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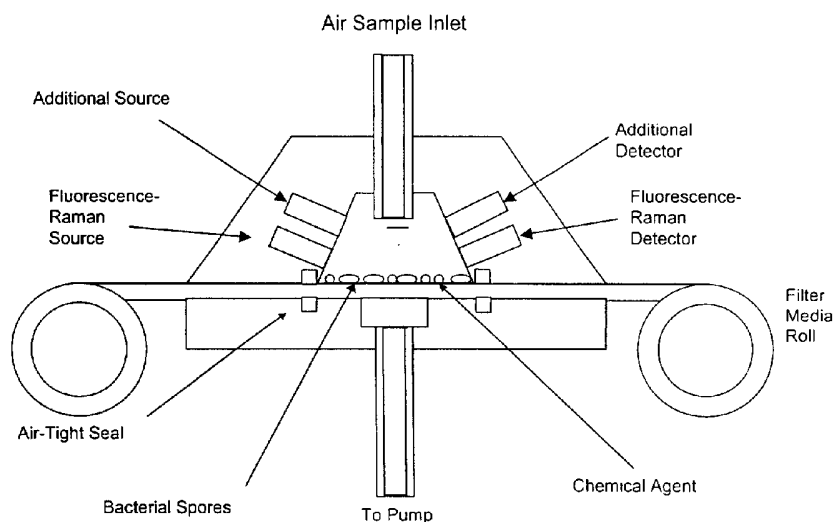VN, YU, ZA, ZM, ZW.

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(54) Title: METHOD FOR DETECTION OF PATHOGENIC MICROORGANISMS

The Integrated Detector Sample Cell

(57) Abstract: Pathogenic microorganisms are detected and classified by spectral imaging of the Raman light scattered by the organisms.

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METHOD FOR DETECTION OF PATHOGENIC MICROORGANISMS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority pursuant to 35 U.S.C. 119(e) to U.S. Provisional
Application Number 60/347,806 filed January 10, 2002, which is incorporated herein by
reference in its entirety including incorporated material.

Field of the Invention

The present invention relates to the field of chemical and biological analysis and more
specifically to the use of Raman and fluorescence imaging spectroscopy to quickly identify
chemical and biological agents.

Background of the Invention

Terrorist deployment of chemical and/or infectious biological agents as weapons of mass
destruction threatens the welfare of the human populace. Public concern has grown, especially
in our nation, as terrorist uses of biothreat agents, such as Anthrax, become reality. . Nightmare
images of tens of thousands of infected and dying innocent victims strike fear in the hearts of
nearly everyone. Biological and chemical warfare is significant, not only in lives lost, but also
in the cost to the US economy. The Centers for Disease Control estimates that the loss of
100,000 lives will have a $29 B economic impact. The mass destruction potential of Biological
Warfare Agents ("BWAs") and Chemical Warfare Agents (CWAs) is thought by many to be
comparable to or even greater than that of nuclear weapons. Nuclear weapons have the potential
to affect a finite area, albeit very large, and the use of such weapons is immediately obvious
after the fact. BWAs and CWAs, on the other hand, have virtually no boundaries and have the
potential to spread silently and unchecked through populations far from ground zero. Likewise,
technology to rapidly detect and quantify very low levels of radioactive contamination is widely
available. Unfortunately, such technology for BWAs and CWAs at similar levels is not
definitive, not widely available and in many cases, is not very rapid.

The psychological impact of this type of threat is also very significant. The public is
becoming increasingly aware of new, emerging pathogens. Fears over the unseen nature of
BWAs and CWAs make for a very effective terrorism weapon in and of itself. In addition to
perception, there is a very real threat due to incredible advances in biotechnology. It is now

possible to alter the most virulent bacterium or virus and to increase both its pathogenicity and resistance to conventional therapy. The molecular biology revolution has now been underway for more than three decades, and the sheer number of persons with technical expertise to potentially create such weapons of mass destruction has consequently increased. In this age of

5   advanced global travel, the likelihood of rapid dissemination of any type of BWA worldwide in a very short period of time is high, and the general public is well aware of this fact.

Conventional means of identifying pathogens using biology tools such as specific antibodies, genetic markers or propagation in culture are fundamentally slow and require hands-on manipulations. Furthermore, as new BWAs and CWAs are engineered, these conventional

10   tools are likely to become less and less effective. As the use of BWAs and CWAs by terrorists becomes a reality, there is an increasing need to develop tools that can rapidly and accurately detect and classify these agents at a molecular level without coming into contact with them. These tools are needed to help expand our understanding of the biological and chemical basis of such warfare agents and the potential impact on the human body. Furthermore, the knowledge

15   gained through such molecular analyses helps identify new targets for therapeutic and preventative agents.

SUMMARY OF THE INVENTION

A spectroscopic imaging system, also described as a chemical imaging system, employing Raman, fluorescence, , UV-visible reflectance/absorption and/or near-infrared (NIR)

20   reflectance/absorption spectroscopic techniques for characterization of BWAs and CWAs is disclosed.

In one embodiment, Raman microscopic imaging spectroscopy and/or fluorescence microscopic imaging spectroscopy can be used to detect, classify, identify and/or visualize BWAs, CWAs and non-threatening compounds. Microscopic imaging spectroscopy detects,

25   classifies and identifies sub-micron size particles, including single bacterium. In addition, Raman microscopic imaging spectroscopy can perform sub-micron size particle detection, classification, identification and visualization of BWAs and CWAs in the presence of non-threatening 'masking' compounds when appropriate data analysis techniques are applied.

In another embodiment, fluorescence and Raman macroscopic imaging spectroscopy can

30   be used to detect, classify, identify and/or visualize BWAs, CWAs and non-threatening compounds. These macroscopic imaging techniques can perform sub-millimeter size particle detection, classification, identification and visualization of BWAs and CWAs (i.e., agglomerated

bacteria and endospore detection and identification). In addition, fluorescence and Raman macroscopic imaging spectroscopy can perform detection, classification, identification and visualization of BWAs and CWAs in the presence of non-threatening 'masking' compounds when appropriate data analysis techniques are applied.

5   In an another embodiment, Raman fiber optic dispersive spectroscopy can detect, classify and/or identify BWAs, CWAs and non-threatening compounds. Moreover, Raman fiber optic imaging spectroscopy can detect, classify, identify and/or visualize BWAs, CWAs and non-threatening compounds when appropriate data analysis techniques are applied.

In order to provide faster real-time analysis, Fiber-Array Spectral Translator (FAST)

10   dispersive spectroscopy is used for rapid detection, classification and identification of BWAs, CWAs and non-threatening compounds. In addition, Fiber-Array Spectral Translator (FAST) imaging spectroscopy can be used for rapid detection, classification, identification and visualization of BWAs, CWAs and non-threatening compounds when appropriate data analysis techniques are applied.

15   The systems described above are applied in a variety of modes. The system is applied as a laboratory or transportable field Raman microscope such as ChemImage's FALCON Raman microscope outfitted with ChemImage's Simultaneous Imaging and Spectroscopy Apparatus. The system is also applied as a UV/Vis/NIR fluorescence, Raman, or UV/Vis/NIR/Mid-IR absorption/reflectance macroscope system such as ChemImage's CONDOR Macroscope.

20   Alternatively, the system is applied as a laboratory or field fiberscope such as ChemImage's RAVEN endoscope. In addition, the system is applied as a laboratory or field Fiber-Array Spectral Translator (FAST) probe.. Each of the modes of application are used separately or in combination with one another to achieve the desired speed and results.

Spectroscopic imaging techniques are applied to sensors designed to detect, classify,

25   identify and/or visualize BWAs, CWAs and non-threatening compounds in ambient air. A schematic of such a sensor is shown in Figure 1. The vacuum created by an air-sampling pump pulls the ambient air through the sample inlet and through the filter. Filter materials could include porous polypropylene or cellulose, in disk or roll form. Particulates in the air sample are trapped on the surface of the filter medium and are held in the field of view of the spectroscopic

30   imaging system. The source, chosen specifically for the type of molecular spectroscopy being used, illuminates the trapped particles and induces either Raman or fluorescence emission from the sample. The imaging detector measures the spatial distribution of emitted light at a series of wavelengths and creates the data file used for further analysis. The inlet to this imaging detector

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can either be an imaging optical fiber or conventional optics. Advanced chemometric techniques along with image analysis routines are used to detect, classify, identify and/or visualize BWAs, CWAs and non-threatening compounds.

The system can be automated through the use of robotics or combined macro/micro instrumentation in order to target BWAs, CWAs and non-threatening agents. Using laser ablation and/or chemical ablation, the system can be automated to eradicate BWAs and CWAs post-targeting

A variety of data processing procedures can be used with the system. A weighted spectral image data subtraction routine can be used to suppress contribution from microscope slide. Alternatively, multivariate image analysis involving principal factor analysis and subsequent factor rotation can be used for differentiation of pure molecular features in BWAs, CWAs and non-threatening 'masking' compounds.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of an ambient air BWA and CWA sensor based on imaging spectroscopic detection.

Figure 2 is a Raman spectroscopic imaging of a mixture of 1μm diameter polystyrene micro-spheres and' Bacillus subtilis var. niger spores (also known as Bacillus globigii (BG) which is an Anthrax simulant).

Figure 3 shows dispersive Raman spectra of three different bacterial spore types including an Anthrax simulant.

Figure 4 is a microscopic fluorescence-spectroscopic image of two different bacterial spore types.

Figure 5 shows the preliminary results of rapid spectroscopic examination of samples sent by the US Armed Forces Institute of Pathology (AFIP), experts in the objective assessment of biothreat detection technologies. These samples include 6 unknown powders and a sample of BG spores.

Figure 5A shows Raman spectra (green laser excitation) of the 6 unidentified powders through the vials.

Figure 5B shows Raman spectra (red laser excitation) of the 6 unidentified powders.

Figures 5C-5D (Sample 1331-002) show Raman, IR and SEM-EDS results on a first of the 6 unidentified powders. The sample is inorganic and most likely talc.

Figures 5E-5F (Sample 1325-002) show Raman, IR and SEM-EDS results on a second of the 6 unidentified samples. The sample is organic and most likely starch, possibly corn starch.

Figures 5G-5H (Sample 1303-002) show Raman, IR and SEM-EDS results on a third of the 6 unidentified powders. The sample is organic and most likely starch, possibly corn starch.

Figures 5I-5N (Sample 1291-006) show Raman, IR and SEM-EDS results on the remaining unidentified powders. There are 3 distinct types of powders in this sample. All 3 have organic content, while 2 of the 3 are fairly rich in aluminosilicates. One of the powders is likely a complex aromatic hydrocarbon.

Figures 5O-5Q show Raman spectra and images of 2 common white powders that can easily be differentiated with Raman Chemical Imaging.

Figure 5R shows Raman spectra of sample BG spores compared with commercially available BG spores. A Raman spectrum of a mixture of the 2 samples is shown, as well. Raman indicates the samples are similar, almost identical.

Figure 5S shows a Chemical Image where the 2 similar spores are differentiated on the basis of autofluorescence differences.

Figure 5T shows a Raman Chemical Imaging where the 2 powders from Figure 5O are mixed with BG spores. The 3 species can readily be discriminated.

Figure 5U shows efforts to detect BG in the presence of a white powder (Baking soda) on an envelope.

Figure 5V shows BG on an envelope imaged while the envelope is moving.

Figure 6 shows the results from additional spore samples selected specifically because the inherent difficulty in differentiating these species. They include Bacillus thuriengensis (BT), Bacillus cereus (BC) and BG. The Raman spectra from the 3 spores are different. These differences suggest a good chance of differentiating anthrax from non-threats. The details follow:

Figure 6A shows raw Raman spectra of BT and the suspension residue. The residue is from the suspension liquid.

Figure 6B shows background corrected spectra of BT and residue. Both the spores spectrum and residue spectrum have been divided by a spectrum of the microscope slide.

Figure 6C shows raw Raman spectra of BC and the suspension residue.

Figure 6D shows background corrected spectra of BC and residue

Figure 6E shows a compilation of sample BT, BC and BG spectra with microscope slide background correction. The spectra are different. The differences are greatest in the fingerprint region.

Figure 6F shows a compilation of the 3 spores after baseline subtraction and normalization to the CH region spectral feature (~2950 cm^{-1}).

Figure 7 shows how RCI can be applied to distinguish between multiple bacterial strains within a single species.

Figure 8 shows how RCI can be applied to distinguish between the same species and strain of bacteria grown under differing conditions.

Figure 9 shows a schematic description of RCI.

Figure 10 shows Raman Chemical Imaging of actual individual *Bacillus anthracis* (Anthrax) spores as well as additional demonstrations of RCI's power to reproducibly distinguish between similar materials.

Figure 11 shows how RCI can be applied to distinguish between viable and non-viable endospores, a critical variable in determining real threat level.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Methods of Raman chemical imaging are extensively covered in the following US patents and Patent applications assigned to the assignee of the present invention: Patent No. 6,002,476; US Non-Provisional Application 09/619,371 filed 7/19/2000; US Non-Provisional Application 09/800,953 filed 3/7/2001; US Non-Provisional Application 09/976,391 filed 10/21/2001; US Non-Provisional Application 10/185,090 filed 6/27/2002; US Non-Provisional Application 10/184,580 filed 6/27/2002; US Provisional Application 60/144,518 filed July 19, 1999; U.S. Provisional Application Number 60/347,806 filed 01/10/2002; U.S. Provisional Application Number 60/144,518 filed 7/19/1999; U.S. Provisional Application Number 60/187,560 filed 3/28/2000; U.S. Provisional Application Number 60/239,969 filed 11/13/2000; U.S. Provisional Application Number 60/301,708 filed 6/28/2001; U.S. Provisional Application Number 60/422604 filed 11/21/02.

The above identified patents and patent applications are hereby incorporated by reference, including referenced material.

Spectroscopy is the study of the interaction of light and matter. Light can be absorbed, reflected, transmitted, emitted or scattered by a substance at characteristic wavelengths (i.e., colors) of the electromagnetic spectrum (incl. gamma ray, X ray, ultraviolet (UV), visible light,

infrared, microwave, and radio-frequency radiation) upon excitation by an external energy source. These characteristic wavelengths can then lead to the identification of the material's elemental and/or molecular composition. Experiments typically consist of a light source, a light-dispersing element (i.e., prism or grating) to create a spectrum and a detection device.

5   In Raman spectroscopy, the photons of interest are scattered by the material. If the incident light is monochromatic (single wavelength) as it is when using a laser source, a small fraction of the scattered radiation differs in frequency (wavelength) from that of the laser. Furthermore, frequencies of the scattered light are unique to the molecular species present. This phenomenon is known as the Raman effect.

10   In Raman spectroscopy, energy levels of molecules are probed by monitoring the frequency shifts present in scattered light. A typical experiment consists of a monochromatic source (usually a laser) that is directed at a sample. Several phenomena then occur including Raman scattering that is monitored using instrumentation such as a spectrometer and a charge-coupled device (CCD) detector.

15   Similar to an infrared spectrum, a Raman spectrum reveals the molecular composition of materials, including the specific functional groups present in organic and inorganic molecules. Raman is useful because each resonance exhibits a characteristic 'fingerprint' spectrum, subject to various selection rules. Peak shape, peak position and the adherence to selection rules can also be used to determine molecular conformation information (crystalline phase, degree of

20   order, strain, grain size, etc.). Unlike infrared spectroscopy, a single Raman spectrometer can be applied to the molecular characterization of organic and inorganic materials simultaneously. Other advantages of Raman over traditional infrared spectroscopy include the ability to analyze aqueous phase materials and the ability to analyze materials with little or no sample preparation. Deterrents to using Raman spectroscopy as opposed to infrared spectroscopy include the

25   relatively weak nature of the Raman phenomenon and interferences due to fluorescence. In the past several years, a number of key technologies have been introduced into wide use that have enabled scientists to largely overcome the problems inherent to Raman spectroscopy. These technologies include high efficiency solid-state lasers, efficient laser rejection filters, and silicon CCD detectors.

30   In fluorescence spectroscopy, photons are emitted from a material following an excitation step in which absorption of photons occurs. Experiments typically include a polychromatic excitation source such as mercury (Hg) or xenon (Xe) lamps or a monochromatic source such as a laser for sample excitation. A portion of the emitted radiation may then be

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directed into a dispersive monochromator to which a detector device such as a CCD is attached. By measuring the fluorescence spectrum from a material, one can deduce qualitative and quantitative information from inorganic and organic species. In comparison to Raman spectroscopy, fluorescence is inherently more sensitive. Detection limits in the parts-per-billion

5   are common. On the other hand, fluorescence is less selective than Raman and there are a limited number of chemical systems that exhibit fluorescence.

Molecular UV/visible and NIR absorption spectroscopies involve the absorption of photons throughout the UV/visible (185-780 nm (54,054 to 12,800 cm^{-1})) and NIR (780 nm - 2.5 μm (12,800 to 4,000 cm^{-1})) spectral regions, respectively. Typical instrumentation include a

10   polychromatic source such as a deuterium or quartz tungsten halogen lamp, a dispersive element such as a monochromator or interferometer and a detection device such as a Si CCD or InGaAs focal plane array detector. Absorption measurements based upon UV-visible or NIR radiation find a wide number of applications for both qualitative and quantitative determination of inorganic and organic species. NIR spectra result from the overtone and combination bands of

15   fundamental mid-infrared (MIR) bands. Like fluorescence, absorption spectroscopies are highly sensitive but only moderately selective.

Spectroscopic methods can be extended to imaging techniques through the use of imaging spectrometers such as liquid crystal imaging spectrometers. The development of this technology in recent years has enabled widefield spectroscopic imaging to develop and mature.

20   Spectroscopic imaging is a versatile technique that is well suited to the analysis of complex heterogeneous materials. Applications of spectroscopic imaging range from the analysis of polymer blends, defect status analysis in semiconductor materials, inclusions in human breast tissue, characterization of corrosion samples and detection, classification and identification of BWAs and CWAs. Spectroscopic imaging provides a potential solution for

25   obtaining both qualitative and quantitative image information about molecular composition and morphology of BWAs and CWAs allowing a more accurate and more rapid analysis than traditional imaging or 'wet' chemical methods.

Spectroscopic imaging respectively combines Raman, fluorescence, UV/visible absorption/reflectance and NIR absorption/reflectance spectroscopies with digital imaging for

30   the molecular-specific analysis of materials. This enabling technology allows images of samples to be recorded at discrete wavelengths (energies). A spectrum is generated corresponding to millions of spatial locations at the sample surface by tuning the liquid crystal imaging spectrometer over a range of wavelengths and collecting images intermittently. Depending on

the materials and the spectroscopic method of choice, depth-related information can also be obtained by using different excitation wavelengths or by capturing spectroscopic images at incremental planes of focus. Contrast is generated in the images based on the relative amounts of Raman scatter, fluorescence emission, UV/visible absorption/reflectance or NIR

5 absorption/reflectance that is generated by the different species located throughout the sample. Since a spectrum is generated for each pixel location, chemometric analysis tools such as correlation analysis, Principal Component Analysis (PCA) and factor rotation, including Multivariate Curve Resolution (MCR) can be applied to the image data to extract pertinent information otherwise missed by ordinary univariate measures.

10 A spatial resolving power of approximately 250 nm has been demonstrated for Raman spectroscopic imaging using visible laser wavelengths. This is almost two orders of magnitude better than infrared imaging that is typically limited to 20 microns due to diffraction. In addition, image definition (based on the total number of imaging pixels) can be very high for spectroscopic imaging based on liquid crystal optics because of the use of high pixel density

15 detectors (often 1 million plus detector elements).

Instantaneous Anthrax Detection System Based Upon Spectroscopic Imaging Instrumentation

There are a number of immediate instrumentation configurations based on spectroscopic imaging that may meet the key instrument requirements outlined previously necessary to be an effective, instantaneous Anthrax detection system. These configurations include platforms

20 based on a microscope, a macroscope, an endoscope, a fiber array spectral translator (FAST), or air sampler designs. Each of these is outlined briefly below.

Microscope-Based System

The spectroscopic imaging microscope combines in a single platform a solid state laser for sample excitation (Raman and laser-induced fluorescence only), a refractive optical

25 microscope base, which is equipped with infinity-corrected microscope objectives, an automated XYZ translational microscope stage and a quartz tungsten halogen (QTH) lamp and/or a mercury (Hg) lamp. Also a part of the microscope system is an analog color charge-coupled device (CCD) detector for ordinary optical image collection and digital image collection, a liquid crystal imaging spectrometer or other imaging spectrometer technology including AOTF,

30 scanned linearly variable or rotated circularly variable dielectric filters, angle-rotated Fabry

Perot dielectric or other bandpass filter, interferometers including Michelson and Zhenac types, or dispersive spectrometers, for spectroscopic image wavelength selection. Also included is either a room temperature or optionally cooled IR FPA for IR image capture or a thermoelectrically cooled (TE) Si CCD detector for UV/visible, Raman and fluorescence image

5   capture, and a remote, dispersive monochromator equipped with a CCD detector for dispersive spectral collection.

UV, visible or NIR illumination is directed to the sample in a reflected light configuration using a QTH source or other broadband white light source, including metal halide, Hg arc lamps or Xe arc lamps or a transmitted light configuration using QTH or other suitable

10   source of a refractive optical microscope platform. In a Raman or laser-induced fluorescence experiment, laser radiation is introduced to the sample through use of a Raman illuminator. Light scattered, emitted, reflected or transmitted is collected from the sample positioned on the automated XYZ translational microscope stage through an infinity-corrected microscope objective.

15   Ordinary optical imagery of the sample can be obtained using a mirror or beamsplitter or prism arrangement inserted into turret wheel of the microscope and collecting an image with an analog or digital color or monochrome charge-coupled device (CCD) or CMOS detector. In spectroscopic imaging mode, the magnified spectroscopic image is coupled through an imaging spectrometer and collected on a NIR or mid-IR focal plane array (FPA) detector (for IR

20   spectroscopic imaging) or a Si CCD detector (for UV/visible absorption/reflectance, fluorescence and Raman spectroscopic imaging. The IR FPA is typically comprised of indium gallium arsenide (InGaAs), but may be comprised of other IR sensitive materials, including platinum silicide (PtSi), indium antimonide (InSb) or mercury cadmium telluride (HgCdTe).

A central processing unit, typically a Pentium computer, is used for spectroscopic image

25   collection and processing. The analog color CCD, IR FPA and/or Si CCD, automated XYZ translational microscope stage controlled via a controller and liquid crystal or other imaging spectrometer (through the appropriate imaging spectrometer controller) are operated with commercial software, such as ChemAcquire (ChemImage Corporation) in conjunction with ChemAnalyze (ChemImage Corporation.).

30   By introducing a polarization sensitive beam splitting element in the optical path prior to the liquid crystal imaging spectrometer, a portion of the signal from the sample may be coupled to a remote dispersive spectrometer. This allows conventional spectroscopic tools to be used to gather spectra for traditional, high-speed spectral analysis. The spectrometers can be of the

following types: fixed filter spectrometers; grating based spectrometers; Fourier Transform spectrometers; or Acousto-Optic spectrometers. A polarization independent interferometer such as a: Michelson interferometer; Sagnac interferometer; Twynam-Green Interferometer; Mach-Zehnder Interferometer, may be used as a filter.

5   Preferably, liquid crystal (LC) imaging spectrometer technology is used for wavelength selection. The LC imaging spectrometer may be of the following types: Lyot liquid crystal tunable filter (LCTF); Evans Split-Element LCTF; Solc LCTF; Ferroelectric LCTF; Liquid crystal Fabry Perot (LCFP); or a hybrid filter technology comprised of a combination of the above-mentioned LC filter types. Additionally, fixed bandpass and bandreject filters comprised

10   of dielectric, rugate, holographic, color absorption, acousto-optic or polarization types may also be used, either alone or in combination with one of the above LC spectrometers.

The spectroscopic imaging microscope can be used as a volumetric imaging instrument through the means of moving the sample through focus in the Z, axial dimension, collecting images in and out of focus and reconstructing a volumetric image of the sample in software. For

15   samples having some volume (bulk materials, surfaces, interfaces, interphases), volumetric spectroscopic imaging has been shown to be useful for failure analysis, product development and routine quality monitoring. The potential also exists for performing quantitative analysis simultaneous with volumetric analysis. Volumetric imaging can be performed in a non-contact mode without modifying the sample through the use of numerical confocal techniques, which

20   require that the sample be imaged at discrete focal planes. The resulting images are processed, reconstructed, and visualized. Computational optical sectioning reconstruction techniques based on a variety of strategies have been demonstrated, including nearest neighbors and iterative deconvolution.

Microscope-based spectroscopic imaging systems have the distinct image of being able

25   to detect, classify, identify and visualize BWAs down to a single bacterium for instance. These systems boast a spectral resolution on the order of 8 cm^{-1} and a spatial resolution of approximately 200 nm with numerical deconvolution methods.

Macroscope-Based System

The spectroscopic imaging macroscope combines in a single platform and illumination

30   subassembly consisting of an illumination source (typically a QTH, Xe, Hg or other metal halide lamp), barrier optical filter(s) and a light-directing module (i.e., direct beam, fiber optic or liquid light guide illumination). An analog color charge-coupled device (CCD) detector is used for

ordinary optical and digital image collection. Wavelength selection is done using a liquid crystal imaging spectrometer or other imaging spectrometer. The imaging detector is either a room temperature or optionally cooled NIR FPA for NIR image capture or a thermoelectrically cooled (TE) Si CCD detector for UV/visible and fluorescence image capture.

5   UV, visible or NIR illumination is directed to the sample in a reflected light configuration using a QTH source or other broadband white light source, including metal halide, Hg arc lamps or Xe arc lamps or a transmitted light configuration using QTH or other suitable source through direct illumination, fiber optics or liquid light guides. Light emitted, reflected or transmitted is collected from the sample positioned on the macroscopic sample base through a

10   macro lens.

Ordinary optical imagery of the sample may be obtained using a mirror or beamsplitter or prism arrangement inserted into the collection stack of the macroscope and collecting an image with an analog or digital color or monochrome charge-coupled device (CCD) or CMOS detector. In spectroscopic imaging mode, the spectroscopic image is coupled through a liquid

15   crystal imaging spectrometer and collected on a NIR focal plane array (FPA) detector (for NIR spectroscopic imaging) or a Si CCD detector (for UV/visible absorption/reflectance, fluorescence and Raman spectroscopic imaging). The NIR FPA is typically comprised of indium gallium arsenide (InGaAs), but may be comprised of other NIR sensitive materials, including platinum silicide (PtSi), indium antimonide (InSb) or mercury cadmium telluride

20   (HgCdTe).

A central processing unit, typically a Pentium computer, is used for spectroscopic image collection and processing. The analog color CCD, NIR FPA and/or Si CCD and liquid crystal imaging spectrometer or other imaging spectrometer (through an appropriate imaging spectrometer controller) are operated with commercial software, such as ChemAcquire

25   (ChemImage Corporation) in conjunction with ChemAnalyze (ChemImage Corporation.).

Preferably, liquid crystal (LC) imaging spectrometer technology is used for wavelength selection. The LC imaging spectrometer may be of the following types: Lyot liquid crystal tunable filter (LCTF); Evans Split-Element LCTF; Solc LCTF; Ferroelectric LCTF; Liquid crystal Fabry Perot (LCFP); or a hybrid filter technology comprised of a combination of the

30   above-mentioned LC filter types. Additionally, fixed bandpass and bandreject filters comprised of dielectric, rugate, holographic, color absorption, acousto-optic or polarization types may also be used, either alone or in combination with one of the above LC spectrometers.

The use of a macroscopic-based system has the advantage of enabling rapid detection of potential BWAs and CWAs over a large area. Previous work has shown the ability image 0.01 mm defects on 200 mm semiconductor wafers using the macroscope system.

Endoscope-Based System

5   Spectroscopic imaging has traditionally been performed in laboratory settings using research-grade light microscope technology as the image-gathering platform. However, spectroscopic imaging is also applicable to *in situ* industrial process monitoring and *in vivo* clinical analysis. The application of spectroscopic imaging outside the research laboratory has been limited by the lack of availability of stable imaging platforms that are compatible with the

10   physical demands of industrial process monitoring and clinical environments. Both industrial and clinical settings often require compact, lightweight instrumentation suitable for the examination of remote areas that are inaccessible to conventional spectroscopic imaging instrumentation.

A robust spectroscopic imaging design employing liquid crystal technology has been

15   developed. The liquid crystal endoscope is the first flexible imaging endoscopic technology that provides real-time video inspection capability with spectral analysis. The endoscope, comprising from two to thousands of independent fibers arranged in a coherent imaging bundle, couples to a video CCD for real-time video imaging of the analysis area. This allows for quick visual screening of the sample. The endoscope tip has been engineered to filter both laser

20   illumination and collected Raman scatter and fluorescence emission (for Raman and fluorescence applications). The light from the laser delivery fiber is filtered so that only the laser wavelength is presented to the sample. The laser is removed from the collected light so that Raman information is visible to within 200 cm^{-1} of the laser line. The distal end of the liquid crystal Raman endoscope is environmentally resistant and can withstand continuous

25   operation at high temperatures and has been demonstrated to operate from 0 – 315°C while maintaining high signal to background (S/B) performance. The distal end can be coupled to a microscope-based system enabling dispersive spectroscopy and spectroscopic imaging to be performed remotely.

The use of an endoscopic-based spectroscopic imaging system has the advantage of

30   being able to detect the presence of suspect BWAs and CWAs in remote locations such as inside a box or envelope.

FAST-Based System

An emerging technology in the field of spectroscopic imaging is the use of fiber optic arrays. We have termed this technology Fiber Array Spectral Translators (FAST) but it is also described as dimension reduction arrays. FAST technology can acquire a few to thousands of

5   full spectral range, spatially resolved spectra simultaneously. This is done by focusing a spectroscopic image onto a two dimensional array of optical fibers that are drawn into a one-dimensional distal array with serpentine ordering. The one dimensional fiber stack is coupled to an imaging spectrograph. Software then extracts the spectral/spatial information that is embedded in a single CCD image frame. Fiber array spectroscopic imaging has been

10   demonstrated in several applications including Raman chemical imaging analysis of micro-composites and biomaterials and time-resolved atomic emission chemical imaging of laser-induced plumes.

The fundamental advantage of this method over other spectroscopic imaging methods is speed of analysis. A complete spectroscopic imaging data set can be acquired in the amount of

15   time it takes to generate a single spectrum from a given material. A current limitation of FAST is the low image definition (number of image pixels) in the object field. Image definition is dictated by the number of elements in the long axis direction of the detector. Alternatives to current designs can include the use of multiple detectors, which has the potential to increase the image definition. Even with limited pixel definition, superimposing color-coded spectroscopic

20   images on high-spatial resolution gray-scale images can provide significant insight into the morphology and chemistry of materials.

Ambient Air Sensor System

The ambient air sensor system consists of two parts, a sampling system and a spectroscopic imaging system. The key to the sampling system is the optics block, shown

25   diagrammatically in Figure 1. This block must support a section of filter medium and provide a complete airtight seal around the periphery of the sampling area. This block must also be easily opened so that either a new filter (discrete filters) or a new section of filter (continuous filters) can be placed in the sampling/optics path.

The sampling system has an inlet, which is open to the atmosphere being tested. Its

30   dimensions are optimized for the sampling flow rate and the anticipated range of particle sizes. For particulate or aerosol sampling, it is important that the inlet have no sharp bends or areas of

14

low linear velocity, which can cause deposition of particulate prior to the collection filter. The sampling system also has a sampling pump, providing the vacuum to pull ambient air through the filter. Anticipated flow rates are in the 0.5 to 2.0 L/min range, and the expected vacuum is in the 100 in.-H_2O (180 mm-Hg) range.

5   The sampling system is typically not run continuously but rather in a series of discrete sampling periods. At the end of each period, it might be necessary to replace the filter medium. This can be done either by the operator or automatically. For continuous sampling, the filter medium can be in a tape-like configuration and new samples of filter can be positioned in the optics block by a tape-drive mechanism, similar to that of an audiocassette.

10   Once the particulates have been trapped on the filter medium, imaging spectroscopy is used to detect and classify the BWA or CWA present. If the excitation source is a laser, coupled to the optics block using conventional or fiber optics, whose light is evenly distributed over the whole sampling area, Raman imaging can be used. In another configuration, a light source comprised of a broadband UV/Vis, filtered UV/Vis, or a UV/Vis laser can be used to excite

15   autofluorescence. The imaging detector can be of the liquid crystal tunable type or another imaging spectrometer type as described earlier and a CCD or other array camera can be used to image the sampling area at multiple wavelengths. Coupling of the detector to the optics block can be through fiber-based or conventional optics. The detector data is processed using chemometric and image analysis tools such as those found in the ChemAnalyze software

20   (ChemImage Corporation).

         The typical operating mode of this type of ambient air monitor is usually as a series of sampling periods during which periodic spectroscopic image measurements are taken. The results from the previous and current sampling periods are interpreted by a system computer which can display results and activate warning and danger alarms, or initiate some action such as

25   turning off a building outside air intake.

Results

         Spectra generated using traditional spectroscopic methods can potentially reveal a wealth of information about molecular properties of BWAs and CWAs. Spectroscopic imaging compounds this information by allowing variations in the composition of these materials to be

30   probed downed to a single bacterium if necessary. Figure 2 shows Raman spectroscopic imaging data on a mixture of 1μm diameter polystyrene micro-spheres and *Bacillus subtilis var. niger* spores (Anthrax simulant). The images on the left side of the figure show a brightfield

reflectance image (top) and a Raman spectroscopic image (bottom) of the bacteria spores/micro-spheres mixture. The bacteria spores and micro-spheres have been color-coded green and red, respectively. The Raman spectra to the right of the images show the spectral "fingerprints" associated with the bacteria spores and the polystyrene micro-spheres, respectively. Despite the

5   morphological similarities between the mixture components, the Raman spectroscopic image reveals the molecularly distinct species. This ability to characterize bacteria spores in the presence of non-threatening 'masking' agents is a critical issue in the detection and identification of BWAs and CWAs. Difficulties exist when trying to differentiate spores from different bacterial species. Figure 3 shows dispersive Raman spectra of three different bacterial

10   spores types. Despite the genetic and morphological similarities, Raman dispersive spectroscopy has been used to sufficiently discriminate among the different bacteria spores.

Figure 4 shows how fluorescence spectroscopic imaging can be used to distinguish between bacteria spore types. The fluorescence spectra in the lower portion of the figure were obtained from the color-coded boxed regions in the concatenated fluorescence spectroscopic

15   images above. It can be seen that *Bacillus subtilis* spores and *Bacillus pumilus* spores exhibit fluorescence peaks maxima at 540 nm and 630 nm, respectively.

Figure 5 shows the results of rapid spectroscopic examination of unidentified samples supplied by the US Armed Forces Institute of Pathology (AFIP). These samples include 4 samples comprising 6 unknown powders and a sample of BG spores.

20   Figure 5A shows Raman spectra (green laser excitation) of the 6 unidentified powders through the vials.

Figure 5B shows Raman spectra (red laser excitation) of the 6 unidentified powders.

Figures 5C-5D (Sample 1331-002) show Raman, IR and SEM-EDS results on a first of the 6 unidentified powders. The sample is inorganic and most likely talc.

25   Figures 5E-5F (Sample 1325-002) show Raman, IR and SEM-EDS results on a second of the 6 unidentified samples. The sample is organic and most likely starch, possibly corn starch.

Figures 5G-5H (Sample 1303-002) show Raman, IR and SEM-EDS results on a third of the 6 unidentified powders. The sample is organic and most likely starch, possibly corn starch.

30   Figures 5I-5N (Sample 1291-006) show Raman, IR and SEM-EDS results on the remaining unidentified powders. There are 3 distinct types of powders in this sample. All 3 have organic content, while 2 of the 3 are fairly rich in aluminosilicates. One of the powders is likely a complex aromatic hydrocarbon.

Figures 5O-5Q show Raman spectra and images of 2 common white powders that can easily be differentiated with Raman Chemical Imaging.

Figure 5R shows Raman spectra of sample BG spores compared with commercially available BG spores. A Raman spectrum of a mixture of the 2 samples is shown, as well. Raman indicates the samples are similar, almost identical.

Figure 5S shows a Chemical Imaging where the 2 similar spores are differentiated on the basis of autofluorescence differences.

Figure 5T shows a Raman Chemical Imaging where the 2 powders from Figure 5O are mixed with BG(AFIP). The 3 species can readily be discriminated.

Figure 5U shows efforts to detect BG in the presence of a white powder (Baking soda) on an envelope.

Figure 5V shows BG on an envelope imaged while the envelope is moving.

Figure 6 shows the results from additional spore samples selected specifically because the inherent difficulty in differentiating these species. They include Bacillus thuriengensis (BT), Bacillus cereus (BC) and BG. The Raman spectra from the 3 spores are different. These differences suggest a good chance of differentiating anthrax from non-threats. The details follow:

Figure 6A shows raw Raman spectra of BT and the suspension residue. The residue is from the suspension liquid.

Figure 6B shows background corrected spectra of BT and residue. Both the spores spectrum and residue spectrum have been divided by a spectrum of the microscope slide.

Figure 6C shows raw Raman spectra of BC and the suspension residue.

Figure 6D shows background corrected spectra of BC and residue

Figure 6E shows a compilation of sample BT, BC and BG spectra with microscope slide background correction. The spectra are different. The differences are greatest in the fingerprint region.

Figure 6F shows a compilation of the 3 spores after baseline subtraction and normalization to the CH region spectral feature ($\sim$2950 cm^{-1}).

Figure 7 shows how RCI can be applied to distinguish between multiple bacterial strains within a single species.

Figure 8 shows how RCI can be applied to distinguish between the same species and strain of bacteria grown under differing conditions.

Figure 9 shows a schematic description of RCI.

Figure 10 shows Raman Chemical Imaging of actual individual *Bacillus anthracis* (Anthrax) spores as well as additional demonstrations of RCI's power to reproducibly distinguish between similar materials.

Figure 11 shows how RCI can be applied to distinguish between viable and non-viable

5   endospores, a critical variable in determining real threat level.

Anthrax spores have been Raman imaged in a secure biohazard laboratory (Figure 10). Different strains of Anthrax spores have been differentiated by Raman Imaging (Figure 7). Additionally, Raman Chemical Imaging has been used to differentiate same species and strain grown under different environmental conditions and/or growth medium (Figure 8). This ability

10   can have useful investigatory applications. And, RCI has been used to differentiate viable from non-viable endospores (Figure 11). Viability of suspect spores is a critical variable in determing the real threat posed. The inventors anticipate that the following pathogenic microorganisms will be susceptible to detection and classification as to species, strains, and viability by the Raman spectral profile and Raman imaging: protozoa; cryptosporidia; Escherichia coli ; Plague

15   (Yersinia pestis; Smallpox (variola major); Tularemia (Francisella tularensis; Brucellosis (Brucella species; Clostridium perfringens;   Glanders (Burkholderia mallei; Melioidosis (Burkholderia pseudomallei; Psittacosis (Chlamydia psittaci; Q fever (Coxiella burnetii; Typhus fever (Rickettsia. prowazekii; Vibrio ; Giardia; . Candida albicans; Enterococcus faecalis ;Staphylococcus epidermidis; Staphylococcus aureus; Enterobacter aerogenes; Corynebacterium

20   diphtheriae; Pseudomonas aeruginosa; Acinetobacter calcoaceticus; Klebsiella pneümoniae; Serratia marcescens; filoviruses (such as Ebola and Marburg viruses), naviruses (such as Lassa fever and Machupo viruses) and alphaviruses(such as Venezuelan equine encephalitis, eastern equine encephalitis, and western equine encephalitis).

Advanced image analysis and chemometric tools take these differences in fluorescence

25   spectra and perform a spatial identification of species, producing the image in Figure 4. The following is a representative algorithm for performing this analysis:

1) Divide the raw image by a background image (taken without the sample)

2) Do cosmic filtering on the resultant image (median filtering for pixels whose value differs significantly from the mean of a local neighborhood)

30   3) Use an alignment procedure to correct for slight movements of the sample during data collection

4) Apply a spatial average filter

5) Perform a spectral normalization (helps correct for varying illumination across the sample)

6) Perform a spectral running average over each set of three spectral points

7) Extract a set of frames corresponding to 550 to 620 nm. The spectra for both bacterial
5   spores (*Bacillus subtilis var niger* and *Bacillus pumilus*) are essentially linear over this range. *Bacillus subtilis var niger* has a positive slope and *Bacillus pumilus* has a negative slope.

8) Create a single frame image in which each intensity value is the slope of the spectral sub-region (from the last image). The slope is determined via a least-squares fit.

10 9) Scale the resulting image between 0 and 4095. Keep track of the point from 0 to 4095 that corresponds to 0 in the prior image (the "Zero point").

10) Create a mask image from a series of steps:

    a) From the aligned image (3rd step), calculate a single frame "brightest" image in which the intensity of each pixel is the maximum intensity value for each spectrum.

15   b) Scale this brightest image between 0 and 4095.

    c) Create a binarized image from the scaled image, in which every pixel whose intensity is greater than 900 is set to 1 in the new image and every pixel whose intensity is less than 900 is set to 0 in the new image. The value of 900 was chosen by an examination of the histogram associated with the scaled image. A future
20   improvement to the algorithm would be to automatically select the threshold by numerically analyzing the histogram for a given image.

11) Multiply the scaled image from step 9 by the mask image from step 10. This restricts the visual display to only areas that correspond to spores. The result is a gray scale image in which intensity values below the zero point defined in step 9 correspond to *bacillus*
25   *pumilus* and the intensity values above the zero point correspond to *bacillus subtilis var niger*.

12) The final RGB image is then created by setting all the "negative" values to red and all the "positive" values to green.

Applications

There is a great need for a spectroscopic imaging instrument that can provide high throughput, non-contact, real-time detection, classification and identification of BWAs and CWAs with high accuracy and with limited or no sample preparation required. The user base

5   for an instrument suitable for objective assessment of BWAs and CWAs will consist of hazardous materials (HAZMAT) teams, government and private facilities where potential threats are high, mail handling facilities, academic, industrial and medical research laboratories, etc.

The benefits to the target users of an instantaneous Anthrax or other microoganism threat detection system will be substantial. Configured in the macroscopic version of the technology,

10   spectroscopic imaging can be employed for rapid assessment of large areas for suspect BWAs and CWAs based on their fluorescence, NIR and/or UV/visible response. Configured in the microscopic mode, positive detection, classification, identification and visualization of suspect BWAs and CWAs can be made. Configured in the endoscopic mode, BWAs and CWAs can be detected, classified, identified and visualized remotely. Configured in FAST mode, BWAs and

15   CWAs can be detected, classified, identified and visualized remotely or at the microscope in real-time. When configured as an air sampler, unambiguous detection of BWAs and CWAs can be done.

When performed in combination the effectiveness of characterizing BWAs and CWAs will likely be enhanced. Benefits will include, but are not limited to, the following:

20   • Rapid large area scanning to detect suspect BWAs and CWAs.

   • Positive detection, classification, identification and visualization of suspect BWAs and CWAs.

   • Non-contact

   • Limited or no sample preparation required.

25   • Remote detection, classification, identification and visualization of suspect BWAs and CWAs.

   • Real-time detection, classification, identification and visualization of suspect BWAs and CWAs in solid or gaseous samples.

Specific applications of a spectroscopic imaging system for instantaneous Anthrax detection will include the following:

- Discrimination of threatening and non-threatening 'masking' agents

- Spatial distribution of BWAs and CWAs

5
- Spatial distribution of BWAs and CWAs down to single bacterial spores

Advantages Over Currently Available Technology

Traditional approaches to detection of BWAs and CWAs include inoculation methods, enzyme-linked immunosorbent assay (ELISA) methods, BioThreat Alert (BTA) test strips,
10 DNA-based tests, DNA chip analyses and mass spectrometry. Inoculation methods involve the inoculation of suspect culture or specimen into an animal that is then observed for development of disease. In addition to animal cruelty issues, there are drawbacks with this approach including the extensive amount of time required to reach a point of detection.

ELISA tests involve antibody detection. This technique is also slow and suffers from
15 high false positive (unrelated antibody reacts with antigen nonspecifically) and worse, high false negatives (interfering compounds present in blood or antibodies not concentrated enough to be detected). Furthermore, a patient can test positive to antibodies long after the patient has recovered.

BTA test strips are small plastic devices that work very much like a home pregnancy
20 test. The test strips contain specific antibodies that change color on the strip indicating the presence of a bio-threat agent. A negative result means the bio-threat agent is not present within the detection limit of the strip. Although results can be obtained in a relatively short period (15 minutes), the incidence of false negatives and false positives is high.

DNA-based tests detect agents by recognizing their genetic sequences. While more
25 sensitive than BTA test strips, DNA-based tests are susceptible to masking agents and involve a lengthy analysis time. DNA chip analysis involves the immobilization of DNA strands on a Si or glass wafer chip. DNA will bind to or hybridize complementary DNA strands in the sample being tested. A specially designed microscope detects where the DNA hybridize. Amplification is achieved by polymerase chain reaction (PCR). Detection of bio-threat agent is reported to be
30 possible within minutes.

The limitations of DNA-based methods are two-fold. First, DNA methods are designed to detect a specific bio-threat agent through its unique DNA sequences. Therefore, each DNA test is specific to one agent and if it is desired to detect additional agents, additional test reagents must be developed. A second limitation revolves to the problems of false negatives and false

5   positives due to environmental contamination. DNA tests are well known to have problems yielding correct results in "real-world" samples.

Mass Spectrometry (MS) uses the pattern of mass fragments when a cell or spore is subjected to an ionization process under high vacuum to characterize organisms. It has the advantage of very sensitive detection but requires a sophisticated sampling system in order to

10   deliver a representative sample to the ionizer. The main limitation of MS is that it requires the use of high vacuum pumps that are inherently delicate and expensive. An additional limitation is that it is a destructive technique, in contrast to Spectroscopic Chemical Imaging, which is a completely no-destructive technique.

Alternative technologies/opportunities include probes or microscopes based on

15   spectroscopic methods including Raman, fluorescence, NIR, etc. for rapid, non-contact and accurate detection of BWAs and CWAs. These types of tools offer the potential to detect, classify, identify and visualize the distribution of BWAs and CWAs in the presence of other BWAs, CWAs and/or non-threatening 'masking' agents in either a local or remote environment – characteristics that no traditional method can provide. Such tools would greatly reduce the

20   incidence of false positives and false negatives and would provide a way to determine if a true bio-threat exists in minutes.

State-Of-The-Art Spectroscopic Imaging Techniques

Several spectroscopic imaging technologies have evolved that compete with widefield tunable filter-based spectroscopic imaging and FAST technology. These techniques include

25   point scanning, line imaging, spectroscopic imaging using interference filters, Fourier-transform interferometry and Hadamard-transform scanning.

Point scanning involves taking a complete spectrum for a single X,Y position of a sample followed by raster-scanning the sample for the remaining X,Y positions. This method offers advantages of high spectral resolution and full spectral resolution, but lacks high image

30   definition capabilities and is extremely time consuming. Line imaging involves collecting data from vertical sections of the sample characterized by a single value of X and all values of Y, followed by subsequent scanning in the X direction. This method has the nearly the same

advantages and disadvantages as the point scanning approach, but can be done more rapidly. Field curvature artifacts are a consequence of line imaging which degrade image quality. The use of single or multiple interference filters can be used to produce a wavelength specific image(s). This method is rapid, cheap and produces high definition images, but lacks spectral

5   resolution and is susceptible to image artifacts.

Fourier-transform interferometers use a mechanically driven interferometer with a CCD-based detection system. Interferograms are imaged with the CCD for subsequent spectral interpretation for each step of the interferometer. This method boasts good spatial resolution but suffers from poor spectral resolution ($\sim$100 cm^{-1}).

10   Hadamard transform chemical imaging techniques couple Hadamard mask spatial multiplexing with CCD-based detection to obtain two spatial and one spectral dimension of data. This method offers S/N advantages for low-light level applications such as Raman spectroscopy in addition to sub-nanometer spectral resolution. However, the technique suffers from fair spatial resolution and poor temporal resolution since the latter involves scanning through

15   numerous coding masks.

The ideal chemical imaging system for characterization would provide fast acquisition times (seconds), high spatial resolution (sub-micron) and good spectral resolution (<200 nm). To date, ChemImage's FALCON™ microscope is the only spectroscopic imaging system that meets these requirements.

20   *Other Spectroscopy-Based Imaging Methods*

Spectroscopic technologies that compete with those previously mentioned including infrared (IR) spectroscopy are not of great concern based on the resolution needed to see individual BWAs and/or CWAs on the order of 250 microns. IR spectroscopy cannot compete due to the difficulty with water absorption in the IR. Typically, BWAs do not image well

25   because of their aqueous nature. The liquid crystal imaging spectrometer chosen for the spectroscopic imaging systems described here surpasses any dispersive grating or acousto-optic tunable filter (AOTF) technology on the market. The spectral bandpass capability of the LCTF is 8 cm^{-1} allowing for the most effective means to obtain image detail.

Obviously, many modifications and variations of the present invention are possible in

30   light of the above teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

We claim:

1. A method for detection of Anthrax (Bacillus anthracis), comprising:

    a) irradiating at least one Anthrax microorganism with light; then

    b) forming an image of the Anthrax microorganism using Raman shifted light from the pathogenic microorganism; then

    c) analyzing the Raman shifted light image for patterns characteristic of Anthrax microorganisms.

2. The method of claim 1, where the step of analyzing includes analyzing the strain of the Anthrax microorganism.

3. The method of claim 1, where the step of analyzing includes analyzing the viability of the Anthrax microorganism.

4. The method of claim 1, where the step of analyzing includes analyzing the growth medium in which the Anthrax microorganism has been grown.

5. A method for detection of pathogenic microorganisms, comprising:

    a) irradiating at least one pathogenic microorganism with light; then

    b) forming an image of the at least one pathogenic microorganism using Raman shifted light from the pathogenic microorganism; then

    c) analyzing the Raman shifted light image for patterns characteristic of pathogenic microorganisms.

6. The method of claim 5, where the Raman shifted light from the region passes through a FAST fiber array spectral translator.

7. The method of claim 5, where the Raman shifted light from the region passes through a Fabry Perot angle tuned filter.

8. The method of claim 5, where the Raman shifted light from the region passes through an acousto-optic tunable filter.

9.   The method of claim 5, where the Raman shifted light from the region passes through a liquid crystal tunable filter.

10.   The method of claim 9, where the Raman shifted light from the region passes
5   through a Lyot filter.

11.   The method of claim 9, where the Raman shifted light from the region passes through an Evan's split element liquid crystal tunable filter.

10   12.   The method of claim 9, where the Raman shifted light from the region passes through Solc liquid crystal tunable filter.

13.   The method of claim 9, where the Raman shifted light from the region passes through liquid crystal Fabry Perot (LCFP) tunable filter.
15

14.   The method of claim 5, where the Raman shifted light from the region passes through a polarization-independent imaging interferometer.

15.   The method of claim 14, where the Raman shifted light from the region passes
20   through a Michelson interferometer.

16.   The method of claim 14, where the Raman shifted light from the region passes through a Sagnac interferometer.

25   17.   The method of claim 14, where the Raman shifted light from the region passes through a Twynam-Green Interferometer.

18.   The method of claim 14, where the Raman shifted light from the region passes through a Mach-Zehnder Interferometer.
30

19.   The method of claim 5, where the Raman shifted light from the region passes through a tunable Fabry Perot Interferometer.

20.   The method of claim 5, where the Raman shifted light from the region passes through at least two filters chosen from the group consisting of Solc, LCFP, Lyot, and Evan's split element filters.

5   21.   The method of claim 5, where the pathogenic microorganisms are anthrax spores.

22.   The method of claim 5, where the pathogenic microorganisms are protozoa.

23.   The method of claim 5, where the pathogenic microorganisms are cryptosporidia
10   microorganisms.

24.   The method of claim 5, where the pathogenic microorganisms are Escherichia coli microorganisms.

15   25.   The method of claim 5, where the pathogenic microorganisms are Escherichia coli 157 microorganisms.

26.   The method of claim 5, where the pathogenic microorganisms are Plague (Yersinia pestis) microorganisms.
20

27.   The method of claim 5, where the pathogenic microorganisms are Smallpox (variola major) microorganisms.

28.   The method of claim 5, where the pathogenic microorganisms are Tularemia
25   (Francisella tularensis) microorganisms.

29.   The method of claim 5, where the pathogenic microorganisms are Brucellosis (Brucella species) microorganisms.

30   30.   The method of claim 5, where the pathogenic microorganisms are Clostridium perfringens microorganisms.

31. The method of claim 5, where the pathogenic microorganisms are Salmonella microorganisms.

32. The method of claim 5, where the pathogenic microorganisms are Shigella microorganisms.

33. The method of claim 5, where the pathogenic microorganisms are Glanders (Burkholderia mallei) microorganisms.

34. The method of claim 5, where the pathogenic microorganisms are Melioidosis (Burkholderia pseudomallei) microorganisms.

35. The method of claim 5, where the pathogenic microorganisms are Psittacosis (Chlamydia psittaci) microorganisms.

36. The method of claim 5, where the pathogenic microorganisms are Q fever (Coxiella burnetii) microorganisms.

37. The method of claim 5, where the pathogenic microorganisms are Typhus fever (Rickettsia prowazekii) microorganisms.

38. The method of claim 5, where the pathogenic microorganisms are Vibrio cholerae microorganisms.

39. The method of claim 5, where the pathogenic microorganisms are chosen from the group of filoviruses (such as Ebola and Marburg viruses), naviruses (such as Lassa fever and Machupo viruses) and alphaviruses(such as Venezuelan equine encephalitis, eastern equine encephalitis, and western equine encephalitis).

40. The method of claim 5, where the pathogenic microorganisms are Giardia microorganisms.

41.   The method of claim 5, where the pathogenic microorganisms are Candida albicans microorganisms.

42.   The method of claim 5, where the pathogenic microorganisms are Enterococcus faecalis microorganisms.

43.   The method of claim 5, where the pathogenic microorganisms are Staphylococcus epidermidis microorganisms.

44.   The method of claim 5, where the pathogenic microorganisms are Staphylococcus aureus microorganisms.

45.   The method of claim 5, where the pathogenic microorganisms are Enterobacter aerogenes microorganisms.

46.   The method of claim 5, where the pathogenic microorganisms are Corynebacterium diphtheriae microorganisms.

47.   The method of claim 5, where the pathogenic microorganisms are Pseudomonas aeruginosa microorganisms.

48.   The method of claim 5, where the pathogenic microorganisms are Acinetobacter calcoaceticus microorganisms.

49.   The method of claim 5, where the pathogenic microorganisms are Klebsiella pneumoniae microorganisms.

50.   The method of claim 5, where the pathogenic microorganisms are Serratia marcescens microorganisms.

51.   The method of claim 5, where the irradiating light is in the ultraviolet spectral region with wavelength less than 410 nm.

52.   The method of claim 5, where the irradiating light is in the visible spectral region with wavelength less than 780 nm and greater than 410 nm.

53.   The method of claim 5, where the irradiating light is in the near infrared spectral

5   region with wavelength less than 2500 nm and greater than 780 nm.

54.   The method of claim 5, where the step of analyzing includes analyzing the strain of the pathogenic microorganisms.

10   55.   The method of claim 5, where the step of analyzing includes analyzing the viability of the pathogenic microorganisms.

56.   The method of claim 1, where the step of analyzing includes analyzing the growth medium in which the pathogenic microorganisms have been grown.

15
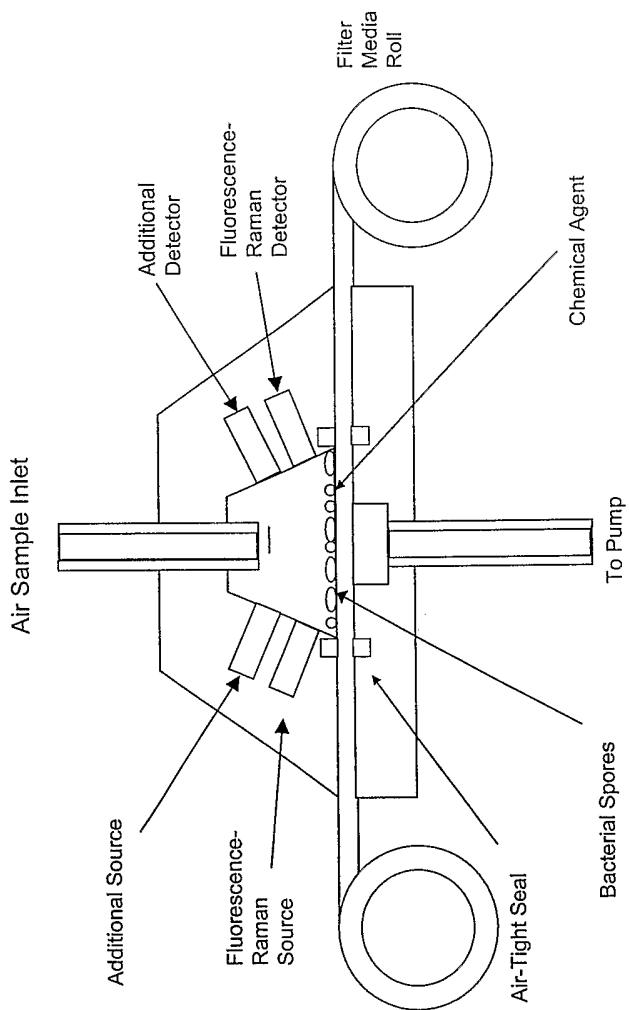
The Integrated Detector Sample Cell

Figure 1

Figure 2

Dispersive Raman Spectra of Anthrax Simulants
Using a ChemIcon FALCON™ Raman Chemical Imaging Microscope

Brightfield Transmittance Image

- Bacteria deposited on microscope slide from aqueous suspension
- Microscope slide background spectrum subtracted from sample spectra

Bacillus stearothermophilus
Bacillus pumilus
Bacillus subtilis var. niger

Raman Shift (cm⁻¹)

Raman Intensity
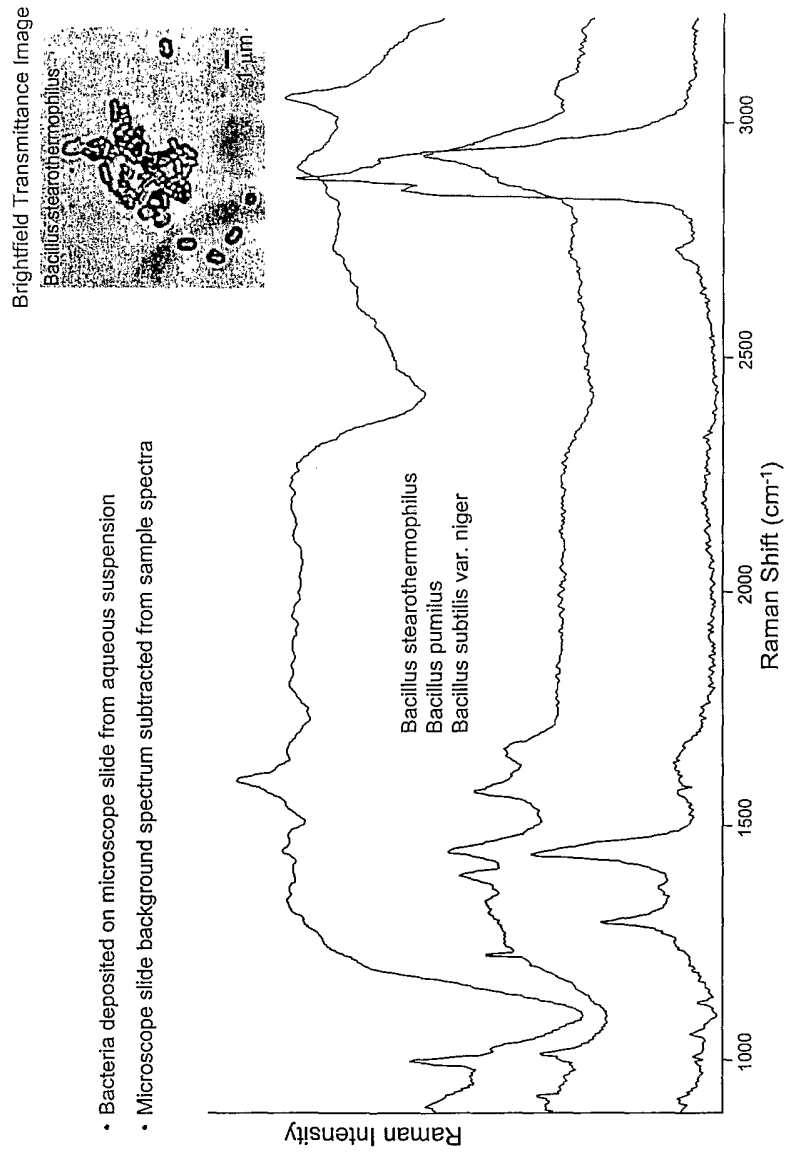
Figure 3

Fluorescence Chemical Imaging of *Bacillus Pumilus* and *Bacillus Subtilis* Mixture

Figure 4

Dispersive Raman Spectroscopy of AFIP Powder Samples
532 nm Laser Excitation – Collected Through Vials (Raw Spectra)

1325-002
1331-002
1303-002
1291-006 (Rod-Shaped Particulate)
1291-006 (Off-White Particulate)
1291-006 (Bright White Particulate)

Dispersive Spectroscopy Parameters
Laser power density (at sample): 86 – 753 W/cm^2
Objective: 10X
Entrance Slit: 25μm
Grating: 150 grooves/mm
CCD Exposure: 1-3 sec., 10 accumulations

Raman Shift (cm^{-1})

Arbitrary Intensity

Figure 5A

Dispersive Raman Spectroscopy of AFIP Powder Samples
789.5 nm Laser Excitation – Collected Directly On Powders (Background Corrected)

Dispersive Spectroscopy Parameters
Laser power density (at sample): 11,888 W/cm²
Objective: 40X
Entrance Slit: 50μm
Grating: 150 grooves/mm
CCD Exposure: 60 sec., 2 accumulations

1325-002
1331-002
1303-002
1291-006 (Rod-Shaped Particulate)
1291-006 (Off-White Particulate)
1291-006 (Bright White Particulate)

Raman Shift (cm⁻¹)

Arbitrary Intensity
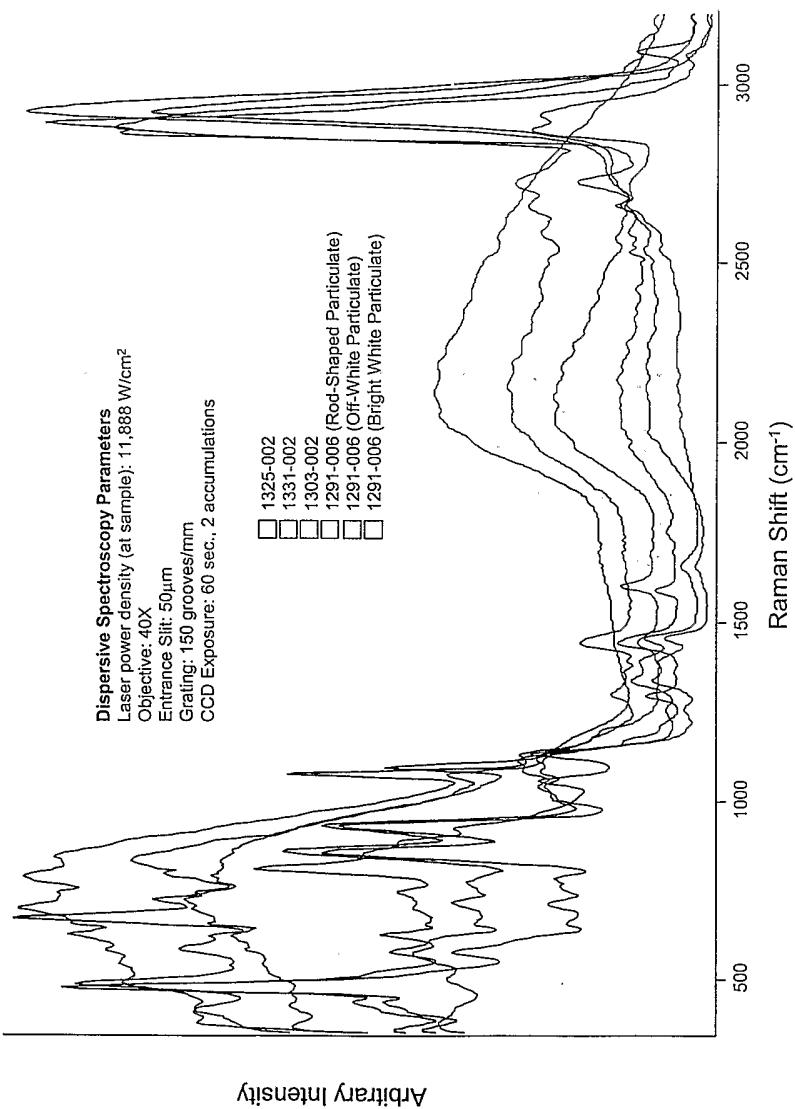
Figure 5B

Dispersive Raman and FT-IR Spectra of AFIP Powder Sample 1331-002
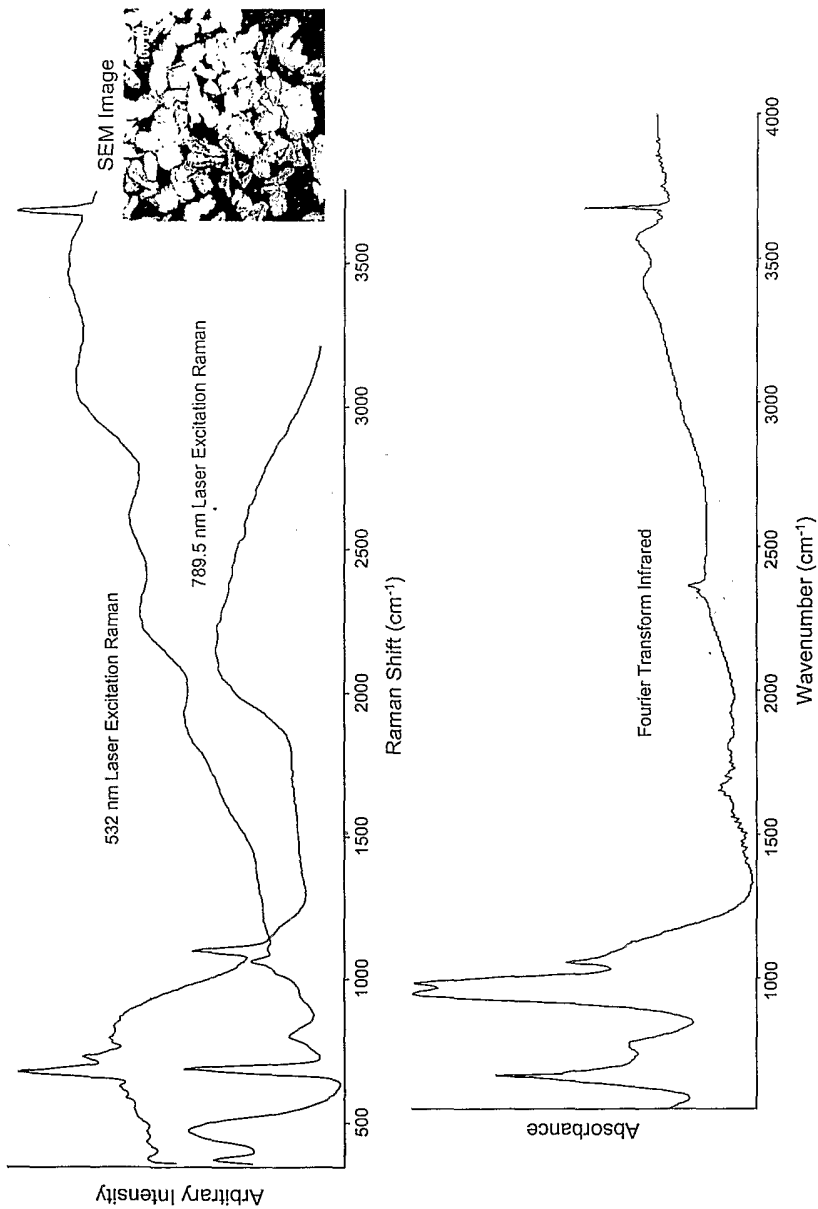
Figure 5C

SEM/EDS of AFIP Powder Sample 1331-002

Backscattered Electron Image

Energy Dispersive Spectrum
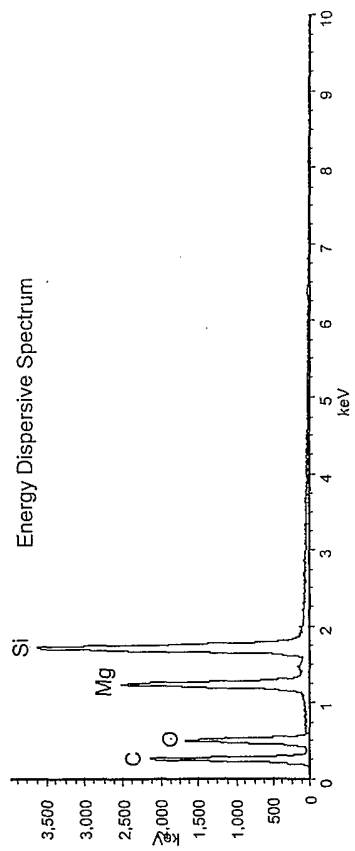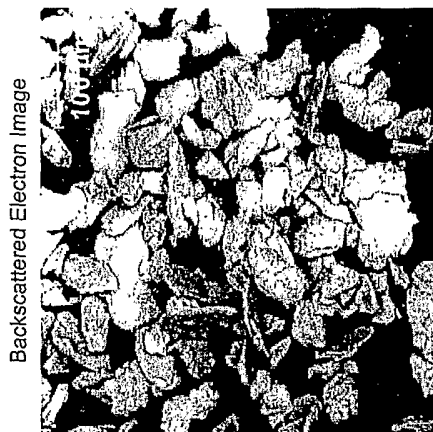
Figure 5D

Dispersive Raman and FT-IR Spectra of AFIP Powder Sample 1325-002
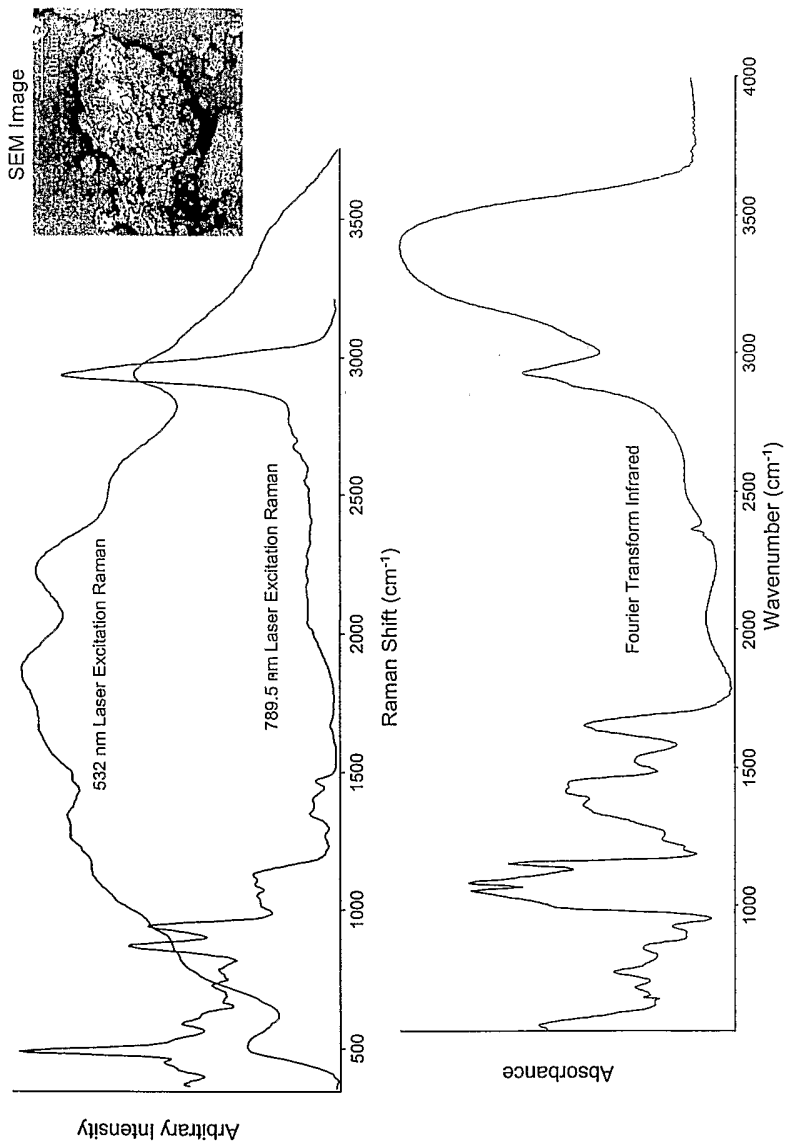
Figure 5E

SEM/EDS of AFIP Powder Sample 1325-002
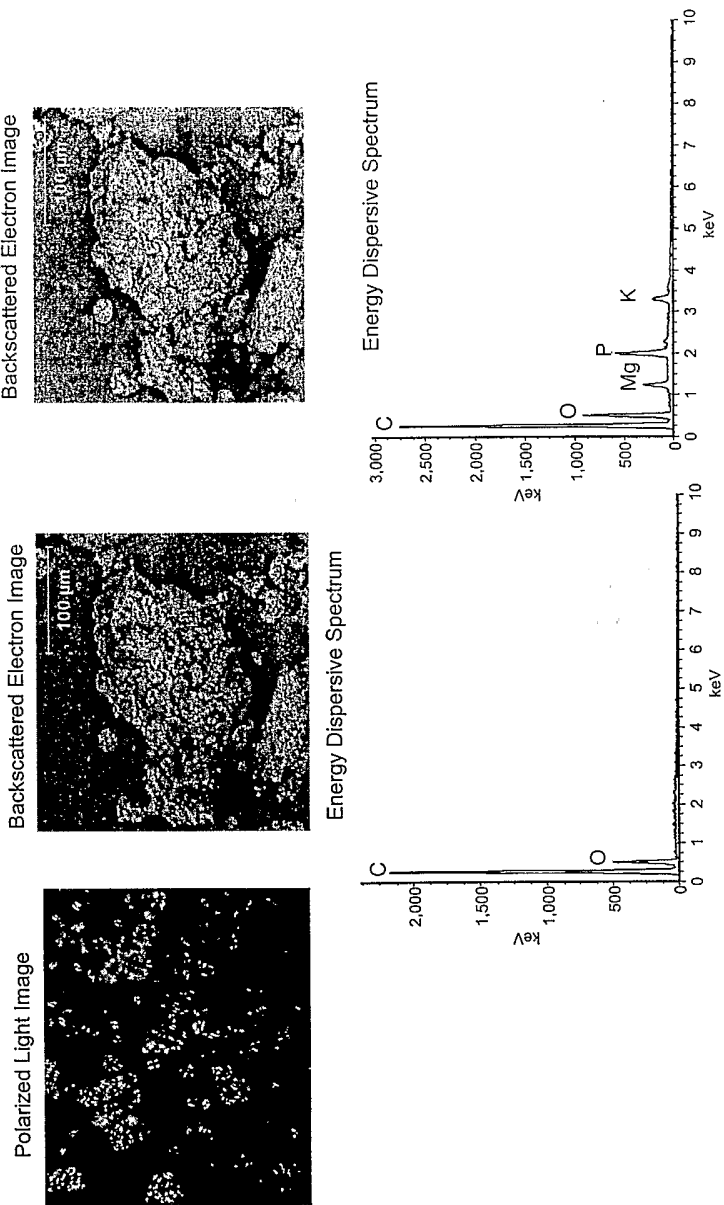
Figure 5F

Dispersive Raman and FT-IR Spectra of AFIP Powder Sample 1303-002
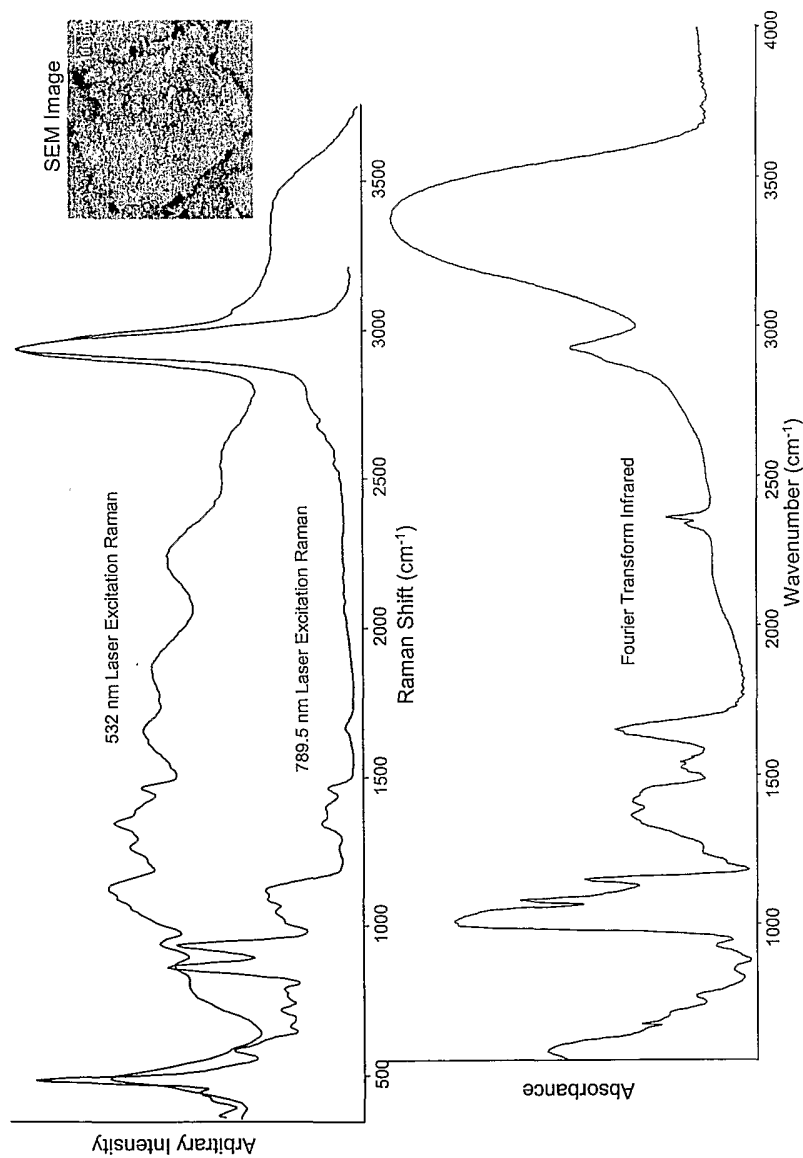
Figure 5G

SEM/EDS of AFIP Powder Sample 1303-002

Figure 5H

Dispersive Raman and FT-IR Spectra of AFIP Powder Sample 1291-006
(Bright White Particulate)
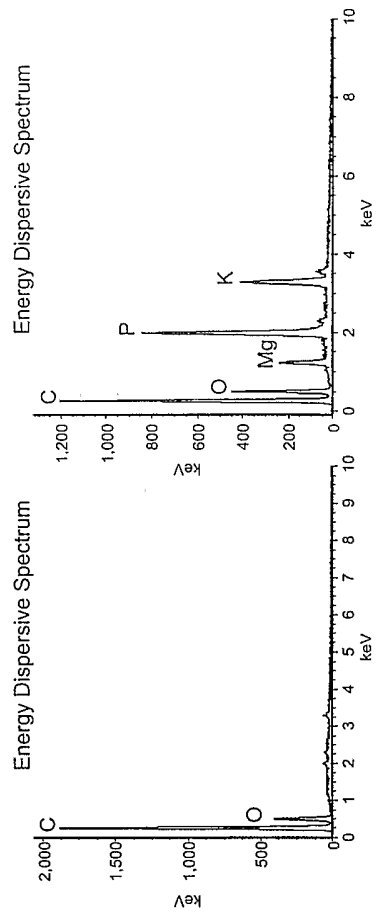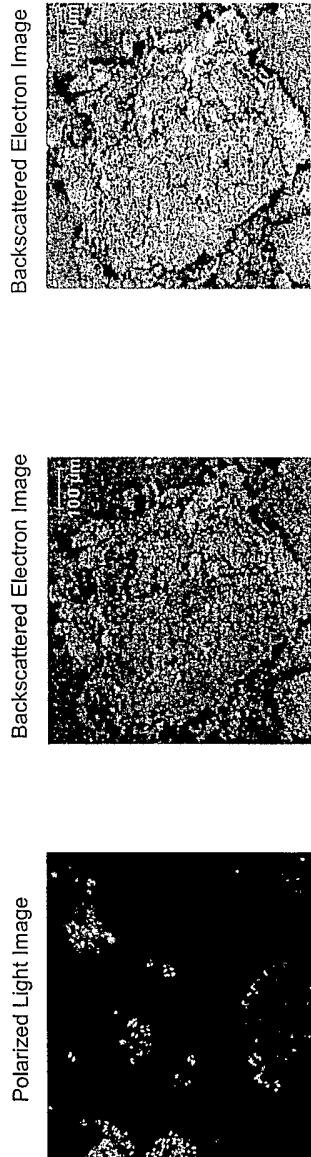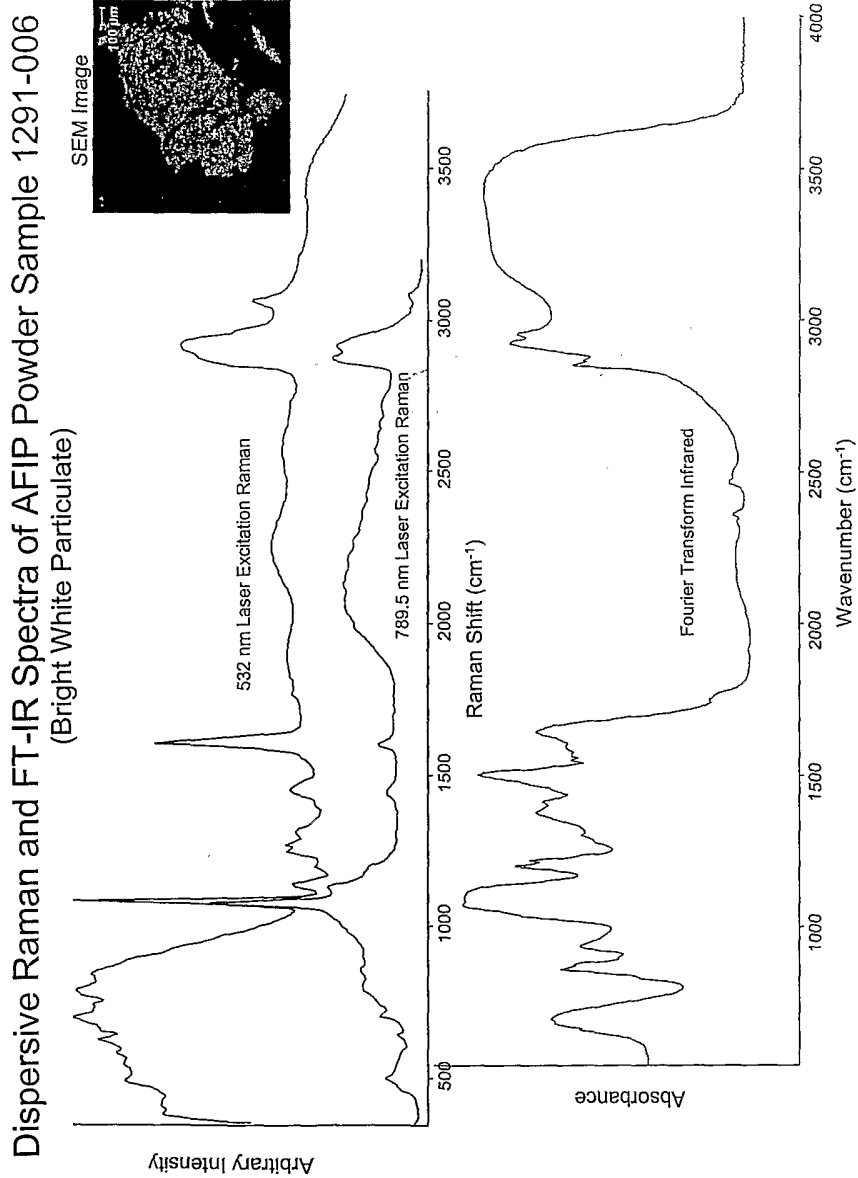
Figure 5I

SEM/EDS of AFIP Powder Sample 1291-006
(Bright White Particulate)

Backscattered Electron Image

Energy Dispersive Spectrum
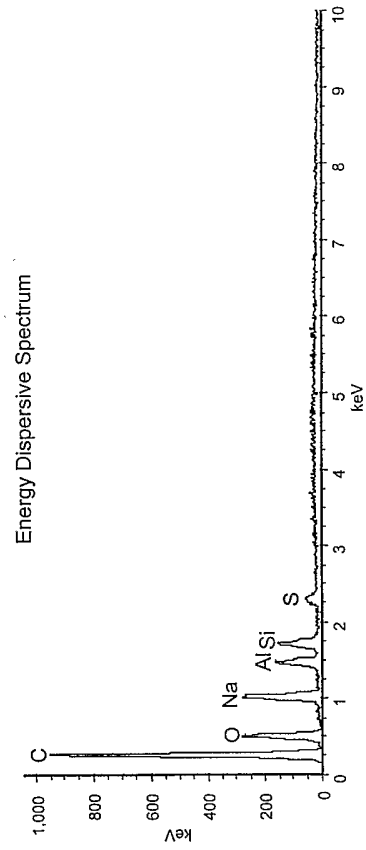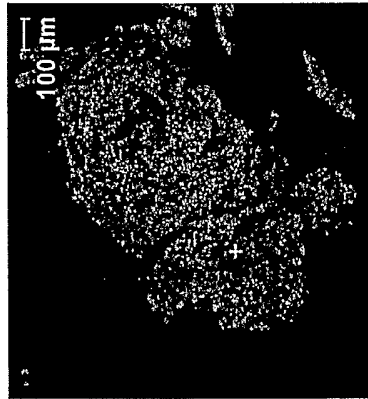
Figure 5J

Dispersive Raman and FT-IR Spectra of AFIP Powder Sample 1291-006
(Off-White Particulate)
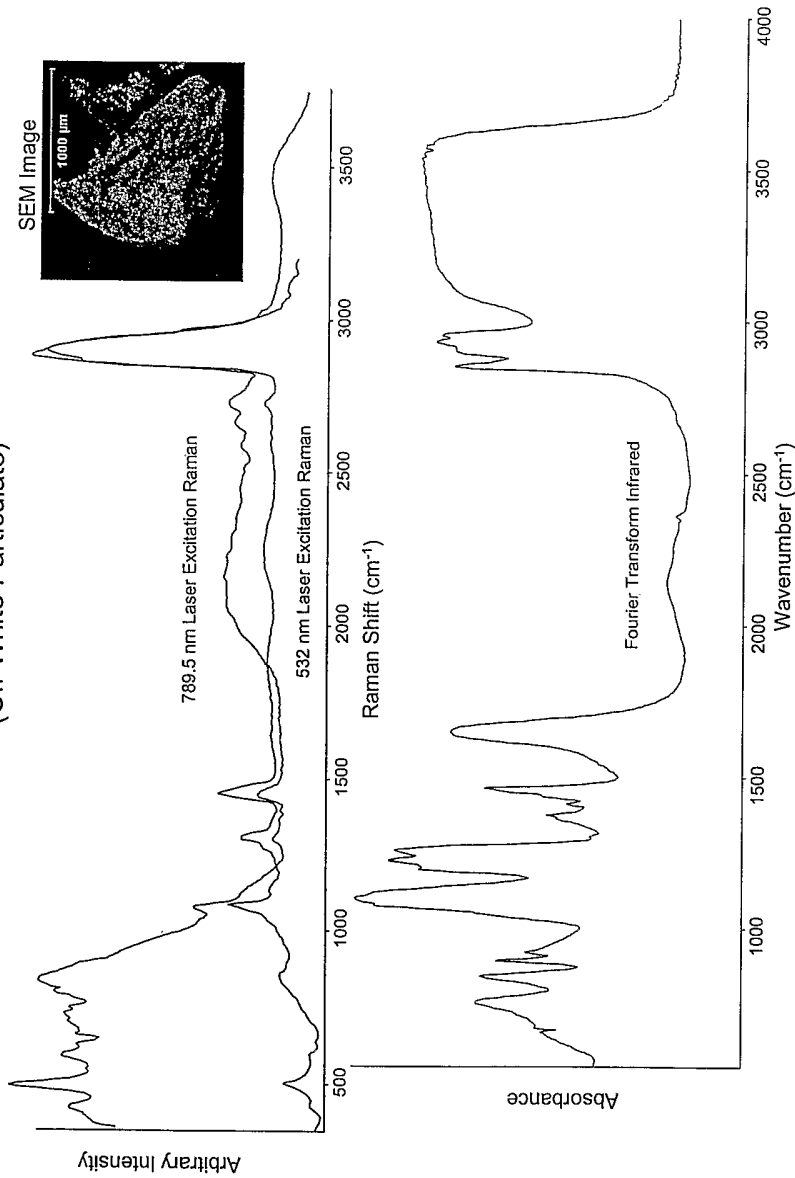
Figure 5K

SEM/EDS of AFIP Powder Sample 1291-006
(Off-White Particulate)

Backscattered Electron Image

Energy Dispersive Spectrum

Figure 5L

Dispersive Raman and FT-IR Spectra of AFIP Powder Sample 1291-006
(Rod Shaped Particulate)
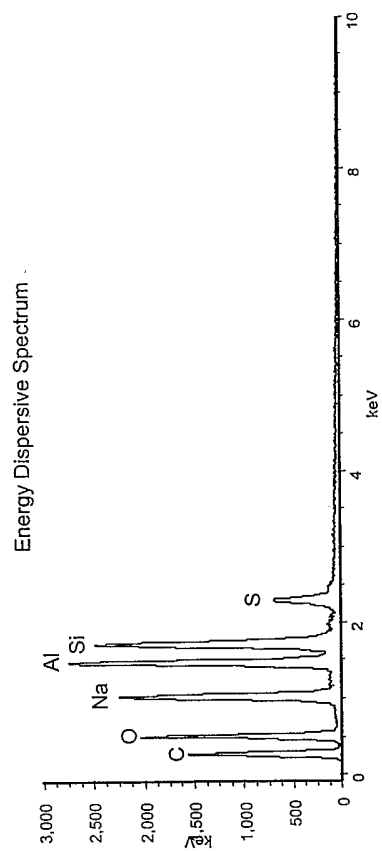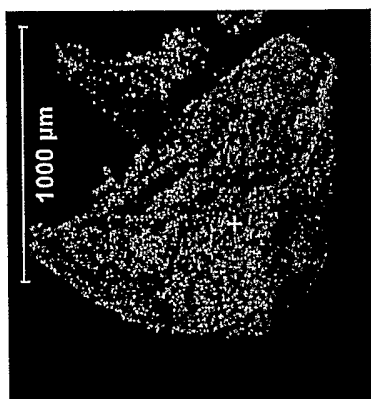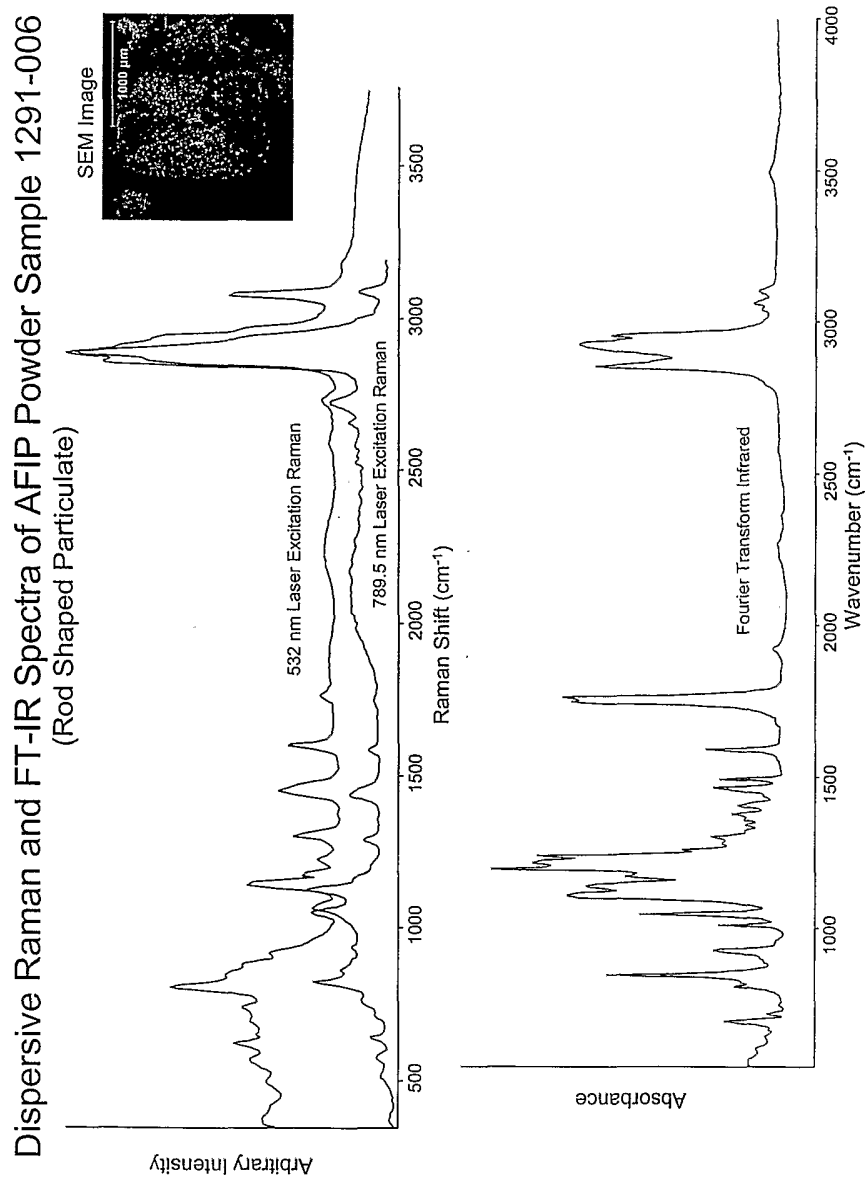
Figure 5M

SEM/EDS of AFIP Powder Sample 1291-006
(Rod-Shaped Particulate)

Backscattered Electron Image

Energy Dispersive Spectrum
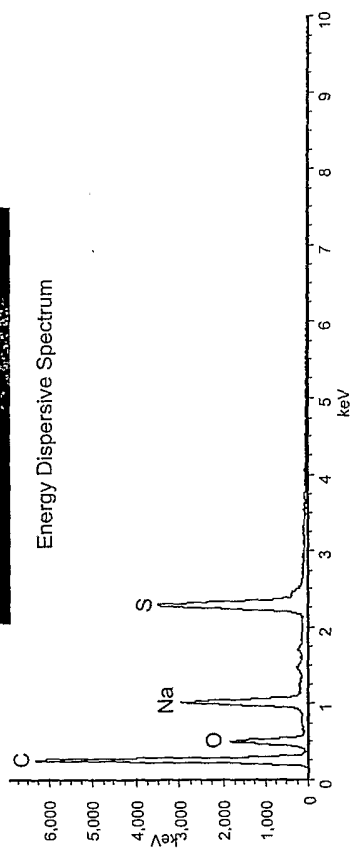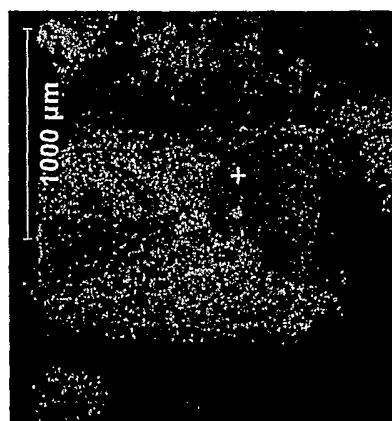
Figure 5N

Dispersive Raman Spectroscopy of Common White Powders

Dispersive Spectroscopy Parameters
Laser power density (at sample): 1675 W/cm^2
Objective: 20X
Entrance Slit: 25μm
Grating: 150 grooves/mm
CCD Exposure: 1-3 sec., 1 accumulations

☐ Sweet-n-Low
☐ Baking Soda

Raman Shift (cm^{-1})

Arbitrary Intensity
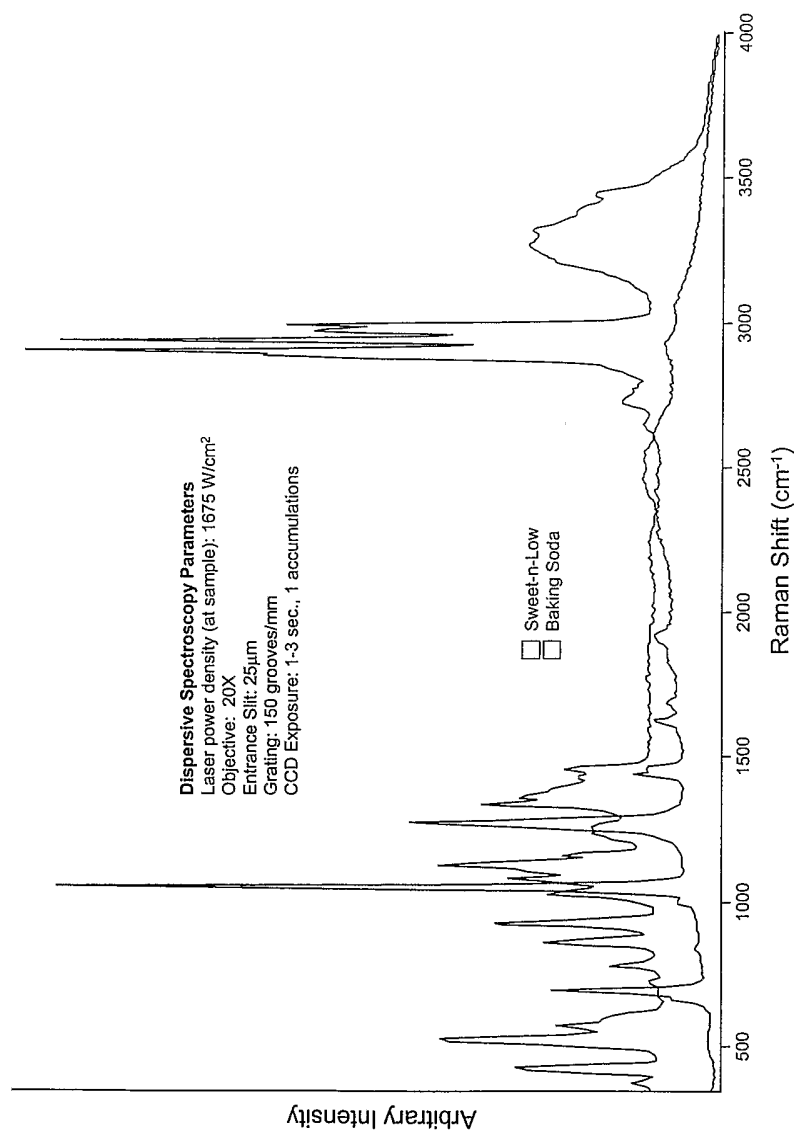
Figure 5O

Raman Chemical Imaging of Common White Powders
C-H Region

Brightfield Reflectance Image   Polarized Light Image   Raman Chemical Image

Imaging Spectrometer Spectra

Baking Soda
Sweet-n-Low

Arbitrary Intensity

Raman Shift (cm⁻¹)
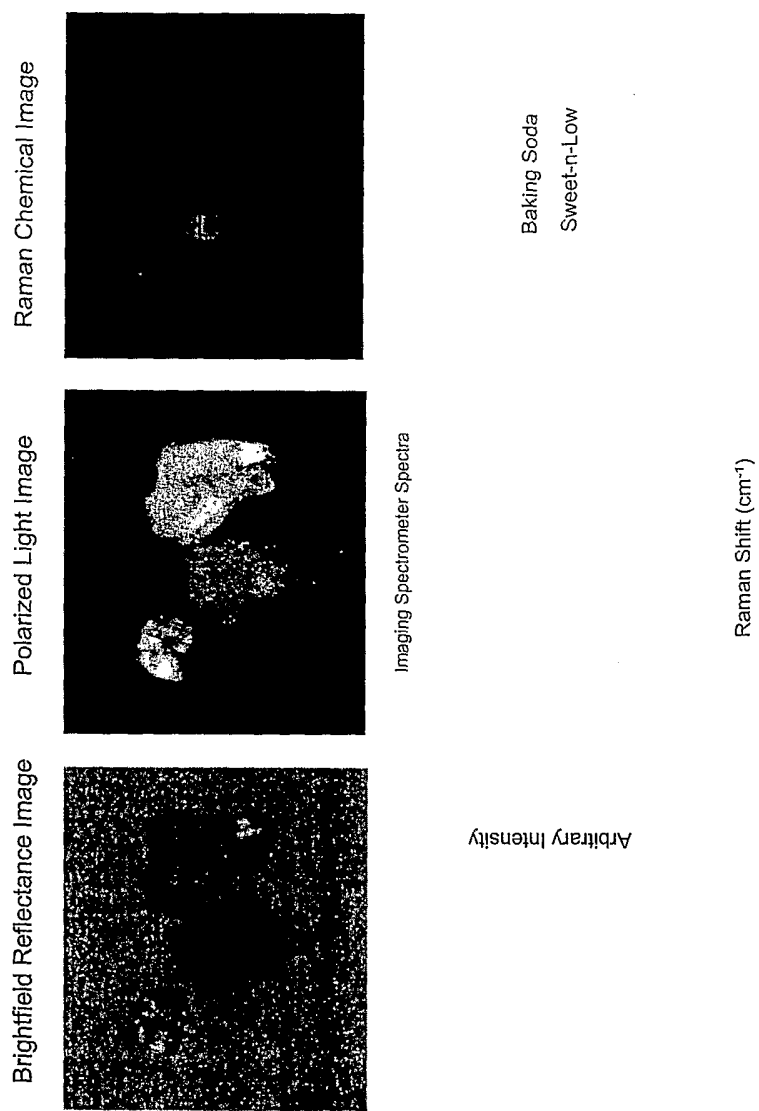
Figure 5P

Raman Chemical Imaging of Common White Powders
Fingerprint Region

Brightfield Reflectance Image

Polarized Light Image

Raman Chemical Image

Imaging Spectrometer Spectra

Arbitrary Intensity

Raman Shift (cm⁻¹)

Baking Soda
Sweet-n-Low
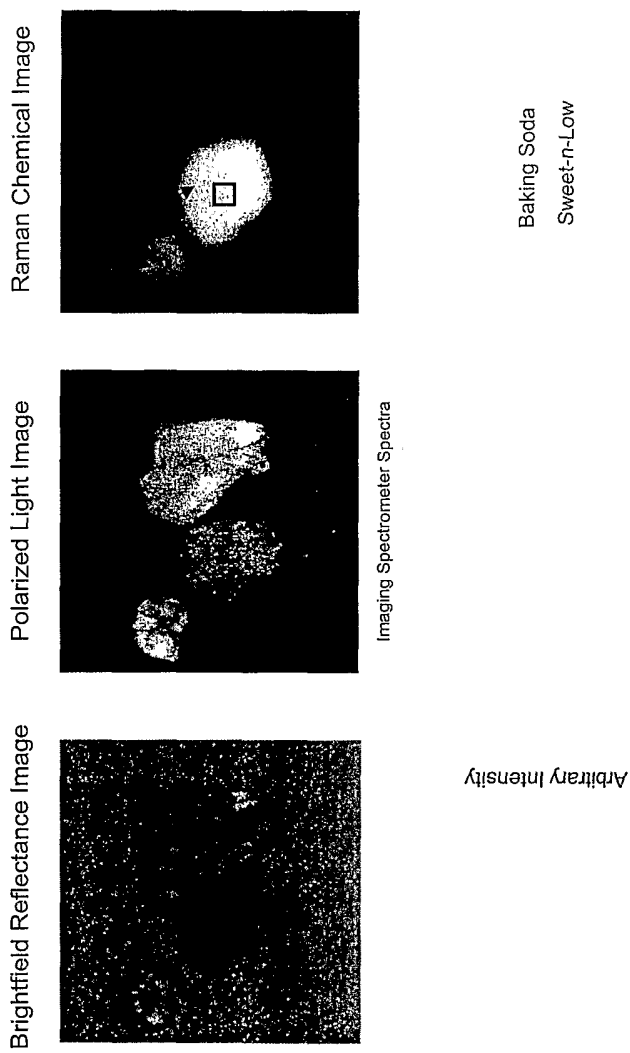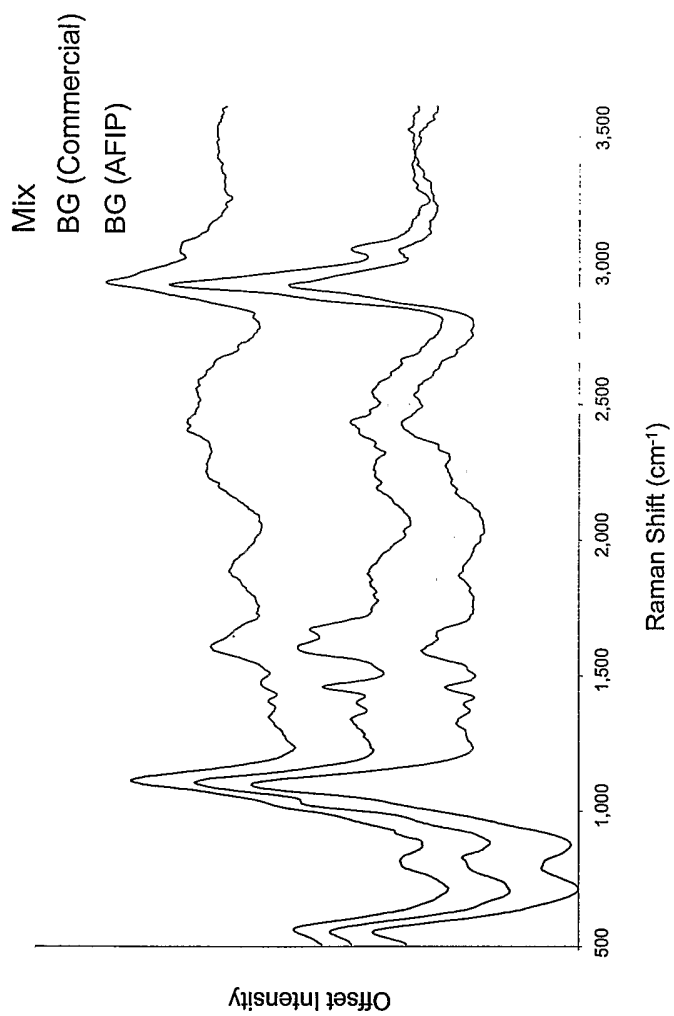
Figure 5Q

Figure 5R

Raman Chemical Imaging of Bacillus Subtillus Spore Mixture
BG (AFIP) and BG (Commercial)
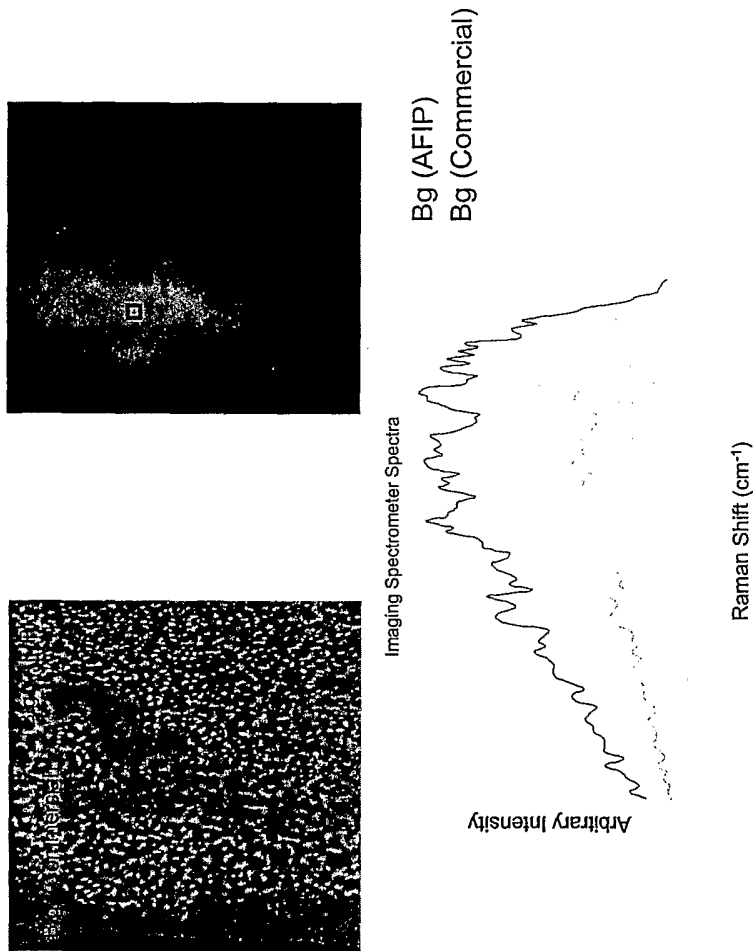
Figure 5S

Raman Chemical Imaging of Spore and Common White Powders Mixture
Bacillus Subtilis var. *Niger* (BG, AFIP sample), Baking Soda, and Sweet-n-Low

Brightfield Reflectance Image

Polarized Light Image

Raman Chemical Image
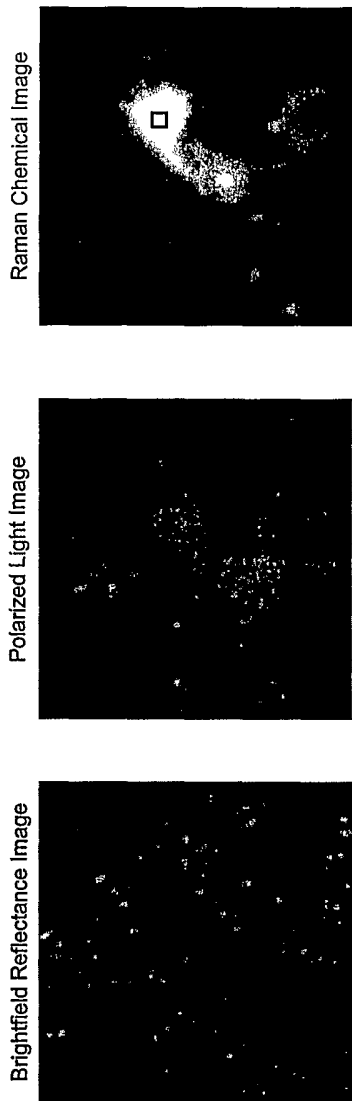
Principal Component Analysis Raman Spectra

BG
Baking Soda
Sweet-n-Low

Arbitrary Intensity

Raman Shift (cm^{-1})
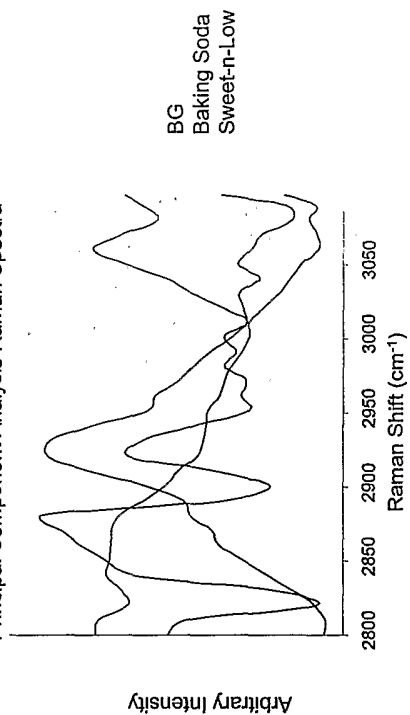
Figure 5T

Macro Fluorescence Imaging of Bacterial Spores
Spores in Powder Mixture

Normalized Background-Divided Fluorescence Spectra

Fluorescence Image of BG Spores on Envelope

RGB Fluorescence Image of BG and Powder Mix on Envelope

Condor Macro Imaging System
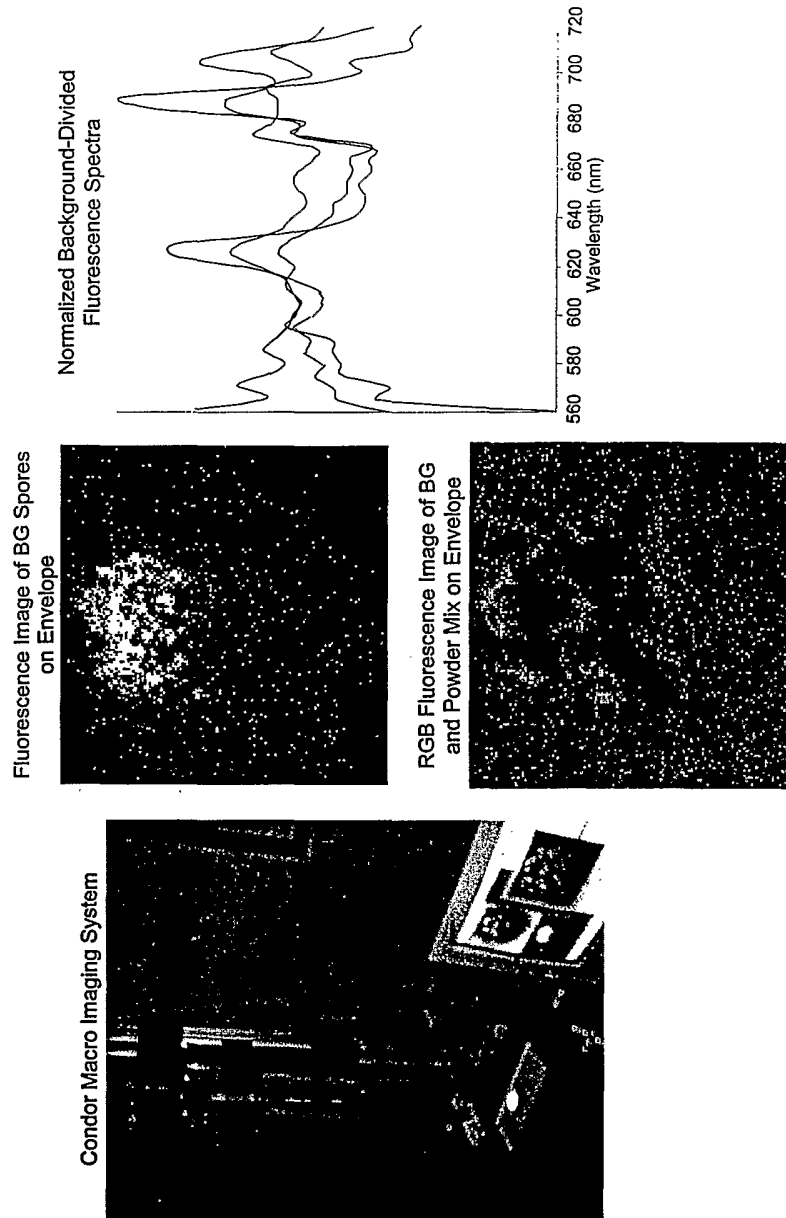
Figure 5U

Macro Fluorescence Imaging of Bacterial Spores

Imaging Moving Objects

Image Alignment Result

Fluorescence Image of Spores On Moving Envelope
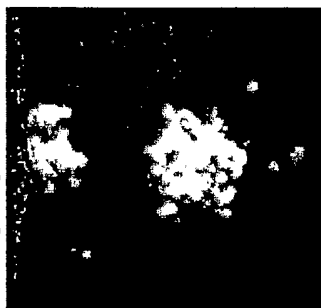
Fluorescence Image of BG Spores on Scrolling Envelope

Figure 5V
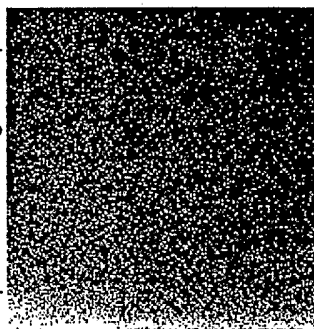
Figure 6A

Figure 6B

Figure 6C

Dispersive Raman Spectra of *Bacillus cereus* Sample
Divided Spectra

Dispersive Spectroscopy Parameters
Laser power density: 13,751 W/cm^2
Objective: 100x, 0.95 NA
Entrance Slit: 50μm
Spectrometer: 0.5m, 150 grooves/mm
CCD Exposure: 25 sec., 3 accumulations

BC
Residue

Arbitrary Intensity

Raman Shift (cm^{-1})
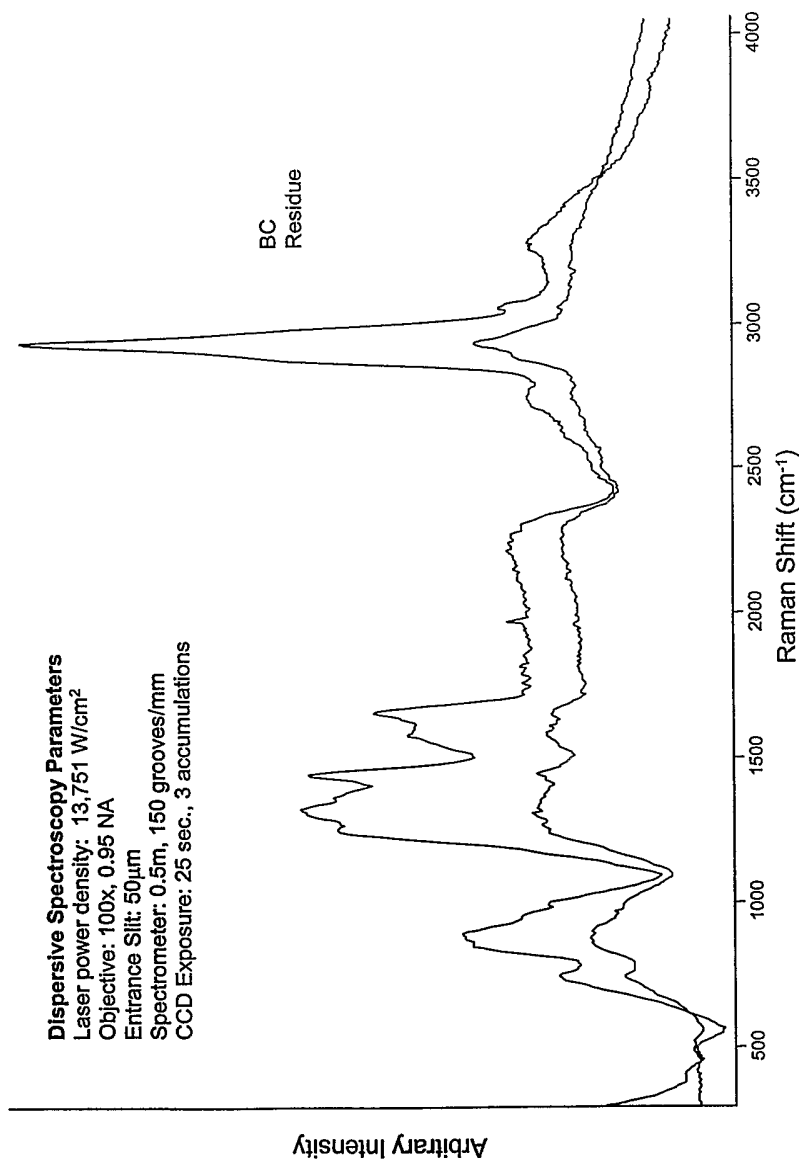
Figure 6D

Dispersive Raman Spectra of AFIP Samples
Divided Spectra

Bacillus thuringiensis
Bacillus cereus
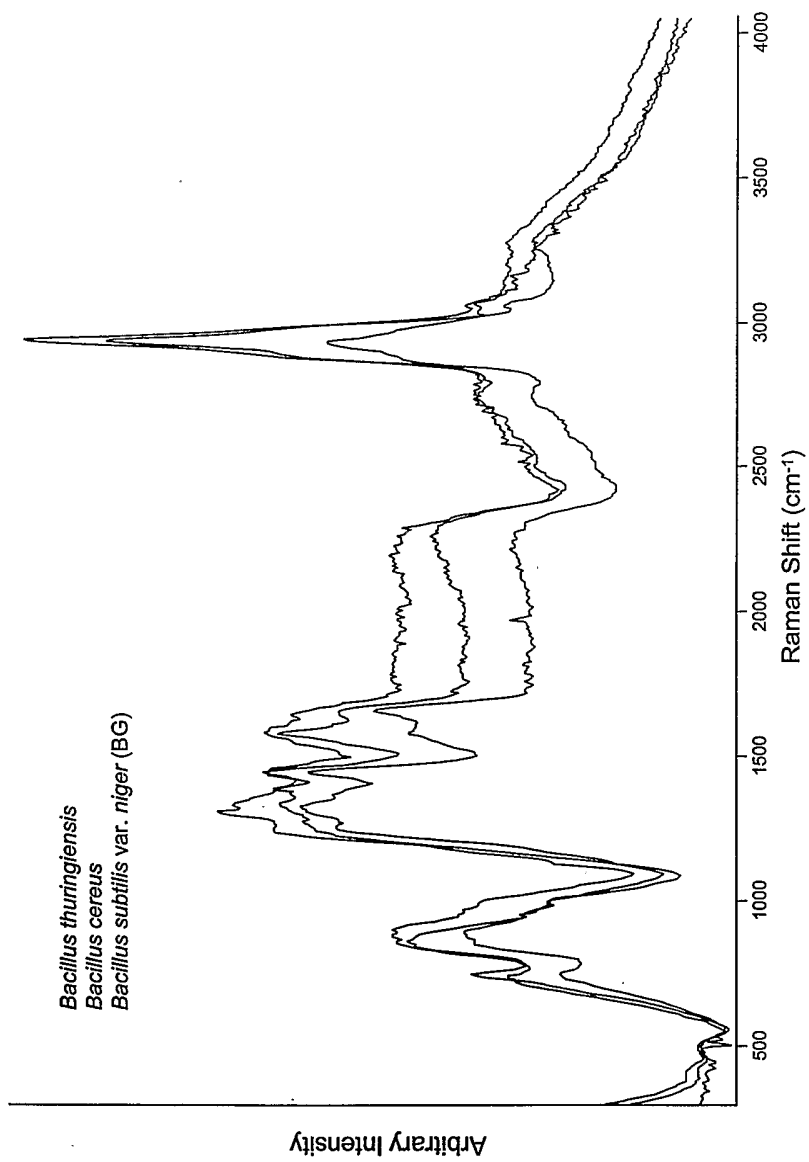Bacillus subtilis var. niger (BG)

Raman Shift (cm⁻¹)

Arbitrary Intensity

Figure 6E

Dispersive Raman Spectra of AFIP Samples
Baseline-Corrected

Bacillus thuringiensis
Bacillus cereus
Bacillus subtilis var. niger (BG)

Raman Shift (cm⁻¹)

Arbitrary Intensity

Figure 6F

Figure 7

G Growth Media
AK2 Growth Media

Figure 8

Figure 9

Bacillus anthracis (BA)
Clostridium sporogenes (botulism sim.)
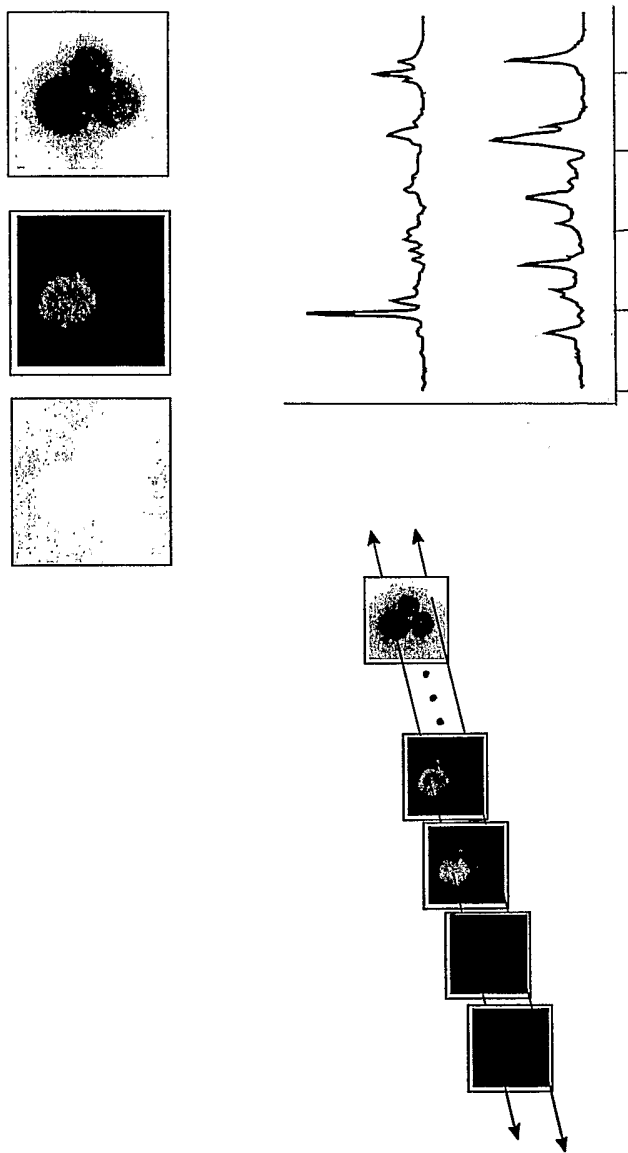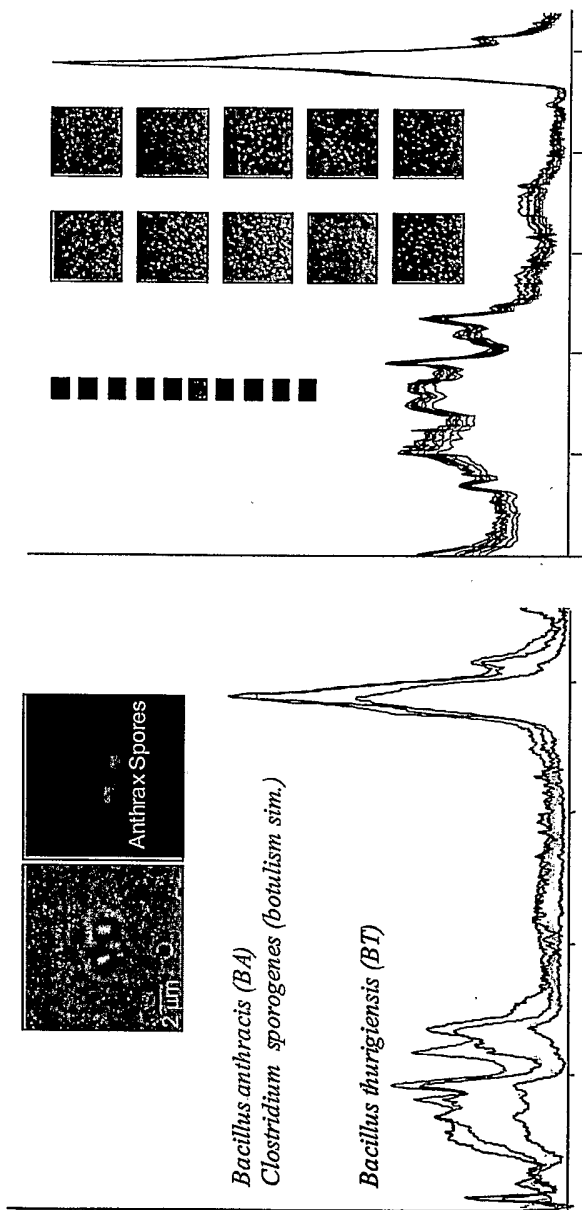
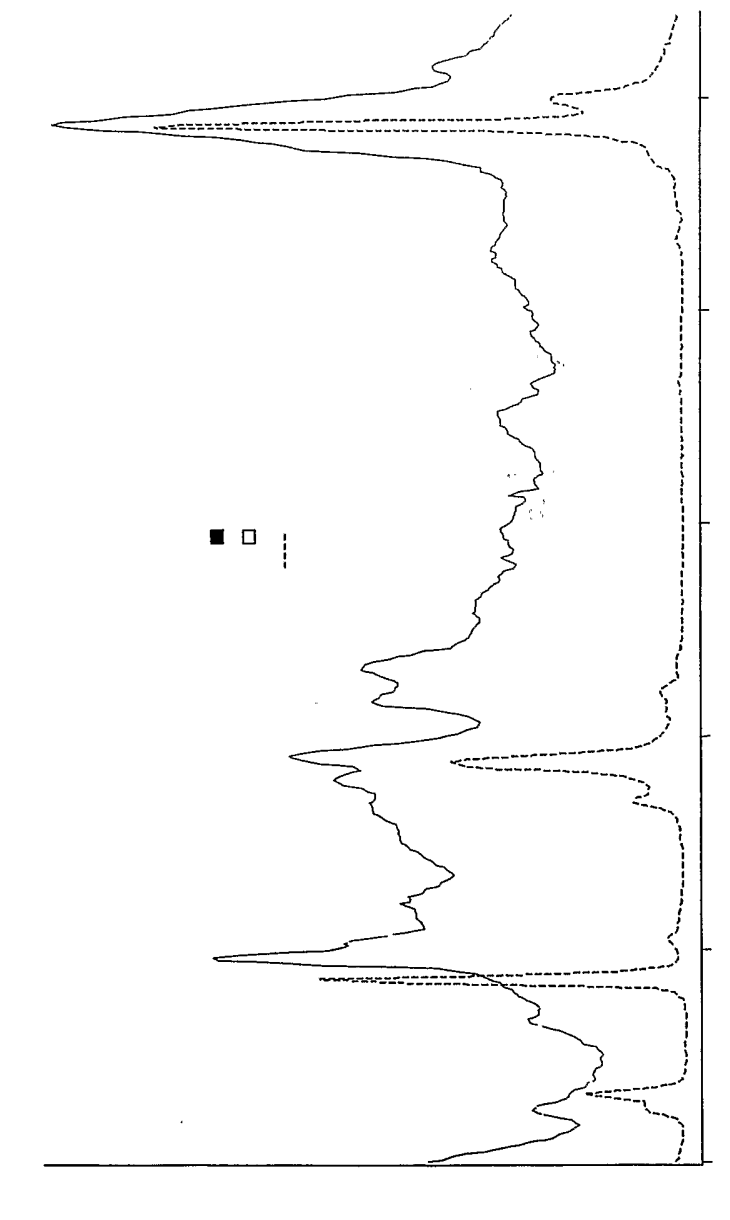*Bacillus thurigiensis (BT)*
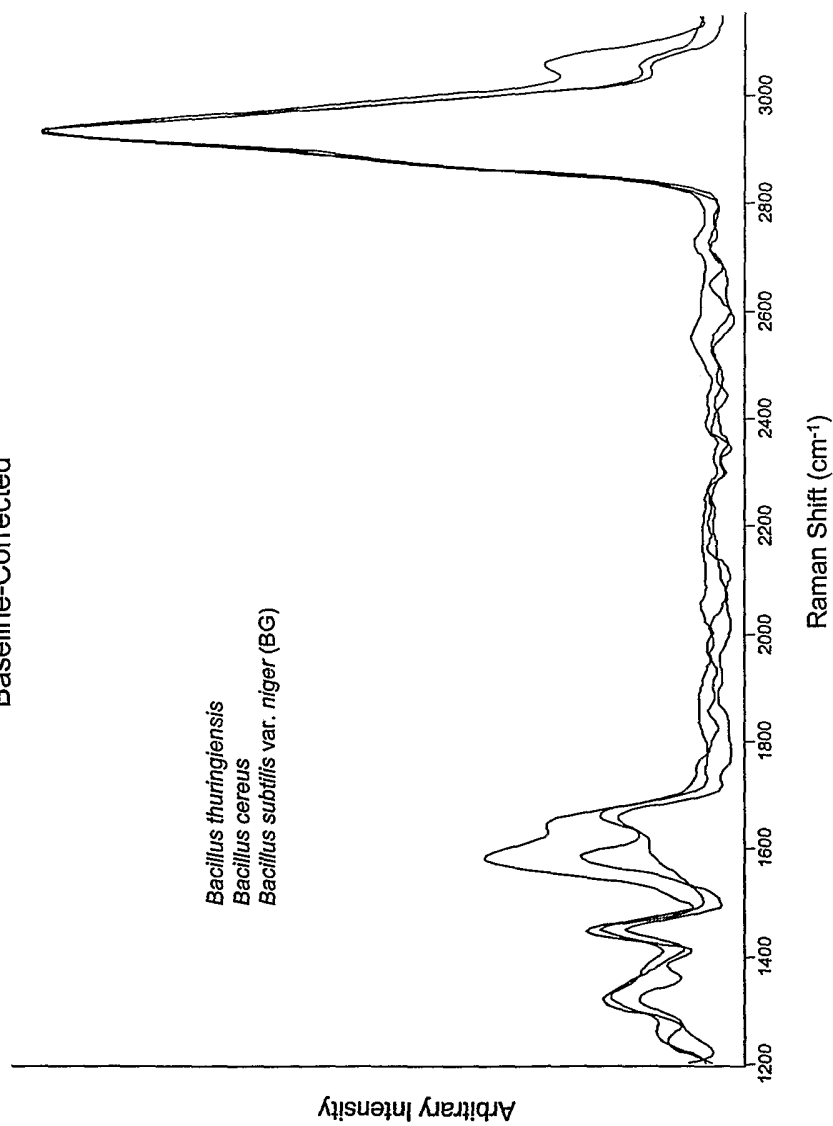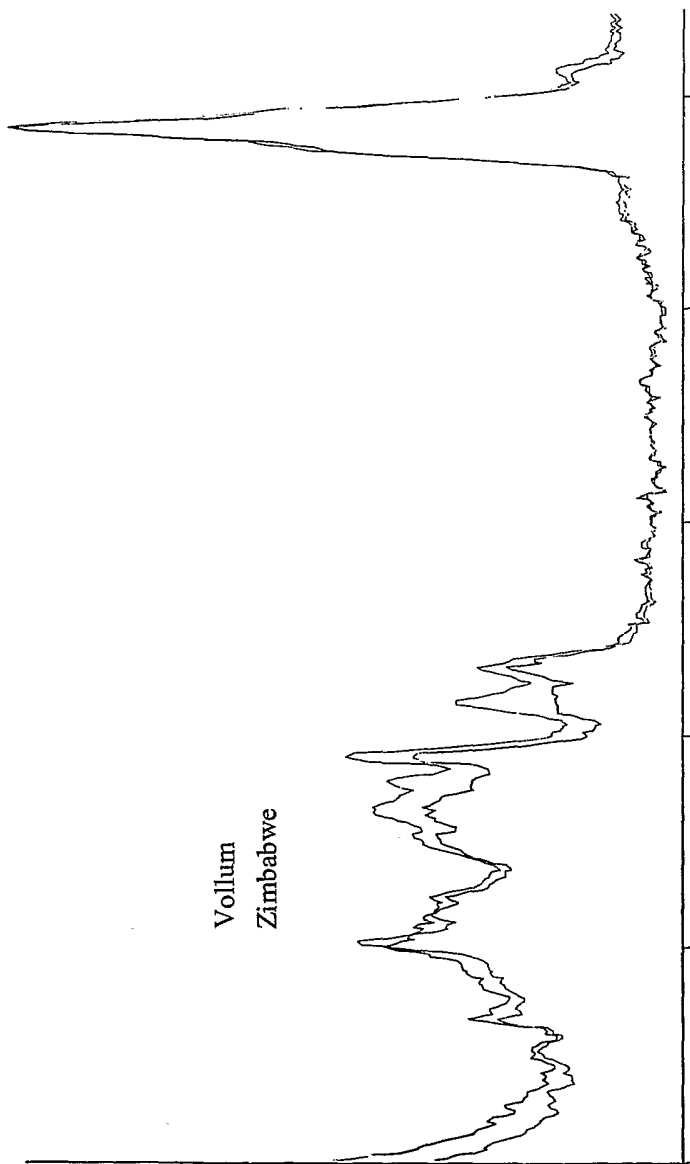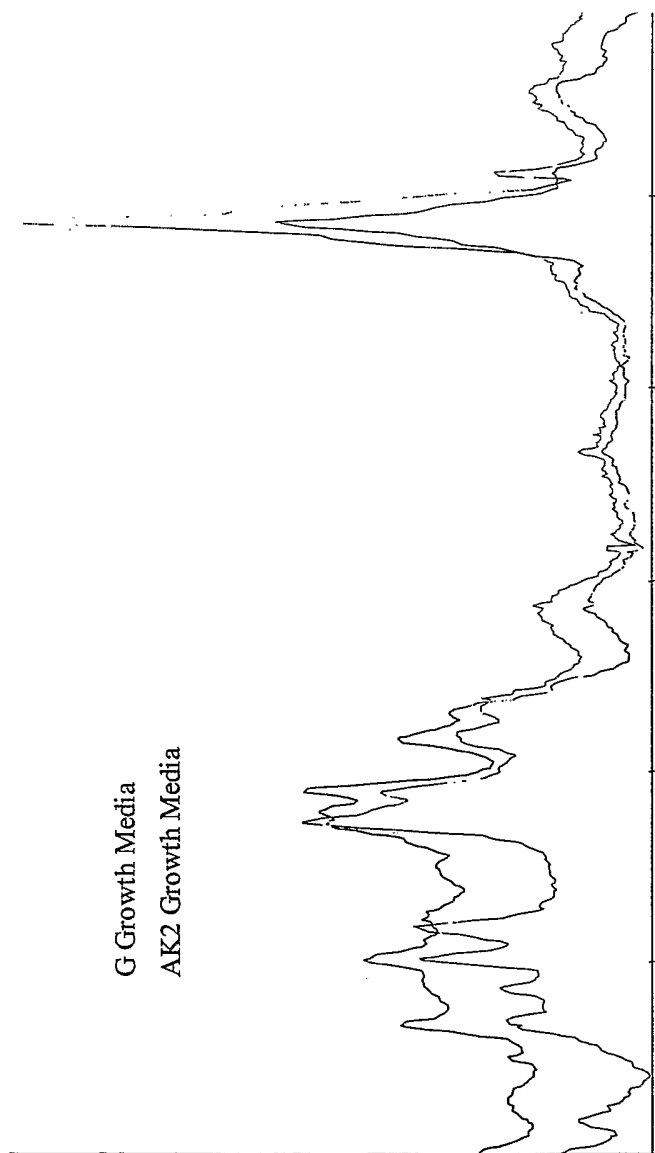
Figure 10

Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/00868

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :   G01J 3/44
US CL   :   356/301

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
    U.S. : 356/301

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,866,430 A (GROW) 02 February 1999 (02.02.1999), see column 1, lines 11-19; column 20, lines 17-31; column 22, lines 35-53; column 35, lines 17-30; and column 56, lines 1-20.	1-6, 8, 21-56 ----------- 7, 9-20
Y	US 6,002,476 A (TREADO) 14 December 1999 (14.12.1999), see column 1, line 47-column 2, line 56.	7, 9-13, 20
Y	US 5,539,517 A (CABIB et al.) 23 July 1996 (23.07.1996), see column 2, lines 63-column 2, line 33; column 5, lines 41-46; column 11, lines 6-16 and 32-40; and column 16, lines 14-19.	14-19

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent published on or after the international filing date
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"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 May 2003 (08.05.2003)	04 JUN 2003

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	F. L. Evans Telephone No. (703) 308-0956

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Form PCT/ISA/210 (second sheet) (July 1998)