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Non-biological measurements on biological agents

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Summary

Chemical and physical analysis of a biological agent can provide information on how and when the agent was produced, and can be used to determine if two agent samples were produced by the same process in the same laboratory. Since the Amerithrax investigation began, a number of novel analytical methods have been developed to answer specific questions about the origin of the *B. anthracis* powders used in that incident. This paper describes the investigation of silica deposition in spores, the development of assays for determining if spores were grown on agar plates, and the validation of sample matching analyses based on elemental composition. A perspective on future needs and directions is provided.

Key Words: Chemical analysis, mass spectrometry, electron microscopy, atomic force microscopy, secondary ion mass spectroscopy, silica, agar, dating, matching

Introduction

The anthrax letters investigation engendered considerable speculation about the potential importance of non-biological measurements on samples of biological agents [1,2]. A variety of mass spectral, spectroscopic, and other instrumental techniques were applied to characterize case samples or to study the properties of surrogate agents with the hope of shedding light on morphological, trace element, isotopic, or other molecular "fingerprints" of the production methods used to create the anthrax powders. It was imagined by some that knowledge of the agent production method might be a key piece of evidence, supplementing that obtained from genetic analysis or other biological properties, and generating valuable investigative leads [3]. At the time, the generation and interpretation of chemical and physical data from biological agents (like other areas of microbial analysis utilized in the investigation) represented a new domain of forensic science.

As might be expected during this formative period, the perceived needs, forensic experience, and decidedly operational mindset of the investigators naturally led to an emphasis on SOP development, and a number of SOPs now exist for analyzing agents by various sophisticated techniques. While these techniques could determine the composition and structural features of agents with exquisite precision and accuracy, consideration of the *meaning* of these data most often revealed the need for extensive exploratory research to identify and understand how specific signatures were related to case-relevant questions about the samples.

Related to this, there was a great deal of concern for analytical validation (standards, calibrations, QA and QC guidelines, etc. [4]) Although this concern was clearly appropriate, there was less awareness of the need for a coherent framework for *inferential validation* (See the accompanying chapter). As the recent NRC report on forensic science illustrates [5], this need is not unique to microbial forensics, but is thought by some to apply to many traditional forensic science techniques as well.

As a result of the need to disentangle longer-term exploratory research and validation activities from near-term operational applications of certain techniques, not all directions proved immediately fruitful. Moreover, perceptions of the relative utility of this kind of evidence evolved substantially over time, and a considerably more mature perspective on many of these issues has been gained in the near-decade since work began in this field. This chapter describes some examples of the progress that has been made through efforts supported by a variety of agencies, and provides a view of current capabilities and future needs.

Determining manufacturing method

It is generally appreciated that the morphological and chemical properties of an agent reflect the methods and materials used to generate it. Thus, it is plausible that certain details about the "recipe" used to make a material may be deduced from chemical or physical analysis. This type of information could be of value to an investigator if it constrained the pool of suspects to those that have access to the equipment, materials, and information necessary to carry out specific "recipes". Two examples of this kind of analysis involve silicon deposition and residual agar in spore preparations.

Silicon deposition in spores

Early in the Amerithrax investigation Michael et. al. observed that silica (SiO_2) could be detected by Scanning Transmission Electron Microscopy (STEM) within the outer coat of the *Bacillus anthracis* spores found in the NY Post and Leahy letters [6]. Comparison with spore preparations made by a variety of standard growth processes led to the conclusion that the amount of SiO_2 observed in the case samples was unusually high, amounting to at least 1% of the spore mass by weight. At the time these observations ostensibly pointed to something unique about the process used to create the Amerithrax materials.

This led to several years of work by Weber, et. al. to understand the factors that govern the concentration and distribution of silica in spores [7]. Samples of *Bacillus anthracis* Ames, and other closely related *Bacillus* species were produced with a wide range of media, production methods, and postproduction treatments. The effect of the silica concentration in the growth medium on the silica concentration in spores was experimentally determined by adding dissolving known concentrations of SiO_2 in different growth media whose native SiO_2 concentration had been determined. Post-production experiments to test the adsorptive capacity of *Bacillus anthracis* spores for SiO_2 in solution were also performed. Silicon concentrations in single spores were determined by high resolution secondary ion mass spectrometry (NanoSIMS) analysis, and bulk silicon concentrations in growth media or spore powders were determined by Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES.) Protocols and standards for quantifying the distribution of elements in single spores were developed in

preliminary work [8,9]

This work demonstrated that a wide range of standard laboratory growth methods produce bacterial spores with silicon levels up to about 3000 μg/g, and that the silicon tends to accumulate preferentially in the spore coat region. This phenomenology was *qualitatively* consistent with the previous observations of Stewart [10] and Michael [6]. The addition of dissolved iron salts to the growth medium enhanced the uptake of silicon, suggesting that silicon uptake in spores might be governed by a general mechanism of silicon adsorption in bacteria reported by Davis [11], Warren [12], Yee [13] and Wightman [14] (referred to hereafter as DWYW). The *qualitative* association between iron and silicon implied by this mechanism was also consistent with the observations of Michael [6].

The average concentration of silicon in the spores was proportional to the silicon concentration in the medium, with an effective spore:solution partition coefficient as high as ≈ 40:1 (i.e. there is preferential binding of dissolved silicon oxides to the spore) at high iron concentrations. Native silicon is commonly found in growth media at concentrations well below saturation. Based on the maximum solubility of SiO_2 in solution (≈ 70 ppm at 35°C [15]) and the partition coefficient between silicon concentrations in the medium and the spores, the maximum level of silicon that is incorporated into spores in a normal fermentation process is ~3000 μg/g (0.3%) by weight. The maximum observed silicon levels among the samples Weber examined [7] were ≤ 3000 μg/g silicon per spore dry weight), consistent with this estimated upper

bound, as shown in Fig. 1.

Preferential deposition of silicon in the spore coat region had previously been observed by Stewart, et. al. in *Bacillus cereus* spores [10]. The concentration of silicon in the *B. cereus* samples was estimated to be ~3000 μg/g (≈0.3%) using an early X-ray spectroscopy method. In their paper, Stewart, et. al. described this as a "surprising" concentration, but, as the attached figure shows, this value is at the high end of the "normal" range of silicon spore concentrations. This upper bound for normal growth methods (0.3%) is significantly lower than the silicon levels inferred to be present in the Amerithrax spores (≈ 1%) by Michael [6]. Their calculation assumed that the Leahy material is predominantly spores with little external material, which is consistent with the appearance of this material in electron micrographs [6,16]. To achieve the 1% level, simple mass balance would require that the initial silica concentration would have to have exceeded saturation by a factor of ≈ 3x even if all the silica in the growth medium had been adsorbed onto spores and cell debris in this material. The observed difference in silica concentration is too high to be explained by statistical fluctuations. Weber, et. al. concluded that the silica concentrations reported for the case samples could not be explained simply by the DWYW mechanism without invoking growth conditions that involve considerable supersaturation of silica in the growth medium, or some sort of "Ostwald ripening" mechanism to increase the size of the silica deposits within the spore, or some as yet unrecognized chemical process.

Two additional observations provide additional support for this conclusion. First,

prolonged exposure of spores to a solution of silicon at saturation was not able to increase spore silicon levels above those seen in spores produced in growth media containing added silicon. As displayed in Fig. 2, in some of these spores SiO_2 actually precipitated onto the spore outer surface during these experiments. Secondly, in addition to the limitation imposed by SiO_2 solubility, the DWYW iron-silicon adsorption mechanism implies an additional bound (Si:Fe ≈ 1:1) on silicon adsorption determined by saturation of all iron binding sites within the spore. The reported silicon to iron ratios in the Amerithrax material [6] is approximately 10:1, significantly too high to be attributable to this mechanism.

Thus, if the estimated silicon concentrations in the Amerithrax spores are correct, they are not consistent with our current understanding of silica deposition, or those materials must have indeed been produced under an unusual set of conditions. If the latter were true, the silica evidence might provide a significant bound on the credible growth and production scenarios that would be consistent with the prosecution narrative in this case.

Presence of agar in spore preparations

Another topic of interest early in the investigation was whether the Amerithrax spore preparations were grown on agar plates rather than in liquid culture. This information might aid an investigation by indicating that certain resources and specific training on agent preparation methods were available to the perpetrator. The effort primarily focused on the idea that when harvesting spores grown on agar plates, agar itself can be entrained into the samples.

Initially Fenselau et. al. [17] developed an assay that uses liquid phase extraction and gas chromatography-mass spectrometry (GC-MS) to identify trace quantities of 3,6-anhydrogalactose (AGal). This method is based on the observation that AGal is a characteristic component of agar, and thus is expected to be a chemical signature indicating the presence of residual agar in a spore preparation. There are several versions of this analytical method reported in the literature [18-20]. Each version uses a chemical extraction procedure that converts the AGal into a unique stable derivative that can be detected using GC-MS instrumentation. The protocol was shown to be nearly quantitative when applied to agar standards [17].

Subsequently, Wunschel et. al. found that spores grown in broth (where AGal is initially absent) yielded detectable amounts of AGal when subjected to the Fenselau protocol, leading to false positive detections [21]. Therefore, they developed an alternative analysis method that permitted them to avoid this problem, and readily detected the AGal marker, despite its partial destruction during hydrolysis. An analysis of a wide range of spore preparations provided preliminary validation that this new protocol could be used to identify agar grown spores with high detection probability and low false positive probability.

Note, however, that strictly speaking, the analysis does not directly answer the question "Was the agent grown on agar medium?" Drawing this inference from detection of AGal in an agent sample would be incorrect, for example, if agar were deliberately added to the

agent after it had been grown in, liquid culture. (Aside from the possibility that a perpetrator might do this to mis-direct investigators, there could be other motivations, for example AGal containing polysaccharides such as carrageenan can be used in certain micro-encapsulation methods for bacteria [22,23].) To rigorously validate such an interpretation, it would be necessary to have some independent method of analysis that could exclude the possibility of post-growth addition of some material that contains AGal. Likewise, a negative result cannot simply be interpreted as excluding the possibility that the agent was grown on agar plates. This could happen if the agent were washed sufficiently well after growth that the residual agar concentration is below the limit of detection (LOD) for the analysis. Clearly, *by itself* agar analysis can only reveal that AGal is present in the sample or determine that it is undetectable. At best it can provide quantitative information like: "This sample has x ± y micrograms of AGal per gram of agent; or "this sample has less than x micrograms of AGal per gram of agent."

Several years after the AGal analysis protocols had been developed, Malkin and co-workers observed that there were measureable differences in the distributions of spore sizes from agar plate and liquid shake flask cultures [24]. Spore dimensions could be quantified using Atomic Force Microscopy (AFM) imaging, and expressed as spore length or calculated volume. A preliminary validation study was performed with a limited number of *B. anthracis* Sterne samples that were provided from several different laboratories, and the results are shown in Fig. 3. These empirical distributions are based on measurements of more than 100 individual spores from each sample.

To understand the inferential power of observing that a spore in a questioned sample has a certain volume it is useful to plot the receiver-operating characteristic (ROC) curve corresponding to the size distribution data, as is done in Fig. 4. The slope (derivative) of the ROC curve at any point is the likelihood ratio corresponding to the value of the metric corresponding to that point. Spore volumes smaller than ≈0.53 μ^3 correspond to points on the ROC curve where the likelihood ratio for the liquid growth hypothesis is higher than 1 (and consequently the measurement does not favor agar growth.) For a single spore measurement, likelihood ratios over the 95% probable size range for shake flask grown spores are fairly modest, in the range 1 to 10. However, as Fig. 4 shows, the ROC curve is considerably sharper, and the likelihood ratios considerably higher if the comparison metric is based on the average of 3 spores. For averages over 10 spores (not shown) the ROC curve is nearly a "perfect" classifier with an area under curve (AUC) value > 0.99 [25].

These results suggested that spore size measurements might provide a viable addition to chemical analysis for determining if an agent was grown on agar plates. In addition to providing an "orthogonal" method, AFM analysis has the advantage that it does not require bulk sample but can work on trace levels of material. Since it does not rely on agar as a signature it is immune to the presence of other sources of AGal, or rigorous washing. However, it was not clear what factors actually determine the difference in size, so interpretation of evidence on the basis of the preliminary validation results alone demanded substantial caution. Therefore a more extensive validation cycle using greater variety of media and inclusion of fermentor grown spores in addition to shake flask

samples was initiated, and is ongoing.

The silica and agar analyses are typical representatives of a variety of assay development efforts that were initiated after Amerithrax. Some were abandoned after initial exploratory results proved puzzling or disappointing, others moved on to SOP development, and a few were subjected to more extensive validation studies. These then form a core of capabilities and exemplars for future casework.

Sample matching

Physical and chemical measurements can be used to compare evidence samples collected by investigators at different locations or at different times. Matching of sample properties can help establish the relatedness of disparate incidents, and mis-matches might exclude certain scenarios, or signify a more complex etiology of the events under investigation. An early report outlining research issues in the chemical and physical analysis of microbial agents for forensics states:

> "Chemical and morphological analysis for sample matching has a long history in forensics, and is likely to be acceptable *in principle* in court, assuming that match criteria are well defined and derived from known limits of precision of the measurement techniques in question. Thus, apart from certain operational issues (such as how to prioritize such measurements in the face of limited sample availability, or how to render samples safe for handling in the analytical laboratory,) instrumental analysis of biological agents for purposes of sample matching alone is unlikely to present fundamental problems that require extensive research and development investments [26]."

In the years after this was written, it became apparent that there *were*, in fact, fundamental issues regarding inferential validation of sample matching protocols for

biological agents. This concern arose from the National Research Council's report on bullet lead analysis and the subsequent abandonment of this method by the FBI [27]. The NRC study contains some valuable insights into the way in which inferences about sample matching should be presented in court, and the statistical framework in which the interpretation of such data is validated. Studies undertaken within the last few years have applied these considerations to matching biological agent samples [28,29] by means of elemental analysis and other methods.

A key feature of the assays that have been developed is that the term "match" is never used to describe test results. The term "match" itself is often difficult to define objectively, and can lead to the adoption of arbitrary or subjective criteria for declaring that two samples are related. Therefore the tests that have been developed adhere to a general paradigm for evaluating and validating the inferential power of sample comparisons that does not rely on defining match criteria. This paradigm consists of:

- Formulating a testable hypothesis concerning common origin of the two samples; for example, were the samples made by the same process in the same laboratory?
- Defining the population of samples for which the test is relevant; for example, the population of all bench-top processes for making dry spore preparations.
- Developing representative sampling frames for the relevant populations; for example, a list of all processes obtained from open sources, and a list of laboratories that can generate surrogate (non-pathogenic) agent samples.
- Performing sampling of exemplars from those frames. Typically this involves having multiple laboratories make samples using multiple processes where the laboratories and processes were selected at random from their respective frames.
- Selecting the chemical or physical signature to be determined by analysis, e.g. the concentrations of a particular set of elements.

- Defining an objective metric derived from the measurement of the signature in two samples that will be used as the basis for the hypothesis test. The metric quantitatively expresses how similar the signatures are in the two samples.

- Evaluating the metric for each pair of samples drawn from the exemplar set, and using the metric to construct a ROC curve estimator for the likelihood ratio at each observed value of the metric.

An example of a ROC curve generated using this method is shown in Fig. 5. In this case, the metric delta is the sum over the squared differences between the concentrations of 11 elements in pairs of samples that are drawn from a population of samples that were made in multiple batches by 7 different processes in 4 different laboratories. The pair population consists of two sub-populations, one consisting of pairs made by the same process in the same laboratory, and the other consisting of pairs made by different processes, or in different laboratories (or both). The ROC curve indicates that if a pair of samples has delta less than ≈ 1.5 then the likelihood ratio is greater than 1, supporting the hypothesis that they were made by the same process in the same laboratory. Over the range of delta values observed for this subpopulation, the likelihood ratio ranges between one and several hundred.

Other hypotheses can be tested by pair comparisons as well, for example that the two samples were made in the same laboratory, but in different batches; or that they were made by the same process, but in different laboratories. The testable hypotheses form a hierarchy of tests in which the corresponding ROC curves are formed from pairs of samples drawn from appropriate sub-populations. The analyst reports the results of comparing two samples by quoting a likelihood ratio for the hypothesis in question.

Constructing a representative sampling frame for agent production process requires a systematic understanding of the many different possible "recipes" for generating agents. Several different frames are possible, including a very general "unit process" frame in which generic recipes are broken down into their basic steps [26,28]. For each unit process in the recipe, there are usually many choices of specific techniques and materials. End-to-end processes are constructed by randomly sampling from the list of specific variants for each unit process. Of course, agents used in bio-crimes could be crudely processed, and in some cases no "processing" as such may be used. Challenges to the validity of the likelihood ratios determined by sample matching tests will ultimately rest on whether the questioned samples were really drawn from the same population as the samples used to generate the ROC curve.

Remaining R&D challenges

There remain a number of significant R&D challenges associated with developing a robust forensic capability for chemical and physical analysis of biological agents. One of the most significant gaps is the ability to date agents. Determining how long before dispersal a biological agent was prepared is important for establishing timelines that are consistent with any narrative that ties together the acquisition, manufacture, transport, and deployment of a device or material. Of course, in situations where the attack agent is only recovered from infected victims, dating its preparation would not be possible *in principle*. On the other hand, if bulk agent is recovered, radiocarbon assay methods provide the only established method for dating. Radiocarbon dating of biological agents using accelerator mass spectrometry [1,30] relies on the fact that common growth

medium components are derived from agricultural products whose radiocarbon content reflects that of the atmosphere at the time they were produced. The radiocarbon content of an organism grown in these media will reflect its production date, assuming that the agent was produced not too long after the medium components were manufactured. However, this technique has a resolution of ±1 year, at best (for recent materials), and is difficult to apply to complex "weaponized" agents, since these can contain petroleum derived components that are radiocarbon depleted and can distort the estimated age of the sample.

It seems likely that other chemical or physical phenomena could be harnessed for dating agents made only months, weeks, or days prior to release, and might be applicable to single particles. One such technique that was proposed involved using nanometer resolution secondary ionization mass spectrometry (nano-SIMS) to map the relaxation of elemental gradients in spores and bacteria after they have been processed [31]. However, relaxation rates of easily measured elements appeared to be too slow to be used this way. Therefore, the development of new agent dating capabilities remains a salient R&D gap.

There is a general need to increase the number of tests that can be applied to trace quantities of agents instead of requiring bulk material, and to replace destructive tests with non-destructive alternatives. Currently, the choice of analytical method that can be applied depends critically on the amount of material available and whether it is contaminated with extraneous material that can compromise the analysis. An initial determination of these qualities has been made for many of the common analysis

methods that are available [8,16,17,21,24,32-40], shown in Table 1. These sample requirement determinations are generalizations, and can depend on the precise object of the analysis. Most of the existing protocols require uncontaminated bulk material and are destructive. Examples include protocols based on solvent extraction of a signature compound, imaging that requires sample fixation, and ablative mass spectroscopy techniques like MALDI and SIMS.

During the Amerithrax investigation, the types of chemical and physical studies that could be considered were generally constrained by available sample size. Because several letters contained substantial quantities of powder [41] the scientific community was able to offer a number of reasonable methods for addressing important questions about this material. However, at some Amerithrax crime scenes bulk powder evidence was not available, so these methods could not be applied. In many imaginable future attack scenarios, the only agent samples that would be available for analysis are those recovered from contaminated surfaces, ductwork or filters from building air conditioning systems, or material on the filter units used in urban air samplers. Such samples would be heavily mixed with other materials, requiring the analyst to identify and isolate the agent particles from the mixture. In the future, it will clearly be important to extend the reach of more kinds of chemical and physical analysis to such situations.

In most cases, chemical and physical analysis of case samples takes place on dedicated instrumentation that is not housed in a biosafety level 3 or 4 environment. As a result, it is desirable to develop inactivation protocols to render samples safe for analysis. In

many bulk analysis procedures, extraction protocols can be designed to render the analytical sample safe, and the extraction can be performed in a biosafety area. However, research needs to be conducted to determine the effect of irradiation or decontamination protocols based on chlorine dioxide (ClO_2) [42] or vaporous hydrogen peroxide (VHP) [43] on signatures that must be determined directly from the agent particles themselves.

Little systematic development of specialized collection methods for instrumental analysis of agent samples has occurred. As more sophisticated analysis methods are developed, it will be important to establish collection and storage methods that do not inadvertently distort subtle structural signatures or alter chemical clues by contamination, or allow them to degrade prior to analysis.

Finally, in many cases it is desirable to use non-pathogenic surrogates for research and development of instrumental analysis methods. Current practice has settled on the use of *B. anthracis* Sterne as a surrogate for virulent *anthracis* strains. There is no standard surrogate non-pathogenic substitute for other pathogens, as yet. The transferability of conclusions drawn from studies on surrogates to the actual agent may not always be straightforward. Therefore, it will be necessary to understand better the limitations of surrogates and how to choose the best surrogate for a given study.

Preparing for future events

The National Bioforensics Analysis Center (NBFAC) is the lead organization for conducting technical (biological, chemical, and physical) analysis of biological agents

used in terrorist or criminal activities affecting U.S. persons or assets [44,45]. In future cases, NBFAC may need to call upon the specialized chemical and physical analysis capabilities of other laboratories ("spoke labs") in order to carry out analyses that utilize sophisticated and expensive instrumentation and unique expertise. It is clearly prudent to have a set of standard procedures in place that can be called upon as needed. On the other hand, there will always be a need to develop certain analyses *ad hoc*, depending on the particular circumstances of a given case. Thus, a pre-existing plan should be flexible enough to accommodate new or unusual samples or situations. In this sense, a "seamless" sample analysis plan involves two major activities: First, to formalize and standardize methodologies as much as possible by anticipating the types of analyses that may be called for and developing Standard Operating Procedures (SOPs) for performing them (i.e. think ahead), and secondly to formalize a mechanism for deciding how to handle new situations for which there are no existing SOPs. When only limited or trace samples are available, a systematic procedure for choosing the most informative analytical tests becomes crucial. It will be important to formalize such considerations to expedite the development of the best analytical plan when an incident occurs. More generally, the formulation of a comprehensive plan involves:

- Developing a mechanism for quickly formulating a consensus analytical plan when a new sample (or set of samples) arises
- Keeping and updating a set of standardized procedures (SOPs) and validation data for analyzing case samples
- Maintaining a set of documented guidelines, requirements, and procedures for sample preparation for each analytical procedure
- Maintaining approved procedures for handling and storing samples
- Developing standardized methods for data analysis, reporting, and presentation

- Maintaining reliable channels for sending & receiving samples
- Maintaining secure conduits for data, information, and discussion
- Developing a mechanism for formulating an on-the-fly validation plan for a new procedure

While many of these items are in place, there has been little or no systematic testing of the existing infrastructure through planned exercises. In the absence of actual casework, comprehensive exercises that include evidence collection, analysis, and expert testimony at mock trials and admissibility hearings are critical for maintaining effective preparedness.

Concluding remarks

The forensic utility of the data that can be obtained from chemical and physical analysis of agents depends critically on establishing rigorous and defensible scientific underpinnings. While much progress has been made over the last decade, it is important to keep in mind that to date, technical microbial forensics is largely untested in U.S. courtrooms and has only begun to be subjected to the scrutiny of domestic and international scientific fora. The most prominent biological terrorism case to date, the anthrax letters incident, will apparently close without a trial [46]. Given the relative rarity of major biological terrorism events, there is some danger of complacency about the strength of our preparations, and uncertainty about research and development priorities.

If there is any lesson to be drawn from past experience, it is that microbial forensic collection and analysis are *not* very effective when they are conducted as an *ad-hoc* activities, by non-specialists, using improvised methods and on-the-fly attempts at validation, without prior review by a knowledgeable community. The utility of microbial forensic analysis rises in proportion to the extent that it is anticipatory, well-planned, driven by a cadre of qualified experts, and adequately resourced. This, in turn, requires adequate attention to the need for long-term fundamental research on many aspects of biological agent composition and structure. It is encouraging that a robust microbial forensic research and development program is now recognized as an essential part of the national strategy to combat biological threats [45]. With sufficient targeted investment the technical challenges associated with chemical and physical analysis can be met, and the expertise gained in this area in the last decade will continue to evolve towards an established capability.

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Table 1. Characteristics of some instrumental analysis methods.

Type of analysis	Method	Destructive or non-destructive?	Can use irradiated sample?	Can use ClO_2 or VHP to decontaminate?	Minimum amount needed	Pristine sample required?
Elemental analysis	**ICP-MS[1]**	**Destructive**	**Yes**	**Yes**	**1 mg**	**Yes**
	MXRF[2]	**Non-des.**	**Yes**	**Yes**	**1 mg**	**No**
	PIXE[3]	**Non-des.**	**Yes**	**Yes**	**1 μg**	**No**
	SIMS[4]	**Destructive**	**Yes**	**Yes**	**Trace**	**No**
Isotopic analysis	**MS[5]**	**Destructive**	**Yes**	**?**	**1 mg**	**Yes**
	SIMS	**Destructive**	**Yes**	**?**	**Trace**	**No**
Organic analysis	**GC-MS[6]**	**Destructive**	**No**	**?**	**1 mg**	**Yes**
	ES-MS[7]		**No**			**Yes**
	MALDI[8]	**Destructive**	**No**	**?**	**1 mg**	**Yes**
	IR[9]	**Non-des.**	**No**	**?**	**1 mg**	**Yes**
	NMR[10]	**Non-des.**	**No**	**?**	**1 mg**	**Yes**
	μ-Raman[11]	**Non-des.**	**No**	**?**	**1 μg**	**Yes**
Microstructural analysis	**SIMS**	**Destructive**	**No**	**Yes**	**Trace**	**No**
	STEM[12]	**Destructive**	**No**	**Yes**	**Trace**	**No**

[1]Inductively Coupled Plasma Mass Spectrometry
[2]Micro X-ray fluorescence
[3]Particle Induced X-ray Emission
[4]Secondary Ion Mass spectrometry
[5]Stable Isotope Mass Spectrometry
[6]Gas Chromatography-Mass Spectrometry
[7]Electro-spray Mass Spectrometry
[8]Matrix Assisted Laser Desorption-Ionization
[9]Infrared spectroscopy
[10]Nuclear Magnetic Resonance spectroscopy
[11]Micro Raman spectroscopy
[12]Scanning Transmission Electron Microscopy

Figures

Figure 1. Silicon concentration in single spores determined by nanoSIMS analysis (data of Weber, et. al., reference 7.)

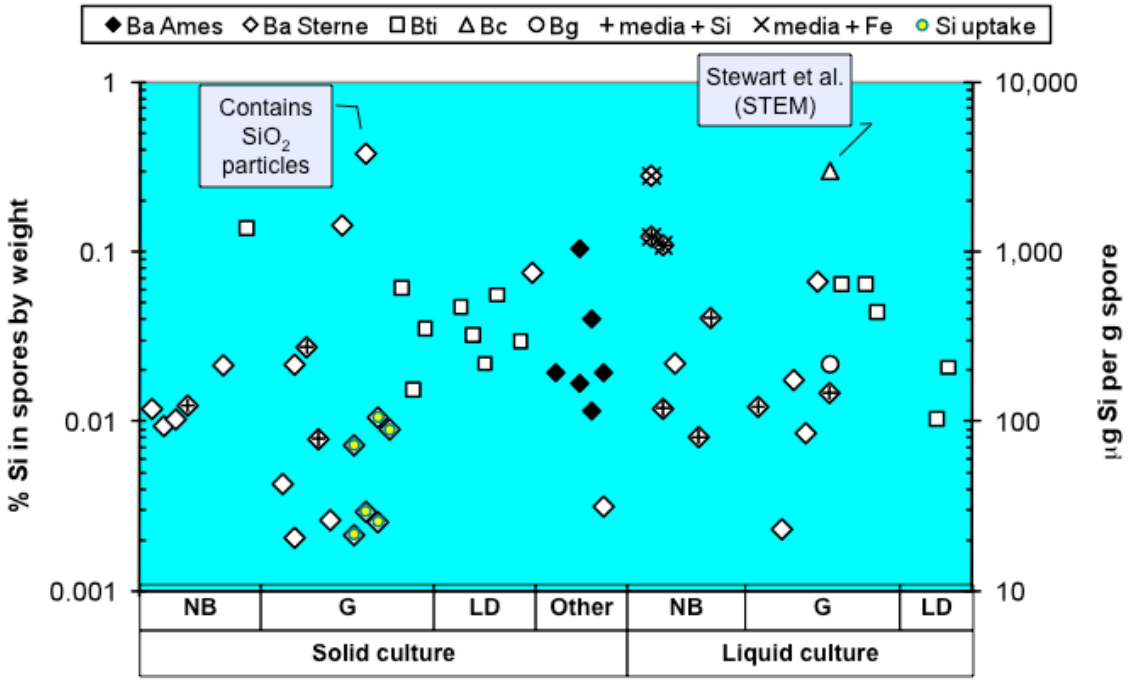
Figure 2. Spores coated with precipitated SiO_2 from a supersaturated solution.

Figure 3. Observed distributions of spore sizes for agar and liquid cultures.

Figure 4. Receiver-operating characteristic for the size based test for agar plate culture. Dashed arrows indicate the approximate position of the spore volume value (0.53 μ^3) that corresponds to a likelihood ratio value of 1.

Figure 5. ROC curve for testing if two samples were made by the same process in the same laboratory.

Figures for *Non-biological measurements on biological samples*



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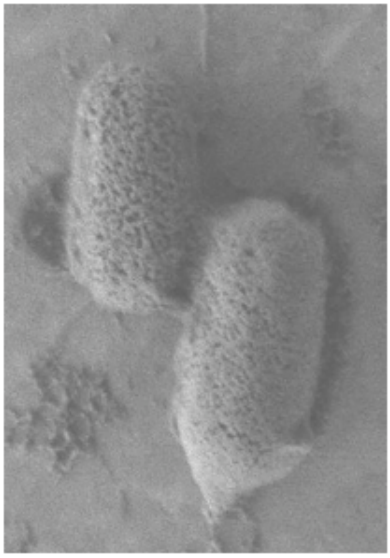
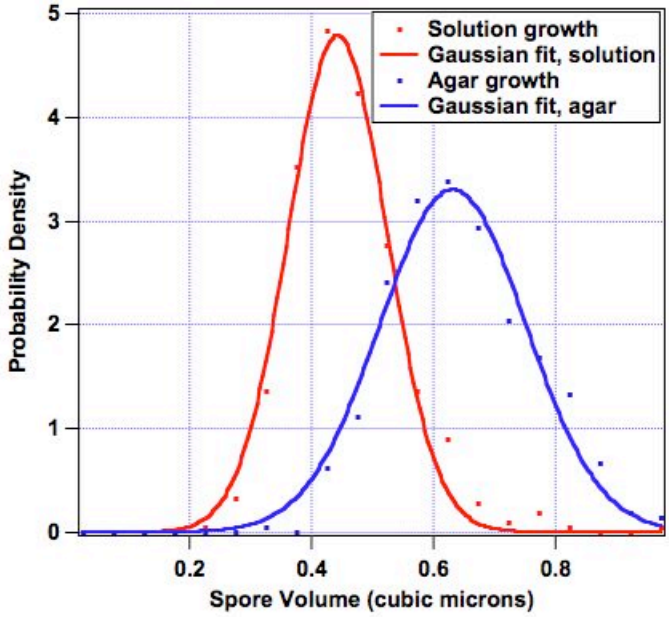


Figure 2. Spores coated with precipitated SiO_2 from a supersaturated solution.



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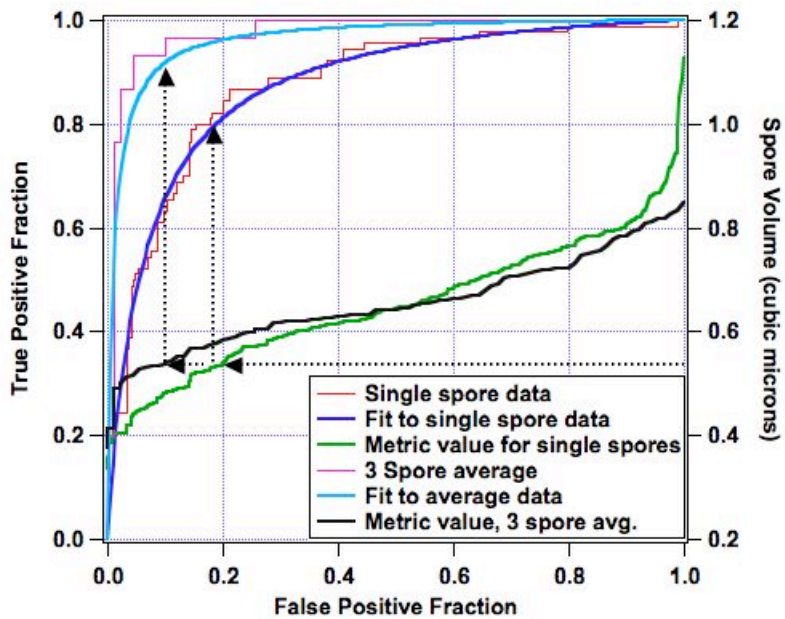

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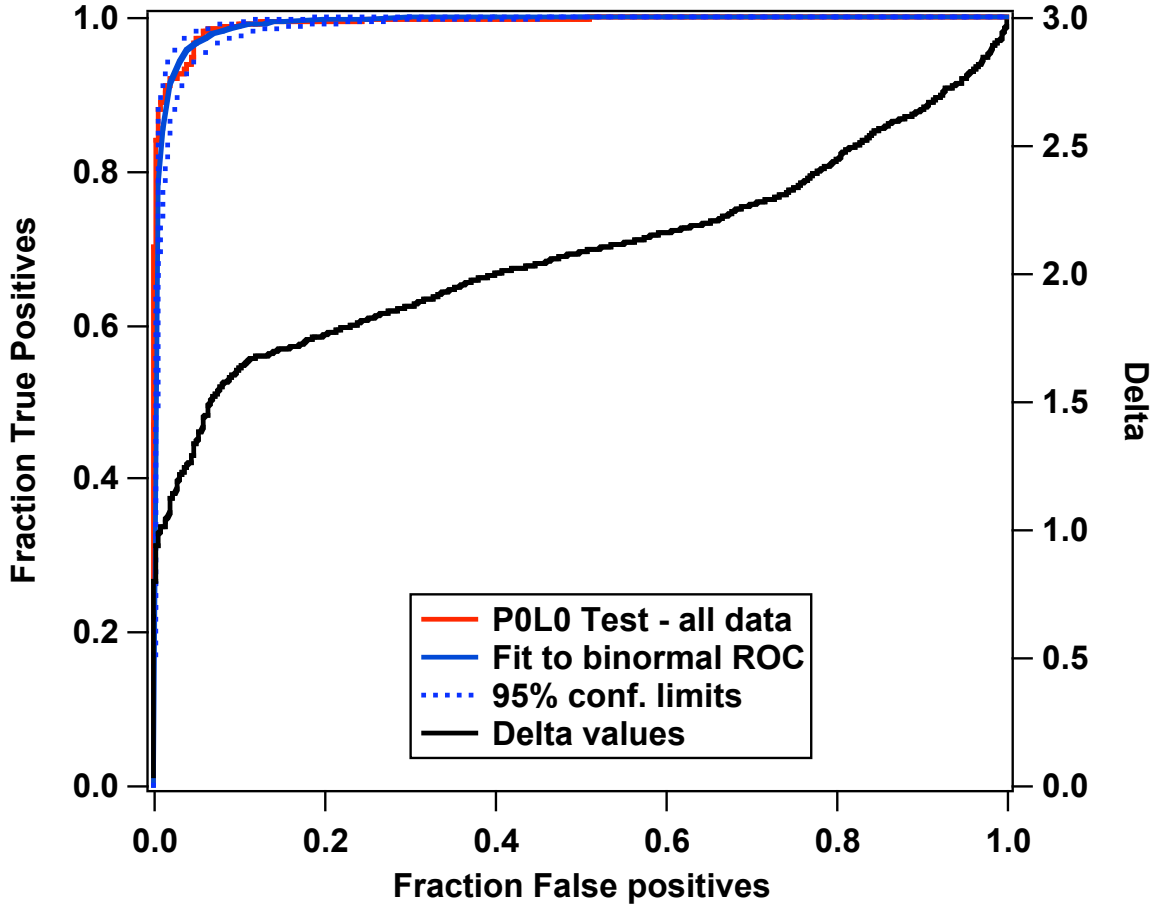

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