Focus on Microscopy: From light microscopy to molecular analysis at the touch of a button

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For scientists and technologists who use microscopes to solve practical problems, the image is everything. It contains structure, function, context and, often, information about viability or failure. However, imaging is not always enough. An innovative, simple-to-use module now expands the microscopists' arsenal to include definitive chemical characterization.

A new solution

Microspectrophotometry, primarily in the UV, visible, and near-IR range (220 and 2200 nm), has been done since the 1930s.[1] The systems were sophisticated, demanding, and called for the most experienced microscopists. As useful as this range has been for document verification, coal analysis, and some biological applications, the spectra generated in this range were limited, leaving microscopists struggling for more conclusive information. Of key importance is a chemical fingerprint that could be used to identify a material, to differentiate it from similar looking materials, or to lead to its source. While the recent convergence of infrared spectroscopy with microscopy provides many of these answers, most new systems were built with an emphasis on spectroscopy and fell short in microscope functionality. IlluminatIR™ (**SensIR Technologies,** Danbury, CT), a new light microscopy accessory based on internal reflection spectroscopy (IRS), is the first infrared spectrometer designed to integrate with existing microscopy technology.

Internal reflection spectroscopy simplifies sample requirements

IRS is derived from Snell's Law of Refraction, the fundamental principle behind all the refracting optics used in conventional microscopy. The basic concept is simple: When a beam of light (or any other type of electromagnetic radiation) approaches an interface between a material of low optical density (low refractive index) and a material of higher optical density (higher refractive index) at an angle, part of the beam will undergo external reflection at the surface and the rest will pass across the boundary, undergoing refraction. The direction and angle of refraction depend on the angle of approach and the relative optical densities or refractive indices on either side of the boundary. According to Snell's Law, the refracted beam in this case will bend toward the normal (*Figure 1*).

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Moving from a higher to lower refractive index produces the opposite effect, as shown in *Figure 2*. This situation contains the founda-

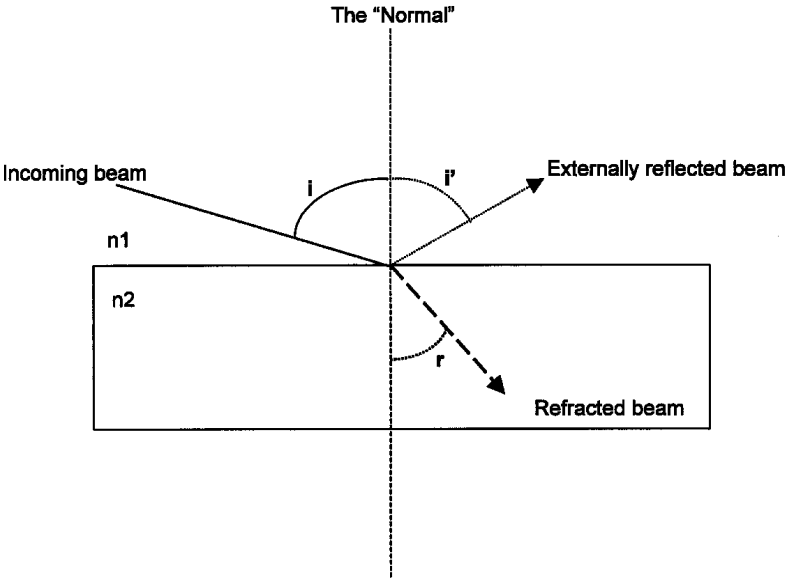

Figure 1 *Snell's Law—moving from low optical density (low refractive index) to higher optical density.*

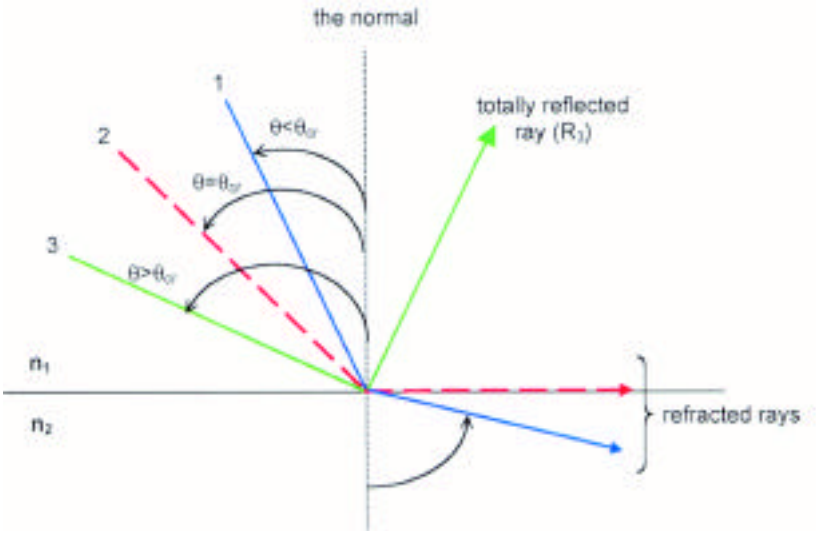

Figure 2 *The case of Snell's Law in which the beam travels from higher refractive index to lower refractive index—the foundation for IRS.*

tion for IRS. The middle beam (beam 2) meets a special criterion. It approaches the interface at just the right angle, based on the relative refractive indices, to cause it to refract or bend so that it travels along the interface, at 90° to the normal. This angle of approach is called the critical angle. (Note that there is also a critical angle in the previous case, but it is not of overriding concern for this explanation.) Any beam approaching the interface at a smaller angle (e.g., beam 1), will cross the interface, on a path bending away from the normal. Any beam approaching the interface at a greater angle (e.g., beam 3), will suffer total internal reflection and bounce off the interface back into the original material.

In the process of reflecting, the beam generates a small, evanescent field at the interface, which penetrates the second material. If the second material absorbs this energy, the intensity for the reflected beam is reduced or attenuated. The absorption is selective, dependent on the chemistry of the second material. Scanning the attenuation over a specific wavelength region (2500–16,250 nm) produces a spectrum that provides a specific molecular fingerprint for the second material.

Because IRS is easy to use and requires minimal sample preparation, internal reflection spectroscopy is becoming one of the more common infrared approaches for analyzing both liquids and solids. The resulting spectrum quickly fingerprints organic as well as covalent inorganic materials. Of special interest to researchers and technicians looking at coatings, ink on various substrates, or tiny particles, the unique properties of the evanescent field allow IRS to "see" just the top layer of the sample and ignore underlying information. Coupled with the imaging power of light microscopy, this approach opens dramatic opportunities to gather correlative information from both material and biological samples.

Figure 3 *IlluminatIR, an accessory that transforms conventional light microscopes into an IRS infrared microprobe.*

Converting conventional microscopes to infrared microprobes

Figure 3 shows the IlluminatIR, an accessory that transforms conventional light microscopes into infrared microprobes. There are three key components to the system: an infrared spectrometer that fits neatly between the microscope and binocular body, a video viewing system, and special infrared objectives.

As seen in this figure, the viewing system uses an integrated LCD screen and a special charge-coupled device (CCD) camera capable of imaging the regular microscope field simultaneously with the infrared image. This feature is especially important because it ensures that the spectrometer measures exactly what the microscope sees. Laboratories that have specific camera needs beyond the integrated system can easily fit both cameras on the microscope by using a dual-viewing "T" tube, available from most manufacturers.

Since IlluminatIR is an add-on module, it retains all the ergonomics and functionality of the original microscope. Conventional bright-field as well as all the normal contrast techniques (darkfield, phase, Hoffman Modulation contrast, polarized light, fluorescence, and Nomarski/DIC) are still available. The system operates equally well in reflected or transmitted light.

Special objectives provide complete range of analyses

As the name implies, internal reflection spectra can be collected from several types of reflected light. For greater flexibility, IlluminatIR comes equipped with two different objectives: a special diamond ATR (attenuated total reflection) objective, useful for a broad range of contact techniques; and

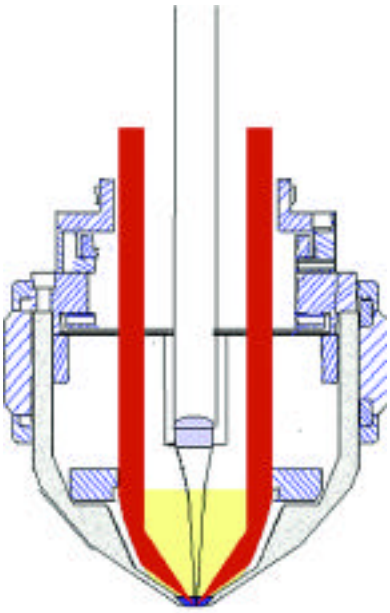

Figure 4 *The ATR objective has two light paths: imaging and infrared measurement.*

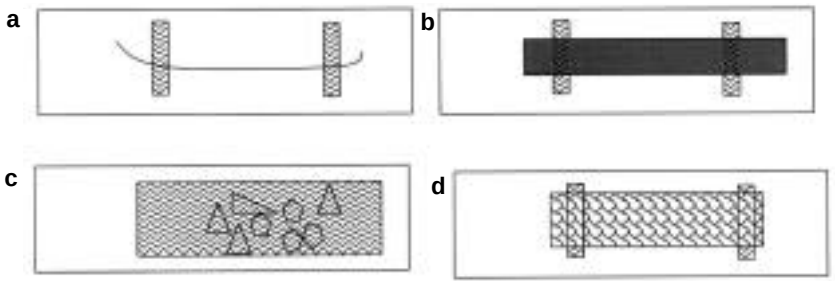

Figure 5 *a) Fibers, b) films, c) crystals, and d) foams can be attached to a micro - scope slide using small strips of double-sided tape.*

an all-reflecting objective for a variety of reflection techniques.

ATR objective

The ATR objective follows Snell's Law precisely, putting highly refractive diamond (refractive index 2.42) on one side of the interface and the test material on the other. The choice of material for this objective presents a serious engineering challenge. It has to transmit adequately in the infrared region, have a refractive index high enough to induce total internal reflection, and, because the front element of this objective needs to make contact with the sample, must be hard and scratch resistant. Diamond was a logical choice. It is easy to clean, is not affected by corrosive materials, and does not scratch or fracture.

As seen in *Figure 4*, the ATR objective actually has two imaging modes: one for conventional viewing (the narrow, inner cone) and one for infrared measurement (the other, red cone). Once the microscopical analysis is completed, the objective is rotated into position and set for viewing mode. Using the normal focus and stage controls, a specific feature to be analyzed can be moved into position and into focus. Note that in the viewing position, the diamond is raised above the sample. A click of the mouse acquires the background spectrum.

Raising the stage brings the diamond internal reflection element into contact with the sample (signaled by a wetting effect). A mouse click acquires the sample's ATR spectrum. Since the system records only the spectrum of the material in contact with the ATR objective, not what is beneath the sample, it can analyze ink on paper or packaging material or the coating on the inside of a soda can without seeing the substrate. As shown in the sample preparation section below, a small piece of the material to be studied can be cut and attached to a microscope slide using double-sided sticky tape for stability and flatness.

Coupling spectroscopy with microscopy adds another dimension in the analysis of both organic or covalent inorganic materials.

All-reflecting objective

An all-reflecting objective enables the IlluminatIR to collect infrared spectra as specular reflection, diffuse reflection, or reflection-absorption spectra (RAS). The type of spectral data depends on the sample. Smooth surfaces produce specular (mirror-like) reflection while fine particles or roughened surfaces scatter light, producing diffuse reflection. Depositing thin layers of the unknown on an infrared-reflecting substrate allows the beam to pass through the test material then reflect, forming the basis for the especially useful RAS. For example, powders can be pressed onto a metal or mirror surface. Alternatively, low-E glass slides can be used for transmitted light microscopy. Light in the normal visible range will pass through the glass for all the conventional microscopy tests. However, the slides reflect in the infrared, creating an opportunity to combine transmitted light microscopy with reflection-absorption spectroscopy.

Practical sample preparation issues

While ATR technology requires minimal sample preparation, it is important to remember that it requires contact between the objective and sample. Fibers, films, and foams can simply be attached to a microscope using double-sided tape (*Figure 5*).

How small a sample area can be measured?

While most microscopists or technologists using this system work in a size range requiring small to mid-range magnifications, a few will always need to push the resolution boundary. The

numerical aperture (NA) of the ATR objective is approx. 0.7, suggesting that the smallest feature resolvable by this NA, using median infrared radiation wavelength of 10 µm, would be on the order of 10 µm. These dimensions are supported by actual testing.

While the optics generate a diffraction-limited spot for analysis, the real limiting factor is the signal-to-noise ratio. Sample morphology, thickness, and dispersion all have significant influence. Since scatter reduces the signal, dispersed fine particles or a thick specimen will produce a poorer signal.

In situations where one material or phase is in close proximity to another, a selection of IR-measuring apertures can be used to isolate the feature of interest. Choose the aperture that gives the largest area on the feature of interest. Even if there is a small amount of overlap to the adjacent area, its contribution will typically be weak, having minor impact on collecting a valid, easily identifiable spectrum.

Converting from imaging dimensions to spectral dimensions

The current industrial climate is fraught with downsizing and job shifting, often requiring one person to learn many skills. For those already using microscopes, the new integrated microscopy/infrared module makes the transition to infrared spectroscopy easy.

To begin with, it is important to establish a translation factor between nanometers, used by microscopists to describe light, to wave numbers (cm^{-1}), used by spectroscopists. Regular light microscopy operates in the near UV (220 nm to approx. 400 nm) through the visible range (approx. 400–700 nm). Occasionally (especially in the semiconductor arena), regular light microscopes are fitted with special gold-coated optics optimized for imaging into the near infrared (800–2200 nm). At this point, the conventional glasses used to make objectives stop transmitting.

Vibrational spectroscopists operate in the far infrared. As shown in all the spectra in this article, that region falls between 4000–650 cm^{-1}. The notation cm^{-1} or reciprocal centimeters simply describes the number of waves (hence, wave number) that travel in that distance. Inverting and dividing by 10,000,000 (the factor for changing centimeters to nanometers), reveals that vibrational spectroscopists operate in the region just adjacent to the microscopist's domain of the near IR: from 2500 to 16,250 nm.

Special tools ease spectral interpretation, speed contamination, and material analyses

Interpreting spectra can be daunting for microscopists unfamiliar with chemical functional groups and their infrared fingerprints. For routine analyses IlluminatIR uses QualID software (**SensIR Technologies**) to automatically search a spectral library for a match, providing an easily traveled bridge between microscopy and spectroscopy.

Just as users can build their own particle atlas using digital images and readout from other microscopical techniques, they can also build their own unique reference collection simply by taking spectra of their own materials. A similar approach to building a customized reference library speeds contamination analysis. Most contamination comes from dust or wear debris found around the laboratory or factory or from a problem with a vendor's product. In many cases, microscopy can significantly reduce the possible suspects to a handful. The analyst can collect those materials, conduct similar microscopical analyses, then take a quick spectrum to provide definite confirmation of the contaminating material and its source. No reference library is necessary, just a spectral match.

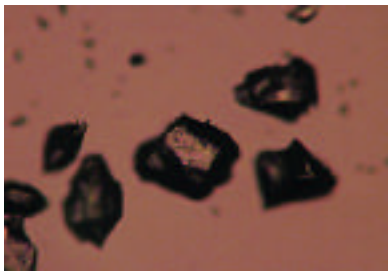

Figure 6 *A contaminant isolated from an injectable drug.*

In rare instances, neither QualID nor spectral matching will solve the problem. In these cases, scientists and technicians familiar with advanced spectral analysis software rely on programs such as Gram's (**Thermo Galactic,** Salem, MA). The IlluminatIR's spectra can be directly exported into those programs and analyzed. For those less experienced in reading spectra, **SensIR** offers Spectra-Fax, a for-fee analytical service. In either case,

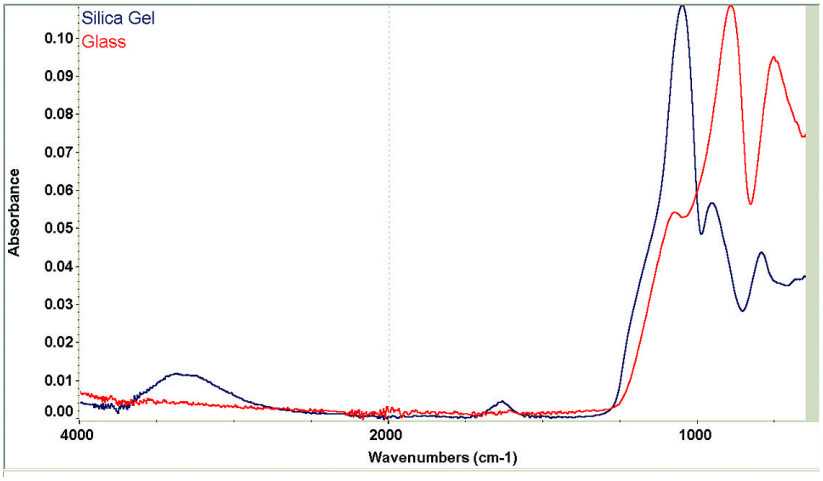

Figure 7 *Spectra of glass (red) and silica gel (blue).*

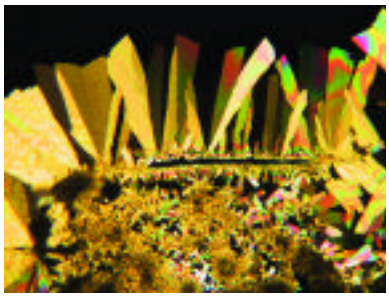

Figure 8 *Ranitidine hydrochloride, a widely available over-the-counter drug. But is it the patent-protected polymorph or its available twin?*

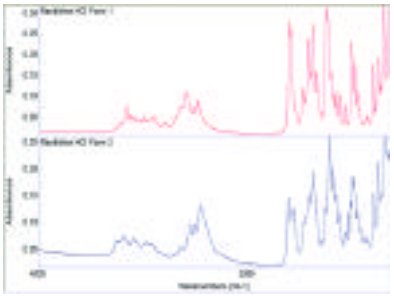

Figure 9 *Spectra of the two polymorphs.*

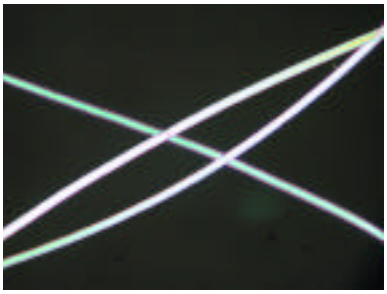

Figure 10 *Photomicrograph of two unknown synthetic fibers.*

once the compound has been identified, its spectrum can be added to the user's reference library.

Applications

In the hands of an experienced chemical microscopist, the microscope is in its glory analyzing crystals and powders. Crystal structure, optical properties, phase information, size distribution, presence of impurities or contaminants, and chemical and physical data can all be collected in a smooth, continuous flow of analytical steps. In a pharmaceutical company, the dispersion and ratio of filler to active ingredient may be the target. For a deodorant manufacturer, the presence or absence of the active crystal form might be critical. For the polymer chemist, it might be the right crystal structure for a catalyst.

Coupling spectroscopy with microscopy adds another dimension in the analysis of both organic or covalent inorganic materials. Because the IR spectrum of a material is a physical constant, materials will produce different spectra depending on their molecular structure as well as their environment: Are they hydrated or anhydrous? Are they fully or partially crystallized? Has the crystalline structure been modified in some way? The combination of imaging and chemical information presents a much more complete picture of the sample.

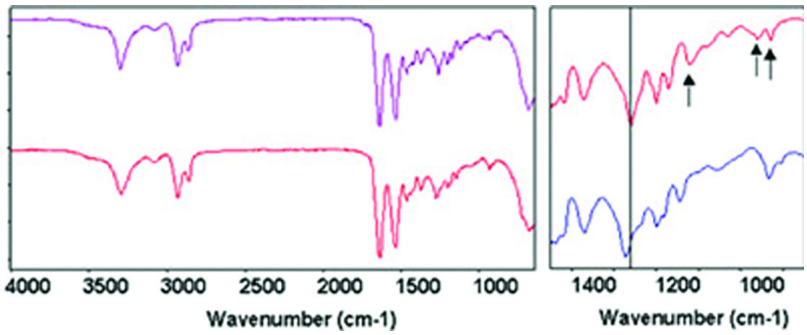

Figure 11 *IR spectra of the two fibers from Figure 10, identifying the fibers as nylon 6 (top spectrum) and nylon 6,6 (bottom spectrum). Inset on right shows the fine details from the 1450–850 cm^{-1} region.*

Silica gel or glass?

Figure 6 illustrates a typical case. A contaminant was retrieved by filtration from an injectable drug. Neither its morphology nor other optical properties could unequivocally determine if it was glass, perhaps from a chipped vial, or some similar material introduced, perhaps, during processing. Both its identity and, equally important, its source, remained a mystery.

Figure 7 shows the spectrum of the contaminant (blue). Comparison with spectra in the IR reference library revealed that it was silica gel (red spectrum), not glass. Closer examination of the process revealed its source—a leaky filter.

Form I or Form II?

In many cases, from drugs to catalysts to paint pigments, there is a close connection between crystal or powder structure and function. In the pharmaceutical industry, crystal structure often determines drug efficacy and bioavailability. Since crystal form may be a critical patent issue, this information can have significant economic impact.

Integrating infrared spectroscopy offers a complementary technique that provides definitive chemical fingerprint in a smooth flow from one technique to another.

The micrograph in *Figure 8* was central to just such a case. Ranitidine hydrochloride is a miracle drug that has relieved the pain of millions of people suffering from chronic gastritis and virtually eliminated the need for surgery for stomach ulcers. Today, the drug is available over the counter, but originally it was formulated as a single polymorph, closely protected by intellectual property laws. When a

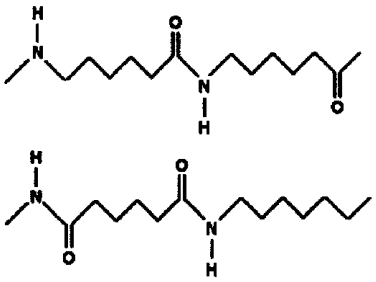

Figure 12 *Molecular structures of nylon 6 (top) and nylon 6,6 (bottom).*

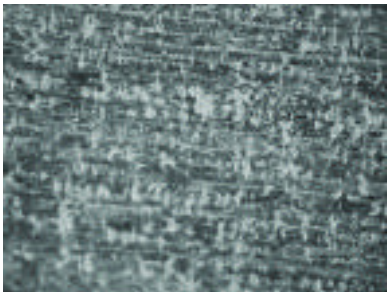

Figure 13 *A micrograph of chromium steel—coated or bare?*

competitor began making a similar product from a different polymorph, a lawsuit ensued, based on the new product's polymorphic form. Polarized light microscopy revealed similar optical properties but could not define which form was in the new product.

The combination of polarized light microscopy with IR spectroscopy provided the answer (*Figure 9*): The new product contained traces of Form II, a polymorph still under patent protection.

Which fiber is it?

Fibers are ubiquitous. To the forensic scientists, they provide key trace evidence from hair, clothing, carpet, or upholstery. In commerce, the difference between one fiber and another may mean the difference between a well-behaved product or a counterfeit.

In many cases, microscopic analysis of morphology provides quick fiber identification, especially for naturally occurring materials such as cotton, wool, and silk. Synthetic fibers may also have tell-tale morphology, such as trilobal structure. Using polarized light takes the analysis to a second level, uncovering unique, identifiable optical properties such as refractive indices and birefringence. (Birefringence is the mathematical difference between the longitudinal and cross-sectional refractive indices.) Microchemical analyses may add still further data, but may be complex and time consuming. Infrared microspectroscopy offers complementary information, is typically faster, and in some cases, may provide answers when microscopy alone cannot.

Figure 10 shows the polarized light images of two types of synthetic fiber, one of which is a ubiquitous textile material. Both are cylindrical and smooth: Their morphology is no help in distinguishing one from the other. Further analysis by polarized light indicates that they have the same refractive indices and birefringence.

At first glance, the IR spectra (*Figure 11*) seem equally fruitless, suggesting that this particular search requires more advanced understanding of spectral fine detail. A closer examination of the 1450–850 cm^{-1} range differentiates the two fibers (inset on right). Dropping a cursor near 1270 cm^{-1} shows a shift of several bands upfield by more than 10 cm^{-1}. Secondly, the top spectrum exhibits additional absorption bands not visible in the bottom spectrum, particularly the doublet at 950 cm^{-1}. Comparing these spectra with reference spectra clearly identifies the fiber related to the top spectrum as nylon 6 and the fiber related to the bottom spectrum as nylon 6,6. *Figure 12* shows just how similar these two molecules really are.

Is this material coated or uncoated?

As shown by *Figure 13*, it is often difficult to tell from just the microscopy information if a coating is present or missing. This situation is especially true when the coatings are thin, transparent, and colorless.

RAS supplies the missing information. *Figure 14a* shows the spectrum of the bare steel while 14*b* shows the radically different spectrum, consistent with that of a proprietary coating.

RAS reveals even more coating information

Another common coating question is whether a polymeric coating actually cured. If it has, it provides the proper protective layer. If not, the contents, such as food, drugs, and chemicals, can either be contaminated or can cause container corrosion. While the micro-

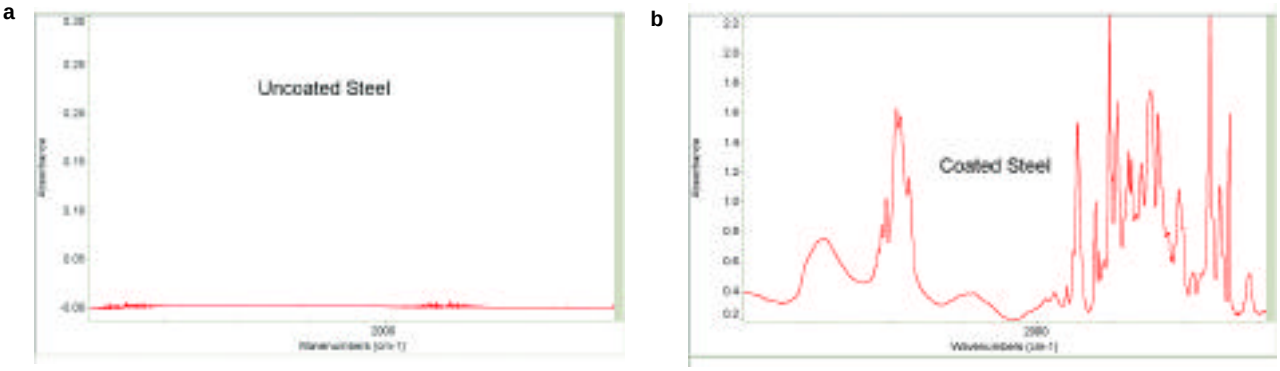

Figure 14 *a) RAS spectrum of bare steel; b) RAS spectrum of proprietary coating.*

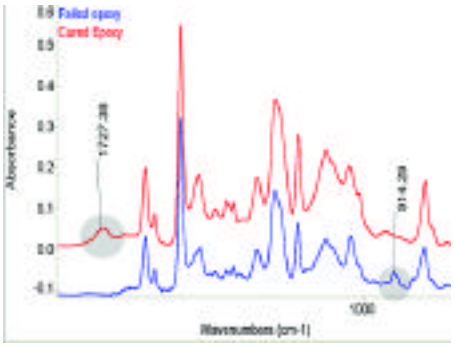

Figure 15 *RAS spectrum of polymeric coating on the inside of an aluminum can.*

scope resolves dimensions and morphology, spectra resolve the chemistry. The difference between cured coating and failed coating is clearly differentiated in *Figure 15*.

Conclusion

While light microscopy is powerful, it faces certain limitations in qualitative analysis capability. Integrating infrared spectroscopy offers a complementary technique that provides definitive chemical fingerprint in a smooth flow from one technique to another. That fingerprint not only unequivocally identifies the unknown material, it often reveals its source as well, quickly solving problems in manufacturing, contamination, and failure analysis, saving time, effort, and money.

Reference

1. Caspersson T. The investigations of nucleic acid distribution in the cell nucleus. Z wiss Mikr 1936; 53:403–19.

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