

Detecting *Bacillus cereus* spores on a mail sorting system using Raman spectroscopy

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The ability of Raman spectroscopy to detect anthrax-causing spores as they pass through a mail sorting system was investigated. A pump was connected to an existing vacuum manifold on a commercial sorter, and a filter designed to capture 0.5–3 μm particles was placed in-line. A standard business letter containing 0.23 g of *Bacillus cereus* spores, a *Bacillus anthracis* surrogate, was placed in a stack of 20 letters and passed through the system. Raman spectra of the filter positively identified the captured material as bacterial spores by the dominant calcium dipicolinate Raman spectral bands associated with the spore core. A limit of detection, using 400 mW of 785 nm laser excitation for a 1-s acquisition, is estimated at 4.5 mg. The ability of a Raman spectroscopy based system to detect and prevent the distribution of a letter containing gram levels of anthrax spores is discussed. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: anthrax; *Bacillus cereus*; mail sorters; homeland security

INTRODUCTION

The October 2001 distribution of anthrax-causing spores through the USA postal system and the subsequent infection and death of several postal and national media employees intensified the need for methods to rapidly detect and identify such biological warfare agents. In particular, instrumentation is required to detect *Bacillus anthracis* spores in mail sorting systems at or near real-time to prevent distribution and save lives. The challenges are formidable considering that the Center for Disease Control estimates that inhalation of 10 000 spores or 100 ng will be lethal to 50% of an exposed population,^{1,2} while a typical mail sorting machine distributes one to five letters in 1 s. Furthermore, a high level of discrimination is required to differentiate spores from a multitude of dust particles and hoax materials.

Positive identification of *B. anthracis* spores or other biological agents requires nucleic acid sequencing (deoxyribonucleic acid, DNA or ribonucleic acid, RNA). Polymerase chain reactions (PCR) or multiplication of the microorganism through culture growth are used to generate the millions of copies that are required to accurately sequence the nucleic

acid. Although *B. anthracis* identification by PCR has been demonstrated by sequencing the capsular protein B gene in under 1 h,³ the CDC required several days to identify the contents of the letter addressed to Senator Tom Daschle as *B. anthracis*, and longer still to verify that it was the deadly Ames strain.¹ Immunoassay methods are also being developed that use competitive binding of the bioagent (as an antigen) and its labeled conjugate for a limited number of antibodies. Early methods were relatively fast (~40 min) and semi-quantitative, but did not employ a well-defined anthrax antigen.^{4,5} An immunoassay has recently been developed that uses B cells with pathogen specific antibodies.⁶ This immunoassay can differentiate pathogens, such as *B. anthracis* or *Yersinia pestis*, and provide a response within 1 min. However, challenges remain in developing a field-worthy sensor in which the viability of the B cells can be maintained. Another method combines laser-light scattering and laser-induced fluorescence.⁷ Light scattering can identify particles within the 2–10 μm diameter respirable range, while fluorescence can determine whether the particles are biological by detecting tryptophan or tyrosine. Unfortunately, many naturally occurring bioaerosols are within this size range and cause false alarms.⁸

A number of other methods are focusing on the detection of calcium dipicolinate (CaDPA) and its derivatives as a *B. anthracis* signature. This is a valid approach in that

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only spore-forming bacteria contain CaDPA, and the most common potentially interfering spores, such as pollen and mold spores, do not. Relatively fast methods have been developed to extract CaDPA chemically and then detect it or its acid form, dipicolinic acid, by mass spectrometry,^{9,10} luminescence^{11,12} or fluorescence.¹³ Yet another method employs a quartz wire to collect the particles, as well as to pyrolyze them using current induced heat, then gas chromatography to separate the volatilized components, and a time-of-flight ion mobility spectrometer to detect DPA.¹⁴ Although these latter methods provide a relatively high degree of discrimination and sensitivity, they still require significant time due to sample handling and analysis.

As an alternative, Raman spectroscopy is attractive in that very small samples can be measured without manipulation. The sample need only be placed at the focal spot of the excitation laser and measured. Moreover, the rich molecular information provided by Raman spectroscopy usually allows unequivocal identification of chemicals and biologicals. As early as 1974 the Raman spectrum of *Bacillus Megaterium* was measured and shown to be dominated by CaDPA.¹⁵ However, the spectrum was of pure spores and took hours to acquire. By 1992, the improvements in Raman instrumentation and the use of resonance enhancement had increased limits of detection dramatically and reduced analysis time to less than 1 h.¹⁶ Here we present a preliminary study aimed at establishing baseline capabilities of Raman spectroscopy to measure spores collected from mail as it passes through a sorting system.

EXPERIMENTAL

Dipicolinic acid (2,6-pyridinedicarboxylic acid, DPA) and sulfur were used as received from Sigma-Aldrich (Milwaukee, WI). Calcium dipicolinate (CaDPA) was prepared from sodium dipicolinate, prepared in turn from DPA according to previous publications.¹⁶ *Bacillus cereus* spores were produced by growing the bacteria on nutrient agar plates at 30 °C for 5 days. The spores were collected by scraping them into distilled water and pelleting them by centrifugation at $12\,100 \times g$ for 10 min. The spores were washed four more times in distilled water by centrifugation. The spore pellet was re-suspended in distilled water and lyophilized, and then scrapped into glass vials for Raman spectral measurements. Approximately 1 g of spore material was produced for this study. This sample had a measured density of 0.081 g ml^{-1} , which indicates a large amount of entrained air.

All measurements were performed using a 785-nm diode laser (Process Instruments Inc. model 785-600, Salt Lake City, UT), a Fourier-transform Raman spectrometer (Real-Time Analyzers, model IRA-785, East Hartford, CT), and a silicon photo-avalanche detector (Perkin-Elmer model C30902S, Stamford, CT). Fiber optics were used to deliver the excitation beam to the sample probe and the scattered radiation to the interferometer (2-m lengths of 200- and

365- μm core diameter, respectively, Spectran, Avon, CT). A 6-mm diameter $f/2$ achromat was used to collimate the laser beam exiting the source fiber optic, while a dichroic filter was used to direct the beam through a 24-mm $f/0.7$ aspheric lens, which focused the beam to a 300- μm spot on the sample. The scattered radiation was collected back along the same optical axis, while a second $f/2$ lens focused the beam into the collection fiber optic. A short pass filter was placed in the excitation beam path to block the silicon Raman scattering generated in the source fiber from reflecting off sampling optics and reaching the detector. A long pass filter was placed in the collection beam path to block the sample Rayleigh scattering from reaching the detector.

A laboratory 'spore capture' test system was designed to evaluate capabilities of various filters, and optimize vacuum and flow parameters. The system consisted of a 2.2-kW, 7.6-amp vacuum (ShopVac, model QSP) equipped with a standard 3.8-cm diameter hose. Five-cm square Plexiglas plates with either a 2- or 4-cm orifice to mount filters were interfaced 0.75 m from the hose end, while 0.635-cm stainless steel and Teflon tubing were interfaced 1.5 m from the hose end and connected to a manometer (Dwyer, Mark II model 25, Michigan City, IN) to measure flow and a gauge to measure pressure (Ashcroft, type 1001T, Ashcroft, CT), respectively. Three filters used in standard air filtration systems to trap 0.5–3 μm diameter particles were tested, one consisted of spun glass fibers (Precisionaire, Inc. model NaturalAire Micro Particle Air Filter, St Petersburg, FL), while the other two filters consisted of electrostatically charged 'synthetic' fibers (Glassfloss Industries, z-line series, Lancaster, OH, and 3M, model Filtrate Ultra Allergen, Maplewood, MN). The filters were cut to fit between the Plexiglas plates.

Process measurements were performed using ID Mail's Dispatcher at their facilities (Fig. 1). Either the laboratory spore capture system or the sorter system vacuum pump was connected to the vacuum manifold of the *Dispatcher*. A filter was placed in-line between the manifold and the ShopVac or sorter system pump to collect samples that consisted of either sulfur or *B. cereus* spores. The system was operated at sorting speeds of 25–125 letters per minute.

RESULTS AND DISCUSSION

Bacillus cereus spores were placed on a glass cover slip and Raman spectra were collected using 400 mW of 785 nm laser excitation at the sample, while a 13-min acquisition time was used to obtain high quality Raman spectra suitable for analysis (Fig. 2). A mild fluorescent background was easily modeled and subtracted. A comparison with the Raman spectrum of CaDPA shows that this component dominates the spore spectrum. Many of the observed spore bands have been assigned to CaDPA,^{15,16} specifically: 1017 cm^{-1} to the symmetric ring stretch, 1397 cm^{-1} to the O—C—O symmetric stretch, 1443 cm^{-1} to the symmetric ring C—H bend, 1573 cm^{-1} to the asymmetric O—C—O stretch,

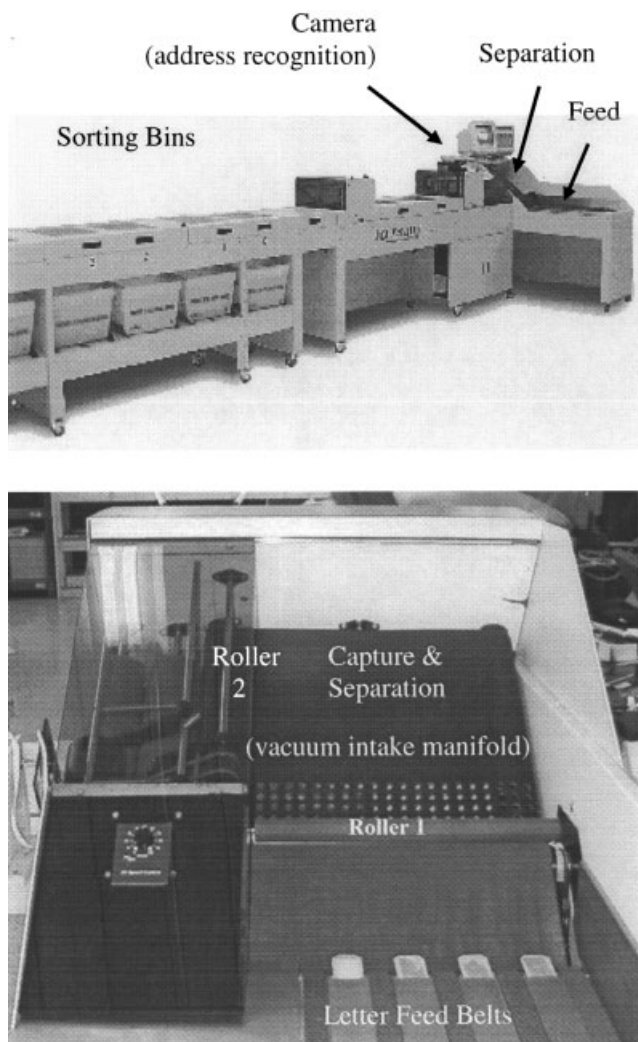


Figure 1. ID Mail's Dispatcher with close-up of Feed and Separation section. Fanned mail is placed on the feed belts, roller 1 draws the bottom-most letter to the capture point, a vacuum manifold flattens the letter, roller 2 then draws the letter to the camera, the address is matched to a bin number, and belts send the letter to that bin.

1604 cm^{-1} to the asymmetric carboxylate stretch, 1660 cm^{-1} to the carbonyl stretches (bound CaDPA), and 3085 cm^{-1} to the symmetric ring C—H stretch. The 1000-cm^{-1} bands in both the CaDPA and spore spectra are coincident in wavenumber with the symmetric ring-stretching mode of DPA, and may indicate some impurity in the CaDPA sample and some DPA in the spores. The broad 2930 cm^{-1} band in the spore spectrum can be assigned to protein C—H stretching modes. Similarly, the added intensity of the 1443 cm^{-1} band likely represents contributions from the protein C—H deformation modes. CaDPA bands at 772 , 1593 , and 1710 cm^{-1} are virtually non-existent in the spore spectrum. Although the 772 cm^{-1} band might be attributed to DPA, again as an impurity in the CaDPA sample, the other

two bands cannot. It is likely that these vibrational modes are modified when CaDPA is incorporated into the surrounding spore structure.

Three filters used in standard air filtration systems to trap $0.5\text{--}3\text{-}\mu\text{m}$ diameter particles were tested. One consisted of spun glass fibers (Precisionaire), while the other two filters consisted of 'synthetic' fibers (Glassfloss and 3M). Since the filters were likely to contribute to the Raman measurements, their spectra were measured. The filter composed of spun glass fiber produced a broad sloping fluorescent background, possibly due to processing or a coating (Fig. 3). The spectra for both synthetic-fiber-based filters were near identical and easily identified as polypropylene–polyethylene copolymers. Although the latter polymer fibers contribute a number of spectral bands, their intensity overall, is substantially lower than the weakly fluorescing glass fiber, and were therefore used.

Development of the vacuum system and characterization of the air filters employed sulfur as a test analyte, since

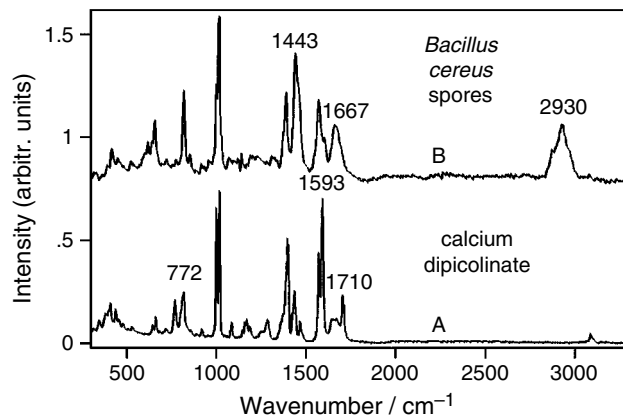


Figure 2. Raman spectrum of (A) calcium dipicolinate and (B) *Bacillus cereus* spores (fluorescence removed). Conditions: 400 mW of 785 nm at the sample, 13 min .

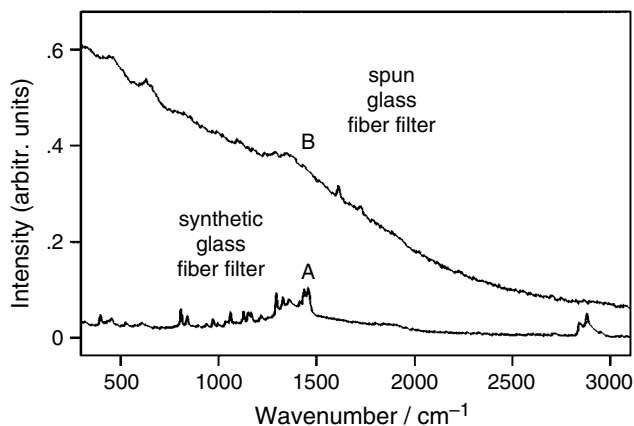


Figure 3. Raman spectra of (A) synthetic and (B) spun glass fiber based filters. Conditions: 500 mW of 785 nm , 30 sec . Note: spectra are on the same scale.

it has a very high Raman scattering cross-section that ensured detection. Fifty-milligram samples were placed in a plastic boat below the vacuum hose. The vacuum was then turned on for 30 s and shut off removing all of the boat contents. The filters were weighed before and after each experiment to determine the particle loading. In general it was found that all three filters were equally efficient at collecting particles, and that the smaller orifice (2 versus 4 cm diameter) and slower flow rates (9 versus 20 m s⁻¹) yielded the best collection efficiencies, which approached 50%. In all cases a pressure drop of 115 mm Hg was measured. Raman spectra corroborate the favorable conditions and identified the highest concentrations to be near the filter centers (Fig. 4). Spectra were signal averaged for 30 s to match the sample collection time.

Due to a limited supply of spores only one 45-mg sample was measured. The collection efficiency of 14.6% was approximately half of that obtained for the sulfur measurements under identical, but not optimized, flow conditions. Nevertheless, a reasonable quality Raman spectrum of *B. cereus* spores was obtained (Fig. 5).

Process measurements were performed at ID Mail using their Dispatcher system. The Dispatcher is a standard mail sorting system and can be used to sort incoming mail by mail stop or floor in large companies or organizations in office buildings. In use (see Fig. 1), stacked mail is placed on the feed belts, roller 1 draws the bottom-most letter to the capture point, a vacuum manifold flattens the letter, roller 2 then draws the letter to a digital camera, the address is read and matched to a bin number, and belts convey the letter to that bin. The system can sort mail at rates from 25 to 125 letters per minute. Initially, the laboratory vacuum system was connected to the vacuum manifold of the Dispatcher to allow matching laboratory air flow conditions. For the initial experiments a standard number 10 business envelope

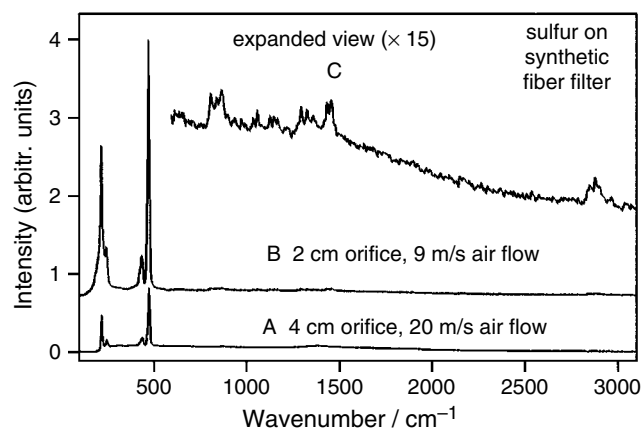


Figure 4. Raman spectra of sulfur on a synthetic filter collected using (A) 4 cm orifice, 20 m s⁻¹ air flow, and (B) 2 cm orifice and 9 m s⁻¹ air flow. (C) X15 expanded view of (B) showing filter spectral contribution. Conditions: 500 mW of 785 nm, 30 s.

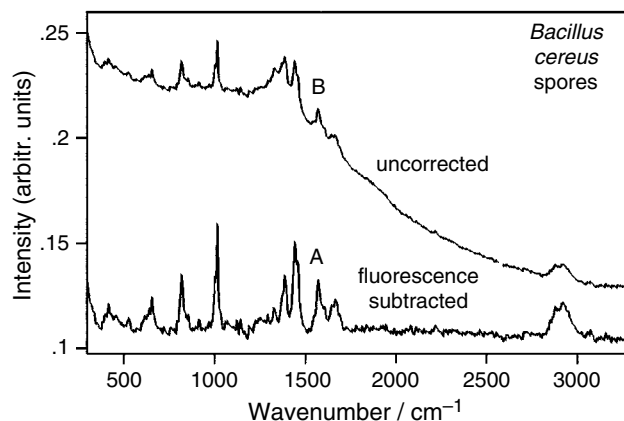


Figure 5. Raman spectrum of *Bacillus cereus* spores collected on a synthetic filter (A) baseline corrected (fluorescence removed) and (B) uncorrected. Conditions: 400 mW of 785 nm at the sample, 13 min.

containing 1 or 2 g of sulfur was placed in the middle of a stack of 20 envelopes. These were fanned onto the moving feed belts with the address facing up and the flap facing down. This is standard operation, as the camera looks down upon the passing mail. Each filter was removed and examined after a set of mail passed through the system. Sulfur was visually observed on the filters at sorting speeds of 25 or 50 letters per minute, flow rates of 9 m s⁻¹ and a 125 mm Hg pressure drop using the laboratory system, but less so using the Dispatcher vacuum pump, which reduced the pressure drop to 100 mm Hg. The filters measured by Raman spectroscopy on-site easily yielded sulfur spectra, even at sorting speeds of 125 letters per minute when the filter showed no visible sign of sulfur (Fig. 6A). At this sorting speed both the filter mass and the sulfur band intensities indicate that approximately 1% of the sulfur from inside the envelope was collected on the filter.

Again, a limited supply of spores allowed preparing only one test letter, which contained 0.226 g of spores. To maximize the chance of obtaining spores, the system was slowed to 50 letters per minute. Approximately 1.45 mg of spores were collected on a filter, or 0.66%, consistent with the sulfur measurements. A Raman spectrum of spores on this filter was obtained using a 5-min acquisition time (Fig. 6B). A signal-to-noise ratio (S/N, where N was the standard deviation for the average value from 2200–2600 cm⁻¹) of 17 for the 1020 cm⁻¹ band suggests a limit of detection (LOD) of 0.26 mg based on a S/N equal to 3. A more modest LOD of 4.5 mg is estimated for a 1-s spectral analysis time using the current collection system. This would be sufficient to detect 5 mg of spores (1%) captured on a filter from a letter containing 0.5 g, and allow isolating the letter in a predetermined bin. However, it would be insufficient to detect spores captured from a secondary letter contaminated by the source letter, if it is assumed that approximately 0.1% is transferred to a nearest neighbor. Nevertheless, it is

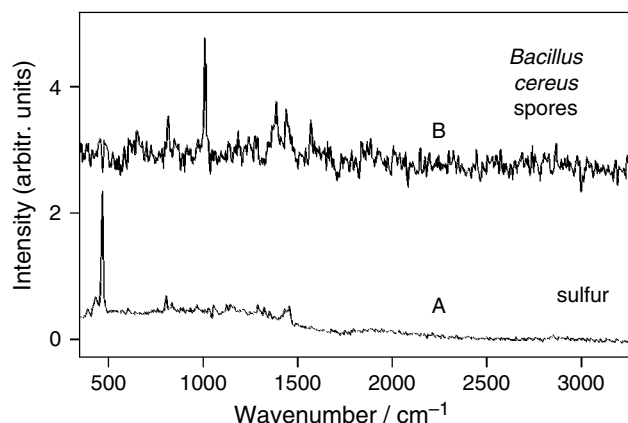


Figure 6. Raman spectra of (A) sulfur and (B) *Bacillus cereus* spores captured on filters at ID Mail. (A) Sample conditions: 11 m s^{-1} flow, $\Delta P = 125 \text{ mm Hg}$, 125 letters per min, spectral conditions: 300 mW of 785 nm, 3 min, (B) Sample conditions: 11 m s^{-1} flow, $\Delta P = 125 \text{ mm Hg}$, 50 letters per min, spectral conditions: 400 mW of 785 nm, 5 min (fluorescence subtracted).

worth noting that the actual amount of spores contributing to the Raman spectrum is considerably less. The 600- μm diameter laser spot represents only 0.09% of the 2-cm diameter orifice or 1.3 μg of the sample. Yet the spores do not distribute evenly across the filter, and laboratory measurements of intensity (e.g. Fig. 2) suggest that the 1020- cm^{-1} band intensity corresponds to as much as 2.5 times this mass. Therefore, approximately 3.3 μg or 330 000 spores contributed to this Raman signal. This has implications pertaining to detection of spores on contaminated surfaces, regarded as secondary sources for infection. For example, a probe directed at a suspicious particle could, as a lower limit, detect 10 μg in 1 s. Based on the measured density, this corresponds to a $1 \times 1 \times 0.12 \text{ mm}^3$ visibly observable particle. In fact, the test letter produced many such particles on the sorter belts and manifold.

CONCLUSION

A simple vacuum system was designed and used to collect *Bacillus cereus* spores from a test letter passing through a mail sorting system. The Raman spectrum indicates that 0.66% of the 226 mg contents were dislodged and collected. The S/N value was used to estimate that 5 mg could be captured on

a filter from a letter containing 0.5 g passing through a mail sorter. A spectral analysis time of 1 s and a sort speed of 50 letters per minute could allow isolating the letter in a predetermined bin, thus preventing further contamination. However, without considerable improvements, such an automated system would not detect secondary contaminated letters. Yet a probe could be used manually to inspect suspect particles on the order of 0.1 mm^3 in 1 s, and to identify the approximately 1 million spores, if present.

Current research is evaluating more efficient spore collection systems, more sensitive detectors, and the use of surface-enhanced Raman spectroscopy to improve detection limits by three to six orders of magnitude.

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