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Detection of biological particles in ambient air using Bio-Aerosol Mass Spectrometry

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ABSTRACT

The Bio-Aerosol Mass Spectrometry (BAMS) system is an instrument used for the real time detection and identification of biological aerosols. Particles are drawn from the atmosphere directly into vacuum and tracked as they scatter light from several continuous wave lasers. After tracking, the fluorescence of individual particles is excited by a pulsed 266nm or 355nm laser. Molecules from those particles with appropriate fluorescence properties are subsequently desorbed and ionized using a pulsed 266nm laser. Resulting ions are analyzed in a dual polarity mass spectrometer. During two field deployments at the San Francisco International Airport, millions of ambient particles were analyzed and a small but significant fraction were found to have fluorescent properties similar to *Bacillus* spores and vegetative cells. Further separation of non-biological background particles from potential biological particles was accomplished using laser desorption/ionization mass spectrometry. This has been shown to enable some level of species differentiation in specific cases, but the creation and observation of higher mass ions is needed to enable a higher level of specificity across more species. A soft ionization technique, matrix-assisted laser desorption/ionization (MALDI) is being investigated for this purpose. MALDI is particularly well suited for mass analysis of biomolecules since it allows for the generation of molecular ions from large mass compounds that would fragment under normal irradiation. Some of the initial results from a modified BAMS system utilizing this technique are described.

Keywords: Bioaerosols, fluorescence, mass spectrometry, MALDI

1. INTRODUCTION

In July of 1993, the religious group, Aum Shinrikyo, aerosolized a liquid suspension of *Bacillus anthracis* from the roof of their headquarter building in Kameido, Tokyo, Japan[1]. Residents in the area reported foul smells, short term loss of appetite, nausea, and vomiting as well as an oily residue on the building side. Environmental health officials requested permission to inspect the building and were denied access by Aum Shinrikyo members. Officials collected residue and air samples, monitored activity at the building and determined that other than the odor, no obvious risks to human health existed. The incident was largely forgotten until the cult attacked the Tokyo subway system with sarin nerve gas in March of 1995. Analysis in 1999 of the 1993 collected samples revealed the presence of the *Bacillus anthracis*, Sterne 34F2 strain, an animal vaccination that is not of significant risk to humans.

In September of 2001, letters containing *Bacillus anthracis* spores was sent via US postal mail throughout the United States. Twenty-two people developed anthrax infections, of which five died of inhalational anthrax. One letter contained 2 grams of the *Bacillus anthracis,* Ames strain estimated to contain between 100 billion and 1 trillion spores per gram[2]. The LD_{50} for anthrax (i.e. the dose sufficient to kill 50% of the persons exposed to it) is believed to lie between 2,500 and 55,000 inhaled *Bacillus anthracis* spores. Thus the contents of that single letter had the theoretical potential to kill millions of people.

Although it is potentially useful to know when *Bacillus* spores are present, it may not always be cause for alarm. It is often of great value to know the species and in some cases even the strain of a potential biological threat. A bioaerosol detector must also be able to differentiate these species from the large variety of background aerosol particles, which are

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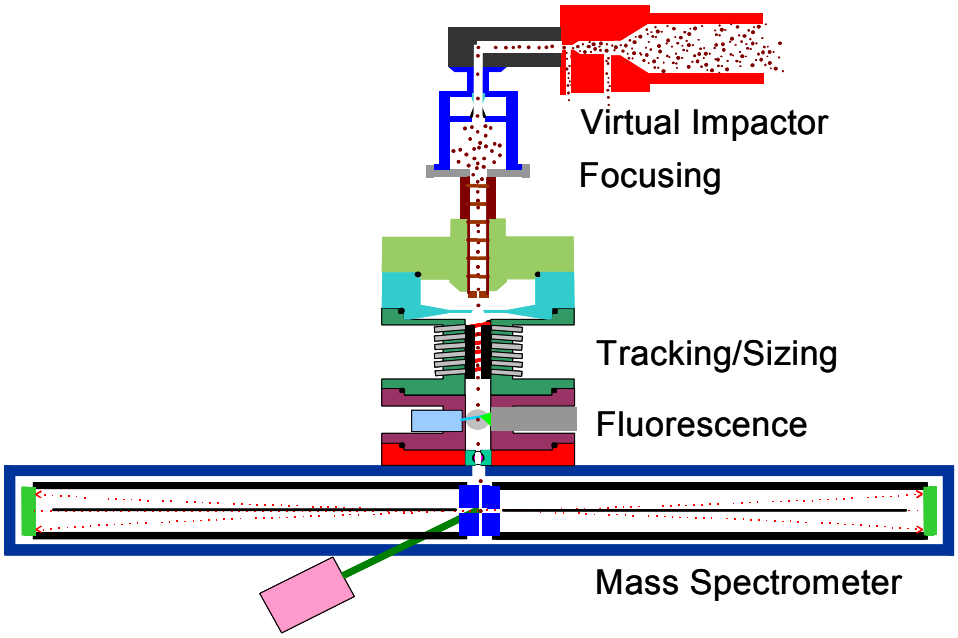


Fig. 1 Basic BAMS system layout.

always present in most environments. In an effort to characterize the ambient aerosols at one important facility, the BioAerosol Mass Spectrometry system developed at Lawrence Livermore National Laboratory was deployed to the San Francisco international airport on two separate occasions. The BAMS system has proven itself to be a powerful detector, but the deployed instrument is unlikely to have the ability to differentiate strains of species. Various modifications have now been implemented and will be described which are intended to push the system toward this ultimate goal.

2. THE BAMS SYSTEM

The basic layout of the BAMS system (Fig. 1) has been described in previous publications[3, 4] and in the accompanying paper[5]. In brief, aerosol is first drawn into a virtual impactor, which concentrates the particles falling within the respirable size range (~1-10μm). The concentrated aerosol is then sampled into the vacuum system of the BAMS instrument. A supersonic expansion into vacuum focuses the particles into a vertically orientated beam. The rest of the BAMS system is composed of stages that generally decrease in speed, but increase in specificity along the particle trajectory. The tracking stage determines a particle's aerodynamic diameter. After that, one or more stages examine a particle's intrinsic fluorescence. During the first deployment, 266nm laser pulses were used for excitation. During the second, 355nm pulses were used. In newer instruments, both stages can be installed in series along with other stages utilizing other non-destructive analysis techniques. Ultimately, molecules from the particles are desorbed and ionized with a single 266nm laser pulse and a dual-polarity mass spectrum is collected. The deployed instruments utilized a commercial, dual-polarity, reflectron time-of-flight mass spectrometer from TSI Inc. A new spectrometer has been designed at UC Davis and LLNL that has improved performance in several important respects.

In time-of-flight mass spectrometry, an initially stationary group of ions are all imparted with the same amount of kinetic energy per charge using an electric field. A small ion carrying the same charge as a larger ion will thus have a higher velocity and cover a fixed distance in less time than the larger ion. By measuring the ion's time of flight over a known path, the mass-to-charge ratio of the ion can be determined. In a reflectron system, the ions traverse a field-free region and are then reflected, using an electrostatic mirror, towards a detector generally located near the point where the ions were first created. This reflection allows a longer flight path to be contained within a given sized flight tube and it also helps correct for any spread of initial kinetic energies that the ions might have started with. Both of these features help improve the resolution of the resulting mass spectra so that ions with similar masses can be more easily distinguished. The longer path length and reflection, however, also provide opportunity for ion losses in a non-optimally designed spectrometer. The new linear mass spectrometer (which does not have a reflectron) was designed to greatly improve the efficiency of ion transport. More details will be given in section 3.4, but is important to note that the new spectrometer has already demonstrated record breaking sensitivity[6].

3. DATA AND RESULTS

3.1. Particle size

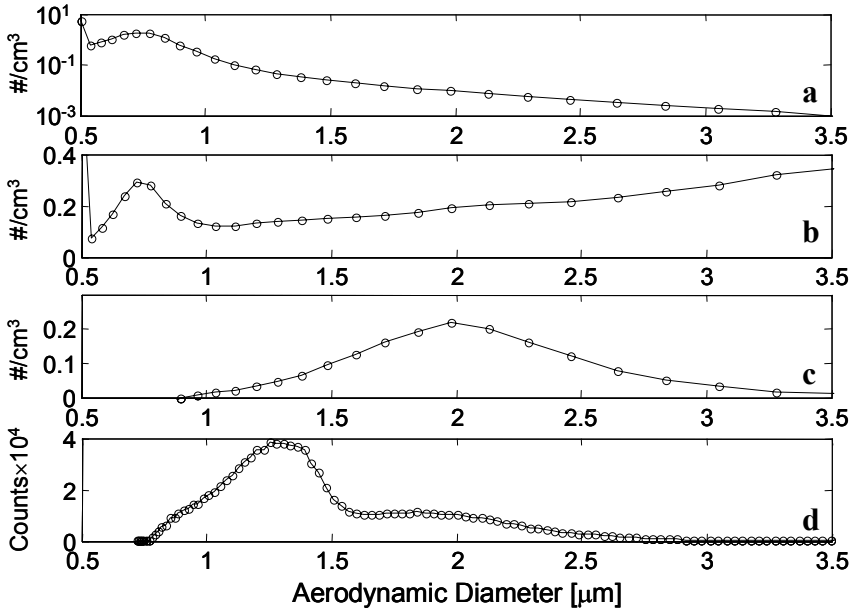


Fig. 2 APS measured size distributions of a) particles during first SFO deployment (note log scale), b) *Bacillus* spores aerosolized with a Sono-Tek nozzle (small particles are agglomerates of various background impurities), and c) *Erwinia* vegetative cells aerosolized with a Sono-Tek nozzle. The size distribution of particles fully analyzed by BAMS during the first deployment is shown in d).

Size is the first parameter obtained from a particle by BAMS. It is a useful quantity that can separate a large fraction of the ambient background aerosol from the particles of interest, which generally fall within the respirable size range (as quantified in the accompanying paper[5]). Nonetheless, once a particle is determined to fall within the respirable size range, knowledge of its size (or specifically it's aerodynamic diameter) provides little additional value for identification. As can be seen in Fig. 2, aerosolized solutions of spores and vegetative cells can contain virtually any sized particle since the individual biological entities can clump with one another and various background impurities. The size distributions shown are a product of the starting solutions and the aerosolization conditions; they do not clearly reveal fundamental properties of the constituent particles. Even if an aerosol consisted purely of individual cells and spores, however, many types of material can obviously produce particles falling within a similar size range. More information is needed from additional stages.

3.2. Fluorescence

Intrinsic fluorescence is a property probed by both existing and proposed rapid detection systems[7-9]. Excitation wavelengths of 355nm and 266nm are convenient choices because of the wide availability of frequency tripled and quadrupled Nd:YAG lasers. Furthermore, 266nm photons can excite fluorescence from important biological molecules such as tryptophan while 355nm photons can excite fluorescence from molecules such as NADH. Two broad fluorescence detection bands are employed by BAMS. For 266nm excitation, the bluer fluorescence band covers wavelengths between 290 and 400 nm. The redder band covers wavelengths of 400 nm and greater. Thus, at least in theory, emission from tryptophan can be collected with minimal interference from NADH emission and vice versa. For 355nm excitation, the bluer collection band covered wavelengths between 373 and 500nm, while the redder band covered wavelengths above 500nm. None of these bands are necessarily optimal for all conditions; more research is needed and planned in this area. Elastically scattered excitation light was not monitored. Consequently, the data shown below is not normalized by the scattered signal as is often done in detectors utilizing fluorescence as the primary means of agent identification.

Fig. 3a shows the 266nm fluorescence data collected from ~1 million particles during the first deployment to SFO. Approximately 6%, 5% and 89% of the particles fall in regions 1, 2 and 3 respectively. Fig. 3b shows typical 266nm data from *Erwinia herbicola* (EH) vegetative cells while Fig. 3c shows data for *Bacillus atrophaeus* (BG) spores. Both samples were grown and aerosolized at LLNL. In the case of EH, 31%, 3% and 66% of the data points fall in regions 1, 2

and 3 respectively. The corresponding percentages for BG are 30%, <1% and 70%. A simple and important conclusion is that "uninteresting" background aerosol particles can be preferentially discarded by considering only region 1 particles in subsequent analysis. Fig. 3d shows 355nm data collected from ~5.5 millions particles during the second SFO deployment. 20%, 6%, 0.2% and 74% of the particles fall in regions 1-4 respectively. Fig. 3e shows 355nm data for EH. 3%, 31%, 2% and 65% of the particles fall in regions 1-4. Region 2 is of the greatest interest for the 355 nm excitation. To first order, it appears that 355 and 266nm excitation wavelengths are about equally effective in isolating vegetative cells from the background aerosol. 355nm excitation, however, at least initially appears much less effective at isolating BG spores. Fig. 3f shows that 99% of spores in one test run fell in region 4. It is important to note though that the test aerosols were composed primarily of single cells and spores, not larger clumps of particles (which generally produce more fluorescence). Subsequent tuning and optimization of the 355nm fluorescence stage have produced more useful results from similar preparations of spores, but that data cannot be fairly compared with data collected during the deployments.

The data presented should not be used to judge the relative value of the 266nm or 355nm fluorescence. A system, however, combining both 266 and 355nm excitation wavelengths should exhibit better discrimination than a system containing only a single excitation wavelength. Data has been collected from a number of agent surrogates and potential background interferents. In a number of cases, the information produced by the two wavelengths is orthogonal. Modified BAMS systems provide the unique opportunity to take correlated measurements of 266 and 355nm fluorescence on individual particles. The value of this is still under investigation; however correlated measurements are likely to provide more information than uncorrelated.

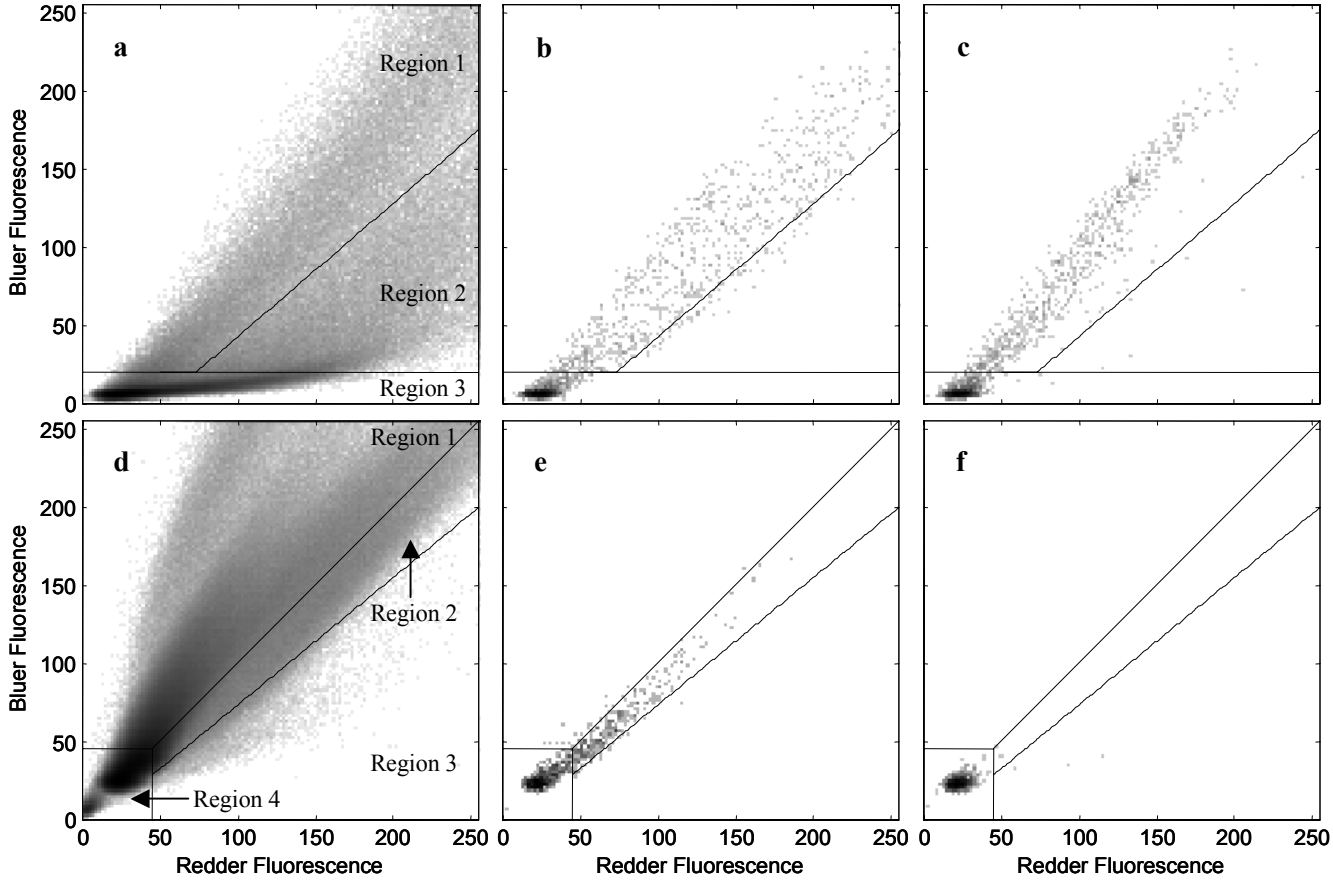


Fig. 3 266nm fluorescence data is shown for a) particles fully analyzed at SFO during the first deployment, b) *Erwinia herbicola* vegetative cells and c) *Bacillus atrophaeus* spores. 355nm fluorescence data is shown for d) particles analyzed in the second SFO deployment, e) *Erwinia herbicola* vegetative cells and f) *Bacillus atrophaeus* spores. The plots are divided into regions, which were set by eye. The gray scale is logarithmic; far more particles fall in the black areas than in surrounding gray areas. Percentages of particles falling in each region are given in the text. All of the spores and cells were aerosolized and measured at LLNL, not at SFO.

Two fluorescence stages, or even a single stage, can be used to remove much of the aerosol background falling within the appropriate size range, but some background particles produce fluorescence signals that are seemingly indistinguishable from those of biological particles. Furthermore, we have little evidence to suggest that accurate species identification will ever be possible based solely on fluorescence. More information is needed.

3.3. Results from the reflectron mass spectrometer

A mass spectrum can be thought of as a chemical fingerprint for a particular type of particle. In most cases, however, it does not reveal the exact chemical composition of the particle; the desorption and ionization process involves many competing factors[10] that cause certain molecules to be preferentially ionized while the ionization of others can be almost completely suppressed. In spite of this complexity, many of the molecules which constitute the spectral patterns of spores and other agent surrogates analyzed in the BAMS system have now been identified[11, 12]. Many of these molecules are clearly biological in nature. It is highly unlikely for a particle to produce an agent-like signature (i.e. spectrum) unless that particle is truly similar in nature to that agent. An anthrax-like spectrum might occasionally be produced by a related *Bacillus* species or perhaps even a more distantly related type of bacteria, but certainly not by things such as common cleaners or combustion byproducts. This is not the case for many less informative analysis techniques such as fluorescence.

The automated identification of potential agent particles analyzed by BAMS is accomplished using an "alarm file". The alarm file is a library of mass spectral types produced by agents or agent surrogates to which unidentified particle spectra are compared. If enough particles are identified as a particular agent in a set period of time, then an alarm can be sounded. The threshold to sound an alarm is never set below a few agent particle identifications in one minute (i.e. alarms are never sounded on the basis of a single particle) and in environments where some fraction of ambient particles are similar to agent particles, the threshold can be dynamically raised and adjusted. An alarm file, known to efficiently recognize a variety of vegetative cell and *Bacillus* spore preparations, was independently developed for other BAMS testing and run against all data collected during the first SFO deployment. The total number of particles analyzed by BAMS every minute, along with minute-by-minute counts of particles identified as spores and cells by the alarm file are shown in Fig. 4. At no point would BAMS have sounded an alarm for agent-like spores or vegetative cells during the seven-week deployment! This is a very low false alarm rate for an instrument with both a response time of one minute or less and proven sensitivity to real agent-surrogate releases not conducted at SFO.

From the one million total particles analyzed at SFO, approximately 300 particles were identified as spores (0.03%). The average concentration of particles measured by an APS with diameters above 0.75 μm was ~5000 per liter. It could thus be estimated that on the order of 1 or 2 particles per liter were either *Bacillus* spores or particles with spore-like constituents. A similar number is obtained for vegetative cells. This type of analysis is susceptible, however, to various

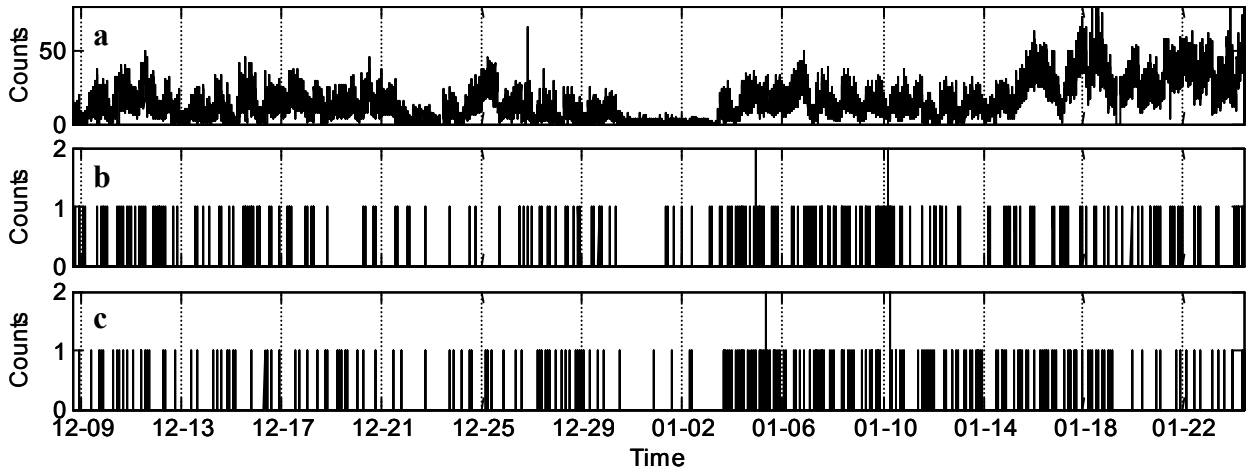


Fig. 4 Minute by minute counts of a) the total number of particles analyzed by BAMS, b) the number of those particles identified as vegetative bacterial cells and c) the number of particles identified as spores. At no point would BAMS have sounded an alarm.

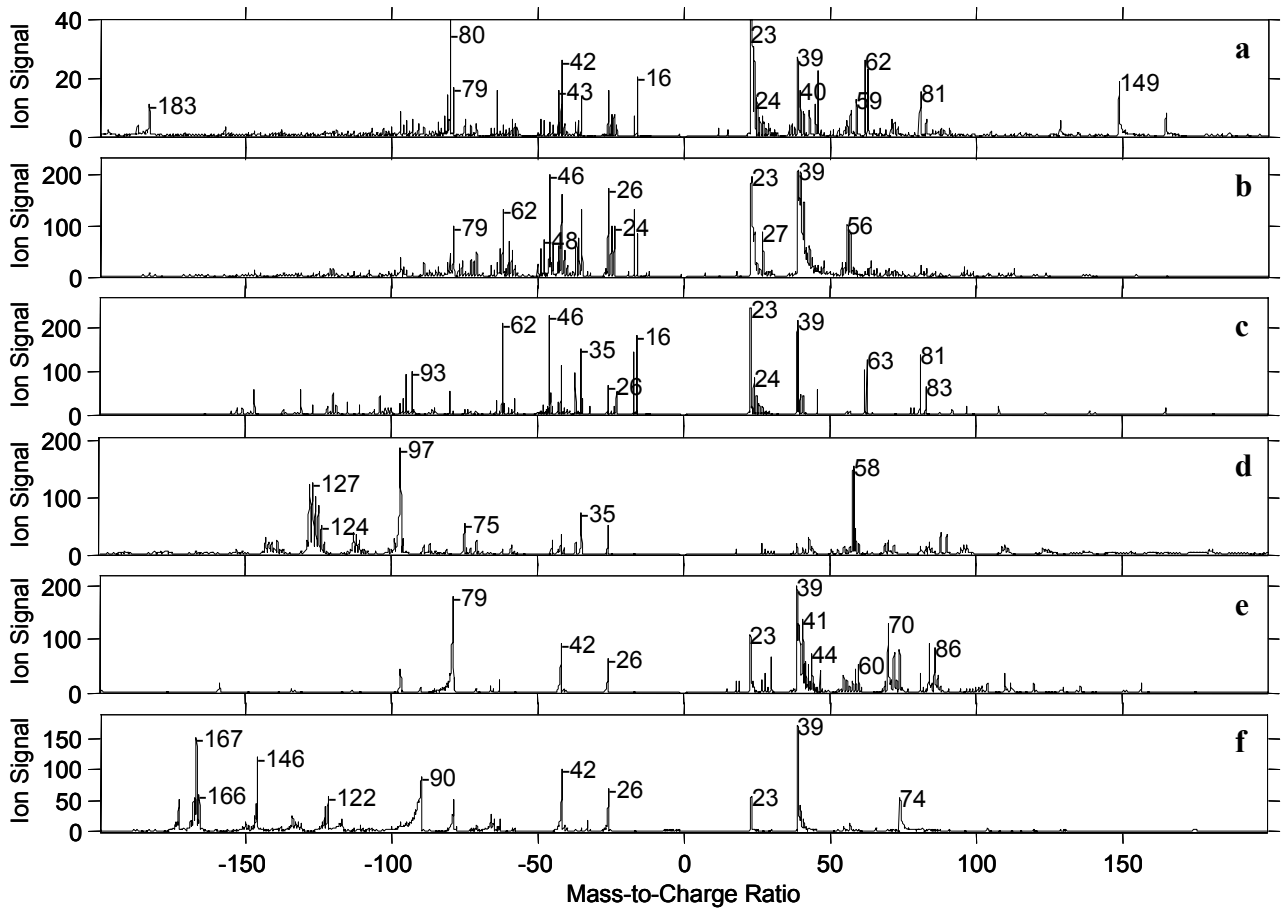


Fig. 5 A typical type of spectrum from the first SFO deployment using 266nm fluorescence excitation is shown in a) for region 1 and b) for region 2. c) shows a typical type of spectrum present in all of the 355nm fluorescence regions. d) shows a more unique spectral type observed in the region 3 of the 355nm data. All of these spectra are averages of multiple individual particle spectra. e) shows an average EH spectrum and f) shows an average BG spectrum for comparison. The BG data was collected on a different BAMS system.

factors that may at times cause either a significant underestimate or overestimate of the true concentrations. The numbers mentioned above should only be considered order of magnitude estimates. Concentrations even ten times higher would still constitute less than 1% of the particles analyzed which is advantageous for a more efficacious rapid detection system.

A separate approach to the analysis of the mass spectral data began with collecting the spectra of the particles falling within each of the fluorescence regions. The spectra from each region were then independently clustered to identify the most common "types" of spectra. These spectral types do not appear visually similar to spore or cell spectra seen previously (Fig. 5). Again, it therefore seems highly improbable that spores and cells could constitute a significant fraction of the ambient aerosol at SFO. This is important because it indicates that a detector can obtain a low false alarm rate in an SFO-like environment, without having to differentiate cells and spores at the species level, and yet still remain sensitive to low concentrations of agents.

In some scenarios, however, the cost of even a single false alarm can be very high. It may well be deemed necessary to identify the species and conceivably even the strain of a potential threat particle. Besides that, an instrument like BAMS has many potential medical and healthcare applications; in that type of setting it would clearly be beneficial to know more than simply that cells are present. The current alarm files and alarm algorithms can and are being improved, but more fundamental difficulty lies in differentiating a wider range of cell and *Bacillus* spore species. Differences between spectra of certain species may not be consistent enough to enable reliable species-level identification given the huge

range of preparation methods, aerosolization techniques and particle-to-particle variations that can be encountered in practice. More information yet is desired from the particles.

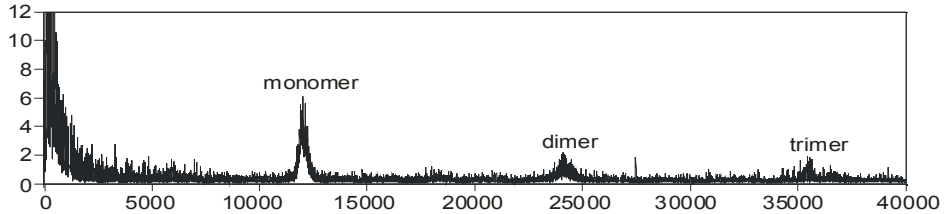
3.4. Results from the linear mass spectrometer and MALDI

Identification of microorganisms using mass spectrometry on a particle-by-particle basis demands sensitive detection due to the limited number of large mass, taxonomically relevant molecules within a single cell. Mass spectra from single bacterial spores and vegetative cells acquired using the commercial TSI reflectron mass spectrometer are generally limited to peaks under *m/z*=300. Thus, one important step to further optimize the BAMS system for single cell analysis is to extend its useful mass range. SIMION modeling of the former reflectron spectrometer system revealed design flaws limiting higher mass ion transmission. While retaining dual polarity capabilities, the reflectron spectrometer in one BAMS system was converted to a linear configuration with delayed extraction and an electrostatic ion guide. Modeling showed that incorporation of the electrostatic ion guide would focus divergent trajectories of larger molecules on to the microchannel plate detector while simultaneously offering the option to gate out lower mass molecules. The energy focusing technique, delayed extraction, was implemented in the new linear design to regain resolving power lost during the conversion from reflectron to linear[13].

Reliable species level classification of individual cells and spores would ideally involve the ionization of taxonomical biomarkers such as the high mass proteins (6-10kD) found in the spore core[14]. To help achieve this goal, the well-established technique of matrix-assisted laser desorption/ionization (MALDI), which is widely used for bulk samples, is being studied and applied to the analysis of individual aerosol particles in BAMS. Over the past decade, MALDI has developed into a powerful tool for mass spectrometric analysis of biomolecules. It is particularly well suited for mass analysis of peptides and proteins since it allows the generation of molecular ions from large mass compounds that would otherwise fragment under normal laser irradiation[15-19]. In MALDI, the analyte of interest is mixed with a large mole excess of a matrix that absorbs efficiently at the laser wavelength and allows the energy from the laser to be dissipated[20]. As a result of the laser irradiation of the sample, the matrix is desorbed entraining with it analyte molecules[21]. Molecular collisions in the resultant plume cause matrix and analyte ionization.

Instrument performance of the new design as well as characterization of single particle ionization was assessed using MALDI techniques of aerosolized peptide and protein particles. Masses two orders of a magnitude higher than previously seen in the reflectron TOF have been seen in the new configuration. To determine the high mass range using single particles, the protein, cytochrome c (*m/z* ~12,000) was studied. A saturated solution of sinapinic acid and cytochrome c yielded a monomer at 12,000m/z, as well as, a dimer at m/z~24,000 and trimer at m/z ~36,000, as seen in (Fig. 6. Achieving this high mass range illustrates the elimination of the instruments low mass bias.

Fundamental aspects of the ionization of molecules from spores can be simulated and studied using standard peptides and proteins (1-12kD) in combination with various matrices, including dipicolinic acid (DPA). In particular, the factors which govern ion creation and transmission can be determined by characterizing single particle ionization in BAMS using MALDI techniques Characterization of the single particle ionization process was assessed using five matrices; 2,6-dihydroxyacetophenone, 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid, ferulic, and sinapinic acid, as well as the peptide, Angiotensin I (1297 *m/z*). Molecular anion formation was correlated to the production of its counter cation. Total angiotensin cation and anion peak area were used as determinants of ion formation for each particle. Specifically, the summation of the total cation peak area, including the protonated, sodiated, and potassiated molecular



(Fig. 6) Average of 27 cytochrome C/sinapinic acid single particle spectra. Monomer, dimer, and trimer peaks were observed at 12kDa, 24kDa, and 36kDa, respectively. Spectra were acquired with a time lag of 350nsec, and guide wire delay, D1, of 3ms[13].

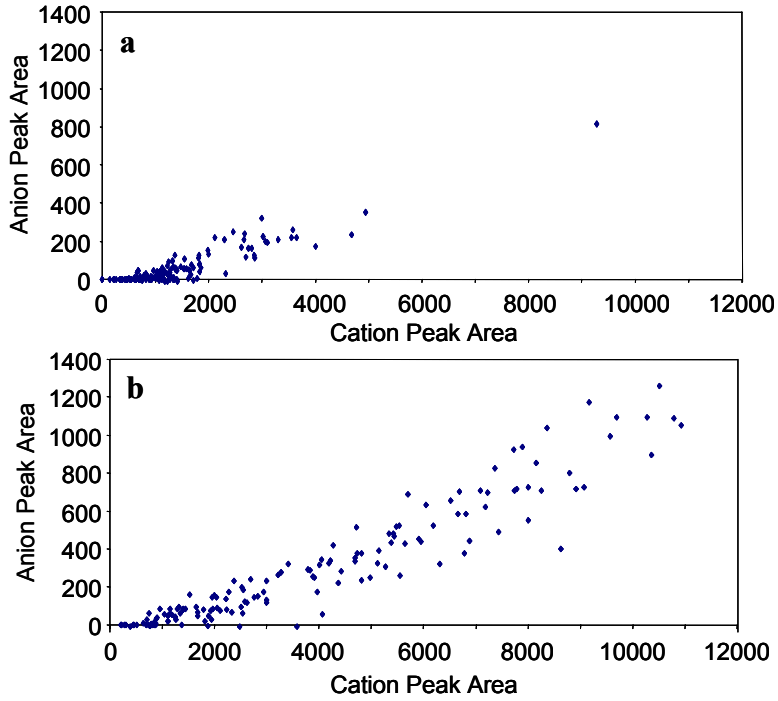


Fig. 7 The total angiotensin cation versus angiotensin anion peak area of 150 particles with a) Sinapinic Acid and b) α-cyano-4-hydroxycinnamic acid used as matrix. The axes units are arbitrary but proportional to the number of ions created and detected.

ion peaks as well as the total molecular anion peak area, namely, the deprotonated species, were determined for each matrix. All matrices studied revealed that an increase in molecular cation formation correlates to an increase in molecular anion formation. The effect is most prominent with α-cyano-4-hydroxycinnamic acid (Fig. 7).

The optimal experimental parameters realized from these studies will ultimately be used to develop dual polarity, high mass signatures of *Bacillus* spores and vegetative cells, which may be coated with a matrix on-the-fly[18, 22]. It is hoped that these modifications and experiments will ultimately make BAMS an even more powerful tool for bioaerosol detection as well as a more useful tool for fundamental research.

4. CONCLUSIONS

Current BAMS systems can already obtain a great deal of information from individual aerosol particles. Size, fluorescence and bipolar mass spectral data all help isolate biological particles. In fact, the vast majority of background particles can be easily distinguished from cells and spores in environments like SFO. Nonetheless, not all spore species can be differentiated and vegetative cell differentiation may be an even greater challenge. Better mass spectra must be acquired and various improvements have been and are being implemented with that goal. Initial results from a linear mass spectrometer system employing MALDI are very encouraging.

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DISCLAIMER

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