line IR microscope systems. Detector mode is changed with a single click of the mouse. The system generates transmission and reflectance spectra in both modes and includes a micro-ATR objective that operates in single point mode, giving Spotlight the versatility to deal with virtually any type of sample."

Figure 1 illustrates both a visible image and a false color infrared R/A image of a 4 μ (micrometer) thick tissue section mounted on the low-E glass slide. The infrared image is based on the peak height of the symmetric stretching vibration of the oxalate anion associated with calcium oxalate. The image clearly and precisely identifies those areas where calcium oxalate is present. The image also exemplifies the benefit of infrared imaging for the detection of mineral inclusions and the study of diseased tissue. In addition to eliminating the staining procedure, interpretation of the results is much less subjective than current methods of mineral inclusion identification. With techniques that rely solely on crystal shape and visual appearance, only physical information is currently being used for identification. With IR imaging, molecular data are used along with the morphological information to facilitate a more accurate identification.

## Moving to ATR analysis

"Once interesting sites have been identified by R/A analysis, ATR analysis can then be used to collect the best data possible," Sommer added. "ATR analysis provides spectra free from many artifacts associated with transmission and R/A analysis and completes the full picture of the components contained in the crystal deposits and tissue." In macro ATR infrared spectroscopy, infrared light is passed into a prismatic crystal of relatively high refractive index in such a way that it is totally internally reflected at the crystal surface. These internal reflections create an evanescent wave, which extends beyond the surface of the crystal into a sample placed on its surface. In a typical experiment, part of the evanescent radiation is absorbed by the sample, producing a characteristic infrared absorption spectrum. The advantages of this method include limited sample preparation, an easily controlled optical path length, and for microanalysis, a magnification factor equal to the refractive index of the internal reflection element. This latter advantage allows spatial domains, which are four times smaller, to be studied.

Figure 2 presents spectra obtained by the R/A method and spectra of the same spot obtained by ATR analysis. The spectrum obtained on the mineral inclusion clearly shows the asymmetric and symmetric stretching modes of the oxalate anion located at 1620 and 1318 $cm^{-1}$, respectively. "These results clearly demonstrate that mixed sample types containing tissue and mineralized deposits are easily analyzed while mounted on a low-energy slide using the ATR method," Sommer said. "An R/A absorption analysis makes it possible to quickly survey a tissue section and provides qualitative information about its components. This new method will save time and training, while simultaneously offering an unbiased analysis of mineralized components that is more accurate and conducive to patient treatments than previous methods. The sensitivity of this method allows tissue sections to remain unstained, alleviating the time-consuming constraints of earlier methods using visual

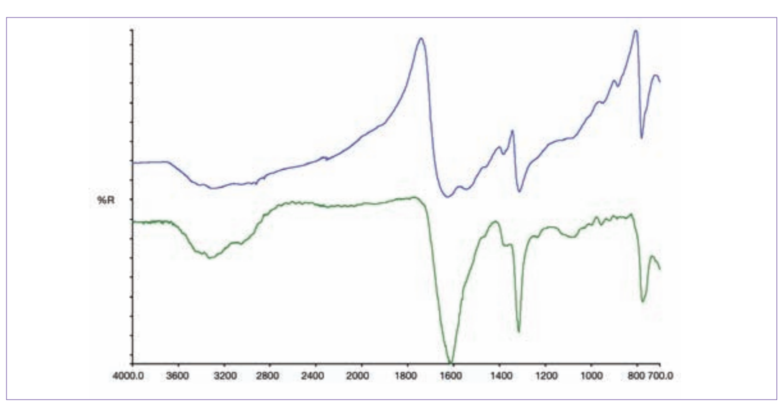


*Figure 2.* R/A (top) and ATR (bottom) spectra of mineral inclusion.

analysis. Parallel studies have already shown that the new method provides substantially greater accuracy than traditional diagnostic methods."

Finally, although it is highly desirable to study mineral inclusions which signal the onset of kidney disease, the study of kidney stones is also an important step in elucidating the chemistry of stone formation. Figure 3 illustrates a visible image of a cross-sectioned kidney stone and infrared images collected within the region bounded by the red box. The infrared images are comprised of more than 130,000 spectra and can be selectively tuned for a given chemical composition. One of these images has been tuned to calcium oxalate and the other to calcium hydroxyapatite. The images clearly show that the chemistry modulates during the growth of the stone from its nucleation site (center) to the point at which it was surgically removed. Like geologists who elucidate the chemistry of the earth by studying sedimentary layers in stone, scientists can determine the chemistry of a stone formation using infrared microspectroscopy.

The AutoImage FT-IR Microscope System and the Spotlight 300 Imaging System referred to in this case study have been superceded by the Spotlight 200 FT-IR Microscope System and Spotlight 400 FT-IR Imaging System respectively.

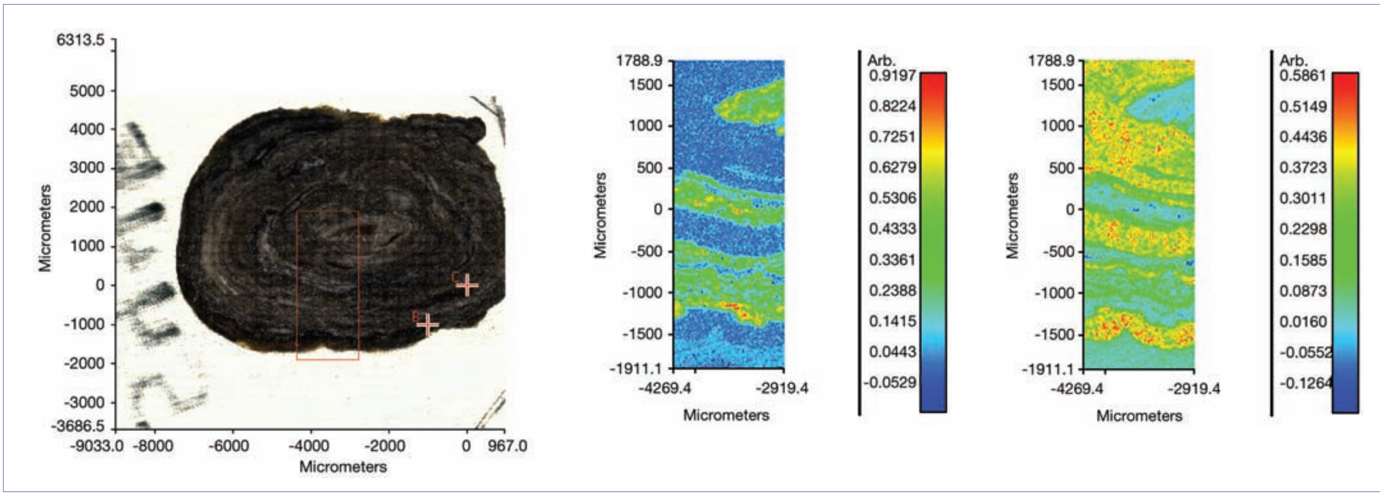


*Figure 3.* Visible and infrared images of a kidney stone.

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