Tutorial

Drug Testing and Analysis

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Historical perspective and modern applications of Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR-FTIR)

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Vibrational spectroscopy has a long history as an important spectroscopic method in chemical and pharmaceutical analysis. Instrumentation for infrared (IR) spectroscopy was revolutionized by the introduction of Fourier Transform Infrared (FTIR) spectrometers. In addition, easier sampling combined with better sample-to-sample reproducibility and user-to-user spectral variation became available with attenuated total reflectance (ATR) probes and their application for *in situ* IR spectroscopy. These innovations allow many new applications in chemical and pharmaceutical analysis, such as the use of IR spectroscopy in Process Analytical Chemistry (PAC), the quantitation of drugs in complex matrix formulations, the analysis of protein binding and function and in combination with IR microscopy to the emergence of IR imaging technologies. The use of ATR-FTIR instruments in forensics and first response to 'white powder' incidents is also discussed. A short overview is given in this perspective article with the aim to renew and intensify interest in IR spectroscopy. Copyright © 2011 John Wiley & Sons, Ltd.

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Infrared spectroscopy

Spectroscopic techniques are indispensable tools in analytical chemistry and a wide variety of methods probing different properties of the analyte exists. Vibrations of the atoms of a molecule, containing a wealth of information about molecular structure and the surrounding environment, are investigated by the complementary techniques of infrared (IR) and Raman spectroscopy. These techniques belong to the classical spectroscopic methods and have been used for many years in drug testing and drug development. However, modern developments in instrumentation and data acquisition have significantly increased the ease and accuracy of data acquisition and expanded the type of samples that can be routinely measured. This perspective will focus on modern applications of attenuated total reflectance – Fourier transform infrared spectroscopy (ATR–FTIR) – that combines the technique of Fourier transforms for data acquisition with the principles of attenuated total reflection that is able to overcome many of the problems and limitations of classical transmission methods. It aims to show that modern IR spectroscopy is a valuable spectroscopic technique that is an excellent complement to other methods such as nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry. As part of the special journal issue *Analysis of Drugs for the Therapy of Anticholinesterase Poisoning*, the list of application examples will conclude with the use of ATR-FTIR spectroscopy in the development of scavengers against poisoning by organophosphorus compounds.

As outlined, IR spectroscopy is vibrational spectroscopy. As the vast majority of molecules exhibit IR absorption in the mid-IR region between 4000 and 400 cm^{-1} (2.5–25 μm wavelength) this is the most looked at spectral region. Different kinds of molecular vibrations exist – among them stretching vibrations (symmetrical and a anti-symmetrical) which go along with a change in bond length between atoms and bending vibrations – where bond angles change. Bending vibrations come in the form of scissoring, rocking (both in plane), wagging and twisting (both out of plane) when looking at a three-atom group like CH_2 (Figure 1). These vibrations can be found in the mid-IR spectrum. In general, a molecule composed of N atoms display 3 N-6 vibrational degrees of freedom (3 N-5 for linear molecules). This corresponds to the number of independent so-called normal modes of vibration. In order for them to be IR active, the electric dipole of the molecule must change. Therefore the stretching vibration in the N_2 molecule is not IR active as the molecule is symmetrical and the dipole moment is not affected by the bond stretching, while in the isoelectronic CO molecule the stretching vibration is IR active as the dipole moment changes. In general polar bonds lead to strong IR bands while the position of the band depends on bond strength and the masses of the involved atoms. Therefore substitution with different isotopes of an element in a molecular moiety leads to a shift of the respective IR band. This effect can be exploited specifically by employing stable isotope labelling of analyte molecules, a method that is also widely employed in NMR spectroscopy and mass spectrometry.[1] Therefore labelling schemes developed for these methods can also be exploited for IR spectroscopy. In addition, the molecular environment and effects like hydrogen bonding also govern the position of the IR band.

Classical dispersive IR spectrometers, that emerged as early as the 1940s, worked by the principle of transmission spectroscopy.

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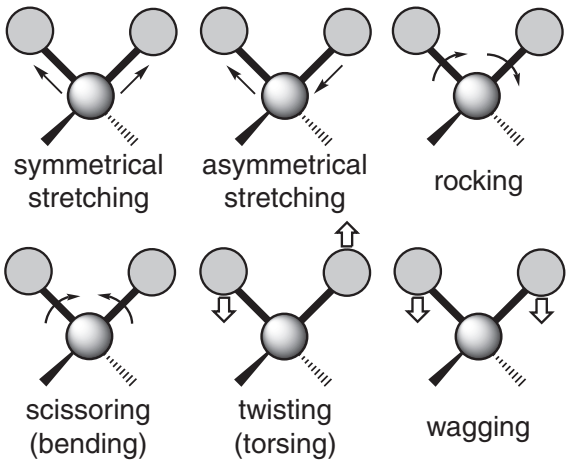

Figure 1. Possible vibrations in a CH_2 moiety. Direction of the arrows is reversed in the back motion of the vibration. Black arrows indicate motions in the paper plane and white arrows indicate movement out of the paper plane.

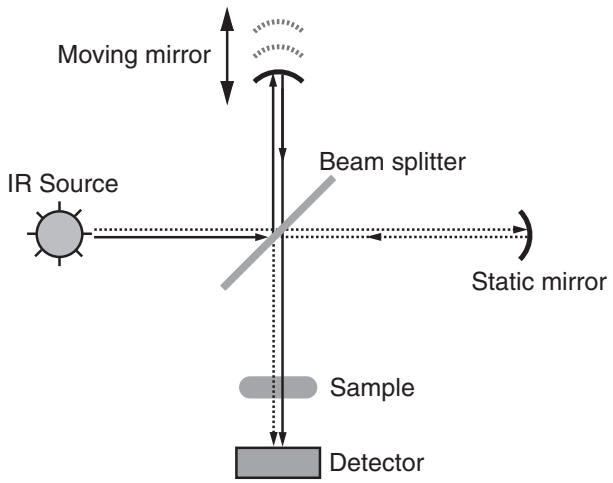

Figure 2. Interferometer used in FTIR spectrometers. IR radiation is passed from the source onto a beam splitter and separated into two beams. One is reflected by a stationary mirror while the other is reflected by a mirror moving with constant velocity. After recombination of the beams they a brought into contact with the sample (by transmission in the depicted case) and then recorded by the detector.

The sample was exposed to IR radiation and it was determined what fraction of the radiation at a certain frequency was absorbed by the sample. Dispersive instruments are also called grating or scanning spectrometers. A diffraction grating is used in these instruments, which is similar to a prism. It separates the wavelengths of light in the spectral range and directs each wavelength individually through a slit to the detector. Each wavelength is measured one at a time and slit and grating are moved to select the wavelength being measured. This is a slow process, and typically only one scan of the sample is made. These kind of instruments are nowadays almost completely replaced by FTIR spectrometers that overcome many of the limitations of the dispersive instruments.

FTIR

An FTIR instrument in its basic form works by the principle of transmission but the way the sample spectrum is recorded is fundamentally different from dispersive instruments in how the IR beam gets from the source to the detector. The core part of an FTIR spectrometer is formed by an interferometer that consists of an IR source, a beam splitter, two mirrors and a detector. These parts are arranged as shown in Figure 2. IR radiation from the source hits the beam splitter and is partly directed towards the two mirrors. One of them is stationary while the other mirror is moved at a constant velocity during data acquisition. The IR beams are reflected by the mirrors, recombined at the beam splitter, and then passed through the sample and reach the detector that records all wavelengths in the IR at the same time. When the two beams reflected by the mirrors recombine, they have travelled different distances and therefore the recombination leads to constructive and destructive interference. The resulting pattern is referred to as an interferogram. What is recorded at the detector after the recombined beam has passed through the sample is the Fourier transform of the IR spectrum of the sample. Data recorded by the instrument is then processed by a computer that performs an additional Fourier transform to back-transform the interferogram into an IR spectrum.[2]

Major advantages of FTIR over dispersive spectrometers include much faster acquisition times that lead to higher throughput. Adding to throughput is also the fact that more energy is reaching the sample and the detector as no diffraction grating is used. Therefore signal-to-noise ratios are much better for FTIR instruments. Each point in the interferogram carries information from all the wavelengths of the IR radiation used for measurement and every complete move of the mirror equals to one scan of the entire IR spectrum. Multiple scans can be combined to enhance the final IR spectrum by averaging. Finally a laser is used in FTIR instruments to control the velocity of the moving mirror. This laser is also used as a reference for wavelength calibration that makes the use of external calibration standards unnecessary. An authorative treatment of the practical and theoretical background of FTIR spectroscopy can be found in the work of Griffiths and De Haseth.[3]

Even though major advantages exist for FTIR spectrometers, they are still based on the principle of transmission spectroscopy in their basic form. This means that sample preparation for solid and liquid samples remains the same as for dispersive instruments. In the case of solid samples, this means to grind the sample to a fine powder and to disperse it in a suitable matrix. This can be either a liquid-like mineral oil (Nujol – a liquid paraffin oil), which is then placed as a thin film between two IR transparent windows made of materials like NaCl, KBr, CsI, ZnSe, AgCl, BaF_2, or CaF_2. Alternatively, the ground material is mixed with KBr and converted under high pressure into a thin KBr disk with a glass like appearance. The problems associated with this kind of sample preparations include the time required for preparing individual samples, the problem of sample-to-sample reproducibility and user-to-user spectral variation. There are obviously also problems with solids that are difficult to grind and prepare in dispersed form. There are fewer problems with liquid samples. They are usually measured in special cells with very short pathlengths. The windows of the cells are again made from IR transparent materials. When measuring aqueous solutions, the cell material must of course be insoluble or only marginally soluble in water and must be compatible with the pH of the solution. In time-resolved spectroscopy, mixing issues can arise due to the thin-film nature of the liquid in the cell. In case of protein solutions the high viscosity due to the required

high concentrations of the biomolecule can add to this. To overcome these issues, flow-cells are available. If aqueous solutions of proteins are measured, issues arise from the strong absorbance of water in the mid-IR region (around 1645 cm^{-1}). This interferes with an important amide band and some protein side chain bands. Pathlengths of 5 μm are required or H_2O must be exchanged by D_2O, which shifts the water band to around 1210 cm^{-1} allowing pathlengths of up to 50 μm.

ATR

An alternative to transmission spectroscopy is the use of attenuated total reflectance (ATR). For ATR the IR beam is passed through a crystal of a material with a high refractive index (high optical density) at an angle greater than the so-called critical angle that leads to total internal reflection of the beam at the crystal surface that is in contact with the sample. Most often this material is either diamond, ZnSe or Ge. This reflectance causes an evanescent wave that extends beyond the crystal's boundaries and penetrates the sample with a depth typically in the range of 0.5–5 μm. IR radiation that is absorbed by the sample will cause the reflected evanescent wave to be attenuated. After one or more reflections in the crystal the IR beam leaves the crystal and is recorded at the detector as depicted in Figure 3. Combination of ATR with FTIR leads to instruments using attenuated total reflectance – ATR-FTIR – allowing better sample-to-sample reproducibility and less user-to-user spectral variation. The first application of ATR used with IR spectroscopy was presented by Harrick in 1960 and Fahrenfort in 1961.[4,5]

In earlier instruments, the crystal face was mounted vertically in the instrument requiring to clamp or push the sample on the crystal. More convenient horizontal ATR (HATR) units are available in newer instruments. The choice of the crystal material depends on the application and costs. While diamond is the most robust and durable material it is also by far the most expensive and the area of the crystal face is limited, also limiting the maximum number of reflections in the crystal. Another popular material is ZnSe, which is much more affordable and the crystal face dimension can easily be in the cm range. But ZnSe is also easily scratched and can only be used in the pH range from 5 to 9.

In situ ATR-FTIR

In normal ATR-FTIR instruments, the sample must be applied to a crystal surface in or on the instrument. To put it in another way: The sample must come to the instrument. In *in situ* ATR-FTIR spectroscopy, one could say that the instrument (the ATR crystal) comes to the sample. To achieve this the IR beam from the IR source in the instrument is guided to the ATR crystal through a flexible or adjustable conduit that allows placing the ATR probe inside of a chemical reactor or any other container with the sample of interest. Two alternative technologies exist to achieve this. The first uses an adjustable conduit that employs mirrors. This kind of conduit is shown connected to an instrument in Figure 4. The alternative is the use of a fiber optical system employing silver halide as the IR conductive material. While the fiber system is more flexible than the conduit the latter can normally achieve better sensitivity although much progress has been made to improve the fiber systems in this regard.

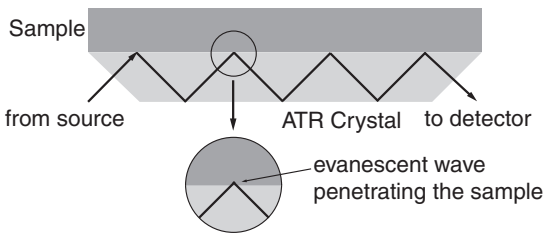

Figure 3. Schematic representation of an ATR crystal with four reflections. The IR beam enters the crystal and is reflected at the crystal boundaries in contact with the sample. An evanescent wave penetrates the sample for a few μm and is attenuated by the IR radiation that is absorbed by the sample. After one or more reflections (in this case four) in the crystal the IR beam is guided back to the detector of the instrument.

Applications

Process Analytical Chemistry (PAC) / Process Analytical Technology (PAT)

The first *in situ* ATR-FTIR spectrometers were developed for real-time process monitoring in the chemical and pharmaceutical industry on the production, scale-up or laboratory scale.[6] While classical sampling methods required to draw samples from a reactor vessel, go through the process of sample preparation and finally run the desired analytical method (which in cases like chromatography can be very time consuming), modern approaches focus on the ability to obtain real-time data on the composition of the reaction mixture including starting materials, products, side products and impurities. Combined with other process parameters such as temperature, pressure, pH, stirring rate, heating or cooling the chemical process can be designed and controlled much more precisely. This is of great importance especially for processes involving valuable reaction components such as those frequently encountered in the pharmaceutical industry. An interesting application was reported by McConnell *et al*. on a reaction step in the formation of a ribonucleotide diphosphate reductase inhibitor that nicely demonstrates the potential of this application.[7] A related application is the monitoring of crystallization processes.[8,9] Unfortunately most applications in industry involve proprietary processes and therefore very few articles are available in the open scientific literature.

ATR-FTIR allows placing the ATR crystal directly into contact with the contents of a chemical reactor. Moving one instrument or using multiple instruments can be applied if the concentrations of reactants differ at different places in the reactor such a flow reactors. Given the great information content of IR spectra about the chemical composition of a reaction mixture, ATR-FTIR is a highly valuable method in PAC/PAT and is used for a great variety of applications.

Quantification of drugs

ATR-FTIR has been used to quantitate drugs in formulations and in polymorph analysis.[10–12] One of the big advantages of ATR-FTIR is that it belongs to the group of non-destructive testing (NDT) methods that do not destroy the testing target during the course of the measurement compared to methods such as high performance liquid chromatography (HPLC) or gas chromatography (GC). However, the main problem associated with the use of spectroscopic data like that from ATR-FTIR is spectral overlap observed with more complex chemical mixtures. In simple mixtures where relevant spectral bands can be isolated and do not suffer from overlap, it is simple to quantitate a component by simply integrating the area for a specific band. The problem of complex mixtures has been successfully

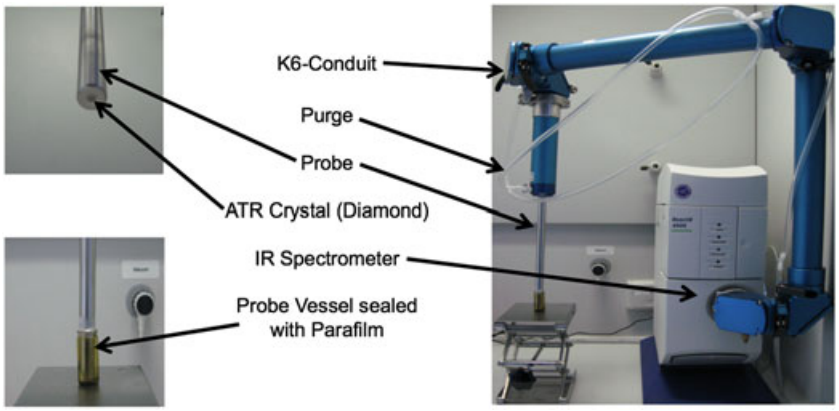

Figure 4. Set-up of an *in situ* ATR-FTIR spectrometer for monitoring the degradation of highly toxic organophosphorus compounds by enzymes and reactive nucleophiles in a small reaction volume and with a sealed reaction vessel (temperature bath omitted for clarity). The conduit is purged with dry nitrogen to remove traces of moisture that would interfere with data acquisition.

addressed by chemometric methods such as partial least squares (PLS) regression and principle component analysis (PCA) used for multivariate calibration.[13] Such an approach has been used to successfully determine drug concentration in starch acetate matrix tablets and it was shown that ATR-FTIR is especially suitable for rough tablet surfaces and it was found that due to the fact that the whole IR spectrum is used for quantification the results are more robust than in the case of univariate analysis.[14]

ATR-FTIR imaging

The combination of ATR-FTIR with IR spectroscopy leads to ATR-FTIR imaging yielding spatial 2-D information of a sample. Main applications in the pharmaceutical field include drug delivery and release. In ATR-FTIR imaging the main advantage is the ability to analyze the spatial distribution of the component materials in blends, granules, other dosage forms or even biomedical samples. It allows to obtain the molecular information from an area of a sample and to visualize the distribution of molecules or functional groups. This is achieved by using spectral information gathered through a microscope lens as the source of contrast in the image. This technique is highly complementary to other imaging method such as visible light or electron microscopy and it avoids the use of added dyes or other labelling methods in the sample.

Dissolution and drug release from pharmaceutical tablets is a new important field of IR spectroscopy. FTIR imaging yields quantitative time-resolved information about the spatial distribution of active ingredients and excipients in tablet formulations when dissolved in water. This allows optimization of controlled drug release. Another interesting application for ATR-FTIR imaging is human skin. The top layer, the stratum corneum (SC), is the major resistance to skin permeation. Understanding this permeation process is of great importance for the development of transdermal drug delivery and ATR-FTIR imaging can be used to gain a deeper understanding of the processes involved in the permeation of a substance through the skin barrier. It should be noted that the SC can also be studied *in vivo* using ATR-FTIR imaging revealing data on hydration, lipid composition or protein composition.

For a more detailed treatment of this interesting technique and the used instrumentation the reader is referred to the review of Kazarian and Chan, the review of Wartewig and Neubert and the recent book publication by Salzer and Siesler.[15–17]

ATR-FTIR with proteins as drug targets

IR spectroscopy with proteins is a demanding task due to the large number of IR active vibrations in the molecule. On the other hand the high information content (chemical structure of vibrating groups, properties of neighboring groups, redox states, bond angles, hydrogen bonding, etc.) of obtainable spectra is an important incentive to overcome potential problems. Simple IR spectra of proteins are dominated by the vibrations associated with the peptide bonds. The amide I band in the range of 1680–1620 cm^{-1} is due to the C = O modes and the amide II band in the range of 1560–1520 cm^{-1} is a joint contribution of CN and NH modes. Also the IR active vibrations of water contribute to the spectra.

Even though such an IR spectrum does not allow the assignment of individual bands to individual sites in the protein molecule, a wealth of important data can still be deduced. The analysis of the secondary structure of a protein is probably the most prominent example and it can be combined with the analysis of folding/unfolding events. Using H_2O/D_2O exchange the flexibility of proteins can be probed. If more detailed information is required about protein function or binding with an effector or drug molecule, the absorbance spectrum does most often not yield enough details. In these cases difference spectra are required that show the changes of a protein in one state compared to another. The problem with difference spectra is that the changes between states are usually small compared to the maximum absorbance and therefore difficult to monitor. Specific isotope labelling can be of high value for this kind of investigations. For detailed treatment the reader is referred to Barth's excellent review.[18] Valuable information can also be found in the contribution of Berthomieu and Hienerwadel.[19]

Forensics and 'white powder' applications

The non-destructive and non-invasive nature of obtaining IR spectra from solids using ATR-FTIR spectrometers have lead to increased interest in the technique in the field of forensics but also for the identification of potential biothreats and toxic chemicals (white powder incidents). When hand-held ATR-FTIR instruments are used, these kind of applications can be carried out directly on the scene. The recorded IR spectra serve as a fingerprint that allows indentification of the identity of a sample or (and sometimes equaly important) can rule out a class of compounds. In so-called white powder incidents, a large number of false-positive responses is triggered and most often the

identification of the powder as a common and harmless products (e.g. flour, icing sugar, or cement) allows to reduce or avoid wide area evacuations and reduces time for first responders during which heavy protective gear is required. In the field of forensics by ATR-FTIR the reader is referred to a recent publication by Elkins and work by Koçak *et al*. that allows a good overview of current state of the art applications.[20,21]

Field-based ATR-FTIR instruments are also used for the detection and more importantly identification of chemical warfare agents including nerve and blister agents.[22] The method allows identification based upon the IR fingerprint of the agents and works very well with pure substances but can suffer from interference and band overlap with other organic compounds that might be present at the same time (like paints, oils, gasoline, lubricants)

Development of scavengers against organophosphorus poisoning

Toxic organophosphorus (OP) compounds that inhibit the enzyme acetylcholinesterase (AChE) pose a credible threat to public health in the form of OP pesticides or as chemical warfare agents (nerve agents like tabun, sarin, soman or VX). Treatment of OP poisoning currently employs the use of atropine as an antagonist of surplus acetylcholine at muscarinic receptors, oximes to reactivate inhibited AChE and benzodiazepines as anticonvulsants.[23] Recent research efforts have focused on new stoichiometric or catalytic scavengers that can turn OP compounds into non-toxic reaction products *in vivo* or that can be used as reactive skin decontaminants.[24,25] We have used *in situ* ATR-FTIR to monitor the degradation of OP nerve agents by the enzyme diisopropyl fluorophosphatase (DFPase) and a number of reactive nucleophiles including oximes (unpublished results).[26–28]

The main advantages of *in-situ* ATR-FTIR compared to older methods like the use of fluoride sensitive electrodes or pH-stat titration are the possibility to carry out tests in small reaction volumes in easily sealed reaction vessels and subsequent easy disposal of the reaction solution and decontamination of the ATR probe. Figure 4 depicts the used set-up with an ATR-probe connected to the spectrometer via an adjustable conduit. The reaction solution is prepared and mixed in a vial, the ATR probe inserted into to the solution and the vessel sealed. After data acquisition the vial is dumped into a decontamination solution and the ATR-probe is cleaned. To monitor OP-degradation the P-O-R stretching vibration was used that is found in all G- and V-type nerve agents. In the OP nerve agent sarin for example this vibration is located at 1060 cm^{-1} in dilute aqueous solution as an isolated peak and shifted to 1030 cm^{-1} in the hydrolysis product *O*-isopropyl methylphosphonate. A calibration curve for quantification was obtained by total hydrolysis of a number of samples containing different concentrations of the OP compound of interest using univariate callibration. The method is easy to apply and complements other modern methods to monitor the degradation of OP compounds such as inverse NMR techniques.[29]

Summary

In summary, ATR-FTIR is a powerful spectroscopic method with a large variety of potential applications ranging from process analytical chemistry over small molecule analysis to the investigation of proteins. The information content of IR spectroscopy is complementary to other spectroscopic techniques and IR imaging techniques are complementary to other microscopic techniques. With the availability of powerful chemometric methods the analysis of complex mixtures became feasible and the availability of new strategies and methods for stable isotope labelling can result in an additional boost for FTIR applications with complex mixtures or complex molecules like proteins. From the early days of dispersive instruments with samples exclusively measured in transmission mode IR spectroscopy has gone a long way of development that certainly deserves renewed or intensified attention by the analytical and pharmaceutical chemist.

Acknowledgments

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