Differentiating bacterial spores from hoax materials by Raman Spectroscopy

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ABSTRACT

The bioterrorism of October 2001 caused by the distribution of anthrax through the U.S. postal system was compounded by the significant delay associated with positive identification of the *Bacillus anthracis* spores and the unknown extent of their distribution along the eastern seaboard. In the ensuing two years, literally thousands of hoaxes, letters containing harmless powders, have been mailed creating additional anxiety. Thus, there is a need for instruments and/or methods that can not only identify anthrax-causing spores to save lives, but also identify hoax materials to eliminate costly shutdowns. Here we present Raman spectra of *Bacillus cereus* spores, an anthrax surrogate, as well as of 30 common substances that might be used as hoax materials. We also examine the choice of laser excitation, 785 nm or 1064 nm, and its impact on the ability to measure visible particles in 5 minutes or less, and to provide a complete answer to the question of suspicious material identity.

Keywords: Anthrax, *Bacillus cereus*, anthrax hoax, mail sorters, Raman spectroscopy, homeland security

1. INTRODUCTION

In the autumn of 2001 bioterrorism became a sobering reality with the distribution of anthrax-causing spores through the U.S. postal system and the subsequent infection and death of several postal and national media employees. Fears grew as spore containing letters were reported from Florida to New York and the extent of distribution remained unknown. The long delay associated with positive identification of the *Bacillus anthracis* spores added to the nation's anxiety. The need for faster detection and identification of anthrax spores became readily apparent. In the ensuing two years, literally thousands of hoaxes, letters containing harmless powders, have been mailed creating additional tension. Thus, in addition to improved methods required to identify anthrax spores, methods are also required to rapidly identify hoax materials.

Positive identification of *Bacillus anthracis* (anthrax) spores requires detecting a unique, identifying sequence of nucleic acid (deoxyribonucleic acid, DNA or ribonucleic acid, RNA). The most definitive method employs culture media to grow the microorganism. However, several days are required to generate sufficient NA quantities to achieve detection.[1,2] In the past decade polymerase chain reactions (PCR) have been developed to substantially reduce this time.[3] PCR employs primers to separate the target NA sequence and polymerases to generate millions of copies of the sequence until it is detectable. The polymerase usually incorporates a detectable absorbent, fluorescent, or radioactive label to simplify analysis. Recently, a complete PCR analysis has been performed in under an hour using a capsular protein encoding gene for *B. anthracis*.[4]

Immunoassay methods have been used to detect the presence of bioorganisms for more than two decades. These methods use competitive binding of the bioorganism (as an antigen) and its fluorescent labeled conjugate for a limited number of antibodies. Although analyses can be performed in less than 30 minutes, immunoassays are only semi-quantitative, and are largely used to screen for analyte presence or absence. As yet there is not a well-defined anthrax antigen,[5,6,7] a limitation that became apparent in the past two years when suspicious materials were falsely identified as anthrax spores, causing hundreds of facilities to close, creating anxiety, and incurring significant costs.[8,9] The false-positive rate was sufficiently high that the Office of Science and Technology Policy recommended that such devices should not be used.[10]

A number of other methods are employing calcium dipicolinate (CaDPA) or its derivatives as chemical signatures for the presence of *B. anthracis.* This approach is valid in that only spore forming bacteria contain CaDPA, and the most common spores, such as pollen and mold spores do not. Relatively fast methods have been developed to extract CaDPA

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and then detect it by fluorescence,[11] or its acid form, dipicolinic acid, as a terbium complex by luminescence.[12,13] Although measurements have been demonstrated in less than 30 minutes, and although these methods may reduce the number of false-positives, their general lack of sensitivity may result in false-negatives.

All of the above approaches, nucleic acid sequencing, immunoassays, and dipicolinic acid detection, are aimed at detecting anthrax spores, and, unfortunately, analysis of hoax materials would only determine that spores are absent.

Another method, Raman spectroscopy, which measures molecular vibrations, may allow identifying anthrax causing spores, as well as suspicious materials. This method is attractive in that very small samples can be measured without manipulation. The sample needs only to be placed at the focal spot of the excitation laser and measured. As early as 1974, the Raman spectrum of *Bacillus Megaterium* was measured and shown to be dominated by CaDPA.[14] Although the spectrum was of pure spores, it took hours to acquire. By 1992, the improvements in Raman instrumentation and the use of resonance enhancement increased limits of detection dramatically and reduced analysis time to less than 1 hour.[15] Recently, we employed Raman spectroscopy to detect *Bacillus cereus* spores on a mail sorting system.[16] The use of 785 nm laser excitation and silicon detection allowed acquiring a spectrum in less than 15 minutes. However, the spectral bands were superimposed on a broad fluorescent background, which reduced measurement confidence with respect to false positives. The possibility of florescence interference could also be problematic when measuring suspicious materials. Fluorescence can, in general, be avoided by using laser excitation at energies less than the electronic absorptions that lead to fluorescence, such as 1064 nm, albeit with a loss in sensitivity. Here, we compare the ability of 785 and 1064 nm laser excitation to analyze *Bacillus* spores, as well as common substances that may be used hoax materials.

2. EXPERIMENTAL

Aspirin, acetaminophen, ibuprofen, dipicolinic acid (DPA) were used as received from Sigma-Aldrich (Milwaukee, WI). The following commercial products were obtained at local stores: aspartame and saccharin containing sweeteners, sugar, bleached flour, whole wheat flour, non-dairy creamer, baby cereal, buttermilk, corn starch, pancake and pudding mix, household cleaner, detergent, chalk, a white business envelope, a brown document envelope, a fiber reinforced envelope, and a coated shipping envelope. Calcium dipicolinate (CaDPA) was prepared from sodium dipicolinate, which was prepared from DPA according to previous publications.[15] Several grams of *Bacillus cereus* spores were obtained from the University of Rhode Island as a lyophilized powder. The bacteria were grown on nutrient agar plates according to published procedures. In all cases the samples were placed into 2-mL glass vials, which were in turn placed in the FT-Raman spectrometer sample compartments. In the case of envelopes, small sections were cut, rolled, and inserted into the vials.

Fourier transform Raman spectrometers were used to perform all measurements (Real-Time Analyzers, models IRA-785 and IRA-1064, East Hartford, CT). The model IRA-785 employed a 785 nm diode laser (Process Instruments Inc. model 785-600, Salt Lake City, UT) and an Si photo-avalanche detector (Perkin Elmer model C30902S-DTC, Stamford, CT), while the model RTA-1064 employed a 1064 nm diode-pumped Nd:YVO_4 laser (Spectra Physics, model J20-V-106C1-CW, Palo Alto, CA) and an InGaAs detector (Germanium Power Devices, model GPD 500, Andover, MA). Fiber optics were used to deliver the excitation beam to the sample probe and the scattered radiation to the interferometer (2 meter lengths of 200 and 365 micron core diameter, respectively, Spectran, Avon, CT). An f/2 achromat was used to collimate laser beam exiting the source fiber optic, while a 4 mm prism was used to direct the beam through an f/0.7 aspheric lens that focused the beam to a 600 micron 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.

3. RESULTS AND DISCUSSION

The Raman spectra obtained for *Bacillus cereus* spores using 785 and 1064 nm laser excitation are shown in Figures 1 and 2. As shown, a moderate fluorescent background is observed using 785 nm laser excitation. Although the fluorescence is easily modeled and subtracted, the limit of sensitivity, defined by the noise, becomes apparent.

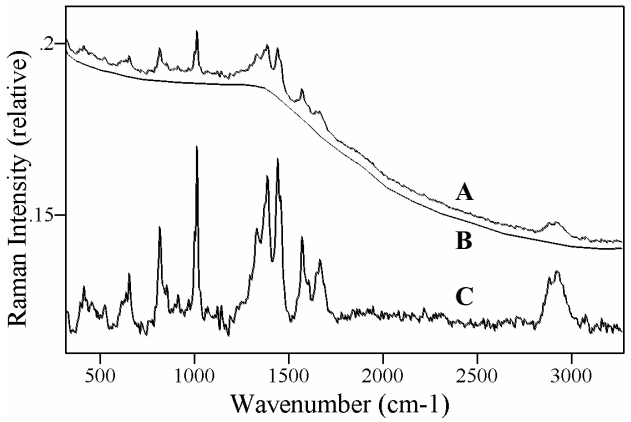

Fig. 1. A) Raman spectrum of *Bacillus cereus* spores using 785 nm laser excitation, B) fluorescent background, and C) corrected spectrum (fluorescence removed). Conditions: 400 mW at the sample, 13-min acquisition time, 8 cm^{-1} resolution.

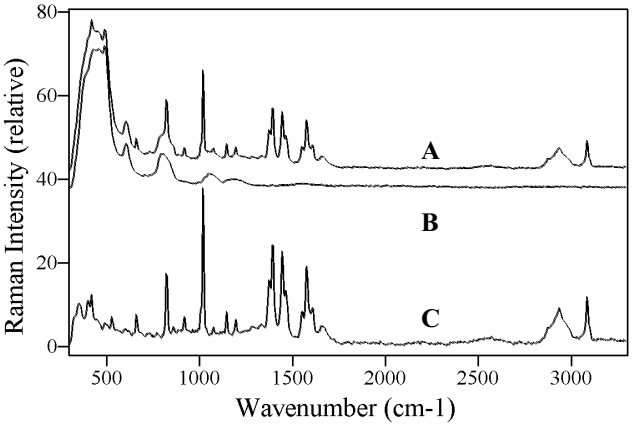

Fig. 2. A) Raman spectrum of *Bacillus cereus* spores using 1064 nm laser excitation, B) silica background, and C) corrected spectrum (silica removed). Conditions: 1000 mW at the sample, 5-min acquisition time, 8 cm^{-1} resolution.

Furthermore, it was found that the fluorescence diminished with increasing laser irradiation due to photobleaching. Although other researchers have used this process to improve sensitivity,[17] it would require variable spectral subtraction during at-site measurements, complicating the analysis procedure. Measurements using 1064 nm laser excitation are free of fluorescence as expected. Surprisingly, the signal-to-noise ratio is better than that obtained with 785 nm laser excitation. Generally, the combination of 785 nm laser excitation and Si detection is 25 times more sensitive than 1064 nm laser excitation and InGaAs detection. The discrepancy is attributed to the fluorescence generated by 785 nm, which adds noise throughout the Fourier transformed spectrum reducing the overall S/N. The current sample system used for 1064 nm excitation employs a fiber optic probe to allow flexibility in measurements, as might be required for measurements in and around a mail sorting system. The band pass filters used in the current probe design do not completely remove the silica Raman spectrum generated in the source fiber. This contribution to the spore spectrum can also be easily subtracted. Although this does reveal a few low frequency bands, it is not required for spectral analysis. Once corrected, both spectra contain the same primary Raman bands. As previously shown,[14,15] these bands are largely due to the calcium dipicolinate content in the spore core (Figure 3). Specifically, the following band assignments have been made: 1017 cm^{-1} to symmetric ring stretch, 1397 cm^{-1} to O-C-O sym. str., 1443 cm^{-1} to sym. ring C-H bend, 1573 cm^{-1} to asym. O-C-O str., 1604 cm^{-1} to asym. carboxilate str., 1660 cm^{-1} to carbonyl str. (bound CaDPA), and 3085 cm^{-1} to sym. ring C-H str. Nevertheless there are some differences between the spectra. For example, the organic content of the spores results in an intense, broad band centered at 2930 cm^{-1} due to C-H stretching modes. Similarly, the increased intensity of the spore band at 1443 cm^{-1} is likely due to C-H deformation modes. Surprisingly, several bands in the

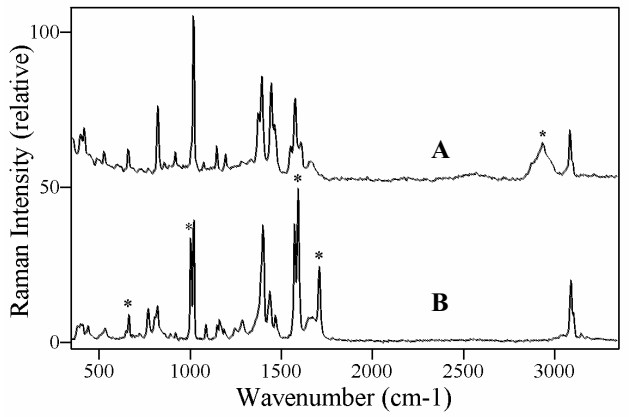

Fig. 3. Raman spectra of A) *Bacillus cereus* spores and B) calcium dipicolinate. Conditions as in Fig. 2.

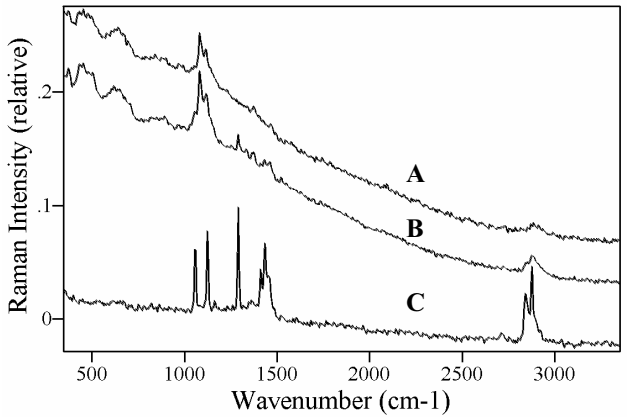

Fig. 4. Raman spectra of envelopes: A) plain white, B) woven, and C) "wax/polyethylene" coated. Conditions: 200 mW at the sample, 5-min acquisition time, 8 cm^{-1} resolution.

CaDPA spectrum are not in the spore spectrum, specifically the bands at 772, 1004, 1593, and 1710 cm^{-1}. Of these bands, DPA has bands at 772 and 1004 cm^{-1} suggesting that DPA is present as an impurity in the synthesized salt. Likewise, NaDPA has bands at 1593 and 1710 cm^{-1} suggesting it is also present as an impurity.

A number of common substances were examined as likely materials to be either used as anthrax hoaxes or simple likely to be found on mail sorting equipment. Mail sorters employ a number of moving components, such as belts and rollers, which abrade envelopes during operation. This generates a significant amount of paper debris that may have the appearance of powder. Paper from four envelopes was examined by Raman spectroscopy. This included plain white paper from a standard business envelope, light brown paper from a document envelope, fiber reinforced paper used in courier envelopes, and coated paper used by private delivery services. The spectra obtained for white paper, fiber reinforced paper, and coated paper using 785 nm laser excitation are shown in Figure 4. The former two papers show mild fluorescence, nevertheless the cellulose bands near 1100 cm^{-1} due to C-C stretches are evident. The brown paper is dominated by fluorescence and no structure is observed (see Figure 9A). The coated paper produces a spectrum that matches polyethylene and is identified as such. The reinforced paper also contains polyethylene bands, which are probably due to a coating on the fibers.

Several hoaxes used common white powders, in particular artificial sweeteners and non-dairy creamer. Figure 5 shows the Raman spectra obtained using 785 nm excitation of aspartame and saccharin artificial sweeteners, and sugar (sucrose), while Figure 6 shows spectra for whole wheat flour, bleached flour, and non-dairy creamer. The spectra of the sweeteners are virtually identical, since they are primarily dextrose with only 3-4% percent of the active ingredient. Dextrose has distinctive ring vibrations at 415 and 515 cm^{-1}, and ether stretches at 855, and 915 cm^{-1}. Although these bands also occur in sucrose, the relative intensities are significantly different. The required purity of these substances yields spectra free of fluorescence. The flours and non-dairy creamer are largely composed of starch, a glucose polymer, and the Raman spectra are similar to dextrose and sucrose, but the bands have broadened. The whole wheat flour has a slight tan color due to xanthophylls, carotenoid pigments, contained in the husk, which have not been removed by bleaching. These pigments are likely responsible for the intense fluorescence that completely obscures the Raman spectrum. In a similar manner, Raman spectra were also measured for baby cereal, buttermilk, corn starch, pancake and pudding mix. Again these food products contain a significant amount of starch and all produce spectra similar to flour. It was found that at 785 nm laser excitation the cereal fluoresced significantly, the buttermilk and mixes fluoresced mildly, and the corn starch did not fluoresce.

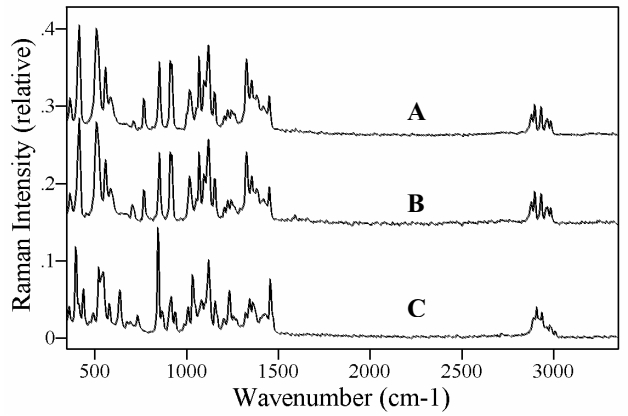

Fig. 5. Raman spectra of sweeteners: A) aspartame, B) saccharin, and C) sugar. Conditions as in Fig. 4.

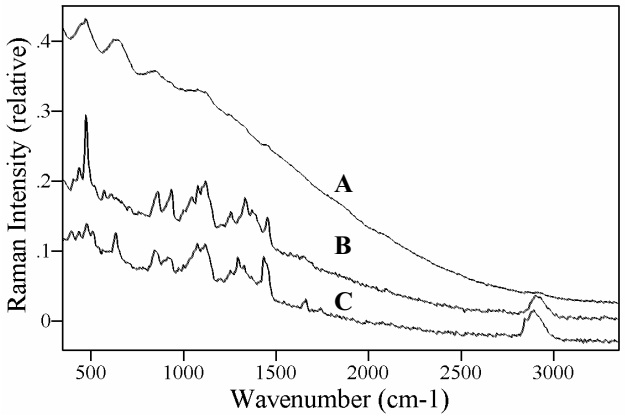

Fig. 6. Raman spectra A) whole wheat flour, B) bleached flour, and C) creamer. Conditions as in Fig. 4.

Other readily available materials that might be used for hoaxes include pain relievers and cleaning powders. Figure 7 shows the spectra obtained using 785 nm excitation for acetaminophen, ibuprofin, and aspirin, while Figure 8 shows the spectra for a household cleaner, chalk, and a detergent. Similar to the sweeteners, the pain relievers must meet stringent purity requirements and the spectra are free of fluorescent interference. The spectra are also unique for each drug and easily identifiable. The household cleaner was light green and was the only colored sample measured. Although it does fluoresce, a carbonate symmetric stretch at 1085 cm^{-1} is apparent. This same vibration appears in the spectrum obtained

from a piece of chalk, which is pure calcium carbonate. Surprisingly this white sample also fluoresces. The Raman spectrum of a detergent powder contains a similar carbonate band, but it is shifted to 1078 cm^{-1}, suggesting that the sodium salt is present. Similar to these substances, two soaps (scented and unscented), bath powder, baby powder, and baking soda were also measured.

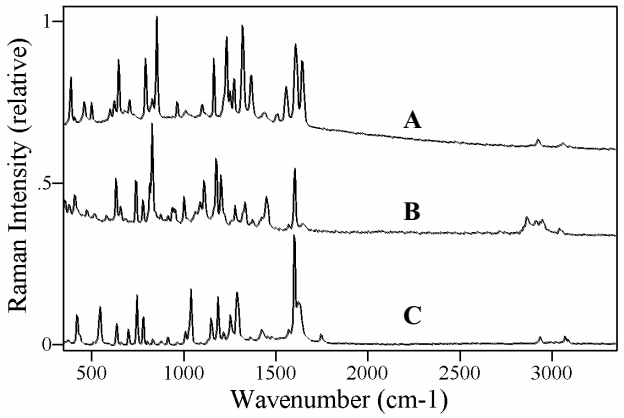

Fig. 7. Raman spectra of pain relievers: A) acetaminophen, B) ibuprofen, and C) aspirin. Conditions as in Fig. 4.

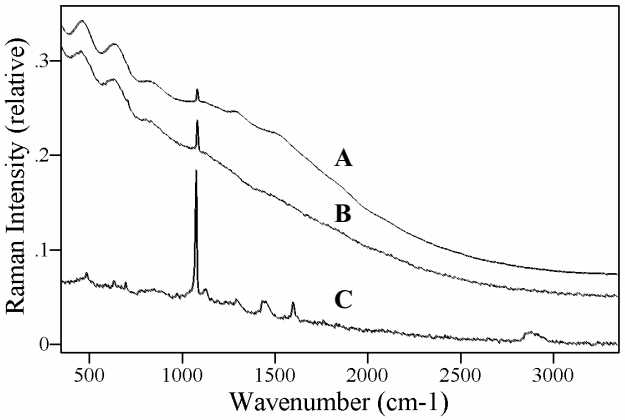

Fig. 8. Raman spectra A) a household cleaner, B) chalk, and C) a detergent. Conditions as in Fig. 4.

All of the common substances were also measured using 1064 nm laser excitation, and were found to be free of fluorescence interference. Figures 9, 10, and 11 compare the spectra of three samples that were dominated by florescence using 785 nm laser excitation. Respectively, 1064 nm excitation provides quality spectra for a brown envelope (cellulose), whole wheat flour (starch), and chalk (calcium carbonate).

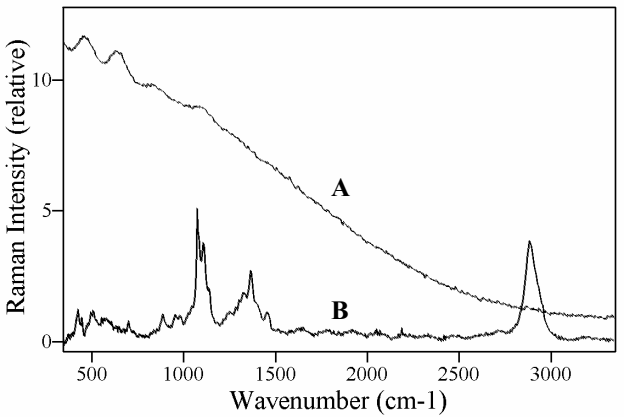

Fig. 9. Raman spectra of a brown envelope using A) 785 nm and B) 1064 nm laser excitation. Conditions for A) as in Fig. 4, B) 1000 mW at the sample, 5-min acquisition, 8 cm^{-1} resolution.

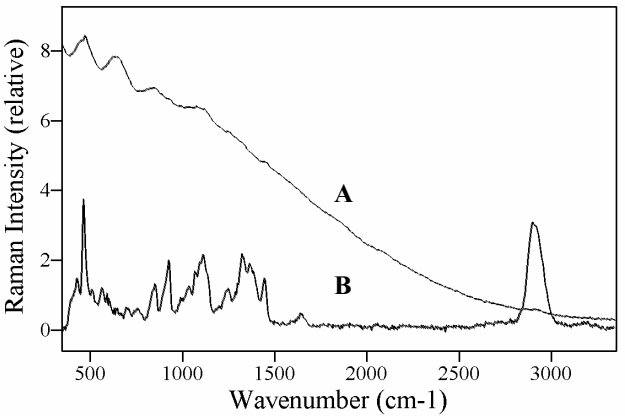

Fig. 10. Raman spectra whole wheat flour using A) 785 nm and B) 1064 nm laser excitation. Conditions as in Fig. 9.

The ability of Raman spectroscopy to differentiate calcium dipicolinate containing spores from common substances is demonstrated in Figure 12. The 5 primary CaDPA bands are compared to those for chalk, brown paper, creamer, saccharin and aspirin in finger print spectral region. It is readily apparent that the 5 bands provide a unique signature suitable for identification. Admittedly, the data base is extremely small. Nevertheless, it is highly unlikely that another chemical will have Raman spectral bands at these same 5 wavenumbers. The on-for-one uniqueness of Raman spectra to chemical composition has been demonstrated for libraries containing hundreds of chemicals by others.[18]

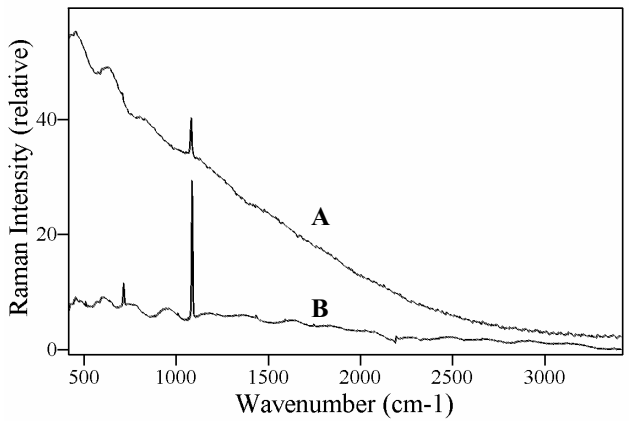

Fig. 11. Raman spectra of chalk using A) 785 nm and B) 1064 nm laser excitation. Conditions as in Fig. 9.

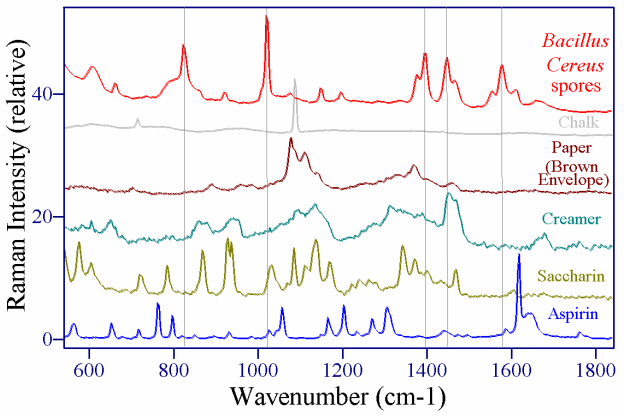

Fig. 12. Raman spectra of *Bacillus cereus* spores, paper (brown envelope), creamer, saccharin, aspirin acquired using 1064 nm laser excitation. Conditions as in previous figures. Note 5 major bands in the "finger-print" region provide a unique signature for *B. cereus* spores.

4. CONCLUSION

Here we examined the ability of Raman spectroscopy to provide a complete answer to the question of the identity of suspicious materials that might be found on mail sorting systems. We have shown that Raman spectroscopy can not only determine if a material is a CaDPA containing spore, but also the composition of the material if it is not a spore. As a preliminary data base, 30 common substances were measured using both 785 and 1064 nm laser excitation. Of these samples the Raman spectra of 6 (mostly food products, but also a brown envelope) were completely obscured by fluorescence, while the Raman spectra of an additional 7 samples contained a fluorescence baseline tilt when 785 nm laser excitation was used. Raman spectra obtained using 1064 nm laser excitation did not contain fluorescence contributions. Of equal importance it was shown that high S/N spectra of *Bacillus cereus* spores could be obtained in 5 minutes using 1 W of 1064 nm laser excitation. The primary *B. cereus* Raman bands are recognizable (S/N > 3) in 5 seconds. In all cases, the measurement area, defined by the excitation laser spot, was 200 micron diameter. This suggests that a single particle, if visible, would produce spectra equivalent to that presented here, and that a Raman spectrometer employing 1064 nm laser excitation could identify hoax materials in 5 minutes, and eliminate costly shutdowns. Such a spectrometer could also identify visible spores that may have spilled from a bioterrorism letter and help limit their further distribution.

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REFERENCES

1 Pasechnik, V.A., C.C. Shone, and P. Hambleton, *Bioseperations*, 3, 267-283 (1993).
2 Jackson, P.J., M.E. Hugh-Jones, D.M. Adair, G. Green, K.K. Hill, C.R. Kuske, L.M. Grinberg, F.A. Abramova, and Keim, P. *Proc. Natl. Acad. Sci.*, 95, 1224-1229 (1998).
3 Glick, B.R. and J.J. Pasternak, Molecular biology: Principles and Applications of Recombinant DNA, ASM Press, Wash. D.C. (1994).
4 Bell, C. A., J. R. Uhl, F. R. Cockerill, "Direct Detection of *Bacillus anthracis* Using a Real-Time PCR Method", ASM 101st General Meeting, Orlando, FL (May 2001)

5 Gatto-Menking, D.L., H. Yu, J.G. Bruno, M.T. Goode, M. Miller, A.W. Zulich, "Sensitive detection of biotoxoids and bacterial spores using an immunomagnetic electrochemiluminescence sensor", *Biosens. Bioelectron.*, 501-507 (1995)
6 Quinlan, J.J. and P.M. Foegeding, *J. Rapid Methods Automation Microbiol.*, 6, **1** (1998)
7 Hindle, A.A., E.A.H. Hall, "Dipicolinic acid assay revisited and appraised for spore detection", *Analyst*, 124, 1599-1604 (1999)
8 Barbaro, M., *Washington Post*, Feb 11, 2003, (http://www.washingtonpost.com/ac2/wp-dyn/A54042-2003Feb10?language=printer)
9 Snyder, L. and J. Pate, Center for Nonproliferation Studies, (http://cns.miis.edu/pubs/week/020520.htm) May 2002
10 Ascher, M.S., US Department of Health & Human Services (http://www.hhs.gov/ophp/presentations/Ascher.doc)
11 Nudelman, R., B.V. Bronk, S. Efrima, "Fluorescence Emission Derived from Dipicolinate Acid, its Sodium, and its Calcium Salts" *Appl. Spectrosc*. 54, 445-449 (2000)
12 Rosen, D.L., C. Sharpless, L.B. McBrown, "Bacterial spore detection and determination by use of terbium dipicolinate photoluminescence", *Anal. Chem.*, 69, 1082-1085 (1997)
13 Pellegrino, P.M., N.F. Fell Jr., J.B. Gillespie, "Enhanced spore detection using dipicolinate extraction techniques." *Anal. Chim. Acta*, 455, 167-177 (2002).
14 Woodruff, W.H. T.G. Spiro and C. Gilvarg, "Raman Spectroscopy *In Vivo*: Evidence on the Structure of Dipicolinate in Intact Spores of *Bacillus Megaterium*," *Biochem. Biophys. Res. Commun.*, 58, 197 (1974)
15 Ghiamati, E., Manoharan, R., S., Nelson, W.H., and Sperry, J.F.,"UV Resonance Raman spectra of Bacillus spores" *Appl. Spectrosc.*, 46, 357- 364 (1992).
16 Farquharson, S., L Grigely, V Khitrov, WW Smith, JF Sperry, and G Fenerty, "Detecting Bacillus cereus spores on a mail sorting system using Raman Spectroscopy", *J. Raman Spectrosc.*, 2003 accepted
17 Esposito, A.P., C.E. Talley, T. Huser, C.W. Hollars, C.M. Schaldach, and S.M. Lane, "Analysis of single bacterial spores by micro-Raman spectroscopy", *Appl. Spectrosc.*, 57, 868-871 (2003)
18 Lavine, B. K., C. E. Davidson and A. J. Moores, "Genetic algorithms for spectral pattern recognition", *Vib. Spectrosc.*, 83-95 (2002)