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Generation of Biomarkers from Anthrax Spores by Catalysis and Analytical Pyrolysis

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GENERATION OF BIOMARKERS FROM ANTHRAX SPORES BY CATALYSIS AND ANALYTICAL PYROLYSIS

by

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ABSTRACT

GENERATION OF BIOMARKERS FROM ANTHRAX SPORES

BY CATALYSIS AND ANALYTICAL PYROLYSIS

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Master of Science

Anthrax spores, in weaponized form, are dangerous biological warfare agents. Handheld technology for the rapid detection of anthrax is greatly needed to improve national security. Methods to detect anthrax spores are diverse, with most taking at least an hour for positive identification. A viable option for rapid detection is analytical pyrolysis (AP), which produces chemicals containing taxonomical information (biomarkers). AP methods are reviewed and critically analyzed to show that reproducible detection of anthrax spores in a rapid manner (<5 min) with a handheld device is not currently possible. A promising alternative to AP is the use of a catalyst to produce biomarkers from anthrax spores with improved selectivity and reproducibility. Catalytic materials having promise for this include platinum, nickel, and superacids. Experiments evaluating several of these materials are described.

A biomarker mass spectral library was created, based on information available in the scientific literature, to facilitate analysis and identification of the biomarkers produced experimentally. The RAMFAC algorithm was used to deconvolute chromatographic peaks to produce clean mass spectra and match them against entries in the biomarker library. While the library is not complete, its use with the RAMFAC algorithm enabled detection of many important biomarkers in experiments involving catalytic breakdown of anthrax spores.

Experimental results from preliminary tests of several catalysts are presented and discussed. Addition of catalysts in the form of platinum nanoclusters and superacids to bacterial spores in a commercial pyrolyzer effected an increase in the amount of biomarkers produced at mild conditions over traditional pyrolysis methods. Electroformed nickel mesh, on the other hand, demonstrated low catalytic activity for the production of biomarkers, likely due to poor contact of the spores with the mesh. Biomarkers similar to those published in the literature were observed, including dipicolinic acid, picolinic acid, propionamide, acetamide, diketopiperazines, fatty acids, furfuryl alcohol, and DNA bases. A statistically designed factorial study was used to determine the importance of temperature, spore loading, and nanocluster loading on the production of three important biomarkers. The relative importance of these variables differs for each of the three important biomarkers, suggesting they are produced by different reaction mechanisms.

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Chapter 1. Introduction and Background

The availability of biological warfare agents throughout the world poses a serious threat to the national security of the United States of America. These agents include bacteria, bacterial spores, toxins, and viruses. Of particular concern is the weaponized form of the bacterium *Bacillus anthracis*, commonly known as anthrax (Hawley and Eitzen Jr 2001; Inglesby 2002; Inglesby *et al.* 2002). Weaponized anthrax is a fine white powder consisting of micron-sized, ellipsoidal bacterial spores. In this form, anthrax is very easily dispersed into the atmosphere; residence times are typically several days, depending on the meteorological conditions. The spores germinate when inhaled into the lungs, deposited onto the skin, or ingested. After germination, the cells proliferate in body tissues and begin to produce deadly toxins (Pasechnik *et al.* 1993). Anthrax can be lethal in very small doses (around 10,000 spores – about 10 nanograms) (Hawley and Eitzen Jr 2001; Pepper and Gentry 2002; Fennelly *et al.* 2004). The toxicity of anthrax, the ease of its dispersal, and the large possible atmospheric residence times combine to make it a very dangerous biological weapon. Methods for the detection and identification of anthrax are therefore crucial in order to prevent or defend against an anthrax attack and facilitate a rapid response to mitigate its effects.

Historically, the method of choice for identifying an unknown sample of bacterial origin has been to grow bacterial colonies from the spores. Following the culture growth, biochemical assays, stains, and microscopic visualization are used to confirm the

presence of anthrax in the original sample (Jackson *et al.* 1998). While this approach works, it takes days to accomplish, and requires significant amounts of equipment. Also, background contaminants can interfere with this method. Therefore, there have been efforts over the last 40 years to develop novel, rapid, and selective detection and identification methods.

The US Armed Forces are interested in technologies that can rapidly detect the presence of anthrax spores, primarily to protect its armed forces from biological attack, as well as to track down and stop terrorists and/or rogue states that are producing or developing biological warfare agents (Hawley and Eitzen Jr 2001). In this application false positives must be avoided, i.e., sensitivity and accuracy must be high (Turnbull 1999) and species-level discrimination is a necessity. A handheld device that meets these requirements is not currently available, despite the great need.

Much effort has been focused on ways to use the many different biochemical compounds contained in bacterial spores in identification algorithms for the rapid detection of anthrax spores. Methods used to remove these biochemical compounds from the microorganism and convert them into detectible chemicals (biomarkers) play a key role in the detection technology. Typical biomarker precursors include fatty acids, proteins, carbohydrates, and deoxyribonucleic acid (DNA). In some cases specific chemicals such as calcium complexed-dipicolinic acid (DPA) may be important.

Several methods and devices to rapidly generate biomarkers from bacterial spores have been developed over the past three decades. Commercially available detection systems are expensive and exhibit limited utility. These include (1) wet detection methods, involving either chemical extractions (e.g., DNA sequencing) or biological

recognition techniques (e.g., antibody based); and (2) dry detection methods, involving the physical decomposition of a sample (e.g., pyrolysis) and detection of the compounds that are released. Other techniques are variations on these, for example the combination of liquid phase chemical extraction with pyrolysis (MIDI 2005). These methods are described in detail in Chapter 2.

The sequencing of DNA gleaned from cellular extracts has been used for the unique identification of bacteria at the species and sub-species level. This technique has been applied to anthrax cells (Bell *et al.* 2002) and spores (Ryu *et al.* 2003); unfortunately, this method takes several hours for bacterial cells, and an additional hour or more for bacterial spores. Analytical pyrolysis (AP), which thermally breaks down precursors to more volatile biomarkers that are detected by analytical techniques, has become a viable alternative for detecting and identifying anthrax spores. Thermal pyrolysis at high heating rates has had success differentiating bacteria at the gram-classification level (Snyder *et al.* 2004; Dworzanski *et al.* 2005). Thermal-hydrolysis methylation (THM), utilizing a derivatizing agent such as a quaternary ammonium hydroxide (TMAH) in concert with pyrolysis, has been able to differentiate bacteria at the species level (Beverly *et al.* 1999b; Hendricker *et al.* 1999; Luo *et al.* 1999; MIDI 2005). With both of these methods, analysis times have improved significantly, with some techniques requiring less than 15 min for sample preparation, pyrolysis, analysis, and identification.

Although AP methods can produce and analyze biomarkers more rapidly than other methods, the required equipment tends to be bulky and require large amounts of power. Moreover, assessment of the reproducibility and general applicability of these

biomarker generation techniques, while critical to their successful development (Xu *et al.* 2003), has been neglected. Thus, while this technology has promise, improvements in biomarker production speed and reproducibility, as well as reductions in detection time, analysis complexity, equipment size, and power consumption, are necessary to advance pyrolysis technology to the required level of performance.

Most of the published studies on the AP of bacterial spores have not chemically identified the pyrolysis products, reactions, and reactants that are involved in the production of biomarkers. The remaining studies have only done so superficially. Finally, previous authors have evidently been unaware of pertinent literature having a direct bearing on the pyrolytic production of biomarkers. Thus, a review detailing and critically analyzing different approaches and results would fill an important need. A comprehensive, critical review addressing the production of biomarkers from bacterial spores by AP is presented in Chapter 2.

The application of catalysis to biomarker production from bacterial spores has significant potential for improving reproducibility, detection time, and portability. A search of the scientific literature has not revealed any reported applications of heterogeneous catalysts to the controlled breakdown or conversion of bacterial endospores, or any other biological material, to biomarkers for detection and identification purposes.

Application of the principles of industrial catalyst design, i.e., catalyst selection and reaction engineering, to this new use of heterogeneous catalysis provides a foundation for this research (Trimm 1980). To breakdown the spores and produce biomarkers, the breaking of carbon-carbon, carbon-nitrogen, and carbon-oxygen bonds

and the formation of carbon-oxygen ester bonds are required. Catalysts that may facilitate these types of reactions include supported nickel and platinum, as well as heteropolyacids (superacids). Literature relevant to the development of a catalytic method for the production of biomarkers from bacterial spores is presented and discussed in Chapter 2.

Chapter 3 describes the scope and objectives of the thesis, which include the literature review discussed above, and additionally limited experimental investigation of three possible catalysts: (1) Pt-nanoclusters, (2) an electroformed fine nickel mesh, and (3) tungstophosphoric acid (TPA), a Keggin-structure superacid. Chapter 4 describes the experimental methods used to investigate the catalytic properties of these materials for the production of biomarkers from bacterial spores. The results of these experiments, presented and discussed in Chapter 5, show that the nickel mesh in its present form is ineffective, while both the Pt-nanoclusters and TPA are effective in producing biomarkers from bacterial spores and merit further investigation.

Conclusions and recommendations are provided in Chapter 6. A complete set of references (over 140) is found at the end of the thesis body. Appendices containing relevant details, data, and methods are attached.

Chapter 2. Literature Review and Critical Analysis

Anthrax as a Biological Warfare Agent

Biological warfare agents can take several forms. They include bacteria, bacterial spores, toxins, and viruses (Hawley and Eitzen Jr 2001). The mode of action of most biological warfare agents is the invasion of host cells (human or animal), followed by replication and destruction of host tissues either by infection or via the action of secreted toxins. Some agents mostly incapacitate, while others have the potential to kill a large fraction of hosts infected. Due to the lethality, availability, longevity, and potential for dispersal of spores, anthrax is an especially dangerous biological agent (Hawley and Eitzen Jr 2001; Pepper and Gentry 2002; Fennelly *et al.* 2004). In this portion of the literature review, the physiology of bacterial spores will be described, followed by more specific information on anthrax spores.

Bacterial Spore Physiology

Bacteria are single-cell prokaryotic organisms that can be roughly divided into two types, gram-positive and gram-negative, based on their response to a chemical staining procedure (the Gram stain). Gram-positive bacteria contain a thick layer of peptidoglycan outside of the cell membrane that is a barrier to loss of the primary dye complex during the decolorization step of the Gram stain. Gram-negative bacteria contain a very thin peptidoglycan layer and therefore do not retain the primary dye complex, but

are stained with the counterstain (Campbell *et al.* 1999). Under favorable conditions, bacteria grow and reproduce in what is called a vegetative state. Under environmental stress, such as nutrient depletion, dehydration, pH changes, or heat, the gram-positive *Bacillus* and *Clostridium* genera produce endospores through a process called sporulation (see Figure 1) (Gould and Hurst 1969; Mock and Fouet 2001). The endospores are formed inside the parent cell and released.

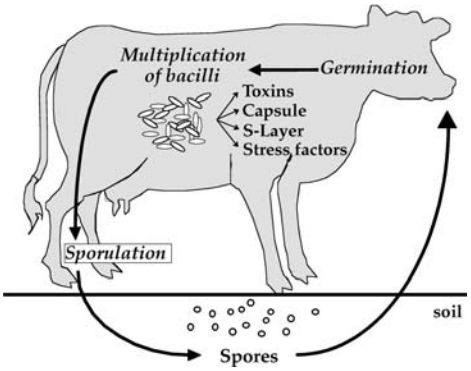


Figure 1. Anthrax (*Bacillus anthracis*) sporulation/germination cycle (Mock and Fouet 2001).

Spores are tough, multi-layered structures, capable of remaining dormant for long periods of time, up to millions of years under ideal conditions (Cano and Borucki 1995). Upon the return of favorable conditions, the spores germinate, producing normal bacterial cells. Spores are very resilient, resisting damage from heat, dehydration, radiation, and a host of other environmental conditions that destroy vegetative bacteria (Atrih and Foster 2002). The sources of this resilience are thought to be the protective layers that are produced during sporulation as well as the high level of dehydration achieved in the core of the spore (the protoplast) (Driks 2003). The spore layers include a thick layer of peptidoglycan (the spore cortex), two thick layers of protein (the spore coats), and for some species a loose layer of glycoprotein (protein and carbohydrate) and lipid that fits

over the outside of the spores, called the exosporium. These layers protect the spore protoplast from chemical and biochemical attack, as well as the degrading effects of radiation and heat. Figure 2 shows the layers of a typical spore (without an exosporium), while Figure 3 is a microscopic image of several spores. Spores are ellipsoidal structures that are around 1.2 μm long and 0.8 μm in diameter (see Figure 3) (Chada *et al.* 2003). Table 1 gives a summary of the various spore layers and their biochemical contents.

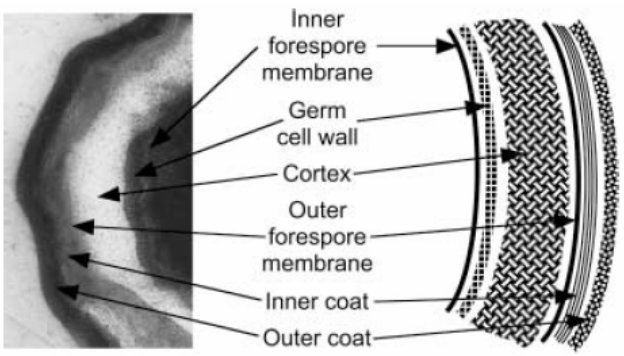


Figure 2. Layers of a typical bacterial spore (Popham 2002).

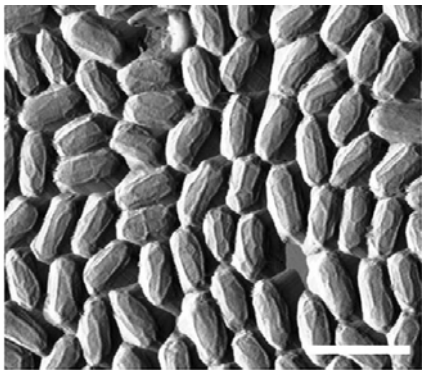


Figure 3. Atomic force microscopy (AFM) images of *Bacillus subtilis* spores; bar = 2.25 μm (Chada *et al.* 2003).

Table 1. Biochemical composition of the layers of the bacterial spore.

Layer	Biochemical Contents
Exosporium (if present)	Lipids (free and bound fatty acids)
Protein Coats	Protein (polypeptides and enzymes)
Cortex	Peptidoglycan (sugar chains and short peptides)
Protoplast	Cytoplasm, DPA, DNA, PHB, plasmids, lipids

The thickness of the individual layers varies between species and genera; in general the spore cortex, made of peptidoglycan, is the thickest layer (approximately 200 nm thick). Peptidoglycan is composed of long chains of glycan (sugars) cross-linked with short peptide fragments. The average length of these glycan chains is about 200 sugar residues (Popham 2002); they consist of alternating *N*-acetyl-glucosamine and *N*-acetyl-muramic acid residues, while the crosslinking peptide fragments are tetra- and pentamers consisting largely of the amino acids alanine and glutamate that. Spore peptidoglycan has a crosslinking extent of around 2.9% (i.e., only about 3 out of every 100 possible glycan linkages are actually crosslinked) (Atrih *et al.* 1996). Parton and Popham *et al.* report the existence of a cross-linking gradient across the spore cortex of *Bacillus subtilis* that ranged from <1% to around 10% (Meador-Parton and Popham 2000).

The proteinaceous spore coats (inner and outer) are the next thickest layers, being approximately 60 and 100 nm in thickness (respectively), depending on the species and the sporulation conditions (Driks 1999; Henriques and Moran 2000). The thickness of the coat layers is dependant on the species, and is the most prominent physical difference between spores of different species (Driks 1999; Henriques and Moran 2000). Much of the protein in the spore coats is highly crosslinked, giving these layers exceptional strength and durability; the amino acids cysteine and tyrosine are especially prevalent in these layers due to their functionality in protein-protein covalent crosslinkages. Enzymes, mostly confined to the spore coats, constitute the other type of protein present in these layers. The enzymes function in sporulation and germination, and generally do not have any structural significance in a dormant spore.

The spore's DNA is protected by small acid soluble proteins, or SASPS, which bind tightly to the DNA. Another important compound in the protoplasm is calcium complexed 2,6-pyridinedicarboxylic acid, more commonly known as dipicolinic acid (DPA – structure shown in Figure 4). This chemical is produced only during sporulation, and is unique to gram-positive bacterial spores of the *Bacillus* and *Clostridium* genera. According to Gould, DPA accounts for between 5 and 15% of the dry weight of the spore (Gould and Hurst 1969). The SASPS and the DPA, combined with the highly dehydrated state of the spore, give the protoplasm a gel-like consistency.

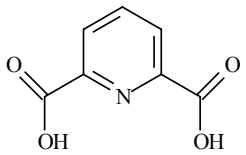


Figure 4. Dipicolinic acid.

Bacterial spores contain relatively low amounts of lipids. These lipids are found in both free and bound form, as shown in Figures 5 and 6 (Deluca *et al.* 1990). In the free form, as fatty acids (FAs), they are long chain hydrocarbons with a carboxylic acid moiety on one end. In the bound form, two or three FAs are joined by ester bonds with a glycerol molecule; the resulting molecules are called di- or triglycerides, respectively (see Figure 6). Lipids are important structural components in membranes and are also energy storage compounds (Lehninger *et al.* 2000). In bacterial spores, lipids make up a significant percentage of the exosporia (Gould and Hurst 1969; Matz *et al.* 1970). They also are present in the inner forespore membrane (see Figure 2) separating the protoplast from the cortex.

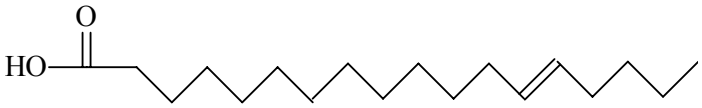


Figure 5. Fatty acid (free form).

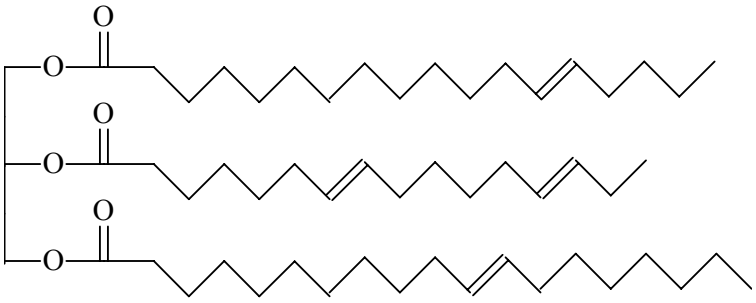


Figure 6. Triglyceride (bound fatty acids).

Chada *et al.* have imaged the surfaces of spores from several different species of *Bacillus* by atomic force microscopy, as seen in Figure 3 (Chada *et al.* 2003). They have discovered the presence of ridges and bumps on the surfaces of several different *Bacillus* spores (Chada *et al.* 2003). They interpret these features as evidence that the spore structure is dynamic, swelling and contracting in response to changing environmental conditions (such as changes in environmental water content and pH); this supports the hypothesis of Driks that spores can, under changing environmental conditions, expand or contract without breaking dormancy (Driks 2003).

The identification of spore biochemical compounds is central to biomarker-based detection methods. A general summary of the biochemicals located throughout the spore layers, and in what relative proportions they are found; is presented by Gould and Hurst (1969). Matz *et al.* gave a description of the chemical composition of the exosporium of *B. cereus,* a close relative of *B. anthracis* (1970). They found that the exosporium makes up approximately 2% of the dry weight of the spore and that approximately 18% of the exosporium is lipid (FAs) (see Table 2). A breakdown of the specific FAs found in the

exosporium of *B. cereus* is given in Table 3. Studies have identified several unique proteins located in the exosporium of *B. anthracis* (Lai *et al.* 2003; Steichen *et al.* 2003; Williams *et al.* 2003). These proteins could play an important role in biomarker production and detection. Additional unique compounds include several carbohydrates and glycoproteins (Fox *et al.* 2003). Significant variation in the FA content and distribution in both gram-positive and gram-negative bacteria have been found, which has prompted development of detection technologies based on FA detection (to be discussed later).

Bacillus and *Clostridium* spores can be found in a soil sample almost anywhere in the world (Madigan *et al.* 2002). Apart from natural diversity, spores of these genera have anthropogenic sources: *Bacillus thuringiensis* and *Bacillus sphaericus* are used extensively as insecticides (Driks 2002) and other members of the genus *Bacillus* are used for the production of antibiotics.

Table 2. *B. cereus* exosporium biochemical composition, adapted from Matz *et al.* (Matz *et al.* 1970).

Compound	% of Exosporium (dry weight)
Protein	**52.1**
Polysaccharide	**20.0**
Lipid	**18.0**
Ash (Ca, P, Mg)	**3.8**
Teichoic Acids	**2.2**
Nucleic Acids (RNA)	**1.2**
Total	**97.3**

Table 3. Exosporium fatty acids of *B. cereus*, adapted from Matz *et al.* (Matz *et al.* 1970).

Fatty Acid[a]	Percentage of Total Fatty Acids in Exosporium	Percentage of Total Fatty Acids in Spore
n-C_{10}	0.5	1.1
n-C_{11}	0.4	0.3
i-C_{12}	3.1	4.8
n-C_{12}	1.0	0.8
n-$C_{13}^{1=}$	1.4	8.1
n-C_{13}	0.8	trace
i-C_{14}	9.0	2.5
i-C_{15}	20.8	0.2
a-C_{15}		5.0
n-C_{15}		1.6
i-C_{16}	2.5	1.7
n-C_{16}	26.7	31.6
a-C_{17}	1.5	trace
n-C_{17}	0.1	trace
Unidentified	3.4	0.4
a-C_{18}	2.6	trace
n-$C_{18}^{1=}$	11.0	12.3
n-C_{18}	13.4	29.0
Total	*99.2*	*99.6*

[a] n denotes normal, I denotes iso (methyl group attached to 2nd carbon from end of chain), a denotes anteiso (methyl group attached to 3rd carbon from end of chain), and 1= denotes one point of unsaturation

Bacillus anthracis Physiology

Bacillus anthracis, more commonly known as anthrax, is a gram-positive bacterium of the *Bacillus* genera. In its vegetative state, the bacterium produces a toxin complex, composed of three polypeptides, which is lethal for man and animals (Pasechnik *et al.* 1993; Mock and Fouet 2001). However, in the vegetative state, anthrax is difficult to disperse and the cells do not live long outside of a host; in fact, contact with air is known to initiate sporulation (Mock and Fouet 2001). To effectively distribute anthrax over a wide area requires the use of spores. In weaponized form, anthrax is a white powder consisting of finely milled spores. Following sporulation, and before

separation, drying, and milling, a chemical surfactant is added to the spores to prevent them from aggregating. This allows them to be aerosolized and remain in the air for days, depending on the meteorological conditions. Weaponized spores are the optimal size (1-2 μm) for penetrating and depositing on the lung alveolar surfaces, where they are phagocytosed, germinate, enter the host's tissues, and produce the deadly anthrax toxin (Hawley and Eitzen Jr 2001; Mock and Fouet 2001; Inglesby 2002; Tsuda *et al.* 2002; Weis *et al.* 2002). The anthrax toxin is a protein complex composed of three polypeptides: protective antigen (PA), lethal factor (LF), and edema factor (EF). These polypeptides work synergistically to kill the host by a complex biomolecular cascade that is not fully understood, the end result being severe edema and shock-like death (Mock and Fouet 2001). The ease of entry of weaponized spores into the human body by inhalation, coupled with their toxicity, makes anthrax a potent biological weapon (Parker 2001). Pepper and Gentry quote a defense intelligence agency report that gives a human LD_{50} value of 8,000 to 10,000 spores by inhalation, which is about 10 nanograms (Pepper and Gentry 2002); the LD_{50} value is the dose required to kill 50% of the infected population. Others have estimated that a lethal dose of anthrax could be between 2,500 and 55,000 spores, which is between 2.5 and 55 nanograms (Brachman 1980). In a very recent study, Fennelly and co-workers have done a rigorous mathematical model of the spore dissemination and infection process to calculate an LD_{50} of between 2,500 and 6,500 spores (Fennelly *et al.* 2004).

B. anthracis differs physiologically from the other species in the *Bacillus* genera in three significant ways. First, the FA content of the anthrax spores and cells differs between species, mostly in terms of relative abundance of the many different FAs. This

difference has been used as a basis for species-level discrimination in many applications, including analytical pyrolysis (discussed later). Second, it is the only species that produces the deadly anthrax toxin complex. The DNA that facilitates the production of this toxin complex is located on separate plasmids (additional DNA loops inside the bacterium). The plasmid pX01 contains the genes that code for the toxin complex, whereas pX02 contains genes necessary for the formation of a capsule surrounding the vegetative cell, which is also required for virulence. These DNA sequences are not present in the plasmids of other *Bacillus* species. In addition to coding for virulence factors, these two plasmids contain genes that are involved in the production of the spore protein layers. Third, anthrax spores generally have thinner protein coats than other members of the *Bacillus* genera (Lai *et al.* 2003).

There are several similarities between *B. anthracis* and closely related species that complicate species-level discrimination between spores. In fact, some consider *B. anthracis* as part of the *B. cereus* group and not a separate species (Turnbull 1999; Helgason *et al.* 2000; Mock and Fouet 2001). Chromosomal evidence and molecular level discrimination studies show that the only significant compositional differences between *B. anthracis* and many *B. cereus* strains are the two plasmids mentioned above, i.e., they share essentially all of the same proteins, enzymes, non-plasmid DNA, etc. The effect of this analysis on the anthrax detection methods has not been discussed in the scientific literature, although successful species- and sub-species-level differentiation has been reported based on profiling of the FAs of spores.

Due to its lethal properties, *B. anthracis* is seldom used in detection studies; rather, surrogates are often used, including *B. cereus*, *B. subtilis* (BG), and *B. anthracis*

strains from which one or both of the two virulence plasmids have been removed (e.g., the Sterne strain, from which one plasmid has been removed).

Anthrax Spore Detection Technology

The rapid detection of a biological warfare agent such as anthrax is of crucial importance to the national security of the US. This was illustrated in 2001 by the deaths of 5 civilians and infection of 6 others following contact with weaponized anthrax spores sent through the US Postal Service (Parker 2001; Weis *et al.* 2002; Fennelly *et al.* 2004). The discovery of various white powders led to several days of tests, and finally the conclusion that the material was anthrax (Parker 2001). More recently (in 2005), an anthrax scare at the Pentagon prompted evacuation and treatment of those feared exposed. However, after three days of testing it was determined that it was a false positive and that there was no anthrax danger (Hsu 2005). In these and other cases, the days required for anthrax identification is too long; the development of faster detection technologies is needed.

The special operations units of the United States Armed Forces are interested in rapid, handheld detection technology. With such portable equipment, they could both protect their personnel from biological attack and inspect suspect bio-weapons production facilities. Portable biological warfare detection technology would also be attractive for domestic applications such as medical diagnostics, forensic investigations, and homeland defense (e.g., first responder kits).

As mentioned above, both wet and dry methods have been developed to generate biomarkers from bacterial spores. The technology for detecting these biomarkers has moved from conventional culture growth/solution-based assay techniques to highly

technical procedures such as DNA sequencing, Raman spectroscopy, gas chromatography (GC), and mass spectrometry (MS). Over the last 20 years, pyrolysis methods have been developed to produce biomarkers from anthrax spores. Two different pyrolysis methods have been developed: (1) those that utilize a curie-point or other heating system to rapidly heat and decompose a biological sample, and (2) those that utilize a methylating agent in tandem with rapid heating to break down and convert the biological material to methyl esters. When either of these pyrolysis methods are coupled with analytical techniques, such as GC-MS, the combined method is called analytical pyrolysis (AP). Tables 4-6 represent summaries of (1) the various non-pyrolytic methods used to detect anthrax spores (Table 4); (2) the AP methods used for the detection of anthrax spores (Table 5); and (3) the AP methods used for the detection of vegetative anthrax cells that are relevant to detection of spores (Table 6). The methods summarized in each of these tables are further discussed in the sections which follow.

Table 4. Non-pyrolytic methods to produce and/or detect biomarkers from *Bacillus* spores and cells.

Method	P.I.	Sample[a]	Conditions & Reagents	Biomarker Type	Time	Notes
Acid methanolysis of lipids (Dworzanski *et al.* 1991)	--	Cells	Extraction w/ anhydrous methanolic HCl or boron trihalides	Derivatized FAs	hours	Destroys some FAs
Transesterification of saponified and extracted lipids; analysis by GC (Moss 1981; Miller 1982)	--	Cells	Saponification w/ dilute alkali in aqueous MeOH @ 100°C, liquid extraction, methylation by BCl_3 @ 85°C	Derivatized FAs	hours	Low yield, can produce amide-bound FAs
Polymerase chain reaction (PCR) (Ryu *et al.* 2003)	Oh	13 *Bacillus* species spores, including BA	Spore lysis[b] followed by DNA extraction and PCR[c]	DNA, Plasmid DNA	~5 hours	Discrimination shown at species and strain level, long times required, non-portable equipment
PCR (Bell *et al.* 2002)	Cock-erill	31 BA strains, 31 *Bacillus* species, and 26 other bacterial cells	DNA extraction followed by PCR on a Roche LightCycler Instrument with a BA detection kit	DNA, Plasmid DNA	~ 3 hours	Highly specific discrimination shown at species and strain level, long times required, non-field-portable equipment
Carbohydrate profiling by GC-MS (Fox *et al.* 1993; Fox 1999; Fox *et al.* 2003)	Fox	BA cells	Carbohydrate extraction and hydrolysis followed by alditol acetate derivatization	Derivatized carbohydrates	hours	Relies on chemical derivatization of unique sugar monomers
Antibody (Ab)-based recognition and enzyme amplification (miniature biochip system) (Stratis-Cullum *et al.* 2003)	Vo-Dinh	BG spores	ELISA-based antigen-Ab interaction using anti-BG Abs immobilized in wells on the chip[d]; spores added & incubated for 45 min	Surface epitopes (not specified)	< 1 hour	Detection limit of 100 spores (17 / L_{air}); no evaluation of species-level discrimination; no biochemical description of the surface epitopes
DPA-triggered terbium luminescence[e] (Lester *et al.* 2004)	Lester	BG spores	Spores autoclaved in the presence of 10 μM $TbCl_3$, 30 s measurement of luminescence	Calcium dipicolinate (CaDPA)	6 min	Aerosols collected from a sprayed plume; detection limit of 50 spores / L_{air}; no species-level discrimination
Raman Spectroscopy of DPA[e] (Farquharson *et al.* 2004b)	Farqu-harson	BC spores	Spores placed in envelope, Raman spectroscopy used to detect CaDPA in collected aerosols	CaDPA	<15 min	Aerosols collected from spore-containing envelope during mail sorting system operation; detection limit of 4.5 mg

[a] BA = *B. anthracis*; BG = *B. globigii*; BC = *B. cereus*; [b] Heat treatment for 1.5 hours or germination in nutrient broth for 1 hour; [c] Performed in an SDS 7900 (applied biosystems), 2 min @ 50°C, 10 min @ 95°C, 50 cycles of 15 sec @ 95°C, and 1 min @ 60°C; [d] Contains excitation source, excitation and collection optics, and integrated circuit photosensing array chip, requires a 635-nm diode laser and other fragile equipment; [e] Designed to detect spores, but not for species-level discrimination; meant for a cheap, rapid spore test, rather than a standalone device

Table 5. Pyrolysis and derivatization pyrolysis methods used to produce biomarkers from *Bacillus* spores.

Method	P.I.	Spore Species[a]	Conditions & Reagents	Biomarker Type	Time	Notes
MIDI application (MIDI 2005)	Standard method	Any	Saponification w/ dilute alkali in aqueous methanol, liquid extraction, followed by esterification w/ TMAH and vaporization by Py	Derivatized FAs	1.5 + hours	Anthrax identification shown to work with spores; yield can be low
Py-MS & THM-MS (Havey *et al.* 2004)	Mowry, Voorhees	BA[b] & BG	10 μg of spores dried on membrane[c], 1 μL of 0.1M TMAH, Py @ 360-440°C for 10 s	*In situ* derivatized DPA, DNA bases, and protein fragments	26 min	<1 Watt power used, small size; no species discrimination shown, membrane degradation observed w/ use of TMAH
Electron monochromator MS (Beverly *et al.* 2000)	Voorhees	BA[b] & 5 others	50 μg of dry spores placed in capillary tube, Py @ 400°C for 10 min	DPA, some FAs	min	Detection limit of 10^5 spores;
Py-MS (Beverly *et al.* 1999a)	Voorhees	BA & 7 other *Bacillus* species	60 μg of spores placed in glass capillary tube, Py @ 400°C for 6 min	FAs, DPA, and poly(3-hydroxybutyrate) pyrolysis products	min	Showed CI[d] is preferred over EI[e], species-level discrimination shown; diglycerides observed
THM-ITMS (Hendricker *et al.* 1999)	Voorhees	BA, 3 others, 56 strains, some w/ spores	150 μg of spores coinjected w/ 5 μL of 1.0M TMAH[f] onto quartz frit, Py @ 450°C for 55 s	*In situ* derivatized FAs, DPA, and protein pyrolysis products	min	Some spore discrimination possible by multivariate analysis
Py-MS & THM-MS (Beverly *et al.* 1996)	Voorhees	BA	10-20 μg of spores dried onto CP[g] wire, overlaid w/ 5 μL of 0.1M TMAH[f], Py @ 510°C for 10 s	*In situ* derivatized DPA	<10 min	Methylated DPA used as spore indicator
Py-MS (Deluca *et al.* 1990)	Voorhees	BG spores	20 μg of spores dried on CP[g] wire, overlaid w/ 10 μL of 0.1M TMAH[f], Py @ 510°C[h]	FAs and *in situ* derivatized FAs	N/A	Fatty acid methyl ester (FAME) profile produced
Py-GC-MS (Snyder *et al.* 2004; Dworzanski *et al.* 2005)[i]	Snyder / Dworz-anski	BA, BC, BM, BG	Water suspended spores dried on quartz filter[j], Py @ 400°C for 7 s[k]	Proteins, FAs, DPA, and peptidoglycan pyrolysis products	min	Gram-type differentiation successful, species differentiation not shown
Py-GC-IMS (Snyder *et al.* 2001)	Snyder	BG spores, EH cells, ovalbumin	Aerosol collection followed by Py @ 350°C for 6 s	DPA pyrolysis products	3 min	Aerosol gram-classification level discrimination

Table 5 Continued.

Method	P.I.	Spore Species[a]	Conditions & Reagents	Biomarker Type	Time	Notes
Py-MS (Tripathi *et al.* 2001)	Snyder	BA, BG, BT, & EH	µL samples dried on quartz wool plug, Py @ 400, 500, 600, or 700°C for 20 s	DPA, peptidoglycan, and protein pyrolysis products	N/A	Decomposition of DPA discussed: higher temps = more, higher flowrates = less; emphasis on biomarkers < 200 Da
Py-GC-IMS (Snyder 1999; Snyder *et al.* 1999)	Snyder	BG spores & EH cells	Aerosols collected on quartz frit, Py in air @ 350°C for 9 s	DPA pyrolysis products (picolinic acid) for spores, for EH no ID of peaks	3-4 min	Lab & field studies, focused on IMS study of picolinic acid
Py-GC-IMS (Dworzanski *et al.* 1997)	Dworz-anski	BG	Aerosols collected on quartz microfiber filter, Py for 7 s[l]	DPA pyrolysis products (picolinic acid)	4 min	Field & lab studies to ID spores by picolinic acid biomarker
Py-GC-IMS (Snyder *et al.* 1996)	Snyder	BA[m], BT, BS; EC & *S. aureus* cells	2 µg loaded on CP[g] wire, Py in air @ 358°C for 6 s	DPA pyrolysis products (picolinic acid)	3-4 min	Spore detection shown by picolinic acid biomarker
Py-MS & FTIR (Goodacre *et al.* 2000)	Good-acre	36 *Bacillus* species/strains (spores & cells)	10^7 spores (10 µg) dried on Fe-Ni foil, Py @ 530°C for 3 s, FTIR done on dried 10^8 cells	DPA	min	Able to differentiate between spores and vegetative cells,
Py-DMS[n] (Krebs *et al.* 2005)	Krebs	BG spores	120 ng spores dried in quartz tube, Py @ 550°C for 100 s	Not identified	min	Spore fragmentation depends on both temp and time of pyrolysis

[a] BA = *Bacillus anthracis* (anthrax); BG = *B. atrophaeus* (formerly *B. subtilis var. niger*, or *B. globigii*); BC = *B. cereus*; BT = *B. thuringiensis*; BM = *B. megaterium*; EH = *Pseudomonas agglomerans* (formerly *Erwinia herbicola*); EC = *Escherichia coli*; YP = *Yersinia pestis* (bubonic plague); VB = *Vibrio cholerae* (Cholera); FT = *Francisella tularensis*; BrM = *Brucella melitensis*; [b] Sterne strain; [c] 1 µm thick silicon-nitride on a 400 µm silicon substrate, etched by deep reactive ion etch to create a membrane, 6.25 mm^2 surface; [d] Chemical ionization; [e] Electron ionization; [f] In methanol; [g] Curie-point, used for inductive heating; [h] Time not specified; [i] For other references see section on thermal pyrolysis below; [j] Amount not specified; [k] The maximum GC temp was 140 °C; in most other studies GC temps reached 300 °C; [l] Temperature not specified; [m] Ames, Sterne, Zimbabwe, and Vollum strains; [n] Differential mobility spectrometry

Table 6. Pyrolysis and derivitization pyrolysis methods used to produce biomarkers from vegetative bacteria.

Method	P.I.	Bacteria[a]	Conditions & Reagents	Biomarker Type	Time	Notes
MIDI application (Basile *et al.* 1998a; Basile *et al.* 1998b; Madonna *et al.* 2001; MIDI 2005)	Standard Method	Any	Saponification w/ dilute alkali in aqueous methanol, liquid extraction, followed by esterification w/ TMAH and vaporization by Py to transfer FAs to analyzer	Derivatized FAs	1.5 + hours	MIDI application for anthrax identification requires 4 mg of cells which must be grown under standard conditions; yield can be low
THM-MS (Xu *et al.* 2000; Xu *et al.* 2003)	Voorhees	BA[b], YP, VC, FT, & BrM	20 µL of cells coinjected into quartz wool plug w/ 1µL of 0.1M TMAH[c], Py @ 500°C for 10 s	*In situ* derivatized FAs	min	Compared results with MIDI app., very similar results, but some loss of biomarkers; multivariate data analysis used
THM-MS (Madonna *et al.* 1999; Madonna *et al.* 2001)	Voorhees	BA, YP, VC, FT, & BrM[d]	100 µg of cells dried on CP[e] Ni filament, overlaid w/ 10 µL 0.1M TMAH[c], Py @ 358°C for 10 s	*In situ* derivatized FAs	min	Complicated multivariate analysis; successful differentiation of eukaryotic vs. prokaryotic cells
THM-ITMS (Hendricker *et al.* 1999)	Voorhees	BA[f], YP, BrM, & FT, 56 strains	150 µg of cells coinjected w/ 5 µL of 1.0M TMAH[g] onto quartz frit, Py @ 450°C for 55 sec	*In situ* derivatized FAs, DNA bases, and protein fragments	min	Separation by species demonstrated, with some strain discrimination possible by multivariate analysis
THM-MS (Beverly *et al.* 1997; Basile *et al.* 1998a)	Voorhees	BA & 14 other gram +/-	Low µg quantities dried on CP[e] wire, overlaid w/ 5 µL 0.1M TMAH[c], Py @ 358°C for 10 s	*In situ* derivatized FAs	<5 min	Observed 40% of fatty acid methyl esters (FAMEs) possible with MIDI app.
THM-MS (Voorhees *et al.* 1997)	Voorhees	20 species, gram +/-	20 µg dried on CP[e] wire, overlaid w/ 5 µL of 0.1-1.0 M TMAH[c], Py @ 510 or 610°C for 6 s or 1000°C for 5 s	*In situ* derivatized FAs, DNA bases, and protein fragments	min	Random prep order w/ replication; analysis of biomarker spectral data; species-level differentiation demonstrated
Py-MS of FAME extracts (Basile *et al.* 1995)	Voorhees	19 gram +/- bacteria	µg quantities of FAME extract[h] dried on CP[e] wire, Py @ 510°C for 10 s	Derivatized FAs	hours	Gram-classification by Py-MS demonstrated

Table 6 Continued.

Method	P.I.	Bacteria[a]	Conditions & Reagents	Biomarker Type	Time	Notes
Py-MS (Deluca *et al.* 1992)	Voorhees	4 gram + and 2 gram - species, some w/ spores	20 µg of cells dried on CP[e] wire, Py @ 600°C for 5 sec	FA pyrolysis products, including glyceride model compounds	min	Random prep and run order, study on glycerides; "pyrolysis conditions may play an important role in determining which species are liberated;" discrimination at genus level
Py-MS-MS (Voorhees *et al.* 1992)	Voorhees	3 species, some w/ spores	10 mg dried on CP[e] wire, Py @ 610°C[i]	DNA, Proteins, FA, and glyceride, pyrolysis products	N/A	Investigated unique pyrolytic biomarker compounds found in bacteria
Py-MS & THM-MS (Deluca *et al.* 1990)	Voorhees	5 species, plus BG spores	20 µg of cells dried on CP[e] wire, overlaid w/ 10 µL of 0.1M TMAH[c], Py @ 510°C[i]	FA and *in situ* derivatized FAMEs	N/A	Successful species-level discrimination of bacteria by FAME profiling
Py-MS (Voorhees *et al.* 1988)	Voorhees	31 species	Growth media w/ bacterial colonies scraped w/ CP[e] wire, Py @ 510°C[i]	Not specified (only mass spectral data presented)	N/A	Investigated growth time and other variables on discrimination; found problems with analysis of large (>20 species) data sets
Py-GC-MS (Snyder *et al.* 2004; Dworzanski *et al.* 2005)	Snyder	BA and others	Water suspended spores dried on quartz filter, Py @ 400°C for 7 s[j]	Proteins, FA, DPA, and peptidoglycan pyrolysis products	min	Gram-type differentiation successful, species differentiation was not demonstrated
Py-GC-ITMS (Smith and Snyder 1992)	Smith / Snyder	BA, BC, BT, BS, EC, & 4 others	20-50 µg sandwich between quartz wool plugs, Py @ 1000°C for 20 s	Intact lipids (mono-, di-, & tri-glycerides)	<10 min	Visual chromatogram comparison allowed species-level discrimination; high MW biomarkers discussed
Py-GC-ITMS (Snyder *et al.* 1990)	Snyder	9 species, 14 strains	5 µg dried on CP[e] wire, Py @ 610°C for 1 s	Intact lipids	min	Some species discrimination demonstrated, some biomarker spectral analysis done
Py-GC-MS (Kurkiewicz *et al.* 2003)	Kurkie-wicz	BG & EC	20-400 µg of cells dried on CP[e] wire, Py w/ 1 µL of 10% NaOH and 5% pyridylcarbinol @ 770°C for 4 s	*In situ* derivatized FAs (FA picolinyl esters)	~40 min	Claim that picolinyl esterification preserves more FA structural information

Table 6 Continued.

Method	P.I.	Bacteria[a]	Conditions & Reagents	Biomarker Type	Time	Notes
THM-MS (Dworzanski *et al.* 1990)	Meuze-laar	EC, BG, *M. tuberculosis*	5 μg dried on CP[e] wire, overlaid w/ 5 μL of 0.1M TMAH[c], Py @ 358, 510, or 610°C for 4 s	*In situ* derivatized FAs	N/A	Observed C_{24}, C_{26}, and C_{32} FAs; < 5 min needed for FAs up to C_{20}
Py-GC-MS (Smith *et al.* 1987)	Morgan	Group A & B Streptococci	60-100 μg dried on Pt coil, Py @ 800°C for 2 s, short GC column	Polysaccharides (carbohydrates) such as glucitol phosphate	min	No correlation of spectral data w/ biomarkers
THM-ITMS (Barshick *et al.* 1999)	Barshick	5 gram - species	10^7 cells dried on quartz wool filter, Py with TMAH (1 μL of 0.1M[c]) @ 550°C for 40 s	*In situ* derivatized FAs	min	Species discrimination shown only w/ CI[k]
Py-MS & FTIR (Goodacre *et al.* 2000)	Good-acre	36 *Bacillus* species / strains (spores & cells)	10^7 cells dried on Fe-Ni foil, Py @ 530°C for 3 s, FTIR done on dried 10^8 cells	DPA	min	Able to differentiate spores and vegetative cells, some cluster differentiation (multivariate analysis) possible
THM-MS with the Block II CBMS[l] (Griest *et al.* 2001)	Griest	BG spores and EH cells	Aerosol collection in quartz tube, Py w/ 1-2 μL of 0.1M[c] TMAH @ 550°C for 16 s	*In situ* derivatized FAs, methylated diketopiperazines	5 min	Used CI[k]; work with tetrabutylammonium hydroxide was attempted
THM-MS with the CBMS[l] (Hart *et al.* 2000)	Griest	5 gram - species	Air sampling (10^6 bacteria), Py with TMAH (2 μL of 0.1M[c]) @ 550°C for 15 s	*In situ* derivatized FAs	min	Testing done only with gram – bacteria, CI[l] used
THM-MS with the CBMS[l] (Luo *et al.* 1999)	Luo	2 bacteria and 2 virus species	Air sampling or injection of 10^8 cells, Py with 2 μL of 1:1 aqueous TMAH:MeOH @ 580°C for 30 sec	*In situ* derivatized FAs and other biomarkers	3 min	CBMS sensitivity = 10^{-8} g toxin or 10^{-7} g bacteria @ 15 ACPLA[m] in 3 min, species discrimination shown
Non-discriminating TMAH-induced thermochemolysis-GC-MS (Poerschmann *et al.* 2005)	Poersch-mann	*Ps. Putida*	25 μg, Py deposited inside 0.53 mm deactivated SS capillary, 1μL of 25% TMAH[c] (10:1 TMAH:sample mass ratio), Py @ 500°C for ms	*In situ* derivatized FAs	<5 min	The authors claim conventional THM is inherently biased; preservation of FA taxonomical info shown w/ Py time of ms

Table 6 Continued.

Method	P.I.	Bacteria[a]	Conditions & Reagents	Biomarker Type	Time	Notes
Py-MS (Shute *et al.* 1984; Shute *et al.* 1988)	Shute	4 species, 53 strains	Cells loaded by scraping[n] growth plate w/ CP[e] wire, Py @ 510°C for 2 s	Carbohydrate, lipid, and protein pyrolysis products	N/A	Investigated the influence of 6 growth media on biomarkers, species discrimination shown
THM-GC-MS (Holzer *et al.* 1989)	Holzer	6 species	10-50 µg of cells dried on CP[e] wire, overlaid w/ 10 µL of 1% trimethylanilinium hydroxide[c], Py @ 360, 510, 610, or 770°C for 10 s	*In situ* derivatized FAs	N/A	THM vs. extraction derivatization[h] showed some differences, no effect of reagent solvent (MeOH or H_2O) observed, detection limit of 5 x 10^6 cells determined
Py-GC-MS (Eudy *et al.* 1985b)	Morgan	20 species of gram +/-	200 µg dried on Pt ribbon, Py @ 800°C for 5 s	Cell pyrolysis products of unidentified origin	N/A	Discrimination of gram+/- shown based on 8 observed biomarkers

[a] BA = *Bacillus anthracis* (anthrax); BG = *B. atrophaeus* (formerly *B. subtilis var. niger*, or *B. globigii*); BC = *B. cereus*; BT = *B. thuringiensis*; BM = *B. megaterium*; EH = *Pseudomonas agglomerans* (formerly *Erwinia herbicola*); EC = *Escherichia coli*; YP = *Yersinia pestis* (bubonic plague); VB = *Vibrio cholerae* (Cholera); FT = *Francisella tularensis*; BrM = *Brucella melitensis*; [b] Ames strain; [c] In methanol; [d] Additionally, 6 fungi species, 3 viruses, 4 types of pollen, and 3 proteins were tested; [e] Curie-point, used for inductive heating; [f] Some spores present; [g] In water; [h] Prepared according to (Miller 1982); [i] Time not specified; [j] The maximum GC temp was 140 °C; [k] Chemical ionization; [l] Chemical/biological mass spectrometer; [m] Agent-containing particles per liter ; [n] Amount collected not specified

Biomarkers and Biomarker Precursors

It has long been known that biomarkers can be used for the discrimination of bacteria at the species level (Abel *et al.* 1963). These biomarkers derive from several different types of biochemical compounds, including biopolymers, lipids, and small organic molecules. The sources of biomarkers and the biomarkers themselves are discussed in this section.

One source of biomarkers is DNA. The polymerase chain reaction (PCR) technique has been used to amplify unknown DNA sequences which are then sequenced and compared with a library of known sequences to identify the source of the genetic material (Bell *et al.* 2002). Because different species of bacteria and viruses have sufficiently different genomes, this method has very high specificity and little error. However, it requires hours of work and specialized equipment to accurately amplify and sequence the nucleic acids to identify the biological agent. This method is only useful for biological agents that contain intact, well characterized genetic material (i.e., it may not work well with heat killed or genetically modified agents, and would not work at all with toxins). Further, for use with bacterial spores, specifically anthrax spores, this method requires the germination of the spores before the DNA can be extracted, which requires an additional 60-95 min (Ryu *et al.* 2003). Therefore, other types of biomarkers have been considered for use in the rapid identification of bioagents.

The carbohydrates located in the exosporium and other layers of the bacterial cells are another source of biomarkers. The carbohydrates in bacteria can be extracted and hydrolyzed, and the resulting fragments derivatized in order to produce volatile biomarkers (Gilbart *et al.* 1987). These biomarkers can be analyzed by GC-MS to

provide a carbohydrate profile of the bacterium, which can then be used for identification and discrimination from other species. Development of this basic approach has led to the identification of several potentially unique biomarkers for anthrax (Fox *et al.* 1993; Wada *et al.* 1996b; Wada *et al.* 1996a; Fox 1999; Fox *et al.* 2003); however, this has not led to the development of field portable devices because this method takes a long time (on the order of hours) and has not been successfully demonstrated with anthrax spores.

Another possible biomarker is dipicolinic acid (DPA). As mentioned in the introduction, calcium-complexed DPA is found in very high concentrations in the bacterial spores, but is not known to be produced anywhere else in nature. In 1976, Tabor and co-workers detected DPA from *Clostridium* spores using gas-liquid chromatography (GLC). Recently, a number of other methods have been developed to detect DPA, including fast chemical extraction of calcium-complexed DPA and detection by mass spectrometry (Beverly *et al.* 1996), luminescence (Eversole *et al.* 1999; Eversole *et al.* 2001; Lester *et al.* 2004) or fluorescence (Rosen *et al.* 1997). Although luminescence and fluorescence methods provide a relatively high degree of discrimination and sensitivity and some techniques are rapid, they still require bench-scale analytical equipment and time-consuming sample preparation procedures. Calcium-complexed DPA in anthrax spores can be rapidly detected by Raman spectroscopy. This has been done in two ways: by detecting the presence of DPA in spore extracts, and by detecting the DPA in whole spores inside sealed envelopes (Farquharson *et al.* 2004a; Farquharson *et al.* 2004b). While this technique is rapid and has promise for use in permanent facilities such as mailrooms and offices (where interfering *Bacillus* or *Clostridium* spores are unlikely to be present in significant concentrations), equipment portability and power requirements

preclude use of this application for handheld detectors. Further, the DPA biomarker is found in all bacterial spores, not just anthrax (i.e., techniques to rapidly detect DPA cannot be used to uniquely identify anthrax). Thus, more unique biomarkers must be used to identify anthrax spores from other species' spores.

The production of unique biomarkers from FAs, proteins, peptidoglycan, and carbohydrates has been shown to be possible by AP. Because of the large amount of taxonomical information contained in them, these biomarkers have promise for the detection of all types of biological agents. AP will be discussed in more detail in the following section. Tables 4-6 present a summary of the various analytical methods that have been used to produce biomarkers from bacterial spores and relevant bacterial cells.

Biomarker Production by Analytical Pyrolysis

Two general classes of AP have been used to produce biomarkers from bacterial spores: (1) thermal pyrolysis, which utilizes a heater to pyrolyze a biological sample and has been successfully used for differentiating bacteria at the gram-classification level; and (2) thermal-hydrolysis methylation (THM), which utilizes a derivatizing reagent in the pyrolyzer to methylate the FAs and has been successfully used to differentiate bacteria at the species and even strain level. In both of these methods, analysis times have been reduced to less than 15 min; each will be discussed in turn.

Thermal Pyrolysis

Thermal pyrolysis is the breaking of chemical bonds with thermal energy. Its main application has been in the analysis of polymers, coals, and other high molecular weight compounds (Wampler 1995). Thermal pyrolysis has more recently found

application in the generation of biomarkers from bacteria and bacterial spores for detection by GC and MS (Wampler 1999; Wampler 2004). In their naturally occurring state, spore biochemicals and biopolymers are of sufficiently low volatility to preclude detection by standard analytical techniques; if thermally broken down to more volatile compounds, the compounds can be more easily detected. The primary reactions typically involve the decomposition of med- and high-molecular weight compounds, such as the thermal fragmentation of proteins into oligopeptides and amino acids. Ideally, pyrolytically-produced fragments from these compounds are swept into the analyzer without further reaction. In practice, secondary reactions may occur; for example, the primary products may react on the walls of the reactor with other molecules such as oxygen or other primary pyrolysis products to form secondary products. Examples of secondary reaction products include diketopiperazines and other cyclized peptides from which many of the functional groups have been cleaved.

Thermal pyrolysis methodology

The use of biomarkers produced by thermal pyrolysis for the detection of biological bacteria was first mentioned in the literature 30 years ago (Meuzelaar and Kistemaker 1973; Anhalt and Fenselau 1975; Risby and Yergey 1976); it has been a subject of research since then (Kossa *et al.* 1979; Irwin 1982; Dworzanski *et al.* 1991; Voorhees *et al.* 1992; Wampler 1995; Voorhees *et al.* 1997; Wampler 2004). The application of pyrolysis to the production of biomarkers from bacterial spores is more recent, and much research effort has been applied in the last ten years. (Beverly *et al.* 1996; Snyder *et al.* 1996; Beverly *et al.* 1999b; Snyder *et al.* 1999; Snyder *et al.* 2004; Dworzanski *et al.* 2005). In this application, the biological polymers (protein,

peptidoglycan, DNA, etc.) and other biomarker precursors (bound FAs, DPA, etc.) native to the spores are broken down and/or converted to more volatile compounds. This is realized by rapidly heating a small sample to elevated temperatures, i.e., 350-650 ºC. The rate of heating is an important variable, which is dependant on the type of pyrolyzer used. In the case of Curie-point (inductively heated) pyrolyzers, the heating rates are on the order of 10,000 °C/s although slower heating rates are possible if desired; for resistively heated pyrolyzers, the heating rate is typically between 50-1000 °C/s. Thus, the temperature rise time to achieve pyrolysis temperature (between 350-700 °C) is usually less than 1-2 seconds, and is often between 100-200 milliseconds.

Snyder *et al.* have developed a pyrolytic method to liberate DPA from the spore protoplast (Snyder 1992; Snyder *et al.* 1996; Snyder *et al.* 1999). Following collection of aerosolized spores on a quartz frit filter or deposition of liquid spore suspensions (low μg amounts of spores) on a small Curie-point wire, pyrolysis at 358 °C for 6 seconds is used to produce biomarkers from the spores; the authors do not provide detailed information on the heating rate or temperature rise time. The pyrolyzate is analyzed by GC-MS, which detects the DPA, among other products that were not identified. The analysis and detection time is typically less than 15 min. The pyrolysis generates by-products (i.e., there were many side reactions - see Figures 12 and 14), which complicate the chromatograms and the data analysis (note that in the literature chromatograms produced by pyrolysis are sometimes call pyrograms). Curie-point pyrolysis developed by Snyder *et al.* has demonstrated capability for detecting the presence of spores and differentiating bacterial at the gram-classification level, but has been unsuccessful at the differentiation of anthrax from closely related species.

Voorhees and co-workers have also developed methods to pyrolytically produce biomarkers, including DPA, from anthrax spores (Deluca *et al.* 1992; Voorhees *et al.* 1992; Beverly *et al.* 1996; Beverly *et al.* 1999b; Havey *et al.* 2004). One method consists of loading 60 μg of spores into a glass capillary tube and heating to 400 °C for 6 min (temperature ramp from 30 to 400 °C at 256 °C/min); the effluent is injected directly into a mass spectrometer and the spectra are analyzed (Beverly *et al.* 1999b). Figure 7 shows electron micrographs of the anthrax spores before and after treatment. It is evident that heating at 400 °C has significant physical effects on the spore, including melting together of some spores and rupture of others into smaller pieces. The authors report that DPA is only observed after heating the spores beyond 250 °C, at which point they begin to melt together and are disrupted.

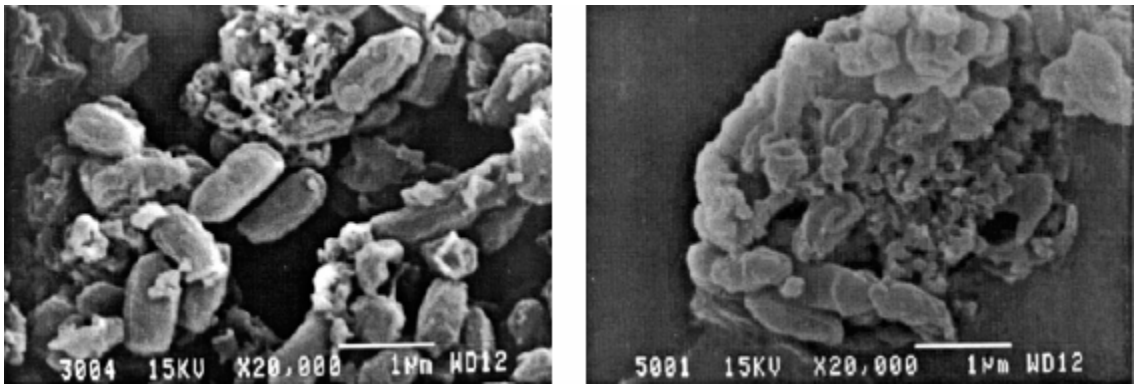


Figure 7. Anthrax spores before (left) and after heating at 400 °C (right) (Beverly *et al.* 1999b).

Another method developed by Voorhees *et al.* involves drying 10-20 μg of spores onto a Curie-point (CP) wire, then overlaying the spores with 5 μL of 0.1 M TMAH (in MeOH) and heating the wire at 510 °C for 10 s with a 50 ms temperature rise time

(Beverly *et al.* 1996). Both methods are able to produce multiple biomarkers including DPA and FAs.

Goodacre & Shann used curie-point pyrolysis and Fourier transform infrared (FTIR) spectroscopy to detect the present of dipicolinic acid in sporulated bacterial samples (Goodacre *et al.* 2000). The sample is applied in a thin, uniform layer over the surface of an iron-nickel foil; curie-point pyrolysis is done at 530 °C for 3 s, with a temperature rise time of 0.5 s. The pyrolyzate is passed through a heated transfer line directly to the MS. The presence of DPA peaks in the mass spectrum is considered to be indicative of the presence of spores, while the absence of these peaks is taken to denote purely vegetative cells.

Biomarker producing reactions during the pyrolysis of spores and cells

A number of studies have addressed the reactions and reaction pathways that occur during the pyrolysis of spores and cells. Snyder *et al.* assessed the microbiological meaning (chemotaxonomy) of the biomarkers produced by curie-point pyrolysis of spores (Snyder *et al.* 2004). The biomarkers were detected by GC-IMS (ion mobility spectrometry) and identified by comparison with both the National Institute of Standards and Technology (NIST) mass spectral database and analytical standards. The samples were dried for 10 s at 120 ºC to remove water and pyrolyzed for 7 s at 400 ºC (temperature rise time not specified) in a helium flowrate of 20 mL/min. The study utilized two different species: gram-positive spores and cells of *B. atrophaeus* (formerly *B. subtilis* var. *globigi*), and gram-negative cells of *Pantoea agglomerans* (formerly *Erwinia herbicola*). Table 7 lists the biomarkers detected and identified by Snyder and co-workers. They concluded that some biomarkers might not be observed due to

inefficient flow paths in their device. Chromatograms of each type of sample are reproduced in Figures 8-10.

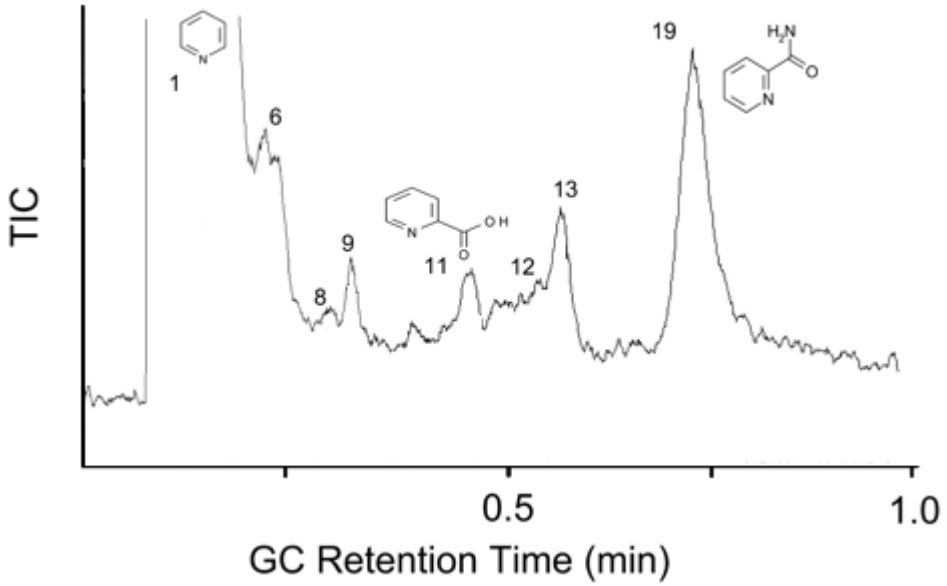


Figure 8. Py-GC-MS chromatogram of *B. atrophaeus* spores - peak numbers correspond to entries in Table 7 (Snyder *et al.* 2004).

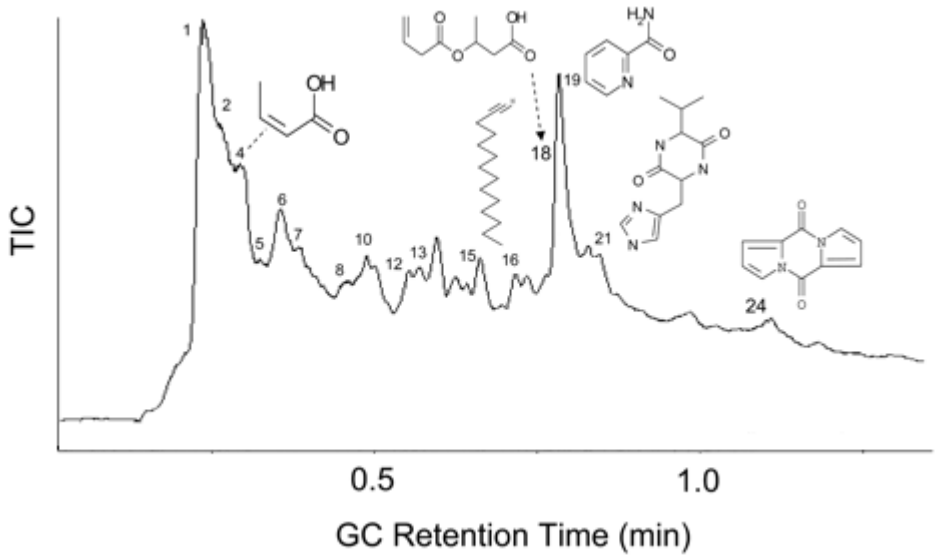


Figure 9. Py-GC-MS chromatogram of *B. anthracis* cells - peak numbers correspond to entries in Table 7 (Snyder *et al.* 2004).

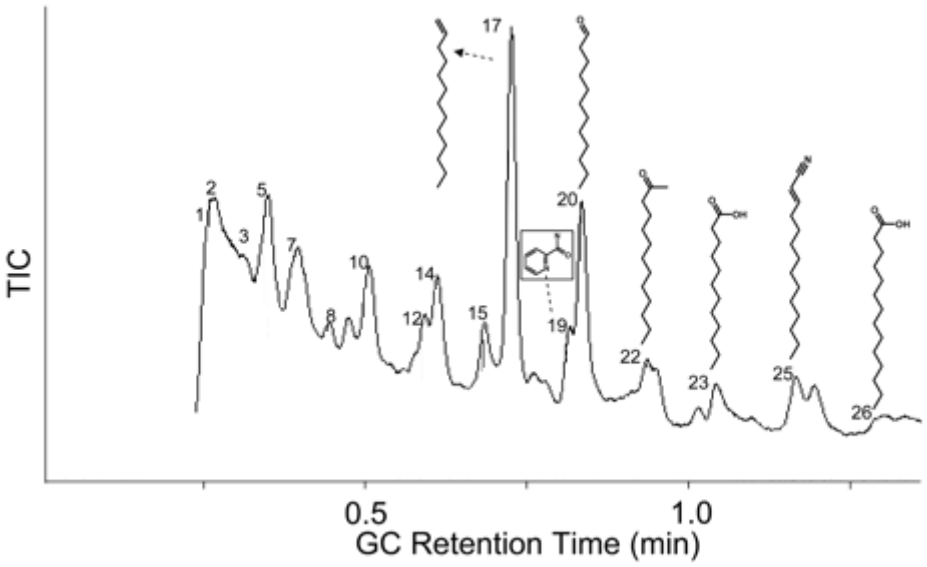


Figure 10. Py-GC-MS chromatogram of *E. Coli* cells - peak numbers correspond to entries in Table 7 (Snyder *et al.* 2004).

In a closely related study, Dworzanski *et al.* investigated the biomarker-producing reactions that occur during the pyrolysis of gram-positive and gram-negative bacterial cell walls (Dworzanski *et al.* 2005). They noted that a major pyrolysis product of both gram-positive bacterial cells and spores is picolinamide (compound 19 in Table 7); they speculated that this comes from DPA in the case of the spores, and from thermal rearrangements of peptidoglycan components in the cells (see discussion below and Figure 11). Other observed biomarkers from gram-negative cells are included in Table 7.

Table 7. Spore biomarkers produced and detected by Snyder and co-workers (Snyder *et al.* 2004; Dworzanski *et al.* 2005).

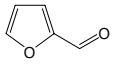
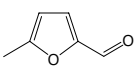
#	Structure/Formula	Name	MW	Precursor
1		Pyridine	79	DPA, proteins, nucleic acids
2		2-Furan-carboxaldehyde (furfural)	96	Carbohydrates
3		5-Methyl-2-furan-carboxyaldehyde (5-methyl furfural)	110	Carbohydrates

Table 7 Continued.

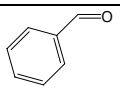
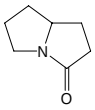
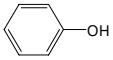
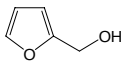
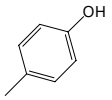
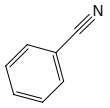
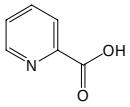
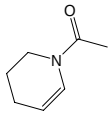
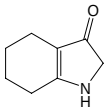
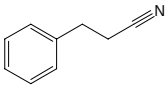
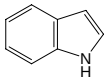
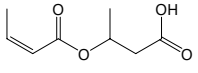
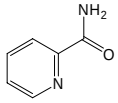
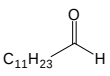
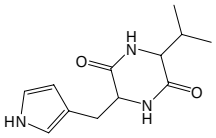
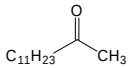
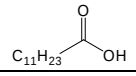
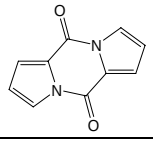
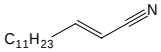
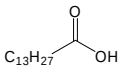
#	Structure/Formula	Name	MW	Precursor
4		2-Butenoic acid (crotonic acid)	86	Poly (3-hydroxybutyric acid)
5		Benzene-acetaldehyde (benzaldehyde)	106	Proteins
6		Hexahydropyrrolizin-3-one (pyrrolizidin-3-one)	125	Proteins
7		Phenol	94	Proteins: tyrosine
8		2-Furanmethanol, tetrahydro (furfuryl alcohol)	102	Nucleic acids (DNA)
9		4-Methylphenol (p-Cresol)	108	Proteins
10		Phenylacetonitrile, (benzonitrile)	103	Proteins
11		Pyridine-2-carboxylic acid (picolinic acid)	123	Pyridine-2,5-dicarboxylic acid (DPA), gram + spores
12		1-Acetyl-1,2,3,4-tetrahydropyridine	125	Proteins
13		4,5,6,7-Tetrahydro-indole-3-one	137	Proteins
14		Benzene propanenitrile	131	Proteins
15		Indole	117	Proteins
16	$(CH_3)_2CH(CH_2)_9CN$	Iso-tridecanenitrile	195	Branched Lipids, lipoproteins
17	$CH_3(CH_2)_{10}CH{=}CH_2$	1-Tridecene	182	Lipopolysaccharides (LPS), gram -
18		2-Butenoic acid, 2-carboxy-1-methylethyl ester (dimer of crotonic acid)	172	Poly (3-hydroxybutyric acid), gram +
19		2-Pyridine-carboxyamide (picolinamide)	122	DPA, Peptidoglycan, gram +
20		Dodecanal	184	LPS, gram -

Table 7 Continued.

#	Structure/Formula	Name	MW	Precursor
21		Diketopiperazine (His-Val)	235	Proteins
22		2-Tridecanone (methylundecylketone)	198	LPS, gram -
23		n-dodecanoic acid (lauric acid)	200	Phospholipids, LPS, gram -
24		Diketopiperazine (Pro-Pro)	186	Proteins
25		2-tetradecenenitrile	207	LPS, gram -
26		n-Tetradecanoic acid (myristic acid)	228	Phospholipids, LPS, gram -

The research of Snyder, Dworzanski and co-workers is the first attempt to identify the reactions that produce the major observed biomarkers. The pyrolysis products and precursors shown in Table 7 represent the first published analysis of this kind. There are several reactions that are briefly described and referenced, including the decarboxlyation of DPA to produce picolinic acid (Compound 11); the decomposition of peptidoglycan to picolinamide (Compound 19 – see Figure 11); the decomposition of multiple biomolecules (DNA, protein, DPA) to pyridine (Compound 1); and pyrolysis of proteins to Compounds 6, 9, 12, and 13 (Snyder *et al.* 2004). Snyder and co-workers describe the pyrolysis of the cell walls of vegetative bacteria to produce the other compounds in Table 7, however, for the sake of brevity this is not discussed here. Visual inspection of the structures and compound numbers in Figures 8-10 gives an indication of which biomarkers belong to gram (+) and gram (-) bacterial cells, as well as to spores.

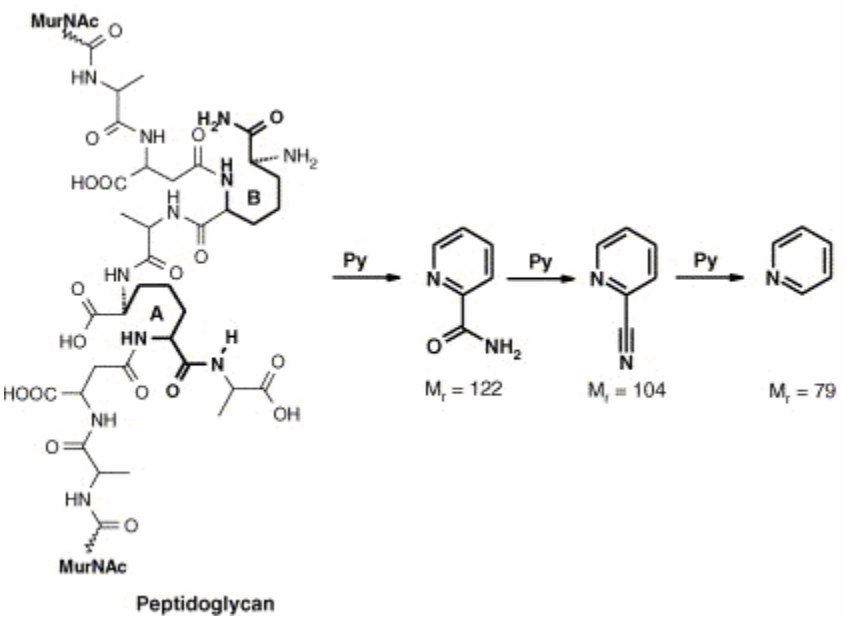


Figure 11. Peptidoglycan pyrolysis products (Dworzanski *et al.* 2005; abbreviations used: GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; meso-DAP, meso-diaminopimelic acid. In the structure on the left side of the figure, the arrows indicate the N to C terminal direction of amino acid residues where the arrow points to the N-terminal moiety; pyrolysis at 400 °C for 6 s, amount and temperature ramp not specified).

Goodacre and co-workers described the degradation (multiple decarboxlyations) of DPA to picolinic acid and pyridine that observed in their study (described in Table 5), according to the reaction pathway shown in Figure 12 (Goodacre *et al.* 2000).

The biomarkers observed from Voorhees and co-workers and other relevant studies are presented in Table 8. While some of the compounds overlap with those observed by Snyder and Dworzanski, most are different.

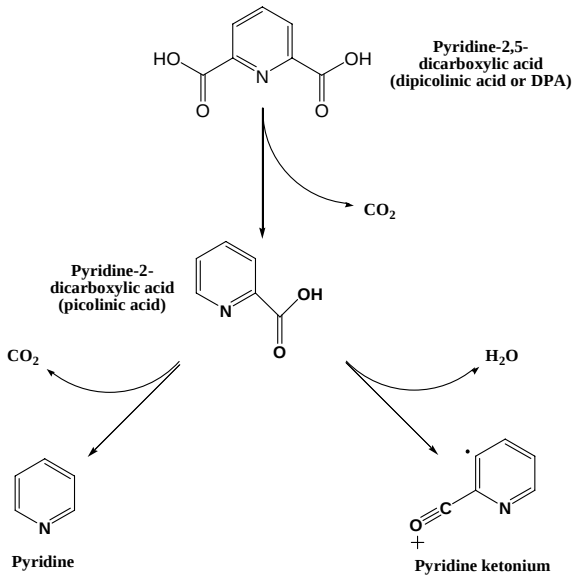


Figure 12. Pyrolytic degradation and electron impact fragmentation pathway of dipicolinic acid; pyrolysis of spores at 530 °C for 3 s, temperature rise time was 0.5 s (Goodacre *et al.* 2000).

Table 8. Spore biomarkers produced and detected by Voorhees and co-workers and other spore pyrolysis studies.

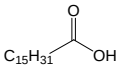
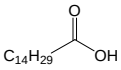
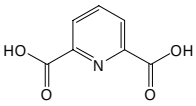
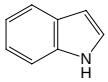
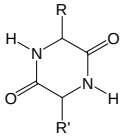
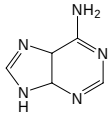
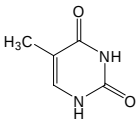
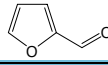
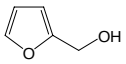
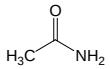
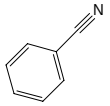
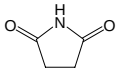
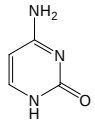
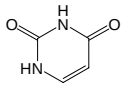
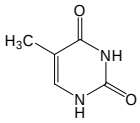
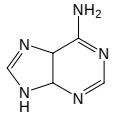
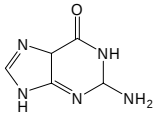
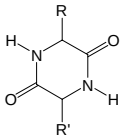
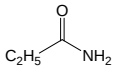
#	Structure/Formula	Name	Precursor (location)	Reference(s)	MW
1		Hexadecanoic acid (palmitic acid)	Free or bound FA (exosporium)	(Beverly *et al.* 2000)	256
2		Pentadecanoic acid	Free or bound FA (exosporium)	(Beverly *et al.* 2000)	242
3		2,6-Pyridine-dicarboxylic acid (dipicolinic acid)	(spore protoplast)	(Voorhees *et al.* 1992)	167
4		Indole	Tryptophan[a]-containing protein (spore coats)	(Eudy *et al.* 1985b; Voorhees *et al.* 1992)	117
5		Diketopiperazines	Oligopeptides, protein (spore coats)	(Voorhees *et al.* 1992)	--
6		Adenine[b]	DNA, ATP (spore protoplast)	(Voorhees *et al.* 1992)	135
7		Thymine[b]	DNA (spore protoplast)	(Voorhees *et al.* 1992)	151
8		2-Furan-carboxaldehyde	DNA	(Eudy *et al.* 1985b)	96

Table 8 Continued.

#	Structure/Formula	Name	Precursor (location)	Reference(s)	MW
9		Furfuryl alcohol	DNA	(Medley *et al.* 1975; Eudy *et al.* 1985b)	98
10		Acetamide	*N*-cetylglucosamine in peptidoglycan (cortex)	(Eudy *et al.* 1985b)	59
11		Benzonitrile	Proteins	(Eudy *et al.* 1985b)	103
12		2,5-Pyrrolidinedione (succinimide)	Protein	(Eudy *et al.* 1985b)	99
13	--	*N*-Acetylmuramyl-L-alanyl-L-iso-glutamine (amino sugar)	Cell wall peptidoglycan (cortex)	(Beverly *et al.* 1999b)	102, 130, 144, 168, 186, 204
14	--	*N*-Acetyl-glucosamine	Cell wall peptidoglycan (cortex)	(Adkins *et al.* 1984)	102, 126, 168, 186, 204
15		Cytosine[b]	DNA (spore protoplast)	(Tripathi *et al.* 2001)	112[c]
16		Uracil[d]	RNA (spore protoplast)	(Tripathi *et al.* 2001)	113[c]
17		Thymine[b]	DNA (spore protoplast)	(Tripathi *et al.* 2001)	127[c]
18		Adenine[b]	DNA, ATP (spore protoplast)	(Tripathi *et al.* 2001)	136[c]
19		Guanine[b]	DNA (spore protoplast)	(Tripathi *et al.* 2001)	152[c]
20		Diketopiperazines	Protein, oligopeptides (spore coats, cortex)	(Tripathi *et al.* 2001)	97, 102, 113-116, 123-126, 146[c,e]
21		Propionamide	Lactyl-peptide bridge and muramic acid in Peptidoglycan (cortex)	(Medley *et al.* 1975)	74

[a] An amino acid; [b] A DNA base; [c] Protonated form (from chemical ionization); [d] A RNA base; [e] For vegetative gram-positive bacteria, masses of 97, 102, 113-116, 123, 129, 130, & 146 were observed

Biomarker producing reactions during the pyrolysis of relevant biopolymers

There have been many studies on the pyrolysis of purified biopolymers in an attempt to determine the sources of many of the biomarkers observed to come from the pyrolysis of biomaterial (e.g., bacterial spores and cells). These biopolymers include proteins, peptidoglycan, poly-D-β-hydroxybutyric acid, and DNA.

Proteins. When placed in a high temperature environment, proteins undergo thermal degradation reactions, which produce smaller oligopeptides and amino acids (primary reaction products). The oligopeptides undergo further reactions, including cyclization (see Figure 13) and loss of certain types of functional groups, (e.g., benzyl, carboxyl, or amino groups; Hendricker and Voorhees 1998) . A summary of the various compounds produced by pyrolysis of each of the 20 common amino acids is presented in Table 9, as well as a scheme of the general fragmentation pathways observed for amino acid pyrolysis (Figure 14).

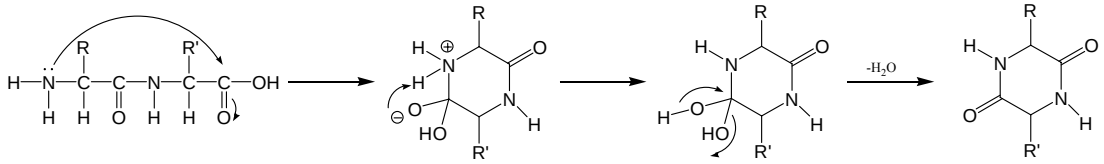


Figure 13. Dipeptide cyclization to produce a diketopiperazine during pyrolysis (Hendricker and Voorhees 1998).

Table 9. Classes of chemicals produced by the pyrolysis of amino acids (Chiavari and Galletti 1992; numbers refer to Figure 14).

#	Chemical Class	Amino acid sources[a]
1	Amine	Leucine, isoleucine, phenylalanine, proline, methionine, and tyrosine
2	Imine	Not specified
3	Nitrile	Leucine, isoleucine, pheylalanine[b]
4	Alkylimine	Leucine, isoleucine, valine
5	Dipeptide	N/A[c]
6	2,5-Diketopiperazine	Proline[d], leucine, isoleucine, valine
7	α-Lactam	Not specified
8	(Substituted) hydrocarbon	Phenylalanine, methionine, tyrosine, tryptophan
9	Cyclized product	Lysine, glutamine, arginine, asparagine

[a] Glycine, serine, alanine, and threonine gave no detectable fragmentation products; [b] Minor source; [c] Undetectable due to low volatility (i.e., would not pass through GC column); [d] Hydroxyproline was similar

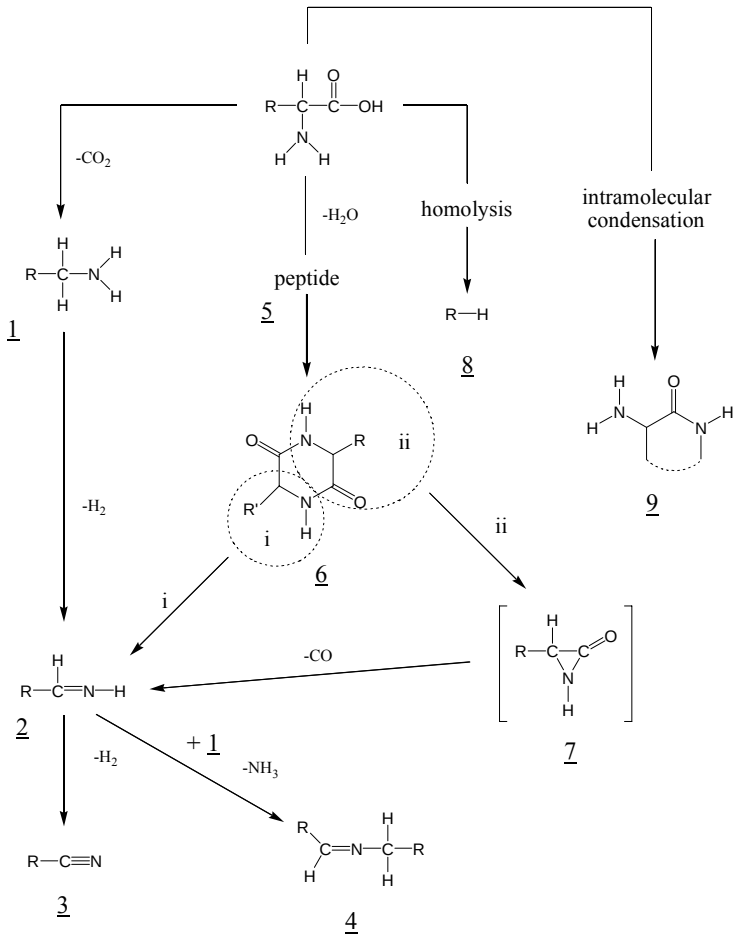


Figure 14. General amino acid pyrolysis pathways (Chiavari and Galletti 1992, R = amino acid functional group, numbers refer to chemicals in Table 9; 150 μg of material heated at 600 °C for 5 s in a quartz tube pyrolyzer).

A representative list of the observed pyrolysis products of amino acids is presented in Table 10; note the presence of a significant number of amino acids that have lost the carboxylic acid moiety. Also present are a number of nitriles, which correspond to the dehydration of the amine and neighboring carbon.

Table 10. Representative compounds produced by amino acid pyrolysis (Chiavari and Galletti 1992).

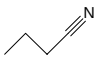
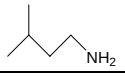
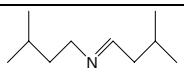
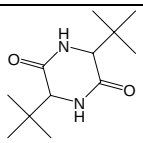
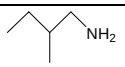
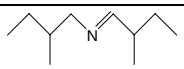
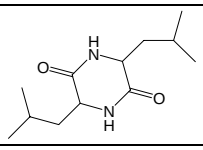
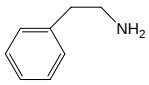
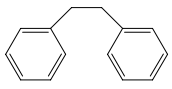
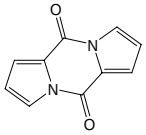
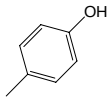
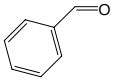
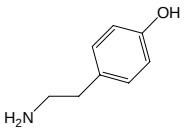
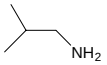
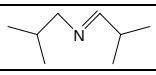
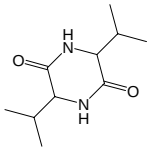
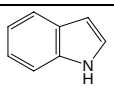
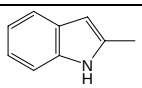
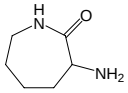
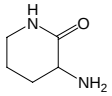
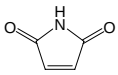
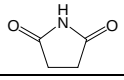
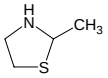
#	Structure/Formula	Name	Amino Acid Source	MW
1		Butanenitrile	Leucine, isoleucine	69
2		3-Methylbutylamine	Leucine	87
3		1-Butaneamine-3-methyl-*N*-(3-methylbutylidene)	Leucine	155
4		3,6-Diisobutyl-DKP[a]	Leucine	226
5		2-Methylbutylamine	Isoleucine	87
6		1-Butaneamine-2-methyl-*N*-(3-methylbutylidene)	Isoleucine	155
7		3,6-(2-Methylpropyl)-DKP[a]	Isoleucine	226
8		Toluene	Phenylalanine	92
9		Benzene-ethane-amine	Phenylalanine	121
10		Diphenylethane	Phenylalanine	182
11		Pyrroline	Proline, lysine, arginine	69
12		DKP	Proline	194
13		3-Methylthio-1-propylamine	Methionine	105

Table 10 Continued.

#	Structure/Formula	Name	Amino Acid Source	MW
14		Phenol	Tyrosine	94
15		4-Methylphenol	Tyrosine	108
16		Benzene-acetaldehyde	Tyrosine	120
17		4-Hydroxyphenethylamine	Tyrosine	137
18		2-Methyl-1-propaneamine	Valine	73
19		2-Methylpropylamine-*N*-(2-methylpropylidene)	Valine	127
20		3,6-Diisopropyl-DKP[a]	Valine	198
21		Indole	Tryptophan	117
22		3-Methylindole	Tryptophan	131
23		3-Aminohexahydroazepinone	Lysine	128
24		Pyrrole	Glutamine	67
25		2,3-Dehydro-2-piperidone	Glutamine	97
26		3-Amino-2-piperidone	Arginine	114
27		Maleicimide	Asparagine	97
28		Succinimide	Asparagine	99
29		2-Methylthiazolidine	Cysteine	103

[a] 2,5-diketopiperazine

Peptidoglycan. As mentioned previously, peptidoglycan is a network polymer consisting of carbohydrate (glycan) strands made up of *N*-acetylmuramic acid and *N*-acetylglucosamine that are crosslinked through the lactyl moiety on the muramic acid by tetra and pentapeptide chains. Pyrolysis of peptidoglycan generates compounds such as acetamide and propionamide from the N-acetyl group of the amino sugars and the lactyl-peptide bridges/muramic acids, respectively (Hudson *et al.* 1982; Eudy *et al.* 1985a).

Poly-D-β-hydroxybutyric acid. Poly-D-β-hydroxybutyric acid (PHB) is a polymer that is produced by bacteria; it stores energy in the form of polymerized short-chain FAs (i.e., repeating units of D-β-hydroxybutyric acid – see Figure 15). PHB makes up a significant fraction (up to 50%, but usually much lower) of the total lipids in vegetative bacteria of the *Bacillus* genus (Asselineau 1966). It is known to be present in vegetative *B. anthracis* cells in significant amounts; however, in *B. atrophaeus* cells it is present in much lower amounts (Snyder, Dworzanski *et al.* 2004 and references therein).

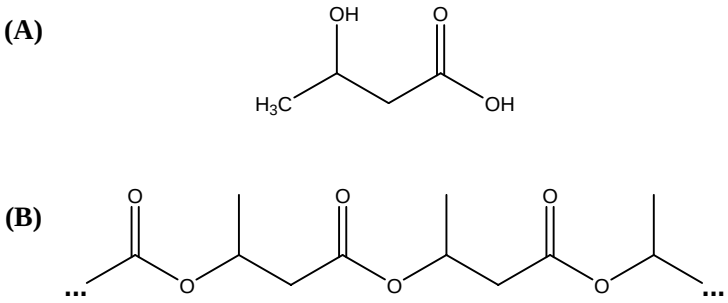


Figure 15. (A) D-β-hydroxybutyric acid; (B) poly-D-β-hydroxybutyric acid (PHB).

Grassie and Murray presented results of a study on the pyrolysis of PHB in a series of publications in 1984 (Grassie *et al.* 1984c; Grassie *et al.* 1984a; Grassie *et al.* 1984b). They found that at temperatures above 170 °C the PHB polymer undergoes degradation by means of a chain scission ester decomposition mechanism that involves a

six-member ring transition state (see Figure 16). The chain scission reaction repeats until volatile oligomers are formed and evolved. The tetramer, trimer, dimer, and monomer (crotonic and isocrotonic acid) of PHB were observed. Significantly, Snyder and co-workers observed crotonic acid and the PHB dimer acid from the pyrolysis of *B. anthracis* cells (Snyder *et al.* 2004), and Beverly and co-workers also observed the dimer, trimer, and tetramer of PHC in both spores and vegetative cells of the *B. anthracis*, *B. cereus*, *B. thuringiensis*, and other species (Beverly *et al.* 1999b).

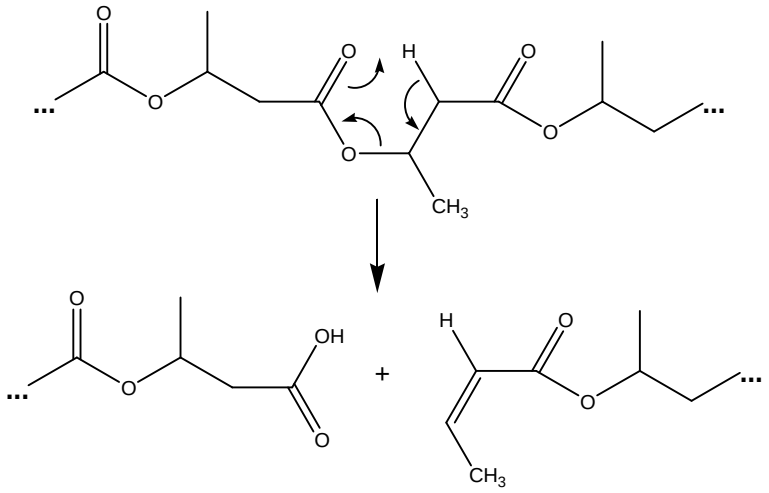


Figure 16. Chain scission ester decomposition mechanism of PHB at T > 170 °C.

Other observed pyrolysis products of PHB include β-butyrolactone, ketene, acetaldehyde, propene, carbon dioxide, and carbon monoxide. The mechanisms for the formation of these compounds are described by Grassie, Murray *et al.* (1984c), but are not presented here due to space limitations. In general, these compounds have not been reported to be observed during the pyrolysis of bacterial spores or cells.

DNA. The pyrolysis of DNA has been studied by Snyder and co-workers (Snyder *et al.* 1987). They observed several biomarkers that agree with studies on the pyrolysis of

bacteria. Biomarkers that have been repeatedly detected in model compound and bacterial studies include each of the DNA bases (adenine, guanine, thymine, and cytosine) and (poly)furanoic compounds. DNA pyrolysis involves the depolymerization of the DNA by thermal cleavage of the phosphodiester bonds that link the bases. Further decomposition of the oligonucleotides produces deoxyribose moieties and base-phosphate conjugates. These conjugates are very non-volatile and only decompose to produce free DNA bases at temperatures higher than 500 °C. The deoxyribose fragments are thought to condense to produce (poly)furanoic compounds. A separate study showed that the pyrolysis of DNA can generate furfuryl alcohol with about 10% conversion (Eudy *et al.* 1985a); this study pyrolyzed 100 μg of purified DNA at 800 °C for 2 s on a platinum ribbon (75 °C/ms ramp). Additionally, RNA was found to produce furfuryl alcohol, although in lesser amounts.

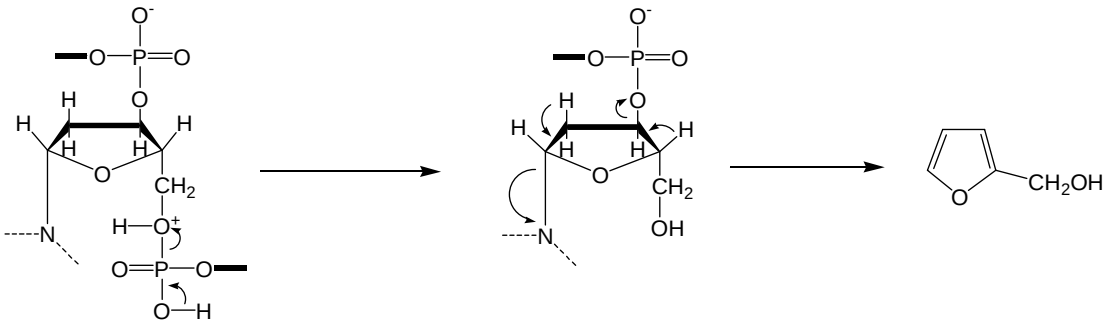


Figure 17. Pyrolytic formation of furfuryl alcohol from DNA (Posthumus *et al.* 1974; Irwin 1982).

Thermal Hydrolysis-Methylation (THM)

It has long been known that the lipid contents of bacterial spores contain a taxonomical information that can be exploited to differentiate closely related bacterial species (Abel *et al.* 1963; Shaw 1974). The development of analytical methods for lipids

has steadily progressed over the years, but applications have been hindered because of their sticky, non-volatile physical nature. However, derivatization (typically methylation) of the lipids significantly increases their volatility and facilitates faster chromatographic analysis. Typically, chemical extraction methods are used to remove the free lipids from the spores, following which the lipids are methylated to create fatty acid methyl esters (FAMEs) that are volatile enough to be detected by GC or MS (Tabor *et al.* 1976). This application has been commercialized by Microbial Identification Systems of Newark, DE and features an automated system for the chemical extraction and derivatization of the FAs; a pyrolysis unit is utilized to vaporize the FAMEs for analysis by GC-MS (MIDI 2005). There are many possible derivatizing agents; a review on the different reagents and their application in producing methyl esters has been provided by Kossa and co-workers (Kossa *et al.* 1979). In general, the preferred reagent for bacterial FA derivatization is tetramethylammonium hydroxide (TMAH) (Challinor 2001).

More recently, a process called thermal hydrolysis-methylation (THM) has been developed, which is capable of not only methylating the *free* FAs with a powerful methylation agent (again TMAH), but also of transesterifying (saponifying and derivatizing) the *bound* FAs (refer to Figures 5 and 6).

THM methodology

THM is typically conducted *in situ* at high temperatures (350-600 °C) in a pyrolyzer similar to that used for thermal pyrolysis. The TMAH is either co-injected with the sample, or is dried onto the pyrolyzer surface with the sample before heating. As in thermal pyrolysis, the volatile chemical produced during heating are passed to a chemical analyzer.

Voorhees *et al.* have developed methods to rapidly produce FAMEs from spore lipids by THM (Deluca *et al.* 1990; Deluca *et al.* 1992; Beverly *et al.* 1996; Hendricker *et al.* 1999; Havey *et al.* 2004). The FAMEs are typically analyzed by GC-MS or direct MS to construct a lipid (or FA) profile. Pattern recognition algorithms are employed to analyze and interpret these profiles in order to identify the presence or absence of anthrax; they have been used to show that FAME profiles are unique for each bacterial spore species and thereby facilitate potentially unambiguous identification (Hendricker *et al.* 1999). Recently Havey and co-workers at Sandia National Labs have collaborated with Voorhees *et al.* to develop a miniature ceramic membrane heating system that is capable of high heating rates (3500 °C/s) to generate FAMEs from bacterial spores by THM at between 360-440 °C (10 s pyrolysis time) (Havey *et al.* 2004). This device has sub-Watt power requirements, but has yet to be field-tested or thoroughly evaluated.

Analysis of FAs by chromatography of necessity takes several minutes; many studies of both bacterial spores and cells have used direct pyrolysis-MS and bypassed the chromatographic step in order to reduce analysis time (Deluca *et al.* 1990; Beverly *et al.* 1996; Voorhees *et al.* 1997; Basile *et al.* 1998a; Hendricker *et al.* 1999; Havey *et al.* 2004). This allows the detection of some molecules which are not volatile enough for GC, but puts a heavy burden on the data analysis and pattern recognition algorithms. The advantages of direct MS include reduced (1) analysis time, (2) analytical equipment, (3) power consumption, and (4) cost.

Biomarker producing reactions during the THM of spores and cells

THM methylates FAs and other carboxylic acid-containing molecules to produce methyl esters. As the native FAs are present in both free and bound forms, there are

likely at least two different reaction pathways. For the free FAs, a simple esterification mechanism could describe the removal of the proton and the substitution of a methyl group. For bound FAs, a more complicated pathway would be required to describe the transesterification that occurs. The locations in the spores or cells of the FAs (both bound and free) that undergo reaction with THM to produce FAMEs have not been defined in previous work, i.e., it is not known whether the FAs originate from the exosporium or from other parts of the spore such as the membranes. A list of the FAMEs produced in the literature of the THM of bacterial spores and cells is presented in Table 11. Additional THM products have been detected, including methylated amino acids, diketopiperazines, and DNA bases (Abbas-Hawks *et al.* 1996; Hendricker *et al.* 1999). A list of non-FA biomarkers is presented in Table 12.

Table 11. Fatty acid biomarkers produced by THM from bacterial spores and cells.

#	FAME[a]	MW	#	FAME[a]	MW
1	C10:0 ME	186	29	aC17:1 ME	282
2	C10:0 2-OH ME	202	30	C17:0 ME	284
3	C10:0 3-OH ME	202	31	cyC17:0 ME	282
4	iC12:0 ME	214	32	C17:1 ω8c ME	282
5	C12:0 2-OH ME	230	33	C18:0 ME	298
6	C12:0 3-OH ME	230	34	C18:1 ME	296
7	iC13:0 ME	228	35	C18:1 ω7c ME	296
8	aC13:0 ME	228	36	C18:1 ω9c ME	296
9	C13:0 ME	228	37	C18:1 ω9t ME	296
10	iC14:0 ME	242	38	C18:1 ω10c ME	296
11	C14:0 ME	242	39	C18:1 w12t ME	296
12	C14:0 3-OH ME	258	40	C18:2 ME	294
13	iC15:0 ME	256	41	C18:3 ME	292
14	aC15:0 ME	256	42	iC19:0 ME	312
15	C15:0 ME	256	43	C19:0 ME	312
16	C15:0 2-OH ME	272	44	cyC19:0 ME	310
17	iC16:0 ME	270	45	iC20:0 ME	326
18	iC16:1 ME	268	46	C20:0 ME	326
19	C16:0 ME	270	47	C20:1 ME	324
20	C16:0 3-OH ME	286	48	C21:0 ME	340
21	C16:1 ω5c ME	268	49	C22:0 ME	354
22	C16:1 ω7c ME	268	50	C22:1 ω13c ME	352
23	C16:1 ω8c ME	268	51	C23:0 ME	368
24	C16:1 ω7c alcohol ME	284	52	C24:0 ME	382
25	iC17:0 ME	284	53	C24:1 ω15c ME	380
26	iC17:1 ω5c ME	282	54	C25:0 ME	396
27	iC17:1 ω10c ME	282	55	C26:0 ME	410
28	aC17:0 ME	284			

[a] i = iso, a = anteiso, ω gives location of double bond, from end of carbon chain, CX:Y gives information about the FA where X denotes the number of carbon atoms in chain and Y denotes the points of unsaturation

Table 12. Non-fatty acid biomarkers produced by THM from bacterial spores and cells.

#	Name	Precursor	Reference(s)	MW
1	Phenyl ion	Aromatic amino acids, protein	(Havey *et al.* 2004)	77
2	Pyridine	DPA	(Havey *et al.* 2004)	79
3	Benzyl ion	Aromatic amino acids, protein	(Havey *et al.* 2004)	91
4	Dimethylated DPA fragments	DPA	(Havey *et al.* 2004)	105, 137
5	Methylated tyrosine fragment	Tyrosine, protein	(Havey *et al.* 2004)	107
6	Indole	Tryptophan, protein	(Havey *et al.* 2004)	117
7	Tyrosine	Protein	(Hendricker *et al.* 1999)	246, 231

Table 12 Continued.

#	Name	Precursor	Reference(s)	MW
8	Phenylalanine	Proteins	(Hendricker *et al.* 1999)	215
9	Tryptophan	Protein	(Hendricker *et al.* 1999)	189
10	Dipicolinic acid dimethyl ester	DPA	(Hendricker *et al.* 1999)	196
11	Cytosine	DNA	(Voorhees *et al.* 1997; Hendricker *et al.* 1999; Havey *et al.* 2004)	111, 125[a], 139[b], 153[c]
12	Thymine	DNA		126, 140[a], 154[b]
13	Adenine	DNA		135, 149[a], 163[b], 177[c]
14	Guanine	DNA		151, 165[a], 179[b], 193[c], 207[d]

[a] First methylation; [b] Second methylation; [c] Third methylation; [d] Fourth methylation

The use of THM for the production of FAMEs from bacterial or spore samples is complicated by the issue of reaction selectivity. As mentioned above, the FA structure (carbon chain structure, hydroxyl substitution, cis/trans double-bond configuration, etc.) contains taxonomical information; the objective of the FAME profiling is to detect this information. However, it has been shown that THM can isomerize or fragment (degrade) FAs, which removes taxonomical information (Downing and Greene 1968; Kossa *et al.* 1979; Challinor 1991). While there are techniques that are known to mitigate or eliminate this problem (Ding *et al.* 1997), the vast majority of THM studies have been carried out without considering the effect of derivatization by THM on the taxonomical information contained in the FAs.

One notable exception to this is the work of Gorecki and Poerschmann (Gorecki and Poerschmann 2001). They have studied the use of THM (500 °C) conducted inside of a deactivated stainless steel capillary in-line with the GC column, a technique they have termed "non-discriminating TMAH-induced thermochemolysis." Essentially because the thermochemolysis is conducted inside a resistively heated capillary tube, the pyrolysis

times are much shorter than the conventional THM pyrolyzer (e.g., several ms vs. 5-10 s; note that this is total pyrolysis time and not temperature rise time). The authors claim that this leads to (1) less secondary pyrolytic reactions than the conventional methods, and (2) preservation of FA chemotaxonomical information that they could be lost in conventional THM techniques.

Biomarker producing reactions during the THM of relevant biopolymers

Two studies have focused on the THM of biopolymers: one on DNA and the other on amino acids and oligopeptides.

DNA. Abbas-Hawks and Voorhees investigated the THM of both free nucleotides and double-stranded DNA, as well as the DNA inside of whole bacterial cells (Abbas-Hawks *et al.* 1996). They found that the methylation of DNA bases can be done on multiple sites on each DNA base; essentially any acidic proton (O-H or N-H) can be replaced with a methyl group (see Figure 18a). There are four possible sites for guanine, three for cytosine and adenine, and two for thymine; the concentration of TMAH has an effect on the number of methylations for each DNA base. This corresponds well to the observations of others (see biomarkers in Table 12). The methylation of oligonucleotides and DNA extracts also produced methylated DNA bases, with the extent of methylation again depending on the TMAH concentration. Abbas-Hawks and Voorhees contend that methylated DNA biomarkers produced by THM are more stable and volatile than the biomarkers produced from DNA during thermal pyrolysis.

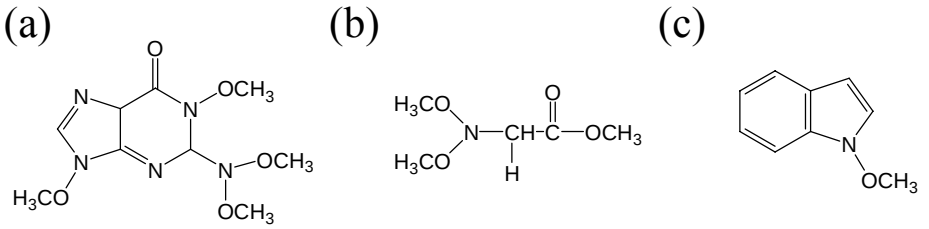


Figure 18. Representative methylated compounds from DNA and protein pyrolysis: (a) tetramethylated guanine; (b) trimethylated guanine; and (c) methylated indole (fragment from tryptophan).

Amino acids and oligopeptides. THM of amino acids and several oligopeptides was studied by Hendricker and Voorhees (Hendricker and Voorhees 1998). They observed methylation of the acidic protons (O-H and N-H) during the THM of individual amino acids (e.g., glycine has three acidic hydrogens at a neutral pH and therefore can be methylated in three locations; see Figure 18b) and also of amino acid fragments (see Figure 18c). They also observed the methylation of diketopiperazines formed by a condensation reaction between either individual amino acids or cyclization of oligopeptides (up to six amino acids in length). The authors observed amino acid dimers following THM, but not during thermal pyrolysis; they postulate that this dimerization is favored during THM because methylation of an amino acid increases its nucleophilicity to the point where peptide bond formation can occur. The formation of dimers occurs readily for amino acids with small side chains (such as aniline or glycine), but is less favored for amino acids with larger side chains (such as tyrosine). For polyamino acids (polypeptides of MW 2000-5000 Da), methylated amino acids and methylated diketopiperazines were observed, indicating that similar reactions would be expected to occur during the THM of proteins and polypeptides.

Reproducibility of Thermal Pyrolysis and THM

Reproducibility of pyrolysis and THM is a very important issue that in general has not received sufficient attention in published studies and reviews on the production of biomarkers from bacteria. Typically, no mention is made regarding the reproducibility of the chromatograms, nor are direct comparisons made to the work of others (most comparisons are of chemicals detected, not chromatograms). However, determination of the reproducibility of the biomarker production method is of great importance in the detection and positive identification of anthrax (Xu *et al.* 2003). Wampler briefly addresses reproducibility in his book, recommending that low-microgram amounts of homogeneous samples be used, and that special attention be paid to the surfaces which contact the pyrolyzate (Wampler 1995). More complete treatments of the possible causes of variability between chromatograms are presented by Irwin (Irwin 1982) and Meuzelaar (Meuzelaar *et al.* 1982), which include factors such as heating rate, sample size, pyrolyzer volume, chromatographic limitations (column stationary phase and maximum temperature), and growth conditions (media, and growth time) for bacteria and other biological material.

Studies on the effects of the pyrolyzer surface on pyrolytic reactions have been done by Kuroda, Muguruma and Meuzelaar. Kuroda studied 4 different metal surfaces (gold, platinum, aluminum, and a Ni-Fe alloy) coated on a curie-point pyrofoil and observed significant differences in the chromatograms obtained from the pyrolysis of lignin (a carbohydrate biopolymer) on each surface (Kuroda and Dimmel 2002). Muguruma noted similar effects for pyrofoils coated with gold and platinum as compared to the Ni-Fe alloy in the pyrolysis of organic polymers (Muguruma *et al.* 1998; Muguruma *et al.* 1999). Meuzelaar noted that the products obtained from pyrolysis in a

quartz tube are very different from those obtained with a filament pyrolyzer (Meuzelaar *et al.* 1982). However, a study that evaluated variation between filament and quartz tube pyrolyzers in the Py-GC-MS of multiple synthetic and biological polymers showed little variation between the pyrolyzers, provided that all analytical variables (such as pyrolysis temperature, gas flow rate, inlet pressure, etc.) are the same between instruments (Stankiewicz *et al.* 1998). This study also evaluated run-to-run variation (very few studies mention this aspect of reproducibility) which showed acceptable reproducibility.

Other studies have shown that the presence of metal oxides in the sample can significantly influence the chromatogram of both biological and synthetic polymers (van Der Kaaden *et al.* 1983; Yoshioka *et al.* 2005).

Of the publications that addressed the issue of reproducibility, most have focused on the effect of growth conditions on the discrimination of bacterial cells by Py-GC-MS or Py-MS; these include work done by (1) Shute (Shute *et al.* 1988) in which batch-to-batch media variation was determined to be insignificant, but different media types produced very different chromatograms which significantly affected species discrimination ability; (2) Gutteridge (Gutteridge and Norris 1980) in which samples of the same species cultured in different media showed drastically different chromatograms, but the time and temperature of culture growth showed less variation between chromatograms; and (3) Voorhees (Voorhees *et al.* 1988) in which incubation time and cell killing methods did not significantly influence the spectrum obtained by Py-MS.

The study of Xu, Voorhees, and Hadfield, mentioned previously, merits further discussion in regards to analysis of reproducibility. THM-MS was used to produce FAME profiles from *B. anthracis*, *Y. pestis*, *V. cholerae*, *Br. melitensis*, and *F. tularensis*

(Xu *et al.* 2003); 20 μg of bacterial cells were loaded with 1 μL of 0.1 M TMAH (in MeOH) onto a quartz wool plug and pyrolyzed in a quartz tube at 500 °C for 10 s (no information on heating rate or temperature rise time provided). Analysis of variance (ANOVA) and a *t*-test using normalized peak areas were used to show that the mass spectral data were obtained in a reproducible manner. Based on this conclusion, they were able to proceed with multivariate statistical analysis to discriminate between the bacterial species.

Application of Catalysis to the Generation of Biomarkers

A catalyst is a material that lowers the activation barrier to formation of the transition state for a given chemical reaction, allowing it to proceed at rates faster than otherwise possible. The catalyst is in principle unchanged during this process, and can assist the reaction many, many times. In their usual form, heterogeneous catalysts consist of small crystallites of metal, metal oxide, or metal sulfide (the active phase) dispersed on a porous ceramic material, called a support. Solid acid catalysts consist of supported metal oxides, zeolites (solid acids), and superacids. Metal and metal oxide acid catalysts have thus far found numerous applications in petroleum refining, chemicals manufacturing, and pollution control. Their major benefits are three-fold (Farrauto and Bartholomew 1997): first, they facilitate rapid reaction at low temperatures and pressures, thus dramatically lowering energy requirements for chemical reactions and processes; second, they offer tremendous increases in selectivity and speed for a desired reaction or set of reactions; third, they reduce the required equipment volume.

A search of the scientific literature indicates no explicitly reported applications of heterogeneous catalysts to the production of biomarkers from bacterial spores. Thus, the

application of catalyst technology to produce biomarkers is a new opportunity. Catalysts can break the same types of bonds that are broken during pyrolysis, i.e., carbon-carbon, carbon-nitrogen, and carbon-oxygen bonds, but at milder conditions. Catalysts used to break hydrocarbon carbon-carbon bonds include solid acids used in the catalytic cracking of heavy hydrocarbons, metal (Ni, Pt, Rh) catalysts used in the steam reforming of hydrocarbons, and a combination solid acid/metal (Ni, Pt) catalyst for the hydrocracking of polynuclear aromatic hydrocarbons (Farrauto and Bartholomew 1997). Materials that catalyze the breaking of carbon-oxygen and carbon-nitrogen bonds include metal oxides and sulfides.

Acid/base catalysts can catalyze reactions similar to the methylation reactions that occur during THM. Superacid catalysts, such as tungstophosphoric acid ($H_3WP_{12}O_{40}$; TPA) in a homogenous (liquid-liquid) application, have been used in transesterification reactions for the breaking and forming of carbon-oxygen bonds (Timofeeva *et al.* 1995; Timofeeva *et al.* 2001; Timofeeva 2003; Timofeeva *et al.* 2003; Bondioli 2004). A study on such a catalyst was done with DPA, a common reagent in the pharmaceutical industry, showing that the Keggin structure superacids have high activity for esterification of DPA (Timofeeva *et al.* 1995). Further, supported TPA can be used in a heterogeneous catalytic application for the transesterification of carboxylic acid-containing molecules (Timofeeva *et al.* 2001; Timofeeva *et al.* 2003).

Critical Analysis of Literature Review

The present state of knowledge for technologies and methods used for the rapid (<10 min) handheld (field-portable) detection of anthrax and other bacterial spores is summarized as follows:

- No proven technology exists, although much work has been done to develop this promising technology.
- One viable approach is the production of biomarkers by thermal pyrolysis or THM.
- Multivariate statistical analysis has been used to discriminate spore species based on the taxonomical information contained in the biomarkers.
- Thermal pyrolysis and THM have been developed to produce biomarkers from spores and cells for gram-type and species-level discrimination, respectively.
- Thermal pyrolysis and THM methods break carbon-carbon, carbon-oxygen, carbon-nitrogen, and carbon-hydrogen bonds to produce volatile compounds; THM forms carbon-oxygen bonds to produce methyl esters.
- Reaction pathways for thermal pyrolysis and THM of representative biopolymers and their constituents have been investigated and in some cases, determined; insights from this work are directly applicable to the thermal pyrolysis and THM of bacterial spores and cells.
- THM is a non-catalytic method, since the TMAH is consumed in the process, which is driven by thermal decomposition and rearrangement of chemical bonds.
- Catalysts can moderate the conditions necessary for these breaking and forming of the bonds specified above and produce biomarkers with greater activities and selectivities; potential catalysts include Pt, Ni, and superacids such as TPA.
- No previous applications of catalysis to the generation of biomarkers from bacterial spores and cells appear in the scientific literature, although catalysis has significant potential for improving selectivity and reproducibility relative to thermal pyrolysis and THM.

The deficiencies and shortcomings of studies on thermal pyrolysis and THM, and the inherent limitations of these methods are summarized as follows:

- The equipment required for thermal pyrolysis and THM is either bulky, power-hungry, unproven, or unsuitable for rapid, handheld applications.
- While some of these methods are rapid (<10 min), in general there is room for improvement of analysis and detection times.
- There is a general lack of attention to reproducibility and statistical significance in the scientific literature; this is especially apparent in the use of many different types of pyrolyzers and very little discussion on what this may or may not mean for the biomarkers observed. One notable exception is the work of Xu *et al.* 2003.
- A summary of the biomarkers produced by thermal pyrolysis and THM of bacterial spores is not readily available from the scientific literature; a mass spectral library for these compounds would be especially useful.
- While some attention has been paid to the identity and origin of chemotaxonomical biomarkers produced by thermal pyrolysis and THM (see Snyder *et al.* 2004), this information is generally lacking in the literature; investigation of reaction pathways, specific biological origin of the biomarker precursors, and comparison of the biomarkers produced in the various thermal pyrolysis and THM studies are needed.
- Previous work has not focused adequately on the differences between spore and gram-positive bacterial biomarkers, other than those biomarkers derived from DPA; there should be significant differences in the distributions of biomarkers produced, based on the physiological differences between spores and cells. A similar need

exists for the investigation of differences in the biomarkers between different species and even strains.

- Pyridine can be produced from both DPA and peptidoglycan as shown in Figures 11 and 12; additionally, it can be produced from protein. This indicates that care must be taken in arbitrarily assigning taxonomical information to compounds observed during pyrolysis of biological material. Rather, detailed analysis and consideration must be used to assign taxonomical importance to chemicals produced during analytical pyrolysis; this level of analysis is rarely provided in the literature.

Information on the contacting of various metal and superacid catalysts with bacterial spores, as well as information on the bond breaking and forming catalytic reactions of Pt, Ni, and TPA with bacterial spores, is not available in the literature. An investigation into these questions would contribute much to the literature, as well as the development of a rapid, handheld detection method based on the production of biomarkers from anthrax and other bacterial spores.

Conclusions

Current, state-of-the-art AP methods and field-portable (handheld) devices have not demonstrated the ability to reproducibly generate biomarkers that can discriminate and identify bacterial spores at the species and sub-species level.

A catalytic process could be expected to lower the energy required to produce biomarkers and enhance formation and selectivity. A literature search has provided data to show that (1) the application of catalysis to the breakdown of bacterial spores has not been previously explored, (2) nickel and platinum catalysts have potential for breaking

carbon-carbon bonds to form more volatile compounds, and (3) heteropolyacid (superacid) catalysts have potential for the transesterification (methylation) of FAs to produce volatile methyl ester biomarkers.

Any experimental work using catalysts to produce biomarkers from anthrax spores must address the issue of reproducibility and statistical significance.

Chapter 3. Thesis Objectives and Scope

Overview

This thesis combines a comprehensive, critical review of the pyrolysis and thermal hydrolysis-methylation (THM) of anthrax spores with experimental observations on the catalytic breakdown of anthrax spores. In this chapter, the objectives and scope of the project are described.

Project Objective

The objective of the project is to contribute to the understanding and document the types of biomarkers that are produced and the chemical reactions that occur during the analytical pyrolysis and catalytic breakdown of anthrax spores.

Scope of the Project

This thesis reviews the chemical reactions reported to occur during the thermal pyrolysis and the thermal hydrolysis-methylation of anthrax spores, as reported in the scientific literature. Relevant details of the application of both of these techniques to bacterial spores are included.

Additionally, this thesis incorporates *limited* experimental results in the form of GC-MS data on the production of biomarkers from anthrax spores by: (1) catalytic decomposition with platinum colloidal nanoparticles, (2) catalytic decomposition with an electroformed fine nickel mesh (200 mesh in^{-1}), (3) heating with tungstophosphoric acid

(TPA) and methanol, and (4) TMAH treatment. The reaction temperatures were between 100 and 350 ºC and the atmosphere was helium at close to atmospheric pressure. Experiments were done using a Frontier Laboratories double shot pyrolyzer (model Py-2020iD). Separation and detection of the volatilized components of the reactions were done using GC/MS. The RAMFAC algorithm was used to determine the chemical identities of the chemicals based on their mass spectral data. A statistically designed set of experiments was completed for work with the Pt-nanoclusters to ensure that the data collected were significant and reproducible.

Project Work Statement

The thesis work consisted of the following tasks:

*(**1**) Search and study the available literature to gain an understanding of, and write a comprehensive, critical review of the production of biomarkers from anthrax spores by AP.*

*(**2**) Conduct a statistically-designed series of tests appropriate for the M.S. degree to collect data on the catalytic production of biomarkers from anthrax spores by platinum catalyzed reactions.*

(***3***) *Analyze and interpret the data from the experiments in (2) using the RAMFAC algorithm and a library of biomarkers constructed from the literature based on (1).*

*(**4**) Compare data and results from the pyrolytic and catalytic reactions; analyze and compare the results from (1) and (3) to further the understanding of each biomarker production method.*

(5) Prepare thesis and publication(s); use (1) – (4) to write the thesis and publication(s).

Original Contributions to the Literature

1. As part of the comprehensive, critical review (Task 1), this project contains a collection of biomarkers data from the thermal pyrolysis and THM of bacterial spores based on journals and books published over a 30 year span. It also furthers the understanding of the available information on the reactions used in the production of these biomarkers by comparing the results of many thermal and THM studies, as well as studies of each of these methods with biopolymers such as protein, peptidoglycan, and DNA. Finally, it includes a review of literature on the effects of the pyrolyzer surface (e.g., metal or other material) on the chemical reactions that have not been cited by any previous studies on the AP production of biomarkers from anthrax spores.

2. The experiments conducted in Tasks 2-5 were the first attempt to develop an understanding of the catalytic reactions that occur between bacterial spores (anthrax spores) and platinum nanoclusters at temperatures between 200 and 350 ºC; this work also included preliminary investigation of nickel mesh and TPA catalysts for the production of biomarkers from anthrax spores.

Chapter 4. Experimental Methods

Spore Preparation

The *B. anthracis* (anthrax) spores used in this study were prepared by DJ Harvey and Dr. Richard Robison of the BYU Microbiology Department. The preparation method was as follows:

Day 1: 10 µL of *B. anthracis* A0256 freezer stock was plated on a Petri dish of Colombia agar. The plate was allowed to grow overnight at 37 °C.

Day 2-5: A colony from the isolation plate was gram stained to check culture purity. Following purity verification a colony was placed into 10 mL of sterile PBS (phosphate buffer solution). 100 µL of the PBS-Bacillus solution was placed into 250 mL of sterile Leighton and Doi broth. The flask was then placed on a shaker (100 revolutions per minute) in an incubator at 32 °C. The flask was left to grow for 3 days (Days 2-5).

Day 5: The solution was removed from the flask, placed into 50 mL conical tubes, and placed in a 65 °C hot water bath for 30 min. After removal from the bath, the tubes were centrifuged in a swinging bucket rotor at 3838 RCF (relative centrifugal force = x g) for 10 min. The supernatant was poured off and the pellet of each tube was resuspended in 5 mL of autoclaved HPLC H_2O. The 5 mL suspensions were then combined into one 50 mL conical tube. The tube was then centrifuged again at 3838 x g for 10 min followed by the removal of the supernatant and resuspension of

the pellet in 20 mL of autoclaved HPLC H_2O. The process of centrifuging, decanting, and resuspension (i.e., spore washing) was repeated a third time, following which the suspension was stored at 4°C overnight.

Days 6-8: The spores were washed once per day and stored at 4°C.

Day 8: Following the final washing, the spore suspension was autoclaved for 90 min at 250°C. Afterwards 100 µL were placed on a Colombia agar Petri dish to check for viability.

Day 9: The viability test was negative and the spore suspension was removed from the Biosafety Level 3 laboratory and delivered to C270 BNSN for testing. In the case of the nanocluster study, the spores used were resuspended in 70% ethanol following the final wash.

A similar procedure was used to prepared the green fluorescent protein (GFP)-labeled *B. anthracis* spores used in the nickel mesh study. These spores emit an intense green color when placed under fluorescent light, which allows visualization of spore loading.

Materials and Chemicals

Electroformed nickel mesh (200 mesh $inch^{-1}$) was obtained from McMaster-Carr (Chicago, IL). Sections of 3 mm x 1 mm were cut and washed with methanol before loading with spores. Chemicals (e.g., solvents such as methanol and ethanol) were obtained from Sigma-Aldrich (St. Louis, Mo) and used without additional purification. Pt-nanoclusters were obtained from Strem Chemicals (Newburyport, MA). The Pt-nanoclusters are metal nanocolloids with polyethyleneglycol-dodecylether ligands; the average cluster diameter is 1.2 nm, ± 0.5nm (Strem 2004).

Biomarker Library Construction

In order to determine the chemical identities of the biomarkers produced during the experiments, a mass spectral library was needed. The NIST database, which contains spectral data for over 200,000 chemicals, is too large to be useful for comparison with the large amounts of data generated, and does not contain all of the compounds of interest. To overcome this problem, a biomarker library was constructed based on information available in the scientific literature and the NIST data base. The chemical identities of biomarkers observed in many Py-MS, Py-GC-MS, THM-MS, and THM-GC-MS studies were extracted form the literature reviewed in Chapter 2 (see Tables 2, 6, 7, 9, 10, and 11). Mass spectral data for these chemicals was taken from the NIST database where available. Appendix A contains a list of the compounds in this library.

Data Collection and Analysis

The catalytic experiments were conducted in a Frontier Laboratories (Fukishima, Japan) double-shot pyrolyzer (Model Py-2020iD, hereafter referred to as the "pyrolyzer"); the pyrolyzer was interfaced with an Agilent 6890-5723 GC-MS (Palo Alto, CA) that detected and recorded products of the reactions done in the pyrolyzer. The GC was operated in split mode (5:1 split) with the inlet flow controlled by a Frontier Laboratories (Fukishima, Japan) selective sampler.

A brief explanation of the experimental procedure is as follows (for more detail refer to Appendix B):

(1) the spore and nanocluster liquid suspension was added to a 60 μL sample cup;

(2) the mixture was left to dry overnight or at an elevated temperature (~40 °C) for two h;

(3) the sample cup was loaded into the pyrolyzer and dropped into the heated zone of the pyrolyzer;

(4) the pyrolysis cup was removed from the heated zone after 2 min;

(5) the compounds evolved during pyrolysis were separated on the GC column and analyzed by the MS.

For the nickel mesh study, the spores were loaded onto the mesh by dipping and air-drying, the loaded mesh was placed into the sample cup, and the sample cup was dropped into the heated zone of the pyrolyzer. In the case of the TPA/TMAH experiments, the reagents were dried together in the sample cup (in air) before lowering the sample cup into the pyrolyzer.

The RAMFAC computer algorithm developed by James Oliphant (adjunct researcher with Palmar Technologies) was used to analyze the data and determine the chemical identities of compounds detected by the mass analyzer of the MS. This algorithm enables extraction and identification of individual chemical species from the GC-MS data, as well as determining quantitatively the amount of the chemical that was detected. See Appendix F for more information on the RAMFAC algorithm.

For experiments involving spores and TPA/TMAH, 4 μg of spores were loaded into a sample cup and dried, together with 0.001 μmol of TPA and/or 0.4 μmol of TMAH (aqueous solutions).

For the wire mesh experiments, low μg amounts of GFP-labeled spores were dried onto the 200 mesh in^{-1} electroformed nickel. Microscopy was used as a secondary means of obtaining information about the loading of the spores on the mesh. Light and fluorescence microscopy were done using a Carl Ziess (Thornwood, NY) Axioskop 2

light microscope, which was equipped with an AxioCam HRc (digital camera) and an AttoArc 2 fluorescent light source (Atto Instruments, Rockville, MD).

Statistically Designed Study

Statistically designed experiments play a crucial role in scientific research. They can be used to: (1) show if collected data are *reproducible* and *significant* and (2) quantify experimental results, allowing comparison to the work of others. Additionally, statistical analyses are often necessary for the publication of data. An experimental study on the Pt-nanocluster/spore method was designed in order to ensure that the data collected during its evaluation were reproducible and significant, and that the appropriate questions could be addressed.

The principal goal of the designed study was to evaluate the relative importance of several variables on the decomposition of the spores, including pyrolysis temperature, spore quantity, and nanocluster amount. Factorial designs with high and low variable values was used, in which the effects of these variables were explored. The high and low values for the variables were as follows: for pyrolysis temperatures, 250 and 300 °C; for spore amount, 10 and 20 μg; and for nanocluster amount, 0.012 and 0.024 μg (see Appendix B for the coating calculations used to determine the desired amount). Three separate factorial designs were prepared, one for evaluation of the pyrolysis of spores, one for evaluation of the pyrolysis of nanoclusters, and one for the evaluation of the effect of mixing spores and nanoclusters. Replication of these factorial designs was not done due to time constraints on the GC-MS.

The response factor(s) used in the statistical analysis plays a key role in determining the effects of the studied variables on the production of biomarkers. For this

study, the response factors were chosen from the biomarkers that were detected in chromatograms from experiments involving spores and spore-nanocluster mixtures. This choice was made following analysis with the RAMFAC algorithm to determine chemical identities, and after consideration of the physical significance of the biomarkers (i.e., whether the biomarker represents a feasible decomposition product of the spores).

The response factors were used to evaluate numerically the effect of the three studied variables on the decomposition of spores and the production of biomarkers. Because the experiments were not replicated, a factorial analysis was not possible; instead statistical regression of the data was done (see Appendix D). This regression analyzed the three factorial designs collectively. This allowed for regression of the data with analysis of variance based on the three variable amounts, which were temperature, amount of spores, and amount of nanoclusters. In analyzing the data, there were collectively three levels of loading for spores and nanoclusters: (1) the high levels, (2) the low levels, and (3) the zero loading levels. With the regression approach, this was accounted for and provided additional information on the effects of all variables. The resulting p-values can be compared with an α value of 0.05 (for a 95% confidence in the significance of the data); variables with p-values less than 0.05 would have a significant effect in the production of the response factor biomarker from the spores. Data modeling included both linear and quadratic effects for spore and nanocluster loading. See Appendix D for more information on the statistical methods used.

Chapter 5. Results and Discussion

Experimental Results

Nickel Mesh Study

Early experiments focused on methods to coat a nickel mesh with spores for catalytic experiments. Microscopy was used to visualize the extent of mesh coverage, and identify what happened to the spores during heating. A small piece of nickel mesh was cut (~3 mm x 1 mm) and dipped in a concentrated GFP-labeled spore solution (~10^8 spores mL^{-1}). Light and fluorescent microscope images of the loaded mesh are presented in Figures 19 and 20, respectively. One of the observed effects was window-paned spores in the corners of the mesh (i.e., spores would aggregate in the corners of the mesh openings). The GFP-labeled spores light up brilliantly under the fluorescent light, which shows that relatively few spores were loaded upon the surface of the nickel wires, relative to the amount of spores agglomerated in the corners between the wires.

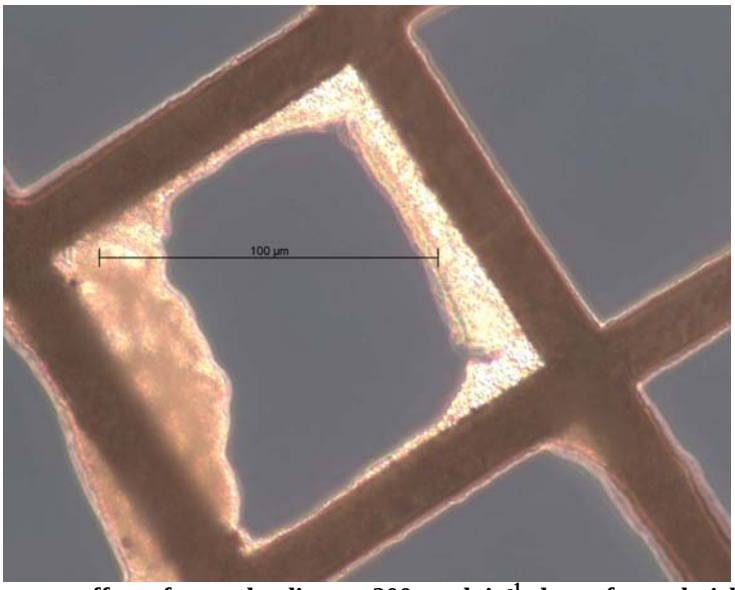


Figure 19. Window-pane effect of spore loading on 200 mesh in^{-1} electroformed nickel (400X).

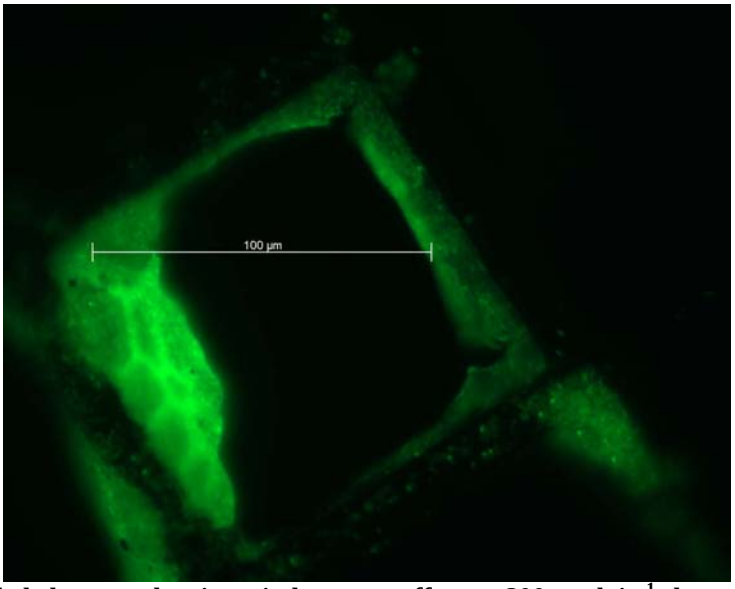


Figure 20. GFP-labeled spores showing window-pane effect on 200 mesh in^{-1} electroformed nickel (400X, fluorescent light).

A lower magnification image, Figure 21, reveals a brilliant outline of the mesh, showing the loading obtained by this dipping process. Upon heating in the pyrolyzer at a 200 °C, the color of the GFP-spores changed from bright green to dull white (Figure 22).

At 150 °C the green fluorescence was still seen, indicating that the degradation of the GFP proteins began to be widespread at a temperature between 150-200 °C.

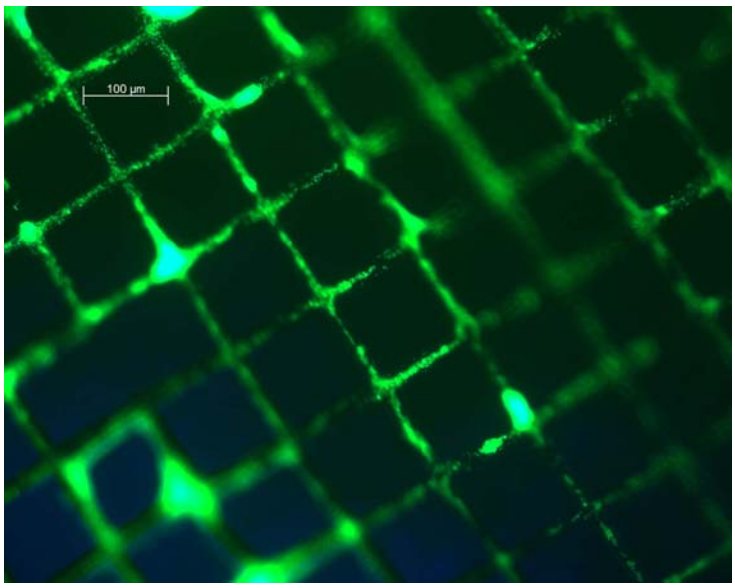


Figure 21. Spore-loaded mesh (100X, fluorescent light).

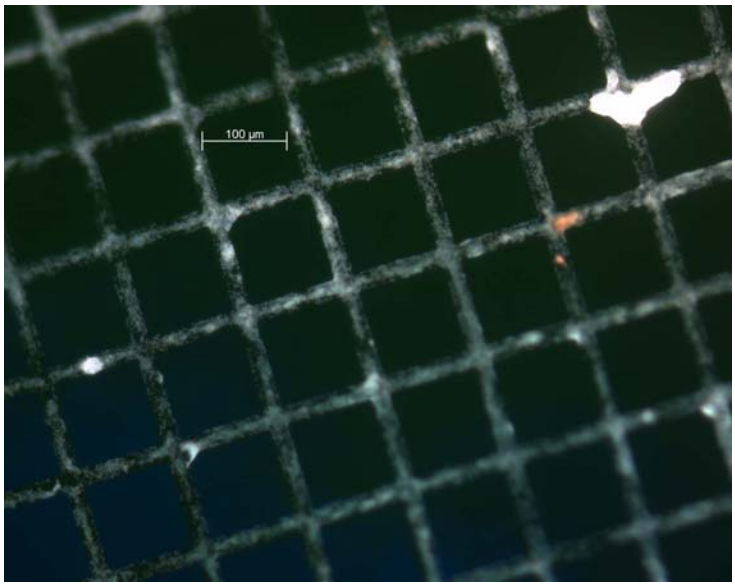


Figure 22. Spore-loaded mesh after heating to 200 °C (100X, fluorescent light).

A representative chromatogram of the mesh/spore heating study is presented in Figure 23, for which pyrolysis was done at 400 °C. This temperature was required in order to obtain detectable biomarkers.

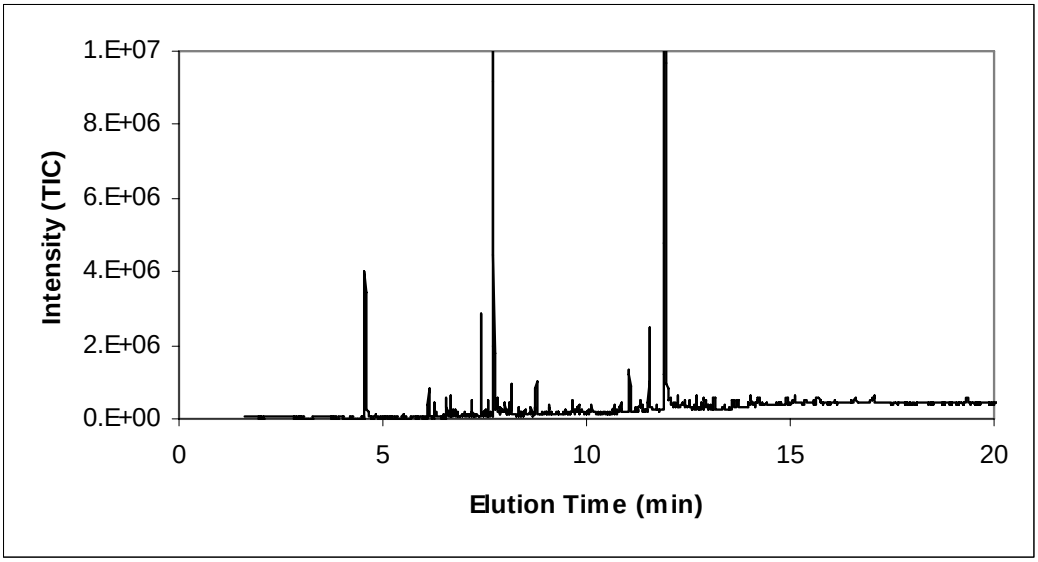


Figure 23. Pyrolysis of autoclaved anthrax spores on nickel mesh at 400 °C.

The chemical identities of the peaks in these chromatograms were obtained by matching the mass spectral data with the biomarker library described in Chapter 4 (see Appendix A). These compounds are listed in Table 13.

Table 13. Biomarkers produced by pyrolysis of the spore-loaded mesh and detected by GC-MS.

#	Name	Retention Time	Precursor
1	Toluene	3.59	Protein
2	1-Butene, 2-methyl	3.93	Protein
3	Pyridine	4.56	DPA, protein, DNA
4	Butanenitrile, 2-methyl	4.96	Protein
5	Ethenylbenzene	5.04	Protein
6	Alanine	5.33	Protein
7	1-Butene, 3-methyl	5.95	Protein
8	Furfural	6.26	DNA, carbohydrates
9	Tyrosine	6.39	Protein
10	Pyrrole	6.49	Protein

Table 13 Continued.

#	Name	Retention Time	Precursor
11	Benzaldehyde	6.60	Protein
12	Picolinic acid	6.75	DPA
13	5-Methyl furfural	6.84	DNA, carbohydrates
14	Valine	7.12	Protein
15	Furfuryl alcohol	7.18	DNA
16	Serine	7.21	Protein
17	Crotonic acid	7.71	PHB
18	Acetamide	7.72	Peptidoglycan
19	Pyrrolidine	7.81	Protein
20	1-Acetyl-1,2,3,4-tetrahydropyridine	8.01	Protein
21	Benzeneacetonitrile	8.48	Protein
22	Phenol	8.75	Protein
23	Benzenepropanitrile	8.99	Protein
24	2-Pyrrolidinone	9.03	Protein
25	*p*-Cresol	9.09	Protein
26	Maleimide	9.66	Protein
27	Picolinamide	10.84	Protein
28	Indole	11.07	Protein
29	2,5-Pyrrolidinedione	11.22	Protein
30	3-Methylindole	11.34	Protein
31	Imidazole	11.54	Protein
32	Guanine	12.04	Protein
33	Glutamine	12.23	Protein
34	C15:0	12.70	Lipids

TPA and TMAH Catalytic Study

A preliminary investigation of the effect of low concentrations of TMAH and TPA on the pyrolysis of spores was conducted in the pyrolyzer. A representative chromatogram of the reaction of TMAH and TPA at 250 °C with spores is presented in Figure 24. For the reaction of just TMAH and spores at the same temperature, a representative chromatogram is presented in Figure 25. It is evident that the addition of TPA to the spores and TMAH increases both the intensity and number of peaks.

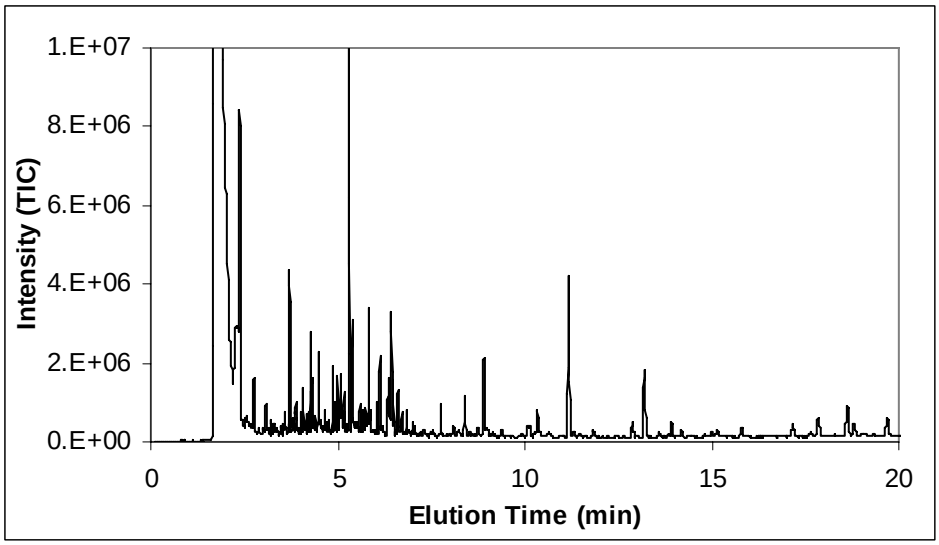


Figure 24. Chromatogram produced by the reaction of spores with TPA and TMAH at 250 °C.

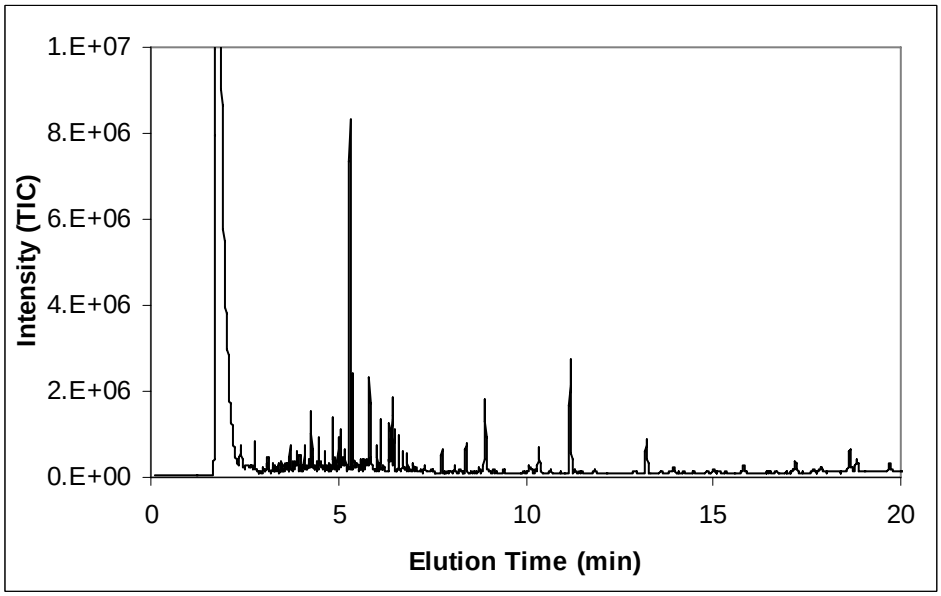


Figure 25. Chromatogram produced by the reaction of spores with TMAH at 250 °C.

The chemical identities of the peaks in these chromatograms were obtained by matching the mass spectral data with the biomarker library described in Chapter 4. These compounds are listed in Table 14.

Table 14. Biomarkers produced by TPA/TMAH catalysis (methylation) of spores.

#	Name[a]	Retention Time	Precursor
1	Acetone	1.65	PHB
2	1-Butene, 3-methyl	1.67	Protein
3	Alanine	2.44	Protein
4	Pyridine	2.77	DPA, protein, DNA
5	Valine	3.06	Protein
6	Benzaldehyde	3.58	Protein
7	C10:0 ME	3.68	Lipids
8	1-Propene, 2-methyl	3.82	Protein
9	2-Pyrrolidinone	4.16	Protein
10	C12:0 ME	4.31	Lipids
11	aC13:0 ME	4.54	Lipids
12	Picolinic acid ME	5.10	DPA
13	C15:0 ME	5.30	Lipids
14	C16:1 w8c ME	6.37	Lipids
15	iC17:0 ME	6.48	Lipids
16	iC18:0 ME	7.7	Lipids
17	Glutamine	10.34	Protein
18	Dipicolinic acid, di-ME	11.17	DPA

Pt-Nanocluster Catalytic Study

The catalytic study was conducted in two phases. First, several experiments with the nanoclusters and spores were conducted in order to obtain exploratory information. Second, a statistically designed experimental plan was prepared to evaluate the relative effects of three important variables on the biomarkers produced from the spores.

Overlaid chromatograms of two spore experiments conducted under the same conditions are presented in Figure 26, which shows visible reproducibility between the two chromatograms.

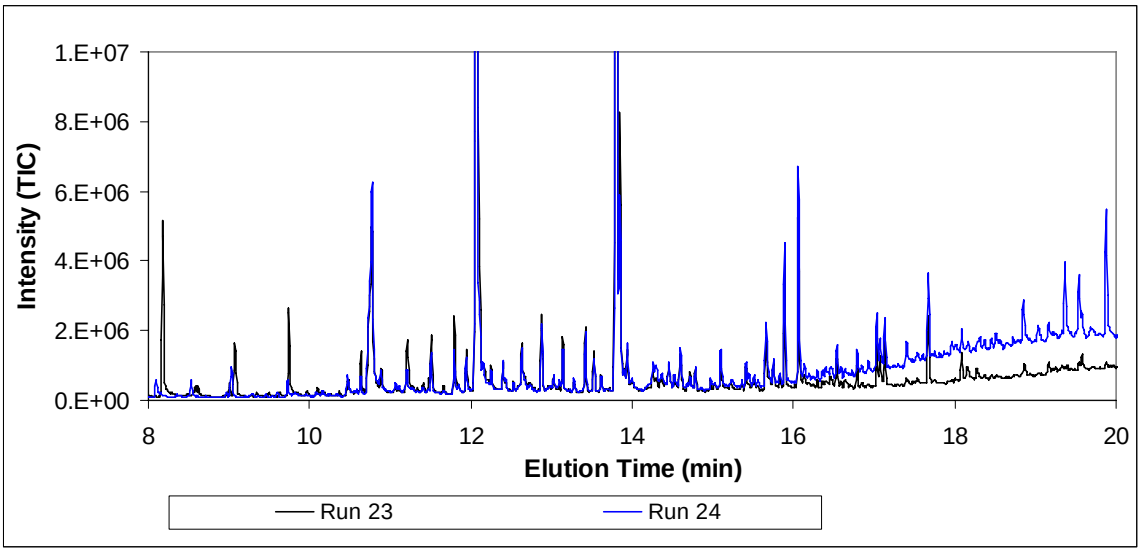


Figure 26. Overlaid chromatograms of spores pyrolyzed @ 250 °C.

Overlaid chromatograms of two nanocluster experiments conducted under the same conditions are presented in Figure 27. Again, visible reproducibility is shown.

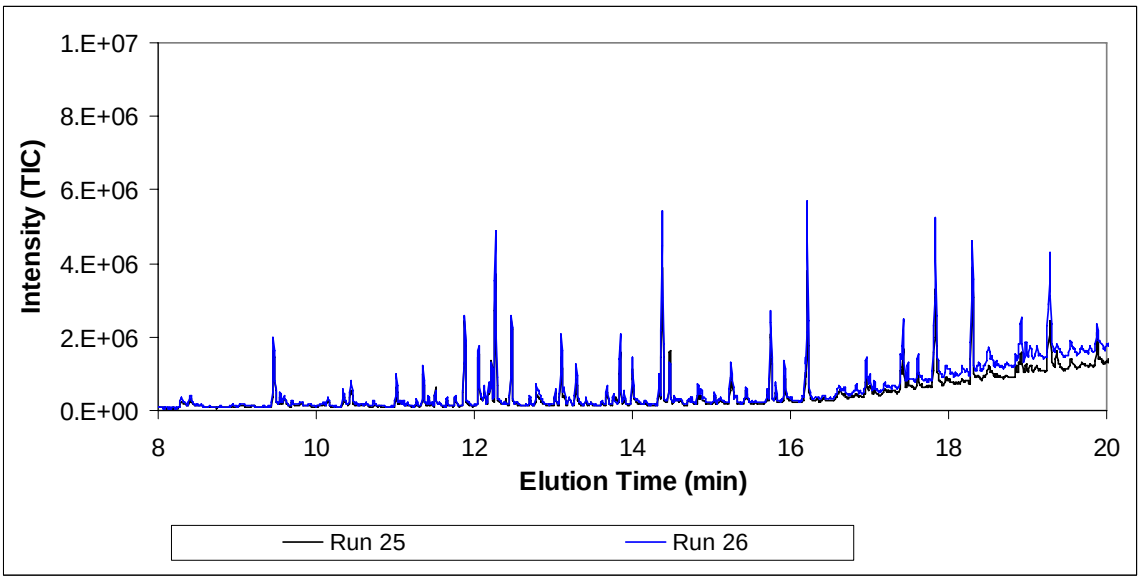


Figure 27. Overlaid chromatograms of Pt-nanoclusters pyrolyzed @ 250 °C.

Overlaid chromatograms (Runs 27, 28, and 29) of the mixture of spores and nanoclusters, prepared and heated under the same conditions, are presented in Figure 28.

The RAMFAC algorithm was used to search these three chromatograms for pyrrolidine and furfuryl alcohol, which are two important pyrolysis products produced from protein and DNA, respectively. The calculated amounts of pyrrolidine for Runs 27, 28, and 29 were 4.00E+6, 4.72E+5, and 5.55E+5, respectively, and a standard deviation of 2.01E+6. Furfuryl alcohol was detected only in run 27, with an amount of 6.43E+5. Picolinic acid was not found in any of the chromatograms.

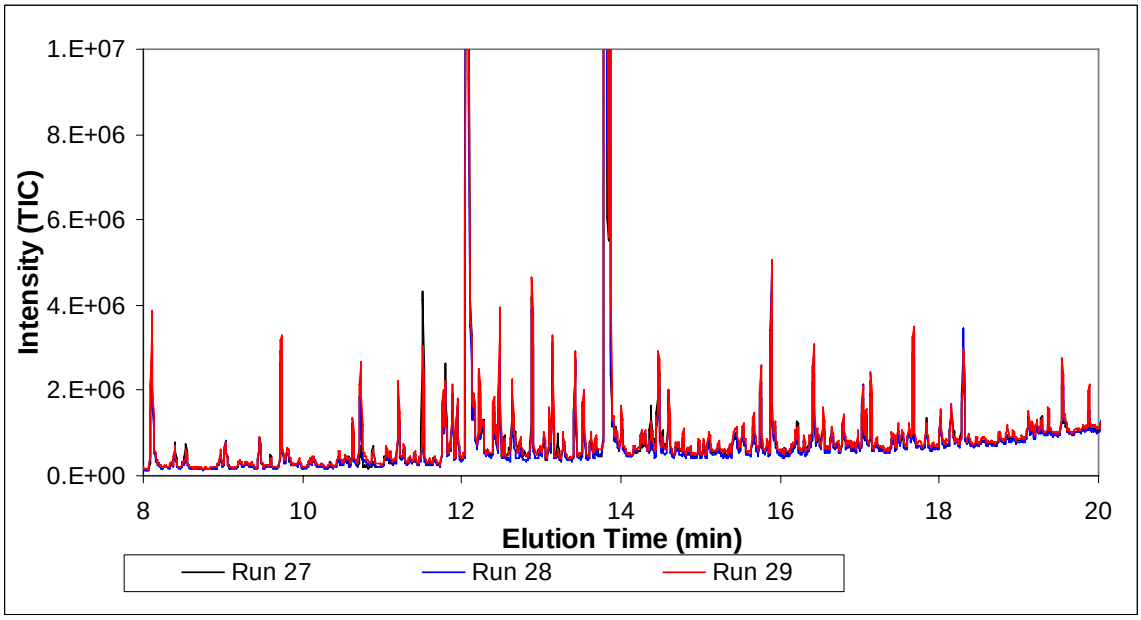


Figure 28. Overlaid chromatograms of mixtures of spores and Pt-nanoclusters pyrolyzed @ 250 °C.

Overlaying the chromatograms of reactions of just spores, just nanoclusters, and their mixture, under comparable conditions (Py @ 250 °C and identical loadings) produces a complicated series of peaks that is difficult to interpret (see Figure 29). Nevertheless, a careful visual analysis enables several important differences to be detected. For example, peak area increases are observed, as compared to the spore-alone chromatogram, at 8.1, 8.4, 9.5, 9.7, 9.8, 11.2, 11.6, 11.9, 12.9, 13.1, 13.6, and 16.5 min.

Peak area losses are also observed, as compared to the spore-alone chromatogram, at 10.8, 15.8, and 15.9. Also, peaks at 9.0 and 16.1 disappear, while a peak at 18.3 appears. These observations indicate that the Pt-nanoclusters influence the decomposition of the spores and that further investigation is merited.

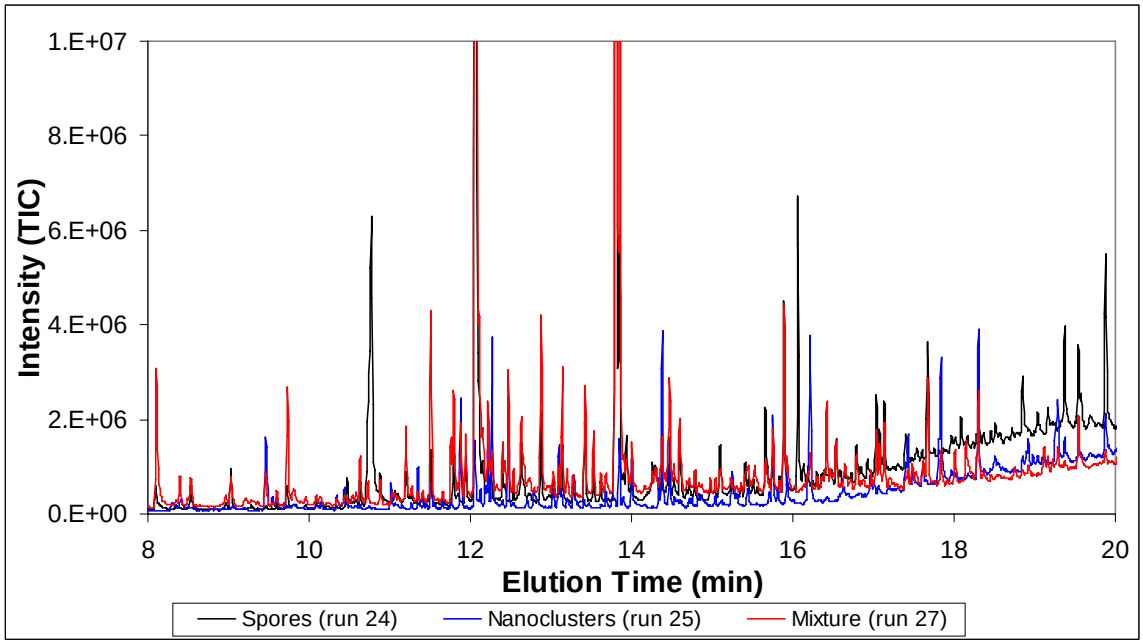


Figure 29. Overlaid chromatograms of spores, Pt-nanoclusters, and their mixture; Pyrolysis @ 250 °C.

A statistically-designed experimental plan was prepared (see description in Chapter 4); a random run order was used for sample preparation. The characteristics of the statistically designed study are listed in Table 15, which shows that Factorial Design Number 1 tested just spores, Number 2 tested just nanoclusters, and Number 3 tested a mixture of spores and nanoclusters. The experiments were not replicated.

Representative chromatograms of the various combinations of the variables in each of the factorial designs are shown in Figures 30-35. The most visible effect on the chromatograms is that of temperature; at higher temperatures many more chemicals are

detected (i.e., increases in both the number of and intensity of the peaks). Visually, the chromatograms obtained for just spores and just nanoclusters are significantly different, as expected. The chromatograms obtained from the mixtures were very complex, and it was difficult to distinguish all significant chemical differences without use of the RAMFAC algorithm.

Table 15. Characteristics of the statistically designed study.

Factorial Design #	Run #	Run Order	Temperature	Spore Loading (µg)	Nanocluster Loading (µg)
1	45	1	300	20	0
	47	3	250	10	0
	48	4	300	10	0
	46	2	250	20	0
2	49	1	300	0	0.024
	50	2	300	0	0.012
	52	4	250	0	0.012
	51	3	250	0	0.024
3	60	8	300	10	0.012
	54	2	300	10	0.024
	55	3	300	20	0.012
	53	1	250	20	0.024
	56	4	250	20	0.012
	57	5	250	10	0.012
	58	6	250	10	0.024
	59	7	300	20	0.024

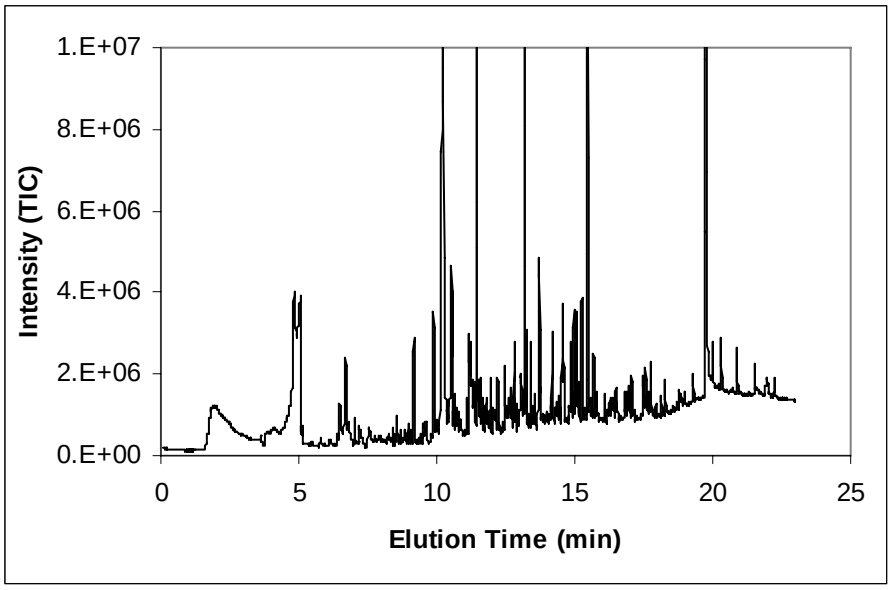


Figure 30. Chromatogram of anthrax spores (20 μg pyrolyzed @ 300 °C, run 45).

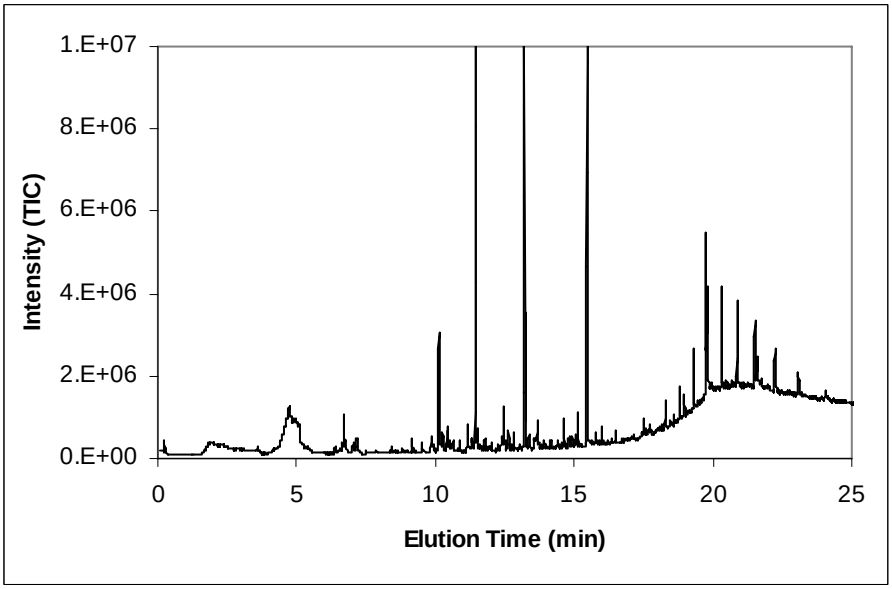


Figure 31. Chromatogram of anthrax spores (20 μg pyrolyzed @ 250 °C, run 46).

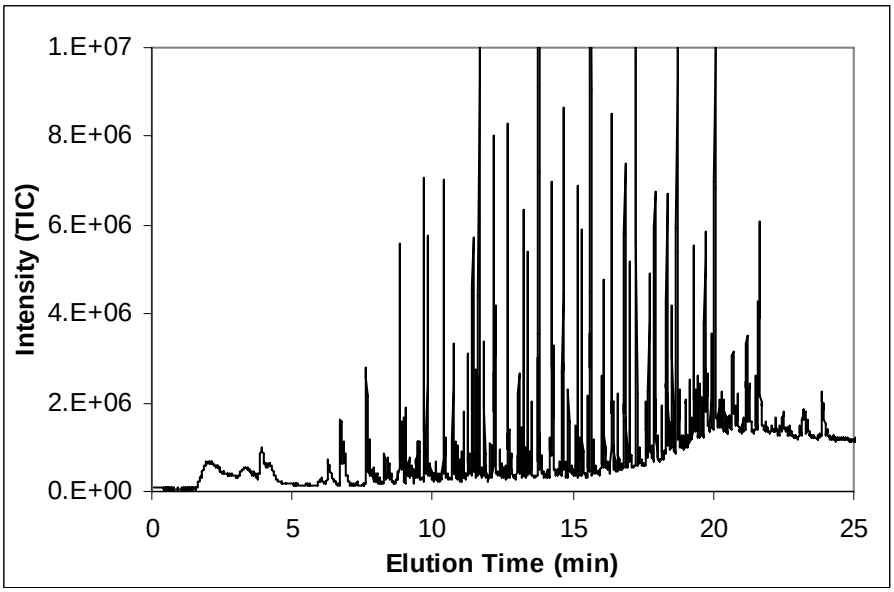


Figure 32. Chromatogram of Pt-nanoclusters (0.024 μg pyrolyzed @ 300 °C, run 49).

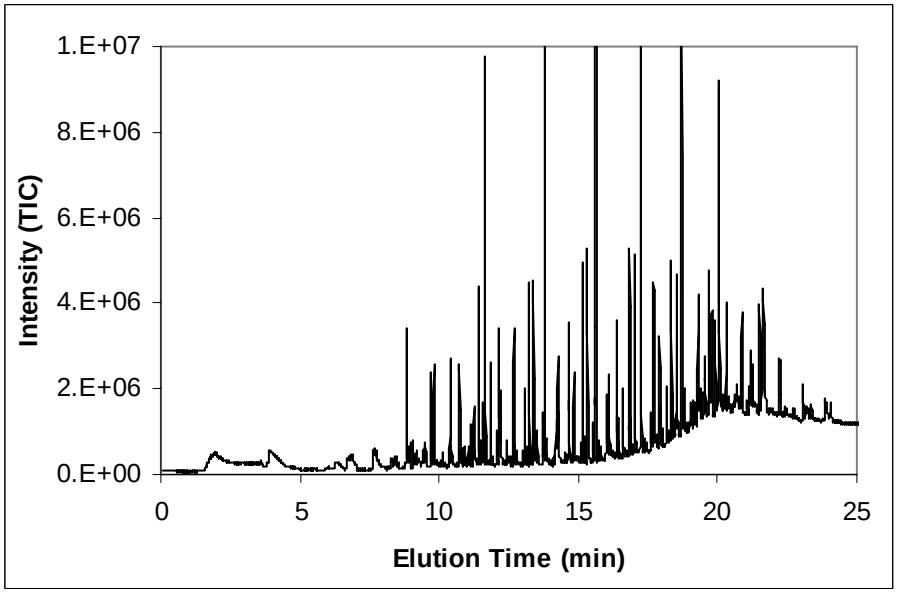


Figure 33. Chromatogram of Pt-nanoclusters (0.024 μg pyrolyzed @ 250 °C, run 51).

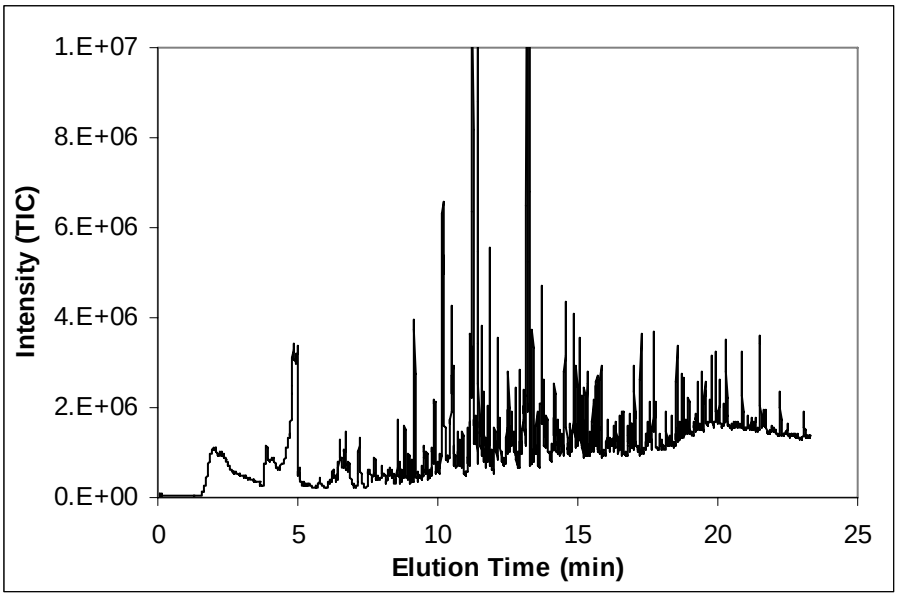


Figure 34. Chromatogram of anthrax spores and nanoclusters (20 & 0.024 µg, respectively, pyrolyzed @ 300 °C, run 59).

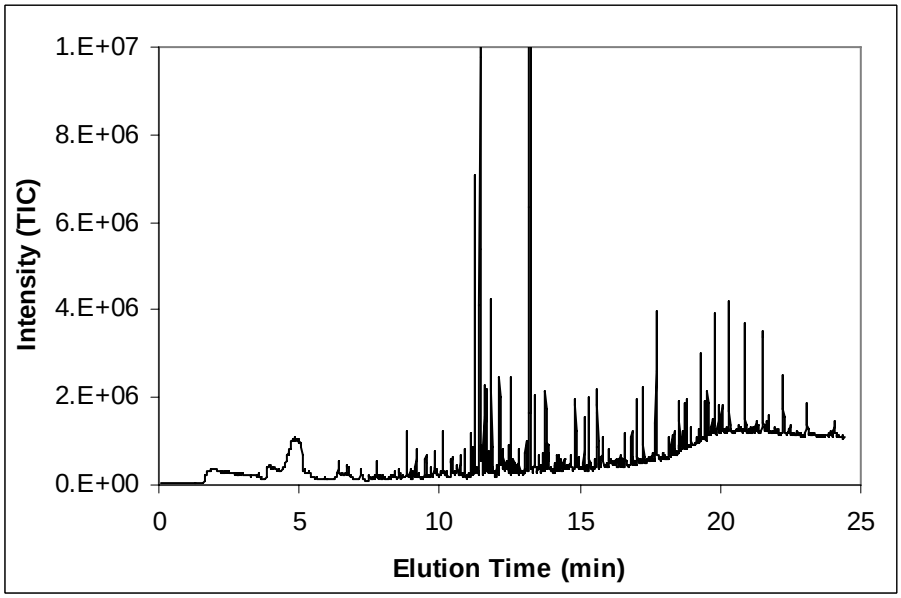


Figure 35. Chromatogram of anthrax spores and nanoclusters (20 & 0.024 µg, respectively, pyrolyzed @ 250 °C, run 53).

The chemical identities of the peaks in these chromatograms were obtained by comparing the mass spectral data with the biomarker library described in Chapter 4 (see Appendix A) using the RAMFAC algorithm. The chemical identities of representative

biomarkers are listed in Table 16. The observation of compounds from the spore protoplast such as picolinic acid (from DPA decomposition – Compound 36), DNA bases (from DNA decomposition – Compounds 17, 18, and 24)), and furfuryl alcohol (from DNA decomposition – Compound 21) suggests that the spores were broken open during the experiments.

Table 16. Representative biomarkers observed in the nanocluster catalytic study.

#	Name	Precursor	Runs 45-49 (just spores)	Runs 53-60 (nanocluster-spore mixture)
1	Glutamine	Protein	Yes	Yes
2	Pyrrolidine		Yes	Yes
3	Butanoic acid, 3-hydroxy (crotonic acid)	PHB	Yes	Yes
4	Picolinamide	Protein, DPA	Yes	Yes
5	C13:0	Lipid	No	Yes
6	Isocrotonic acid	PHB	Yes	Yes
7	*p*-Cresol	Protein	Yes	Yes
8	1-Butene, 3-methyl	Protein	Yes	Yes
9	C12:0	Lipid	Yes	Yes
10	Acetaldehyde	Protein	Yes	Yes
11	Acetone	PHB	Yes	Yes
12	Butanal, 3-methyl	Protein	Yes	Yes
13	Propanenitrile, 2-methyl	Protein	No	Yes
14	Pyridine	Protein, DPA	No	Yes
15	Indole	Protein (tryptophan)	Yes	Yes
16	Imidazole	Protein	Yes	Yes
17	Guanine	DNA	Yes	Yes
18	Thymine	DNA	Yes	Yes
19	Isoleucine	Protein	Yes	No
20	Butanenitrile	Protein	Yes	Yes
21	Furfuryl alcohol	DNA	Yes	Yes
22	2,5-Pyrrolidinedione	Protein	Yes	Yes
23	Propionamide	Peptidoglycan	Yes	Yes
24	Adenine	DNA	Yes	Yes
25	Acetamide	Peptidoglycan	Yes	Yes
26	3-Methylindole	Protein	Yes	Yes
27	1-Butene, 2-methyl	Protein	Yes	Yes
28	Proline	Protein	Yes	Yes
29	Tyrosine	Protein	Yes	Yes
30	4-Hydroxy-phenylethylamine	Protein (tyrosine)	Yes	Yes
31	Maleimide	Protein	Yes	Yes
32	2,5-Diketopiperazine	Protein	Yes	Yes
33	Butanenitrile, 2-methyl	Protein	Yes	Yes
34	Benzene propanenitrile	Protein	Yes	Yes
35	1-Tridecene[a]	Protein	Yes	Yes

Table 16 Continued.

#	Name	Precursor	Runs 45-49 (just spores)	Runs 53-60 (nanocluster-spore mixture)
36	Picolinic acid	DPA	No	Yes
37	Muramic acid[a]	Peptidoglycan	Yes	Yes
38	Asparagine	Protein	Yes	Yes
39	Pro-Gly-DKP[b]	Protein	Yes	Yes
40	Threonine	Protein	Yes	Yes
41	1-Butaneamine-2-methyl-N-(2-methylbutylidene)	Protein	No	Yes
42	Dodecanal	Lipid	No	Yes
43	1-Propene	PHB	No	Yes
44	Ketene	PHB	Yes	Yes
45	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro	Protein	Yes	Yes

[a] Also observed in the nanoclusters; [b] Diketopiperazine

Figure 36 shows many of these biomarkers' peak locations in the chromatogram of run 59, which is a mixture of spores and nanoclusters pyrolyzed at 300 °C. Also, Figure 37 shows biomarkers' locations in the chromatogram of run 45, which was the pyrolysis of just spores at 300 °C. It is evident that the identified biomarkers constitute a minority of the peaks in both chromatograms.

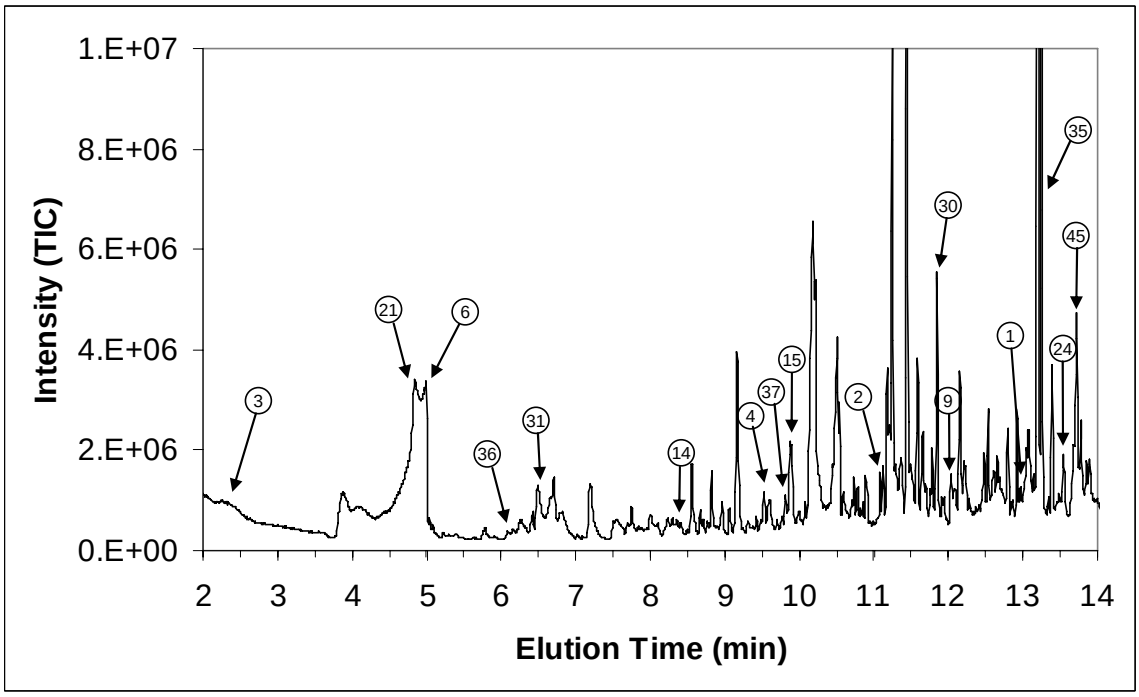


Figure 36. Chromatogram of run 59 (20 μg spores, 0.024 μg nanoclusters, pyrolysis @ 300 °C, see Figure 34 for full chromatogram); peak labels correspond to Table 16.

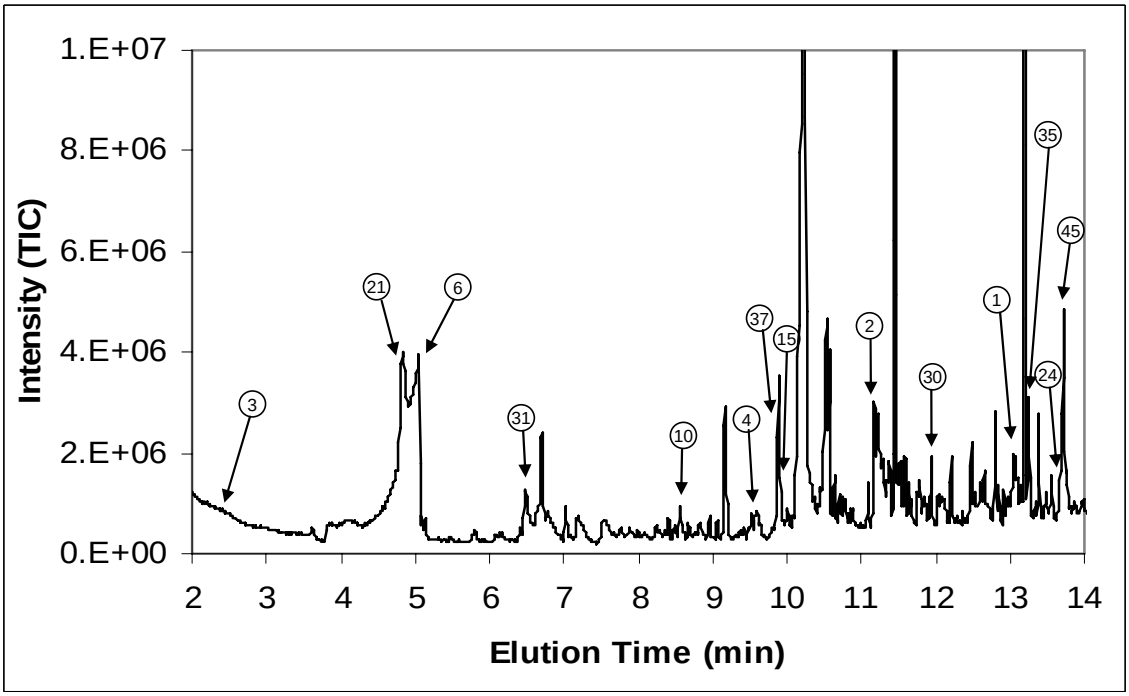


Figure 37. Chromatogram of run 45 (20 μg spores, pyrolysis @ 300 °C, see Figure 30 for full chromatogram); peak labels correspond to Table 16.

Furfuryl alcohol (Compound 21 from Table 16) was selected as a response factor, based on its presence in each of the experimental runs involving spores and its physical importance as a measure of spore break down (it comes from DNA, present in the core of the spore). Pyrrolidine (Compound 2 from Table 16) was chosen as a second response factor because it is also present in every run involving spores, although its physical meaning is less pronounced. Picolinic acid (Compound 36 from Table 16) was chosen as a third response factor, because of its presence in some of the experimental runs, and because of its physical importance as a measure of spore break down. The response factor amounts used in analysis of the factorial study are listed in Table 17; these amounts are the direct output of the RAMFAC algorithm, and while lacking physical units, are comparable between runs. Figures 38-40 present the amounts graphically for each response factor.

Table 17. Response factor values used in regression analysis of the factorial studies.

Run #	Furfuryl Alcohol Amount[a]	Pyrrolidine Amount[a]	Picolinic acid Amount[a]	Temp (°C)	Spore Loading (µg)	Nanocluster Loading (µg)
45	1.5E+07	0	0	300	20	0
46	1.2E+07	4.6E+06	0	250	20	0
47	1.0E+07	3.0E+06	0	250	10	0
48	8.4E+06	3.5E+07	0	300	10	0
49	0	0	0	300	0	0.024
50	0	0	0	300	0	0.012
51	0	0	0	250	0	0.024
52	0	0	0	250	0	0.012
53	7.3E+06	2.9E+06	0	250	20	0.024
54	4.4E+06	1.3E+08	7.0E+05	300	10	0.024
55	1.2E+07	3.1E+06	1.3E+06	300	20	0.012
56	1.0E+07	2.7E+06	0	250	20	0.012
57	7.7E+06	2.0E+06	0	250	10	0.012
58	6.7E+06	2.7E+07	0	250	10	0.024
59	8.7E+06	6.4E+07	1.2E+06	300	20	0.024
60	7.9E+06	2.9E+06	1.4E+06	300	10	0.012

a Determined by the RAMFAC algorithm (see Appendix F)

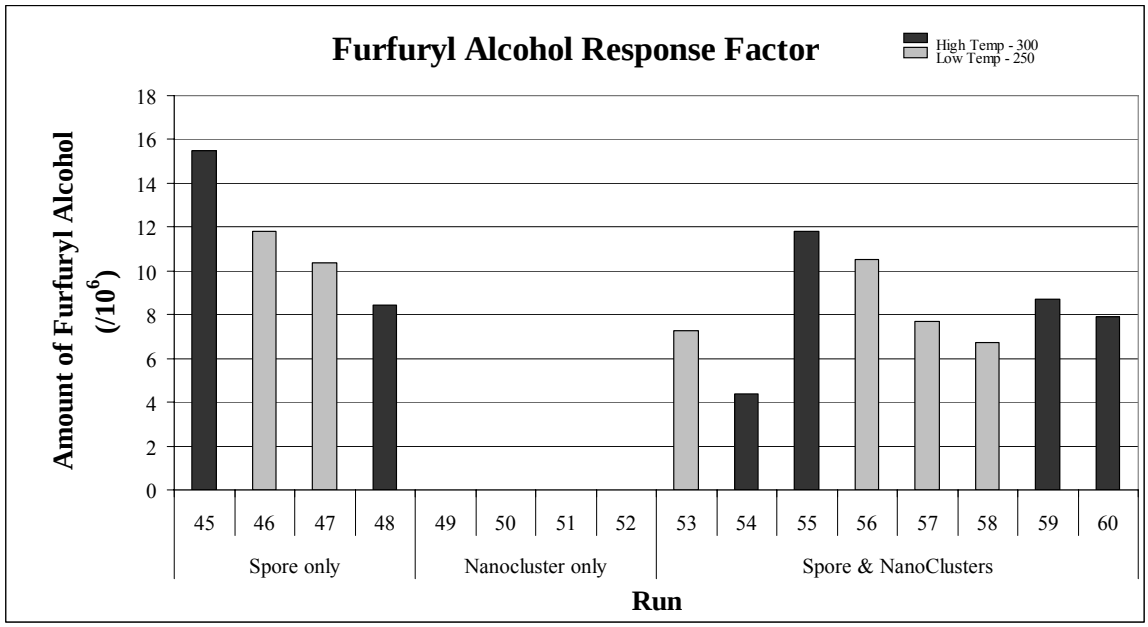


Figure 38. Amounts of the furfuryl alcohol response factor.

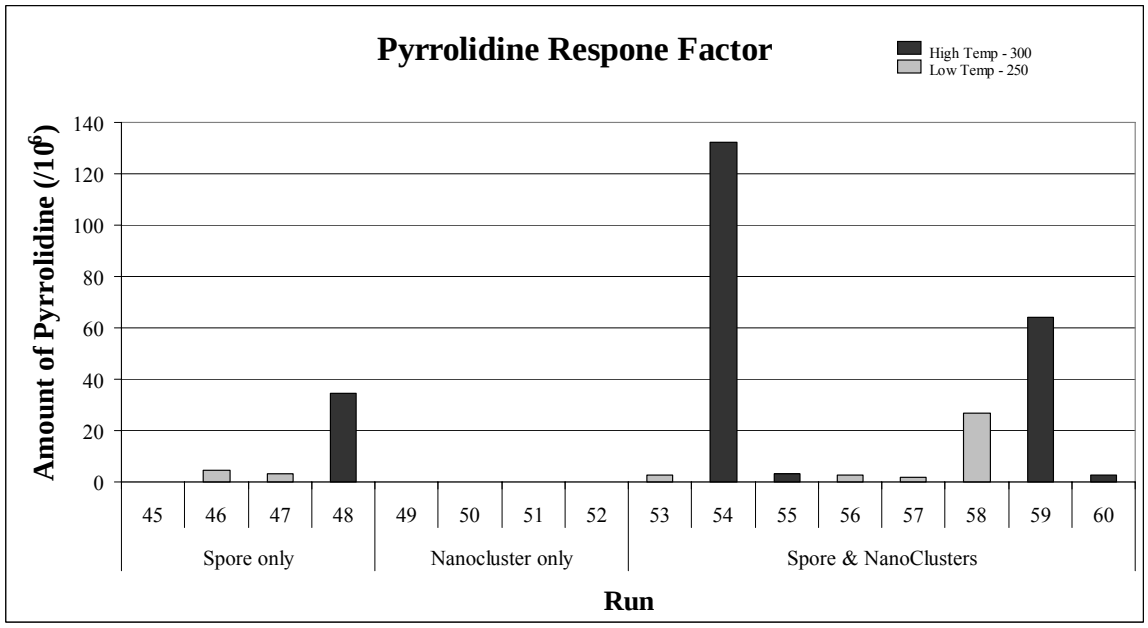


Figure 39. Amounts of the pyrrolidine response factor.

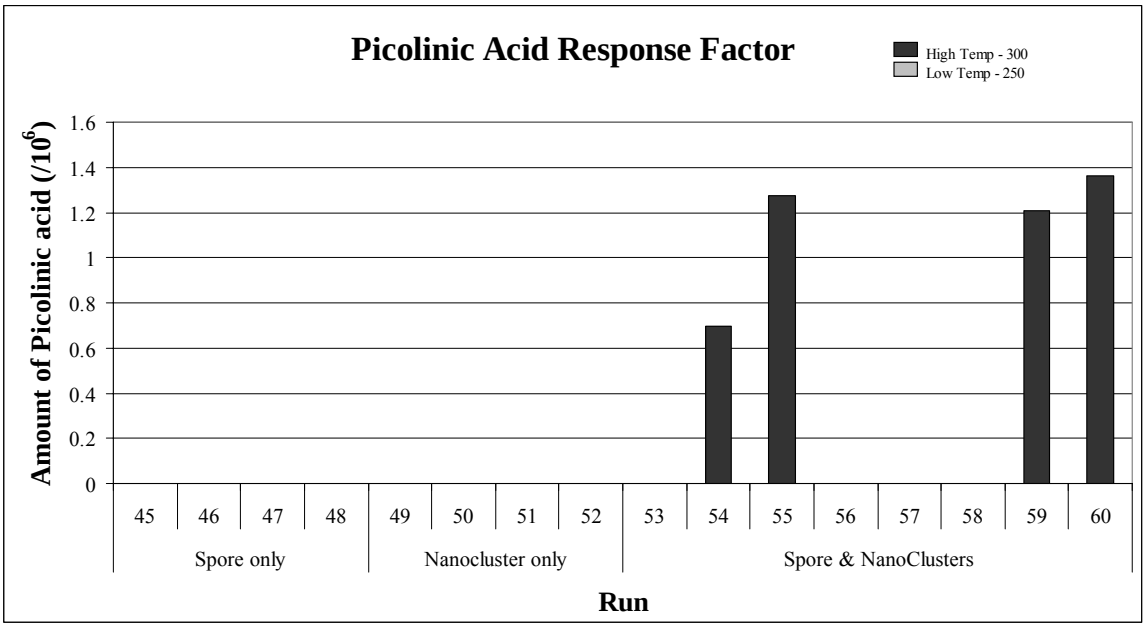


Figure 40. Amounts of the picolinic acid response factor.

In order to determine the effect of the three variables studied, statistical analysis was required. Because the experiments were not replicated at each level of the variables, it was not possible to determine the standard deviation and a factorial analysis was not possible. Instead, a statistical regression (similar to ANOVA) was done with the data in Table 17, and the results are presented in Table 18. There are statistically significant results in each of the response variable models. Specifically, spore loading is significant at $\alpha = 0.05$ when regressed linearly for pyrrolidine, but is significant for furfuryl alcohol when regressed quadratically; both are significant at $\alpha = 0.05$ for picolinic acid. Additionally, nanocluster loading is not significant at $\alpha = 0.10$ for pyrrolidine, but is significant at $\alpha = 0.05$ for furfuryl alcohol. Temperature is only significant at $\alpha = 0.05$ for picolinic acid; at $\alpha = 0.10$ it becomes significant for furfuryl alcohol.

Table 18. Statistical regression of factorial experiments.

Response Factor	Variable	P value[a]
Pyrrolidine	Temperature	0.0912*
	Spore loading (linear)	0.0684*
	Spore loading (quadratic)	0.0286**
	Nanocluster loading (linear)	0.2108
	Nanocluster loading (quadratic)	0.8760
Furfuryl alcohol	Temperature	0.67836
	Spore loading (linear)	1.15E-6**
	Spore loading (quadratic)	0.06190**
	Nanocluster loading (linear)	0.00105**
	Nanocluster loading (quadratic)	0.64849
Picolinic acid	Temperature	0.0150**
	Spore loading (linear)	0.0441**
	Spore loading (quadratic)	0.02877**
	Nanocluster loading (linear)	0.0822*
	Nanocluster loading	0.0969*

[a] For a value to be significant, the p-value must be less than 0.10 (for 90% confidence) indicated by '*' or 0. 05 (for 95% confidence) indicated by '**'

Discussion

Experiments with a nickel electroformed mesh showed that contact of a catalytic surface with the spores is an important issue in the development of a catalytic method for production of biomarkers from spores. A temperature of 400 °C for pyrolysis of the spore-containing mesh was required to observe detectable quantities of biomarkers, while only 250 °C was required for spores dried in the sample cup (without the Ni-mesh). This could be the result of poor contact of the majority of the spores with the nickel surface due to the window-paning effect, which may have also lowered heat transfer to the spores. It could also result from the different surfaces – nickel for the mesh and quartz for the sample cups. It is clear that good contact between the spores and the heated surface is required for reactions to occur. The biomarkers detected were likely produced pyrolytically, with little or no enhancement by catalysis. Further studies of spores dried in the deactivated sample cup showed the presence of significant amounts of biomarkers from pyrolysis at lower temperatures (300 °C). Thus, achieving good contact between the

spores and the catalyst is an issue that must be considered in any attempt to catalytically break down the spores.

The TMAH and TPA experiments showed that use of these reagents, after drying in tandem with the spores, created many methylated biomarkers, including several FAMES and both the dimethyl ester of dipicolinic acid and the methyl ester of picolinic acid. This is a significant finding, as these results agree with published literature on the THM of spores. However, as these experimental results were only preliminary, and no investigation of reproducibility was done, further study of the TPA catalyst is recommended.

Experiments using Pt-nanoclusters mixed and dried with anthrax spores produced many biomarkers that matched with the biomarker library (see Table 16). These biomarkers reflect breakdown of many different biological compounds in the spores, including peptidoglycan, lipids, DNA, protein, and polyhydroxybutyric acid (PHB). While many of these same biomarkers are observed in the pyrolysis of spores alone, the amounts of products observed as well as the absence of some larger fragments in the experiments with nanoclusters suggest that the nanoclusters increase the extent and selectivity of the reactions that occur upon heating. Some of the biomarkers in Table 16 appear only with the nanocluster-spore mixture, including pyridine, picolinic acid, and 2-propene. Additionally, some compounds that are present in the pyrolysis of spores were not observed in the mixture – for example, isoleucine. Because pyridine and 2-propene are known end products of protein degradative reactions (see Chapter 2), and isoleucine is an intact amino acid, this information suggests that the nanoclusters catalyze increased breakdown of the spores. Further, picolinic acid was only observed at the high level of

temperature (300 °C), showing that temperature has a significant effect on the breakdown of spores. The experimental data did not provide enough information to firmly establish if the nanoclusters are truly catalytic, or if instead they simply serve another function, such as to enhance the rate of heat transfer to specific locations on the outside of the spores, which enhances the pyrolysis of the spore biochemical compounds. Nevertheless, the results are consistent with a catalytic effect. Observation of picolinic acid, DNA bases, and furfuryl alcohol suggests that the spores were broken open in at least some of the Pt-nanocluster experiments.

These same experiments also produced compounds that did not match with the biomarker library. While it is not possible to estimate the absolute number of compounds due to limitations of the RAMFAC algorithm, it is likely that they fall into three classes: (1) nanocluster ligand pyrolysis products, (2) nanocluster-spore catalysis products that were not identified because they have not been previously reported, and (3) column degradation products. The second class could include catalytically produced biomarkers that would not have been included in the library because they have not previously been reported.

The amounts of chemicals calculated from the RAMFAC algorithm have a large associated standard deviation. Further, in some cases the chemicals were not detected reproducibly, e.g., furfuryl alcohol was detected in Run 27, but not in Runs 28 or 29. It was not possible to determine if (1) these problems arose from the RAMFAC algorithm, or (2) the chemicals were not generated reproducibly during the experiment.

This difficulty in reproducibly detecting the amounts of the chemicals present in the chromatograms in Runs 27, 28, and 29 contrasts with the earlier observation that the

chromatograms were visibly reproducible (see Figure 28). However, as Figure 36 shows, the biomarkers that were identified account for relatively few of the peaks in the chromatogram, highlighting the need for additional work on the biomarker library.

Regression analysis of the amounts of furfuryl alcohol, pyrrolidine, and picolinic acid (the response factors) determined by the RAMFAC algorithm revealed that the controlled experimental variables were found to have varying levels of statistical significance among the three response factors. This may indicate that there are different chemical reaction pathways that generate the three response factors, which depend on the experimental variables in different ways. Temperature and spore loading were found to be significant at $\alpha = 0.05$ for the generation of picolinic acid; the nanocluster loading only became significant at $\alpha = 0.10$. Further experimental work is needed to determine the full effect of the nanoclusters on the spore break down during heating at mild temperatures and at various loadings of spores and nanoclusters.

Chapter 6. Conclusions and Recommendations

In weaponized form, anthrax is a highly potent biological weapon. The use of analytical pyrolysis (AP) to identify biological warfare agents, especially bacterial spores, hinges on the generation and detection of chemicals that contain taxonomical information (biomarkers). A need exists for biomarker-based methods and technologies to rapidly and reproducibly detect anthrax spores. To build the foundation for developing this technology, a comprehensive, critical review of the extensive literature that has been published on the AP production of biomarkers from bacterial spores was presented in Chapter 2. This review (1) addressed current and past methods for the production of biomarkers from bacterial spores (i.e., pyrolysis and THM), (2) enumerated the chemical reactions thought to occur during pyrolysis and THM of spores and relevant biopolymers, (3) summarized relevant literature on the effect of pyrolyzer design on the reactions and biomarkers produced; and (4) critically analyzed the methods and technologies used to produce biomarkers from bacterial spores, showing that no proven method or technology exists for rapid, handheld, reproducible detection of anthrax spores. This literature review and analysis contributes to an understanding of the pyrolytic reactions that occur during the production of biomarkers from bacterial spores, providing the first substantive review of this topic in the published literature.

A biomarker library was created based on the literature review and analysis presented in Chapter 2. The compounds in this library are listed in Appendix A. This

library was successfully used to identify the chemical identities of many compounds present in the chromatograms produced in the experimental work with spores and various catalysts. However, there were still many peaks that were not identified, showing that the library is not complete. This less than complete collection is explained by a lack of available mass spectra for (1) compounds identified in the literature as being produced during the AP of spores, (2) compounds not specifically identified in the literature that might come from the AP of spores, and (3) compounds that might be catalytically produced from spores. When finished, this library should have utility in many other areas of current research in the production of biomarkers from bacterial spores, and will constitute a significant contribution to the scientific literature.

The use of a catalyst to produce the required biomarkers from bacterial spores more rapidly and under milder conditions shows promise for fulfilling portability and reproducibility requirements for a handheld biological weapons detector. This conclusion is supported by preliminary data under relatively mild conditions on Pt-nanoclusters and superacid catalysts showing significant production of biomarkers from bacterial spores. The results were analyzed, interpreted, and compared with the comprehensive review.

On the other hand, the course nickel mesh had very little catalytic effect on the pyrolysis of spores and, in fact, may have hindered pyrolysis by reducing heat transfer to the spores due to the window-pane loading of the spores on the mesh.

Experiments showed that TMAH and TPA, together with anthrax spores, could produce methyl esters of fatty acids, and of dipicolinic and picolinic acids at relatively mild conditions, which is suggestive of a catalytic role.

The production of biomarkers from spores at mild temperatures was especially effective in the presence of Pt-nanoclusters; the degradation of protein, peptidoglycan, and DNA in particular appeared to be enhanced by the addition of the Pt-nanoclusters. The extent to which this was a catalytic effect or augmented heat transfer to specific locations on the spores surface was not elucidated.

Results of statistically-designed factorial studies showed that temperature and loading of spores and nanoclusters influenced the production of biomarkers, although the effects were dependant on the specific biomarker used as a response factor. The biomarker amount, as calculated by the RAMFAC algorithm, was found to have a relatively large standard deviation. Further, chemicals were not reproducibly identified as being present in the chromatograms of samples prepared and run under what were presumably identical conditions. The reasons for these reproducibility problems were not elucidated, but may include errors made by the RAMFAC algorithm, non-reproducible reactions taking place during the experiments, or the presence of large amounts of background spectra (i.e., noise) in the data.

It is recommended that the experimental work with the Pt-nanoclusters be pursued further. Increasing Pt-concentration and decreasing ligand concentration should be explored. Additionally, comparison of this work with experiments with Ni-nanoclusters could identify differences in the chemical reactions catalyzed by the two metals. It is likely that Ni-nanoclusters would exhibit more carbon-carbon bond breaking activity than platinum. Further studies might be done on other forms of the nickel mesh such as materials with higher surface area-to-volume ratios since this would lead to better contact with the spores.

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Appendices

Appendix A: Biomarker Library

A biomarker mass spectral library was created, using information available in the scientific literature (see Chapter 2) and the NIST library of mass spectral data. The following table shows all of the compounds that were entered into the library and their respective chemical abstract services (CAS) number, as well the compounds for which mass spectral data was available (marked with "*").

#	Name	CAS #
1	Glutamate – 1*	56860
2	PAME (2-Pyridinecarboxylic acid, methyl ester)*	204359
3	Glutamate – 2*	617652
4	iC16:0 (Pentadecanoic acid, 14-methyl-)	4669-02-7
5	Glutamine*	6899-04-3
6	C15:0 (pentadecanoic acid)*	1002-84-2
7	Ethylbenzene*	100-41-4
8	Ethenylbenzene*	100-42-5
9	Phenylacetonitrile (benzonitrile)*	100-47-0
10	Benzene-acetaldehyde (benzaldehyde)*	100-52-7
11	4-methylphenol (p-Cresol)*	106-44-5
12	2,5-Diketopiperazine*	106-57-0
13	3-Methylbutylamine*	107-85-7
14	Toluene*	108-88-3
15	Phenol*	108-95-2
16	Butanenitrile*	109-74-0
17	1H-Pyrrole*	109-97-7
18	C10:0 ME (Decanoic acid, methyl ester)*	110-42-9
19	Pyridine*	110-86-1
20	Muramic acid – 1*	1114-41-6
21	C12:0 ME (Dodecanoic acid, methyl ester)*	111-82-0
22	C16:1 w8c ME (9-Hexadecenoic acid, methyl ester,)*	1120-25-8
23	C20:0 ME (Eicosanoic acid, methyl ester)*	1120-28-1
24	C16:0 ME (Hexadecanoic acid, methyl ester)*	112-39-0
25	Dodecanal*	112-54-9
26	C18:0 ME (Octadecanoic acid, methyl ester) *	112-61-8
27	C18:1 w10c ME (9-Octadecenoic acid (9Z)-, methyl ester)*	112-62-9
28	Aziridinone, 3-	113702-17-3
29	2,5-Pyrazinedione, 1,6-dihydro-6-(1-methylethyl)-	113702-18-4
30	4-Imidazolidinone, 2,5-bis(1-methylethyl)-	113702-19-5
31	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-2-methyl-3-(1-methylethyl)-	113702-21-9
32	1-Azabicyclo[3.2.0]heptan-7-one, 6-methyl- (9CI)	113702-23-1
33	3H-Pyrrolo[1,2-a]imidazol-3-one, 1,2,7,7a-tetrahydro-2-methyl-	113702-24-2
34	Pyrrolo[1,2-a]pyrazine-1,4-dione, 2,3,8,8a-tetrahydro-3-methyl-	114311-01-2
35	Pyrrolo[1,2-a]pyrazine-1,4-dione, 2,3,8,8a-tetrahydro-3-(1-methylethyl)-	114311-02-3
36	3H-Pyrrolo[1,2-a]imidazol-3-one, hexahydro-2-(1-methylethyl)- (9CI)	114311-03-4
37	3H-Pyrrolo[1,2-a]imidazol-3-one, 1,2,7,7a-tetrahydro-2-(1-methylethyl)- (9CI)	114311-04-5
38	1-Propene*	115-07-1
39	1-Propene, 2-methyl-*	115-11-7

40	Indole*	120-72-9
41	Benzene-acetaldehyde*	122-78-1
42	4-Ethylphenol*	123-07-9
43	2,5-Pyrrolidinedione (Succinimide)*	123-56-8
44	Pyrrolidine*	123-75-1
45	C14:0 ME (Tetradecanoic acid, methyl ester,)*	124-10-7
46	Carbon dioxide*	124-38-9
47	C18:1 w9t ME (10-Octadecenoic acid, methyl ester, (E)-) *	13038-45-4
48	aC15:0 (Tetradecanoic acid, 12-methyl-)	135096-46-7
49	Benzeneacetonitrile*	140-29-4
50	*n*-Dodecanoic acid (lauric acid)*	143-07-7
51	2-Pyridine-carboxyamide (picolinamide) *	1452-77-3
52	3-Ethylindole	1484-19-1
53	Phenylalanine*	150-30-1
54	2-Ethylpyrrole*	1551-06-0
55	iC17:0 (Hexadecanoic acid, 15-methyl-)	1603-03-8
56	C16:1 w5c ME (12-Hexadecenoic acid, methyl ester,)	170932-92-0
57	2-Butenoic acid, 2-carboxy-1-methylethyl ester	172471-84-0
58	C18:1 w9c ME (10-Octadecenoic acid, methyl ester, (Z)-)	17257-43-1
59	C11:0 ME (Undecanoic acid, methyl ester)*	1731-86-8
60	C13:0 ME (Tridecanoic acid, methyl ester)*	1731-88-0
61	C17:0 ME (Heptadecanoic acid, methyl ester)*	1731-92-6
62	C19:0 ME (Nonadecanoic acid, methyl ester)*	1731-94-8
63	3-Amino-2-piperidone	1892-22-4
64	Butanenitrile, 2-methyl-*	18936-17-9
65	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-*	19179-12-5
66	1-Acetyl-1,2,3,4-tetrahydropyridine*	19615-27-1
67	PHB trimer (Crotonic acid, ester with 3-hydroxybutyric acid bimol. ester)	22128-60-5
68	PHB tetramer (2-Butenoic acid, 3-[3-(2-carboxy-1-methylethoxy)-1-methyl-3-oxopropoxy]-1-methyl-3-oxopropyl ester)	22128-61-6
69	2-Methylthiazolidine*	24050-16-6
70	C23:0 ME (Tricosanoic acid, methyl ester)*	2433-97-8
71	1-Tridecene*	2437-56-1
72	C24:0 ME (Tetracosanoic acid, methyl ester)*	2442-49-1
73	iC15:0 (Tetradecanoic acid, 13-methyl-)	2485-71-4
74	aC17:0 ME (Hexadecanoic acid, 14-methyl-, methyl ester)*	2490-49-5
75	Phenol, 4-ethenyl-	2628-17-3
76	iC18:0 (Heptadecanoic acid, 16-methyl-)	2724-58-5
77	iC19:0 (Octadecanoic acid, 17-methyl-)	2724-59-6
78	C18:1 w7c ME (12-Octadecenoic acid, methyl ester, (12Z)-)	2733-86-0
79	C18:1 w12t ME (7-Octadecenoic acid, methyl ester, (E)-)	28010-28-8
80	Pyrroline	28350-87-0
81	C14:0 3-OH (Tetradecanoic acid, 3-hydroxy-)	28715-21-1
82	1H-Imidazole*	288-32-4
83	Butanoic acid, 3-hydroxy-*	300-85-6
84	Alanine*	302-72-7
85	Serine*	302-84-1
86	β-butyrolactone (2-Oxetanone, 4-methyl-)*	3068-88-0
87	Asparagine*	3130-87-8
88	Hexahydropyrrolizin-3-one*	32548-24-6
89	Leucine*	328-39-2
90	Cysteine*	3374-22-9
91	1-Butanamine-3-methyl-N-(3-methylbutylidene)*	35448-31-8
92	Crotonic acid (2-Butenoic acid)*	3724-65-0
93	Diphenylethane	38888-98-1
94	3-Methylthio-1-propylamine*	4104-45-4
95	Isoleucine*	443-79-8
96	1H-Pyrrole-2-carbonitrile	4513-94-4
97	Ketene (Ethenone)*	463-51-4
98	Muramic acid – 2	484-57-1
99	5H,10H-Dipyrrolo[1,2-a:1',2'-d]pyrazine-5,10-dione	484-73-1
100	Dipicolinic acid (DPA)*	499-83-2
101	Histidine*	4998-57-6
102	Isocrotonic acid (2-Butenoic acid, (2Z)-)*	503-64-0
103	C17:0 (Heptadecanoic acid)*	506-12-7
104	2,5-Piperazinedione, 1,6-dimethyl-	50627-39-9
105	C12:0 2-OH ME (Dodecanoic acid, 2-hydroxy-, methyl ester)*	51067-85-7
106	iC12:0 ME (Undecanoic acid, 10-methyl-, methyl ester)*	5129-56-6
107	iC13:0 ME (Dodecanoic acid, 11-methyl-, methyl ester)	5129-57-7
108	iC14:0 ME (Tridecanoic acid, 12-methyl-, methyl ester)*	5129-58-8

109	iC15:0 ME (13-Methyltetradecanoic acid methyl ester)	5129-59-9
110	iC16:0 ME (Pentadecanoic acid, 14-methyl-, methyl ester)*	5129-60-2
111	iC18:0 (Heptadecanoic acid, 16-methyl-, methyl ester)*	5129-61-3
112	aC13:0 ME (Dodecanoic acid, 10-methyl-, methyl ester)*	5129-65-7
113	aC15:0 ME (Tetradecanoic acid, 12-methyl-, methyl ester)*	5129-66-8
114	Valine*	516-06-3
115	4-Hydroxyphenethylamine*	51-67-2
116	C16:0 3-OH ME (Hexadecanoic acid, 3-hydroxy-, methyl ester)*	51883-36-4
117	Benzene-ethane-amine	525584-89-8
118	1H-Indole, 3-ethenyl-	53654-36-7
119	Pyrrolidine, 1-(2-amino-3-methyl-1-oxobutyl)-	54124-67-3
120	Tryptophan*	54-12-6
121	Maleimide*	541-59-3
122	C14:0 (tetradecanoic acid or myristic acid)*	544-63-8
123	1-Butanamine-2-methyl-N-(2-methylbutylidene)*	54518-97-7
124	DPAME (2,6-Pyridinedicarboxylic acid, dimethyl ester)*	5453-67-8
125	1H-Indole-3-carbonitrile*	5457-28-3
126	iC19:0 ME (Octadecanoic acid, 17-methyl-, methyl ester)*	55124-97-5
127	C25:0 ME (Pentacosanoic acid, methyl ester)*	55373-89-2
128	Tyrosine*	556-03-6
129	C14:0 3-OH ME (Tetradecanoic acid, 3-hydroxy-, methyl ester)*	55682-83-2
130	2,5-Piperazinedione, 3,6-bis(1-methylethyl)- *	5625-44-5
131	1-Butene, 3-methyl-*	563-45-1
132	1-Butene, 2-methyl-*	563-46-2
133	Propanamide, 2-methyl- (isobutyramide)*	563-83-7
134	Glycine*	56-40-6
135	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(1-methylethyl)-	5654-87-5
136	C16:0 (Hexadecanoic acid)*	57-10-3
137	C18:0 (Octadecanoic acid)*	57-11-4
138	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-2,3-dimethyl-	57224-38-1
139	2H-Pyrrole, 3,4-dihydro- (1-pyrroline)	5724-81-2
140	C26:0 ME (Hexacosanoic acid, methyl ester)*	5802-82-4
141	3H-Indol-3-one, 1,2,4,5,6,7-hexahydro-*	58074-25-2
142	Butanal, 3-methyl-*	590-86-3
143	aC17:0 (Hexadecanoic acid, 14-methyl-)	5918-29-6
144	Methyl undecyl ketone (2-tridecanone)*	593-08-8
145	*N*-Acetylmuramyl-L-alanyl-L-isoglutamine	59331-38-3
146	Methionine*	59-51-8
147	Nicotinic acid*	59-67-6
148	Acetamide*	60-35-5
149	2,5-Piperazinedione, 3-methyl-	6062-46-0
150	C21:0 ME (Heneicosanoic acid, methyl ester)*	6064-90-0
151	Proline*	609-36-9
152	C16:1 w7c ME (10-Hexadecenoic acid, methyl ester, (Z)-)	61012-44-0
153	1H-Indole-3-ethanamine*	61-54-1
154	2-Pyrrolidinone*	616-45-5
155	Aspartic acid*	617-45-8
156	Glutamic acid*	617-65-2
157	5-methyl-2-furan-carboxyaldehyde (5-methyl furfural)*	620-02-0
158	Butanenitrile, 3-methyl-*	625-28-5
159	C10:0 3-OH ME (Decanoic acid, 3-hydroxy-, methyl ester)	62675-82-5
160	Carbon monoxide*	630-08-0
161	C13:0 (Tridecanoic acid)*	638-53-9
162	C22:1 w13c ME (10-Docosenoic acid, methyl ester)	639820-35-2
163	Benzene propanenitrile*	645-59-0
164	C19:0 (Nonadecanoic acid)*	646-30-0
165	iC20:0 ME (Nonadecanoic acid, 18-methyl-, methyl ester)	65301-91-9
166	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-methyl-	65556-33-4
167	Thymine*	65-71-4
168	2-Propanone (acetone)*	67-64-1
169	1-Propanamine, 2-methyl-N-(2-methylpropylidene)- *	6898-82-4
170	iC17:0 ME (Hexadecanoic acid, 15-methyl-, methyl ester)*	6929-04-0
171	Lysine*	70-54-2
172	1H-Pyrrole-3-carbonitrile*	7126-38-7
173	C10:0 2-OH ME (Decanoic acid, 2-hydroxy-, methyl ester)*	71271-24-4
174	Cytosine*	71-30-7
175	C15:0 ME (Pentadecanoic acid, methyl ester) *	7132-64-1
176	Arginine*	7200-25-1
177	Threonine*	72-19-5
178	C12:0 3-OH ME (Dodecanoic acid, 3-hydroxy-, methyl ester)*	72864-23-4

179	Adenine*	73-24-5
180	Isoleucine*	73-32-5
181	Guanine*	73-40-5
182	Methanethiol*	74-93-1
183	Acetaldehyde*	75-07-0
184	*N*-Acetylglucosamine*	7512-17-6
185	C17:1 w8c ME (10-Heptadecenoic acid, methyl ester)	75190-82-8
186	1H-Indole-3-acetonitrile*	771-51-7
187	2-Methyl-1-propanamine*	78-81-9
188	Propanenitrile, 2-methyl-*	78-82-0
189	Propanal, 2-methyl-*	78-84-2
190	Propionamide*	79-05-0
191	Threonine*	80-68-2
192	3-Methylindole (skatole) *	83-34-1
193	2,3-Dimethylindole*	91-55-4
194	C15:0 2-OH ME (Pentadecanoic acid, 2-hydroxy-, methyl ester)	928-77-8
195	C22:0 ME (Docosanoic acid, methyl ester)*	929-77-1
196	2-Methylbutylamine*	96-15-1
197	Butanal, 2-methyl-*	96-17-3
198	2-Hydroxymethyl-furan*	97-99-4
199	Furfuryl alcohol*	98-00-0
200	2-furan-carboxaldehyde (furfural)*	98-01-1
201	Pyridine-2-carboxylic acid (picolinic acid)*	98-98-6
202	Iso-tridecanenitrile	
203	Diketopiperazine (His-Val)	
204	2-Tetradecenenitrile	
205	C16:1 ME (structure not defined)	
206	cyC17:0 ME (structure not defined)	
207	C18:1 ME (structure not defined)	
208	cyC19:0 ME (structure not defined)	
209	C24:1 ME (structure not defined)	
210	C16:1 w7c alcohol ME (structure not specified)	
211	iC17:1 w10c ME (structure not found)	
212	iC17:1 w5c ME (structure not found)	
213	iC17:1 ME (structure not specified)	
214	cycloC19:0 ME (structure not specified)	
215	C24:1 w15c ME (structure not found)	
216	aC17:1 ME (structure not identified)	
217	iC16:1 ME (structure not specified)	
218	C20:1 ME (structure not defined)	
219	C18:2 ME (structure not defined)	
220	C18:3 ME (structure not defined)	
221	C16:1 fatty acid (structure not defined)	
222	CyclopropylC17:0 fatty acid (structure not defined)	
223	CyclopropylC19:0 fatty acid (structure not defined)	
224	C18:1 fatty acid (structure not defined)	
225	methylated cholesterol	
226	18:1 fatty acid	
227	24:1 fatty acid	
228	15:0 fatty acid	
229	17:0 fatty acid	
230	Pyridine-2-carboxylic acid (PA)	
231	Pyridine ketonium	
232	Heptose	
233	Diphosphatidyl glycerol (cardiolipin)	
234	Phosphatidyl glycerol	
235	Phosphatidyl serine	
236	Phosphatidyl ethanolamine	
237	Coenzyme Q	
238	Phosphatidic acid	
239	lysophosphatidyl ethanolamine	
240	KDO	
241	3,6-Diisobutyl-2,5-diketopiperazine	
242	3,6-(2-Methylpropyl)-2,5-diketopiperazine	
243	Vinyl-1-methylthioether	
244	Propane-1-methylthioether	
245	1-Propanamime-N-(3-methylthiopropylidene)	
246	Methylamine-N-(2-methylpropylidene)	
247	Ethylamine-N-(2-methylpropylidene)	
248	2-Methylpropylamine-N-(2methylpropylidene)	

249	3-Isopropyliden-2,5-diketopiperazine
250	3,6-Diisopropyl-2,5-diketopiperazine
251	Intramolecular deyhdration product
252	1,2-Dipyrrylethane
253	1,3-Dipyrrylpropane
254	Diketodipyrrole
255	3-Aminohexahydroazepinone
256	2,3-Dehydro-2-piperidone
257	2,3-Dehydromethyl-2-piperidone
258	n-Methylpyridine
259	n-Methylthiazolidine
260	Methylethylthizaole
261	Val-Pro DKP pyrolysis product

Appendix B. Pt-Nanocluster Experimental Procedure

Pyrolyzer Experiment # _____ Date: _____

☐ **Preparation**

1. Ensure that sample cup and wire are clean (flame out if necessary)
2. Weigh pyrolyzer sample cup and record: _____; tare analytical balance
3. Don appropriate personal protective equipment (PPE)

☐ **Add desired volume of nanocluster stock solution to the sample cup**

1. Vortex the stock nanocluster solution
2. Remove desired volume with a calibrated micropipette
3. Transfer desired volume to a pyrolyzer sample cup from the micropipette
4. Record volume added: _____of batch: _____

☐ **Remove desired amount of spores from the stock solution and place into pyrolyzer sample cup**

1. Vortex the stock spore solution
2. Remove desired volume with a calibrated micropipette
3. Transfer desired volume to a pyrolyzer sample cup from the micropipette
4. Record volume added: _____of batch: _____

☐ **Mix spores and nanoclusters in the pyrolyzer sample cup and let dry**

1. Triturate sample until well mixed - let dry, at elevated temperature if necessary
2. Record dry sample cup weight: _____; calculate spores and nanoclusters added: _____

☐ **Prepare the pyrolyzer and GC-MS**

1. Be sure that the MS is operating within the tuned parameters (i.e., no leaks), at the appropriate pressure ($<2 \times 10^{-5}$ torr)
2. Condition the column and all heated zones of the pyrolyzer and GC-MS at the appropriate temperature

☐ **Load the sample cup into the pyrolyzer and wait for pressure equilibration, vent once**

☐ **Select the desired pyrolyzer GC methods**

1. Load the appropriate methods for the pyrolyzer and GC

2. Select and record a file name: _____
3. Record the methods used:
    a. GC: _____
    b. Py: _____
4. Prep the system (make sure all variables are at their set point)

☐ **Perform the pyrolysis and GC-MS analysis**

1. Introduce the sample cup to the pyrolyzer heated zone and begin the pyrolyzer cycle
2. After the pyrolysis time has expired, remove the sample cup from the heated zone
3. Be sure that the data are appropriately recorded on the computer and backed up

☐ **Restore the system and materials to their original condition**

1. Recondition the column and heated zones, if necessary
2. Clean the pyrolyzer sample cup and suspension wire by flaming it out with the butane torch
3. Dispose of all chemicals and biological hazardous waste appropriately; clean-up area with Clorox solution

The chromatography and mass spectrometry conditions for the Pt-nanocluster study were as follows:

(1) Column flow was held at a constant 1 mL min^{-1}, transfer from the pyrolyzer was done at a split ratio of 5:1, except for run 56 which was done (accidentally) at a split ratio of 20:1.

(2) The temperature program was held constant at 50 °C for 2 min (during pyrolysis), and was then ramped at 15 °C min^{-1} to 310 °C and held for 10 min or until no further compounds eluted.

(3) For the dry samples no solvent delay was used; for wet samples a solvent delay of 3 min was used.

(4) The mass spectrometer scanned an m/z range of 35-500.

Appendix C. Pt-Nanocluster/Spore Coating Calculations

The following calculations were done in Mathcad 2001i:

Spore and nanocluster concentration calculations
22 March 2005 - Present

Objective: to determine the necessary concentration of spores and nanoclusters to facilitate uniform, total coating of the spores upon drying of the solution.

Spores are ellipsoids, with the literature giving dimensions as 1.2 μm long and 0.8 μm in diameter (Chada, Sanstead et al. 2003). Finding the exterior surface area of a spore:

Formula for finding the surface area of an ellipsoid (prolate spheroid) from a google search:

$$\pi\left(2a^2 + \frac{b^2}{e}\ln\left(\frac{1+e}{1-e}\right)\right)$$ where the eccentricity, e, is: $$\left(1-(b^2/a^2)\right)^{1/2}.$$

- *a* is the major axis length
- *b* is the minor axis length

$a := \frac{1.2}{2}\mu m$ $b := \frac{0.8}{2}\mu m$ $\mu m \equiv 10^{-6} m$ $ecc := \left[1 - \left(\frac{b^2}{a^2}\right)\right]^{\frac{1}{2}}$

$A_s := \pi \cdot \left(2 \cdot a^2 + \frac{b^2}{ecc} \cdot \ln\left(\frac{1+ecc}{1-ecc}\right)\right)$

$A_s = 3.56\mu m^2$ for a sphere or 1μm diameter; $A_{s2} := \pi \cdot (1\mu m)^2$ $A_{s2} = 3.142\mu m^2$

these are in fair agreement, which indicates that this surface area is probably realistic

Next, the number of clusters that will evenly coat the surface of these spores must be determined. There are a number of assumptions that must be made to come up with an approach. The first is that the cross-sectional area of the clusters will represent the area that they take up on the spore. While this may or may not be representative of what actually happens, this is a good enough first approach. Later, considerations such as wetting angle and surface tensions may be applied, but for now this is good enough.

For the 1st nanoclusters, the diameter is 1.2 nm.

$d_{nc} := 1.2nm$ $nm \equiv 10^{-9} m$ The cross-sectional area of a cluster is then approximately:

$A_{nc} := \pi \frac{d_{nc}^2}{4}$ $A_{nc} = 1.131nm^2$

Dividing the surface area of a spore multiplied by the desired fractional coverage (η) by the area of the cluster gives a rough approximation as to how many clusters are required per spore:

$\eta := 100\%$ $n_{ncps} := \frac{\eta \cdot A_s}{A_{nc}}$ $n_{ncps} = 3.148 \times 10^6$ which is realistic

Next, setting up some relationships that give the amount of clusters required per amount of spores at a given concentration:

$C_s := 0.5 \cdot 10^9 \cdot mL^{-1}$ $V_s := 40\mu L$ $\mu L \equiv 10^{-6} L$ $n_{spores}(V_s) := C_s \cdot V_s$ $n_{spores}(V_s) = 2 \times 10^7$

$n_{nc}(V_s) := n_{spores}(V_s) \cdot n_{ncps}$ $n_{nc}(V_s) = 6.296 \times 10^{13}$ this is the number of nanoclusters required for 40 μL of spores

Now I need to determine how much nanocluster powder is required to get this many nanoclusters

$pm \equiv 10^{-12} m$

$d_{nc} = 1.2 \times 10^{-9} m$ $V_{nc} := \frac{\pi d_{nc}^3}{6}$ $V_{nc} = 0.905 nm^3$

The crystal structure of platinum is that of a crystal close pack - ccp, with a cell length of 392.42 pm and 4 atoms per cell (webelements.com.) Knowing the dimensions of a platinum atom allows use of the crystal structure of Pt to calculate how many Pt atoms there are per cluster:

$L_{cell} := 392.42 pm$ $V_{cell} := L_{cell}^3$ $V_{cell} = 0.06 nm^3$ $n_{atomspercluster} := \frac{V_{nc}}{V_{cell}} \cdot 4$ $n_{atomspercluster} = 59.889$

$n_{ptatoms}(V_s) := n_{atomspercluster} \cdot n_{nc}(V_s)$ $n_{ptatoms}(V_s) = 3.77 \times 10^{15}$ $moles_{Pt}(V_s) := \frac{n_{ptatoms}(V_s)}{N_{av}}$

$moles_{Pt}(V_s) = 6.261 \times 10^{-9} mol$ $m_{Pt}(n_{spores}) := moles_{Pt}(n_{spores}) \cdot MW_{Pt}$ $m_{Pt}(V_s) = 1.221 \times 10^{-3} mg$

$MW_{Pt} \equiv 195.078 \frac{gm}{mol}$ $N_{av} \equiv 6.022141 10^{23} mol^{-1}$

now take into the account that only 10% of the nanocluster powder is platinum: $\eta_2 := 10\%$

and to account for non-specific binding (guess of 50%) $\eta_3 := 50\%$

Given a nanocluster solution of the following concentration, how much of it will it take to coat the spores:

$m_{nc}(V_s) := \frac{m_{Pt}(V_s)}{\eta_2 \cdot \eta_3}$ $m_{nc}(V_s) = 0.024 mg$ $m_{nc}(V_s) = 0.000024 gm$

$C_{nc} := \frac{1.5mg}{1mL}$ $m_{nc2}(V_{nc}) := C_{nc} \cdot V_{nc}$ $V_s := 30\mu L$ $m_{nc}(V_s) = 0.0183 mg$

Given

$m_{nc}(V_s) = m_{nc2}(V_{nc})$ $V_{nc\_f} := Find(V_{nc})$ $V_{nc\_f} = 12.213\mu L$ $V_{nc\_f} \cdot C_{nc} = 0.0183 mg$

Appendix D. Statistical Methods

The data from the factorial studies (Table 17) were fitted with a linear regression model using the statistical program R 2.0.1 (R Core Development Team, 2004). The model followed the equation:

$$Y_i = \beta_0 + \beta_1 temp_i + \beta_2 n1_i + \beta_3 n2_i + \beta_4 s1_i + \beta_5 s2_i$$

where: i = 1-15 observations

Y_i = the response factor amount for either furfuryl alcohol, pyrrolidine, or picolinic acid

β_0 = Intercept to be estimated

β_1 - β_5 = Regression Coeffiencts to be estimated

$temp_i$ = (250*C, 300*C)

$n1_i$ = (1 : 0.024 µg nanocluster,
0 : 0.012 µg nanocluster
-1 : 0.00 µg nanocluster) linear

$n2_i$ = (-1, 2, 1) quadratic nanocluster

$s1_i$ = (1 : 20 µg spore,
0 : 10 µg spore
-1 : 0 µg spore) linear

$s2_i$ = (-1, 2, 1) quadratic spore

A regression model may combine several elements, such as containing linear and quadratic terms, and still be treated as a general linear regression model. Because the true curvilinear response function is unknown and complex it is common practice to add a quadratic effect to the model, which gives a good approximation to the true function.

In this model, the predictor variables for nanoclusters and spores were fit with orthogonal linear and quadratic effects. Orthogonal effects are uncorrelated and provide equally spaced levels of a predictor. Parameter estimates for the regression coefficients

including p-values are listed in the results from the statistical program R and presented in Appendix E.

R Development Core Team (2004). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Appendix E. Statistical Regression Output

The amounts of each chemical determined by the RAMFAC algorithm were analyzed using a statistical regression in R (a statistical program, see Appendix E) to determine the effects of each variable on each response factor individually. The output of the regression, based on the data in Table 17, is presented below and summarized in Table 18:

PYRROLIDINE

```
 summary(mod.pyr<-lm(pyrrolidine~temp+s1+s2+n1+n2,exp2) )

Call:
lm(formula = pyrrolidine ~ temp + s1 + s2 + n1 + n2, data = exp2)

Residuals:
     Min       1Q   Median       3Q      Max
-36866216 -11882906  -7346676  11454381  63514682

Coefficients:
             Estimate   Std. Error  t value  Pr(>|t|)
(Intercept) -151409023   81013325   -1.869   0.0912 .
temp            598861     293251    2.042   0.0684 .
s1             6251295    9926579    0.630   0.5430
s2            13247929    5183987    2.556   0.0286 *
n1            13273045    9926579    1.337   0.2108
n2             -829844    5183987   -0.160   0.8760
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 29330000 on 10 degrees of freedom
Multiple R-Squared: 0.543,      Adjusted R-squared: 0.3145
F-statistic: 2.376 on 5 and 10 DF,  p-value: 0.1144
```

FURFURYL ALCOHOL

```
> summary(mod.fur<-lm(furfuryl~temp+s1+s2+n1+n2,exp2))

Call:
lm(formula = furfuryl ~ temp + s1 + s2 + n1 + n2, data = exp2)

Residuals:
    Min      1Q  Median      3Q     Max
-1565614 -1100342   189390   805480  2148352
```

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4908489	3857619	1.272	0.23202
temp	5965	13964	0.427	0.67832
s1	4895646	472675	10.357	1.15e-06 ***
s2	518795	246846	2.102	0.06190 .
n1	-2152606	472675	-4.554	0.00105 **
n2	-115998	246846	-0.470	0.64849

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 1396000 on 10 degrees of freedom
Multiple R-Squared: 0.9444, Adjusted R-squared: 0.9167
F-statistic: 34 on 5 and 10 DF, p-value: 5.782e-06

PICOLINIC ACID

> summary(mod.pic<-lm(picolinic~temp+s1+s2+n1+n2,exp2))

Call:
lm(formula = picolinic ~ temp + s1 + s2 + n1 + n2, data = exp2)

Residuals:

Min	1Q	Median	3Q	Max
-380657	-270782	-122634	321764	483730

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-2931646	1068752	-2.743	0.0207 *
temp	11348	3869	2.933	0.0150 *
s1	301482	130954	2.302	0.0441 *
s2	76795	68389	1.123	0.2877
n1	253007	130954	1.932	0.0822 .
n2	125270	68389	1.832	0.0969 .

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 386900 on 10 degrees of freedom
Multiple R-Squared: 0.6379, Adjusted R-squared: 0.4568
F-statistic: 3.523 on 5 and 10 DF, p-value: 0.04271

Appendix F. RAMFAC Algorithm

The RAMFAC (*Ra*pid *M*ultivariate *Fac*torization) is a algorithm written primarily by James Oliphant that facilitates the deconvolution of mass spectral based-chromatographic data and identification of individual spectra. It is based on the numerical transformation of the mass spectral data using the householder transformation. Unlike the AMDIS program, it does not use peak fitting to separate the individual compounds in the chromatographic data. Rather, consecutive subtractions of various mass spectra from the individual peaks, in the spectral dimension, facilitates separation of the spectra of various compounds. These compounds can then be matched with a separate spectral database in order to determine the chemical identity of the compounds. In this way, it is possible to identify each of the compounds that are present in the chromatogram in terms of both retention time, amount (total ion count), and chemical identity. Various matching routines are available, including the creation of a new library based on the spectral data (i.e., no matching), matching with the NIST database, and matching with the biomarkers library described in Chapter 4. A matching correlation (from 0-1) is calculated based on the similarity (or difference) between the actual spectral data and the data in the library match recognition can be set a different levels. For this study, matches were considered good at a correlation value of 0.80.