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Differentiation of *Bacillus* Endospores from Gas Chromatography-Mass Spectrometry of Biomarkers Produced by Thermochemolysis Methylation

Tai Van Truong
Brigham Young University - Provo

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Tai V. Truong

A dissertation submitted to the faculty of
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Doctor of Philosophy

Milton L. Lee, Chair
H. Dennis Tolley
David V. Dearden
Steven R. Goates
Delbert J. Eatough

Department of Chemistry and Biochemistry

Brigham Young University

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ABSTRACT

Differentiation of *Bacillus* Endospores from Gas Chromatography-Mass Spectrometry

of Biomarkers Produced by Thermochemolysis Methylation

Tai V. Truong

Department of Chemistry and Biochemistry, BYU

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Methods for fast, simple detection of biomarkers to detect and differentiate closely related *Bacillus* endospores including *Bacillus anthracis* (BA), *Bacillus thuringiensis* (BT), *Bacillus atrophaeus* (BG), and *Bacillus cereus* (BC) using thermochemolysis and methylation (TCM), coiled wire filament (CWF), solid phase micro extraction (SPME) and gas chromatography-mass spectrometry (GC-MS) were developed. The main biomarkers detected and used for differentiation include dipicolinic acid methyl ester (DPAME), fatty acid methyl esters (FAMEs), 3-methyl-2-butenoic acid methyl ester (3M2BAME), 2-butenoic acid methyl ester (2BAME), and several methylated sugars.

TCM of endospores was performed based on hydrolysis and methylation at elevated temperature after the endospores were mixed with sulfuric acid (H_2SO_4) with or without addition of tetramethylammonium hydroxide (TMAH) in methanol (MeOH). TCM products were then introduced into a heated GC injector port using a coiled wire filament (CWF) or solid phase microextraction (SPME) for detection and differentiation of the endospores by GC-MS.

The CWF, which consisted of a tiny platinum helical wire coil attached to a retractable plunger that moved the coil in and out of a syringe needle housing, allowed for sampling to be accomplished by dipping the CWF in an endospore sample suspension, evaporating the suspension liquid, and then introducing the CWF into the injection port to enable on-line TCM.

New SPME techniques, including half-half extraction, coated-needle extraction (CNE), and a new home-made polymer coated needle were used to speed up solid phase micro extraction of biomarkers produced from TCM. These simplified the detection of anthrose and other

biomarkers. TCM with a CWF and TCM with SPME produced high intensity profiles of DPAME, FAMEs, 2BAME, 3M2BAME and methylated sugars.

While the presence of DPAME can be used for the general detection of endospores (*Bacillus and Clostridium*) and the presence of 3M2BAME for the detection of BA, specific saturated and unsaturated C15, C16, and C17 fatty acid methyl esters and methylated sugars provide additional information for differentiating various *Bacillus* species grown at different temperatures and in different media. DPAME was detected in samples containing as few as 2,500 and 6,000 endospores using TCM-CWF with and without a concentration step, respectively. GC-MS peak area percent reproducibility for FAMEs using TCM and CWF varied from 3 to 13% (RSD). Better than 97% correct predictability of *Bacillus* species identity was obtained from a blind experiment consisting of 145 samples using DPAME and specific FAMEs. Conventional SPME and a modified form of "in-needle" extraction allow for detection of the biomarkers in less than 35 min. The detection limits with SPME sample introduction injection were approximately 5×10^3 endospores.

Using these approaches, differentiation of *Bacillus* endospores and other biological agents grown under different conditions were based on the following characteristics: (1) presence of DPAME and specific FAMEs (iso or anteiso C15:0 and iso or anteiso C17:0) in *Bacillus* endospores, (2) unique presence of 3M2BAME (anthrose by-product) in BA, (3) absence of 2-butenoic acid methyl ester in BG, and (4) presence and absence of specific methylated monosaccharides in various *Bacillus* species. *Clostridium* endospores and non-sporulating bacteria, such as *Yersinia pestis* (YP) and *Francisella tularensis* (FT) could also be easily distinguished from *Bacillus* endospores based on the presence and absence of several specific sugar derivatives and fatty acid methyl esters (FAMEs), such as iso or anteiso C15:0 and iso or anteiso C17:0, and > C18 FAMEs which were simultaneously produced during TCM.

Keywords: *Bacillus*, Endospores, Anthrax, Differentiation, Thermochemolysis methylation, Solid phase micro extraction, Coiled wire filament, Coated-needle extraction, Gas chromatography-mass spectrometry, Dipicolinic acid, Fatty acid, Sugars, Anthrose

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LIST OF ABBREVIATIONS

2BAME	2-butenoic acid methyl ester
3HBA	3-hydroxy butyric acid
3HBAME	3-hydroxy butyric acid methyl ester
3M2BAME	3-methyl-2-butenoic acid methyl ester
AsFs	Asymmetry factors
ATOFMS	Aerosol time-of-flight mass spectrometer
Au	Area unit
BA	*Bacillus anthracis*
BC	*Bacillus cereus*
BG	*Bacillus atrophaeus*
BM	*Bacillus mycoides*
BP	*Bacillus pseudomycoides*
BT	*Bacillus thuringiensis*
BW	*Bacillus weihenstephanensis*
CART	Classification and regression trees
CB	Chemical and biological
CI	Chemical ionization
CNE	Coated-needle extraction
Col	Columbia
COC	Cold on-column

CWF	Coiled wire filament
DB	Prototype database
DPA	Dipicolinic acid
DPAME	Dipicolinic acid methyl ester
DPG	Dugway Proving Ground
EI	Electron impact ionization
EIM	Extracted ion monitoring
ESI	Electrospray ionization
FAMEs	Fatty acid methyl esters
FAs	Fatty acids
FFAP	Free fatty acid phase
FT	*Francisella tularensis*
Fuc	Fucose
Gal	Galactose
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
Glu	Glucose
HMDS	Hexamethyldisilane
HPLC	High performance liquid chromatography
ID	Inner diameter
LD	Leighton-Doi
MALDI TOF MS	Matrix assisted laser desorption time-of-flight mass spectrometry

MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
NTD	Needle trap device
OD	Outer diameter
P3HBA	Poly(3-hydroxybutyric acid)
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Poly(ethylenglycol)
PMMA	Poly(methyl methylacrylate)
PTV	Programmed temperature vaporizer
RaS	Raman spectroscopy
Rha	Rhamnose
S/S	Micro-syringe and split/splitless
SASPs	Small acid-soluble proteins
SAW	Surface acoustic wave
SBSE	Stir-bar sorptive extraction
SDME	Single drop microextraction
SEP	Sample enrichment probe
SERS	Surface-enhanced Raman scattering
SIM	Selected-ion-monitoring
SPDE	Solid phase dynamic extraction
SPME	Solid phase micro extraction

TCM	Thermochemolysis methylation
TEAAc	Tetraethylammonium acetate
TMAH	Tetramethylammonium hydroxide
TMCS	Trimethylchlorosilane
TMSH	Trimethylsulfonium hydroxide
Xyl	Xylose
YP	*Yersinia pestis*

LIST OF SYMBOLS

K_{fs}	Fiber coating/sample matrix distribution constant
V_f	Fiber coating volume
V_s	Sample volume
C_o	Initial concentration of analyte in the sample
C_{wo}	Initial concentration of analyte in the water solution
K_{aw}	Henry's law coefficient
K_{fg}	Fiber coating/gas partition coefficient
V_w	Water volume
V_g	Headspace volumes
ω	Angular velocity
r	Radial distance from the center of the vortex

1 INTRODUCTION

1.1 SIGNIFICANCE OF DETECTION AND DIFFERENTIATION OF *BACILLUS* ENDOSPORES FROM EACH OTHER AND FROM OTHER BACTERIAL SPECIES

Biological warfare and bioterrorism are no longer hypothetical after the events of September 11, 2001. Microbiological agents, especially *Bacillus anthracis* (BA), were used by countries such as Russia, North Korea, and Japan during the Cold War. BA is a Gram-positive spore-forming soil bacterium that causes anthrax,[1-4]and is a member of the *Bacillus cereus* (BC) group (also known as the ‘‘group 1 *bacilli*”) which includes *Bacillus cereus* (BC), *Bacillus thuringiensis* (BT), *Bacillus mycoides* (BM), *Bacillus pseudomycoides* (BP), and *Bacillus weihenstephanensis* (BW).[3,5]

BA is a particular organism of interest because it can inflict a serious and often fatal disease in mammalian livestock and humans. Although more than 400 distinct strains of BA are known to exist, genetic variation of the species is represented by 89 strains,[6] ranging from highly virulent (sometimes used for biological warfare and bioterrorism purposes) to benign (those used for vaccines). Vegetative cells of virulent strains produce three exotoxin proteins, including the protective antigen, the edema factor, and the lethal factor, and they surround themselves with a capsule comprised of poly-D-glutamic acid. BA is the only known bacterium with these virulence factors.[7] The BC group represents a highly homogenous subdivision that includes the three closely related species, BA, BC, and BT, and the more distantly related BM and BW.

BC is a foodborne agent responsible for some 5% of food poisonings in the United States, and BT is a commercially available biological larvicide that is harmless to humans.[7] Differentiation of these species using molecular assays has been problematic due to their high

level of genetic similarity. However, the three closest species of this group each produce unique compounds related to their pathogenesis. *B. atrophaeus* (BG) is not closely related to BA; however, it has been used for decades as an important model organism, especially as a nonpathogenic surrogate for BA in bio-weapons research.[8,9] Historically, BG has been called different names including *B. niger*, *B. globigii*, and *B. subtilis*.[8,9] It is a naturally occurring soil bacterium, capable of producing subtisin, a bacteriocin which kills closely related species or competitive BG strains.[7] BA, BC, BT, and BG were selected for study in several recent reports regarding the differentiation of closely related *Bacillus* species.[10,11]

Differentiation of BA from its non-lethal relatives is essential for correctly identifying a bioterrorism event and controlling the outbreak of disease resulting therefrom. Recent studies have indicated that BA is genetically similar to other members of the genus *Bacillus*. A distinguishing characteristic of the spores of BA and related species is the exosporium, an outermost layer that is not present in spores of many other *Bacillus* species.[5] *Bacillus* cells produce spores in response to nutrient deprivation,[12] which are remarkably resistant to physical stress, such as high temperatures, harsh chemicals radiation, desiccation, physical damage, and even the vacuum of outer space.[4,13-15] *B. anthracis* endospores share a high degree of similarity with those in the *B. cereus* group; however, it is a fully virulent *Bacillus* that carries two plasmids, pXO1 and pXO2, which contain genes to produce the lethal factor and edema factor toxins, and a poly-γ-D-glutamic acid capsule.[5,16-18] When disseminated as spores, *B. anthracis* has been described as the ultimate biological weapon because of its virulence and persistence.[14,19] The anthrax disease in mammals can be contracted by ways of gastrointestinal (intestines), inhalation (lungs) or cutaneous (skin) entry by spores.[20] Inhalational untreated death

rate is highest (>90%) and can kill most victims if they are not treated within 24-48 h, while cutaneous and gastrointestinal untreated death rate is ~20 and 25-60% respectively.[12,13]

B. cereus, a soil-dwelling saprophyte, is best known for causing two distinct forms of food poisoning that develop symptoms of vomiting or diarrhea,[21] eye infections, and other nongastrointestinal infections.[22] Differently from the two mentioned species, *B. mycoides* is a non-pathogenic species with distinctive rhizoidal colony formations,[23] and *B. thuringiensis*, usually defined as containing parasporal crystal proteins, has a toxic effect to insects or other invertebrates and has been used as an insecticide.[24,25]

The bioterrorism attacks in Autumn of 2001, which caused five deaths and 22 infected cases, has resulted in widespread panic and brought the US postal system to the brink of collapse. These incidences have demonstrated the danger of anthrax as a biowarfare agent to terrorize civilian populations[13,26] and have further emphasized the need for fast and efficient methods to detect and reliably differentiate biowarfare agents.

1.2 SPORULATION OF *BACILLUS* SPECIES

Sporulation of *Bacillus or Clostridium* in the environment is a process that these genera perform themselves to preserve and to propagate the genetic information contained within the bacteria,[20] following a mechanism for both spatial and temporal escape from local conditions that are unfavorable to rapid growth.[27] Unlike most adaptive responses in bacteria, sporulation takes many hours and includes major changes in cellular morphology as well as in biochemistry and physiology.[28] After sporulation, spores can survive under harsh conditions for even decades or hundreds of years.[27,29,30]

Generally, spores of *Bacillus* or *Clostridium* are usually prepared by cultivating the bacteria at 32ºC or 37ºC in a liquid nutrient broth-based sporulation medium at a high growth

rate to high cell density until some essential nutrient, such as the carbon source, is exhausted from the medium.[20,31] In our research, different media were used to investigate media effects on biomarkers which are used for detection and differentiation. Recent studies, which were focused on gene expression, found that growth phase often plays an important part in regulating expression of virulence-associated genes.[32-35] In sporulation, the construction of endospores in response to harsh conditions takes cells about 8 h to accomplish; this process is conducted by a tightly controlled genetic program.[36-38]

Many members of Gram-positive bacteria, including *Bacillus* and *Clostridium,* produce endospores similar to that of *B. subtilis* in which sporulation occurs through the following stages (see Figure 1.1): (1) the cell creates an internal protoplast with its own copy of the chromosome after it commits to sporulation; (2) the cell builds a septum that is positioned off one cell side and generates a smaller forespore compartment and a larger mother cell compartment; (3) soon afterward, an endocytosis-like event converts the forespore into a protoplast, possessing two membranes; (4) in this stage, a unique organelle composed of two peptidoglycan structures called germ cell and cortex is synthesized to produce and functionized cell wall and cortex; and (5) finally, a thick protein shell is formed that encases the forespore before the spore is released.[37,39]

1.3 SPORE COMPOSITION

A spore has a very different structure with a number of unique features and constituents compared to a growing cell. Starting from the inside and proceeding outward, the spore layers include central core, inner membrane, germ cell wall, cortex, outer membrane, coat, and exosporium (see Figures 1.2 and 1.3). The spore structure and its chemical composition play major roles in spore resistance.[40]

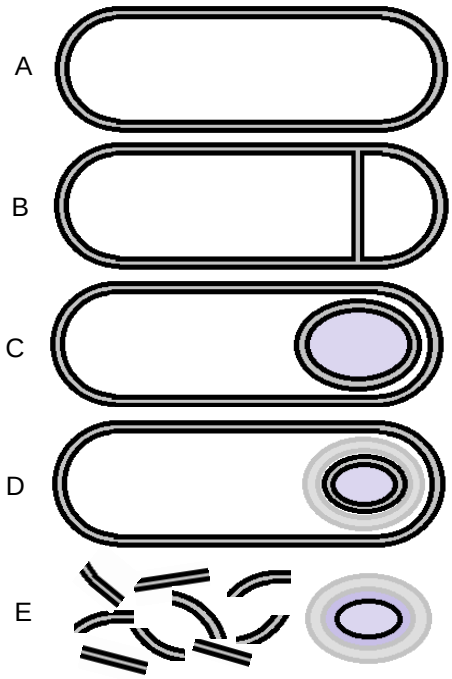

Figure 1.1. Stages of sporulation: (A) stage 1, cell commits to sporulation; (B) stage 2, cell builds a septum; (C) stage 3, cell generates a smaller forespore compartment and a larger mother cell compartment; (D) stage 4, cell builds a germ cell wall and cortex, then coats the protein; and (E) the spore is released. The hatched line around the spore in panels D and E is the coat.[37]

1.3.1 Core

The spore core contains a relatively dehydrated mixture of DNA, enzymes, proteins, ribosomes, and pyridine-2,6-dicarboxylic acid or dipicolinic acid (DPA).[41,42] DPA is a small molecule, extremely important in spore resistance. The presence and high concentration (5-15% of the dry spore weight) of this compound in both *Bacillus* and *Clostridium* cores enable the use of it as a strong biomarker for detection of endospores. DPA is mainly in the core, where it is most likely chelated with divalent cations, largely Ca^{2+}.[43,44] Popham et al.[44] found that the amount of DPA in spores of their wild-type and mutant strains was relatively constant, and they also found that heating of the mutant spores (*dacB* and *spmA*, two wildtype strains of BS*)* at

70ºC resulted in significant losses of DPA. For example, after 45 min at 70ºC, the wildtype *dacB* deletion mutant, *spmA* deletion mutant, and *dacB* insertion mutant spores lost 11, 24, 33, and 51% of their DPA, respectively. Some loss of DPA from spores upon sublethal heating was previously observed.[45,46] Another important component in the spore core is the protein component, which includes small acid-soluble proteins (SASPs) that can be exploited as biomarkers for identification of the spore species by HPLC-MS.

1.3.2 Membranes

Bacillus and clostridium endospores have two membranes: an inner one that lies between the core and cortex, and an outer one that resides between the cortex and the coat. These membranes contain a significant amount of protein, including enzymes.[47] They serve as permeability barriers for the spores.[48] Under attack by chemicals, such as 0.3-1 M HCl at 24ºC or 70%v/v ethanol at 65ºC, the permeability barrier of the inner membrane can be damaged. The same effect also occurs with interaction of oxidizing agents, such as hydrogen peroxide, ozone, and sodium hypochlorite.[49,50] This indicates that the endospore structure can be destroyed to release internal substances by attack with several common chemicals.

1.3.3 Cortex

The main biomarkers that exist in the cortex and can be exploited as biomarkers for detection and differentiation of endospores are two types of peptidoglycan. Both of these consist of alternating N-acetylglucosamine and N-acetylmuramic acid sugar residues cross-linked by peptides.[51] A key function of the cortex is to maintain the low water content of the core during spore survival by applying pressure to counteract the osmotic or turgor pressure.[52]

1.3.4 Coat

This structure consists largely of protein with smaller amounts of complex carbohydrate, lipid, and a significant amount of phosphorous in some species.[53] Different spores possess different coat complexities, such as thickness, number of different proteins, and the manner in which they are assembled.[54] The role of the coat is to protect the core from degradative enzymes, surfactants and other toxic molecules.[55,56]

1.3.5 Exosporium

This thin, loose-fitting, and external layer that is not considered to be a significant permeability barrier consists of protein, polysaccharide, neutral lipid, and phospholipid molecules.[57] Spore-specific antigens and some enzymatically active proteins are contained in this structrure.[58] Anthrose has been reported to exist in a distinctive oligosaccharide in the BA exosporium and in lower abundance in some strains of *B. thuringiensis* and *B. cereus.*[59] The exosporium of *Bacillus cereus* was extricated from dormant spores for analysis of intrinsic constituents by Matz et al.[57] The results indicated that the exosporium is chemically complex, but not unique; it consists mainly of protein (52%), amino and neutral polysaccharides (20%), lipids (18%), and ash (4%). Seventeen common amino acids were also identified by chromatographic methods, and these amino acids were present in usual proportions, except for low levels of methionine, histidine, cystine-cysteine, and tyrosine. Glucosamine was the only amino sugar, while glucose and rhamnose were the principal neutral sugars. In the lipid fraction, cardiolipin (the only phospholipid) was present at 5.5%, while 12.5% belonged to neutral lipids. More than 19 fatty acids were also found, in which normal C16 and C18 were predominant constituents. Calcium and phosphorus were found in the ash. Small amounts of teichoic, ribonucleic, and dipicolinic acids were also detected, but considered to be contaminants.[57] Besides bio-subtances

in the endospores, investigators found that silica, which is considered to be a "quasi-essential" element for most living organisms, was deposited in a spore coat layer of nanometer-sized particles in BC. This presence enhances acid resistance, and became the focus of attention when anthrax spores were mailed to U.S. senators in the fall of 2001. The presence of silica is considered to be a cheap and easy way to disperse spores when the container is opened, because silica can prevent spores from sticking together. Thus, if silica is found in BA spores, this suggests that they were weaponized.[60]

1.4 BIOMARKERS OF *BACILLUS* SPECIES AND OTHER BIOLOGICAL THREAT AGENTS

1.4.1 Small acid soluble proteins

Intact *Bacillus* spores contain a large amount (almost 15% by mass) of proteins from the SASP family. These primarily DNA-binding proteins with less than 10 kDa in size, provide

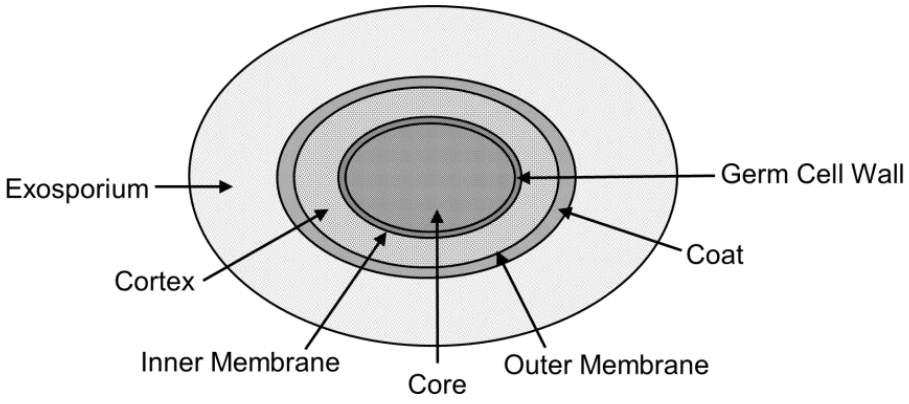

Figure 1.2. Spore structure. The labeled spore layers are not drawn to scale, and the size of various structures, in particular the exosporium, vary significant between spores of different species.[40]

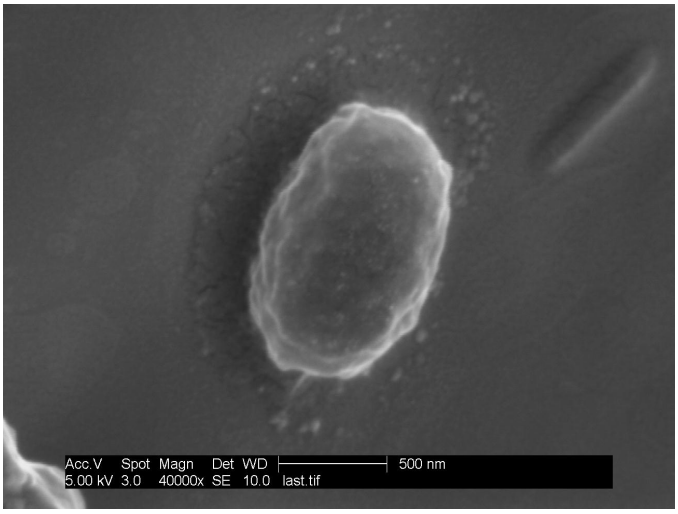

Figure 1.3. SEM of one BA endospore.

spore protection against a number of external factors.[61] These SASPs can be selectively and rapidly extracted with acid from intact spores without the need for spore disruption by physical and/or additional chemical means. SASPs have been used as biomarkers for BA identification by MS-based (MALDI and ESI) methods.

1.4.2 Fatty acids and beta-hydroxy butyric acid

Free fatty acids (FAs) are released from phospholipids and other complex lipid cellular components (comprising approximately 6% of the dry weight of a bacterial cell).[30] Fatty acid composition generally varies from species to species and with growth conditions. Christopher et al.[62] determined that cellular fatty acid methyl ester (FAME) profiles were reliable enough to be used to distinguish among spore samples by culturing BC T bacterial spores in 10 different medium formulations. The largest dissimilarities in FAME profiles were found among spores grown on media with disparate nitrogen and protein sources (meat peptone, yeast/casein peptone, yeast, brain heart infusion/gelatin digest, and beef extract/meat peptone). The dissimilarities were

likely caused by the distinct differences in fatty acid precursors (amino acids and α keto acids) inherent in each medium formulation.[62] This information confirmed that changes in FAME profiles from *Bacillus* species may occur when being grown under different conditions.

Methylated FAs are popular derivatives for fatty acid or lipid analysis using GC. There are many derivatization methods, each with specific advantages and disadvantages. Several tranesterification methods have been used by analytical chemists. Methylation with diazomethane is a fast and easy method to produce FAMEs. However, some negative aspects of diazomethane (i.e., carcinogenic and explosive hazards) limit the wide application of this method. Some other methylation methods that have been used for a long time include MeOH-[OH^-]-catalyzed and MeOH-[H+]-catalyzed methylation. Fatty acids can be methylated in anhydrous MeOH in the presence of HCl or H_2SO_4[63] (or other acid such as dichloroacetic, trifluoroacetic, benzene sulfonic, polyphosphoric, and *p*-toluene sulfonic).[64] Conventional bacterial fatty acid analysis such as MIDI is based on this derivatization method; however, it takes at least 1 h and a number of laboratory tools for sample preparation, including harvesting, saponification, methylation, extraction, and washing with base. Acid methylation of FAs was conducted as described by Christie[65]and Susana[66] with 1% sulfuric acid or 5% HCl in methanol, and heptadecanoic acid was added as an internal standard (1 mg/mL) before derivatization. The final solution was dried under nitrogen, dissolved in 1 mL of hexane, and stored at low temperature until GC analysis. However, the method was time consuming (2-3 h) and laborious because there were 5 steps needed before GC-MS.[66] Figure 1.4 shows the mechanism of methylation using H_2SO_4 as catalyst.

In recent years, thermally-assisted hydrolysis and methylation with tetramethyl

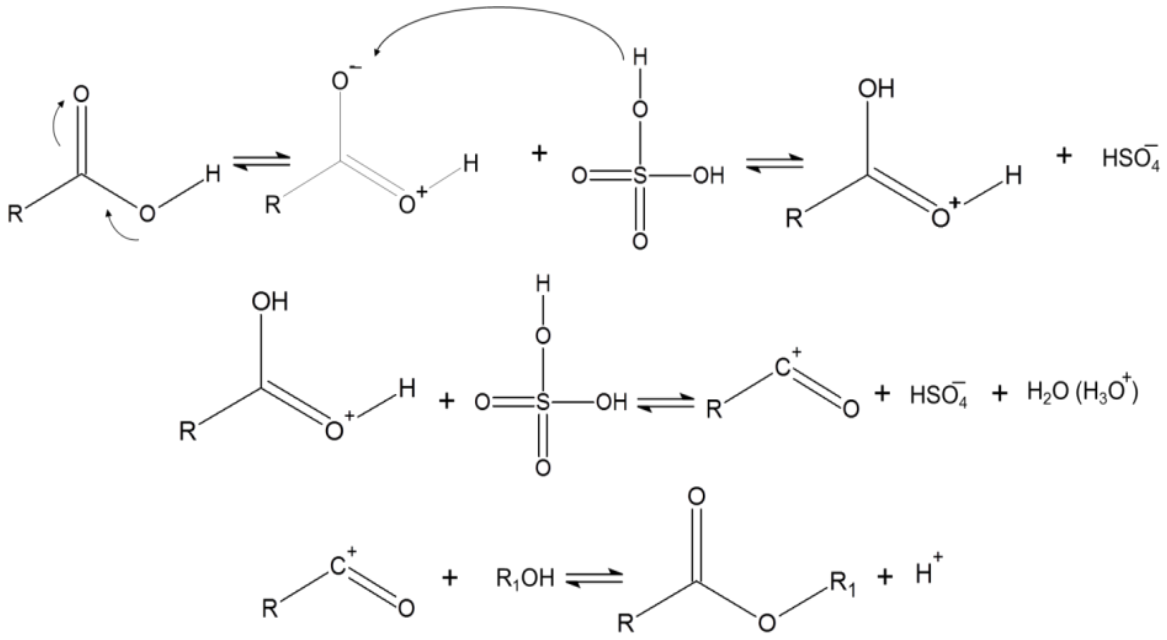

Figure 1.4. Mechanism of fatty acid methylation using H_2SO_4.

ammonium hydroxide [$(CH_3)_4N^+OH^-$,TMAH] in a GC injector has been reported.[64,67] The mechanism of methylation using TMAH is shown in Figure 1.5.

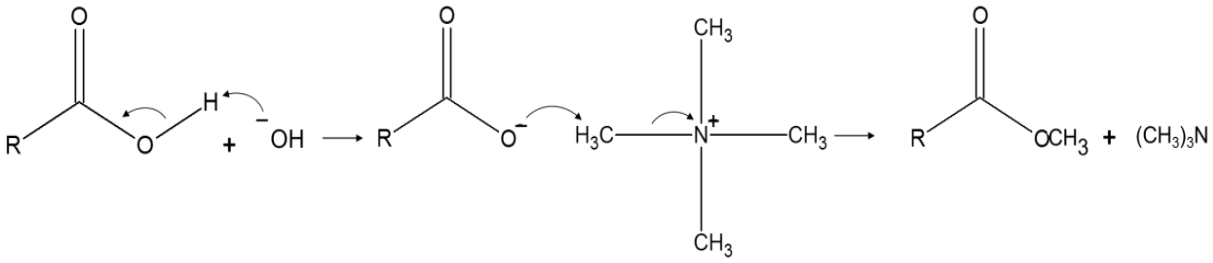

Figure 1.5. Mechanism of fatty acid methylation using TMAH.

1.4.3 Dipicolinic acid

DPA is synthesized only in the mother cell compartment of the sporulating cell, and is taken up into the forespore and subsequently excreted in the first minutes of spore germination by mechanisms that are not well understood.[40] In the spore core, DPA is present as a calcium-dipicolinate complex (see Figure 1.6), which is easily hydrolyzed with an acid such as H_2SO_4 to release DPA. As mentioned before, the high concentration and unique presence of this component in *Bacillus* and *Clostridium* spores enable differentiation from other non-sporulating micro-organisms.

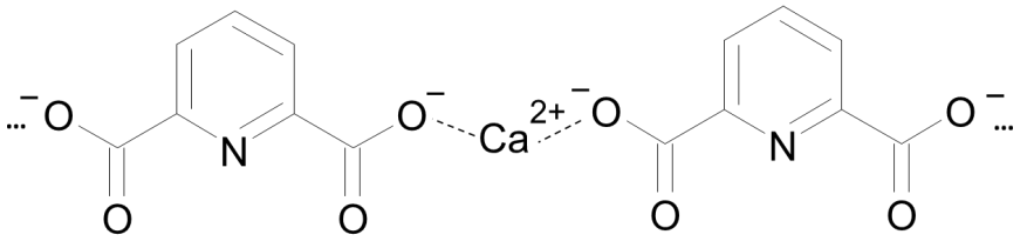

Figure 1.6. Complexing of Ca^{2+} with DPA to form a calcium-dipicolinate complex.

1.4.4 Sugars

Monosaccharides and amino monosaccharides. There are two common types of derivatives which are GC-compatible for sugar analysis: trimethylsilyl ether derivatives, which are formed by the reaction of sugars with hexamethyldisilane (HMDS), trimethylchlorosilane (TMCS), or similar reagents in non-aqueous organic solvents such as pyridine or dimethyl sulfoxide;[68] and acetate forms including tri-fluoroacetic acid alditol derivatives, trifluoroacetate alditol derivatives, acetic acid nitrile derivatives and acetic acid oxime derivatives. These derivatizations are usually formed in pyridine, butylene oxide or

methyl imidazole solvents. The trimethylsilyl derivatives are normally separated in GC with medium polar stationary phases, and tailing peaks appear if strong polar stationary phases are used. However, for acetate derivatives, polar stationary liquids such as poly(ethylenglycol) (PEG) or free fatty acid phase (FFAP) are the best choices.[69] The determination of sugars by GC-MS requires their conversion into less polar, thermostable, and volatile structures. Among the major derivatization methods of acetylation, silylation, and methylation, the latter one is the most widely used for GC-MS, which produces permethylated alditol acetates.[70] After the first *O*-methylated sugar method conducted by Purdie and Irvine[71] by treating dry carbohydrates dissolved in methanol with methyl iodide (MeI) in the presence of silver oxide, there have appeared many methods to obtain per-*O*-methylated carbohydrates.[72] Two popular methods used for saccharide analysis include: (1) reductive hydrolysis followed by alditol acetate derivatization, and (2) hydrolysis followed by reduction and alditol acetate derivatization.[73] Until the 1960s, these two methylation methods, with some modifications, were used for preparative and analytical *O*-methylation of carbohydrates. In 1964, Hakomori[74] performed per-*O*-methylation of polysaccharides in one step, by adding a solution of sodium methylsulfinyl carbanion (Na dimsyl) and methyl iodide to the carbohydrate dissolved in dimethyl sulfoxide.

Methyl derivative analysis is important in structural polysaccharide and monosaccharide chemistry. It involves exhaustive hydrolysis and methylation of these saccharides to form a mixture of monomeric methylated sugars in their monomethylated or polymethylated forms. These mixtures are then separated by GC for indentification and quantitation.[75] Fully methylated polysaccharides are often not soluble in hot aqueous solutions; however, they can be demethylated by an acid such as HCl, which can demethylate better than formic acid and H_2SO_4.[75] The *O*-methylation of carbohydrates by treating with TMAH (see representative

reactions of α-D-mannopyranose and an N-acetyl amino sugar in Figures 1.7 and 1.8, respectively) is based on substitution of the proton in a carbohydrate hydroxyl with a methyl group by stepwise reaction. The first step involves the deprotonation of a carbohydrate hydroxyl group in an equilibrium reaction with the formation of an alkoxide and water. The elimination of water from the system helps to complete conversion of the carbohydrate hydroxyl to the corresponding alkoxide.[75]

Generally, per-*O*-methylation of carbohydrates with a free hemiacetal group such as d-glucose, and d-fructose, gives isomers generated by two cyclic forms and the presence of anomeric centers. All of these isomers can be separated, leading to multiple peaks for one compound in the chromatogram. Sugars that do not have hemiacetal groups, such as sucrose, give only one peak.[72] The number of peaks that can be identified by GC-MS will, therefore, be higher than the real number of sugars in the samples.

***Anthrose*.** In bioanalysis, unique biomarkers are important for detection as well as for differentiation of bio-organisms. In 2004, Daubenspeck et al.[76] reported a unique tetrasaccharide in the major glycoprotein BclA on the surface of *B. anthracis* spores. The non-reducing end of this carbohydrate is capped with a highly specific monosaccharide that was named anthrose [2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucopyranose]. This component was reported to be absent in spores of related strains of bacteria[14,77] such as *B. cereus and B. thuringiensis.*[78] The anthrose-containing tetrasaccharide chain was claimed to be highly specific for *B. anthracis*, which makes it a unique biomarker for the detection of these spores.[79]

1.5 BIOMARKER DETECTION METHODS

Much effort has been made to develop bacterial identification methods based on biomarker detection. Conventional methods that have been employed for a long time are

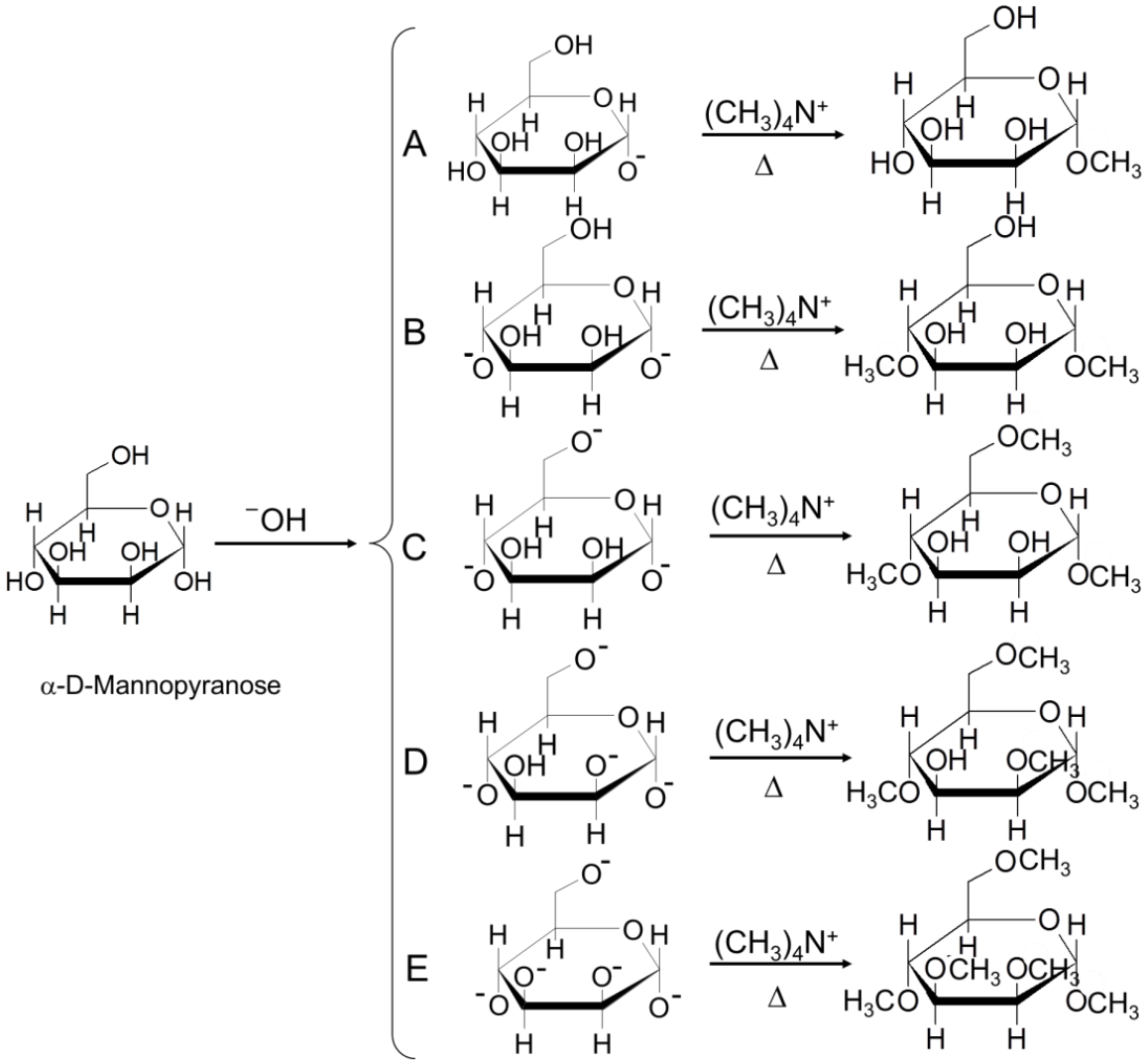

Figure 1.7. Methylation of sugars (e.g., α-D mannopyranose) to (A) mono, (B) di, (C) tri, (D) tetra, and (E) per-*O*-methylated carbohydrate forms using TMAH.

bacteriological assays, which are reliable, but time-consuming, laborious and not useful for field application. Currently, there are several other advanced approaches, that have been proposed and used for detection of *B. anthracis*, including immunological assays,[80] polymerase chain reaction (PCR), a primer-mediated enzymatic DNA amplification,[81-85] molecular fingerprinting,[86,87] and mass spectrometric (MS) analyses.[88] In these methods, the detection targets have mainly focused

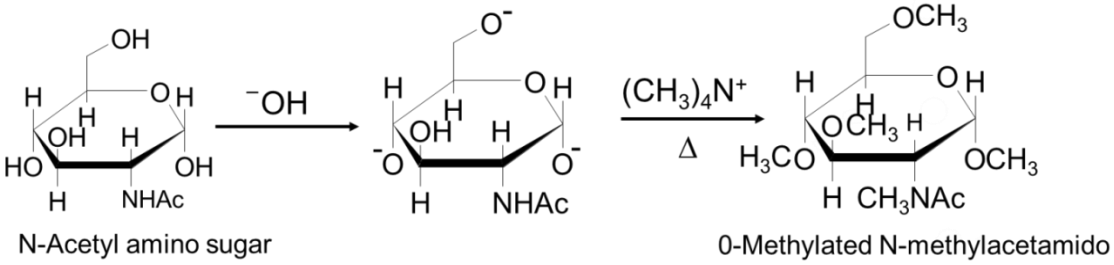

Figure 1.8. Methylation of amino sugars (e.g., N-acetyl aminosugar) to *O*-methylated N-methylacetamido using TMAH.

on the plasmids pXO1 and pXO2 (using PCR), gene polymorphisms, specific gene sequences (Ba813, rpoB, bclA) (using molecular fingerprinting), and SASPs (using MS) in the spores. However, these methods involve complicated protocols such as cell disruption, nucleic acid extraction and protein purification, and they cannot support convenient, rapid and real-time detection of *B. anthracis* as well as common microorganisms.[89-93] These disadvantages do not allow for field application using hand-portable systems. More detail for each are given in the following sections.

1.5.1 PCR and antibody methods

These methods rely on previously knowing the genetic sequence, without which they provide limited information. PCR-based methods, which assess size variation of intact products or restriction digests, are the most common molecular techniques for species discrimination. In PCR, the total DNA including plasmid DNA is extracted from the original specimen and used as template in a PCR assay.[94] However, both PCR and DNA sequencing techniques as mentioned before are time-consuming and laborious, especially when dealing with comparison of numerous strains.[12] PCR requires expensive reagents (primer and enzyme), molecular fluorophores, and considerable sample processing prior to analysis.

In the last two decades, immunoassays have been employed effectively to identify and differentiate microorganism spores/cells.[95] However, Immunoassays, which rely on the interaction between antibodies, can detect 10^5 organisms or cells in approximately 15 min and specific antibodies are needed for each desired agent.[96,97]

1.5.2 Vibrational spectroscopy methods

Vibrational spectroscopy provides highly specific chemical information content and, therefore, is capable of uniquely identifying target analytes. Both Fourier transform infrared and Raman spectroscopies have shown the ability to differentiate bacterial spores.

Raman spectroscopy. Raman spectroscopy (RaS) is a well-known technique for its specificity in chemical and biological analysis. RaS offers some distinct advantages over other spectroscopic methods for homeland security applications. RaS is well suited for applications in aqueous environments because of the small Raman scattering cross-section of water.[24] In contrast, application of near-infrared and mid-infrared spectroscopies is limited because of their inherent spectral congestion and competitive absorption of water. By 1992, improvements in Raman instrumentation and the use of resonance enhancement dramatically improved detection limits and significantly reduced analysis times (to less than 1 h).[25,26]

After September 11, 2001, a Raman spectroscopy system was used to detect *Bacillus* spores in letters when they passed through a mail sorting process by measuring the dominant calcium dipicolinate Raman spectral bands associated with the spore core. However, this method required large endospore sample sizes because its detection limit, using 400 mW of 785 nm laser excitation for 1 s acquisition, was estimated at 4.5 mg, equivalent to 4.5×10^9 spores.[24] Current research using surface-enhanced Raman scattering (SERS) coupled with Raman integrated tunable sensor technology provides great promise for the simultaneous detection of many

different chemicals as well as biological species. A portable Raman device was also developed that can quickly provide both qualitative and quantitative analysis without disturbing the sample. Using SERS, the detection limits and sensitivities of this technique can allow for trace analysis of various chemical and biological (CB) agents.[98]

FTIR spectroscopy. Fourier transform infrared has been used successfully to distinguish between sporulated and vegetative *Bacillus* species.[99,100] Valentine and others have demonstrated that vegetative bacteria and endospores have unique IR signatures that can be used to identify them and, in some cases, differentiate even at the strain level.[101] However, application of near-infrared and mid-infrared spectroscopies is limited because of inherent spectral congestion and competitive absorption of water.[96]

1.5.3. LC-MS

Recently, liquid chromatography with MS detection has been employed to detect conserved proteins that have been used as biomarkers for bacterial identification and discrimination[88] and for stablishing phylogenetic correlations between closely related species or sub-species.[102] In LC-MS, matrix assisted laser desorption time-of-flight mass spectrometry (MALDI TOF MS), which was pioneered by Karas and Hillenkamp in 1987,[103,104] has been effectively used. In addition, a new aerosol time-of-flight mass spectrometer (ATOFMS) was developed. The combination of ATOFMS with laser-induced fluorescence selection and MALDI mass spectrometry allows for real-time analysis of single bioaerosol particles.[105] In reality, MALDI is a popular technique for bacterial identification based on the analysis of extracts from bacterial vegetative cells or spores.[12] MALDI-MS has been used for indentifying the protein biomarkers for some human pathogens.[106,107]

Besides MALDI, electrospray ionization (ESI) has also been used. It was demonstrated to be a more comprehensive method, allowing for the analysis of intact proteins such as SASPs[26,30] that are normally released by treatment with 2 N HCl.[108] ESI is typically employed in tandem with high performance liquid chromatography (HPLC), usually for proteomic applications in combination with a nano electrospray configuration.[109] Unlike in ESI, generally, only singly charged ions are observed with MALDI[110]in single MS and tandem MS modes after these proteins are converted into peptides by enzymatic digestion.[12,111] MS is rapid (a run takes minutes vs. days for classical microbiology or toxicology experiments), sensitive (biothreats can be identified in a sample containing fewer than 10^4 intact organisms, or less than several femtomoles of a toxin), robust, automatable, and can be used to provide date for bioinformatics algorithms.[4]

1.5.4 Photoluminescence

DPA in spores was detected based on DPA-triggered terbium photoluminescence assay.[112] The binding of a chelate (DPA) to Tb^{3+}, when excited with UV light, can transfer energy to the emissive state of Tb^{3+}, leading to great emission intensity enhancement (> 20,000 times). The original Tb3+ excitation spectrum (λ_{ex} = 270 nm) is changed to a characteristic dual-peak spectrum (λ_{ex} = 2 73, 279 nm) upon complexation with DPA.[112] This specific change was used to detect DPA from endospores.

1.5.5 GC-MS

Although the methods introduced above have advantages in detection of biomarkers, they cannot be easily used for fast and in-field analyses due to their complex sample preparation and instrumental operation (HPLC-MS). Several of them (FTIR, Raman, and photoluminescence) fail to provide enough identification information for the differentiation of *Bacillus* species from

each other and from other organisms. GC-MS overcomes most of the limitation of these other methods.

Gas chromatography with MS detection. Gas chromatography coupled with MS using different ionization methods, including electron impact ionization (EI) and chemical ionization (CI), and different mass analysers, such as quadrupole, ion trap, and TOF, can be used for detection of biomarkers.[110] MS fragmented ions (one time or several times for MS^n) can be used effectively to identify targets in biosamples. With chromatographic retention times, mass spectra provide complementary information for positive identification. For detection of DPA, a major EI fragment ion at m/z 137 produced by sequential losses of H_2CO and CO from the molecular ion was used, combined with full scan MS, in which less intense m/z 165 and m/z 164 ions were also observed from the fragmentation of ($M+^{\bullet}$- H_2CO) and ($M+^{\bullet}$ - $^{\bullet}OCH$), respectively. Another prominent ion present in the spectrum is m/z 105, which results from loss of CH_3OH from the remaining ester substituent on the pyridine ring. Finally, a minor ion at m/z 77 in the EI fragmentation pathway for DPA is from m/z 105 after losing CO.[113] The use of the NIST mass spectral library simplifies the identification of target compounds, including most biomarker derivatives.

Pyrolysis gas chromatography with MS detection. Compositions of bacterial pyrolyzates have been studied using various techniques to obtain characteristics of thermal cleavage products and to elucidate the identities and origins of thermally generated compounds. Pyrolysis combined with MS can provide identification information from many biomaterials such as proteins, nucleic acids, and fatty acids. With *Bacillus* and *Clostridium* endospores, DPAME formed normally from DPA and TMAH, can be detected easily with the coupled technique. This was used for the differentiation of Gram-negative and Gram-positive

organisms. However, thermally generated products at high temperature in pyrolysis do not provide enough information to distinguish bacterial species or even to differentiate between prokaryotic and eukaryotic organisms.[114]

GC-MS field detection. TCM has been reported to be a feasible technique for detecting biomarkers using field-portable pyrolysis quadrupole ion trap mass spectrometry. This combination was employed for the differentiation of four pathogenic bacteria: *Bacillus anthracis, Francisella tularensis, Brucella melitensis,* and *Yersinia pestis.*[115]

The combination of pyrolysis and TCM using TMAH allows both efficient cleavage of polar bonds and subsequent methylation of COOH, OH, and NH_2 groups, thereby releasing of less polar, GC-amenable compounds.[116] This combination was successfully applied to a number of natural macromolecules including biopolymers, humic substances, and kerogens.[117] There have been reports in which PY-TMAH-GC-MS was developed to identify some key prebiotic molecules such as carbohydrates,[118] lipids,[119] chiral hydroxy carboxylic acids,[120] and the 20 protein amino acids.[116,121]

However, sample complexity is a problem, and further simplification is needed for pyrolysis and TCM to be really useful for field application. To partially solve this problem, we developed several non-traditional sample introduction tools whose operational principles relate to pyrolysis and solid phase micro extraction. These devices are presented in Section 1.6.

1.5.6 Solid phase micro extraction (SPME)

SPME provides an attractive alternative over traditional sample introduction by combining sampling, preconcentration, and direct transfer of the sample into a GC injector. It also minimizes the introduction of impurities and contamination of the GC system.[122]

SPME is based on partition equilibrium of analytes between the sample environment and a stationary phase. The stationary phase, normally called the SPME fiber coating, can be a liquid polymer, a solid sorbent, or a combination of both coated on a fused silica or metal fiber. After being collected in the sorbent, analytes are desorbed inside an injector and carried by a carrier flow into a suitable separation and detection system, usually a gas or liquid chromatographic system.[123] SPME has been developed and improved by Pawliszyn and co-workers. The main use of SPME is in GC; therefore, the extracted analytes are thermally desorbed in the GC injector.[124,125] SPME can be used for a number of matrices, including water, air, and soil.[126,127] It can also be used for other matrices, such as emulsions or suspensions in which the targets can be selectively isolated by a suitable stationary phase.

The theory of SPME is quite simple; the amount of analyte extracted by the coating can be described by

$$n = \frac{K_{fs} V_f V_s C_o}{K_{fs} V_f + V_s} \qquad (1.1)$$

where K_{fs} is the fiber coating/sample matrix distribution constant, V_f is the fiber coating volume, V_s is the sample volume, and C_o is the initial concentration of the target in the sample. In the case that the sample volume is very large ($K_{fs}V_f << V_s$), the equation can be simplified to and the determination of unknown concentrations of analytes in water by SPME can be facilitated with different approaches such as with external and internal standard calibration.[128] Simply speaking, in water medium and at equilibrium conditions, the amount of a target analyte is proportional to the fiber coating volume and the coating/water partition coefficient, K_{fw}, that is normally

$$n = K_{fs} V_f C_o \qquad (1.2)$$

experimentally established or determined by using another approach as described by Pawliszyn et al.[126,129]

For headspace SPME, at equilibrium, the amount extracted by the fiber coating is described by Equation 1.3, where K_{aw} and K_{fg} are the Henry's law and fiber coating/gas partition coefficients, respectively; V_w, V_f, and Vg are the volumes of the water, fiber coating and headspace, respectively; and C_{wo} is the initial concentration of the analyte in the water solution. The K_{aw} value can be calculated from Equation 1.3 if K_{fg} is known. Similarly, once the K_{aw} value is established, K_{fw} can be determined using Equation 1.4. Alternatively, K_{fw} can be determined by direct SPME water extractions (Equation 1.5). A simple, yet highly reliable method of establishing or accurately estimating K_{fw} for a number of analytes in aqueous matrices would allow widespread application of SPME for water sampling. In addition, rapid and accurate knowledge of K_{fw} values would ensure proper experimental design, including selection of appropriate sample and fiber volumes, estimation of the detection limits, and quantitation of target analytes for which standards are not available.[129]

$$n = \frac{C_w^o K_{fg} K_{aw} V_f V_w}{K_{fg} K_{aw} V_f + K_{aw} V_g + V_w} \quad (1.3)$$

$$n = K_{fg} K_{aw} \quad (1.4)$$

$$n = \frac{C_w K_{fw} V_f V_w}{K_{fw} V_f + V_w} \quad (1.5)$$

1.6. NON-TRADITIONAL SAMPLE INTRODUCTION

1.6.1 Coiled wire filament sampling probe

A simple device for field sampling and concentration of analytes for subsequent introduction into an injection port for GC analysis consists of a tiny, coiled platinum wire filament (CWF) that is attached to a retractable plunger wire, which fits inside a syringe needle housing. Sampling is accomplished by dipping the end of the CWF in a liquid sample, which is drawn into the wire coil by capillary action, and introducing it into the injection port either before or after allowing the solvent to evaporate. The CWF can be used with or without a nonvolatile chemical coating. A major advantage of this sampling device is that nonvolatile sample matrix components remain on the wire coil, reducing the required injection port and liner cleaning frequency and contamination of the head of the chromatographic column. The coil itself can be easily cleaned between analyses by rinsing and/or burning off residual material in a small flame. The sampling coil facilitates specifically designed chemical reactions in the injection port, such as thermochemolysis and methylation (TCM). Applications demonstrated in Chapter 2 include: (1) direct introduction of samples with little or no pre-treatment, (2) simultaneous thermochemolysis and methylation of lipid-containing samples such as bacteria and bacterial endospores for analysis of biomarkers, and (3) solid phase micro-extraction (SPME) using temporary wire coatings.

The CWF allowes for significant reduction in sample preparation time, in most cases to less than in few minutes. The peak shapes examined for polycyclic aromatic hydrocarbon analytes (PAHs) were significantly better (asymmetry factors <1.3) when using the CWF sampling technique compared to splitless and on-column injection techniques (asymmetry factors >1.3). Extraction efficiencies for SPME (especially for high boiling point components

such as PAHs), improved by an average of 2.5 times when using the CWF compared to the performance of commercially available SPME fibers. Coiled wire filaments and GC injection port liners were used for more than 100 *Bacillus* endospore TCM analyses without the need for cleaning or replacement. Detailed results and discussion are presented in Chapter 2.

1.6.2 Coated-needle extraction

Coated-needle extraction (CNE) was performed using a conventional syringe needle, was investigated in our laboratory to improve the efficiency of SPME by speeding up the extraction and shortening the equilibrium time because of dynamic movement of sample fluid across the surface of the stationary phase layer coated inside a tiny needle. A similar method called solid phase dynamic extraction (SPDE) has been in use since the early 2000s by Chromtech GmbH (Idstein/Germany). SPDE and CNE allow for dynamic extraction of samples with a significantly larger amount of sorbent material and larger surface area (compared to SPME). They are normally employed, combined with rapid sample flow over the coating, thereby ensuring short extraction time. Both methods offer time and labor savings for the analysis of many different samples in either both liquid or headspace. Operation of SPDE and CNE simply requires a gas-tight syringe (different volumes available) equipped with an extraction phase coated needle. The coatings can be applied in various thicknesses and surface areas. The sorption process is carried out in a liquid or headspace sample by repeatedly moving the plunger in and out, which allows a distribution balance to be reached between the sample matrix and the sorbent phase. After sorption, the syringe is moved to the GC system, the needle is inserted into the heated GC inlet, and the analytes are thermally desorbed. Different from our method, Chromtech first withdraws carrier gas into the syringe barrel from a fiber bake-out station (this station also is used to dry the coating and syringe) before inserting the needle into the injection port for desorption. The

additional gas flow from the syringe forces the analytes into the inlet, thereby ensuring sharp peak shapes and minimizing carryover.

An automated SPDE method was also developed and used for different field applications, such as forensic (amphetamines in hair detection)[130] or medical tests (i.e., gamma hydroxyl butyric acid in urine, and trichloroethanol, metabolite of chloral hydrate and propofol in subtherapeutic concentrations in blood) were analyzed by Barbora Maralíková et al. CNE can replace conventional SPME for sampling and sample introduction. Detailed results and discussion are presented in Chapter 6.

1.7 SAMPLES

1.7.1 Growth conditions and cell/spore characteristics

When cultured under most growth conditions, *B. anthracis* cells have a distinctive morphology. The vegetative cells which appear as approximately 5-8 µm square ended rods have the propensity to form chains. By using India Ink exclusion, BA capsules can be microscopically visualized. Although formation of pellicles during static incubation and adherence to solid surfaces can occur, BA generally grows as planktonic cells in liquid media.[5,131,132]

Different from related *Bacillus* species, BA is best distinguished by its ability to synthesize the anthrax toxin proteins and the poly-γ-D-glutamic acid capsule. Theresa[5] reported that optimal synthesis of the toxin proteins occurs during culture at 37°C in media containing glucose as the carbon source and bicarbonate. When compared with growth at lower temperature, Sirard also indicated that the temperature affects toxin gene expression; cells grown at 37°C exhibit 4 to 6 fold higher levels of toxin gene transcripts than ones growing at 28°C.[133] After sporulation, the exosporium of spores of BA is a distinguishing characteristic, an outermost layer that is not present around spores of many other *Bacillus* species. The exosporium has been

visualized using electron crystallography and atomic-force microscopy. [134,135] BA is a facultative anaerobe that can multiply readily in a variety of common laboratory media using multiple sugars and amino acids as carbon sources.[131] Therefore, many studies of carbohydrate metabolism have been performed to determine relationships between sugar utilization and toxin synthesis. The requirements of nutrition for BA are still not clear and appear to vary between strains. Several acid amines such as methionine and thiamine are required for growth, but growth in glucose-salts media containing methionine and thiamine requires supplementation with multiple amino acids, which most likely serve as sources of nitrogen.[136] At a minimum according to Hoffmaster,[137] the medium must contain glucose, ferric chloride, thiamine, glutamic acid, glycine, methionine, proline, serine, threonine, ammonium sulfate, magnesium sulfate, manganese sulfate, potassium phosphate, and sodium citrate; and the optimal growth temperature for BA reported by Dai and Passalacqua[139] is 37ºC (the bacterium is unable to grow at temperatures above 43ºC).[138,139]

1.7.2 *Bacillus* endospore samples

The same procedures and conditions were used for culturing bacterial endospores of BA, BC, BG, and BT. Leighton-Doi (LD) and Columbia agar (Col) plates were the two main media; a salt dextrose medium (magnesium sulfate heptahydrate, manganese sulfate monohydrate, ferrous sulfate heptahydrate, potassium chloride, calcium chloride dihydrate, and dextrose) was added to help in the formation of spores. Isolation plates were prepared on untreated Col, and a sample of pure culture was then used to cover the salt dextrose plates, which were then incubated at 32ºC or 37ºC for two weeks. After verification that 90-95% sporulation had occurred (through phase contrast microscopy), the spores were collected from the agar plates and heat treated to kill

any remaining vegetative spores. Spores were washed once a day for three days before GC-MS analysis.

1.8 THERMOCHEMOLYSIS AND METHYLATION

Thermochemolysis and methylation (TCM) appeared a long time ago as a rapid and effective tool for the molecular characterization of various classes of organic compounds in soils such as lipids, lignin units and carbohydrates.[140,141] Generally, *in situ* TCM can produce derivatives amenable to GC from different chemical groups of biological materials including fatty acids, dipicolinic acid, DNA, RNA, carbohydrates, ubiquinones, phospholipids, amino acids, peptides and proteins.[110]

TCM combined with GC-MS can be used to detect fatty acids, n-alkanols, ω-diacids, and ω-hydroxyacids via their methyl esters. In addition, different ligneous subunits and permethylated deoxy aldonic acids derived from carbohydrates, as introduced above, can also be produced by TCM and detected by GC-MS. For fatty acids and sugars, by using TCM, all saturated and unsaturated (one double bond) compounds from C8 to C18, and branched iso and anteiso C15 and C17 components, which are typical of *Bacillus* endospores,[142] can be produced. For sugars, TCM produces a series of permethylated deoxy aldonic acids or permethylated deoxy aldonic acids from glucose/mannose (Glu, the most abundant), galactose (Gal), xylose (Xyl), rhamnose (Rha) and fucose (Fuc).[140]

Reagents for TCM. Tetramethylammonium hydroxide (TMAH) was recently introduced for the qualitative characterization of organic matter from natural waters using TCM.[143] Such characterizations were usually of a qualitative nature, and any semi-quantitative assessments of individual compounds were often achieved by measuring relative peak areas and assuming unity as a response factor. TCM with TMAH is an analytical technique has first introduced for the

characterization of synthetic phenolic polymers.[144,145] It has subsequently been used to assess the molecular composition, degradation state and taxonomic source of bio- and geo-macromolecules in natural materials such as lignin, cellulose, cutin, suberin and humic substances.[118,146] Upon heating with TMAH, macro-molecules are chemolytically hydrolyzed and methylated, yielding low molecular weight compounds that are amenable to separation and detection by GC-MS.[147] Different from conventional high temperature pyrolysis using TMAH, in normal pyrolysis, more structural units are preserved because, in high temperature pyrolysis, the sample is heated up to 400ºC or higher.[148] In the case of off-line reaction, the heating time can be quite long (30 min) and the TMAH concentration can be high (1:1 sample/TMAH 25% w/w).[116] Recently, TCM was used combined with coiled-wire-filament sample introduction and GC-MS to detect diverse biomolecules present in bio-organisms[149] and plant materials.[150] TCM in this method was performed at lower temperature than that used in conventional pyrolysis GC-MS (300-350ºC vs. 400-600ºC).[151] In thermochemolysis, heat is primarily used to drive the reaction between the methylating reagent and the analyte functional groups, while at the same time assisting in base-catalyzed hydrolysis of ester and ether bonds, and to a lesser extent thermal fragmentation.[150] Similar to TMAH, tetraethylammonium acetate (TEAAc)[152] or trimethylsulfonium hydroxide (TMSH) can be used for TCM. However, TEAAc is specifically used for derivatization of free fatty acids, while TMAH can be used for free and covalently bound fatty acids, and at low temperature.[150] Actually, TMAH is not only a methylating agent, but it is also a strong base, and likely induces a variety of reactions. It can be used for off-line TCM at 250ºC (for 30 min) or 300ºC (for 10 min), and for much shorter time (1 min) at 300ºC when using on-line TCM with a coiled wire filament.[153,154] When using TMAH as a reagent for profiling fatty acids as methyl esters, there was no significant discrimination for saturated, monounsaturated and cyclopropane

FAs when using temperatures up to 550°C; the same result was also found for *cis*/*trans* ratios of monounsaturated FAs.[157] These results are comparable with FAME profiles obtained by classical preparation methods (including solvent extraction, alkaline saponification, and methylation). However, they differ greatly with results from conventional pyrolysis systems (both Curie-point or flash pyrolyzers) for which much longer TCM times are used.[155]

1.9 INSTRUMENTATION USED FOR THIS RESEARCH

The instrumentation primarily used for work reported in this dissertation was an Agilent 6890-5973 GC-MS, it was used in combination with Chemstation software. MS was equipped with an electron ionization (EI) source and quadrupole analyzer. Chromatographic columns including medium-polar and polar columns from Restek, Phonominex, and Agilent. CWF, SPME, and CNE were used for injection of samples for specific analyses.

1.10 OVERVIEW OF THIS DISSERTATION

Effective methods for identifying different biomarkers for the detection and differentiation of BA from related *Bacillus* species and other biological agents by GC-MS techniques requires a combination of various techniques. These techniques can include both traditional and non-traditional approaches. The challenge addressed by this dissertation is the detection and differentiation of BA from its closely-related species as quickly and simply as possible.

In Chapter 1, issues involved in developing methods to detect and differentiate BA endospores from other species have been presented. They include information on the composition and key biomarkers of *Bacillus* endospores, and the current methods used to detect and differentiate them. This chapter also briefly introduces the TCM methods and tools developed in this work to better analyze for these biomarkers quickly and accurately.

Chapter 2 introduces a new sample introduction device, a coiled wire filament (CWF) that was built and used for the introduction of solid samples into the GC injector in order to simplify and improve on-line thermochemolysis methylation for detection of endospore biomarkers. This device can also be used for solventless sample injection in general. The contents of Chapter 3 describe a new TCM method using the CWF for differentiation of four *Bacillus* species, BA, BC, BG, and BT, grown at different temperatures and different media. TMAH was used for TCM, and the CWF sample injection method and GC-MS were used for profiling the biomarker derivatives for detection and discrimination of endospores from DPAME and specific FAMES. Additional biomarkers were detected and exploited for the detection and differentiation of *Bacillus* species endospores as described in Chapters 4 and 5. DPAME, FAMEs and methylated sugars produced from TCM using H_2SO_4 at high temperature (120-140°C) followed by TMAH addition led to the identification of the new biomarkers. The CWF combined with a concentration step during sample preparation allowed for significant decrease in the detection limits and improved the differentiation of not only the four *Bacillus* species but also *Clostridium* and other microorganisms, such as YP, and FT. Chapter 5 deals more with the detection of anthrose, a unique biomarker of BA. Optimization of the TCM procedure using H_2SO_4 at high concentration and temperature, and evaluation of SPME with different fibers was done to maintain the detection sensitivity and to detect new biomarkers. In this approach DPA, FAs, 3-hydroxy butyric acid and an anthrose by-product, 3-methyl-3-hydroxy butyric acid, were detected and used for positive detection of to BA. Chapter 6 describes the development and application of CNE, which could replace conventional SPME for many applications. Methods for production, as well as, advantages and disadvantages of the device and approach compared to conventional SPME for the detection of biomarkers and other analytes are presented in this

chapter. Finally, Chapter 7 presents the general conclusions of this research and outlines additional research that could be done to improve and extend this work.

1.11 REFERENCES

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2 SAMPLE INTRODUCTION IN GAS CHROMATOGRAPHY USING A COILED WIRE FILAMENT

2.1 INTRODUCTION

In addition to the well-known hypodermic syringe in which a liquid or gas is sampled via a plunger-in-needle device, several other syringe-based devices intended for sample introduction into a standard gas chromatograph (GC) have been reported. Hypodermic syringes have been used as a means to introduce solids[1,2] or condensed vapors[3,4] sometimes with the inclusion of derivatization reagents inside the needle.[5] Needles packed with quartz wool were used to trap particulate matter and desorb volatiles inside the GC inlet.[6,7] A syringe-like device holding a removable capillary tube was used to sample "dirty" samples that had adhered onto the inner surface of the capillary following low-temperature removal of the solvent.[8] A method for concentrating an analyte on the tip of a syringe needle while inside a GC inlet was recently patented.[9] Several versions of syringes with an extendable solid body housed within a septum-penetrating needle have been reported. Hollowed out plungers possessing a small side vent were developed for capillary uptake and carrier gas expulsion of a sample.[10,11] Other extendable bodies intended mostly for solid samples have included straight[12] or flattened and twisted[13,14] wires or plungers with grooves[15] as well as tubes with "tongues,"[16,17]needles with troughs,[18] or needles with "windows."[19] Sorbent materials have been installed on the inside of capillary tubes or on fibers or hollow bodies that can be extended from within syringe needles,[20-22] including helical-shaped metal wires extendable from within a syringe.[23-26] In this chapter, a new syringe-type sampling probe in the form of a coiled wire filament (CWF) is described. The CWF was designed for flexibility in GC sample introduction, allowing injection of both liquid and solid

(i.e., solvent-less) samples, solid phase micro-extraction (SPME), and reaction-facilitated volatilization inside the heated injection port. Various types of samples ranging in complexity were analyzed and simultaneous thermochemolysis and methylation for differentiation of bacterial endospores is described. Additionally, the ease with which this device performs SPME is demonstrated using temporary coatings of polymeric materials. Because the CWF greatly expands the types of samples that may be analyzed directly by GC, it is a complement to SPME and other solid-probe-type sample introduction techniques.

2.2 EXPERIMENTAL

2.2.1 Chemicals, materials, and samples

HPLC grade methanol, dichloromethane, and *n*-hexane were obtained from EMD (San Diego, CA, USA). Tetramethylammonium hydroxide pentahydrate (TMAH, >97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). H_2SO_4 (98%) was from Mallinckrodt Chemical (Phillipsburg, NJ, USA). Polycyclic aromatic hydrocarbons (PAHs) were obtained from a variety of sources. Apiezon N was from Kurt J. Lesker (Gilbert, AZ, USA). Pt-Ir wire (90% Pt, 10% Ir, approximately 90 µm diameter) was obtained from California Fine Wire (Grover Beach, CA; www.calfinewire.com). A solution of PAHs was prepared by dissolving naphthalene, 1-methylnaphthalene, biphenyl, 2,6 dimethylnaphthalene, 2,3-dimethylnaphthalene, acenaphthene, fluorene, diphenylmethane, and anthracene in dichloromethane at concentrations between 10 and 50 ppm each. This sample was analyzed using CWF, conventional splitless, and cold on-column injection techniques for comparison. Vegetative bacteria or endospores were produced in a biosafety level-3 facility located on the Brigham Young University campus. *Yersinia pestis* was grown on Columbia agar at 32ºC, *Francisella tularensis* was grown on

enriched Mueller-Hinton agar at 32ºC, and endospores of *Bacillus thuringiensis* (BT) were grown in Leighton-89 Doi broth at 32ºC.

2.2.2 Coiled wire filament (CWF)

The 90 µm O.D. Pt-Ir wire was deflection coiled by Motion Dynamics (Fruitport, MI; http://www.motiondc.com). Pt-Ir was chosen because of its increased stiffness relative to platinum, which allowed for better control over the coiling process. This family of materials generally exhibits high strength and oxidation resistance.[27] The length of the coiled section was 1.2 cm, the coil outer diameter was approximately 360 µm, and the coil pitch was approximately 180 µm (Figure 2.1). A 0.5-cm straight "nib" was left on one end of the coil so that it could be inserted inside the empty socket of a Supelco SPME assembly and mechanically held in place by compressing the socket against the nib with a DMC crimping tool (Orlando, FL; www.dmctools.com).

2.2.3 Sample introduction using the CWF

During sampling, a small volume (~0.65 µL) of the sample was withdrawn onto the coiled wire by dipping the coil at least partially into the sample. Capillary forces rapidly drew the sample into the CWF and prevented any loss during sample transfer to the injection port. After drying in air (~20 s), the coil was retracted inside the syringe, the syringe was inserted inside the GC injection port, and the coil was extended. Immediately, the GC program was begun and the coil was left inside the injection port for 60 s. The coil and needle were solvent- or flame-cleaned (e.g., with a butane lighter or Bunsen burner) prior to subsequent use (the melting temperature of 90-10 Pt-Ir is approximately 1800ºC). [28]

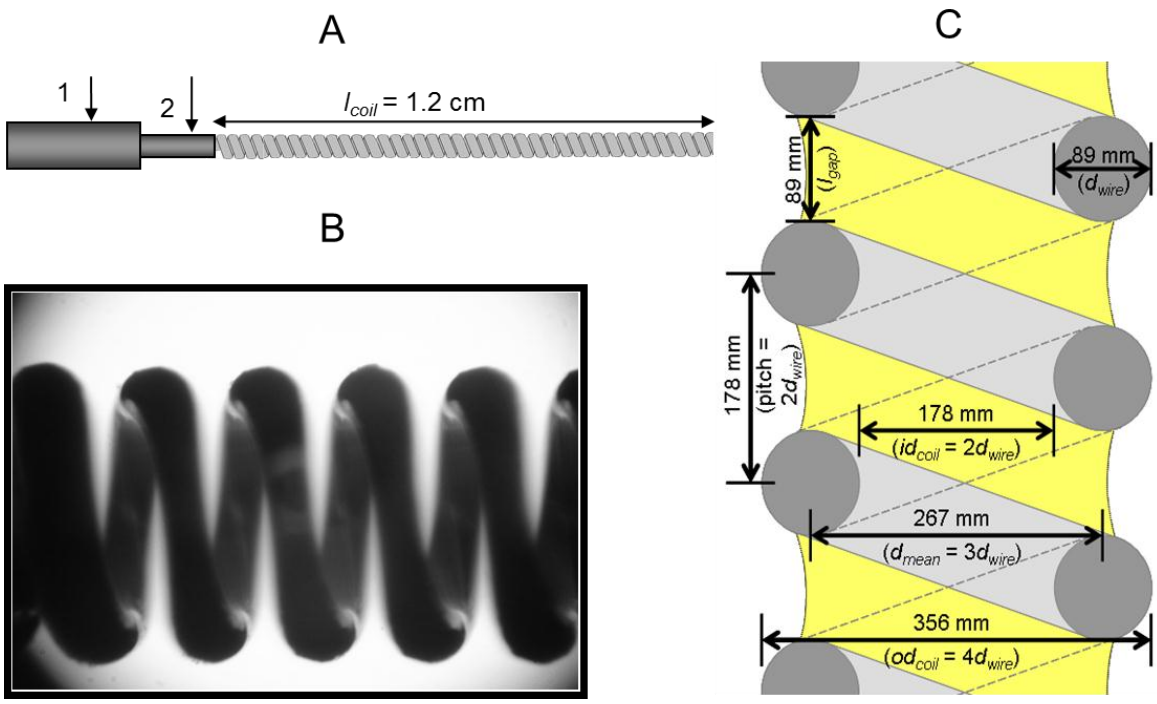

Figure 2.1. (A) Schematic drawing of a coiled wire filament. The wire filament is held in place by a socket (2), which may be extended from or retracted inside an SPME needle (1). The coil diameter is 0.356 mm and the pitch is 0.178±0.013 mm. (B) Photograph of an actual coiled wire filament produced by deflection coiling. (C) Cutaway schematic of the coil showing its dimensions.

2.2.4 Thermochemolysis methylation of (TCM) bacteria and bacterial endospores

A 500-µL volume of methanolic H_2SO_4 (1%, v/v) was added to a cell/spore pellet as received from the microbiology laboratory (i.e., 108-1010 cells/spores, slightly wet from the residual rinse water that had been used to clean them). The mixture was allowed to react for approximately 1 min at room temperature. A 200-µL volume of the cell/spore mixture was combined with 40 µL of 2.0 M methanolic TMAH and 40 µL of internal standard (50 ppm chrysene in a mixture of methanol and dichloromethane, 10:1, v/v). Sample introduction into the heated GC injection port for completion of the TCM reactions and for GC-MS analysis was performed as described in the previous chapter.

2.2.5 GC-MS Analysis

An Agilent 6890 gas chromatograph (Agilent, San Jose, CA) with split/splitless injector was fitted with a 0.75 mm i.d. Restek liner to accommodate the sampling device. An Agilent 5793 MS with electron ionization and quadrupole analyzer was used as the detector (230ºC quadrupole temperature, 150ºC source temperature, 30-500 mass range, 1670 EM voltage, 35 µA emission current, and 70 eV ionizing voltage). Two GC capillary columns were used: J&W/Agilent DB-5, 30 m × 250 µm i.d. × 0.25 µm film thickness (column A) and J&W/Agilent DB-5, 10 m × 100 µm i.d. × 0.4 µm film thickness (column B). Typical chromatographic operating parameters were as follows: the injector was maintained at 290ºC and operated at constant inlet pressure (12-75 psi, depending on the column length and i.d.) with 2 min splitless time, the column was heated from 60 to 305ºC at 14ºC/min, and the transfer line to the mass spectrometer was maintained at 270ºC.

2.3 RESULTS AND DISCUSSION

2.3.1 Sampling volume reproducibility using the CWF

Two individual machine-coiled wires were dipped into each of two solutions (methylene chloride or methanol) spiked with chrysene, allowed to dry, and injected into the GC. A total of nine injections were made for each wire with each solvent, and flaming of the wire was carried out between each three consecutive samples. A calibration curve was produced by transferring between 0.3 and 0.9 µL of the same methylene chloride or methanol solutions via microsyringe to a CWF in order to correlate peak areas with sample volumes. Using the calibration data, the average volumes taken up were 0.66 µL (±0.03) µL and 0.64 (±0.02) µL, respectively, for the above solvents (numbers in parentheses are 95% confidence intervals on the mean). It is more useful, however, to consider the overall confidence intervals, since they indicate the error of the

method. Overall, 95% confidence intervals were ±0.13 and ±0.09 µL, respectively, giving ±19 and ±14% as expected deviations from the means reported above. Thus, 19 out of 20 liquid samples taken up by the wire should be between 0.53 and 0.79 µL or 0.55 and 0.73 µL, depending on whether the solvent is methylene chloride or methanol, respectively.

2.3.2 Coiled wire filament vs. conventional injection techniques

The CWF injection technique was compared against two conventional injection techniques, i.e., splitless injection and on-column injection. Sample volumes (1 µL) of a solution of 9 PAHs at concentrations between 10 and 50 ppm each were introduced according to each technique. In the case of CWF injection, a 1 µL volume of sample was placed directly on the wire coil using a syringe, and the solvent was evaporated before introducing the CWF into the injection port. Representative chromatograms are shown in Figure 2.2 for which the peak asymmetry factors (AsFs) are also reported. Visual inspection of the chromatograms and comparison of the AsFs reveal that the chromatographic peaks are more symmetrical when using CWF injection compared to the other injection techniques due to the rapid rate of sample volatilization; the lack of thermal, diluent, and other band-broadening effects associated with solvent evaporation;[29] and the use of a narrow inlet liner (note that the time scale for the on-column injection chromatogram is slightly expanded because of the lower initial temperature required for this technique).

2.3.3 Thermochemolysis methylation and analysis of bacteria and bacterial endospores

Biomarker derivatives have been studied for a number of years for the purpose of differentiating microorganisms.[30-32] FAMEs (C8-C20), picolinic acid methyl ester (PAME), dipicolinic acid methyl ester (DPAME), 3-hydroxy butyric acid, various amines (derived from proteins and DNA, the amine derivatives observed were 1,3,5-trimethyl-2,4(1H,3H)-

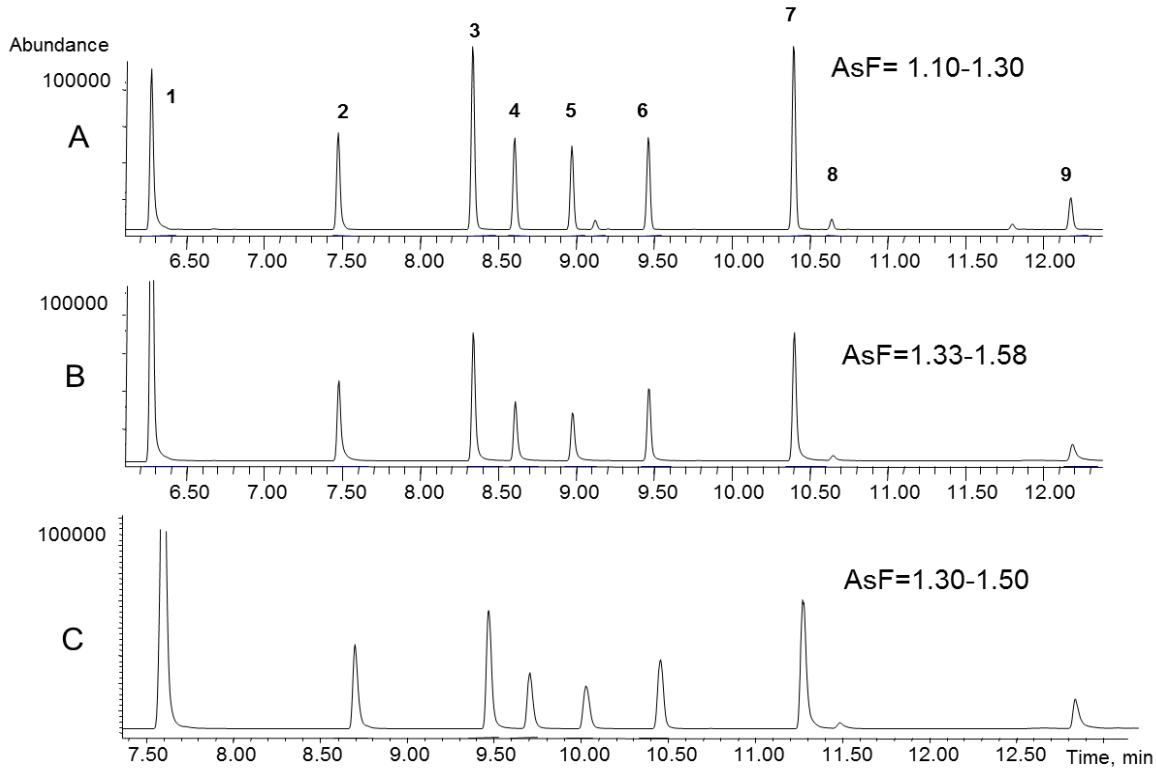

Figure 2.2. Chromatograms of standard PAHs obtained using (A) CWF, (B) splitless, and (C) on-column injection techniques. Conditions: column A; (A) and (B) 60ºC initial temperature, 14ºC/min rate, 305ºC final temperature, 290ºC injector temperature, 12.4 psi He pressure; (C) 60 ºC initial temperature, 14ºC/min rate, 305ºC final temperature, 50ºC initial injector temperature, 14ºC/min injector rate, 290ºC injector final temperature, 12.4 psi He pressure. Peak identifications: (1) naphthalene, (2) 1-methylnaphthalene, (3) biphenyl, (4) 2,7 dimethylnaphthalene, (5) 2,3-dimethylnaphthalene, (6) acenaphthene, (7) fluorene, (8) diphenyl methane, and (9) anthracene.

pyrimidinedione; 3-methyl dihydro-2,4(1H,3H)-pyrimidinedione; 1,3-dimethyl-2,4(1H,3H)-pyrimidinedione; N,9-dimethyl-9H-purine-6-amine; N,N,9-trimethyl-9H-purine-6-amine; 6-amino-1-methylpurine; l-proline-1-methyl-5-oxo-methyl ester; and 4-methylproline methyl ester. Specifically, µ-d-allopyranoside, methyl-6-deoxy-2-*O*-methyl and methyl-2,3,4-tri-*O*-methyl-6-deoxy-µ-d-mannopyranoside isomers), and carbohydrates are examples of biomarker compounds that have been used to differentiate various bacteria and bacterial endospores. In this

study, two bacterial species (Y. pestis and F. tularensis) and an endospore sample (BT) were treated in methanolic sulfuric acid at room temperature and subsequently derivatized with TMAH in the GC injection port.

Extracted ion chromatograms of representative reaction products of these samples are shown in Figure 2.3. Investigation of the reaction conditions was carried out in order to optimize the yield of FAMEs (from C15:0 to C18:0) and DPAME, two key biomarker types. In order to expedite the analysis, the GC oven temperature program was ramped from 75 to 270ºC at 10ºC/min. The responses of iso C15:0 FAME (chosen as a representative FAME) and DPAME at injector temperatures of 270, 280, 290, 300, and 310ºC (injection time of 1 min) and then at various injection times (i.e., time that the CWF is held in the injector) from 1 to 40 s at 290ºC showed maximum intensities at 290ºC and injection times ≥20 s. DPA demonstrated a lower degree of methylation relative to iso C15:0 fatty acid at short injection time and low injection temperature because (1) DPA has two sites of methylation, while fatty acids only have one; (2) DPA is located in the central part of the spore, which is protected by outer protein and peptidoglycan structures,[33] while fatty acids are present in the spore exosporium, outer membrane, and inner membrane[33,34] and, thus, are more accessible to the TMAH reagent; and (3) DPA is complexed with Ca^{2+} in the spore,[35] which inhibits the thermochemolysis reaction.[36] From these studies, the optimum reaction conditions were found to be 290ºC and 1 min. Once the reaction conditions were established, the reproducibility of the method was assessed by repeated injections of a BT sample (10^{10} spores/mL). Table 2.1 lists the mean peak areas and standard deviations of eight major FAMEs using the mean percent contribution of each FAME to the total m/z 74 response (the principal electron ionization fragment of FAMEs). The standard deviation ranged from 0.3 to 1.5, indicating that the method of injection is quite reproducible.

Use of the CWF for simultaneous thermochemolysis and methylation simplifies the detection of biomarkers.

Table 2.1. Reproducibilities of BT endospore FAMEs for 10 replicate injections

FAME	m/z 74 Mean Peak Area (%)	Std. Dev.
Iso C14:0	7.5	0.42
n C14:0	6.6	0.34
Iso C15:0	33.7	1.46
Anteiso C15:0	9.1	0.29
n C16:1	3.6	0.21
n C16:0	8.1	1.11
Iso C17:0	14.3	0.52
n C18:0	2.7	0.12

2.3.4 SPME using temporary polymer coatings.

A sorbent polymer could be easily applied to the CWF prior to sampling by dipping the filament into a solution of the polymer and allowing the solvent to evaporate in the air. The geometry of the polymer coating depended on the concentration of the polymer solution (i.e., approximately 5% solution yielded a thin polymer coating on the wire, while 10-20% solution led to entrained polymer in the wire coil). Thermal conditioning was performed at a suitable temperature to stabilize the polymer. The coated filament was then used to collect the sample of interest either by dipping it into a liquid sample or introducing sorbent materials were also explored such as PDMS, epoxy, mixtures of epoxy and Apiezon N, polyethylene glycol 35,000, and polyethylene glycol-polypropylene glycol copolymer (Ucon 75- H-90000) (data not shown). The extraction efficiency of SPME was generally improved by an average factor of 2.5 for PAHs when using a polymer-coated CWF compared to a 100 µm coated commercial fiber.

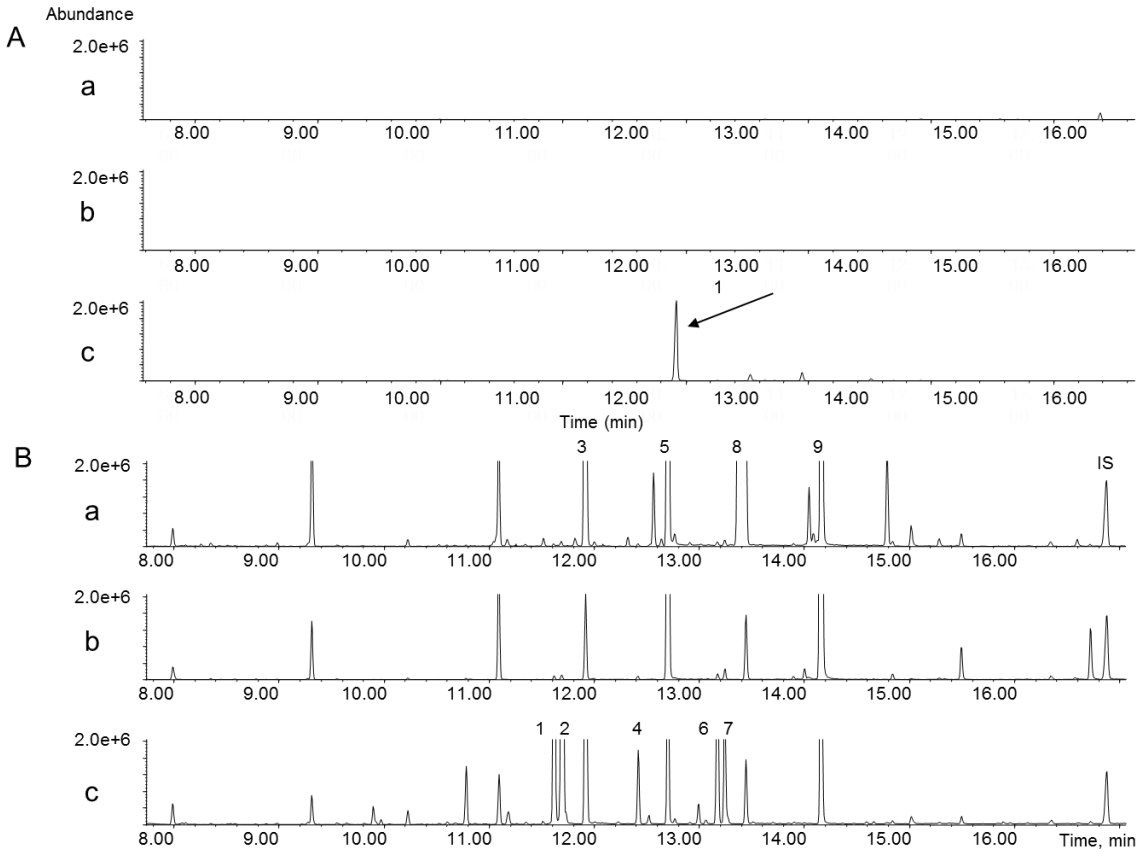

Figure 2.3. Extracted ion chromatograms for (a) Yersinia pestis, (b) Francisella tularensis, and (c) BT endospores. Conditions: column A, 60ºC initial temperature, 14ºC/min rate, 305ºC final temperature, 290ºC injector temperature, 12.4 psi He pressure, splitless mode. Peak identifications: (A) m/z 137 showing that DPAME is only present in BT spores and (B) m/z 74 showing FAMEs: (1) iso C15:0, (2) anteiso C15:0, (3) C15:0, (4) iso C16:0, (5) C16:0, (6) iso C17:0, (7) anteiso C17:0, (8) C17:0, (9) C18:0, and internal standard (IS).

2.3.5 General considerations

There are a number of drawbacks to the CWF design. The fixed sub-µL sampling volume may be too small for some applications (although concentration may be accomplished by repeated dipping and drying). With the reproducibility being about ±20% of its nominal volume, it is not as reliable as are other sample introduction tools that completely enclose the liquid sample. Thus, an internal standard is recommended for quantitation. Volatile compounds are lost more readily during solvent evaporation than less volatile species, leading to discrimination that

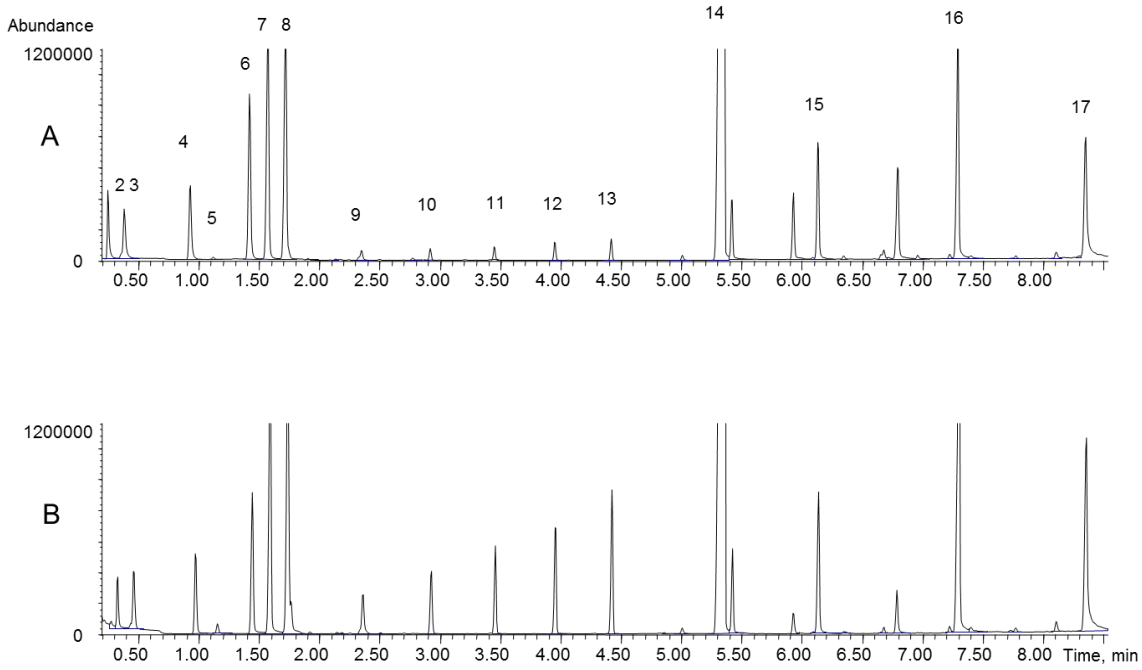

Figure 2.4. Chromatograms of volatile hydrocarbons using a polymer-coated filament and commercial SPME fiber. Conditions: column B, 45ºC initial temperature, 1 min initial time, 30ºC/min rate, 270ºC final temperature, 270ºC injector temperature, 75.0 psi He pressure, 10/1 split ratio. Extractions of aqueous samples were carried out at 20ºC for 20 min using (A) a Supelco 65 µm DVB /PDMS commercial SPME fiber and (B) an Apiezon N-coated CWF. Peak identifications: (1) hexane, (2) and (3) benzene + CCl_4, (4) toluene, (5) octane, (6) chlorobenzene, (7) o-xylene, and (8) p-xylene, each at 1-10 ppm concentration; (9-13) C10-C14 straight-chain alkanes, each at 1 ppm concentration; and (14) fluorene, (15) anthracene, (16) pyrene, and (17) chrysene, each at 0.1-0.5 ppm concentration.

affects quantitative analysis even with an internal standard. Sample carry-over may occur if the wire is improperly cleaned or if particularly dirty, viscous, or "sticky" samples are analyzed. However, our experience is that the CWF assembly stays surprisingly clean since most of the nonvolatile residue remains on the coil during and following evaporation of liquid and volatiles. The convex external geometry of the coil does not allow it to physically contact the inner walls of the needle housing or GC inlet liner by more than just a small area, reducing the likelihood of contamination. The rate of sample heating is somewhat buffered by the presence of the passively heated wire, since mechanisms of heat transfer are convective and conductive wherever contact

is made with the sides of the liner. Compared to resistively heated devices that may produce a more rapid heating rate, relatively little (if any) violent flashing of solvent or analytes is thought to occur. The geometry of the coiled wire is such that, when external flow is present, altered flow patterns and reduced boundary layer thickness at the external portion of the coil increase the rates of heat and mass transfer. This effect has been exploited for improved SPME sampling by Ciucanu who reported better results with small coiled wires coated with PDMS sorbent polymers compared to straight SPME fibers.[23-26] Similarly, incorporation of spiral inserts into GC inlets has been utilized as a means for improving mixing between sample/carrier solvent vapors and carrier gas in GC injection systems.[37-38] Some of the syringe-based introduction devices previously mentioned (i.e., references 13-19) were similar in size to the CWF, although their geometry rendered them less effective for retaining a liquid sample since they did not possess an internal cavity with a high degree of surface area for contacting a liquid. While the syringe-compatible helical SPME of Ciucanu[23,25,26] was close in size and geometry to the present CWF, in only one case[26] was it used for direct sampling from liquids, and the primary mechanism of sample capture by this SPME device was analyte absorption into the PDMS coating; physical capture of liquid solvent, if it occurred, was not addressed. Only the syringe-based capillary devices of Harris,[10] Golovistikov,[11] and Amirav and Dagan[8] possess the same capability of reliable liquid sample collection and introduction, but with the key disadvantage of having small orifices for sample collection and expulsion and, where applicable, carrier gas entry. The small access holes slow pre-injection solvent removal by evaporation, inhibit sample expulsion via diffusion mechanisms, challenge sampling of viscous fluids, and complicate removal of residues remaining inside the probe. The flow-through devices[10,11] require special GC inlet modifications or inserts to operate properly.

2.4 CONCLUSIONS

The CWF is the first syringe-like capillary-based collection and introduction tool for GC injection to incorporate a physical geometry for collecting virtually any liquid sample (including those with fine, suspended solids) for chromatographic analysis without using a solid-wall tube or pipette as a means of sample transfer. Because of the open design, the solvent evaporation step that precedes introduction into the chromatograph is rapid. By eliminating the solvent, a narrow sample band can be introduced into the column inlet, improving resolution and sensitivity, plus allowing for a higher initial oven temperature than when using traditional solvent injection methods. Since nonvolatile residues remain mostly on the wire, deposition of solids on the inside surface of the liner (and thus production of active sites) is minimized, allowing the introduction of complex samples (especially those of biological origin). The CWF can serve as a reactor on which TCM, derivatization, and other reactions may occur. Inert, high-melting materials such as Pt alloys are useful because they may be cleaned rapidly and repeatedly in a flame and reused hundreds of times. The ease of sampling by dipping the CWF in a liquid is a distinct advantage for field applications where simplicity and robustness are important. Several authors have emphasized that solvent-less sample introduction devices are especially well-suited for analysis of samples containing extracts of compounds of biological origin such as steroids, fatty acids, and amines[13,39,40] as well as whole biological samples.[41] This is not only because of the absence of solvent and ability to quantitatively transfer a minute amount of sample, but also because selection of a satisfactory extraction procedure is difficult to do without knowing the sample properties, such as for samples containing very diverse molecules.[41] It is expected that new applications of the CWF will appear as analysts explore the use of this technique for the almost limitless sample types.

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3 DIFFERENTIATION OF *BACILLUS* ENDOSPORE SPECIES FROM FATTY ACID METHYL ESTER BIOMARKERS

3.1 INTRODUCTION

Bacillus anthracis (BA) is a gram-positive, rod-shaped bacterium that is a member of the *Bacillus cereus* (BC) group in the genus *Bacillus*. It has been of particular interest because it causes anthrax, a serious and often fatal disease of mammalian livestock and humans. With more than 400 distinct strains of BA known to exist, genetic variation of the species is represented by 89 strains,[1] which range from benign to highly virulent. BA veretative cells of the highly virulent BA produce three exotoxin proteins (i.e., the protective antigen, the edema factor, and the lethal factor) and surround themselves with a capsule comprised of poly-D-glutamic acid.[2]

The *Bacillus cereus* group represents a highly homogenous subdivision that includes the three closely related species, BA, BC, and *Bacillus thuringiensis* (BT), and the more distantly related *B. weihenstephanensis* (BW) and *B. mycoides* (BM). Differentiation of *Bacillus* species using molecular assays has been difficult because of their high level of genetic similarity. Excepting *B. atrophaeus* (BG) which is not closely related to BA, has been used for decades as an important model organism, especially as a nonpathogenic surrogate for BA in bio-weapons research.[3,4] Historically, BG has been called different names including *B. niger*, *B. globigii*, and *B. subtilis*.[3,4] It is a naturally occurring soil bacterium, capable of producing subtisin, a bacteriocin which kills closely related species or competitive BG strains.[2] BA, BC, BT, and BG were selected for study in several recent reports regarding the differentiation of closely related *Bacillus* species.[5,6]

Bacterial endospores are differentiated bodies formed in response to nutrient deprivation that are resistant to adverse environmental conditions, including radiation, heat, toxic chemicals, and pH extremes.[7,8] Mature spores are almost completely metabolically inactive and have a highly ordered structure that provides the protection required for survival over long periods, even in the face of harsh environmental conditions. [9] Endospores are comprised of an inner core (spore core) surrounded by a cytomembrane (core wall), a cortex of peptidoglycan, outer membrane, and an exterior spore coat.[10,11] Spores of many bacterial species (including BA, BT, BC, and BG) are surrounded by a loosely attached exosporium.[12] A typical desiccated bacterium generally consists of 70% protein, 10% RNA, 5% DNA, 6% lipid, and 5% polysaccharide.[13] Any molecule or molecular fragment from these chemical classes that are unique to a particular bacterium can be considered to be a biomarker of that microorganism. DNA and dipicolinic acid (DPA) are contained in the spore core. DPA is present in a 1:1chelate with Ca2+ (Ca-DPA), which comprises 5-15% (dry weight) of *Bacillus* spores.[14] Ca-DPA plays a major role in the maintenance of spore dormancy and environmental resistance.[15] The Ca-DPA concentration in spores can vary with spore species/strain, cell growth medium, sporulation conditions, and size.[14] While a number of techniques have been reported for general detection of bacterial endospores, most are not applicable for both detection and differentiation at the species level in the field, which requires them to be simple, robust, and portable. Those that are applicable include nucleic acid-based methods such as polymerase chain reaction (PCR),[16] various antibody-based methods,[17,18] and chemical separation/detection with coupled systems such as gas chromatography-mass spectrometry (GC-MS).[19] GC and MS alone can indicate the presence of a biomarker compound in a sample, however, they cannot provide unambiguous detection without being coupled together. GC-MS analysis of bacteria or bacterial endospores requires rather harsh

sample pre-treatment, such as thermal reaction, hydrolysis, extraction, and/or derivatization before analysis. As early as 1990, pyrolysis coupled with GC-MS was employed to detect biomarkers in the field.[20] Micro-volume Curie-point pyrolysis GC-MS allowed for characterization of various lipid moieties in microorganisms. Gram-positive bacilli and gram negative species were discriminated by the pyrolysis patterns of their lipid components.[21-23] A hand-portable system comprised of a micro-pyrolyzer, micro GC, and surface acoustic wave (SAW) array detector was recently described for rapidly identifying microorganisms based on detection of fatty acid methyl esters (FAMEs) produced from pyrolysis and tetramethylammonium hydroxide (TMAH) methylation.[24] Unfortunately, the SAW detector could not provide conclusive structural identification. A prototype field-portable pyrolysis GC-ion mobility spectrometer was described by Snyder et al., which was more useful than pyrolysis-GC alone, but not as diagnostically useful as when coupled to an MS detector.[25] Although a number of methods using GC and/or MS have been reported for the detection of *Bacillus* endospores, most are not convenient for field application because of instrumentation size, weight, and electrical power requirements, or the methods are not simple and reproducible. Pyrolysis methods suffer from poor reproducibility and harsh reaction conditions that destroy potentially useful biomarker compounds.

In this chapter, I report a successful approach for fast, simple detection and differentiation of biomarkers of four related *Bacillus* species based on thermochemolysis methylation (TCM) using a coiled wire filament (CWF) sample introduction device followed by GC-MS. Dipicolinic acid methyl ester (DPAME) and FAME profiles of specific strains of BA, BT, BC, and BG spores grown under various conditions were reproducibly generated and utilized to differentiate these microorganisms from each other.

3.2 EXPERIMENTAL

3.2.1 Chemicals and materials

HPLC grade methanol (MeOH) and dichloromethane (CH_2Cl_2) were obtained from EMD (San Diego, CA, USA). Tetramethylammonium hydroxide pentahydrate (TMAH, > 97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). H_2SO_4 (98%) was from Mallinckrodt Chemical (Phillipsburg, NJ, USA). Pt-Ir (90-10) wires (90 mm) were from California Fine Wire (Grover Beach, CA, USA). The Pt-Ir wires were formed into coils by Motion Dynamics Corporation (Fruitport, MI, USA).

3.2.2 Endospore growth

The development of a GC-MS methodology for discriminating between *Bacillus* endospore species consists of three steps: (1) identification of a list of candidate biomarkers, (2) reduction of the list to discriminatory biomarkers, and (3) confirmation that the discriminatory biomarkers are effective. For this process I used three groups of samples. For the first two sample groups (i.e., sample groups 1 and 2), four distinct *Bacillus* species were cultured: *B. anthracis* (BA, Sterne 1043), *B. thuringiensis* var. kurstaki (BT), *B. cereus* (BC, ATCC 14579), *and B. atrophaeus* (BG, ATCC 51189). Sample group 3 consisted of 25 samples each of BA and BT weaponized endospores, which were provided by Dugway Proving Ground (DPG), Dugway, UT. Except for the weaponized samples, all endospore suspensions were prepared in a biosafety level 2 facility located on the Brigham Young University campus. The first group included endospores grown at three different temperatures (24, 28 and 32ºC) on four different media (casamino acid, Columbia salt dextrose, Leighton-Doi, and semisynthetic). Altogether, 600 samples constituted this group. The purpose of this sample group was to optimize the analytical methods as well as to identify potential biomarkers. Our initial TCM methods did not include

sulfuric acid treatment. We learned early in this study (i.e., during the analysis of this first group of 600 samples) that addition of sulfuric acid greatly improved the yield of DPAME, while retaining the characteristic FAME profiles. Thereafter, the addition of sulfuric acid became part of the ‘‘standard’’ procedure.

The second sample group was designed to explicitly confirm the validity of the candidate biomarkers obtained from studies using the first sample group. The best sample preparation and analysis conditions for DPAME and FAMEs were selected for the analysis of endospores in this group using the results obtained from the analysis of sample group 1. In addition, this group was designed to examine if the candidate biomarkers were robust against changes in incubation temperature and/or growth media, neither of which would be known in the field. Samples of each spore type were prepared by incubation at either 32 or 37ºC on agar plates containing either Leighton-Doi or Columbia salt dextrose growth media (LD and Col, respectively). Altogether, there were 270 samples prepared in this group.

The purity of each organism was verified by isolation plating on Columbia agar plates followed by gram staining and inspection under a microscope by phase-contrast microscopy (Zeiss Axioskop 2 equipped with an AxioCam HRc digital camera, Göttingen, Germany). LD and Columbia salt dextrose plates were then inoculated with an isolated colony from the Columbia agar plates. Spore cultures were grown for approximately 10 days in an incubator at 32 or 37ºC. Additional microscope inspection ensured sufficient sporulation, that is, the spore to vegetative cell ratio was greater than 9:1, before the spores were harvested. Sporulation of BC was not very successful on Columbia media at 37ºC, so these samples were not analyzed. On the other hand, even though sporulation of BC on Columbia media at 32ºC was not as successful as for the other *Bacillus* species, it was deemed sufficient to include these spores in the study.

Spores were collected from culture plates and suspended in 10 mL of autoclaved HPLC water in 50-mL polypropylene centrifuge tubes. The spore suspensions were then placed in a 65ºC water bath (LAUDA-Brinkman RM20, Delran, NJ) for 45 min to kill any remaining vegetative cells. The endospores were separated from vegetative debris by centrifugation (Beckman GS-15R centrifuge, Brea, CA) at 3800 x g for 10 min, followed by removal of the supernatant containing the vegetative debris. The remaining endospore pellet was then re-suspended in autoclaved HPLC water. This process (centrifugation and resuspension, i.e., ‘‘washing’’) was repeated daily for three days with suspensions being stored at 4ºC between washings. The endospore concentration was then determined by direct counting (Bright-Line hemacytometer, Horsham, PA). From the final spore suspensions, 1-mL samples were placed in1.5-mL Eppendorf tubes and centrifuged at 16,000 x g (Eppendorf 5415 C, Brinkman, Westbury, NY) for 4 min. The supernatant was removed and the Eppendorf tubes containing spore pellets were prepared for analysis.

3.2.3 *Bacillus* endospore sample preparation

In the final TCM procedure, centrifuged endospore pellets in polypropylene Eppendorf tubes (containing between 10^8 and 10^{10} spores) were re-suspended by adding 200 mL acidic MeOH (1% v/v H_2SO_4). The tubes were then vortexed or shaken well for 1 min to ensure total suspension. Then, 40 mL of 2.0 M TMAH (in MeOH) and 40 mL of internal standard (50 ppm chrysene in MeOH-CH_2Cl_2, 10:1 v/v) were added. The solution/suspension was well mixed prior to sampling for GC-MS analysis. All sample preparation steps were performed at room temperature, and the volumes and concentrations of reagents were proportionally adjusted at times to accommodate some variation in the concentrations of samples. This procedure was used for the later studied samples in group 1 and all of the samples in group 2. The initial samples

analyzed in group 1 and the weaponized samples comprising group 3 were analyzed before it was determined that adding sulfuric acid improved biomarker yields, especially DPAME. Otherwise, the methods were nearly the same, and produced similar distributions of biomarkers.

3.2.4 Coiled wire filament sample introduction

A CWF (Figure 3.1) was used both for sample introduction into the GC-MS system and for TCM reaction. Sampling was accomplished by dipping the CWF in the sample suspension. Details of the CWF and its use for TCM of microorganisms were recently published.[26] A major advantage of this sampling device is that nonvolatile sample matrix components remained on the wire coil after the sample was heated, reducing the required injection port and liner cleaning frequency and lessening contamination of the head of the chromatographic column. The coil

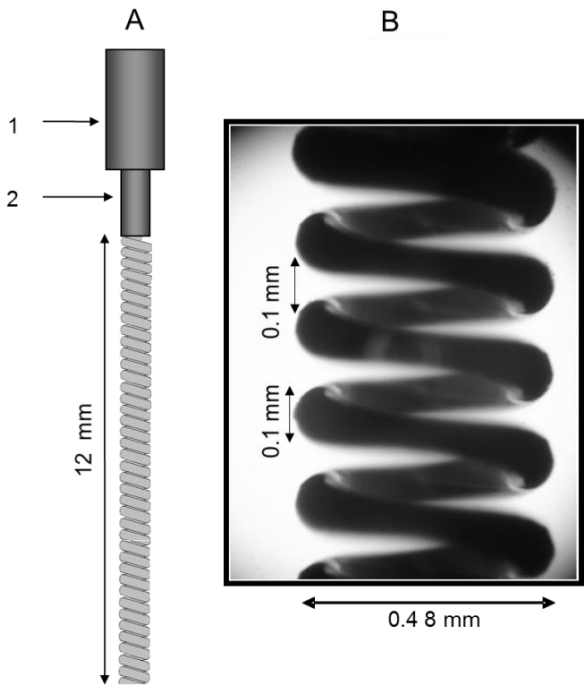

Figure 3.1. (A) Schematic drawing of a coiled wire filament. The wire filament is held in place by a wire filament socket (2), which may be extended from, or retracted inside, an SPME needle (1). (B) Photograph of an actual coiled wire filament produced by deflection coiling.

itself was easily cleaned between analyses by washing with a suitable solvent and/or burning off residues in a small flame. The CWF helped to improve the reaction yield by concentrating the reagents and target compounds into a small volume on the filament. In addition, the use of a narrow i.d. liner (0.75 mm) also helped to improve the reaction efficiency as well as reaction velocity. Target compound vapors were transported to the column faster in a narrow i.d. liner compared to a conventional liner.

3.2.5 GC-MS

An Agilent 6890 gas chromatograph (Agilent, San Jose, CA) with split/splitless injector was fitted with a 0.75 mm i.d. Restek liner. The GC was coupled to an Agilent 5973 MS with electron ionization and quadrupole analyzer. Helium was used as carrier gas. Two fused silica capillary columns were used in this study: 30 m x 250 mm i.d. x 0.25 mm film thickness (column A, DB-5, J&W Scientific, Folsom, CA) and 10 m x 100 mm i.d. x 0.4 mm film thickness (column B, Rtx-5, Restek, Bellefonte, PA). Typical chromatographic operating parameters included 290ºC injector temperature, 270ºC transfer line temperature between the GC and MS, and column temperature programs and inlet pressures as indicated in the figure legends. Even though the endospore samples were basic (i.e., pH 9-10) after addition of TMAH, hundreds of runs could be performed without any noticeable column deterioration. Relative retention times of separated compounds were computed with reference to the internal standard, chrysene.

The MS operating parameters for all experiments were: 230ºC quadrupole temperature, 150ºC source temperature, 30-500 m/z mass range, 1670 EM voltage, 35 mA emission current, and 70 eV ionizing voltage. Extracted ions m/z 74 and 137 were used to reconstruct the FAME

and DPAME chromatograms, respectively. For SIM, m/z 74 and 87 ions were used for FAMEs, and m/z 137, 138 and 139 ions were used for DPAME.

3.3 RESULTS AND DISCUSSION

3.3.1 Repeatability and detection limits

The repeatability of the new TCM method was examined by repeated injections of a BT endospore (Col, 32ºC) sample. Data from 10 replicates were used to calculate the percentages of peak areas for 5 specific FAMEs. The results indicate that the method is quantitatively repeatable (see Table 3.1), which can be attributed to: (1) well-controlled TCM reaction conditions, (2) consistent volume of sample taken up by the CWF due to capillary action, (3) constant drying time, and (4) use of an internal standard. The addition of methanolic H_2SO_4 prior to TMAH helped in the extraction of DPA from the Ca-DPA complex in the spores and/or hydrolysis of the spore structure.[27] Furthermore, H_2SO_4 is a catalyst for methylation and a water scavenging chemical that may help to release substances from the spores. The detection limit for DPAME was approximately 6,000 endospores using selected-ion-monitoring (SIM, m/z 137, 138, and 139 ions).

Table 3.1. Repeatabilities of specific FAME peak area percentages using extracted ion (m/z 74) chromatograms from 10 replicate injections of a BT endospore sample.
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FAME	Mean Area (%)	Std. Dev.
Iso C15:0	33.8	1.5
Anteiso C15:0	9.1	0.3
n C16:1	3.7	0.2
Iso C17:0	14.4	0.5
Anteiso C17:0	4.6	1.5

This is one to three orders of magnitude higher than the detection limits of PCR, which varies from ten to a few hundred endospores.[28-31] The GC-MS detection limit depended on bacteria species, growth conditions (i.e., growth medium and temperature), state of endospore dehydration, and whether total ion-current (TIC, m/z 137 extracted ion) or SIM was used.

3.3.2 Differentiation of *Bacillus* endospore species

DPA is unique to endospore-forming bacteria, the most familiar examples being *Bacillus* and *Clostridium*. The presence of DPAME indicates the likely presence of at least one of these microorganisms in the sample being analyzed. Unfortunately, DPA alone is not diagnostic beyond indicating the general presence of spores. Our approach relies on detection and differentiation based on the presence, absence, and intensities of both DPAME and FAMEs. Statistical methods such as principal component analysis (PCA), cluster analysis, or classification and regression trees (known as CART) have often been used to identify the most predictive sources of variance among different *Bacillus* species.[6] Much of the variability in our samples could be attributed to different growth, sample preparation, and analysis conditions. Rather than using sophisticated empirical pattern recognition methods, which would necessitate adjustment for experimentally induced variation, we utilized expert elicitation coupled with a survey of the literature described below.[32] While purely statistical methods use all of the empirical information in the sample, they do not readily incorporate known scientific relationships. Elicitation, on the other hand, allows for utilization of expertise, which implicitly incorporates previous experience as well as data obtained from the current sample. It is essential to confirm candidate biomarkers obtained by elicitation using an independent set of experiments. Table 3.2 and Figure 3.2 indicate the FAMEs selected for detection (i.e., DeFAMEs) and FAMEs selected for differentiation (i.e., DiFAMEs), of the four *Bacillus* species in sample

Table 3.2. List of FAMEs identified in Figure 3.2. FAMEs for detection (DeFAMEs) are indicated by an asterisk (*); FAMEs for differentiation (DiFAMEs) are indicated by two asterisks (**).

No.	FAME	No.	FAME
1	Iso C13:0*	12	n C16:1(Δ11)
2	Anteiso C13:0*	13	n C16:1(Δ^9)**
3	n C13:0*	14	n C16:0
4	Iso C14:0	15	Iso C17:1**
5	n C14:0	16	Anteiso C17:1**
6	Iso C15:0*	17	Iso C17:0*
7	Anteiso C15:0*	18	Anteiso C17:0*
8	n C15:0*	19	n C17:0*
9	Iso C16:1**	20	Iso C18:0
10	Anteiso C16:1**	21	n C18:1
11	Iso C16:0	22	n C18:0

group 2. Of these, iso C13:0, iso C15:0, iso C16:1, anteiso C17:1 FAMEs (absent from the NIST mass spectral library) were predicted based on their intensities, mass spectra, and elution orders, and from comparison of FAMEs detected from previously published papers. These identifications agreed with results obtained by different authors, such as Kaneda.[33] The results indicate the general rule (due to biosynthetic mechanisms) that saturated fatty acid pairs differing in 2 carbons, such as iso C15:0 and iso C17:0, and anteiso C15:0 and anteiso C17:0, are always present in lipids of bacteria, although they may have different abundances in different species.[33] Kaneda also confirmed the presence of iso C13:0, anteiso C13:0, and anteiso C17:1 in lipids of BC by GC analysis.[34] Kämpfer,[35] and Väisäanen and M. Salkinoja-Salonen[36] identified lipids of many different Bacillus species and indicated the presence of almost all fatty acids shown in Table 3.2. Of these, iso C16:1 and anteiso C16:1 were found in BC, BG, and BT. In the *Bacillus*

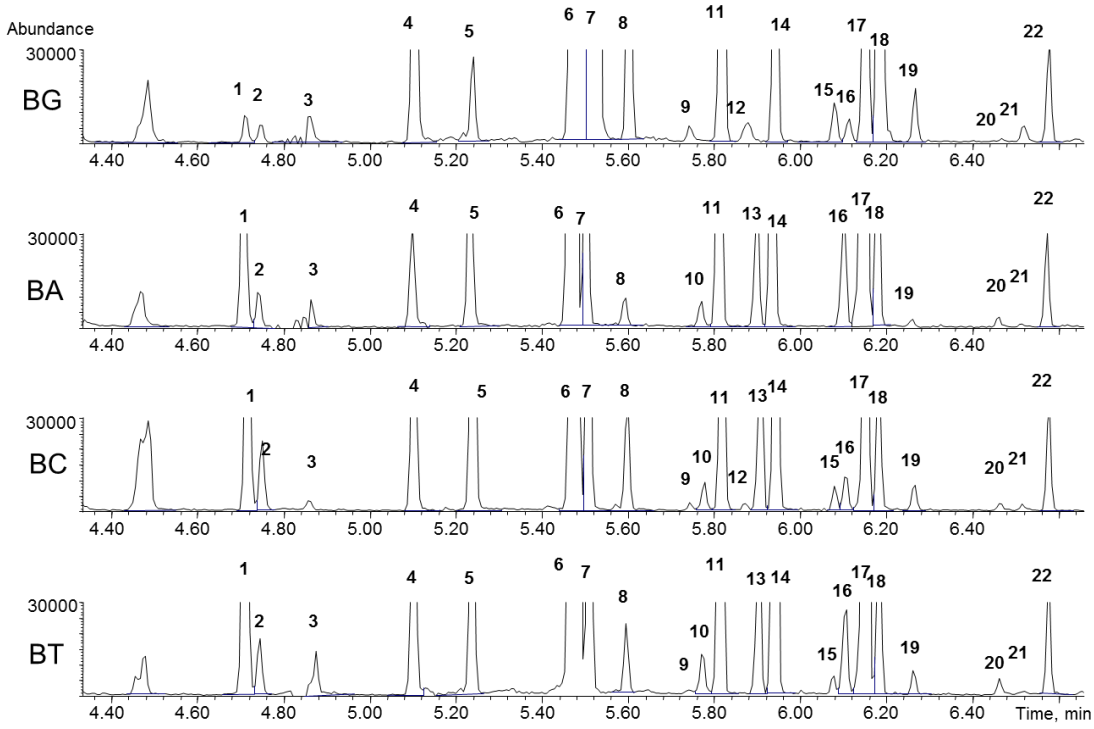

Figure 3.2. Extracted ion (m/z 74) chromatograms of *Bacillus* species (BG, BA, BC, and BT) grown in LD medium at 32ºC. Numbered peaks are identified in Table 3.2. Conditions: column B, temperature programmed from 60ºC to 300ºC at 33ºC/min, 75 psi He inlet pressure.

species, some fatty acids such as iso C15:0, anteiso C15:0, iso C17:0, and anteiso C17:0 have especially high intensities that can be used effectively for differentiation from non-*Bacillus* species. In other words, these fatty acids, similar to DPAME, can be used for detection of *Bacillus* species. Within *Bacillus*, various fatty acids were reported to be unique to certain organisms or groups of organisms. For example, Song et al. reported that fatty acid iso C17:1(Δ7) did not appear in BA but was present in BC, BT, and BG. These results were consistent for the several strains studied of each species.[37] Conversely, Whittaker found that anteiso C17:1 fatty acid was present in BA, but was substantially less pronounced or non-

existent in BC.[38] In the same study, Whittaker confirmed the finding of Song et al. that fatty acid iso C17:1(Δ7) was present in BC but absent from BA.[38] It should be noted here, however, that while individual studies tend to be internally consistent regarding intensities of individual FAMEs in the same species, and especially consistent for the same strains, there is generally little agreement between authors. These discrepancies can be at least partially attributed to the strong effect of growth conditions on fatty acid profiles and to the misidentification of unsaturated FAMEs (see ref. 33 for a detailed discussion).

In our research, discriminating biomarkers were found by studying selected-ion chromatograms of m/z 74 for FAMEs and m/z 137 for DPAME. Observations from GC-MS analyses of the presence, absence, and intensities of FAME peaks for the variety of samples analyzed in sample group 1 include: (1) DPAME is an indicator of either *Bacillus* or *Clostridium* endospores; (2) n C16:1(Δ9) and iso C16:1 FAMEs are from BA, BC, and BT, but not from BG endospores; (3) anteiso 17:1 FAME is from all *Bacillus* endospores; (4) iso C16:1 and iso C17:1 FAMEs are from BC, BG, and BT, but not from BA endospores; (5) i15:0 > a15:0 FAMEs (referring to peak areas) in BA, BC, and BT endospores the reverse being true for BG; (6) iso C17:0 > anteiso C17:0 FAMEs (referring to peak areas) in BA, BC and BT endospores the reverse being true for BG; (7) iso C15:0, anteiso C15:0, iso C17:0, and anteiso C17:0 FAMEs are the main components (highest intensity peaks) of all *Bacillus* species; and (8) C15:0 FAMEs > C14:0 FAMEs (referring to peak areas) in all *Bacillus* endospores. From these data, a flow chart for organism differentiation was constructed (Figure 3.3) to simplify the discrimination procedure. Generally, BG can be recognized based on the absence of C16:1(Δ9) FAME which exists only in BA, BC, and BT spores. BA is differentiated from BT and BC by the absence of iso C17:1 FAME. Peak areas of these and other FAMEs, as well as potential sugar biomarkers,

are currently being investigated in order to more easily distinguish BC from BT. One would expect that if such methods allow differentiation between very closely related species, they should also allow differentiation between *Bacillus* and non-*Bacillus* species. *Clostridium* is differentiated from *Bacillus* by very low intensity (or absence) of iso C15:0, anteiso C15:0, iso C17:0 and anteiso C17:0 FAME peaks compared to the C14:0 and C16:0 FAME groups, although a high intensity DPAME peak is detected in both (data not presented here). We expect similar differentiation ability between *Bacillus* and other non-*Bacillus* species.Confirmation of the biomarkers used to differentiate BA, BG, and BC/BT endospores was accomplished in two ways using the 270 endospore samples from sample group 2: (1) using an automated statistical decision-making model and (2) manual inspection by an expert. The automated differentiation algorithm has relevance to potential field applications. The basis for the algorithm is the decision tree shown in Figure 3.3, which utilized DiFAMEs to differentiate between BA, BG, and BC/BT. As indicated by Figure 3.3, detection of an additional biomarker is required for reliable differentiation between BC and BT. The parameters of the decision tree (Figure 3.3) were estimated using 125 random samples of BA, BG, BC, and BT taken from the 270 samples of group 2 previously described, accounting for within-species variation in peak areas and relative retention times. The algorithm was then tested using the remaining 145 samples. Only two of the 145 samples were misidentified, for an overall success rate of 98.6%, and a 97.4% success rate for BA. These results (Table 3.3) suggest that automated differentiation may be sufficiently reliable for field applications. In the manual confirmation step, which in part was used to validate the automated step, we were able to correctly detect and differentiate all but one of the 270 BA, BT/BC, and BG endospore samples grown in Col and LD media at 32ºC and 37ºC (see Table 3.4, rows 1-4). Upon further investigation, it was discovered that the single misclassification

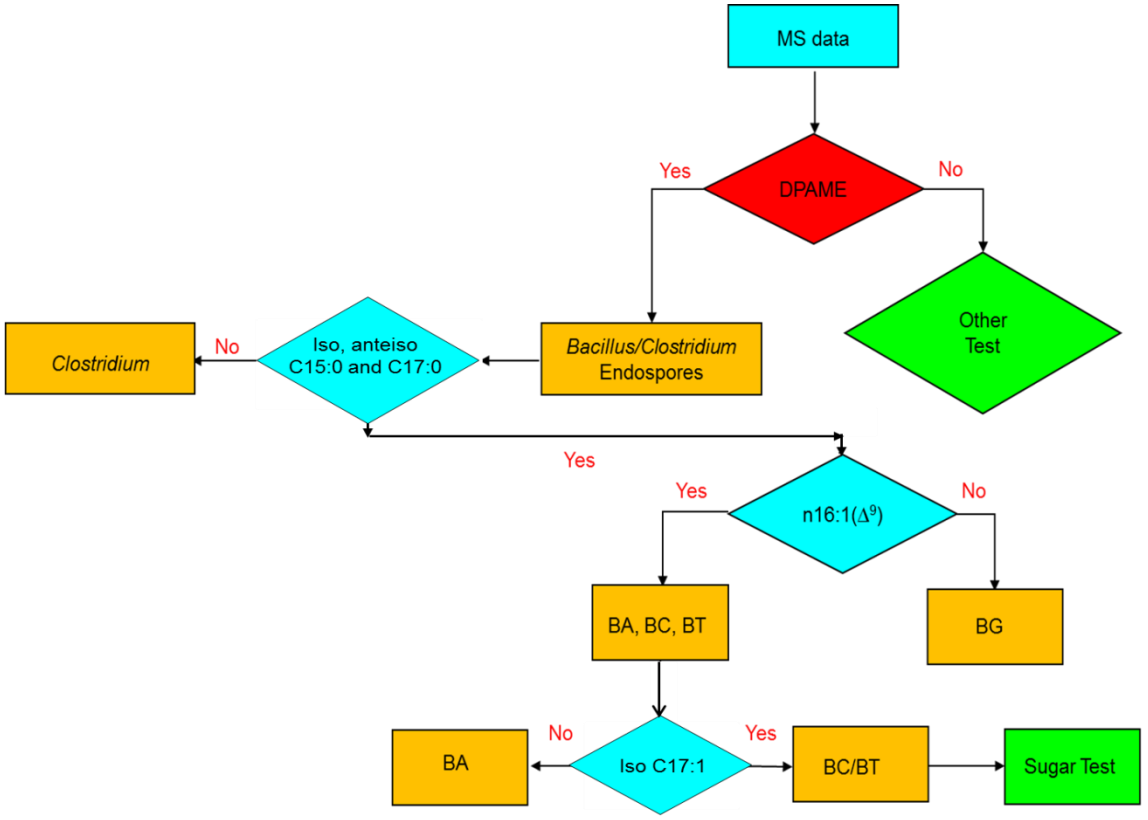

Figure 3.3. Differentiation flow chart for *Bacillus* and *Clostridium* endospores using DPAME and FAMEs.

Table 3.3. Automated differentiation results for BA, BG, and BC/BT endospores.

Species	Total predicted	Total correct	Percentage correct
BG	41	40	97.6
BA	38	37	97.4
BC or BT	66	66	100

was a case in which an extremely high peak left so much residue in the column that the next chromatogram was affected. This emphasizes the need for a blank run between each test run.

Table 3.4. Manual differentiation results for BA, BG, and BC/BT endospores grown under different conditions.

Sample Group	Growth temp., (ºC)	Growth media	Species examined	Number of observations			Correctly predicted percentages		
				BA	BG	BC/BT	BA	BG	BC/BT
2	32	Col	BA, BG, BC/BT	16	16	32	100	93.8	100
2	32	LD	BA, BG, BC/BT	19	19	38	100	100	100
2	37	Col	BA, BG, BT	18	18	18	100	100	100
2	37	LD	BA, BG, BC/BT	19	19	38	100	100	100
3	Unknown	unknown	BA, BT	25	0	25	100	NA	100

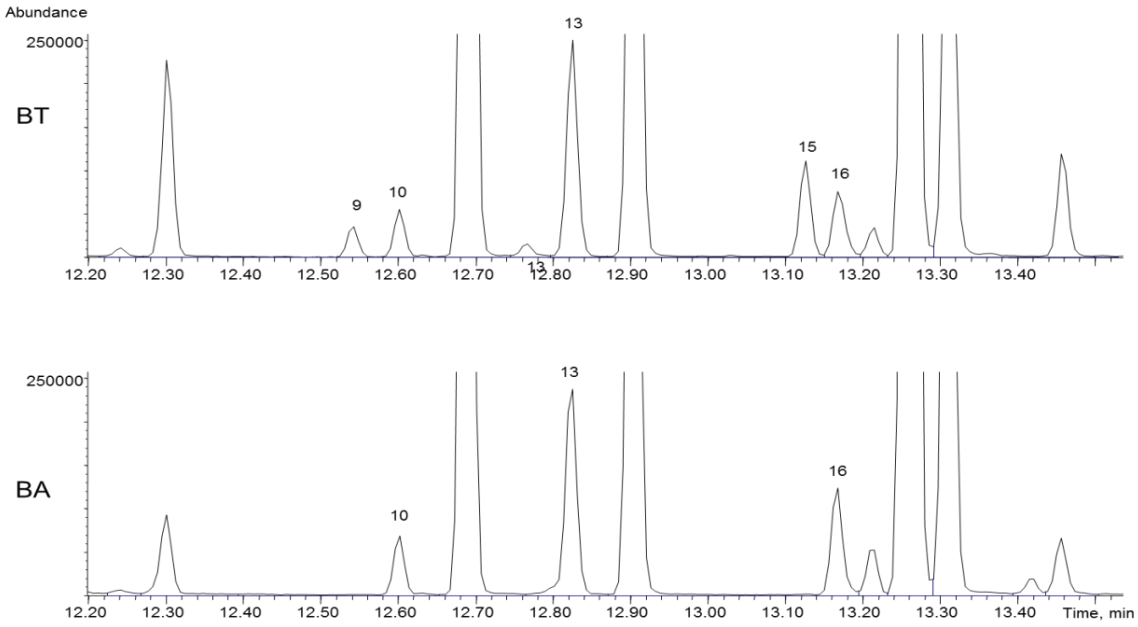

Figure 3.4. Extracted ion chromatogram (m/z 74) of FAMEs from weaponized BA and BT samples. Conditions: column A, temperature programmed from 60ºC to 300ºC at 14ºC/min, 12.5 psi He inlet pressure.

Importantly, this observation was one of the two missed by the automated procedure. As a final illustrative example, chromatograms displayed in Figure 3.4 were produced from weaponized BA and BT endospores (sample group 3) provided by Dugway Proving Ground. It should be emphasized that these samples were prepared by methods entirely unknown to us in a different laboratory by different personnel. Furthermore, they were analyzed using our earlier TCM procedure that did not include the addition of sulfuric acid. Even so, straightforward differentiation between these organisms was possible based on DiFAMES, especially iso C16:1 (peak 9) and iso C17:1 (peak 15) FAME peaks (see Table 3.4, row 5 for differentiation results). Both were correctly classified.

3.4 CONCLUSIONS

Conventional methods for fatty acid characterization of endospores and vegetative cells are laborious and time-consuming, usually taking more than 1 h.[37] In comparison, TCM with CWF sample introduction is fast and simple, making it easily adaptable to field analysis. By screening the TCM products for DPAME and specific FAMEs, BA can be easily detected and differentiated from other microorganisms, including closely-related *Bacillus* species, when cultured under well-defined conditions.

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4 DIFFERENTIATION OF *BACILLUS* ENDOSPORES FROM SUGAR BIOMARKERS USING COILED WIRE FILAMENT SAMPLE INTRODUCTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

4.1 INTRODUCTION

4.1.1 Detection of bacterial species from chemical biomarkers

Fast detection and differentiation of pathogenic bacteria (category A), such as *Bacillus anthracis* (BA), *Clostridium botulinum*, *Yesinia pestis*, and *Francisella tularensis* are important to counter the possibility of a bioterrorism attack. Differentiation of endospores from various *Bacillus* species, including BA, *B. thuringiensis* (BT), *B. atrophaeus* (BG, formerly *B. globigii*), and *B. cereus* (BC) is of special interest because of the historical use of BA in such activities and similarities in chemical compositions (e.g., up to 99.5% similarity in DNA sequence) of the various *Bacillus* species. These microorganisms must be differentiated not only from each other but also from other microorganisms and other biologically-derived materials. Successful techniques could be applied to many other problems encountered in forensic investigations, environmental pollution, food contamination, etc.

Specific signature molecules (i.e., biomarkers) can be used to detect and identify target microorganisms. While DNA, proteins, fatty acids, and sugars are common biomarkers that have been studied for detection and differentiation of biological agents, dipicolinic acid (DPA), specific sugars and fatty acids are biomarkers that can be used to confirm the presence of sporulating bacteria, in the first case (i.e., *Bacillus* and *Clostridium*), and more specifically BA, in the second case.[1-5]

As introduced in Chapter 1, a variety of techniques have been used to detect biomarkers. Polymerase chain reaction (a primer-mediated enzymatic DNA amplification method), immunoassays, and vibrational spectroscopy (i.e., Raman and infrared) can provide information concerning biological warfare agents within a relatively short time.[6-8] However, they typically require specific reagents and conditions for sample preparation that are inconvenient to use in the field and for fast analysis. Furthermore, some methods have high detection limits (e.g., 4.5×10^9 BA endospores for the Raman technique) and require significant operator skill.[7-9] Recently, detection of bacterial endospore by luminescence was reported by Cable based on high affinity binding of DPA to Tb^{3+} [i.e., Tb^{3+}(macrocycle) dipicolinate] that triggers intense green luminescence under UV excitation.[10]The characteristic band shape of the luminescent center in these experiments remained unchanged down to 6.0 nM, corresponding to 2.1×10^4 spores per mL.[10] However, this method is limited in providing differentiation information since it is based solely on DPA as a biomarker.

Again, mass spectrometry (MS) has been employed for many years in microorganism analysis.[11-13] Due to inherent advantages of soft ionization and low fragmentation, matrix-assisted laser desorption ionization (MALDI) MS has been used to detect high molecular mass biomarkers (i.e., biomacromolecules such as proteins).[14] Currently, there is a growing interest in applying MS techniques for rapid classification of bacteria, especially by the use of MS-based proteomics methods.[15-26] Protocols have been developed for bacterial protein extraction and mass analysis, especially by matrix-assisted laser desorption ionization (MALDI) time-of-flight MS.[27]

Liquid chromatography-electrospray ionization-tandem mass spectrometry of tryptic peptides derived from whole cell digests[28] or chromatographic profiles of proteins[29] have recently been used for differentiation. Peptides were identified and matched to a prototype database (DB)

of reference bacteria with fully sequenced genomes to obtain their phylogenetic profiles. In using protein profiles, discrimination and identification from mass spectra of mixtures of microorganisms and those subjected to different growth conditions have been based on differentiation software.

However, microorganism identification by mapping of bacterial protein mass profiles or by matching to protein masses predicted from the genome is often limited by low mass accuracy, insufficient mass resolution, and poor spectral reproducibility.[30] Generally, MALDI-MS has been used to characterize and differentiate microorganisms at species and strain levels.[31] Unfortunately, it cannot be employed easily for in-field applications.

GC-MS and HPLC-MS, and MS/MS with quadrupole or ion-trap analyzers have been the most popular techniques used for sugar detection. Electron ionization (EI), selected-ion-monitoring (SIM), and extracted ion monitoring (EIM) MS modes were used.[32]

Generally, sugar monomers released from whole cells by hydrolysis, must be derivatized before being analyzed by GC. Alditol acetates are often preferred derivatives for neutral sugars and aminosugars (acidic carbohydrates do not produce chromatographic peaks due to the underivatized carboxyl group) because each sugar produces a single stable derivative, which leads to simple chromatograms.[31] However, the detection of sugars using HPLC-MS or GC-MS is typically time-consuming and laborious and not suitable for field applications. Sample preparations for these conventional methods take from hours to days to finish.[33-39]

Gas chromatography-mass spectrometry (GC-MS) also has a major technique limitation; It cannot be used directly for biological agent detection without first using reactions to break down the microorganism structure and release the biomarker compounds as volatile analytes. Such reactions include, pyrolysis, thermolysis, hydrolysis, acetylation, methylation, and

silylation, and the main resulting biomarkers include dipicolinic acid methyl ester (DPAME), fatty acid methyl esters (FAMEs), 3-hydroxy butyric acid methyl ester (3HBAME), methylated sugars and amino sugars, and methylated amines.[40-44]

4.1.2 Sugar biomarkers

Sugars, generally speaking, comprise a class of biomarkers that have been extensively investigated. Fox et al. showed that vegetative cells and spores of BA and BC exhibit distinct carbohydrate profiles, and spores commonly exhibit different carbohydrate profiles from those of vegetative cells.[17] They reported that BA contains high levels of galactose but not galactosamine, whereas BC contains galactosamine. BA spores also contain rhamnose, but not fucose, whereas BC spores contain both rhamnose and fucose.[18] Both BA and BC spores contain 3-*O*-methyl rhamnose, whereas BC spores also contain 2-*O*-methyl rhamnose. Differentiation using carbohydrate profiles does not depend on whether or not organisms are in the vegetative or spore stage of growth.[20,21] Sugars are present in the spore as constituents of DNA and RNA, peptidoglycan, and various glycosylated proteins.[45]

As previously mentioned, notable differences in spore sugars exist in the exosporium. Sugars present in spores may generally include ribose, galactose, glucose, galactosamine, and glucosamine as well as others. [46] Again, the saccharide content is notably different in spores of different species.[47-52] Tornaben indicated that the main difference between the vegetative forms of BA and BC for most of the strains studied by the author was the relatively high level of galactose and low to no galactosamine in BA and, conversely, the relatively low level of galactose and high galactosamine in BC.[52] However, both contained the major sugars typically identified in Gram-positive bacteria, such as ribose, glucose, muramic acid, glucosamine, and mannosamine. Furthermore, it was shown that bacilli (detected using both GC-MS and LC-MS)

can exhibit three distinct carbohydrate profiles, depending on their growth conditions and existing forms: (1) vegetative cells grown under phosphate-rich conditions, (2) vegetative cells grown under phosphate-limiting conditions and (3) spores. The combination of GC-MS and LC-MS carbohydrate profiles provides powerful information for characterization of the physiological status of the cell and identification of the species.[32]

4.1.3 Sugar biomarkers from TCM

Conventional sample preparation for sugar analysis using GC-MS includes many steps, such as homogenization, centrifugation, hydrolysis, and derivatization, which may take many hours or days.[53] In our research, TCM of bacterial endospores is initiated in methanolic H_2SO_4 solution and completed inside the GC heated injection liner. DPAME and FAME profiles from GC-MS analyses of TCM products were used for detecting and differentiating BA from other *Bacillus* species.[54] DPAME from approximately 3 x 10^3 spores was used to detect *Bacillus* and *Clostridium* endospores while typical FAMEs, such as iso C15:0, anteiso C15:0, iso C17:0, and anteiso C17:0, were used to differentiate *Bacillus* from *Clostridium.* In addition, the presence and absence of certain FAMEs (i.e., C16:1Δ^9a, iso C17:1, and anteiso C17:1) allowed the differentiation of endospores from four *Bacillus* species, i.e., BA, BG, BC and BT.[54] The limitation of this approach is relatively high detection limit, since the distinguishing FAMEs are in low concentrations in the endospores.

To overcome this limitation, more useful biomarkers from TCM for differentiation were investigated. In addition to DPAME and FAMEs, 2BAME and simple sugar derivatives (as methylated monosaccharides and amino sugars) were found and investigated for differentiating BA, BC, BG, BT and *Clostridium* endospores from each other.

4.1.4 Beta-hydroxy butyric acid

Beta-hydroxy butyric acid or 3-hydroxy butyric acid (3HBA) can be generated from poly(3-hydroxybutyric acid) (P3HBA), which is a polymer that is produced by bacteria. It stores energy in the form of polymerized short-chain FAs (Figure 4.1 shows units of

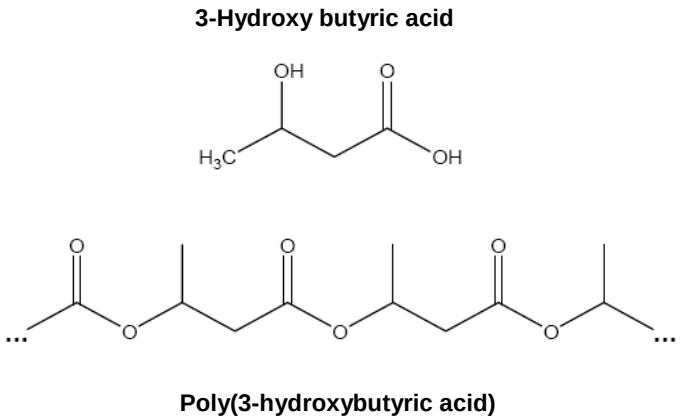

Figure 4.1. Chemical structures of 3-hydroxybutyric acid and units of poly(3-hydroxybutyric acid).

3HBA in P3HBA). P3HBA makes up to 50% (but usually much lower) of the total lipids in vegetative bacteria of the *Bacillus* genus.[55] It is known to be present in very low amounts in vegetative BG cells, but in significant amounts in BA cells.[56]

Grassie and Murray, in a series of publications in 1984, presented results of a study on the pyrolysis of P3HBA.[57-59] These authors found that at temperatures above 170ºC, P3HBA undergoes degradation by means of a chain scission ester decomposition mechanism that involves a six-member ring transition state (see Figure 4.2). This decomposition produced tetramer, trimer, dimer, and monomer (crotonic and isocrotonic acids) of P3HBA. In 1999 and 2004, Beverly,[60] Snyder,[56] and their coworkers found the dimer, trimer, and tetramer of P3HBA in both spores and vegetative cells of BA, BC, BT, and other species. Under TCM conditions

performed in our laboratory, the high temperature and high concentration of H_2SO_4 should lead to P3HBA as the predominant species in its methyl ester form.

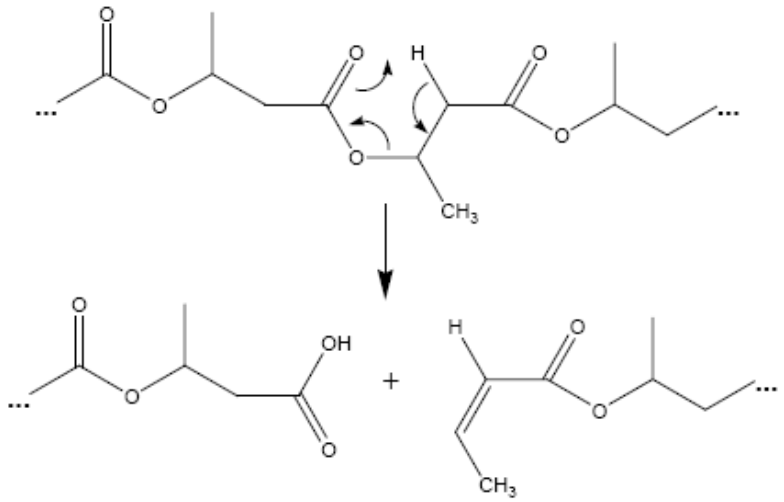

Figure 4.2. Chain scission ester decomposition mechanism of P3HBA.

There is an additional issue that may affect the detection of 3HBA. P3HBA can be converted to 3HBA by enzymes, e.g., peptidases or artificial catalysts, which are known to accelerate hydrolysis reactions. P3HBA can also be hydrolyzed in hot alkali as well as in strong acid to produce 3HBA.

If *Shewanella spp*., a group of facultative anaerobic Gram-negative marine bacteria widely distributed in marine and freshwater environments,[61] and *Pseudomonas syringae pv. tabaci* 6605 are present in the sample, 3HBAME and 2BAME can be produced by TCM because decomposition (by nucleophilic acyl substitution) of the anthrose-like saccharide structures that exist in these organisms (see Figures 4.3 C and 4.3 D) under acidic conditions leads to carboxylic

acids and the ammonium ion. In cases of basic hydrolysis, the products will be carboxylate ions and ammonia. Therefore, intentional or incidental presence of 3HBA can affect the detection of *Bacillus* endospores if it is used as a specific biomarker. 3H3MBAME can be produced under our TCM conditions when *Shewanella spp*. is present due to the presence of its anthrose-like structure (see Figure 4.3 B); however, SP injection using a CWF and low concentration of H_2SO_4 does not produce a significant amount of this biomarker.

4.1.5 Microwave heating for TCM

Although a heater block or small oven can be used to heat up samples during TCM, a microwave oven can also be used to speed up reactions.[65-69] Microwave heating was used to enhance the intensity of sugar profiles and improve the detection limit of DPAME due to

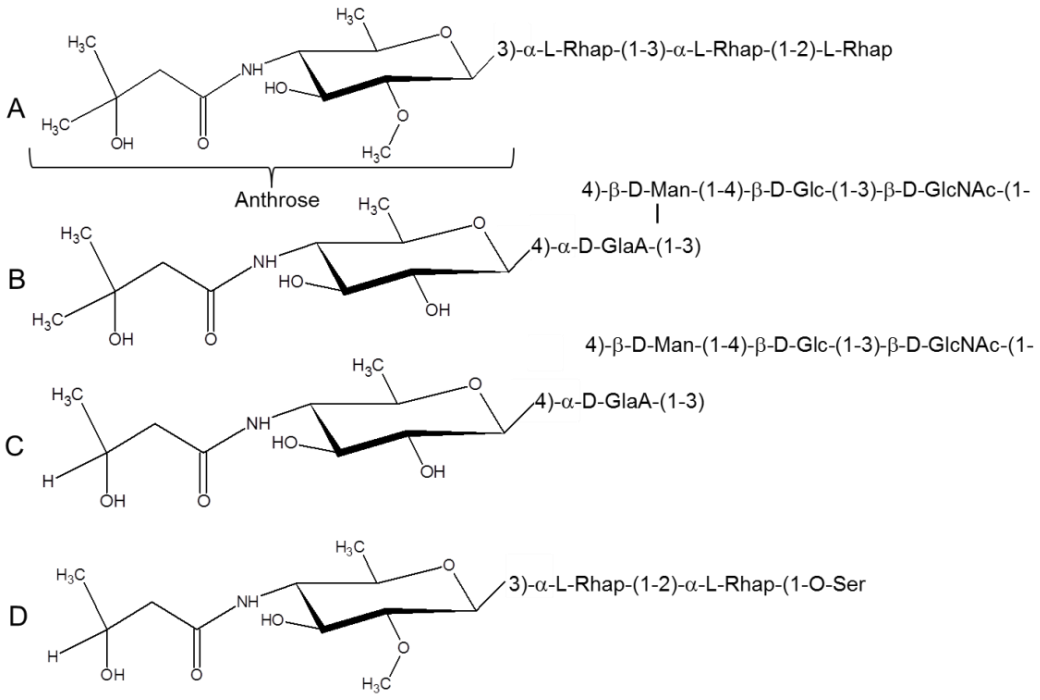

Figure 4. 3. Structures of saccharides of (A) BA BclA glycoprotein;[62] (B, C) *Shewanella spp*. MR-4 CPS (both structures are present and the ratio depends on the growth conditions);[63] and (D) *Pseudomonas syringae pv. tabaci* 6605 flagellin glycan.[64]

its high efficiency of hydrolysis and cleavage, and reduction in sample preparation time. Microwave treatment has been used to minimize the sample preparation time. For example, in 1993, Lagana et al. described the analysis of sialic acids using a microwave digestion system.[70] More recently, household microwave ovens have been used for chemical analysis of proteins, lipids, fatty acids, phospholipids, and sugar structures in the laboratory.[71-79] In addition to the heat generated, a microwave oven produces oscillations along the axes of asymmetrical molecules, which helps to damage the organisms and release trapped substances.

For TCM in our laboratory, a household microwave oven was used for endospore sample preparation. The power and time was controlled to suit the sample properties. From 10-20 s at high energy was required for 40-100 µL sample suspension in methanolic H_2SO_4 (1-2% v/v) for sugar detection. Without a microwave, conventional acidic hydrolysis in high HCl concentration (6 M) and heating time (110°C) of 20-24 h were required. A microwave oven reduced the same process to just minutes.[75] Saki et al., by analyzing the profiles of glycosphingolipid constituents obtained using the microwave oven method, reported quantitative and qualitative results comparable to those obtained by time-consuming conventional methods.[80] The use of a household microwave oven provided almost the same results as those obtained using a hot plate.

4.2 EXPERIMENTAL

4.2.1 Chemicals and materials

HPLC grade methanol (MeOH) and dichloromethane (CH_2Cl_2) were obtained from EMD (San Diego, CA, USA). Tetramethylammonium hydroxide pentahydrate (TMAH, > 97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). H_2SO_4 (98%) was from Mallinckrodt Chemical (Phillipsburg, NJ, USA). Pt-Ir (90-10) wires (90 µm) were from California Fine Wire

(Grover Beach, CA, USA). The Pt-Ir 90/10 wires were formed into coils by Motion Dynamics (Fruitport, MI, USA).

4.2.2 GC-MS system and conditions

An Agilent 6890 gas chromatograph (San Jose, CA) with split/splitless injector was fitted with a 0.75-mm i.d. Restek liner to accommodate the sampling device. The GC was coupled to an Agilent 5793 MS with electron ionization and quadrupole analyzer. Two polar GC capillary columns (10 m and 30 m x 0.25 mm ZB-FFAP) and a moderately polar column (5 m x 0.1 mm x 0.4 µm film thickness MS5 were used. Typical chromatographic operating parameters included 250-290ºC injector temperature, 2-min splitless injection time, temperature program from 50-60ºC (initial) to 250-290ºC (final) at different rates depending on the analysis, and 230-270ºC transfer line temperature between the GC and MS. The MS operating parameters for all experiments were: 230ºC quadrupole temperature, 150ºC source temperature, 30-500 m/z mass range, 1670 EM voltage, 35 µA emission current, and 70 eV ionizing voltage. Extracted ions 74 m/z, 137 m/z, and 43 m/z were used to reconstruct chromatograms for FAMEs and methylated sugars, DPAME, and 3HBAME, respectively.

4.2.3 SP sample injection

A coiled wire filament was used for both sample introduction and TCM. Sampling was accomplished by dipping the wire coil in the sample suspension or by applying drops to the coil. Multiple dipping and evaporation steps or multiple drops could be applied to increase the number of spores sampled.[54,81] A detailed description of the coiled wire SP injection technique was given in Chapter 2.

4.2.4 Microbiological samples

The same procedures and conditions for culturing the bacterial endospores were used as described in Chapter 3. Leighton-Doi (LD) and Columbia agar plates were prepared; a salt dextrose medium (magnesium sulfate heptahydrate, manganese sulfate monohydrate, ferrous sulfate heptahydrate, potassium chloride, calcium chloride dihydrate, and dextrose) was added to help in the formation of spores. Isolation plates of BA, BG, BT, and BC were prepared on untreated Columbia agar, and a sample of pure culture was then used to cover the salt dextrose plates, which were then incubated at 32ºC or 37ºC for two weeks. After verification that 90-95% sporulation had occurred (through phase contrast microscopy), the spores were collected off the agar plates and heat treated to kill any remaining vegetative spores. Spores were washed once a day for three days before GC-MS analysis.

4.2.5 Endospore growth

Four distinct *Bacillus* species were cultured. Unless otherwise specified, all endospore suspensions were prepared in a biosafety level 2 facility located on the Brigham Young University campus. Spores of BA (Sterne 1043), BT var. *kurstaki*, BC (ATCC 14579), and BG (ATCC 51189) were prepared in different sample batches. Multiple batches of each spore type were prepared (as summarized below) by incubation at either 32ºC or 37ºC on agar plates containing either Leighton-Doi or Columbia salt dextrose growth media (LD and Col, respectively). The purity of each organism was verified by isolation plating on Columbia agar plates followed by gram staining and inspection under a microscope by phase-contrast microscopy (Zeiss Axioskop 2 equipped with an AxioCam HRc digital camera, Göttingen, Germany). LD and Col salt dextrose plates were then inoculated with an isolated colony from the Col agar plates. Spore cultures were grown for approximately 10 days in an incubator at 32ºC or

37ºC. Additional microscope inspection ensured sufficient sporulation; that is, the spore to vegetative cell ratio was greater than 9:1 before the spores were harvested. Sporulation of BC was not very successful on Col media at 37ºC, so BC spores grown on Col media were not studied at this temperature. Furthermore, sporulation of BC on Col media at 32ºC was not as successful as for the other *Bacillus* species; however, in light of the additional spore isolation methods discussed later, BC sporulation on Col media at 32ºC was deemed sufficient to include in the study.

Spore matter was collected from culture plates and suspended in 10 mL of autoclaved HPLC water in 50-mL polypropylene centrifuge tubes. The spore suspensions were then placed in a 65ºC water bath (Lauda-Brinkman RM20, Delran, NJ) for 45 min to kill any remaining vegetative cells. The endospores were separated from vegetative debris by centrifugation (Beckman GS-15R centrifuge, Brea, CA) at 3800 x g for 10 min, followed by removal of the supernatant containing the vegetative debris. The remaining endospore pellet was then re-suspended in autoclaved HPLC water. This process (centrifugation and re-suspension, often called "washing") was repeated daily for three days with suspensions being stored at 4ºC between washings. The endospore concentration was then determined by direct counting (Bright-Line hemacytometer, Horsham, PA). From the final spore suspensions, 1-mL samples were placed in 1.5-mL Eppendorf tubes and centrifuged at 16,000 x g (Eppendorf 5415 C centrifuge, Brinkman Instruments, Westbury, NY) for 4 min. The supernatant was removed and the Eppendorf tubes containing spore pellets were prepared for analysis. Altogether, there were 22 batches prepared, comprising 270 samples.

Additionally, two BA and BT weaponized endospore batches, comprising 50 samples, were provided by Dugway Proving Ground (DPG), Dugway, UT. These samples, prepared under unspecified conditions, constituted the third sample group.

4.2.6 New biomarker detection experiments

Different experiments were performed to improve differentiation and detection of *Bacillus* endospores grown at 32ºC and 37ºC in Col and LD media. The same protocol used for previous *Bacillus* endospore batches was applied (see Chapter 3). The only exception is that a step was added that included heating the mixture of suspended endospores and H_2SO_4 prior to adding TMAH solution. Besides improving the detection limit of DPAME, sugar derivatives were detected.

4.2.7 Concentration and TCM for detection of DPAME and sugar derivatives

In this method, endospore samples were concentrated twice before heat treatment and after adding TMAH solution. To concentrate, methanol (in methanolic H_2SO_4) was eliminated by applying a vacuum or purging an inert gas through the sample suspension. This method allowed the initial use of a large volume of low concentration H_2SO_4 solution.

In the final step of this method, a large (up to 50% or higher) sample volume could be introduced into the GC-MS, because the remaining sample volume after concentration was small (5-10 µL). The concentration step and coiled wire sample introduction method allowed for detection of the smallest concentration of endospores. This method is necessary for analyzing sampling materials, such as impact filters, that require a large volume of methanolic H_2SO_4 to collect endospores on a large surface. Figure 4.4 pictorially shows the stepwise procedure, which is described in detail as follows:

1. Sample transfer: the sample (200 µL endospore suspension or Teflon filter after spore collection) is placed in a conical bottom vial (1-mL). If the sample is on a Teflon filter, the filter is rolled and placed into a transfer pipette tip. The pipette tip is subsequently inserted into the conical bottom vial, and the vial is placed in an ultrasonic bath. A 200-µL volume of H_2SO_4 (with concentration from 0.05 to 0.1% v/v, depending on the sample concentration) is used to re-suspend the spores from the filter. During sonication, the transfer pipette plunger is pressed and released repeatedly to help release the spores from the filter surface.

2. Concentration: N_2 gas or vacuum is used to evaporate the solvent until 10-20 µL of suspension remains in the vial.

3. Heating: A hot plate is used to heat the sample after capping the vial with a Teflon/silicone septum.

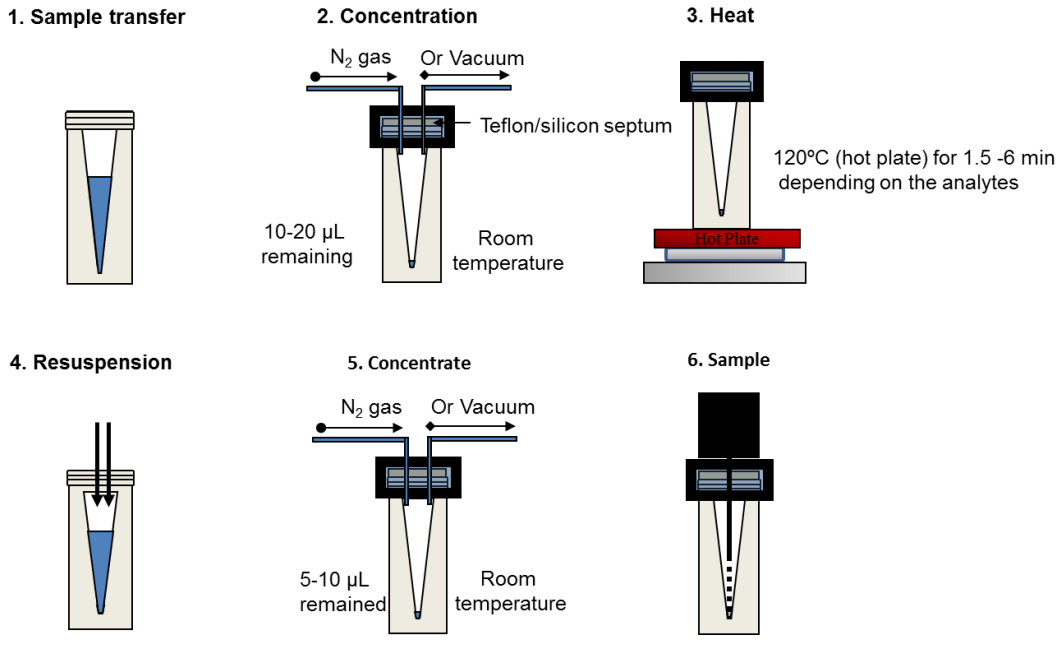

Figure 4.4. Stepwise procedure for TCM and concentration using CWF.

4. Re-suspension: The sample is suspended after it cools by adding TMAH (0.2-0.4 M) and internal standard solutions (both prepared in MeOH solvent) until the pH of the suspension is from 8.5-9.

5. Concentration: The sample is concentrated by an N_2 gas purge or vacuum until 5-10 µL remain in the vial bottom.

6. Sampling: A coiled wire filament (CWF) is inserted into the vial to capture the sample, or a drop of sample is applied onto the wire coil surface using a conventional micro syringe. Injection into the GC-MS is performed after solvent evaporation.

The biomarker detection limit was an important aspect of the method development; therefore, optimization of the main steps of the analysis was performed. Besides optimization of GC injection, GC separation, and MS detection, the conditions of sample preparation were also investigated.

4.3 RESULTS AND DISCUSSION

4.3.1 Concentration

A concentration step is necessary prior to heating to increase the H_2SO_4 concentration when low H_2SO_4 concentration and large volume of solution are used. This is required to accommodate enough volume for collecting endospores on a filter or impactor surface. Methanolic H_2SO_4 has been popular for methylating compounds, and its efficiency is improved at high temperature and under anhydrous conditions. In addition, H_2SO_4 is also a good digestion reagent, which helps significantly to release DPA from $Ca(DPA)_2$ in the spore core.

4.3.2 β-Hydroxy butyric acid from *Bacillus* endospores

β-Hydroxy (or 3-hydroxy) butyric acid methyl ester (3HBAME) and/or 2-butenoic acid methyl ester were detected by GC-MS following TCM. As discussed previously, they originate

from poly(3-hydroxy butyric acid) in *Bacillus* endospores. The suggested mechanisms for their formation are given in Figure 4.5.

Using the coiled wire filament for sampling, only 3HBAME is typically detected because 2BAME is lost during solvent evaporation. With SPME sampling, both compounds can be detected. The presence of 3HBAME in BA, BT, and BC but not in BGwas confirmed previously, even without any heat treatment (see Chapter 3). With heat treatment and SPME

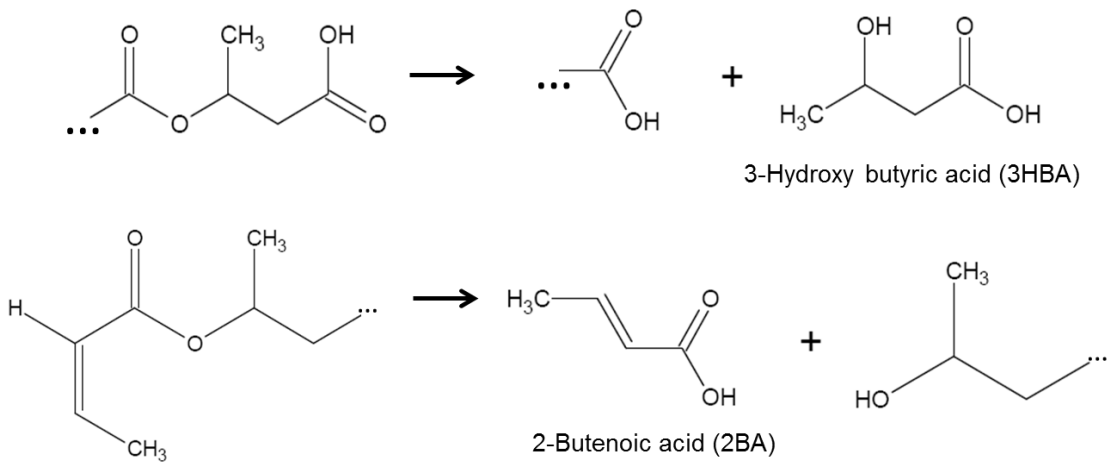

Figure 4.5. Mechanisms of TCM 3-hydroxy butyric acid and 2-butenoic acid.

sampling, 2BAME was consistently found in three of the *Bacillus* endospore species, but with different peak intensities. Figure 4.6 shows selected ion plots for 3-hydroxy butyric acid methyl ester in BA, BC, BT and BG, which agrees with the results reported by Snyder. [82,83] This information can be used for distinguishing BG from the three other *Bacillus* endospore species.

4.3.3 TCM method for FAMEs, DPAME, 3HBAME, and methylated sugars

As described, this method was developed to detect biomarkers in low concentration samples. The use of TMAH improved the methylation reaction over methylation in methanolic H_2SO_4 solution. Furthermore, the CWF helped to eliminate solvent and minimize sample residues remaining in the GC liner. To simplify the experiment by eliminating the first concentration step, ~1 x 10^9 BA endospores were suspended in 1 mL of 2% H_2SO_4, and then 50 µL of this suspension were transferred to a conical bottom vial and heated in a microwave for

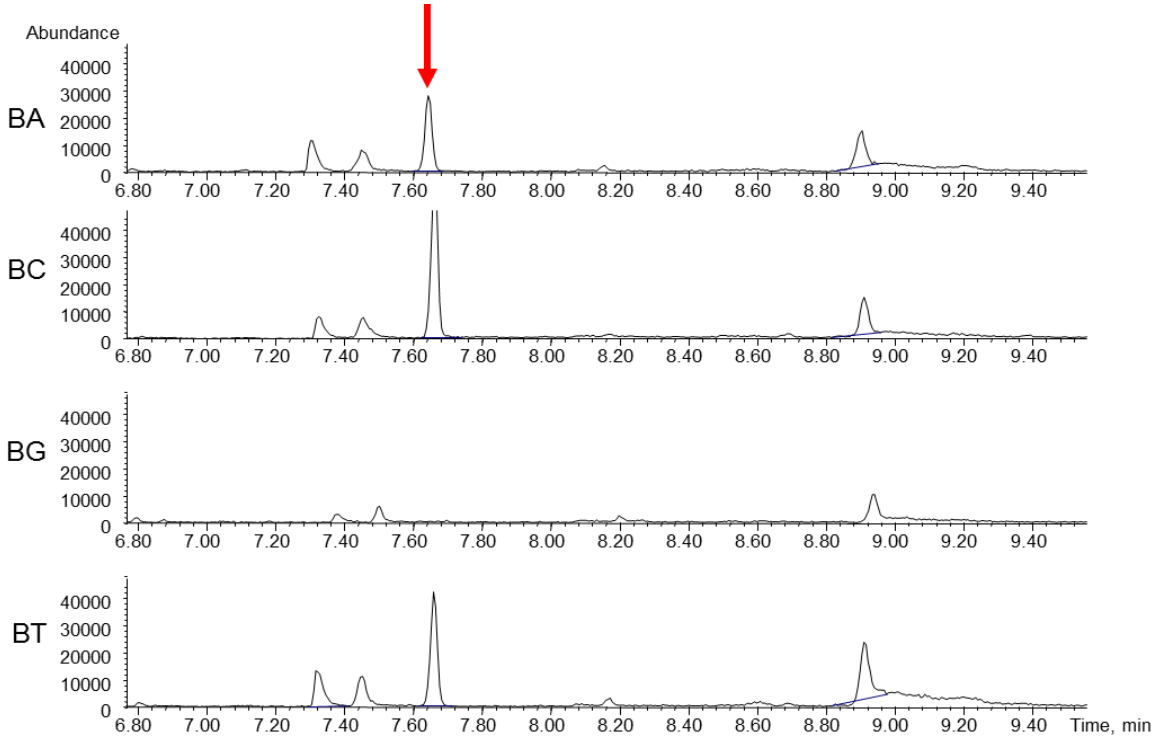

Figure 4.6. Extracted ion chromatogram (m/z 43) of 3-hydroxy butyric acid methyl ester (red arrow) from BA BG, BC, and BT endospores, cultured in LD medium at 32ºC. Conditions: 5 m x 0.25 mm x 0.25 µm film thickness MS-5 column, temperature programmed from 60°C to 300ºC at 8º/min, 30 psi He inlet pressure.

10 s. The sample was cooled down to room temperature, and 10 µL of 1 M TMAH plus 100 µL of 100 ppm chrysene (internal standard) solution were added. The solvent was evaporated under

vacuum until the volumes reached ~10 µL; ~2 µL were then placed onto the coiled wire, and the solvent was evaporated in air before injection. As a result, many sugar derivatives were detected after heat treatment in methanolic H_2SO_4. Figure 4.7 and Table 4.1 show the chromatogram obtained and list the sugar derivatives identified from BA endospores. The red numbered peaks indicate sugar derivatives; however, many small peaks were not labeled due to the lack of space in the figure. These methylated derivatives indicate that TCM with methanolic H_2SO_4 heat treatment followed by TMAH methylation inside the GC liner converted most of the hydroxyl groups (OH) into methoxy groups (OCH_3). However, some information was possibly compromised; for instance, it would be impossible to know whether a methoxy group of a sugar derivative was a methylated form of a sugar OH group or an original methoxy group on the sugar (for example, rhamnose). The sources of the sugar derivatives in Table 4.1 were predicted based on the literature of *Bacillus* endospore sugars. and the structures shown in Table 4.2 were found by searching the NIST mass spectral library and choosing the structure with the highest matching level. The names in red indicate a high probability of correct prediction. Although some compounds could not be differentiated from their geometric isomers by mass spectra alone, their chromatographic retention times can be used to provide additional confirmation. Peak pairs such as 4 and 5; 8 and 11; 9 and 10; 15 and 22; 17 and 18; 25 and 28; 29 and 30; and 32, 34, and 35 have almost the same mass spectra, and predicted structures are actually isomers. Their chromatographic retention times could be used to provide additional confirmation. The corresponding structures of the sugars numbered in Table 4.1 are shown in Table 4.2. Figure 4.7 is an example selected-ion chromatogram for sugars that have m/z 74 ions. Figure 4.7 also indicates that by using the polar FFAP column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) 32 sugar derivatives, produced from saccharides, acetyl amino sugars, and amino sugars, were

separated. As mentioned earlier, methylation under these experimental conditions occurred at most hydroxyl locations on the sugar structures. In addition, significant interactions between the derivatives and the GC stationary phase (nitroterephthalic acid modified polyethylene glycol)

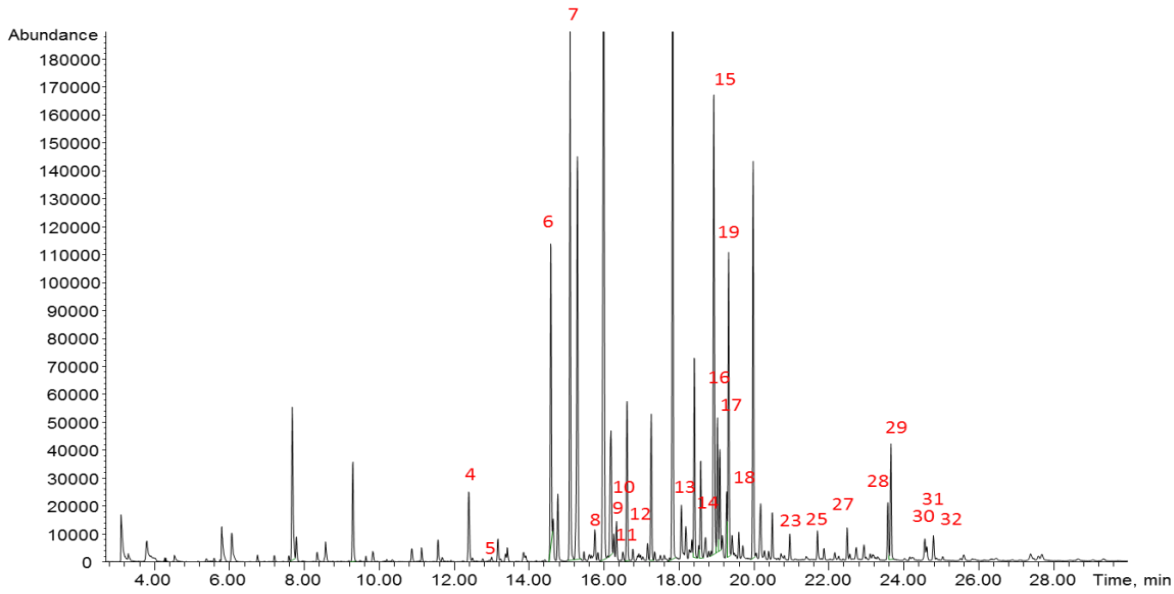

Figure 4.7. Extracted ion (m/z 74) chromatogram of the TCM products from BA endospores prepared using 50 µL of H_2SO_4 (2%), heated by microwave at high energy for 10 s, and adjusted to pH 8-9 using 1 M TMAH. Conditions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 50ºC at 8 ºC/min to 180ºC, and 10ºC/min to 250ºC at 20 psi He carrier gas.

helped to provide valuable identification information. Structures similar to rhamnose and fucose were found for peaks 4, 5, 6, 7, 15, and 22, with high intensities, which agree with many previous reports. Acetylated acetyl amine sugars such as acetylated acetylglucosamine and acetylgalactosamine in their methylated forms (peaks 27, 29, and 30) were also found. These may originate from acetylglucosamine and acetylgalactosamine, or glucosamine and

Table 4.1. Names, retention times, and base ions of methylated sugars and other specific derivatives tentatively identified from TCM products from BA.

Peak No.	Name	RT (min)	Base ion	Proposed Precursor
1	2-Butenoic acid methyl ester	2.61	69	Poly(3-hydroxybutyric acid)
2	3-Hydroxy butanoic acid methyl ester	7.74	43	Poly(3-hydroxybutyric acid)
3	Phosphoric acid trimethyl ester	9.20	110	Nucleotides and Phospholipids
4	Methyl 2,3,4-tri-*O*-methyl-6-deoxy-β-D-mannopyranoside	11.57	88	Rhamnose/Fusose
5	Methyl 2,3,4-tri-*O*-methyl-6-deoxy-β-D-mannopyranoside	13.01	88	Rhamnose/Fusose
6	L-Glucose, 6-deoxy-3-*O*-methyl	14.77	74	Rhamnose/Fusose
7	Methyl 6-deoxy-2-*O*-methyl-β-d-allopyranoside	15.10	74	Rhamnose/Fusose
8	Methyl 2,3,5,6-tetra-*O*-methyl-α-D-galactofuranoside	15.75	101	Unknown
9	Methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside,	16.26	88	Glucose/Galactose/Manose
10	Methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside	16.33	88	Glucose/Galactose/Manose
11	Methyl 2,3,5,6-tetra-*O*-methyl- α-D-galactofuranoside	16.49	101	Unknown
12	Methyl 2,3,5-tri-*O*-methyl- α-D-xylofuranoside	18.35	101	Unknown
13	Methyl 2,4,6-tri-*O*-methyl-β-D-glucopyranoside	18.51	71	Glucose/Galactose/Manose

14	Methyl 2,4-di-*O*-methyl-α-D-glucopyranosiduronamide	18.71	101	Unknown
15	Methyl 6-deoxy-2-*O*-methyl-β-d-allopyranoside,	18.93	74	Rhamnose/Fusose
16	Methyl 2,4,6-tri-*O*-methyl-β-D-glucopyranoside,	19.03	71, 101	Glucose/Galactose/Manose
17	Methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside	19.10	88	Glucose/Galactose/Manose
18	Methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside	19.27	88	Glucose/Galactose/Manose
19	Methyl 2,4,6-tri-*O*-methyl-β-D-glucopyranoside,	19.32	71, 101	Glucose/Galactose/Manose
20	Methyl 2,3,5,6-tetra-*O*-methyl-α-D-galactofuranoside	19.41	101	Unknown
21	Methyl 2,4,6-tri-*O*-methyl-β-D-glucopyranoside	19.60	71, 101	Glucose/Galactose/Manose
22	Methyl 6-deoxy-2-*O*-methyl-β-d-allopyranoside	19.71	74	Rhamnose/Fusose
23	Methyl 3,4,6-tri-*O*-methyl-α-d-glucopyranoside	20.49	71	Glucose/Galactose/Manose
24	Methyl 2,3,4-tri-*O*-methyl-β-D-lyxopyranoside	20.72	88, 101	Unknown
25	Methyl 2-deoxy-3,4,6-tri-*O*-methyl-2-(N-methylacetamido)-α-D-glucopyranoside	20.95	88	Acetyl(glucose/galactose/manose)amine
26	Methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α-d-glucopyranoside	22.15	73, 115	Acetyl(glucose/galactose/manose)amine
27	Methyl 2-(acetylmethylamino)-2-deoxy-3,4-di-*O*-methyl-α-D-glucopyranoside, acetate	22.49	87, 142	Acetyl(glucose/galactose/manose)amine
28	Methyl 2-(acetylmethylamino)-2-deoxy-3,4,6-tri-*O*-methyl-α-D-glucopyranoside,	23.57	87, 142	Acetyl(glucose/galactose/manose)amine
29	Methyl 6-*O*-acetyl-2-acetamido-2-deoxy-3,4-di-*O*-methyl-α-D-glucopyranoside	23.65	73, 115	Acetyl(glucose/galactose/manose)amine

30	Methyl 6-*O*-acetyl-2-acetamido-2-deoxy-3,4-di-*O*-methyl-α-D-glucopyranoside	24.54	73, 115	Acetyl(glucose/galactose/manose)amine
31	Methyl 2-(acetylmethylamino)-2-deoxy-3,4,6-tri-*O*-methyl-α-D-galactopyranoside,	24.61	87, 142	Acetyl(glucose/galactose/manose)amine
32	Methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α-D-galactopyranoside	24.79	73, 115	Acetyl(glucose/galactose/manose)amine
33	Methyl 2-(acetylamino)-2-deoxy-4,6-di-*O*-methyl-α-D-galactopyranoside	27.38	71, 128	Acetyl(glucose/galactose/manose)amine
34	Methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α-d-glucopyranoside	27.59	73, 115	Acetyl(glucose/galactose/manose)amine
35	Methyl 2-(acetylmethylamino)-2-deoxy-3,4,6-tri-*O*-methyl-α-D-galactopyranoside	27.68	87, 129	Acetyl(glucose/galactose/manose)amine

Table 4.2. Structrures of methylated sugars and other specific derivatives of BA as suggested by a NIST library search.

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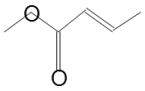
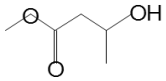
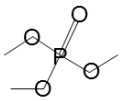
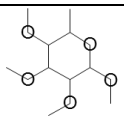
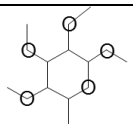
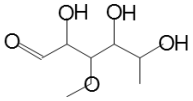
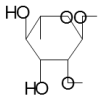
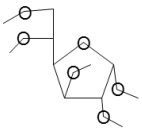
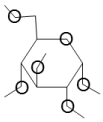
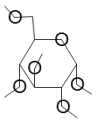
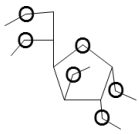
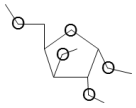
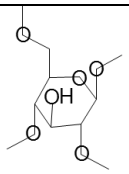
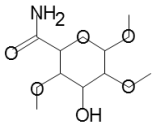
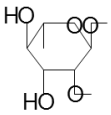
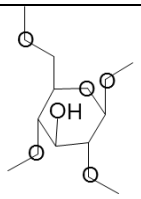
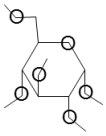
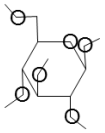
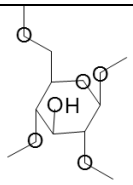
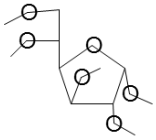
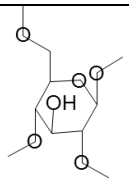
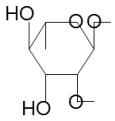
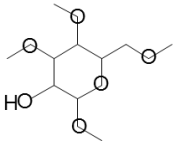
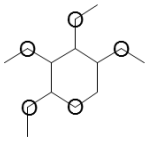
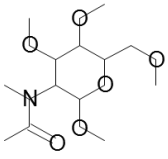
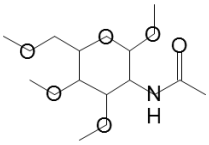
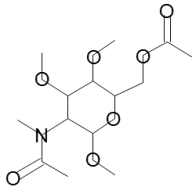
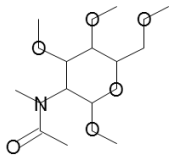
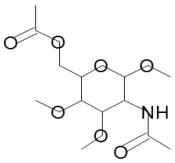
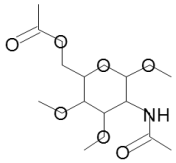
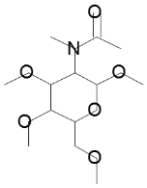
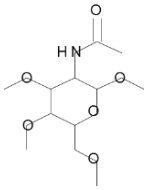
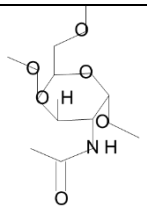
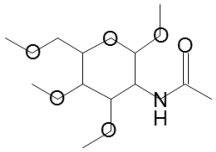
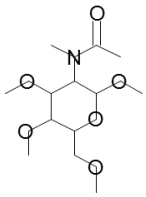
1		2	
3		4	
5		6	
7		8	
9		10	

11		12	
13		14	
15		16	
17		18	
19		20	
21		22	
23		24	
25		26	

27		28	
29		30	
31		32	
33		34	
35			

galactosamine, after being acetylatedon the meta-hydroxy group, or acylated and acetylated by acetic acid produced from amino acids via nucleophilic acyl substitution and acetic anhydric from acetic acid in strong H_2SO_4 medium. The structures of peaks 13, 25, 26, 31, and 32-35 indicate that acetyl amine sugars were present, or galactosamine or glucosamine present in *Bacillus* endospores were acylated. Peaks 8, 17, and 23 most likely originate from a complicated series of methylation, ring opening and cyclization reactions.

TCM with addition of TMAH further improved the methylation efficiency. Without the addition of TMAH, chromatograms with low peak intensities were obtained (data not shown). Interaction between TMAH and the target compounds occurred on the surface of the Pt/Ir wire coil. While the catalytic effect of Pt has not yet been confirmed, the high thermal conductivity of this coiled wire, with its advantages as introduced previously in Chapters 2 and 3, indicate that this method is promising for fast detection of diverse biomarkers of microorganisms.

4.3.4 Sugar derivative profiles of the four *Bacillus* species

Further TCM experiments were carried out for the four *Bacillus* species endospores using a short (5 m), weakly polar column (5% phenyl, 95 methyl, polysiloxane) for fast GC-MS.

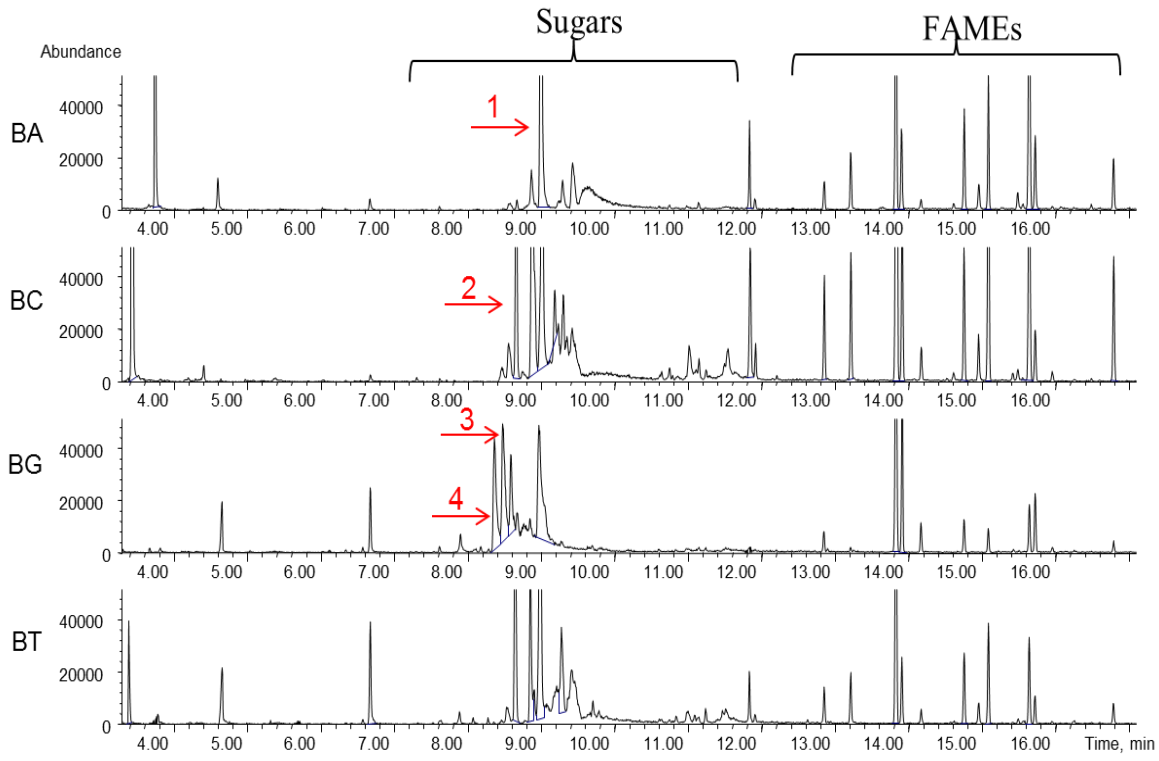


Figure 4.8. Extracted ion (74 m/z) chromatograms of the TCM products (FAMEs and sugar derivatives) from BA, BC, BG, and BT, grown in LD media at 32ºC. Conditions: 5 m x 0.1 mm i.d. x 0.4 µm film thickness MS 5 column, temperature programmed from 40ºC at 8ºC/min to 290ºC at 30 psi He carrier gas.

Extracted ion (m/z 74) chromatograms shown in Figure 10 indicate distinct sugar derivatives for these *Bacillus* species grown in LD medium at 32°C. Figure 4.9 indicates high reproducibility for differentiation of BA and BT endospores.

Comparison of the sugars detected in the various *Bacillus* species shows that BA contains a trace of a rhamnose-like methylated and/or acetylated sugar (peak 2 in Figure 4.8) while BT and BC have much higher levels. BG possesses rhamnose-like methylated and/or acetylated sugars (peaks 3 and 4 in Figure 4.8) that were not found in the others. Using the weakly polar, short column, most of the methylated sugars were eluted before the main FAMEs (see Figure 4.8). Although the peaks are not separated completely, they are clearly recognized and different enough for distinguishing BA from other species. The separation of these compound classes was

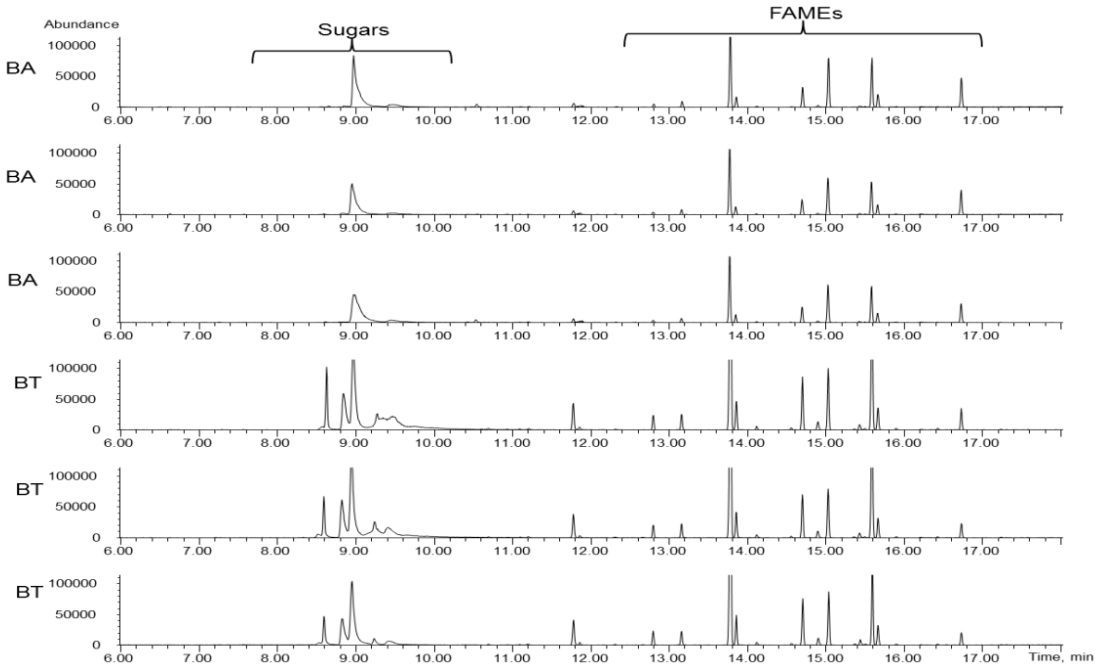


Figure 4.9. Extracted ion (74 m/z) chromatograms of the TCM products (FAMEs and sugar derivatives) from BA and BT grown in CA at 32ºC. Conditions: 5 m x 0.1 mm i.d. x 0.4 µm film thickness MS 5 column, temperature programmed from 40ºC at 8ºC/min to 290ºC at 30 psi He carrier gas.

improved significantly when using the longer FFAP column, because they moved toward the ends of the chromatograms due to their stronger interactions with the stationary phase. Figure 4.10 shows differences in the monosaccharide profiles from BA, BC, BG, and BT grown in LD medium at 32ºC. The peaks identified by red arrows are compounds that can be used for differentiating one species from the other. BG can be easily recognized based on the presence of peaks 1 and 2 (unique structures similar to numbered peaks 15 or 22 in BA, and peak 1 coelutes with a FAME). There are more peaks in BT, such as peaks 3, 4, 5, and 6 whose structures are similar to numbered peaks 12, 15, 19, and 22 in BA, respectively, and peak group 7 contained different acetylated and acylated species. These structures did not appear in BA. Peak 6 group 7 were prominent in BT, much lower in BC, and absent in BG.

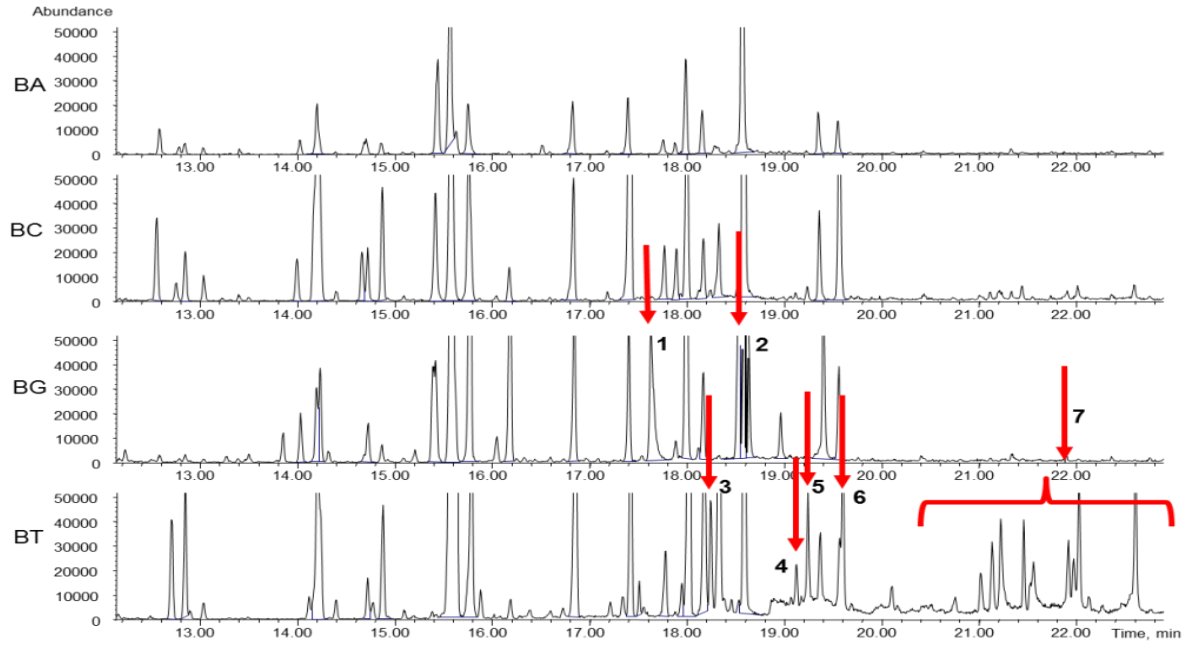


Figure 4.10. Extracted ion (m/z 74) chromatograms of the TCM products (FAMEs and sugar derivatives) from BA, BC, BG, and BT, grown in LD at 32ºC. Conditions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 50°C at 10°/min to 250°C at 15 psi He carrier gas.

A similar result was also found for endospore samples grown in Col medium at 32ºC (see Figure 4.11). The group 7 peak intensities were lower than when the spores were grown in LD medium. The presence and absence of the other compounds were almost the same. The FFAP column provided excellent separation of all types of biomarker derivatives produced from TCM without any noticeable chromatographic degradation after hundreds of sample analyses.

Encouraged by these promising results, I investigated the differences in FAME and sugar biomarkers of *Yersinia pestis* (YP) and *Francisella tularensis* (FT) cells, two bacterial bio-threat agents, using the same method. Preliminary results, as shown in Figure4.12, indicate that compound groups (methylated sugars and FAMEs) were easily differentiated from and from each other, not only in peak intensities but also by the presence or absence of peak. For example, iso and anteiso C15:0 and C17:0 never appear in YP and FT but are present in BA and the other *Bacillus* endospores. Many other saturated and unsaturated FAMEs (>C18) appear in FT, but are fewer and lower in intensity in BA and YP. On the other hand, the sugar group showed an opposite trend, i.e., there were more compounds and with high intensities in BA compared to FT and YP. The presence of DPAME only in BA further differentiated them from BA. Comparing YP with FT (see Figure 4.13), intensities and number of high molecular weight saturated and unsaturated FAMEs (> C18, i.e., group 6 containing C20, C22, C24, and C26), were higher for FT. On the other hand, 2-hexyl cyclopropane octanoic acid methyl ester (peak 3), 2-octyl cyclopropane octanoic acid methyl ester (peak 4), and C15:0 and C17:0 FAMEs (peaks 1 and 2, respectively) were found in YP but not in FT.

4.3.5 Discrimination of *Clostridium* and *Bacillus* species endospores

Differences in sugar derivatives and FAMEs between *Bacillus* and *Clostridium*

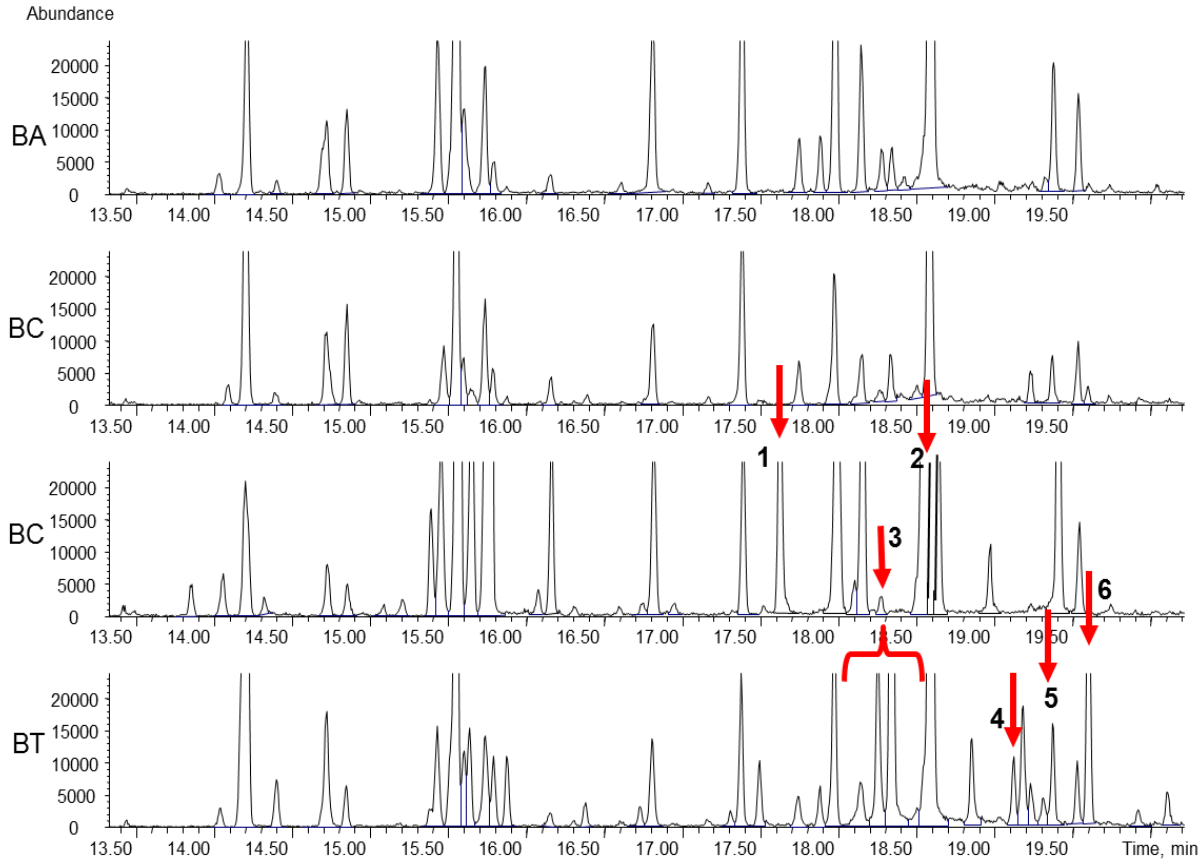


Figure 4.11. Extracted ion (m/z 74) chromatograms of the TCM products (FAMEs and sugar derivatives) from BA, BC, BG, and BT, grown in Col at 32ºC. Conditions: 30 m x 0.25 mm i. d. x 0.25 µm film thickness FFAP column, temperature programmed from 50°C at 10°/min to 250°C at 15 psi He carrier gas.

endospores using the CWF method are shown in Figures 4.14 and 4.15. Discrimination of *Clostridium* from *Bacillus* species endospores based on these profiles was fast and easy, as indicated above by using specific FAMEs (iso and anteiso C15: and C17:0) and methylated sugars produced by TCM and separated on FFAP or shorter MS 5 columns. FAMEs formed without heat treatment (see Figure 4.15) were used for BA and *Clostridium* endospore discrimination, and FAMEs plus sugar derivatives formed without heat treatment (see Figure

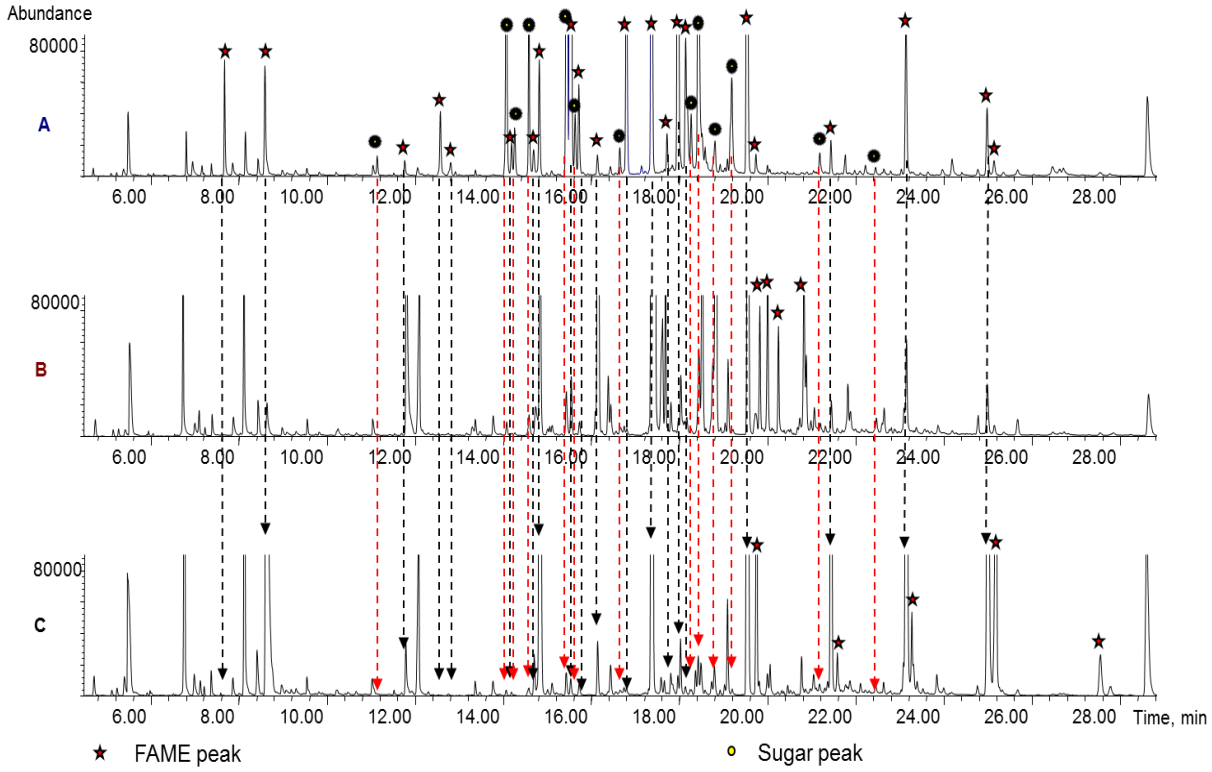


Figure 4.12. Extracted ion (m/z 74) chromatograms of the TCM products (FAMEs and sugar derivatives) from (A) BA, (B) *Yesinia pestis, and* (C) *Francisella tularensis* grown in LD at 32ºC. Conditions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 50°C at 8°/min to 180°C, and 10°/min to 250°C at 20 psi He carrier gas.

4.14) were employed for BA, BG, and *Clostridium* endospore differentiation. Generally, retention times and mass spectral profiles allowed for a rapid differentiation.

4.3.6 Detection limit of the CWF concentration method

Experiments performed with the CWF method indicated that the detection of biomarkers was improved significantly. Although FAMEs and methylated sugars were simultaneously

detected, the DPAME unique biomarker appearing in samples containing a couple of thousand endospores was used to calculate the detection limit for *Bacillus* endospores. The chromatogram

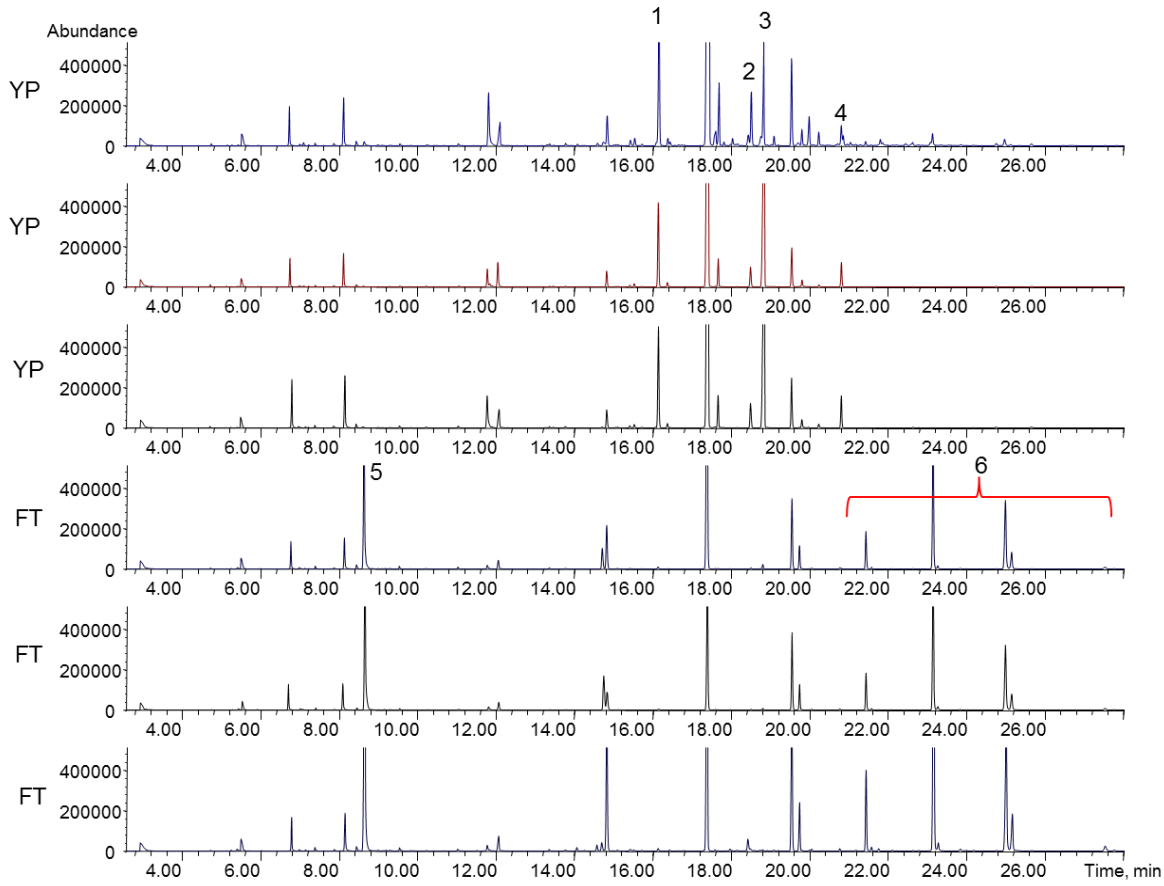


Figure 4.13. Extracted ion (m/z 74) chromatograms of the TCM products from *Yesinia Pestis* and *Francisella tularensis* grown in LD at 32ºC. Conditions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 50°C at 8°/min to 250°C at 20 psi He carrier gas. Peak identification: (1) C15:0 FAME, (2) C17:0 FAME, (3) cyclopropane octanoic acid 2-hexyl methyl ester, (4) 2-octyl cyclopropane octanoic acid methyl ester, (5) C10:0 FAME, (6) group of > C18 saturated and unsaturated FAMEs.

shown in Figure 4.16 shows the DPAME peak produced from analyzing a BA sample containing approximately 50,000 endospores. Because a large (up to 50% or higher) amount of sample may be introduced into the GC-MS system using a concentration step and CWF sample introduction,

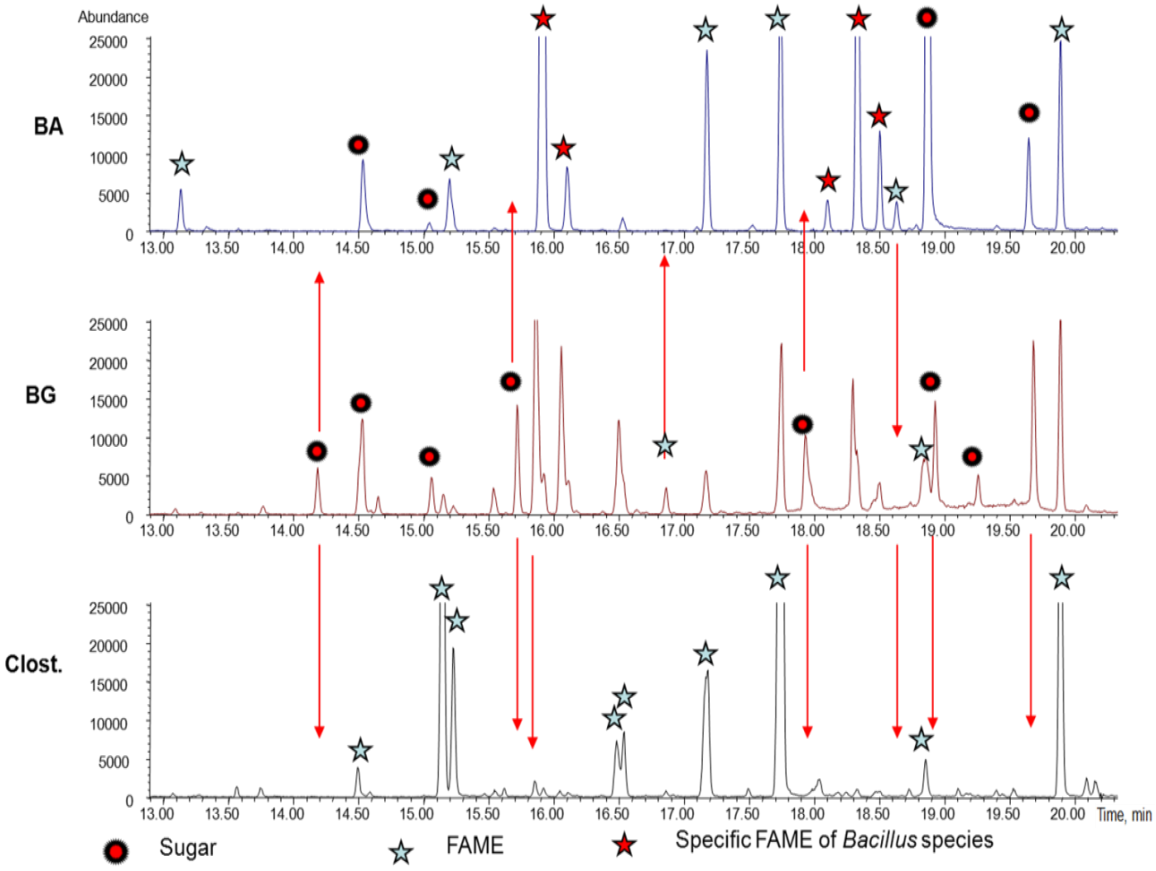


Figure 4.14. Extracted ion 74 m/z chromatogram of FAMEs and MMDs produced by thermochemolysis methylation with heat treatment. Conditions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 50°C at 10°/min to 250°C at 20 psi He carrier gas.

this allows for detection of a small amount of endospores in the sample. Approximately, 2500 spores was the detection limit for *Bacillus* endospores using the CWF concentration method. However, for detecting BA, 3-hydroxy-3-methyl butyric acid should be combined with DPAME, and the detection limit should be then calculated by using the less sensitive biomarker.

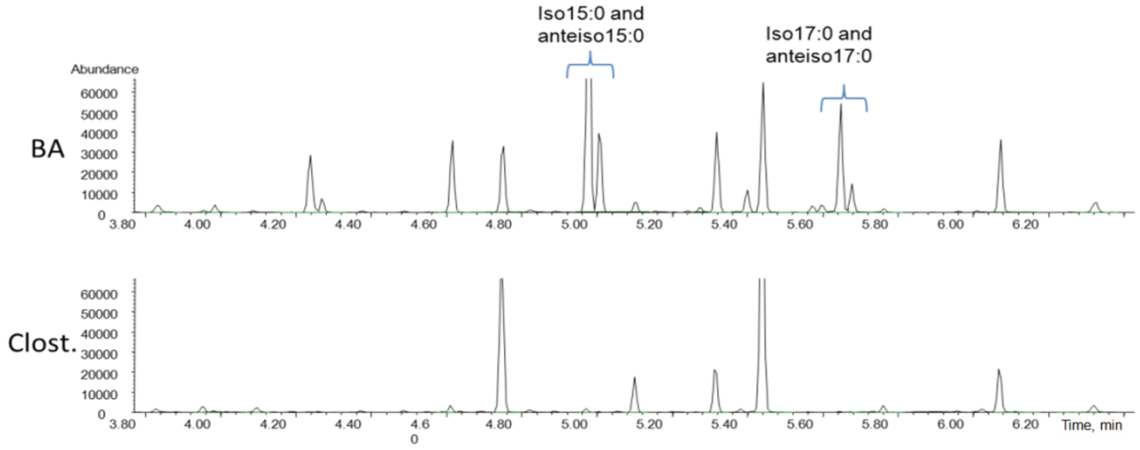


Figure 4.15. Extracted ion 74 m/z chromatograms of FAMEs (iso C15:0, anteiso C15:0, iso C17:0, and anteiso C17:0) of BA and *Clostridium* endospores. Conditions: 5 m x 0.1 mm x 0.4 µm film thickness MS 5 column, temperature programmed from 50°C at 20°/min to 250°C at 30 psi He carrier gas.

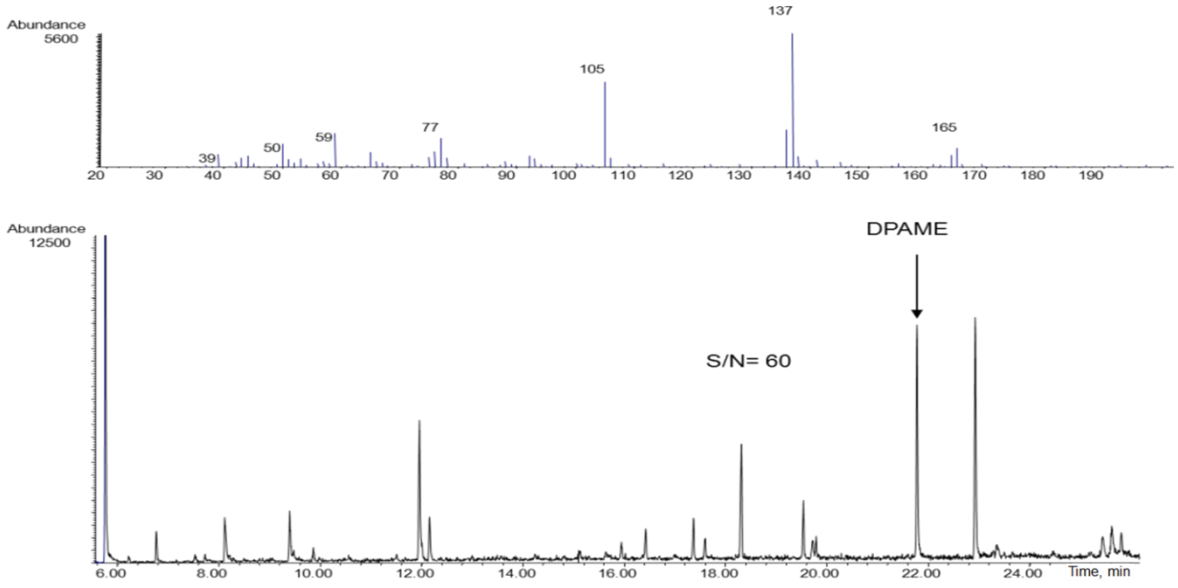


Figure 4.16. Mass spectra and extracted ion m/z 137 chromatogram of DPAME in a BA sample (containing approximately 50,000 endospores) using the CWF method. The final volume was approximately 5 µL; 2.0 µL of final suspension was injected using the CWF (sample drop placed om coiled wire). Conditions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 50°C at 10°/min to 250°C at 20 psi He carrier gas.

4.4 CONCLUSIONS

H_2SO_4 heat treatment and concentration improved the TCM yields, the number of biomarker derivatives, and the detection limits of the endospores. The analysis time was reduced very significantly from hours to minutes, especially when using a household microwave oven.

SP using a CWF can be employed to detect biomarkers that allow the differentiation of *Bacillus* and *Clostridium* endospores, and YP and FT. Methylated sugars, DPAME, 3HBAME and specific FAMEs produced from TCM with H_2SO_4 heat treatment improve the detection of BA endospores from clean samples. Experimental results also indicated that the detection of biomarkers was improved significantly,i.e., DPAME can be detected in samples containing a few thousand endospores. The CWF is a promising tool for on-or-off-line TCM and GC-MS in biomarker detection.

In addition to DPAME and iso C15:0, anteiso C15:0, iso C17:0, and anteiso C17:0 fatty acid methyl esters, carbohydrate profiling has been demonstrated to be a powerful tool for differentiating closely related *Bacillus* species. The differentiation may not depend solely on whether or not organisms are in the vegetative or spore stages of growth.

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5 IDENTIFICATION OF *BACILLUS* ENDOSPORES FROM SUGAR BIOMARKERS USING SOLID PHRASE MICRO EXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

5.1 INTRODUCTION

As introduced in Chapter 4, rapid detection and differentiation of pathogenic bacteria (category A), such as *Bacillus anthracis* (BA), *Clostridium botulinum*, *Yesinia pestis*, and *Francisella tularensis* are important to determine the possibility of a bioterrorism attack. These microorganisms must be differentiated not only from each other, but from other microorganisms and other biologically-derived materials in different types of samples in the field. In addition to profiles of the classes of biomarkers, such as fatty acids and sugars, specific markers, including dipicolinic acid (DPA) and anthrose can be used to confirm more certainly the presence of BA.[1-5]

Although, a variety of techniques, as introduced in Chapter 1 and 4, such as polymerase chain reaction (PCR, a primer-mediated enzymatic DNA amplification method), immunoassays, vibrational spectroscopy (i.e., Raman and infrared)[6-8] and, recently luminescence as reported by Cable[9] have been used to detect dipicolinic acid, they typically require specific reagents and conditions for sample preparation that are inconvenient to use in the field and for fast analysis. Furthermore, some methods have high detection limits[7,8,10] and are based solely on DPA as a biomarker, which limits their ability to provide differentiation information.

Mass spectrometry (MS) has been employed and considered a useful technique in microorganism analysis for many years.[11-13] Matrix-assisted laser desorption ionization (MALDI) MS,[14] electrospray ionization-tandem MS have been used to detect high molecular mass biomarkers (i.e., biomacromolecules such as proteins and tryptic peptides[15-30]). Generally,

MALDI-MS has been used to characterize and differentiate microorganisms at species and strain levels. [30,31] On the other hand, it cannot be employed easily for in-field applications.

Gas chromatography-mass spectrometry (GC-MS) as mentioned before also has a major limitation. It cannot be used directly for biological agent detection without first using reactions such as pyrolysis, thermolysis, hydrolysis, acetylation, methylation, and silylation to break down the microorganism structure and release the biomarker compounds as volatile analytes. If optimized, electron ionization (EI), selected-ion-monitoring (SIM), and extracted ion monitoring (EIM) MS in GC-MS with quadrupole or ion-trap analysers can be used effectively for biomarker detection;[32] DPAME, FAMEs, methylated sugars[33-37] and sugar by-products have been exploited for detection and differentiation of bacterial organisms.

As explained in Chapters 4 and 5, TCM of bacterial endospores can be conducted in short time using methanolic H_2SO_4 solution in high concentration and elevated temperature. A coiled wire filament injection method facilitated the reaction inside the heated GC injection liner. DPAME and FAME profiles from GC-MS analyses of TCM products were sufficient for detecting and differentiating BA from other *Bacillus* species.[39] DPAME was used to detect *Bacillus* and *Clostridium* endospores while typical FAMEs such as iso C15:0, anteiso C15:0, iso C17:0, and anteiso C17:0 were used to differentiate *Bacillus* from *Clostridium.* Although, the presence and absence of certain FAMEs (i.e., C16:1Δ^9a, iso C17:1, and anteiso C17:1) can be used for differentiation of endospores from BA, BG, BC and BT,[39] they are not abundant biomarkers and can be difficult to detect. Since anthrose is part of an abundant tetrasaccharide unique to BA, we investigated the production of unique fragments of anthrose in TCM products that could be used as more specific biomarkers.

Anthrose, a non-reducing terminal sugar, 4,6-dideoxy-4-[(3-hydroxy-3-methyl-1-oxobutyl) amino]-2-*O*-methyl-D-glucopyranose, was found in a polysaccharide with the structure shown in Figure 4.1. This tetrasaccharide settles on the surface of the exosporium glycoprotein BC1A BA and consists of three L-rhamnose sugars and a D-sugar. It is an antigen

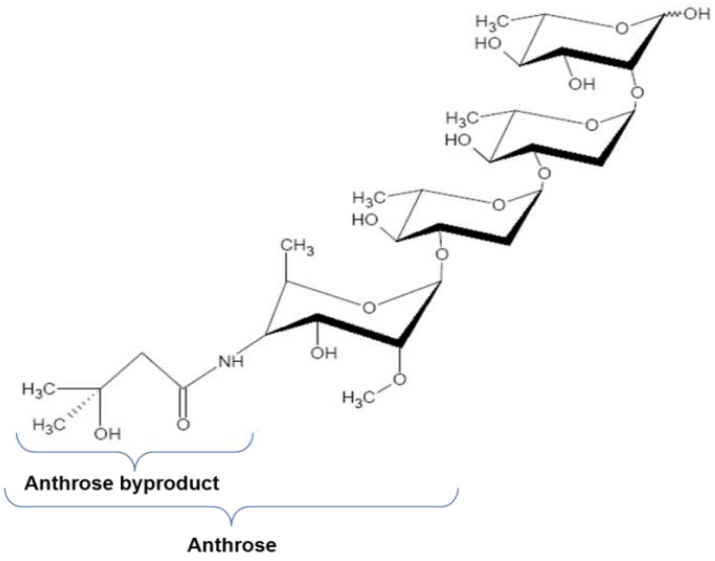

Figure 5.1. Structure of BA endospore tetrasaccharide containing anthrose and rhamnose.

that was discovered and elucidated in 2004 by Turnbough.[40,41] A unique characteristic of this antigen is its D-sugar, anthrose, which is not present in related strains of bacteria.[42] Daubenspeck[41] also indicated that anthrose was found in *B. anthracis* and not in either BC or BT, two species that are the most phylogenetically similar to it. Other authors also indicated that anthrose may be used as a biomarker for species-specific detection of BA spores or as a new target for therapeutic intervention.[41,43,44]

In 2008, Dong, a coauthor of Daubenspeck's publication, after doing additional experiments, confirmed that anthrose also exists in BT Al Hakam and BT subsp. *Kurstaki* although in lower concentrations (42, 3, and 8%, respectively, compared to the BA Stern strain). Therefore anthrose biosynthesis is not restricted to BA as previously suggested.[1]

3-Hydroxy-3-methylbutyric acid (3H3MBA) or 3-hydroxy-3-methylbutyrate has been known as a metabolite of the essential amino acid, leucine, which is synthesized in the human body. 3H3MBA from endogenous production occurs in liver, muscle, and possibly other tissues. In fact, 3H3MBA is also currently marketed as a calcium salt because it has been shown to increase strength and lean mass gains in humans undergoing resistance-exercise training.[45-47] anthrose can also be converted to 3H3MBA by enzymes, e.g., peptidases or artificial catalysts, and anthrose can also be hydrolyzed in hot and strong acid as well as in alkali.

3-Hydroxy butyric acid (3HBA), which was discussed in Chapter 4, can also be detected by TCM using SPME. It is known to be present in significant amounts in BA cells, but in very lower amounts in vegetative BG cells.[48]

There is an additional issue that may affect the detection of 3M3HBA; i.e., although anthrose is not present in *Shewanella spp*.,[49] 3-methyl-2-butenoic acid methyl ester may be produced by TCM due to the anthrose-like saccharide structures that exist in these organisms (see Figure 5.2 A). Therefore, the presence of *Shewanella spp* in a sample can simultaneously produce not only 3HBAME or 2BAME (see Figure 5.2 B), but also 3M2BAME as described in Chapter 4. Another source of 3M2BAME is the Ca salt of 3M3HBA, available under the name 3-hydroxy-3-methylbutyric acid calcium salt monohydrate (2010 TCI© American) as a commercial dietary supplement which promotes fat loss and increases strength and fat-free mass in studied

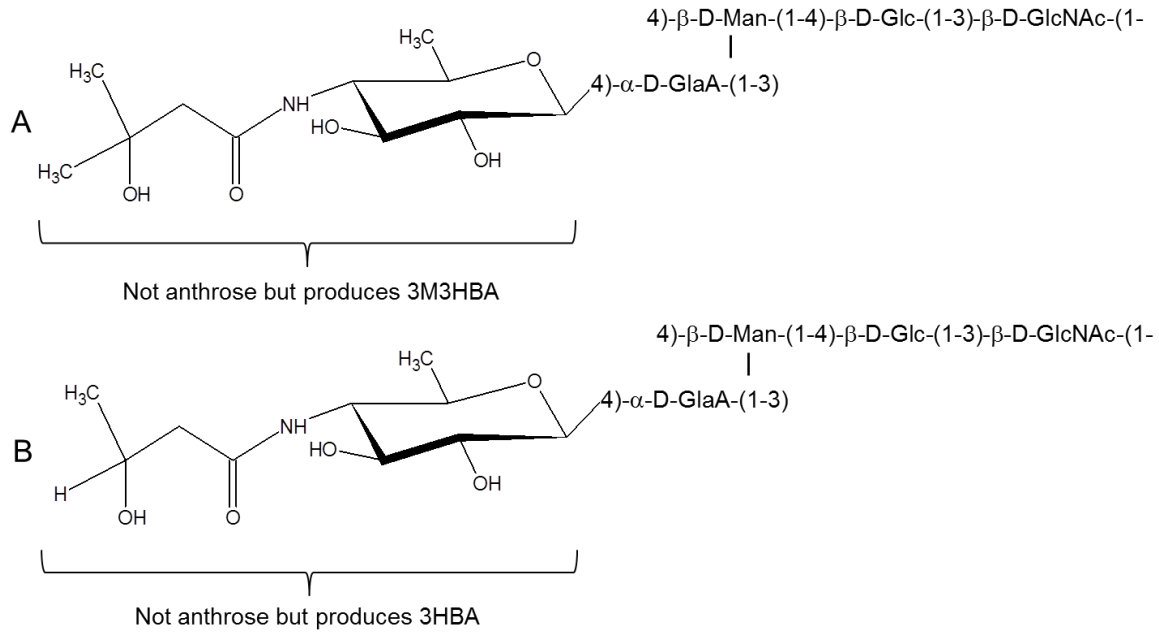

Figure 5.2. Structures of saccharides of *Shewanella spp*. MR-4 CPS (the ratio of the two structures depends on the growth conditions).[44,52] (A) Structure can produce 3M3HBAME, (B) structure can produce 3HBAME.

populations.[50,51] The intentional or incidental presence of this compound in samples would affect the detection of BA endospores if 3H3MBA was used as a specific biomarker.

5.2 EXPERIMENTAL

5.2.1 Chemicals and materials

HPLC grade methanol (MeOH) was obtained from EMD (San Diego, CA, USA). H_2SO_4 (98%) was from Mallinckrodt Chemical (Phillipsburg, NJ, USA). SPME fibers (fused silica, 23-gauge), 2-cm long, 50/30 µm DVB/CAR/PDMS, 65 µm DVB/PDMS, 75 µm CAR/PDMS, and 100 µm PDMS) were obtained from Supelco (Bellefonte, PA, USA).

5.2.2 GC-MS system and conditions

An Agilent 6890 gas chromatograph (San Jose, CA) with split/splitless injector was fitted with a 0.75 mm i.d. Restek liner to accommodate the sampling device. The GC was coupled to an Agilent 5793 MS with electron ionization and quadrupole analyzer. Two polar GC capillary columns (10 m and 30 m x 0.25 mm ZB-FFAP) were used. Typical chromatographic operating parameters included 250-260ºC injector temperature, 2-min splitless injection time, temperature program from 50 (initial) to 250-255ºC (final) at different rates depending on the analysis, and 230-250ºC transfer line temperature between the GC and MS.

The MS operating parameters for all experiments were: 230ºC quadrupole temperature, 150ºC source temperature, 30-500 m/z mass range, 1670 EM voltage, 35 µA emission current, and 70 eV ionizing voltage. Extracted ions 74 m/z, 137 m/z, 83 m/z, and 69 m/z were used to reconstruct the FAME, DPAME, 3M2BAME, and 2BAME chromatograms, respectively.

5.2.3 Microwave heating for TCM

As discussed in Chapter 4, a microwave oven can be used to speed up reactions in the preparation of organic compounds.[54-58] Microwave heating was used to enhance the intensity of biomarker profiles and improve their detection limits. The same household microwave oven was used in this study as described in Chapter 4. The power and time were modified to suit the sample properties. From 10-30 s at high energy were required for 40-100 µL sample suspensions in methanolic H_2SO_4 (4-5% v/v). The use of a household microwave provided almost the same results as those obtained using a hot plate.

5.2.4 Microbiological samples

The same procedures and conditions for culturing the bacterial endospores were used as described in Chapter 4. The spores were collected off the agar plates and heat treated to kill any

remaining vegetative spores. The spores were washed once a day for three days before GC-MS analysis.

5.2.5 Endospore growth

Unless otherwise specified, all endospore suspensions were prepared in a biosafety level 2 facility located on the Brigham Young University campus. Four distinct *Bacillus* species were cultured following the same procedure described in Chapter 4.

5.2.6 New biomarker detection experiments

Different experiments were performed with *Bacillus* endospores grown at 32ºC and 37ºC in Columbia and LD media to determine unique biomarkers for differentiation. The main goal was to detect a new biomarker derived from anthrose by a simple and fast method without interfering with the detection of other biomarkers. The protocol used for previous *Bacillus* endospore batches (see Chapter 4) was modified. TCM in this method used higher H_2SO_4 concentration, no addition of TMAH, but addition of KOH or phosphate buffer to adjust the pH before SPME sampling. Besides DPAME and FAMEs, 3M2BAME (the by-product of anthrose) and 2BAME became new biomarkers that were investigated to improve the detection and differentiation of BA from the other bacteria.

5.2.7 Concentration and TCM with SPME sampling

This method was simpler than the previously described method for CWF sampling. However, the H_2SO_4 concentration was higher (~4-5% v/v in MeOH after the first concentration step). The heating time and temperature were almost the same (~5-8 min at 120ºC for 20-40 µL samples) depending on the sample size and vial volume. Figure 6 shows the stepwise procedure, described as follows:

Sample transfer and concentration. The same procedure as outlined for the coiled wire

method was performed, except both a conical bottom (1.5-2 mL size) or flat bottom (3 dram) vial was used. Sample was concentrated by evaporating the solvent using N_2 purging gas or vacuum until 50-100 µL of sample remained in the 1.5 mL vial, achieving 4-5% v/v H_2SO_4 concentration.

pH adjustment. The sample pH was adjusted to 3-5, after the heating step when the sample cooled, by adding KOH solution (~0.1 N), or a buffer, such as phosphate (1 M) as indicated from pH paper (or methyl orange or bromophenol blue indicator).

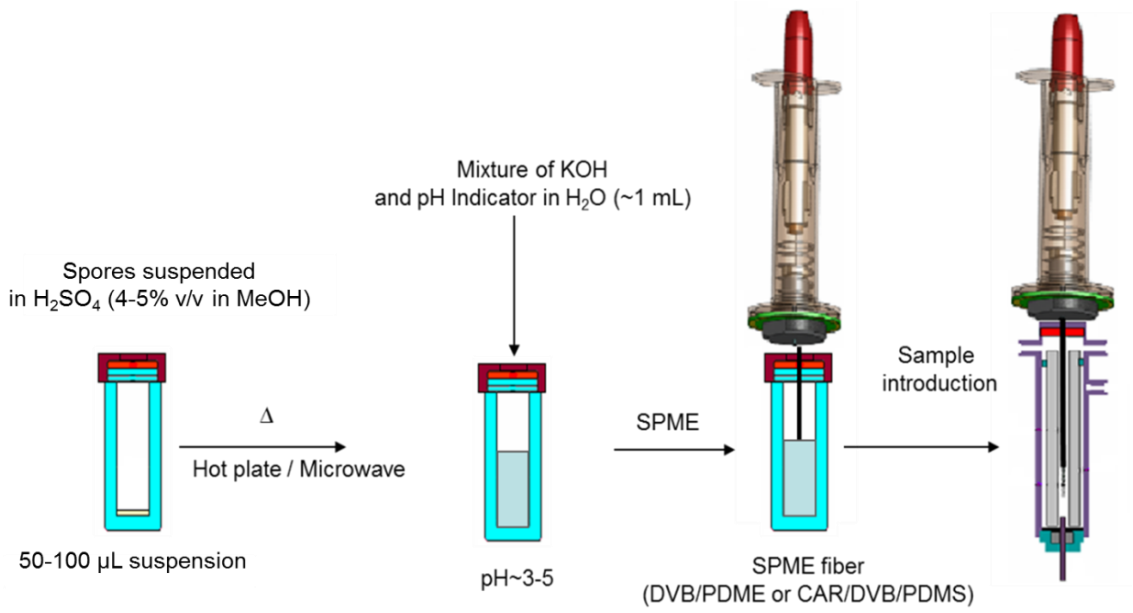

Figure 5.3. Stepwise procedure for TCM and concentration for SPME sampling.

SPME. A suitable SPME fiber (DVB/PDMS or DVB/CAR/PDMS) was used at room or higher temperature with stir bar vibration or similar action to speed up the extraction. At room temperature, the extraction was performed for 20 min. For injection, the fiber was heated inside the GC-injector (preheated at 250ºC) for not less than 30 s.

The biomarker detection limit is an important aspect of the method development; therefore, optimization of the main steps of the analysis was performed. I addition to optimization of GC injection, GC separation, and MS detection, the conditions of sample preparation were also investigated, such as determination of (1) the best fiber for SPME, (2) the optimum pH for SPME, and (3) the heating time. The fiber was determined by examination of four commercial fibers: DVB/PDMS, CAR/PDMS, PDMS, and DVB/CAR/PDMS. In our experiments, SPME was performed by exposing the whole length of the fiber coating to the liquid sample or by simultaneously sampling both the liquid sample and the headspace above the sample (called half-half extraction) at ambient (20ºC) or higher temperature with stirring (~500 stir bar speed). The optimum pH for SPME was determined by preparing and analyzing the sample after its pH was adjusted to values from 2 to 12 by using different concentrations, but the same volume, of KOH solution.

Estimates of measurement precision. Experiments were conducted using different samples in the same batch of each the four *Bacillus* species (BA, BC, BG, and BT) cultured under the same conditions to estimate the precision of peak area intensities of the methylated biomarkers detected by GC-MS after TCM and SPME. The graphs shown in a number of figures in the following section include error bars that represent the outer limit values of 3 to 6 measurements averaged for each data point.

5.3 RESULTS AND DISCUSSION

5.3.1 Concentration

A concentration step was often necessary prior to heating the sample for TCM to increase the H_2SO_4 concentration, especially when a low concentration and/or large volume of solution were used. This was required when endospores had to be washed off a filter or impactor surface.

Methanolic H_2SO_4 is popular for methylating compounds, and its efficiency is improved at high temperature and under anhydrous conditions. In addition, H_2SO_4 is also a good digestion medium, which helps significantly to release DPA from $Ca(DPA)_2$ in the spore core.

5.3.2 Determination of anthrose via its TCM by-product derivative, 3M2BAME

As previously described, a method was developed to detect anthrose, a unique terminal tetrasaccharide found in BA endospores. Use of SPME for extraction of target biomarkers has several advantages. SPME can be used to extract biomarkers from both vapor and liquid phases. Specifically, 3M2BAME, which is hard to detect using the coiled wire TCM method can be easily seen. SPME also allows for simultaneous detection of FAMEs, DPAME, and 3HBAME for differentiation.

The primary focus in this work was the determination of 3M3HBA, which forms methyl derivatives of 3M3HBAME and 3M2BAME. DPAMS, FAMEs and 2BAME are detected simultaneously by SPME sampling and GC-MS (see Figure 5.4). 3-Hydroxy-3-methyl butyric acid is an intermediate TCM product of anthrose via nucleophilic acyl substitution. From the structure of the tetrasaccharide shown in Figure 5.1, we can see that anthrose contains an amide group that can be converted into 3-hydroxy-3-methyl butyric acid by TCM. The amide group in anthrose is hydrolyzed to a carboxylic acid and an amine by acid catalysis (H_2SO_4) at high temperature. The mechanism of the conversion follows a series of reactions including cleavage of the tetrasaccharide, nucleophilic acyl substitution of anthrose, and methylation. Figure 5.5 outlines this proposed mechanism.

According to this mechanism, 3M2BAME should be the main product when ~5% v/v H_2SO_4 is used and ~20 µL sample is heated at 120ºC for 5 min. 3-Hydroxy-3-methyl butanoic acid methyl ester becomes the major product when a lower H_2SO_4 concentration or lower heating

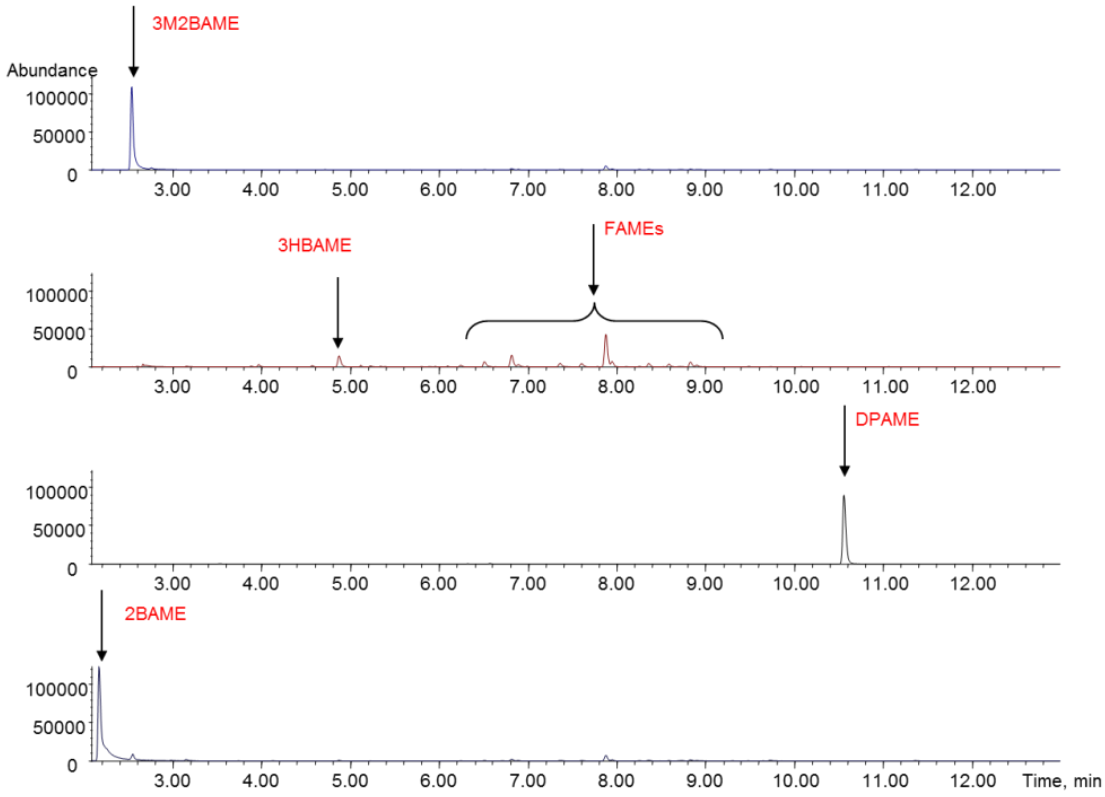

Figure 5.4. Extracted ion chromatograms of the TCM products [3M2BAME (m/z 83), 3HBAME and FAMEs (m/z 74), DPAME (m/z 137), and 2BAME (m/z 69)] from BA endospores grown in LD medium at 32ºC, (2 x 10^7 endospores) suspended in 20 µL H_2SO_4 5% v/v in MeOH. The suspensions were heated on a stirrer/hot plate heater at 120ºC. SPME conditions: DVB/CAR/PDMS, 2 cm fiber, 20 min, 20ºC, pH ~ 3.5, stir-bar mixing at ~500 rpm. GC conditions: 10 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 50°C at 20°/min to 250°C; 6 psi He carrier gas.

temperature (and/or short heating time) are used. The detection of 3M2BAME is an advantage over detection of 3H3MBAME or anthrose because of its high volatility, easy separation by conventional GC and characteristic base peak (m/z 83), which is a selective ion in MS of the biomarker derivatives of the sample. However, as mentioned before, the contamination of 3-hydroxy-3-methyl butyric acid or its Ca salt in samples may cause false positive results.

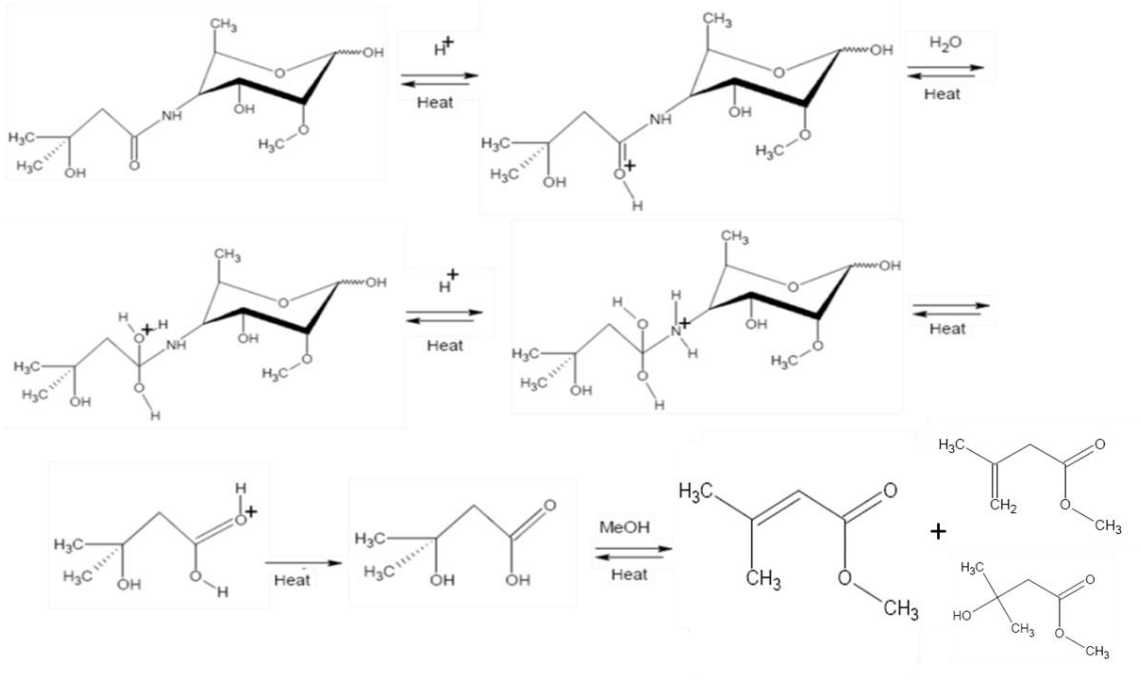

Figure 5.5. Reaction mechanism for generation of 3M2BAME by TCM. The tetrahedral intermediate produced is deprotonated, and the amine is protonated, thus creating a good leaving group. Then the protonated amine is cleaved. Finally, deprotonation of the acid generates the ammonium cation and the carboxylate ion.

Although Dong[1] reported that anthrose was not specific to BA, our analyses of BA, BG, and BT endospores grown in Leighton-Doi and Columbia media at 32ºC and 37ºC indicated that 3M3HBA [detected as 3M2BAME (major) and 3M3BAME or 3M3HBAME (minor)] only appeared at significant levels in BA. This difference was clear when high concentration samples were used (see Figure 5.6). These results suggest the hypothesis that anthrose is only present in certain species such as BT Al Hakam, BT subsp. *kurstaki*, and BC T. Although anthrose alone might not be a definitive biomarker for detection and differentiation of BA from certain other *Bacillus* species, false positive detection should be reduced by using this biomarker along with other selected biomarkers such as DPAME, specific FAMEs and methylated sugars.

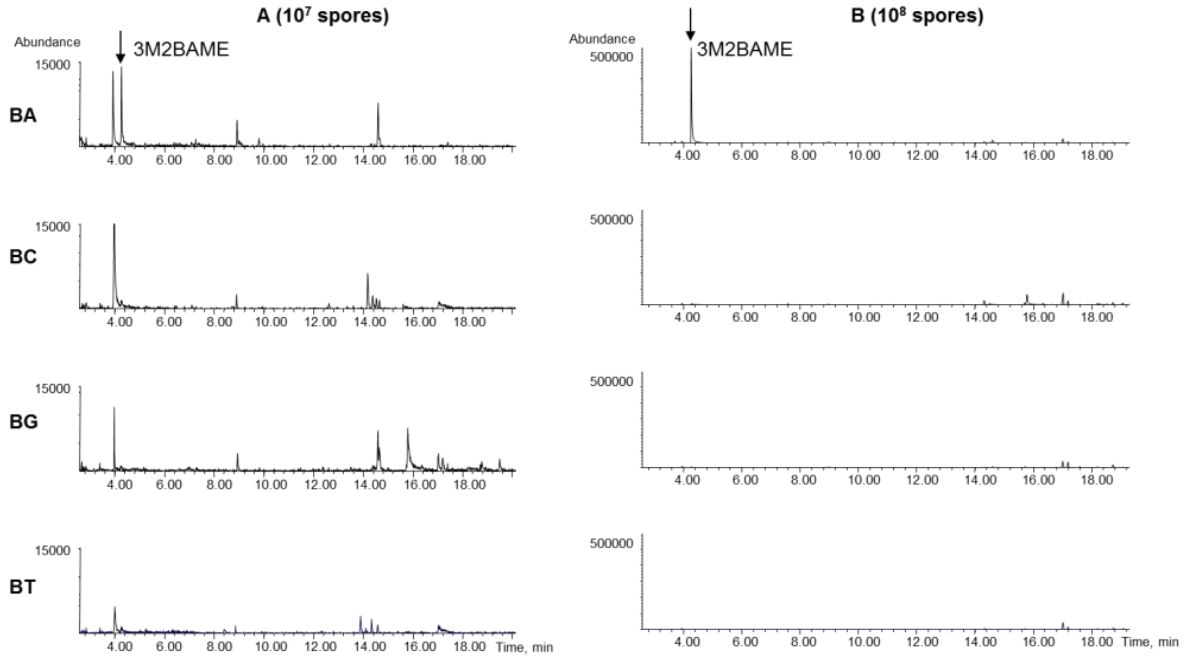

Figure 5.6. Extracted ion chromatograms of the TCM product, 3M2BAME (m/z 83), from BA, BC, BG, and BT endospores grown in LD medium at 32ºC, (10^7 and 10^8 endospores) suspended in 20 µL H_2SO_4 5% v/v in MeOH. The suspensions were heated on a stirrer/hot plate heater at 120ºC. SPME conditions: DVB/CAR/PDMS, 2 cm fiber, 20 min, 20ºC, pH ~ 3.5, stir-bar mixing at ~500 rpm. GC conditions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 60°C at 10°/min to 250°C, 20 psi He carrier gas.

TCM combined with SPME sampling provides a fast, simple method compared to the method reported by Dong[1] for detection of anthrose, which involves the determination of monosaccharides by suspending dry spore samples in 0.4 mL of 1.5 N methanolic-HCl and placing the suspension in a heating block at 80ºC for 16 h. The samples are then dried by vacuum centrifugation, followed by re-N-acetylation to derivatize the aminosugars. Trimethylsilylation is then done after the sample is placed under vacuum to totally eliminate water. The silylated derivatives could then be detected by GC-MS.[1]

In our approach, TCM in strong acidic medium produced 3-methyl-2-butenoic acid methyl ester from BA. The relatively high volatility of 3M2BAME required SPME sampling instead of using the coiled wire filament. The SPME procedure still must be optimized for all target biomarkers, which will involve determining the best SPME fiber and extracting conditions.

5.3.3 Examination of the effect of TCM conditions on biomarker yields

Since TCM and SPME can be used to detect important biomarkers for differentiation, i.e., 3M2BAME, DPAME, certain FAMEs and 2BAME, experiments were conducted to determine the effect of heating time on the yield of biomarkers of the four *Bacillus* species grown in LD medium at 32ºC. The endospores (~10^9) were suspended in 1 mL of MeOH, and 50 µL of this suspension were mixed with 50 µL of methanolic H_2SO_4 (10% v/v) solution to give a total volume of 100 µL. The mixture was heated on a hot plate at 120ºC for periods of time from 5 to 30 min in 5 min intervals. A DVB/PDMS fiber was used for SPME sampling of the contents of the TCM vial while the sample was stirred using a hot/stirrer plate, at 20°C for 20 min. The peak areas of the biomarkers from the extracted-ion chromatograms as, shown in Figure 5.7. were plotted against the heating times. The necessary heating time to achieve maximum endospore digestion, biomarker release, hydrolysis and methylation depended on the amount of endospores, volume of suspension, H_2SO_4 concentration, and the type of glass vial used. We began testing the digestion time with 5 min (the typical time used for heat treatment when 10-20 µL suspension was used). A higher concentration of H_2SO_4 (5% v/v) was also used compared to the normal concentration of 0.5 % v/v because we omitted the concentration step. It was found that shorter than 5 min and longer than 30 min heating times produced low derivative yields.

Figures 5.7 A-D indicate the relationships of the peak areas of 3M2BAME, iso C15:0 acid methyl ester (representative of FAMEs), DPAME, and 2BAME to heating times for BA (6 samples), BC (3 samples), BG(3 sample), and BT (3 samples). These observations can be summarized as: (1) DPAME always gives a higher peak area than the other three biomarker derivatives; (2) DPAME and iso C15:0 FAME can be produced after a short heating time (note: they were even found at room temperature after 1 min of mixing endospores and methanolic H_2SO_4), while the other biomarkers required more time (energy) for conversion; (3) 3M2BAME and 2BAME were found in low intensities at all heating times (5-30 min) from BG and BT, while BC and BA produced significant intensities (approximately 500,000 Au at heating times from 20-25 min and lower for 10-15 and 25-30 min for BC, and from 5,000,000 to 7,000,000 Au for BA); (4) there were no significant differences in peaks areas of 3M2BAME and 2BAME for BG and BT as a function of heating times. These differences were quite clear in BC and absolutely significant in BA, and the peak areas increased with heating time from 5 to 20 min. In BA, these two biomarkers achieved their highest intensities after approximately 25 min; heating longer than this resulted in decomposition, which reduced the peak intensities. Therefore, differentiation can be achieved by using DPAME as a biomarker for confirming the presence of endospores, iso C15:0 or/and anteiso C15:0 as biomarkers for confirming the presence of *Bacillus* endospores but not *Clostridium* endospores, and 3M2BAME as biomarker for confirming the presence of *Bacillus anthracis.*

The relative intensities of DPAME and 3M2BAME and the presence of typical FAMEs are useful factors to confirm the presence of BA in various samples. The higher the ratio of 3M2BAME, the higher is the probability of the presence of BA. In addition, 2BAME was

present in low intensity in BT and BC compared to BA, and was absent in BG, which agrees with the results reported in Chapters 3 and 4 where 3-hydroxy butyric acid methyl ester instead

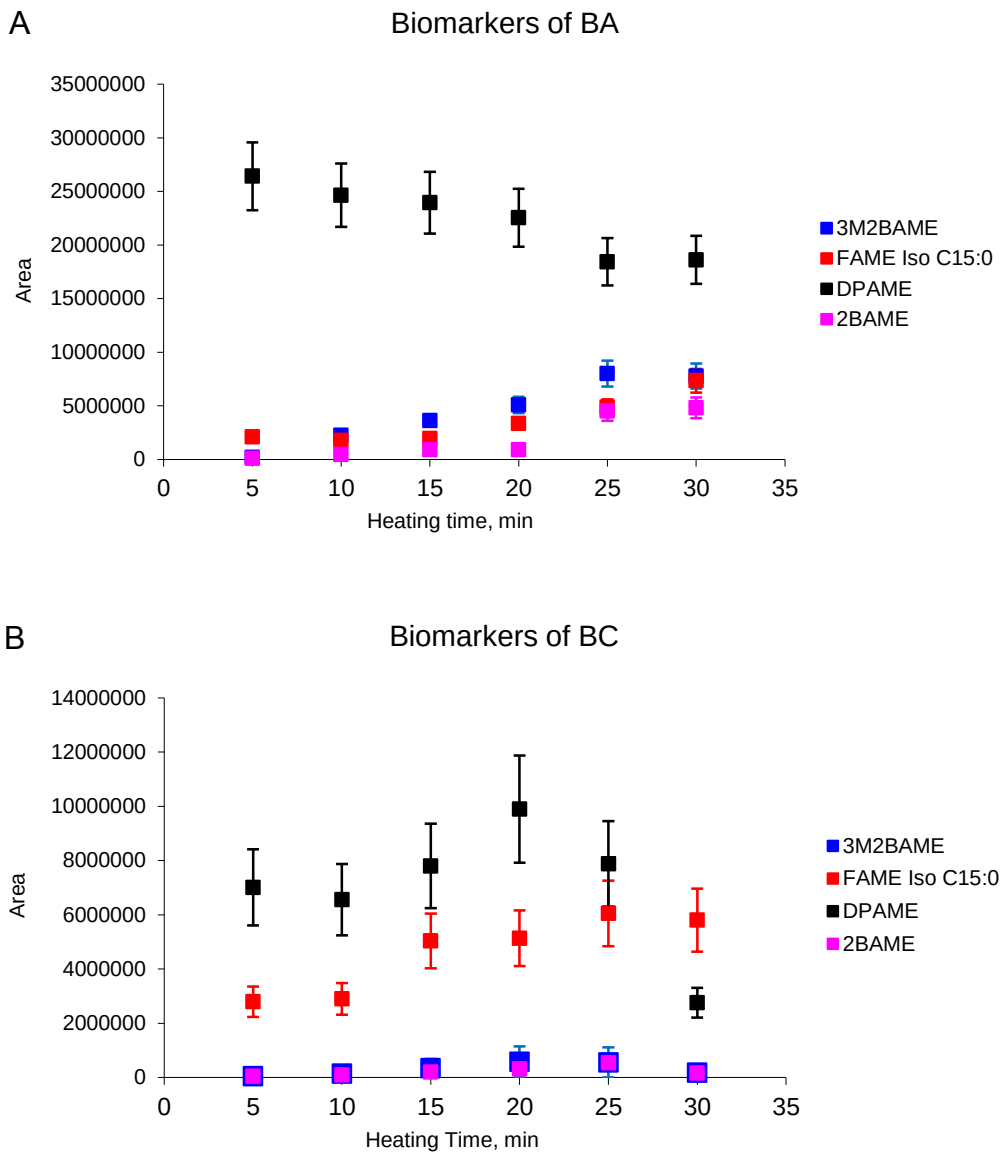

Figure 5.7. Peak areas of the four main biomarker derivatives from (A) BA and (B) BC vs. heating times at 120ºC TCM-SPME.

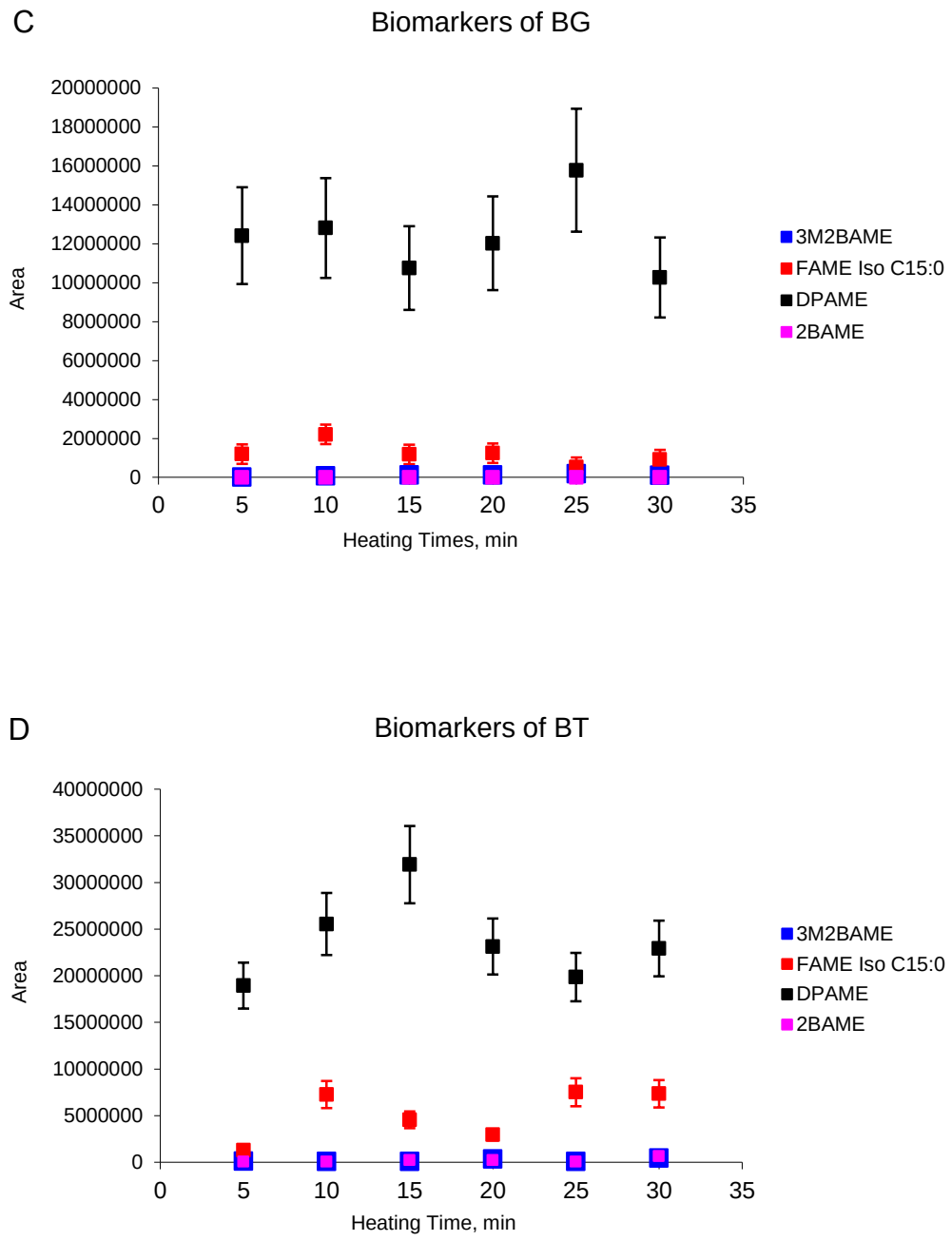

Figure 5.7. (cont.) Peak areas of the four main biomarker derivatives from (C) BG, and (D) BT vs. heating times at 120ºC TCM-SPME.

of 2BAME was also produced from 3-hydroxy butyric acid.

A further comparison of biomarker intensities of the *Bacillus* species are shown in Figures 5.8 A-C. These figures show the relative intensities DPAME, 3M2BAME, and 2BAME from BA, BC, BG, and BT. The DPAME concentrations from *Bacillus* endospores depend on the species, growth and storage conditions. Insignificant variations in peak area vs. heating times (see Figure 5.8 A) indicate that the hydrolysis and methylation of CaDPA occurred easily under the TCM conditions. The abundances of 3M2BAME and 2BAME in BA were greatest after 25-30 min heating, while the other biomarkers gave nearly the same, but smaller, peak intensities for all periods of heat treatment.

In general, 3M2BAME and 2BAME can be used effectively for detection and differentiation of BA from other species. In cases of low spore counts (less than 10^6 spores), these two biomarker derivatives cannot be detected in TCM products from BC, BG, and BT. 3M2BAME appeared in BA in much higher intensity compared to BC, BG, and BT (see Figure 5.8 B), while 2BAME (see Figure 5.8 D) was not seen in BC. These results indicate that BA and BG can be distinguished from other species based on the high intensity of 3M2BAME and the absence of 2BAME.

The required TCM heating times depend on the amount of sample; a longer heating time is needed for a larger sample. An investigation of the optimum time for TCM was performed. We found that 5 min heating at 120ºC for a 20 µL sample gave similar results to 25 min heating of a 100 µL sample at the same temperature (see Figure 5.9).

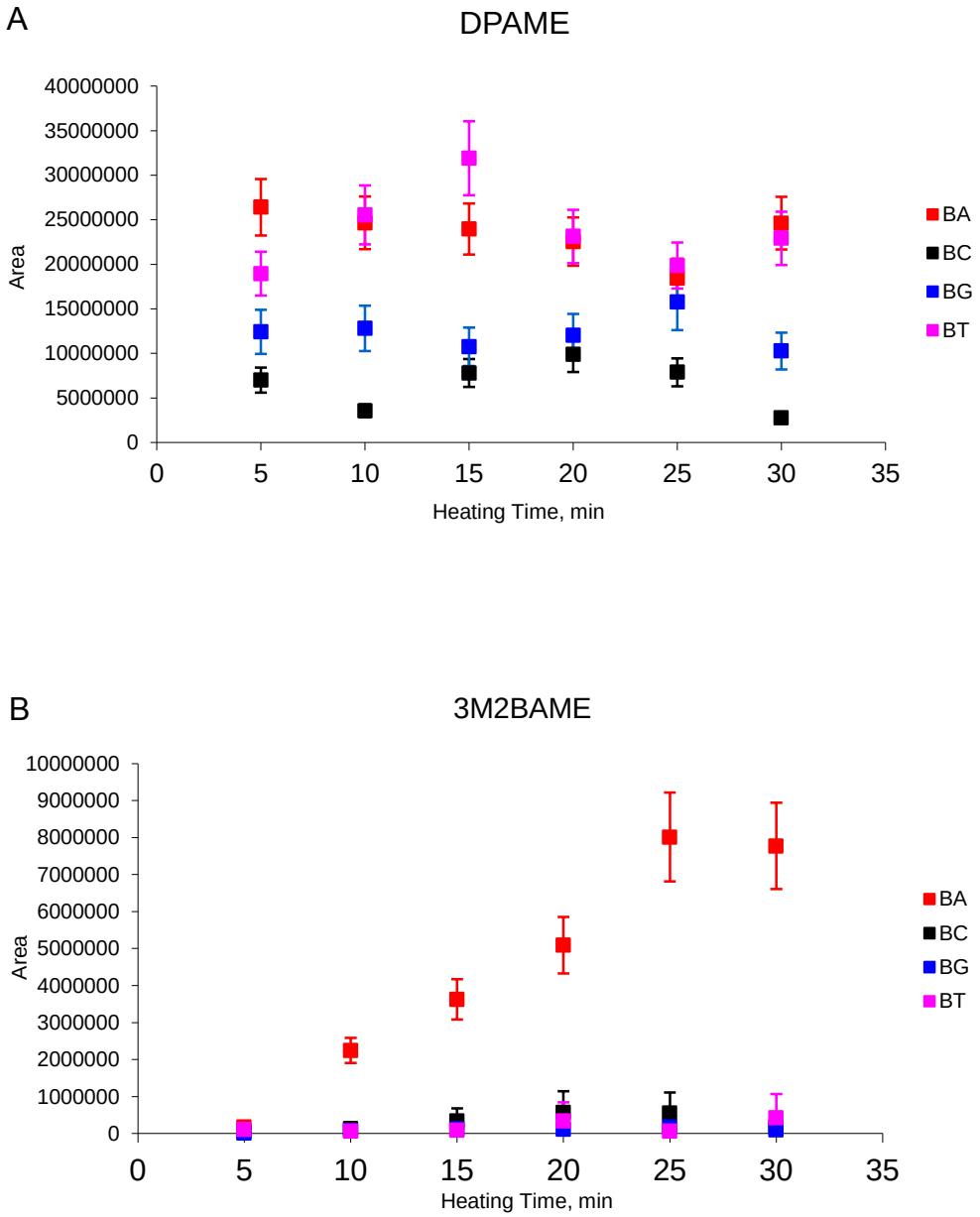

Figure 5.8. Relative peak areas for (A) DPAME and (B) 3M2BAME from BA, BC, BG, and BT vs. heating time at 120°C TCM-SPME.

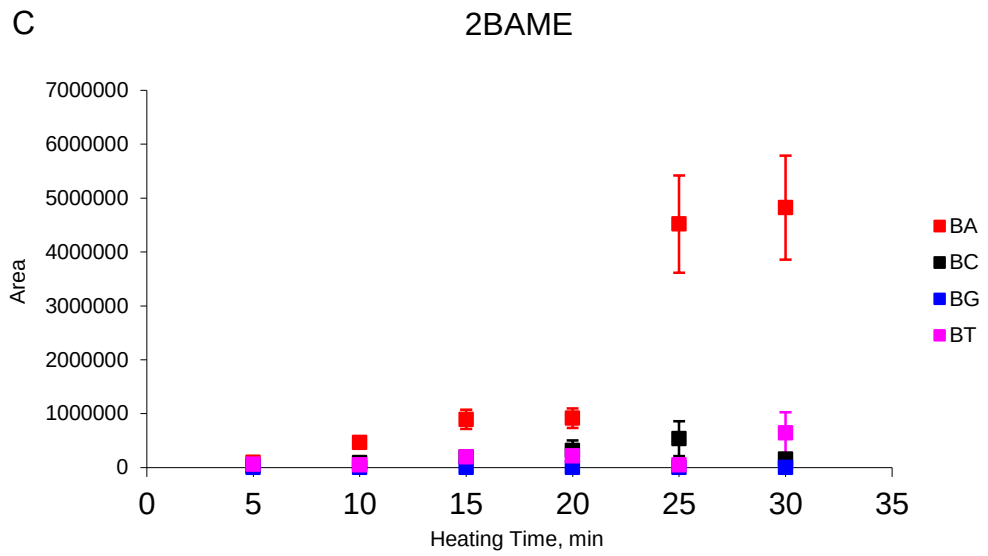

Figure 5.8. (cont.) Relative peak areas for (C) 2BMAE from BA, BC, BG, and BT vs. heating time at 120°C TCM-SPME.

5.3.4 Determination of optimum pH

The pH of the solution is also an important factor that affects the extraction efficiency. To investigate this effect, we used the same sample preparation and SPME methods, but varied the sample pH. After heating, the pH of the solution was adjusted from 2-10 by adding the same volumes of KOH solutions prepared with different concentrations. The pH values were determined using pH paper. A 2 cm long DVB/CAR/PDMS fiber was used for extraction. The relationship of pH to peak intensity for the four main biomarkers as shown in Figure 5.10 indicates that the extraction of FAMEs, 2BAME, and 3M2BAME was not significantly affected when the pH changed from 3-7. However, the DPAME extraction efficiency dropped rapidly when the pH was higher than 6.5, and the FAME peak intensities tended to decrease when the

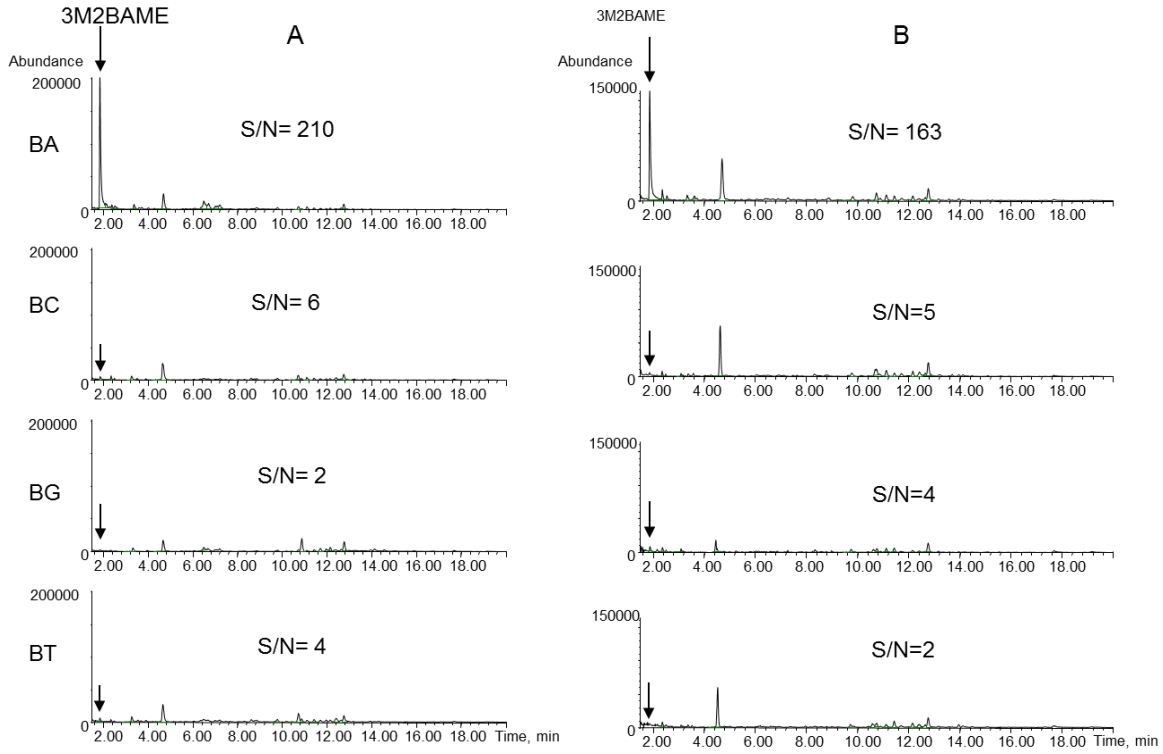

Figure 5.9. Extracted ion chromatograms of the TCM products [3M2BAME (m/z 83)], from BA, BC, BG, and BT endospores grown in LD medium at 32ºC, (5 x 10^7 endospores) suspended in (A) 20 µL H_2SO_4 5% v/v in MeOH, heated at 120ºC for 5 min and (B) 100 µL H_2SO_4 5% v/v heated at 120ºC for 25 min using a stirrer/hot plate heater. SPME conditions: DVB/CAR/PDMS, 2 cm fiber, 20 min, 20ºC, pH ~ 3.5, stir-bar mixing at ~500 rpm. GC conditions: 10 m x 0.25 mm i.d. x 0.25 µm film thickness FFAP column, temperature programmed from 60°C at 10°/min to 250°C, 20 psi He carrier gas.

pH was lower than 2.5. After TCM and pH adjustment, the solubility of the target compounds in the sample solution and in the SPME fiber coating may be dependent on the pH. These interactions could be ionic, dipole induced dipole, hydrogen bonding, or dispersion.

5.3.5 Determination of the best SPME fiber for biomarker detection

Determination of 3M2BAME, FAMEs (iso C15:0, anteiso C15:0, iso C17:0, and anteiso C17:0), DPAME, and 2BAME is necessary for fast identification and differentiation of BA from

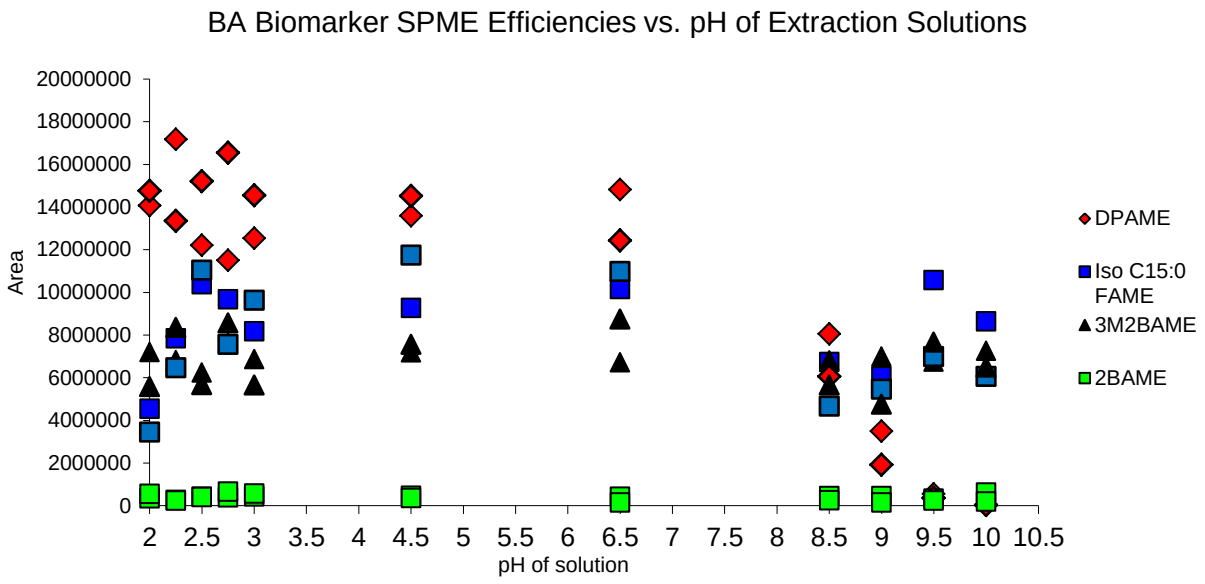

Figure 5.10. Effect of pH on extraction efficiency. SPME conditions: DVB/CAR/PDMS, 2 cm fiber, 20 min, 20ºC, with stirring at ~500 rpm. GC conditions: 60ºC to 250ºC at 10ºC/min, 1 min final time; 10 m x 0.25 mm x 0.25 µm film FFAP column.

other bacteria in complex samples. By comparison of extraction results (see chromatograms shown in Figure 5.11) for four commercial fibers, I found that DVB/PDMS and DVB/CAR/PDMS provided the best extraction efficiencies for the three main biomarkers.

Although the DVB/PDMS coated fiber provided almost the same extraction efficiency, I preferred to use the DVB/CAR/PDMS coated fiber because it was longer and easier to control for half-half extraction. In addition, because (1) DVB plays a major role in extraction of 3M2BAME and DPAME because of induced dipole interaction with the targets analytes, (2) PDMS provides better van der Waals interactions suitable for long chain FAMEs, and (3) CAR moderately absorbs FAMEs and DPAME but strongly absorbs 3M2BAME, DVB/CAR/PDMS fibers are the best for simultaneous detection of these biomarkers.

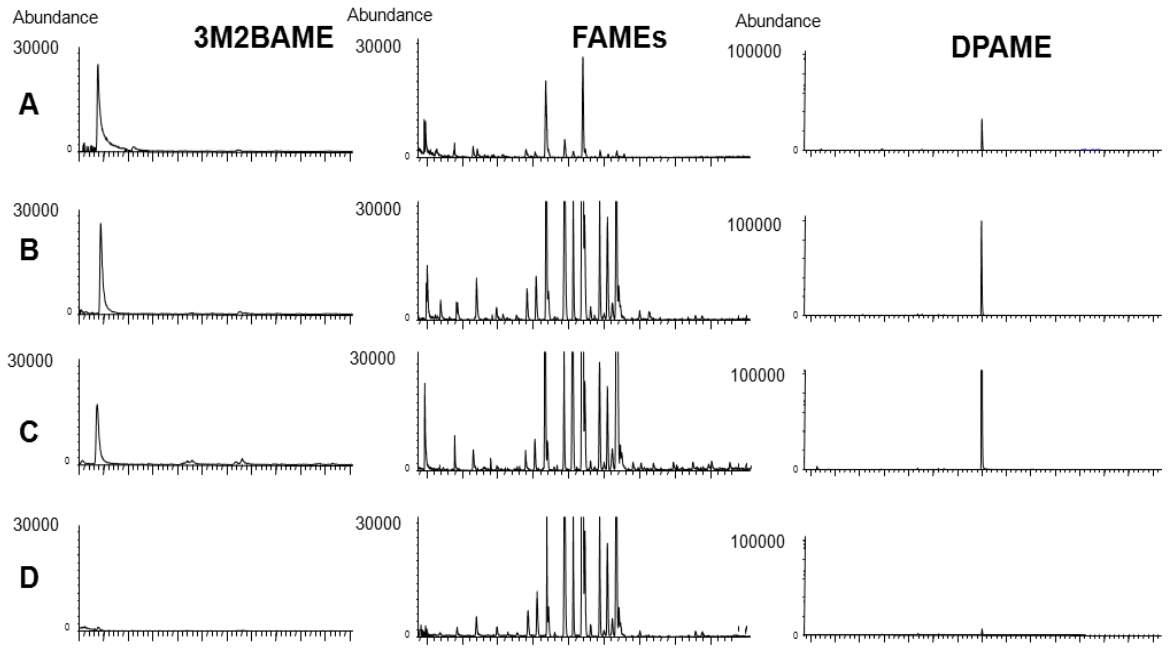

Figure 5.11. Extracted ion chromatograms of 3M2BAME (m/z 84), FAMEs (m/z 74), and DPAME (m/z 137) from BA endospores detected using (A) 75 µm CAR/PDMS, (B) 65 µm DVB/PDMS, (C) 50/30 µm DVB/CAR/PDMS, and (D) 100 µm PDMS fibers. SPME conditions: DVB/CAR/PDMS, 2 cm fiber, 20 min, 20ºC, with stirring at ~500 rpm. GC conditions: 60ºC to 250ºC at 10ºC/min, 1 min final time; 10 m x 0.25 mm x 0.25 µm film FFAP column.

5.3.6 Investigation of biomarker derivative peak intensities vs. extraction time

The effect of extraction time on peak intensities of the biomarker derivatives of BA grown in LD medium at 37ºC was investigated. Approximately 10^8 endospores were suspended in 20 µL of methanolic H_2SO_4 (5% v/v) in a 1 mL conical vial. Heat treatment at 120°C was applied for 5 min using a hot/stirrer plate. After being cooled to room temperature (20ºC), the pH was adjusted to 3-4 by using 0.1 N KOH solution (in H_2O). A 2 cm DVB/CAR/PDMS coated SPME fiber was used for different extraction (half-half mode) times, including 1 min, 5 min, 10 min, and 20 min at 20°C in the stirring mode (~500 rpm). Figure 5.12 presents the GC-MS

extracted ion peak areas of the five biomarker derivatives [i.e., 3M2BAME (m/z 83), 3HBAME and FAMEs (m/z 74), DPAME (m/z 137), and 2BAME (m/z 69)]. These values are similar to results obtained in previous experiments. The slopes of peak intensity vs. extraction time were different for the different target compounds; however, they did not differ significantly from 1 min to 20 min. The equilibrium times (at 20°C and ~500 rpm stirring) for these target biomarkers were longer than 20 min, and the ratios of 3M2BAME to DPAME peak intensities did not vary significantly. In our experiments, the ratios did not vary significantly with extraction times and were around 1 for the method conditions described above. Any variability probably resulted from inconsistencies in the vials (i.e., different geometry, bottom surface, and wall thickness), locations where the vials were placed on the hot plate surface, and differences in air circulation (hood door open or closed). Although the peak intensity ratios of 3M2BAME to DPAME may differ depending on bacteria strain, growth conditions and storage conditions, these ratios may still be independent of extraction time. In this experiment, sampling using a DVB/CAR/PDMS SPME fiber in the half-half mode improved the extraction efficiency for volatile components; therefore, 2BAME and 3BAME gave higher relative intensities than observed when using a DVB/PDMS SMPE fiber due to the presence of CAR in the fiber coating. In addition, variations in bacteria growth conditions and sample preparation for TCM may change the biomarker concentrations.

The results also indicated that short extraction time (~ 1 min), even without using a stirrer (for field application), can be applied to detect BA (from BC, BT, BG and complex matrices) due to the small difference in the intensity ratio of 3M2BAME to DPAME. However, with low concentration endospore samples, longer SPME extraction is necessary. Target analyte sensitivities measured using SPME depend on the extraction time; an increase in extraction time

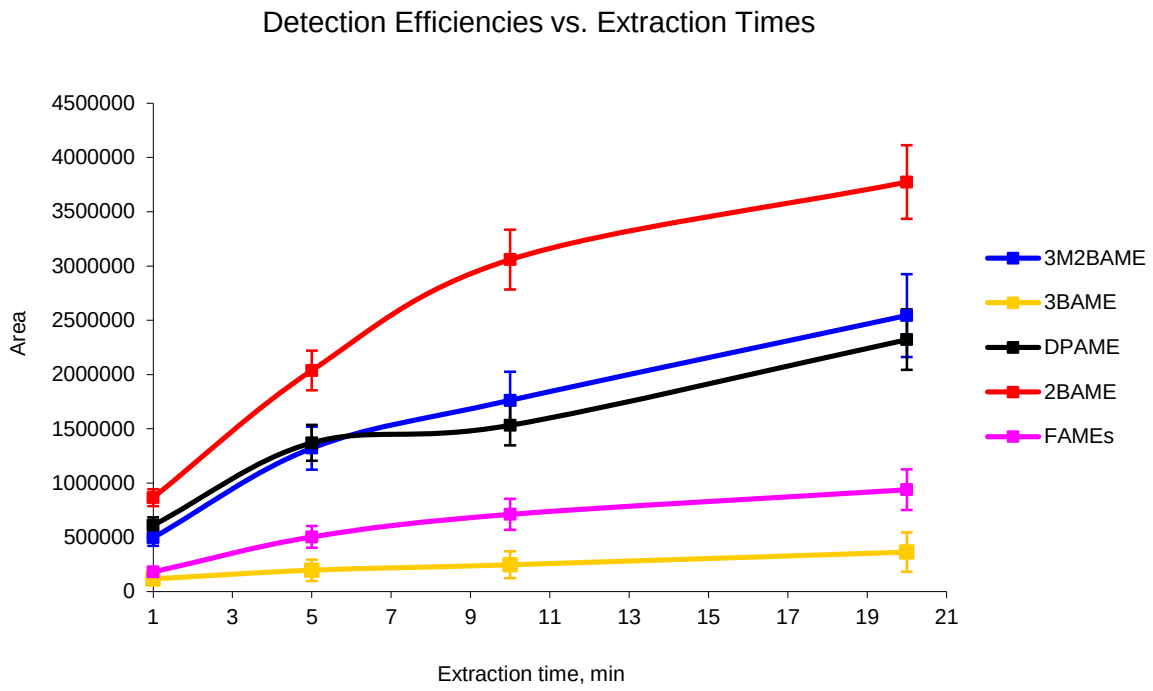

Figure 5.12. Peak intensities of BA biomarker derivatives vs. extraction time.

improves the detection limits until partition equilibrium is reached. SPME requires a relatively long time (ten minutes to hours depending on the properties of the target compounds, the fiber coating, and extraction conditions, such as temperature, stirring speed, sample volume, and sample matrix) to achieve equilibrium. In many cases, achieving equilibrium is not required for either identification or quantitation, however, the extraction time should be long enough to collect enough sample for detection.

5.3.7 β-Hydroxy butyric acid from *Bacillus* endospores

β-Hydroxy butyric acid (3-hydroxy butyric acid) methyl ester and/or 2-butenoic acid methyl ester were detected by GC-MS following TCM. As discussed previously, they originated

from poly(3-hydroxy butyric acid) in *Bacillus* endospores. The suggested mechanisms for their formation were given in Figure 4.5. 2BAME is the major product from TCM due to the dehydration of 3HBME and 3HBA in high H_2SO_4 concentration. At 5% v/v H_2SO_4 and 120-140ºC, 3HBA was converted more to 2BAME than 3HBAME. The results shown in Figure 5.8C indicate that BG does not produce 3HBA, which agrees with the results presented in Chapter 4 and from previous reports.[59,60] This property can be used to distinguish BG from the three other *Bacillus* endospore species.

5.3.8 Discrimination of *Clostridium* and *Bacillus* species endospores

The presence and absence of iso and anteiso C15:0 and C17:0 in the *Bacillus* species and *Clostridium,* respectively, can be shown by both CWF sample introduction and SPME. As reported in Chapter 4, differentiation was easily accomplished due to the dominance of these FAMEs in the *Bacillus* species chromatograms (see Figures 4.14 and 4.15). In SPME, these target biomarkers are easily extracted in DVB/CAR/PDMS, DDVB/PDMS, and PDMS coated fibers.

5.3.9 Discrimination of *Bacillus* species *endospores* from YP and FT

Differences in FAMEs between the *Bacillus* species and *Yersinia pestis* (YP) and *Francisella tularensis* (FT), two bacterial bio-threat agents, can be easily determined using TCM and SPME. Iso and anteiso C15:0 and C17:0, which are characteristic of BA and the other *Bacillus* endospores never appear in YP and FT. Many other saturated and unsaturaed FAMEs (>C18) appear in FT, but are fewer and lower in intensity in BA and YP. On the other hand, the presence of DPAME only in *Bacillus* species endospores further differentiates them from the two bacteria.

5.3.10 Identification of unknown endospore samples

Unknown spore samples can be identified as illustrated in the following example shown in Figure 5.13. A high intensity m/z 137 ion peak with correct retention time indicates that it is a

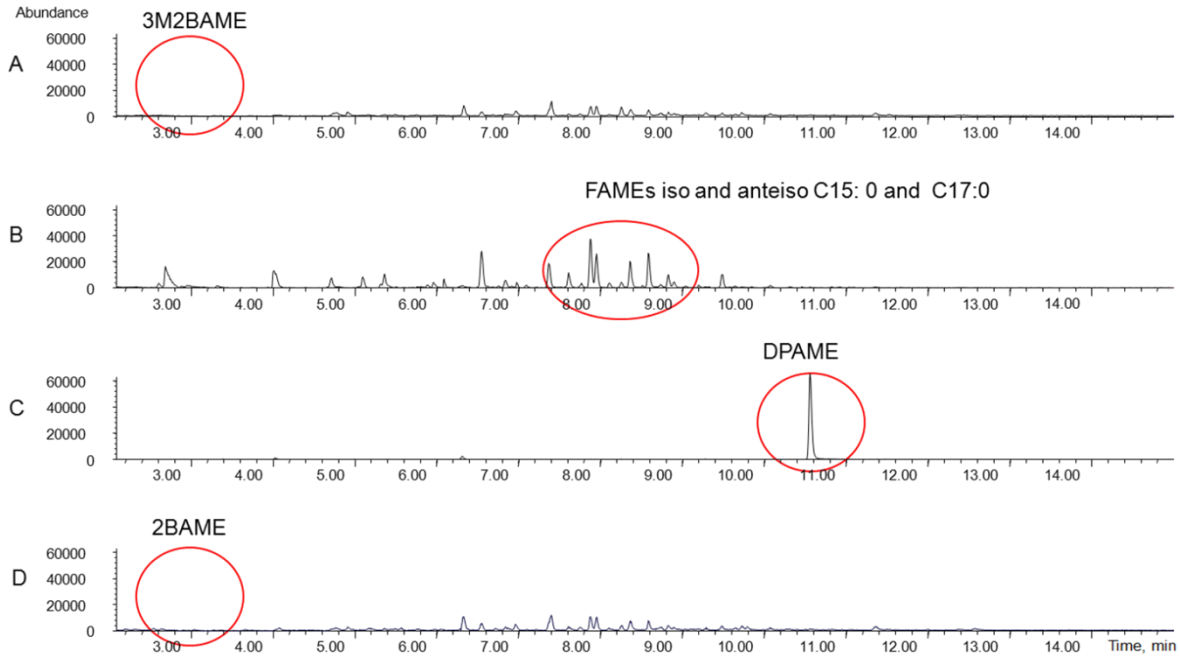

Figure 5.13. Extracted ion chromatograms of an unknown spore sample. (A), (B), (C), and (D) are m/z 83, 74, 137, and 69, respectively. SPME conditions: DVB/CAR/PDMS, 2 cm fiber, 20 min, 20ºC, with stirring at ~500 rpm. GC conditions: 60ºC to 250ºC at 20ºC/min, 1 min final time: 10 m x 0.25 mm x 0.25 µm film FFAP column.

sporulating bacterial form. The presence of iso C15:0, anteiso C15:0, iso C17:0, and anteiso C17:0 (ion m/z 74) confirms that the sample is not *Clostridium*. The absence of m/z 83 and m/z 69 peaks indicates that the sample is not BT or BC. Therefore, the sample is most likely BG.

5.3.11 Detection limits using DVB/CAR/PDMS extraction

Experiments for determining the detection limits of TCM and GC-MS using the DVB/CAR/PDMS SPME fiber were carried out. Different sample concentrations (lowest

number of spores was approximately 10,000) were prepared by the method described for pH 4-5 SPME extraction. The detection limits of the method based on 3M2BAME and DPAME (using Chemstation software) of the lowest spore count sample (see Figure 5.14) was around 5000 spores, respectively. This result was considered for a clean BA sample, and may not be applicable for a real sample involving a complex matrix.

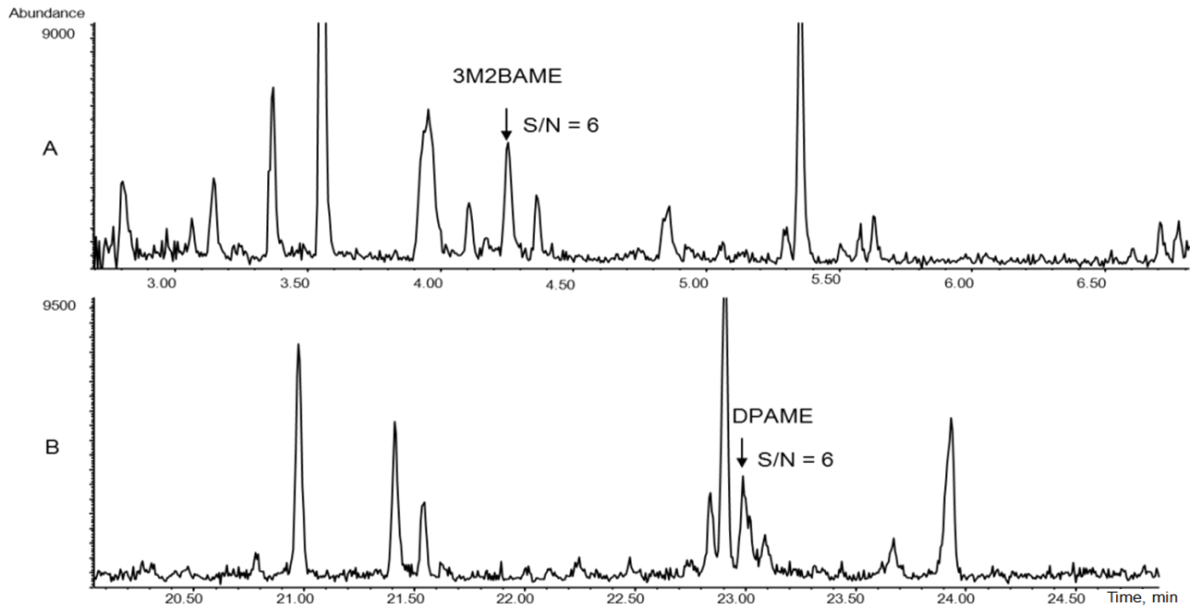

Figure 5.14. Extracted ion chromatograms of 3M2BAME (m/z 83) and DPAME (m/z 137) of 10,000 BA spores. SPME conditions: DVB/CAR/PDMS, 2 cm fiber, 20 min, 20ºC, pH ~ 3.5, stir-bar mixing at ~500 rpm. GC conditions: 30 m x 0.25 mm i.d. x 0.25 µm film FFAP column, temperature programmed from 60°C at 10°/min to 250°C, 20 psi He carrier gas.

5.3.12 Flow chart for differentiation

Based on the results described in this chapter, a flow chart was devised for detection and differentiation of the *Bacillus* species and *Clostridium* from each other. In this flow chart shown in Figure 5.1, DPAME is the first observation followed in order by anthrose (by-product, 3M2BAME), iso and anteiso C15:0 and C17:0, specific methylated sugars, and 3HBA (3HBME or 2BAME). BT and BC refer to specific BT and BC species that contain anthrose, such as BT

Al Hakam, BT subsp. *kurstaki* , and BC T. Since YP and FT are non-sporulating, they can be detected and differentiated based on specific FAMEs as introduced earlier. Differentiation of BC, BS and BG on the left site of the flow chart can be achieved using methylated sugars.

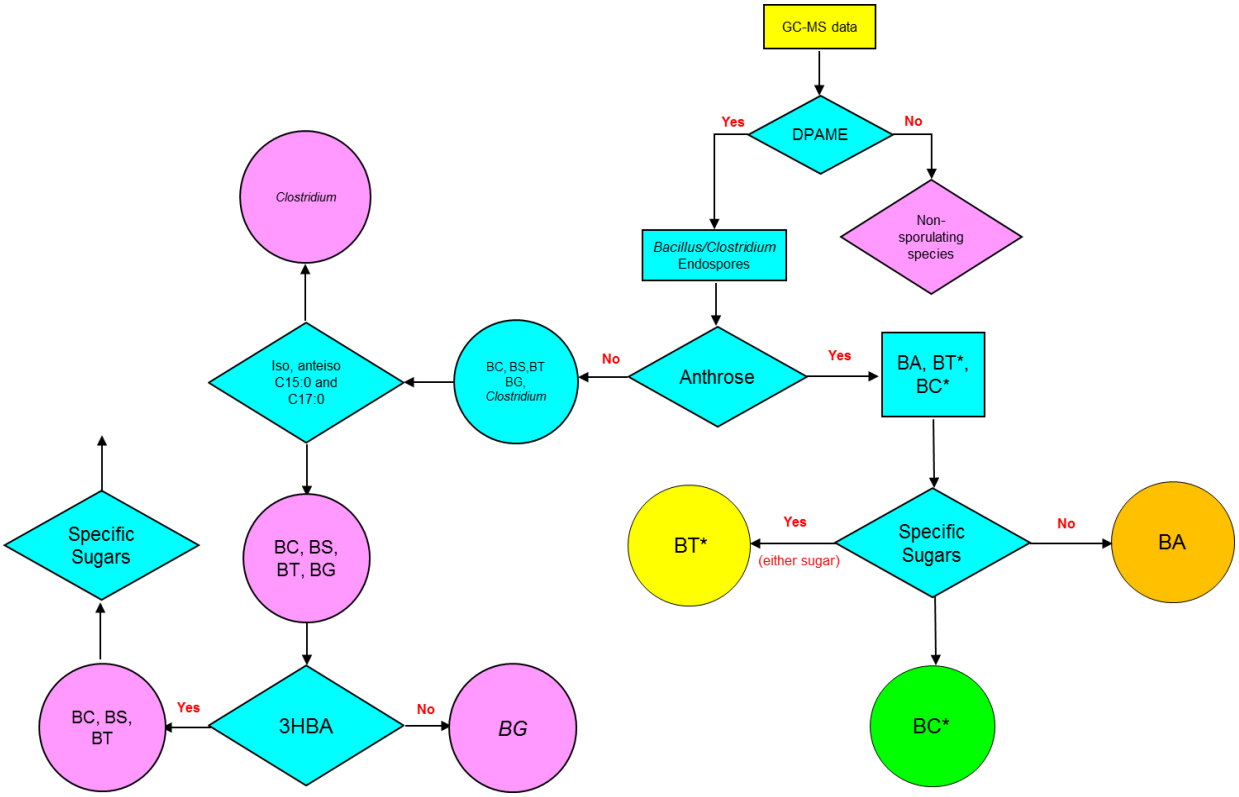

Figure 5.15. Differentiation flow chart for *Bacillus* and *Clostridium* endospores.

5.4 CONCLUSIONS

TCM using H_2SO_4 heat treatment and concentration produce definitive biomarkers of *Bacillus* species. The analysis time is reduced from hours to minutes, especially when using a microwave oven. The simplicity of the SPME method should allow for field applications using a portable GC-MS. Anthrose detected by TCM-SPME improves detection of BA.

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6 COATED-NEEDLE EXTRACTION

6.1 INTRODUCTION

In gas chromatography (GC), sample introduction plays an important role, especially for quantitative analysis. Because of this, the GC injector and the injection methods must be properly designed and utilized in order to satisfy the analysis requirements. From the beginning of the GC technique, injectors have experienced many modifications and improvements, with the objective of achieving the best quality analysis in terms of quantitation and identification. Gas, liquid, and solid samples require quite different approaches to be transferred properly into the GC system. Solid samples cannot be introduced using a micro-syringe unless they are first converted into gases or liquids. On the other hand, solid probe introduction requires evaporation directly from the probe in the GC inlet. The diversification of samples requires a variety of introduction devices to accommodate the characteristics of the target analytes, the sample matrices, and the analytical purposes.

In considering sample introduction, it is a major mistake to ignore the sample preparation requirements. In reality, the analytical results are not reliable without the proper sample preparation method. The combination of appropriate sample preparation with appropriate sample introduction are key to ensuring good quality of analysis. Today, analytical trends include simplification, miniaturization, and speed, as well as making chemical analysis environmental friendly. These goals extend to sample preparation and injection, as well as to the final analytical measurement step.[1-6]

6.1.1 Sample introduction techniques for GC

As in any analytical method, sample preparation is the starting point for GC analysis. It is

an essential step to eliminate interferences, isolate and/or concentrate target analytes, and/or convert them into forms amenable to GC separation and detection. Sample introduction, regardless of the rest of the analytical process, plays such an important role in analysis quality that the following claim was made: "introduction of sample into a GC column probably calls for more attention of the analyst than any other part of the technique."[7] Micro-syringe and split/splitless (S/S) injection modes are standard designs that are principally used to facilitate direct injection of liquid or gas samples. S/S injection via a small hypodermic syringe has been popular with analysts because of its simplicity and wide applicability. The programmed temperature vaporizer (PTV) injector is a form of S/S injection during which temperature can be programmed at high rates for heating and cooling. This design minimizes discrimination effects in the S/S injection port, and allows for large volume injection when combined with solvent venting, and trapping or re-focusing techniques. However, it is not the best choice for very volatile components which can be lost during solvent venting, and it may not be suitable for fast analysis due to the longer than desired heating/cooling cycle time. Finally, cold on-column (COC) injection, a less popular injection technique compared to the other techniques, is designed to minimize discrimination and sample decomposition that occurs in the heated injection liner. Excellent peak resolution is obtained with COC injection if the initial sample band is narrow, which depends on the initial temperature, pressure of the injection port, and solvent properties. COC injection however, should only be used for clean samples, or a retention gap must be employed to protect the main column from contamination.[8-15]

Contamination in the vaporizing injection port or column caused by non-volatile compounds occurs normally with micro-syringe injections. Minimizing or eliminating this

contamination requires a carefully designed sample preparation stage. Isolation of the target analytes from the sample matrix before GC injection is important, especially for fast, simple, and robust analysis.

6.1.2 Solid phase micro extraction (SPME)

As introduced in Chapter 1, SPME provides an attractive alternative to traditional GC injection methods by combining sampling, preconcentration, and transfer of the target analytes into the GC injector. It also minimizes contamination of the GC system.[16]

6.1.3 Advantages and disadvantages of SPME

The main advantages of SPME compared to liquid extraction are reduction in solvent use; combination of extraction, concentration, and analysis into one step; and the ability to examine small samples with low concentrations. When applied under controlled conditions, such as temperature, time, salting out, and vibration, SPME can provide high sensitivity and reliable results. By using different polarity coatings, nonpolar and polar analytes in a wide range of matrices can be determined. SPME can be easily employed for both GC and LC sample introduction.[17]

Unfortunately, SPME has its own limitations. Commercial fiber coatings (especially polar ones) are limited to relatively low operating temperature, and they can be unstable and swell in organic solvents. Fused silica fibers, which represent the most widly used support for SPME, are fragile. Some of these problems have been mitigated by covalent bonding of the polymer phase to the fused-silica substrate and by extensive cross-linking, making them solvent resistant and thermally stable up to approximately 350ºC.[18-20]

6.1.4 SPME-like devices

There are many devices which have been designed following the principles of SPME to

satisfy different demands of analysis or to overcome the disadvantages of conventional SPME. A sample enrichment probe (SEP), consists of a thin rod of an inert material with one end covered with a short sleeve of polydimethylsilicone rubber. The SEP was designed for high-capacity sample enrichment of analytes from gaseous and aqueous samples for analysis by GC and its hyphenated techniques.[21]

Stir-bar sorptive extraction (SBSE) involves a magnetic stir-bar that provides rotating movement above a stirring plate in the sample, which is covered with a thin glass jacket, and finally a layer of polydimethylsiloxane sorbent into which target analytes are extracted.[22] The stir bar has a much larger volume of sorbent than the conventional SPME fiber, so an additional step is needed (thermal-desorption or liquid extraction) to transfer the target analytes to the GC system.[22] Since the amount of analyte extracted using SBSE is proportional to the volume of the extraction phase, the detection limits are greatly improved.[18,23,24] In addition, since the extraction rate is controlled by the diffusion of analyte from the sample matrix through the boundary layer to the extraction phase, it takes much longer to achieve equilibrium. Generally, SBSE is considered to be superior to SPME in terms of sensitivity and accuracy for trace level determinations in difficult matrices.[25]

Thin-film microextraction uses a thin membrane, which enhances extraction without long equilibrium times.[18] Example applications involving this extraction type are: (1) a cross-linked commercial PDMS membrane[26] for extraction of PAHs from headspace, and (2) an in-house prepared membrane of PDMS/cyclodextrin[27] used for extraction of both nonpolar PAHs and polar phenolic compounds. The operation of membrane extraction is simple. In the previously mentioned application, a sheet of PDMS membrane was attached to a deactivated stainless steel rod and positioned inside the sample container. It was important to ensure that the membrane

was shaped like a flag. The samples were normally stirred, after which the membrane was rolled around the rod and placed in the GC injector port for thermal desorption.

In membrane PDMS, the extraction rate was much higher than when using the coated rod formats of SPME using thick coatings because of the larger surface area to extraction-phase volume ratio of the sorbent. This larger surface area allowed for a larger amount of analyte to be extracted within a short period of time. As a result, higher extraction efficiency and better sensitivity could be achieved without sacrificing analysis time. In liquid membrane SPME extraction, a linear relationship was found between the initial rate of extraction and the surface area of the extraction phase. However, in headspace extraction, the rates were lower because of the resistance to analyte transport at the sample matrix/headspace barrier.[26] For field application, thin-film micro-extraction is more convenient than stir bar extraction.[28]

In-tube solid-phase micro extraction (in-tube SPME) uses an open tubular fused-silica capillary column as an extraction device. Organic compounds in aqueous samples are directly extracted into, and concentrated in a coating inside the capillary. Following the principal of SPME, analytes move toward equilibrium distribution by repeated draw/eject cycles of the sample solution. After extraction, the target analytes are transferred to the GC or LC system for analysis.[29] In-tube SPME was successfully applied to the analysis of drugs in biological fluids. It was claimed to be an ideal sample preparation technique for LC because it is fast, is solvent-free, inexpensive, and easy to automate. In-tube SPME can perform continuous extraction, concentration, desorption, and injection, and has been primarily used in combination with LC and LC-MS.[30] Several commercial GC columns, such as Omega Wax 250,[31] HM/DB-5 (zylon fiber packed in a DB-5 capillary, 5% phenylpolydimethyl siloxane),[32] polypyrrole, poly(methacrylic acid-ethylene glycol dimethacrylate) monolith coated, and cyclodextrin coated

capillaries have been used for in-tube SPME determination of drugs in biological fluids.[33] Some materials have properties which allow for extraction without any coating. Polyimide, the standard material used to enhance the mechanical stability of optical fiber, has been employed for Cr(III) extraction after derivatization with TFA[34] and for extraction of chlorinated hydrocarbons and PCBs.[35] HB type pencil lead, despite giving severe carryover, has been applied to the determination of several pesticides.[36] Another graphitic material has been applied to determination of surfactants.[37] Anodized aluminum wire was found to be useful for extraction of aliphatic alcohols and BTEX from gaseous samples. These devices may not be considered as authentic SPME types, but as probes for direct sampling.[38]

6.1.5 Coated-Needle Extraction

As introduced in Chapter 1, coated-needle extraction (CNE) was investigated to speed up extraction and shorten the equilibrium time because of the dynamic movement of sample fluid past the surface of a stationary phase layer coated inside a tiny needle. A very similar technique called solid phase dynamic extraction (SPDE) was developed and commercialized in the early 2000s by Chromtech GmbH (Idstein/Germany), and Pawliszyn, inventor of the SPME method, also developed and used SPDE and various other devices[39] whose operational principles were similar to SPDE, such as in-tube SPME or in-needle capillary adsorption;[40-51]; single drop microextraction (SDME); and needle trap device (NTD). These devices allowed dynamic extraction of samples in a significantly larger amount of sorbent material and surface area compared to SPME. Both liquid and headspace or air samples could be sampled.[52-54]

In our early work with CNE, the coating was a physically deposited stationary phase located inside a conventional 22 or 23-guage stainless steel needle tip. Sticky polymers such as PDMS, epoxy, and apiezon N were physically coated inside the needle and stabilized by heating.

Polymer or elastomer and curing agent solutions (for crosslinking polymers) prepared in CH_2Cl_2 were drawn inside the needle tip by capillary action when dipping the needle into the polymer solution. After solvent evaporation, the polymer coating was formed by inserting a wire through the needle while blocking the needle tip to avoid pushing the polymer out of the needle. The polymer layer then is heat-treated in an inert gas environment of such as He or N_2 to further eliminate solvent, and/or to accelerate crosslinking to immobilize the polymer layer. The wire was kept in the needle during heating until the solvent was completely evaporated and the crosslinking was complete. A conditioning step was finally applied before the coated needle could be used for extraction.

6.2 EXPERIMENTAL

6.2.1 Chemicals and materials

HPLC grade methanol (MeOH) and dichloromethane (CH_2Cl_2) were obtained from EMD (San Diego, CA, USA). H_2SO_4 (98%) was from Mallinckrodt Chemical (Phillipsburg, NJ, USA). SPME fibers, 23G 50/30 µm DVB/CAR/PDMS, 2 cm long, were obtained from Supelco (Bellefonte, PA, USA). Activated carbon (100 mesh) was from Columbus Chemical Industries (Columbus WI, USA). Sylgard 184 elastomer and curing agent for making PDMS was from Dow Corning (Midland. MI, USA). Stainless steel needles (type 304, 22 G and 23 G) were from Small Parts (Seattle, WA, USA), a Hamilton 23 G needle was obtained from Hamilton (Reno, NV, USA). A 1-way, F-luer to M-luer, nylon body-HDPE plug stopcock on-off valve was obtained from Kimble Chase (Vineland, NJ, USA). Poly(methyl methylacrylate) (PMMA) was from Scientific Polymer Products (Ontario, NY), polystyrene (PS, MW 2360) was from Aldrich Chemicals (Milwaukee, WI, USA), Ucon 75 was from Alltech (Deerfield, IL, USA), SE 54 was from Supelco, and epoxy was from ITW Devcon (Davers, MA, USA). Alkanes and polycyclic

aromatic hydrocarbons (PAHs) used for standards were obtained from a variety of sources. *Bacillus* endospore samples were prepared as described in Chapter 4.

6.2.2 GC-MS system and conditions

An Agilent 6890 gas chromatograph (Agilent, San Jose, CA) with split/splitless injector was fitted with a 0.75 mm i.d. Restek liner to accommodate the sampling device. The GC was coupled to an Agilent 5793 MS with electron ionization and quadrupole analyzer. Two GC capillary column types were employed, 10 m and 30 m x 0.25 mm i.d. x 0.25 µm film thickness polar FFAP stationary phase (Phenomenex, Torrance, CA, USA) and 5 m x 0.1 mm i.d x 0.4 µm film thickness weakly polar Mtx 5 and Rtx 5, 5% phenyl, 95% methyl polysiloxane (Restek, Bellefonte, PA, USA); 30 m, and 15 m x 0.25 mm i.d. x 0.25 µm film thickness HP-MS5 (Agilent, San Jose, CA, USA). Typical chromatographic operating parameters included 250-300ºC injector temperature, 2 min splitless injection time, temperature program from 50ºC (initial) to 250-290ºC (final) at different rates depending on the analysis, and 230-270ºC transfer line temperature between the GC and MS. The MS operating parameters for all experiments were: 230ºC quadrupole temperature, 150ºC source temperature, 30-500 m/z mass range, 1670 EM voltage, 35 µA emission current, and 70 eV ionizing voltage. Extracted ions 74 m/z, 137 m/z, 83 m/z, and 69 m/z were used to reconstruct the FAME, DPAME, 3M2BAME, and 2BAME chromatograms, respectively.

6.2.3 Coating of polymers inside needles

Coating experiments involved two main materials: (1) sticky polymers including PDMS, epoxy, Apiezon N, Ucon, and SE54, and (2) mixtures of activated carbon and PDMS (C/PDMS), polystyrene (PS), and polymethylmethacrylate (PMMA). PDMS and C/PDMS were mainly focused to developed and used for the extraction of hydrocarbons, PAHs and *Bacillus* endospore

biomarkers. A simple assembly consisting of a coated needle, valve, and gas-tight syringe is shown in Figures 6.1 and 6.2. Coupling between the three parts were ideally zero-dead-volume connections. Liquid and headspace sampling as illustrated in Figure 6.3 are the simplest ways to use the sampling system.

6.2.4 Coated-Needle Extraction (CNE)

SPME experiments using both conventional SPME and several CNE devices were conducted to determine the feasibility, efficiency and comparability between the two extraction techniques for both headspace and liquid sampling. Different injection modes including with and without purging, split/splitless operation, inlet pressure program, high injection temperature and exhaustive extraction were investigated to evaluate carryover when using this technique. The use

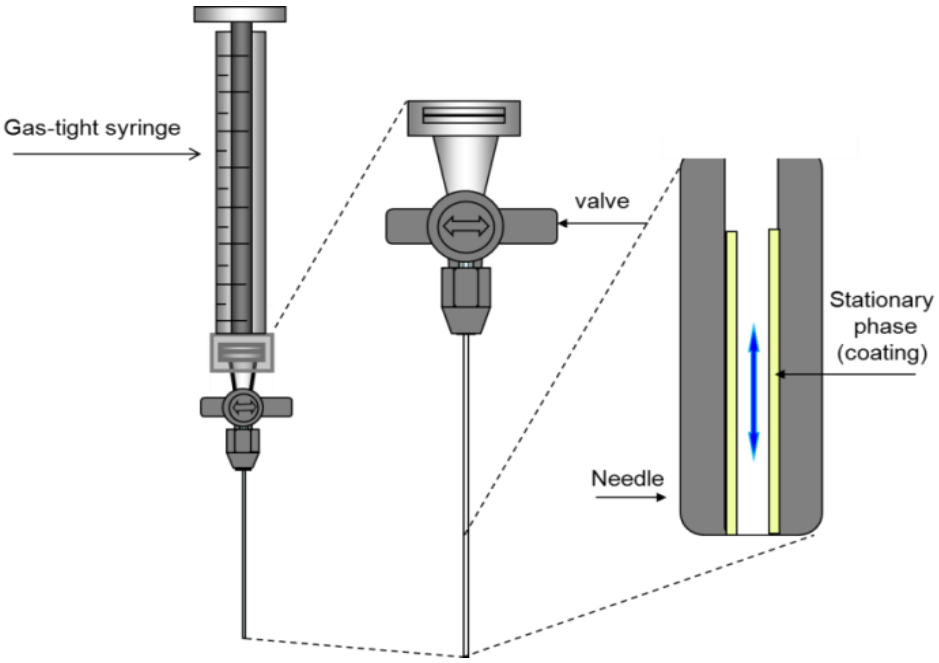

Figure 6.1. Schematic drawing of the home-built CNE device.

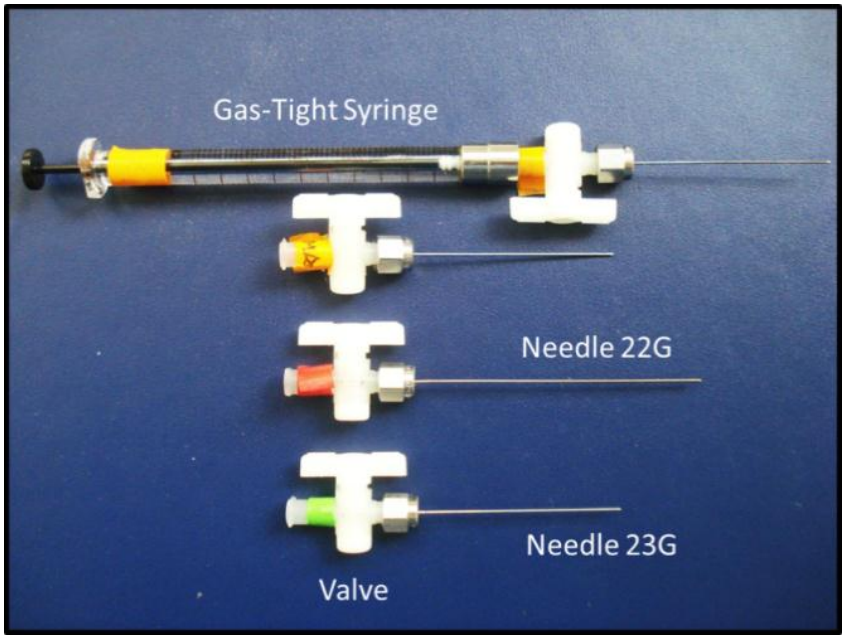

Figure 6.2. Photograph of needles coupled to on-off valves and to the syringe body.

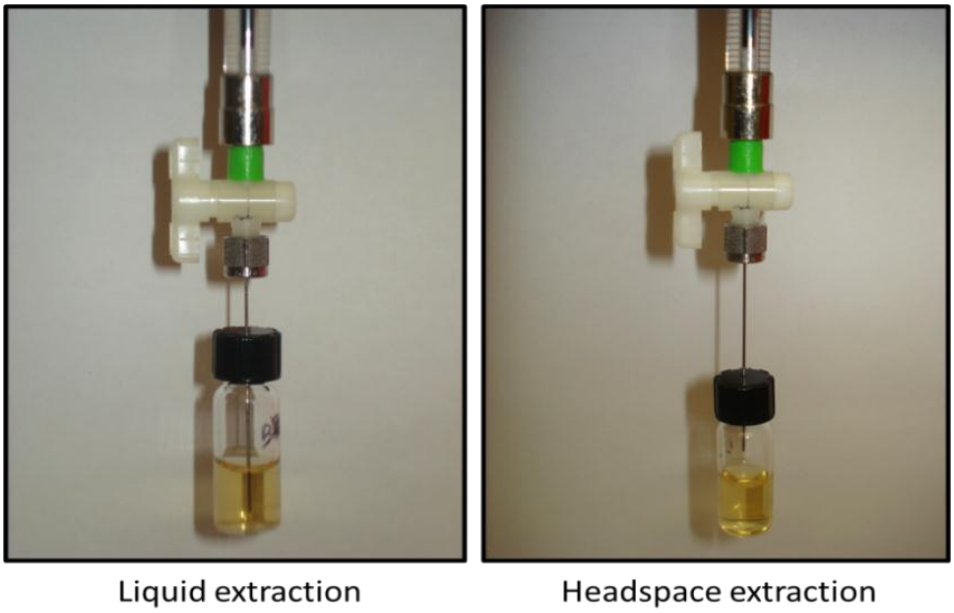

Figure 6.3. Photographs of liquid and headspace sampling using CNE.

of home-made C/PDMS coatings for *Bacillus* endospore biomarker detection was evaluated to simplify and speed up our current SPME method for differentiating *Bacillus* species endospores from each other and from other biological threat agents. Purging and non-purging injection involved four modes that are illustrated in Figure 6.4. Modes 1and 3 are similar to conventional

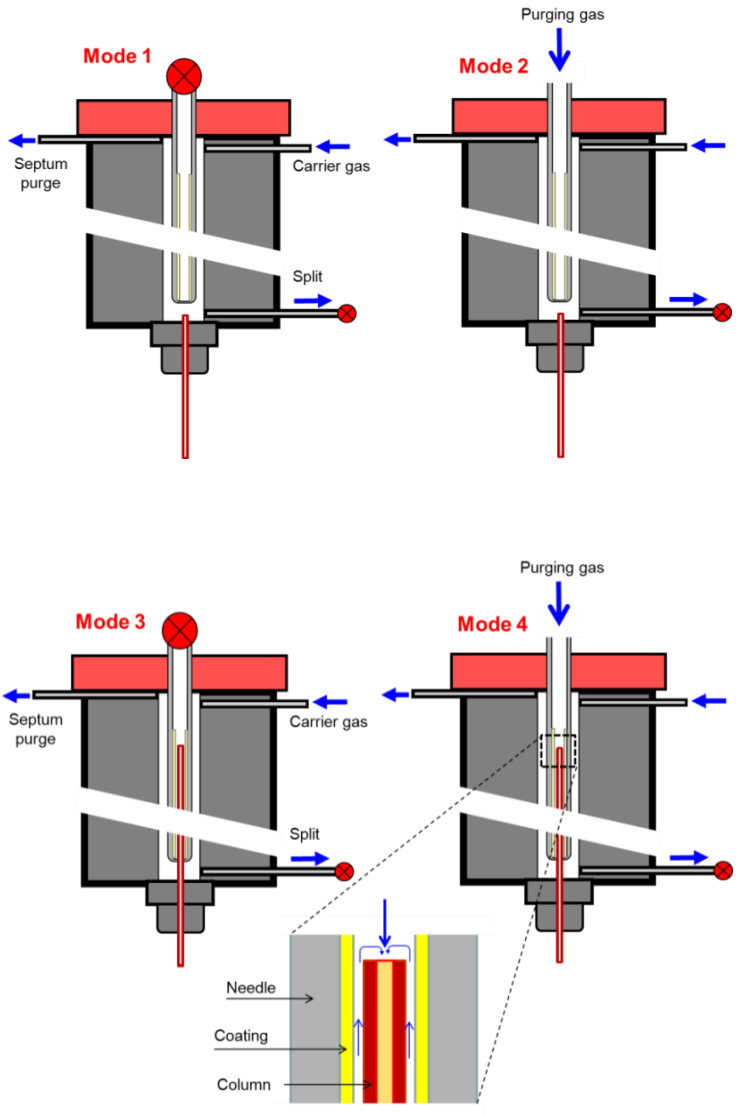

Figure 6.4. Schematic drawing of the various injection modes using CNE.

SPME injection (split or splitless) except the column end is inserted into the DNE needle in mode 3. Modes 2 and 4 are the same as modes 1 and 3, respectively, however purging gas is provided via a separate source from the GC carrier gas. In mode 4, the carrier gas comes to the column from two directions, the first is from the needle tip (at the liner base) and the second is from the purging gas (at the liner head).

6.2.5 Carryover measurements in CNE

Carryover was evaluated for the four sampling modes by conducting repetitive injections of a sample after one extraction. The order of injections was first with sample and then repeated injection without extracting again or cleaning the needle to determine the percentage, if any of the targets remaining in successive runs.

6.3 RESULTS AND DISSCUSION

6.3.1 Fabrication of in-needle polymer coatings

PDMS coating. PDMS is a commonly used silicon-based organic polymer. It is a popular material in analytical chemistry due to its unique mechanical, chemical, and optical properties. The cross-linking that forms a relatively hard rubber film occurs without aid once the PDMS elastomer and curing agent are mixed; the polymerization progress can be greatly accelerated with heat. The mixing ratio is normally 10:1 PDMS elastomer to curing agent, and the curing procedures determine the chemical, mechanical, and optical properties of the final solid. An advantage of PDMS is that when cross-linked, it acts like a rubbery solid that cannot be permanently deformed by stress or strain, but will return to its original shape when being released. The elasticity of PDMS is highly dependent on the amount of cross-linking agent integrated into the polymer. Rigidity increases with concentration of cross-linking agent. With little or no cross-linking agent, the polymer remains a viscous liquid.

Generally, PDMS is considered to be chemically inert. However, most organic solvents can penetrate the PDMS surface. When organic solvents are absorbed into the polymer, the volume of the polymer increases, or swells, to account for the volume of the introduced chemicals. However, neither chemical absorption nor physical swelling is permanent; the absorbed chemicals can easily diffuse out of the polymer during drying. Since PDMS is notably hydrophobic, water cannot easily penetrate its surface. These properties of PDMS make it desirable for use in extraction processes.

Polymer coatings were easily made inside the stainless steel needle as a result of the adhension of the polymer to the needle surface. Surface forces for elastomers, which are responsible for adhesion between a cured elastomer and a rigid smooth surface, can arise from: (1) van der Waals forces, (2) hydrogen bonds and (3) electrostatic forces. Although the forces providing the major source of bonding are not clearly defined, most observers are in favor of van der Waals forces. Good adhesion is obtained between oxidized metal (Fe_2O_3, Cr_2O_3, Ni_2O_3 MnO_x, etc. sites on the surface of the stainless steel and PDMS micro-fabrication.[55]When a primer is used, oxidized Si, instead, creates very good adhesion. Furthermore, due to the high flexibility of PDMS and low surface energy, good adhesion is obtained between cured PDMS and polished surfaces with an average roughness of less than 0.33 μm. Adhesive strengths up to180 kPa were observed in this case.[56]

During fabrication of PDMS coatings, coating and curing occur at the same time, and the processes are accelerated at higher temperatures. Heating was carried out in a controlled manner from low to high temperature to carefully eliminate the solvent and slowly promote cross-linking. The presence of an inert gas avoid oxidation or degradation of the polymer during heating. After inserting the metal wire, the needle was heated first in an oven at 80ºC for 10 min,

then in a GC injector (equipped for heating and column conditioning) pressurized with He (by connecting a pressure restrictor to the outlet port) at approximately 15 psi with flow through the injector at approximately 2-3 mL/min. Temperature was programmed from room temperature to 200ºC at 5º/min and held for 20 min at the upper temperature. At this point, the coating was solidified and had strong adhesion with the needle wall and the center wire. The wire was removed from the needle when cool by twisting to first release the physical bonding between the wire and the coating, and then slowly withdrawing the wire from the needle. Before use, the needle was connected to the GC injection port and conditioned at a suitable temperature as in the

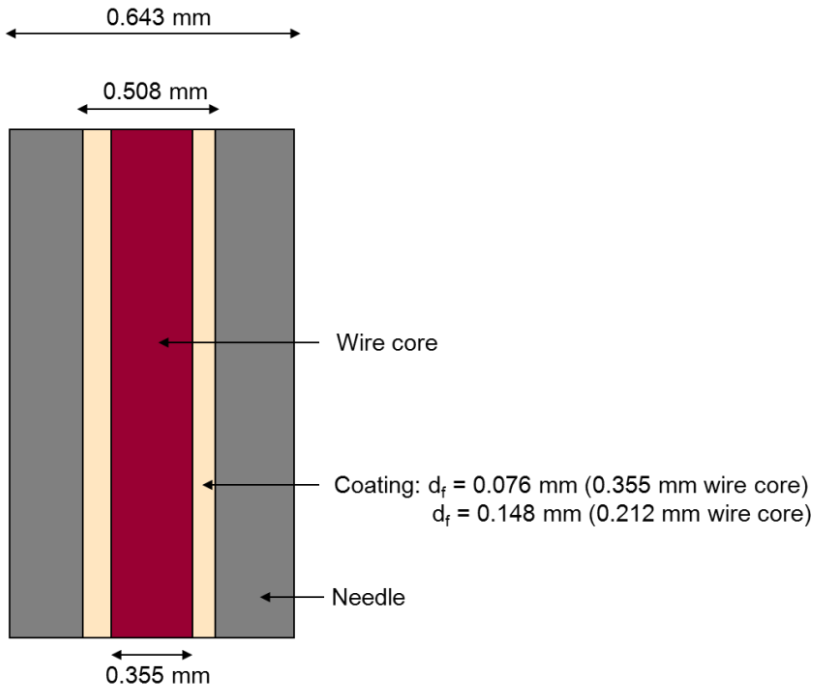

Figure 6.5. Schematic drawing of an ideal section of coated 23G needle.

heating step, except the needle was connected to a He gas source at 15 psi and was entirely inserted into the GC injection port.

A coating as depicted in Figure 6.5 was expected, if the wire was centered inside the needle. The wire must have smooth a surface and be absolutely straight to be easy removed, leaving a uniform surface. The wire was centered by manual adjustment under a microscope before heating. Heating normally required longer time and a significantly higher temperature than for eliminating the solvent. The film thickness could be changed by using different diameter wires. The PDMS coating in the needle was used at high temperatures (310°C) many times without degradation. This result agrees with the report that the thermal decomposition temperature of thick (500 μm) and thin (50 μm) crosslinked PDMS membranes is approximately 310 and 450ºC, respectively. The increased thermal stability is attributed to the enhanced cross-linked network resulting from fully reordered polymer chains.[57]

Other polymer coatings. The fabrication of epoxy, Ucon, SE54, and Apiezon N coatings were carried out similarly to the PDMS procedure in terms of polymer concentration, introduction, and wire form alignment; however, the heat treatment time and temperature

Table 6.1. Heat treatment conditions for fabrication of polymer coatings.

Polymer	Epoxy	Ucon	SE54	Apiezon N
Temperature program, ºC	50-250 (10/min)	50-250 (10/min)	50-280 (10/min)	50-290 (10/min)
Time at final temperature, min	20	10	10	10

program varied from one to the other (Table 6.1). These polymers could be used after this treatment without any significant traces of solvent or polymer fragments. PDMS, on the other

hand, require longer time to eliminate residues of elastomer and curing agent. When conditioning at 280 and 300ºC, the coating could be used only after at least 60 and 30 min, respectively.

6.3.2 Fabrication of activated carbon/polymer coating

An active sorbent was added to the PDMS coating for volatile biomarker compounds from bacteria, including the three main biomarkers 3-methyl-2-butenoic acid methyl ester (3M2BAME), fatty acid methyl esters (FAMEs), and dipicolinic acid mthyl ster (DPAME). Activated carbon (C) (also called activated charcoal or activated coal) was mixed with PDMS, PS, and PMMA to improve the extraction efficiency for 3M2BAME. Fabrication and conditioning of the C/polymer coatings were performed as described previously for other polymer coatings. Mixtures of activated carbon and polymer were prepared in dichloromethane by mixing well, and degassing in an ultrasonic bath for 10 min. The extraction efficiencies for this type of coating changed with the concentration of activated carbon; volatile and medium polar compound extraction efficiencies normally increased with activated carbon concentration. However increasing the concentration weakened the adhesion of the coating on the needle surface.

Because PDMS provides adhesion of the coating with the needle surface, activated carbon should be used only at a concentration that does not reduce significantly the contact area of the PDMS on the needle, but provides interaction of analytes with both sorbents to provide good extraction efficiencies for all targets. Microscope observations (Figure 6.6) and other experiments indicated that the concentration of activated carbon should not be higher than 10% or lower than 3% w/w (of mixture which contained 20% PDMS in diclorometh ane). Therefore, I decided to use activated carbon and PDMS at 5% and 20%, respectively, as concentrations for fabrication of coatings.

6.3.3 Needle-to-needle coating uniformity

Ideally, the coatings should be uniform in thickness and position inside the needles as shown in Figure 6.5. The wire core form should be made of stainless steel or hard metal whose surface is smooth to minimize deformation or damage that may occur during removal of the core from the needle. Nonuniform coatings as depicted in Figure 6.7, caused by misalignment of the

Figure 6.6. Microscope images of activated carbon/PDMS films formed with 3, 5, and 7% (w/w) solution concentrations at magnification of 100X.

wire during coating, may not affect significantly the extraction and desorption efficiencies because the coating thickness is very small (<0.2 mm) compared to its length (>1 cm) and the coating volume and surface areas are constant from needle to needle. In addition, the high injector temperature and the low initial GC oven temperature help to overcome any differences in peak widths due to desorption rate changes, if any, from one needle to another. However, the extraction or equilibrium time may be longer with a nonuniform coated needle compared to one with a uniform coating due to greater film thickness at some locations. To minimize misalignment of the wire, it was adjusted before heating the polymer by checking its location under a microscope. In reality, it could not perfectly aligned.

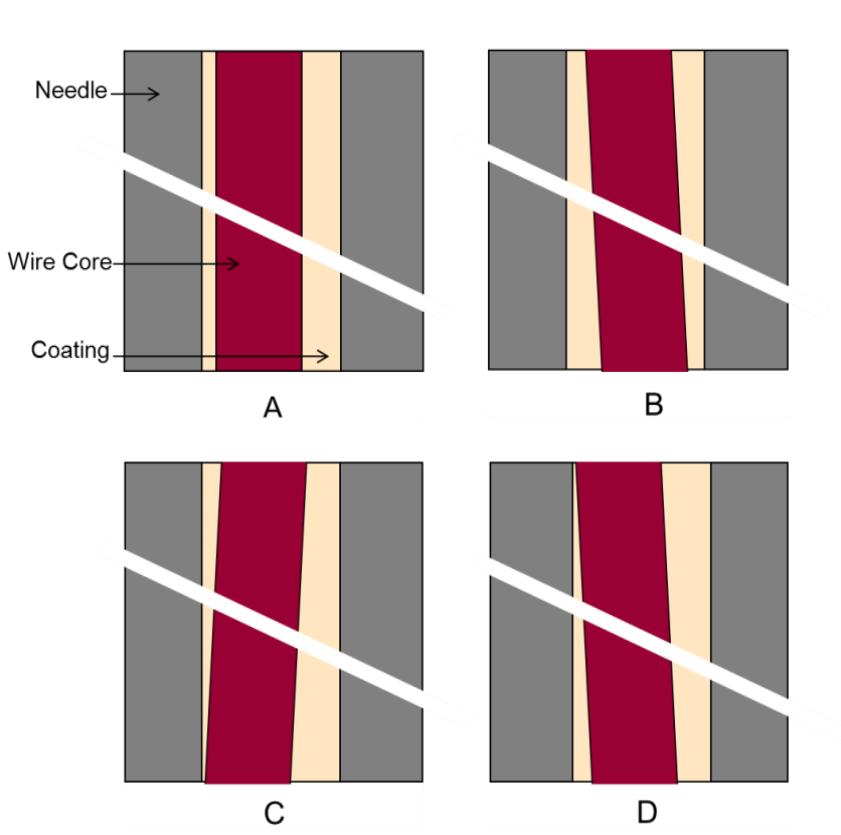

Figure 6.7. Schematic drawing of nonuniform coatings caused by misalignment of the wire core during fabrication. (A) wire core is parallel with the needle but not centered, (B) wire core is not parallel with the needle but is centered, (C) and (D) wire core forms different angles with the needle and is not centered.

6.3.4 Coated-Needle Extraction (CNE)

Extraction equilibrium.The diffusion for CNE compared to conventional SPME is dependent on equilibrium between the stationary phase and the surrounding medium. Therefore, absorbed chemicals will remain in the polymer as long as a similar concentration of that chemical exists in the surrounding medium at the stationary phase surface. If the concentration in the medium decreases, then diffusion will cause the absorbed chemical to naturally flow out of the stationary phase until a new equilibrium is met.

Equilibrium in SPME, both conventional and in-needle, depends on many factors including temperature, stirring conditions, type and concentration of salt addition, and fiber properties.[58,59] Although the concentration of the sample has no impact on the equilibration time,[60] the mass absorbed depends significantly on agitation conditions; the sensitivity increases by an order of four from no to perfect agitation. Equilibrium time increases with film thickness, and mass absorbed is proportional to the coating volume or film thickness (surface area of the coating is constant). Diffusion coefficients vary from one component to another; therefore, their equilibrium times are different. A change in distribution constant with temperature is indicated in Equation 6.1; a high extraction temperature increases the extraction rate, but also enhances the release of analytes and decreases the amount extracted at equilibrium.[60]

$$K_{fs} = K_o \exp\left[\frac{-\Delta H}{R}\left(\frac{1}{T} - \frac{1}{T_o}\right)\right] \qquad (6.1)$$

Generally, extraction sensitivity depends on the fiber coating thickness, K_{fs}, the nature of the matrix, and temperature; extraction selectivity is affected by type of coating, and target analyte properties; and extraction speed increases with agitation level and temperature. In my research, I did not focus on all parameters, but fixed room temperature, no salting out, and no vibration.

CNE of liquid samples. CNE of liquid phase does not require vibration or agitation of the sample as in conventional SPME. Fast sample flow generated using a gas-tight syringe provided high enough movement for accelerating the extraction. The extraction efficiency increased with the number of syringe strokes until equilibrium was established. CNE shows the same behavior as conventional SPME, including the responses to temperature, salting out, agitation and matrix.

However, CNE demonstrated shorter equilibrium time compared to conventional SPME due to higher relative movement between the two phases. Consequently, at equilibrium, the average density of target analyte absorbed by the coating in CNE may be smaller than in conventional SPME if the movement is too high. Fortunately, since the surface area and sorbent volume of the former is larger than the latter, the total amount of target analytes absorbed is still high in CNE.

Headspace extraction and gas samples. Headspace extraction can be performed similarly to conventional SPME under all conditions, except the gas phase is repeatedly forced through the coated needle using a gas-tight syringe as shown in Figure 6.8. An advantage of CNE headspace extraction is the formation of vacuum inside the sample vial during half of the sampling cycle (Figure 6.8A) or by using a vacuum system (Figure 6.8B). If the headspace volume is as small as the syringe volume and there is no leak in the system, a significant vacuum will be formed. This effect enhances the distribution of the targets in the headspace and, thus, the extraction efficiency. However, care should be taken to limit the velocity of the gas phase because the reduced pressure on the surface of the stationary phase during extraction, according to Bernoulli's law, decreases the absorption efficiency. A simple way to minimize this affect is to reduce the needle temperature to a value that is lower than the sample solution and the syringe. This is also the case for liquid extraction; however, due to the higher viscosity of liquids compared to gases, the velocity almost never reaches a high enough value even with a very short syringe stroke.

Circulation of sample in SPME and CNE. The circulation of sample in SPME usually creates a forced (rotational) vortex. In a forced vortex, the rotation of the fluid behaves essentially like a solid body without shear. The motion can be considered as a dish of fluid on a

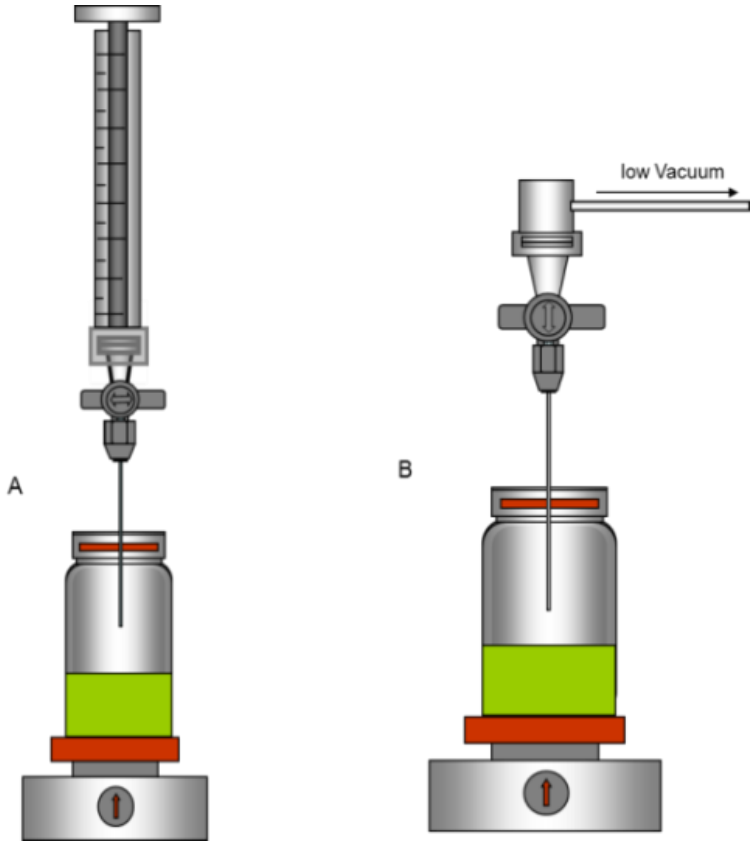

Figure 6.8. Schematic drawings of headspace CNE using (A) a gas-tight syringe and (B) a vacuum system.

turntable rotating at ω radians/s; the fluid has a vorticity of 2ω everywhere, and the free surface (if present) is a parabola. The tangential velocity is given by:

$$V = \omega r \qquad (6.2)$$

where ω is the angular velocity and r is the radial distance from the center of the vortex. The relative velocity of sample solution at the surface of the fiber was calculated based on the cross-sectional diameter of the fiber, rotational speed of the stir bar and

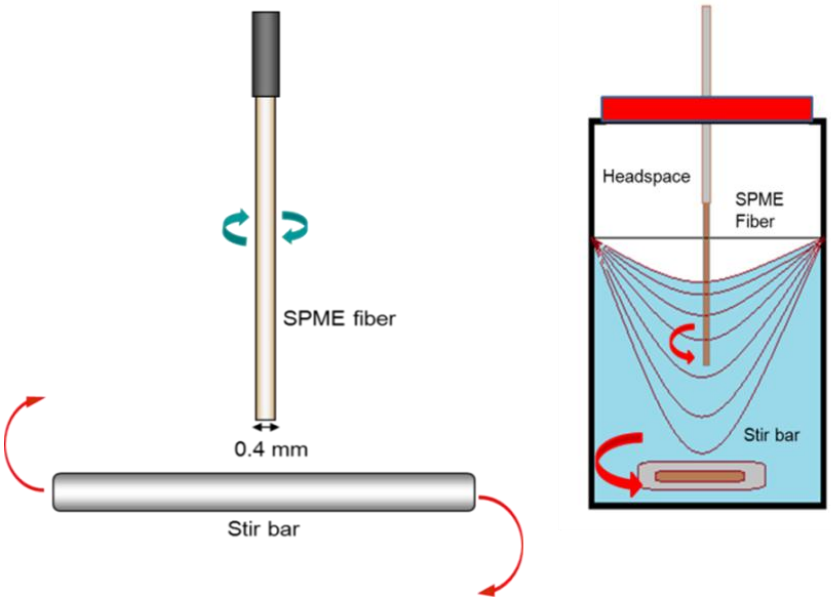

Figure 6.9. Schematic drawing of stirring in conventional SPME using a stir bar and half-half extraction.

ignoring the shear between the liquid and stir bar and the vertical velocity caused by the vorticity (see Figure 6.9). In CNE, due to the small needle inner diameter, sample movement through it was considered to be streamline flow, and the relative velocity of the sample was calculated based on the flow rate and diameter of the needle. The plots in Figure 6.10 indicate the changes in relative velocity vs. the sample flow rate.

From the relative velocity, the time for a half extraction cycle in CNE to extract a 1 mL sample was calculated, and is presented by plots in Figure 6.11. The results shown in the plots indicate that in CNE (where a half cycle takes from 10-20 s), the relative velocity varies approximately between 1,500 and 3,000 mm/s. For simple matrices, for which extraction can be performed faster (half cycle of 5-10 s) the relative velocity varies between 3,000-6,000 mm/s. These are much higher than in conventional SPME (see yellow areas of the plots in Figure 6.10).

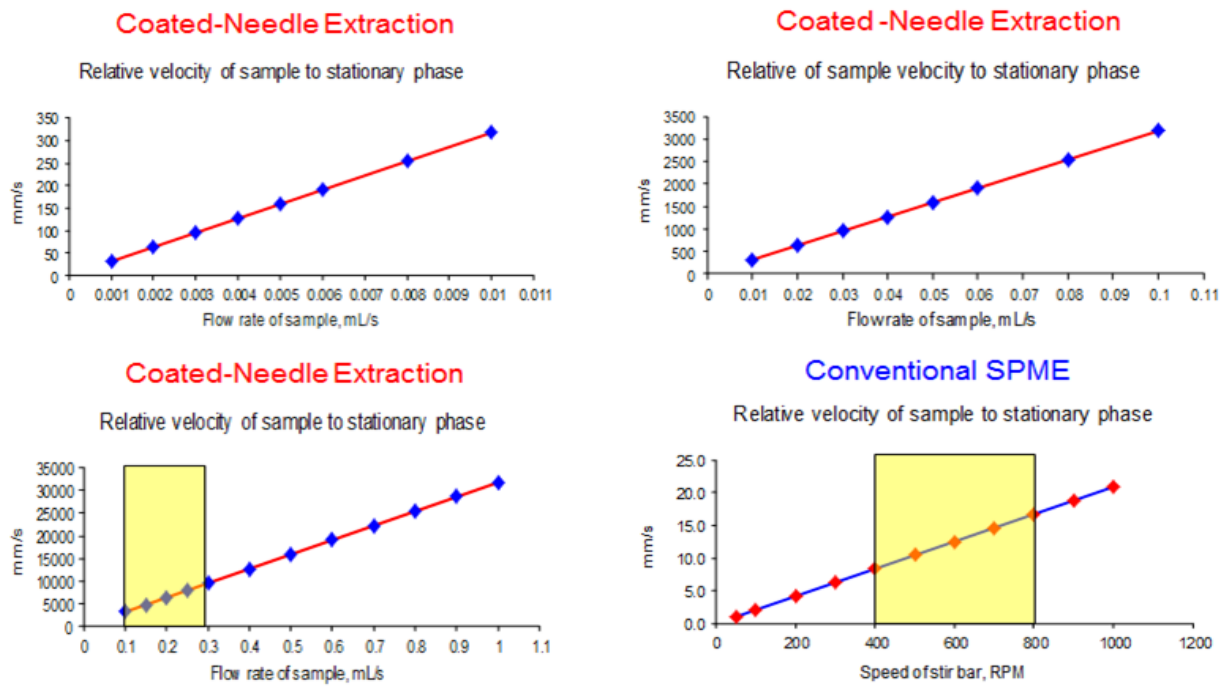

Figure 6.10. Changes in relative velocity vs. sample flow rate in CNE.

6.3.5 Carryover in CNE

Caryover in CNE is more significant than for conventional SPME due to the vapor inside the needle having only a small orifice at one end of the needle from which to escape. A small amount of analyte may remain inside the needle after the injection is finished. Although, carryover can be easily eliminated by cleaning the CNE assemply after each injection, various methods of injection were evaluated to determine if one would be better than another for satisfying different analytical requirements.

I have classified the in-needle injections methods into 4 modes, which can be classified themselves into two types: non-purging and purging. Mode 1 is non-purging, while modes 2-4

are purging types, which have a carrier gas supply from an external source or from the GC carrier gas when the column is inserted into the needle. Figure 6.12 presents schematic

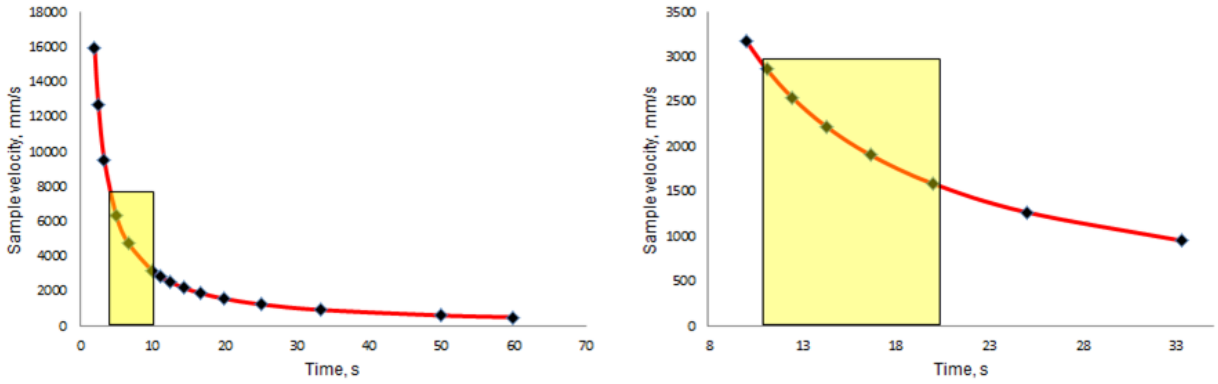

Figure 6.11. Relative velocity vs. time for half extraction cycle for CNE of 1 mL sample.

drawings and photographs of examples of non-purging and purging injection modes (using an external carrier gas source) for CNE.

Non-purging injection was performed simply by closing the valve during injection, while purging injection was carried out by replacing the syringe with a carrier gas source, such that the pressure was higher than the GC inlet pressure ($\Delta P \sim 5$ psi). The valve was opened after the needle was completely inserted into the injector, and it was closed before the needle is taken out. Splitless injection was normally used for both injection types, however, the split mode could also be useful for high concentration samples or in the case of high carryover. Since carryover when using the purging injection mode is smaller than for the non-purging injection mode, the sensitivity of the former is always higher than that of the latter. Figure 6.13 shows GC-MS total-ion chromatograms of the same sample extracted using the same CNE assembly and condition but using mode 1 and 3.

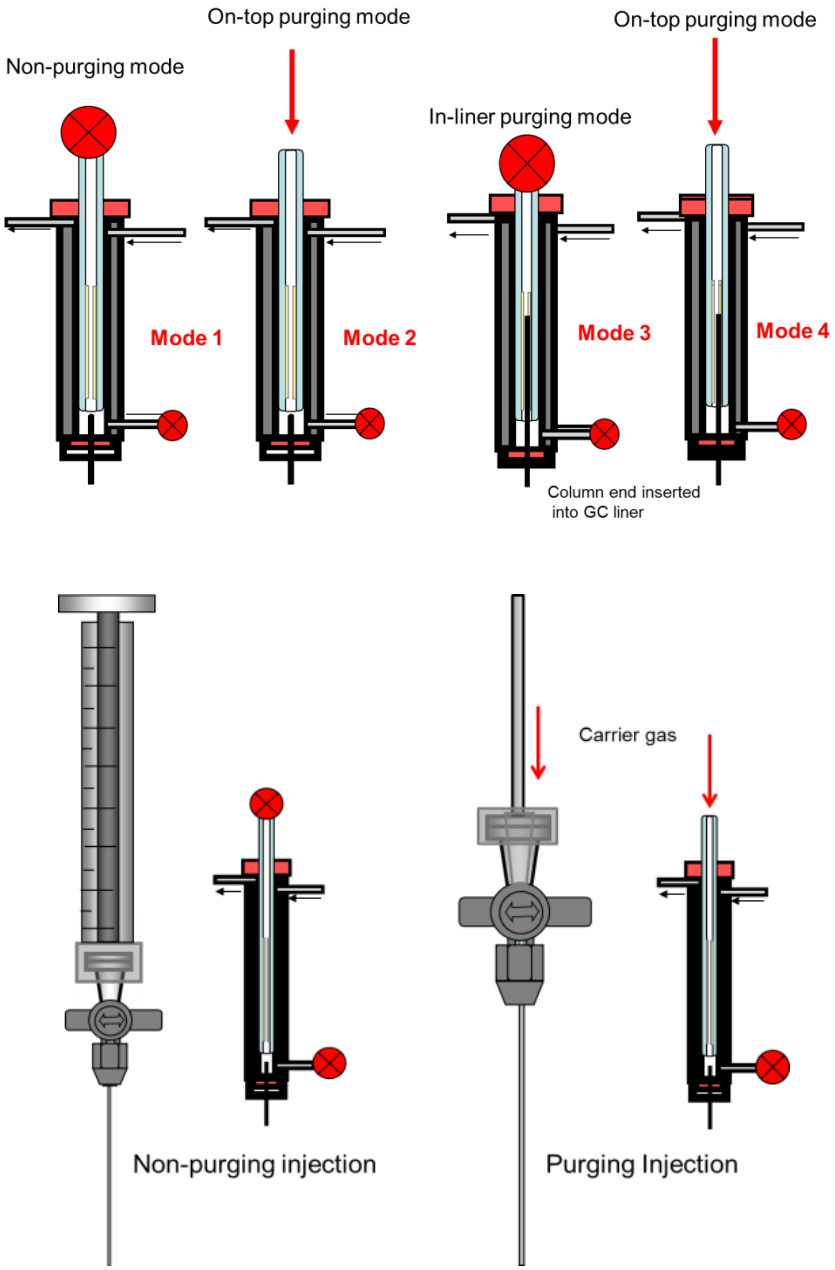

Figure 6.12. Schematic drawings of modes 1, 2, 3, and 4 and non-purging and purging injection modes for CNE.

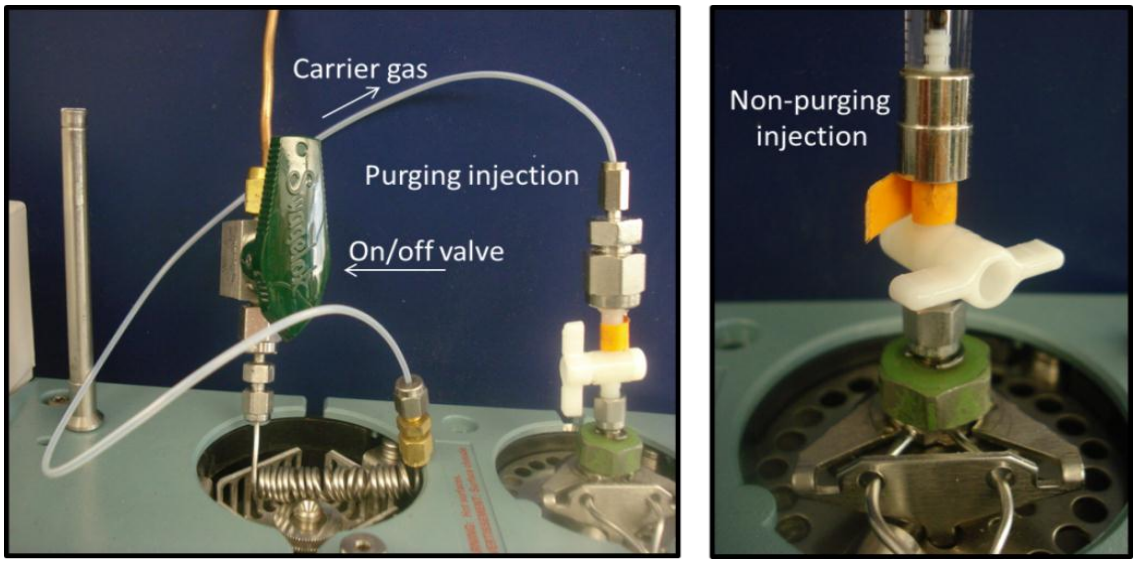

Figure 6.12. (cont.) Photographs of non-purging and purging injection modes for CNE.

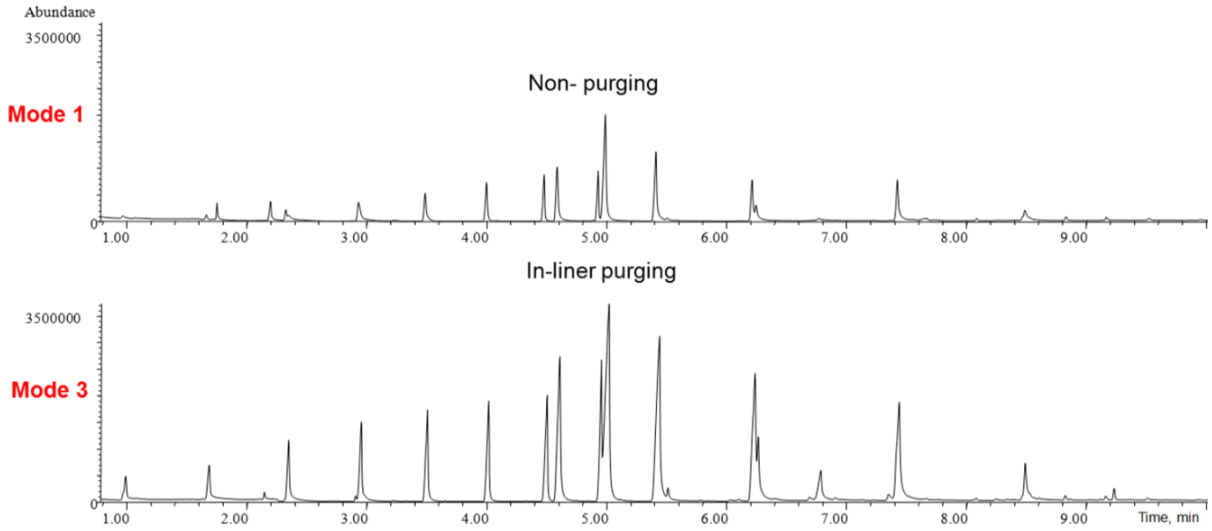

Figure 6.13. TIC chromatograms of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted by CNE (22G needle) using non-purging (mode 1) and in-liner purging (mode 3). Extraction conditions: liquid extraction, PDMS coating, 2 min (10 cycles), 20ºC, and 1 mL sample. GC conditions: Restek Rtx 5 MS, 5 m x 0.1 mm i.d. x 0.4 µm film thickness column, temperature programmed from 60ºC to 300ºC at 20ºC/min, 20 psi He inlet pressure, 1 min injection time, 280ºC injector temperature, 1.5 min splitless time.

Carryover in mode 1. As described above and in section 6.2.4, mode 1 injection was performed simply by inserting the needle with the valve close into the heated GC injector. Split or splitless injection could be used in this mode. Without a needle purging gas, the target analytes were transferred into the column only by vapor expansion and diffusion. Therefore, injection time, temperature, pressure in the injector, and liner volume were the factors that affected carryover. Carryover in mode 1 decreased with an increase in injection time. As shown in Figure 6.14, carryover was greatest at 2 s, smallest at 300 s and almost zero at longer than 300 s. The higher boiling point components (such as PAHs) led to higher carryover. Carryover percentages of PAHs such as anthracene, pyrene, and chrysene were even higher than 100% (see Figure 6.15) for 2 s injection, However this condition is rarely used with SPME; typical times range from 10 to hundreds of seconds. Unfortunately, 300 s (5 min) injection is not suitable for fast analysis when the GC run time is short. Therefore, minimizing carryover is necessary to satisfy the analysis requirements.

Carryover in mode 2 (purging). Purging using an external carrier gas source or one shared with the GC [for which, as mentioned, the purging pressure is set higher ($\Delta P \sim 5$ psi) than the GC inlet pressure] is convenient for mode 2. Figure 6.16 shows a diagram of the CNE device and purging system used for mode 2. Before injection, the purging gas was turned on while the valve remained off. After the pressure was established, the SPME needle was inserted into the injector, the valve was immediately turned on and only switched off just before the needle was taken out of the injector. A carryover run was performed immediately after each sample run, following the same method of injection. With this injection mode using splitless injection,

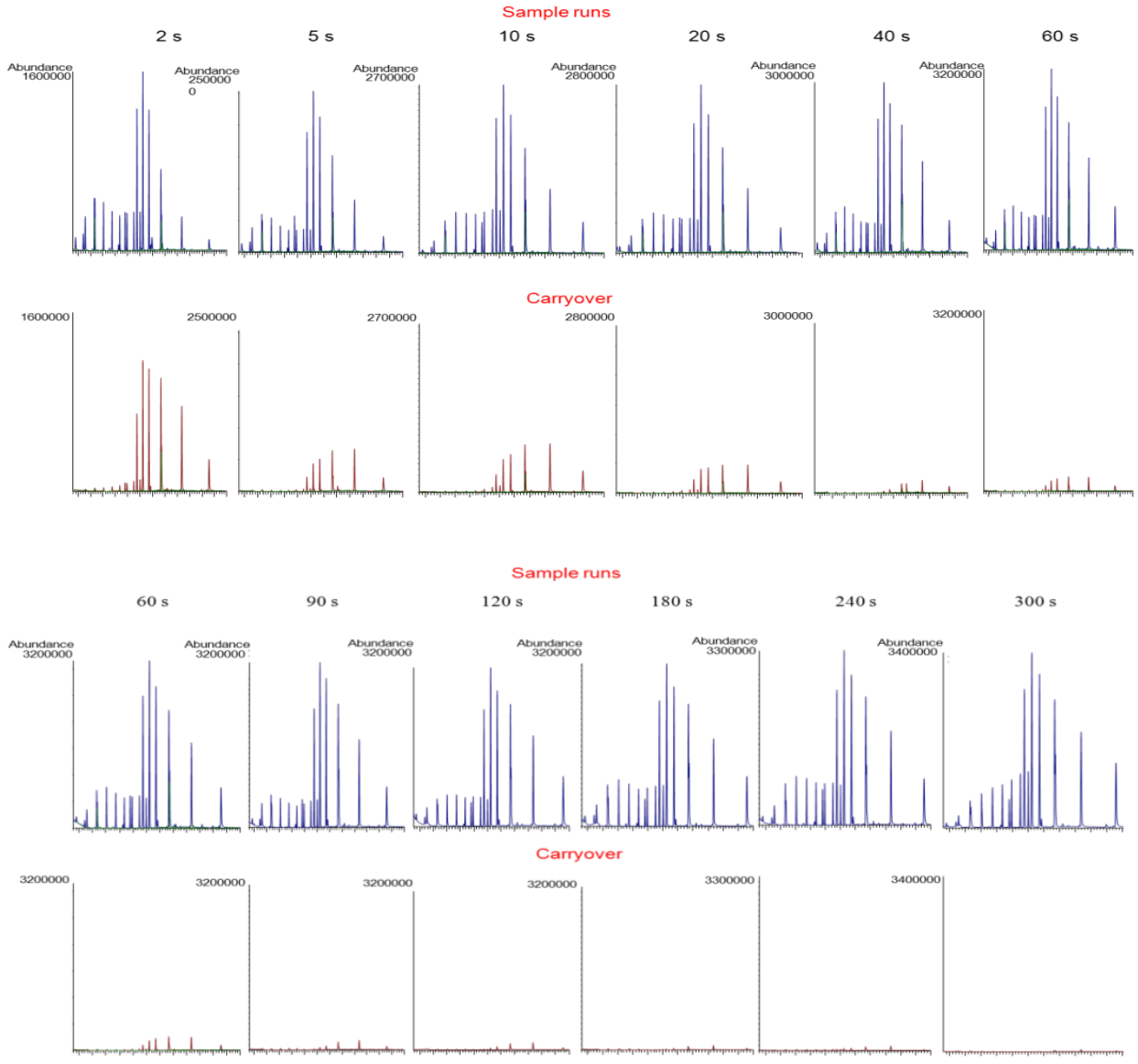

Figure 6.14. TIC chromatograms showing carryover of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted by CNE (23G needle). Extraction conditions: PDMS coating, liquid extraction, 2 min (10 cycles), 20ºC, and 1 mL sample. GC cconditions: HP-MS 5, 30 m x 0.25 mm x 0.25 µm film thickness column, temperature programmed from 60ºC (hold 1 min) to 290ºC (hold 10 min) at 25ºC/min, 12 psi He inlet pressure, injection mode 1, different injection times from 2 s to 300 s, 280ºC injector temperature, 1.5 min splitless time.

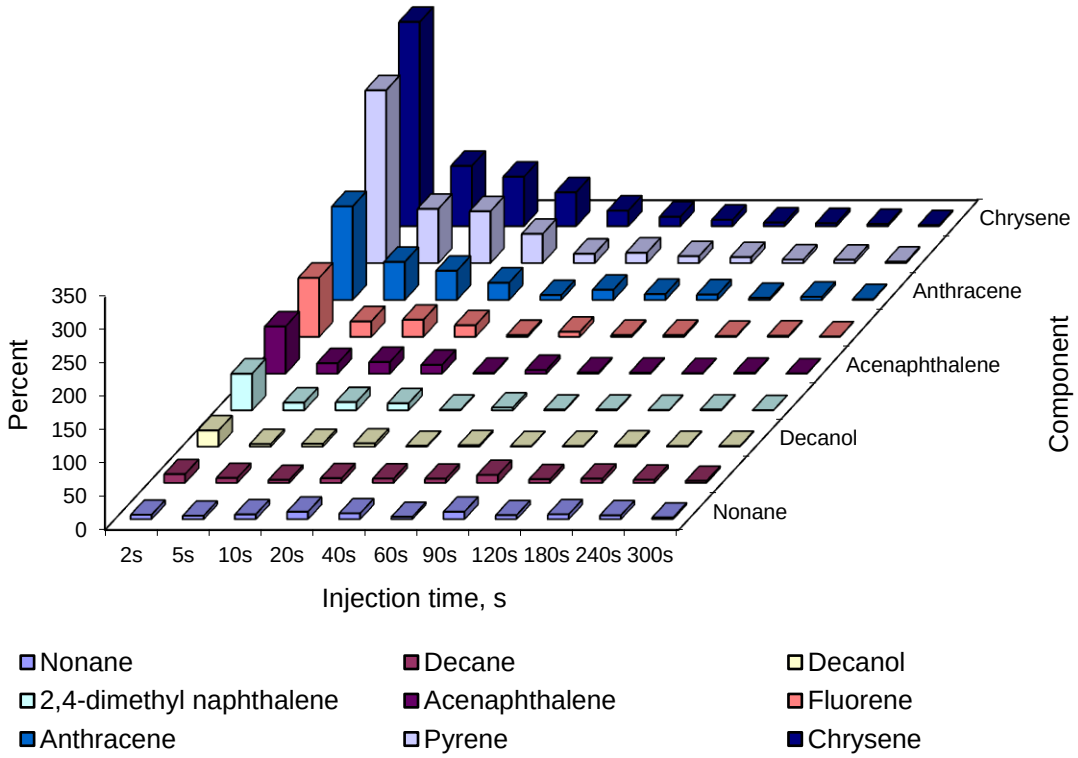

Figure 6.15. Bar plot of carryover percentages of hydrocarbons and PAHs using CNE and injection mode 1.

carryover almost disappeared after only 60 s injection at 280ºC. Purging using a separate gas source provided the best results for CNE.

Carryover in mode 3 (in-needle purging). Mode 3 operation is simpler than mode 2 in design, but requires attention during injection to make certain the column is inserted carefully into the end of the extraction needle. Use of a large i.d. needle such as 22G or a small o.d. column allows for easier injection. In this experiment, the extraction needle was 23G (0.508 mm i.d) and the column was 0.25 mm i.d. x 0.35 mm o.d. fused silica. In this case, the coating thickness should be smaller than 50 µm and the split between the needle and the coating should

be larger than 30 µm. The coating thickness in the experiment was approximately 40 µm (See Figure 6.17).

Injection was performed by inserting the CNE needle completely into the injector and rotating it approximately ~1/4-1/2 turn to allow the column end to enter the needle; the needle was held it in the injector for 1 min**.**

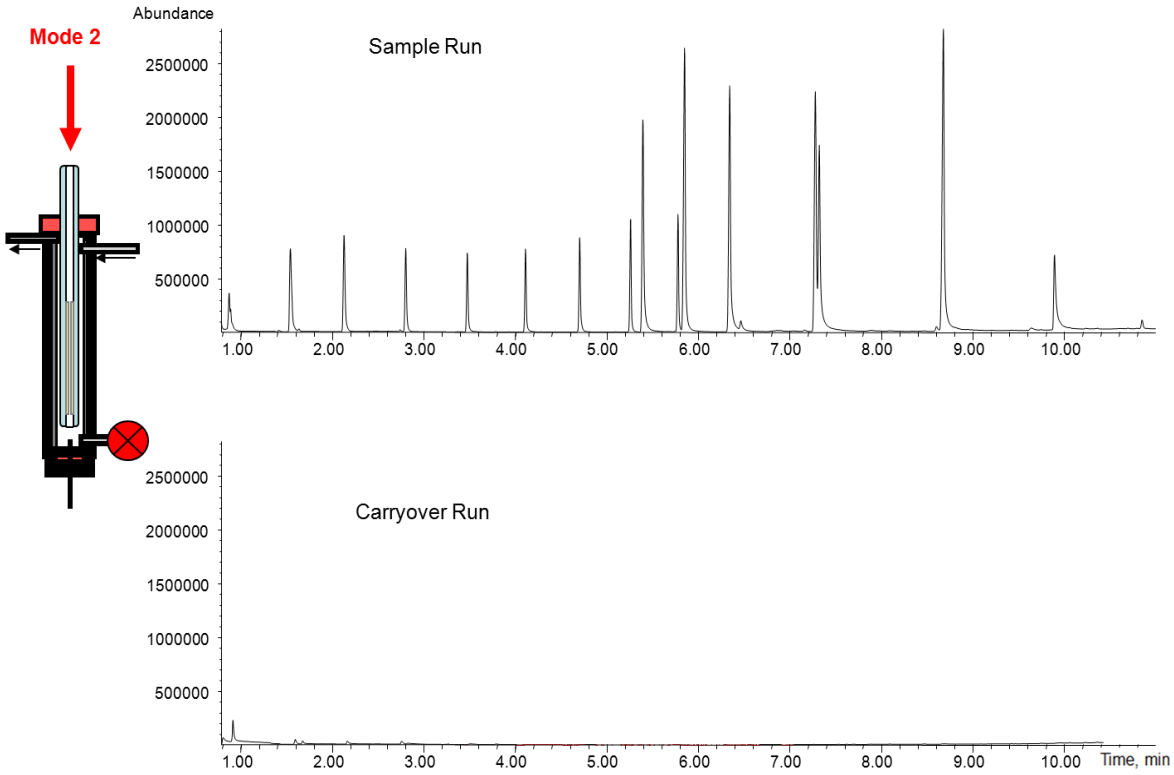

Figure 6.16. TIC chromatograms showing carryover of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted by CNE (23G needle). Extraction conditions: SPME coating, liquid extraction, 2 min (10 cycles), 20ºC, and 1 mL sample. GC conditions: HP-MS 5, 15 m x 0.25 mm i.d. x 0.25 µm film thickness column, temperature programmed from 70ºC (hold 1 min) to 300ºC (hold 5 min) at 25ºC/min, 7 psi He inlet pressure, injection mode 2, 1 min injection time, 280ºC injector temperature, 1.5 min splitless time.

Injections were easiest if a Restek MTX 0.1 mm i.d., 0.22 mm o.d. metal column was used. In this case, 100 µm coating thickness could be used, which still left a 35 µm space between the column and the needle, allowing the carrier gas to easily flow through the column. When comparing carryover, this mode was better than mode 1 but worse than mode 2. Figure 6.18 indicates that the carryover nearly disappeared after the fourth run (after running the sample and repeating three subsequent carryover runs without cleaning the needle). This result indicates that the sample could be more completely transferred into the column if the injection time was

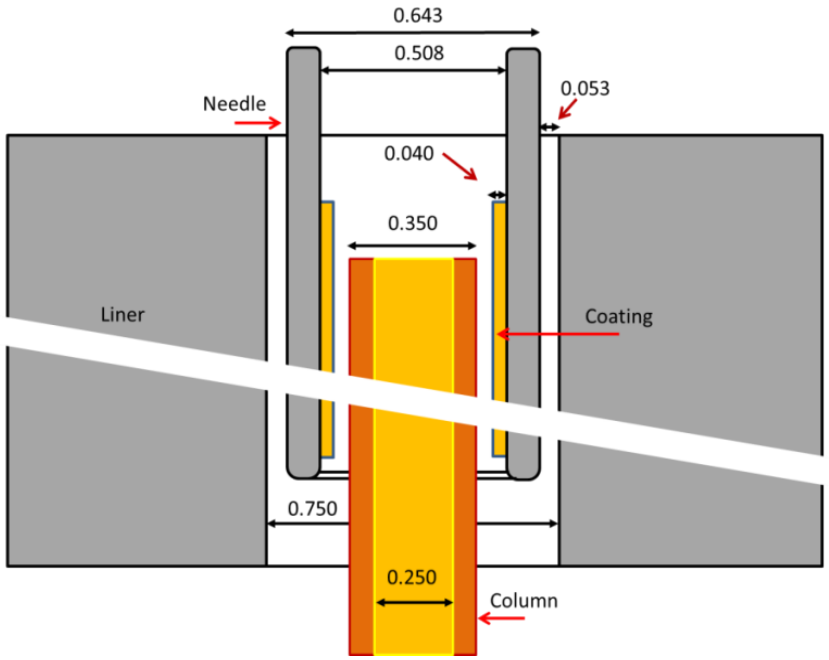

Figure 6.17. Schematic drawing of the injector using mode 2 with needle fully inserted, conditions: 23G needle and standard GC column (0.35 mm o.d., 0.25 mm i.d. column), labeled length units are in mm.

longer than 1 min. An injection time of 2 min could be used which would leave approximately <0.1% (calculated from the largest component remaining in the needle) carryover.

Carryover in mode 4 (purging). Mode 4 is a combination of modes 3 and 2, and allows for short time injections without carryover. The chromatograms shown in Figure 6.19 indicate no carryover after 1 min injection. This is reasonable because all of the analytes are in the flow path of the carrier gas through the needle. However, the peak shapes in this mode tail more than the previous modes. The explanation for this is since the inlet pressure is almost unchanged, the flow rate through the column is almost constant, and is the sum of two flows, one from the purge from

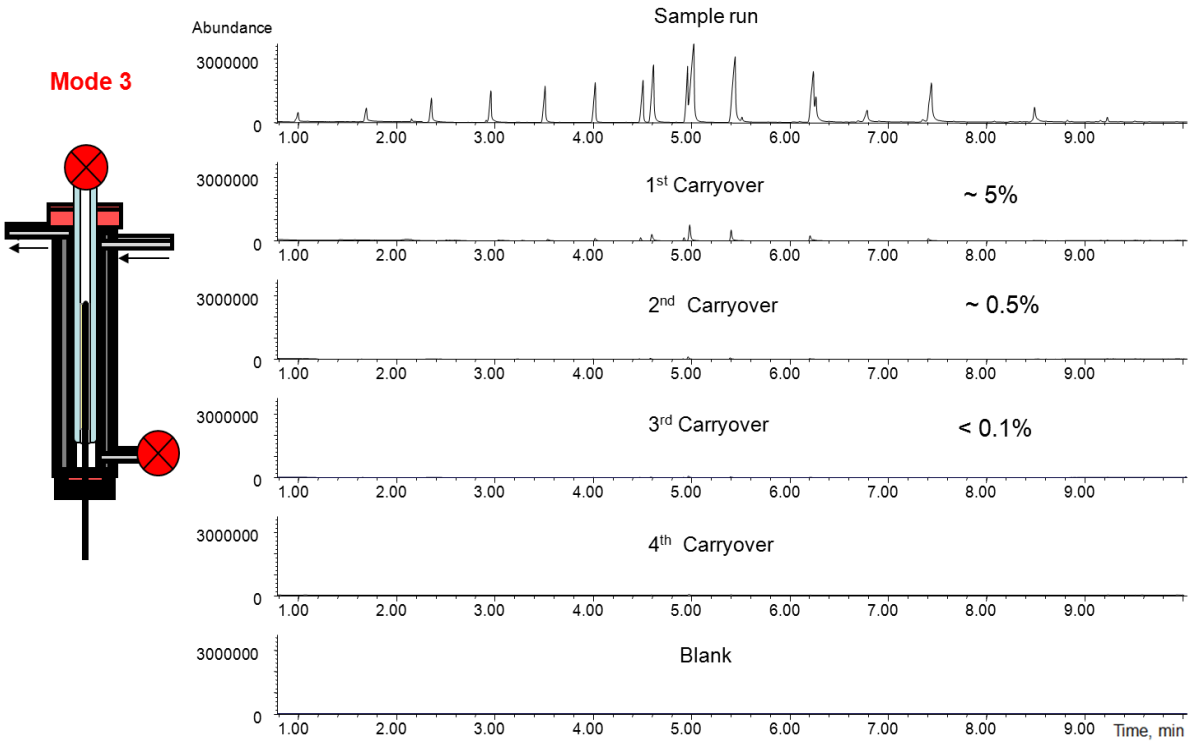

Figure 6.18. TIC chromatograms showing carryover of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted by CNE (23G needle). Extraction conditions: PDMS coating, liquid extraction, 2 min (10 cycles), 20ºC, and 1 mL sample. GC conditions: RTX 5, 5 m x 0.1 mm i.d. x 0.4 µm film thickness column, temperature programmed from 55ºC to 300ºC at 30ºC/min, 30 psi He inlet pressure, injection mode 3, 1 min injection time, 280ºC injector temperature, 1.5 min splitless time.

the top and the other from the purge in the needle. The latter flow rate is lower than that in mode 3, which makes the sample band in the column wider and tailing if there is no change in the temperature program. However, this disadvantage can be easily overcome by applying a higher pressure in the injector at the moment of injection.

In-needle purging and purging through the GC liner (mode 3) are convenient. There are no additional modifications needed for this injection method if a narrow (0.75 mm) i.d. liner is used as a guide to center the needle (23 G or 22 G) so that the capillary column end can easily be inserted into the needle. The results shown in Figure 6.19 indicate that

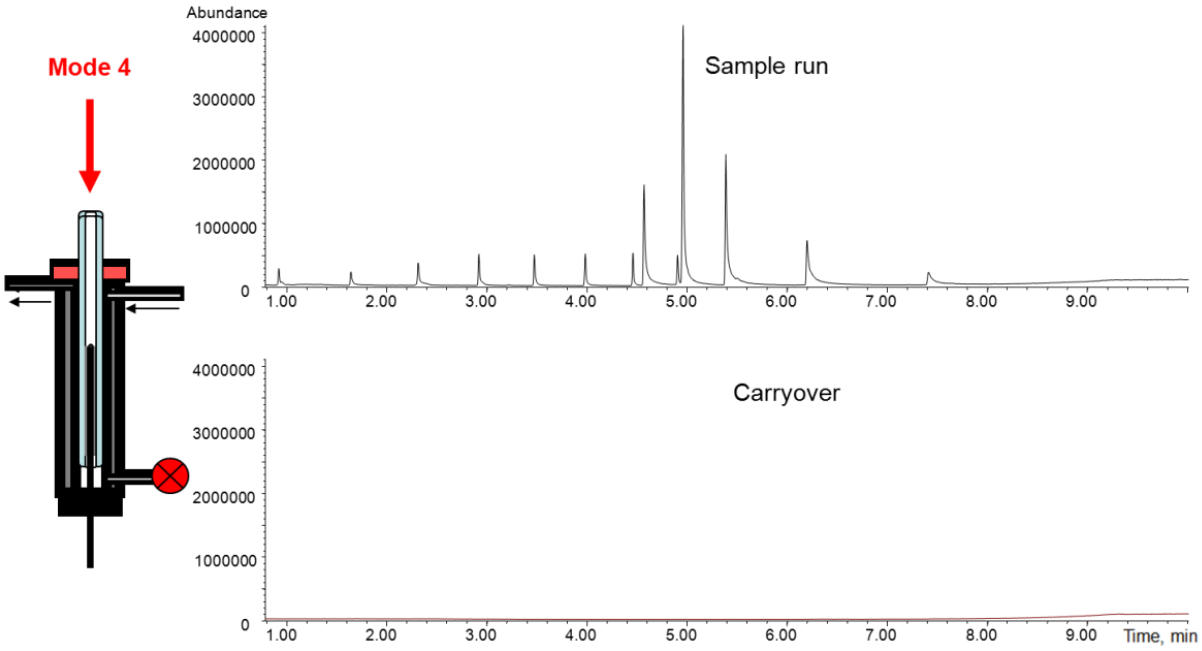

Figure 6.19. TIC chromatograms showing carryover of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted CNE (23G needle). Extraction conditions: PDMS coating, liquid extraction, 2 min (10 cycles), 20ºC, and 1 mL sample. GC conditions: RTX 5, 5 m x 0.1 mm i.d. x 0.4 µm film thickness column, temperature programmed from 55ºC to 300ºC at 30ºC/min, 30 psi He inlet pressure, injection mode 4, 1 min injection time, 280ºC injector temperature, 1.5 min splitless time.

carryover was reduced significantly after 2 min injection. A challenge of this method is how to insert the needle in the injector over the column end without damaging the needle coating. I found no problem in this regard using PDMS and activated carbon/PDMS coatings. The column tip did not damage the hard surface of the coating easily. However, a safer design should be developed so that this injection mode is feasible for any type of coating. A possible solution is to employ a retention gap which has a smaller o.d. than the conventional GC column, and use an extraction needle with a crimped tip, which would allow the column end to easily move in without contact with the coated surface. Figure 6.20 shows a conceptual drawing of this modification in which a 23G or 22G needle and 0.32 mm o.d. retention gap are used. The needle

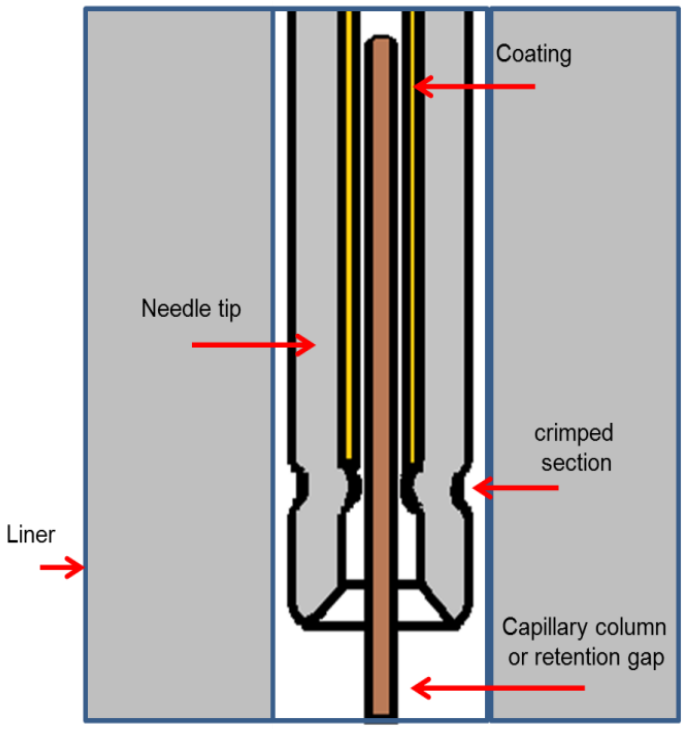

Figure 6.20. Schematic drawing of a crimped-tip needle used for injection with a retention gap.

would be coated as described earlier. The retention gap would be easily inserted into the needle as a result of the cone shape of the inner edge of the needle tip. The crimped section would guide the retention gap end to the center of the needle and minimize contact with the coating. The retention gap should be made of deactivated fused silica capillary, with or without a stationary phase coating. In addition, the outer edges of the retention capillary ends could be rounded (by mechanical or chemical methods) to ease the entry of the retention gap into the needle. Theoretically, the retention gap would also protect the GC column from contamination and improve peak shapes in many cases if there are any incompatibilities between the sample solvent and the stationary phase.

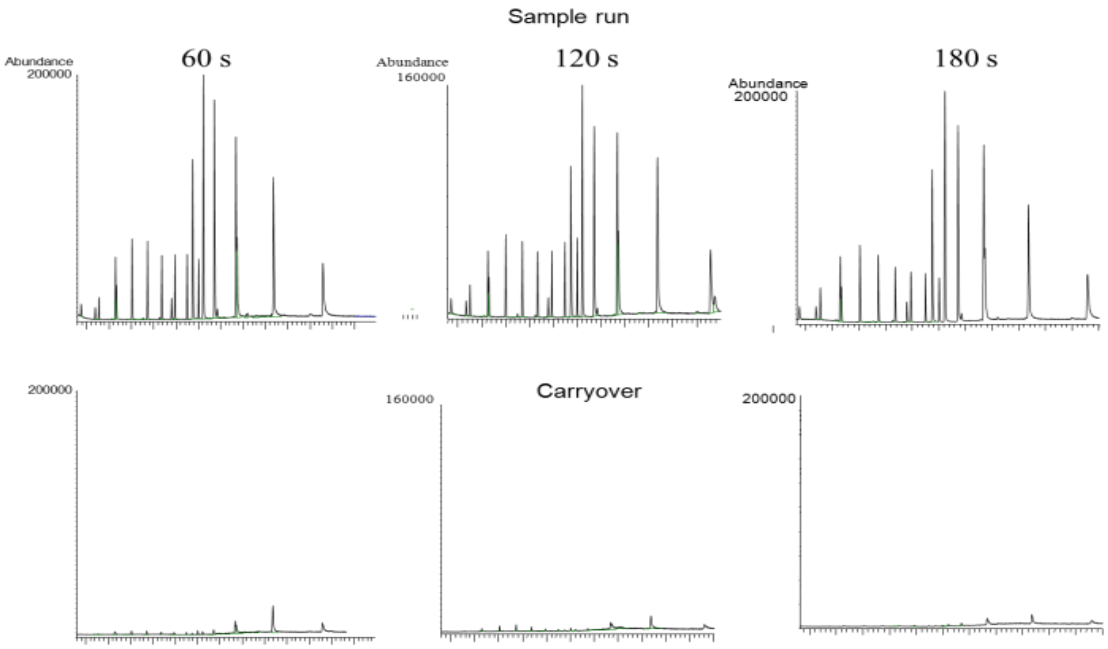

Figure 6.21. TIC chromatograms showing carryover of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted CNE (23G needle). Extraction conditions: PDMS coating, liquid extraction, 2 min (10 cycles), 20ºC, and 1 mL sample. GC conditions: MTX 5, 5 m x 0.1 mm i.d. x 0.4 µm film thickness column, temperature programmed from 55ºC (hold 1 min) to 300ºC (hold 5 min) at 30ºC/min, 30 psi He inlet pressure, injection mode 1, 1-3 min injection time, 280ºC injector temperature, 20:1 split ratio.

6.3.6 Other solutions to further reduce carryover in CNE

In addition to use a purging gas, there are several ways to further reduce memory effects: (1) use split injection, (2) minimize any dead volume in the CNE design, (3) increase the injection temperature (up to >300°C), and (4) use a pressure program during injection.

Carryover when using split injection. In split injection, the high flow rate of the carrier gas flowing through the injector liner helps to increase migration of the target vapor due to a pressure decrease at the needle tip according to Bernoulli's law. Figure 6.21 shows chromatograms of sample and carryover for 60, 120, and 180 s injection times. These carryover percentages are smaller than those obtained when using the splitless mode as shown in Figure 6.14. However, the improvement is not very significant because the carrier gas velocity is not high enough to create the desired pressure decrease at the needle tip.

Carryover for zero-dead volume and split injection. Minimizing dead volume in the CNE assembly significantly decreases the memory effect. Although the space between the valve and the needle is tiny, it is still large enough to trap some analytes. The temperature in this space during injection is normally lower than at any other point in the needle, which reduces evaporation and diffusion of the target analytes and causes high carryover. Without purging, increasing the injection time and temperature are effective ways to minimize carryover. Figure 6.25 shows chromatograms of sample and carryover when zero dead volume was combined with a small split ratio (10:1). To establish zero dead volume in the CNE assembly, the space between the needle and the value rotor must be made small as possible. To accomplish this, Teflon tubing was inserted into the male fitting connection of the plastic valve until it contacted the valve rotor (during which the flow control handle was closed). A stainless steel nut was then used to tightly fix the needle inside the male fitting of the valve. The dead volume in this configuration was zero

when the three parts, i.e., needle, Teflon tubing, and male fitting, were perfectly matched to each other. Figure 6.23 shows a schematic drawing of the three parts of the zero dead volume CNE assembly.

Carryover when using high injection temperature. A higher injector temperature evaporates the target and analytes faster to form a larger vapor volume. High temperature also increases diffusion, thus minimizing any target vapor remaining inside the needle. These issues all contribute effectively to elimination of carryover. However, only PDMS

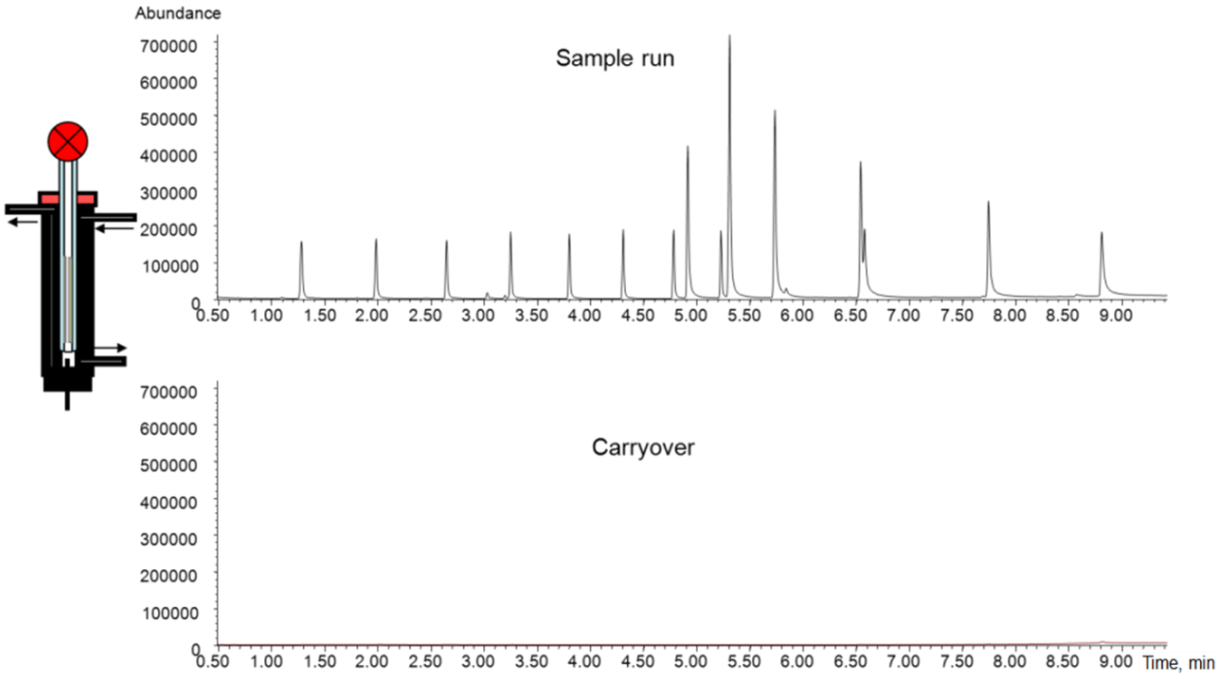

Figure 6.22. TIC chromatograms showing carryover of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted CNE (23G needle). Extraction conditions: PDMS coating, liquid extraction, 2 min (10 cycles), 20ºC, and 1 mL sample. GC conditions: MTX 5, 5 m x 0.1 mm i.d. x 0.4 µm film thickness column, temperature programmed from 55ºC (hold 1 min) to 300ºC (hold 5 min) at 30ºC/ min, 30 psi He inlet pressure, injection mode 1, 1 min injection time, 280ºC injector temperature, 10:1 split ratio.

and activated carbon/PDMS coating can be used at high temperature (280-300ºC). The others are normally employed at temperatures under or equal to 270ºC. The results of an experiment combining high injector temperature (300ºC), zero dead volume, and split injection mode (10:1) indicated that improvements were made in sensitivity for high boiling point components (e.g. PAHs), as well as minimizing carryover of analytes (see Figure 6.24).

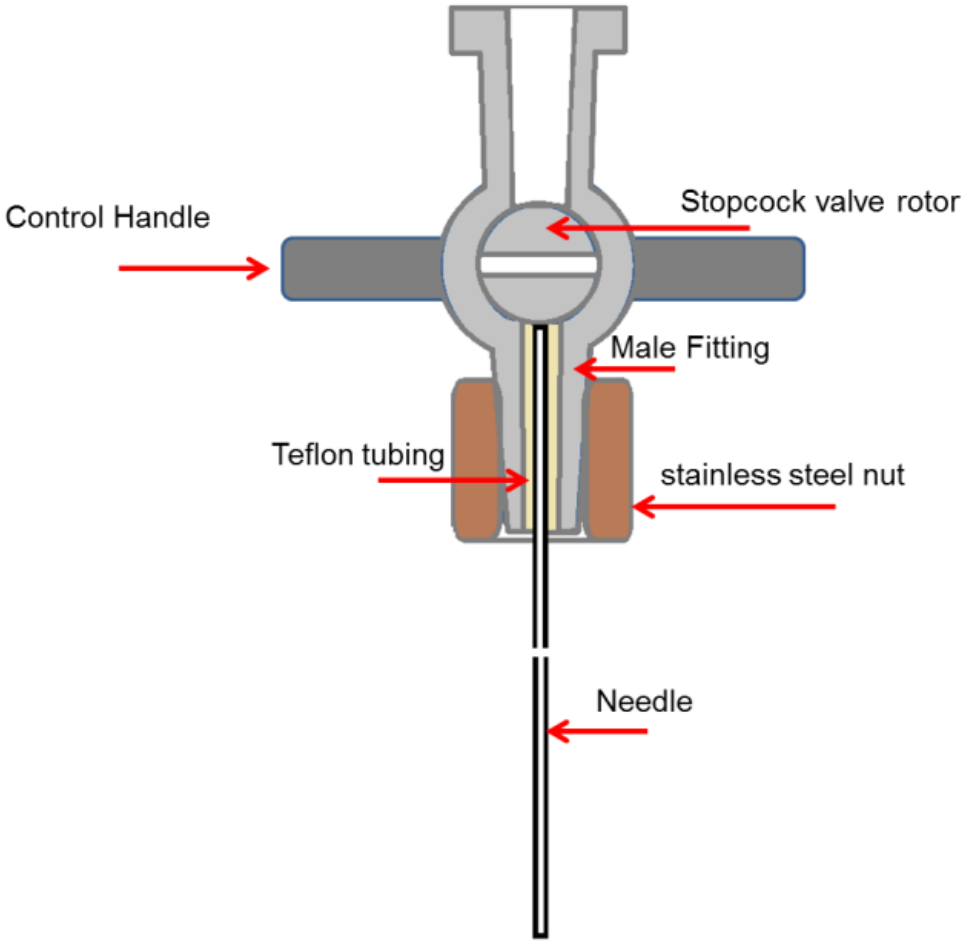

Figure 6.23. Schematic drawing of a zero dead volume CNE assembly.

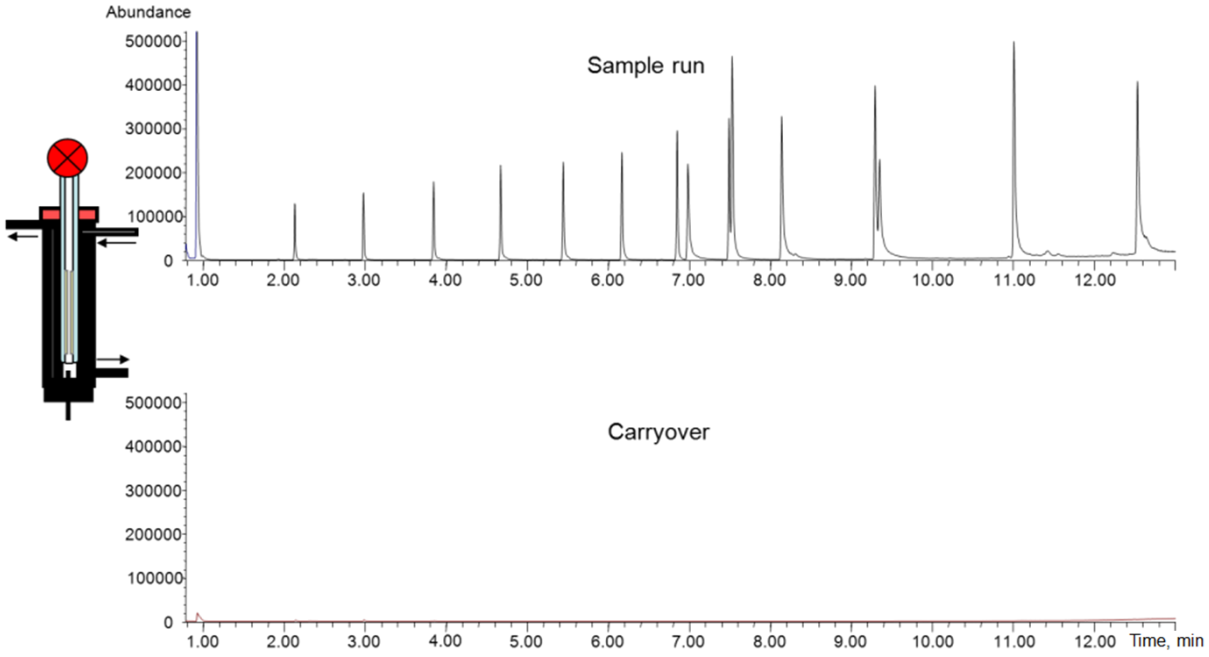

Figure 6.24. TIC chromatograms showing carryover of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted CNE (23G needle). Extraction conditions: PDMS coating, liquid extraction, 2 min (10 cycles), 20ºC, and 1 mL sample. GC conditions: HP-MS 5, 15 m x 0.25 mm i.d. x 0.25 µm film thickness column, temperature programmed from 50ºC to 300ºC at 20ºC/min, 20 psi He inlet pressure, injection mode 1, 1 min injection time, 300ºC injector temperature, 10:1 split ratio.

Injection pressure program. Pressure programming (high initial and low final pressures) during GC injection has normally been used to improve peak shapes from compression of the sample vapor volume and fast transfer of the compressed volume into the GC column. A pressure program for CNE sample introduction also helps to expel sample vapor out of the needle due to the fast pressure drop in the liner while the pressure inside the needle (formed by the initial inlet pressure and evaporation of sample) is still elevated. The preliminary results conducted with a pressure program from 30 psi to 12 psi at a ramp rate of 18 psi/min and 280ºC

injection temperature (see Figure 6.25) indicates significant improvements in carryover and PAH sensitivity.

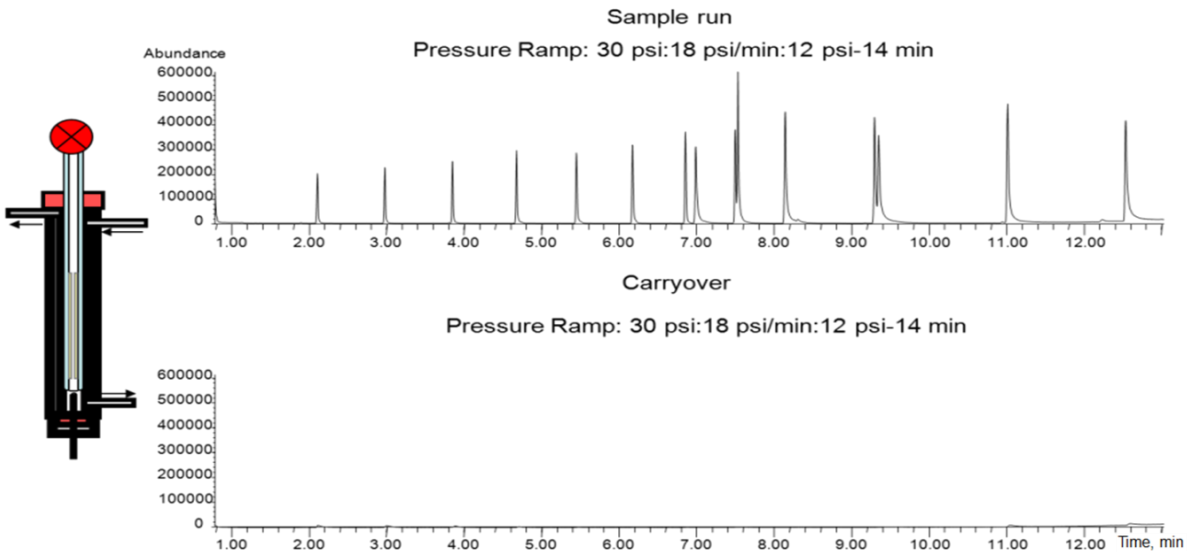

Figure 6.25. TIC chromatograms showing carryover of hydrocarbons, alcohols, and PAHs (0.4-1 ppm in H_2O) extracted by CNE (23G needle). Extraction conditions: PDMS coating, liquid extraction, 2 min (10 cycles), 20ºC, and 1 mL sample. GC conditions: HP-MS 5, 15 m x 0.25 mm i.d. x 0.25 µm film thickness column, temperature programmed from 50ºC to 300ºC at 20ºC/min, He inlet pressure ramp, injection mode 1, 1 min injection time, 280ºC injector temperature, 10:1 split ratio.

A combination of several conditions at the same time can be used to minimize carryover to an acceptable level. Each condition contributes a different effect that depends on the CNE sample introduction mode, the characteristics of the sorbent, and the analyte properties. These factors can be ranked with decreasing order of importance as follows: injection temperature and time, zero-dead volume, pressure program and, finally, split injection.

6.3.7 CNE for *Bacillus* endospore biomarker detection

CNE coatings for Bacillus endospore biomarker detection. Different CNE coatings evaluated include activated carbon (C)/PDMS, C/Poly styrene (PS), C/Poly (methyl metharylate) (PMMA), silicone SE 54, epoxy, and Ucon (75-H-9). They were investigated to determine which was best for *Bacillus* endospore biomarker detection. The experimental results from the investigation, which was conducted using the same method and sample, are shown in Figure 6.26. The peak intensities for 3M2BAME, FAMEs (represented by iso C15:0 and iso C17:0), and DPAME indicate that C/PDMS and C/PMMA were most suitable for the target compounds.

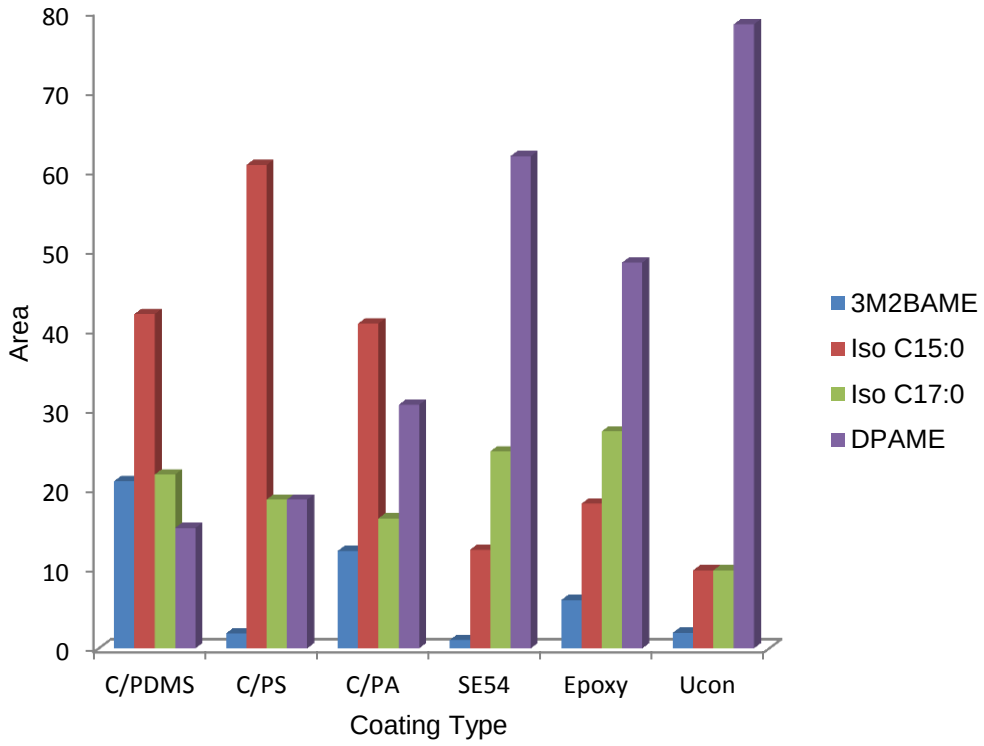

Figure 6.26. Relative peak intensities of the main biomarkers of BA as a function of CNE coating.

Generally, the best coating among the types investigated were C/PDMS, and C/PMMA; however, C/PDMS provided better thermal and chemical stability and it was easier and cheaper to fabricate, C/PMMA and the other polymers were degraded rapidly in the injection conditions used for biomarker detection. The addition of DVB, which was predicted to be the most suitable material, has not yet been done due to chemical toxicity and complicated polymerization.

Activated carbon/PDMS coating for Bacillus endospore biomarker detection.

Activated carbon physically binds analytes by van der Waals or London dispersion forces. It does not bind certain chemicals well, including alcohols, ammonia, strong acids and bases, metals and most inorganic compounds. Since the target *Bacillus* biomarkers are not in these groups, C/PDMS is suitable for the extraction of *Bacillus* samples treated by TCM in H_2SO_4 medium. Extraction and injection of hydrocarbons and PAHs using the C/PDMS coating were compared with results from conventional SPME using 30 µm PDMS fiber, and the results are presented in Figure 6.27. The large volume and surface area of the CNE coating and the fast movement (many fold higher than that in conventional SPME) of the sample fluid back and forth through the needle increased the quantities of absorbed compounds. As a result, the sensitivities were improved and the extraction time was reduced. The high chemical and temperature resistance of the C/PDMS coating is necessary for TCM in which sulfuric acid, phosphate buffer (pH<6) and basic substances such as KOH are used in high concentrations. A C/PDMS coating was investigated for 1 min headspace and liquid extractions of BA endospore suspension at pH=3.5 (KOH was used to adjust the pH) at 60ºC. The extracted ion chromatograms of the three main biomarkers are shown in Figures 6.28 and 6.29. Both extractions were better from headspace than from liquid, while DPAME was detected almost equally from both phases at elevated temperature (50ºC). 3M2BAME is a very volatile compound with high abundance in the

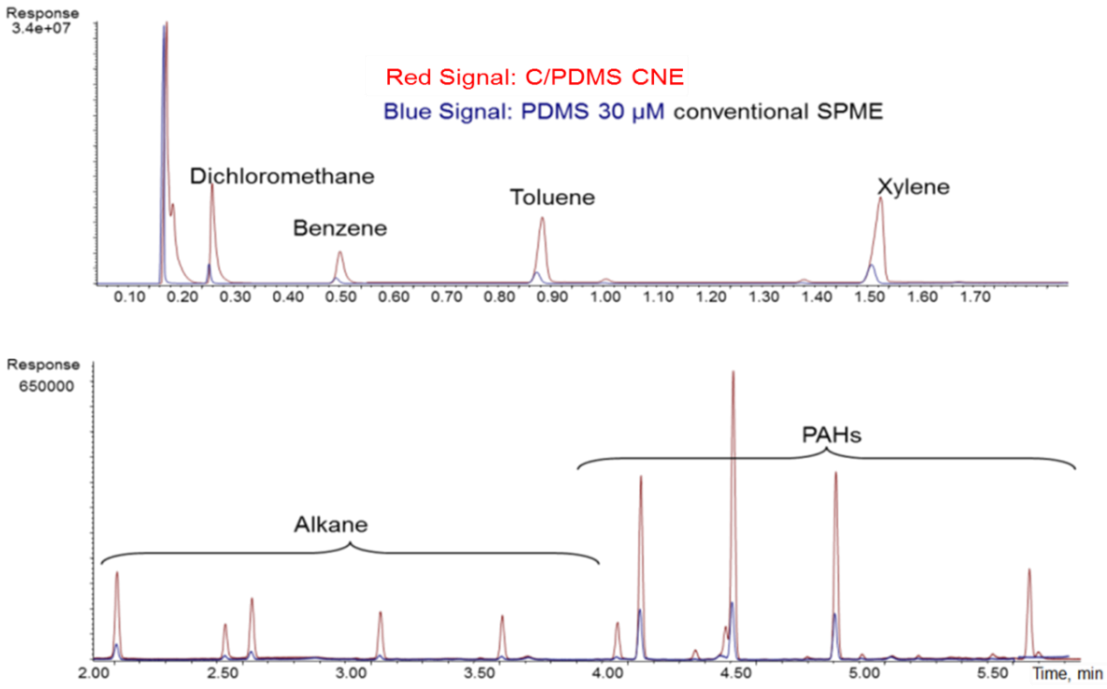

Figure 6.27. FID chromatograms of hydrocarbons and PAHs (5 ppm in H_2O) extracted using a C/PDMS coated-needle (23G needle) and conventional 30 µm PDMS SPME fiber. Extraction conditions: liquid extraction, 30 s (2 cycles), 20ºC, and 1 mL sample. GC conditions: Restek MTX 5, 5 m x 0.1 mm i.d. x 0.4 µm film thickness column, temperature programmed from 60ºC to 300ºC at 20ºC/min, 27 psi He inlet pressure, injection mode 1, 1 min injection time, 300ºC injector temperature, 30:1 split ratio, hand stirring for conventional SPME.

headspace at elevated temperature. FAMEs have sufficient volatilities to exist in the headspace at levels high enough to provide good extraction sensitivities. Higher extraction efficiencies were obtained from the headspace than from the liquid phase. DPAME, on the other hand, partitions into the liquid phase better than in the headspace at ambient temperature due to its polarity. The relatively low peak intensity of DPAME from the gas phase compared to the other two shown in Figure 6.28 indicates that DPAME was present in higher concentration in the liquid phase.

Comparison of C/PDMS coated CNE and conventional DVB/CAR/PDMS SPME for BA biomarker detection. Experiments comparing C/PDMS CNE with conventional

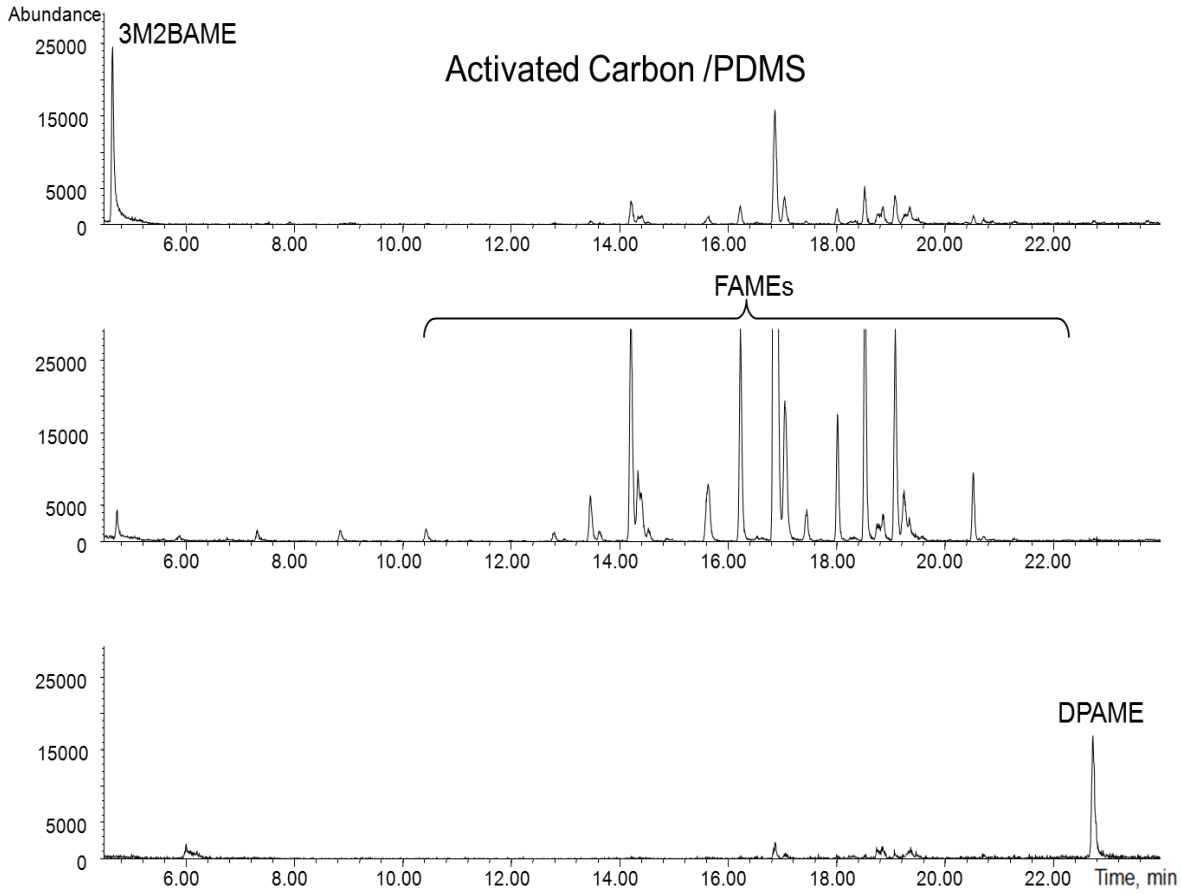

Figure 6.28. Extracted ion chromatograms of 3M2BAME (m/z 83), FAMEs (m/z 74), and DPAME (m/z 137) from 10^7 LD BA spores. Extraction conditions: C/PDMS CNE, headspace extraction, 1 min (12 cycles), 50ºC, and 0.5 mL sample. GC conditions: FFAP, 30 m x 0.25 mm i.d. x 0.25 µm film thickness column, temperature programmed from 60ºC to 180ºC at 8ºC/min, from 180ºC to 250ºC (hold 16 min) at 10ºC/min, 12 psi He inlet pressure, injection mode 1, 2 min injection time, 280ºC injector temperature, 2 min splitless time.

DVB/CAR/PDMS SPME for BA biomarker detection were conducted for headspace and liquid extractions. The TCM sample was extracted at 20ºC for 2 min in the headspace and in the liquid phase using a C/PDMS coated CNE device (approximately 2 cm long and 75 µm thick), and compared to extraction under the same condition using a conventional 2 cm long DVB/CAR/PDMS fiber. Stirring was applied for the latter at 500 rpm; 10 s and 5 s cycle were applied for liquid and headspace CNE, respectively. The chromatograms shown in Figures 6.30

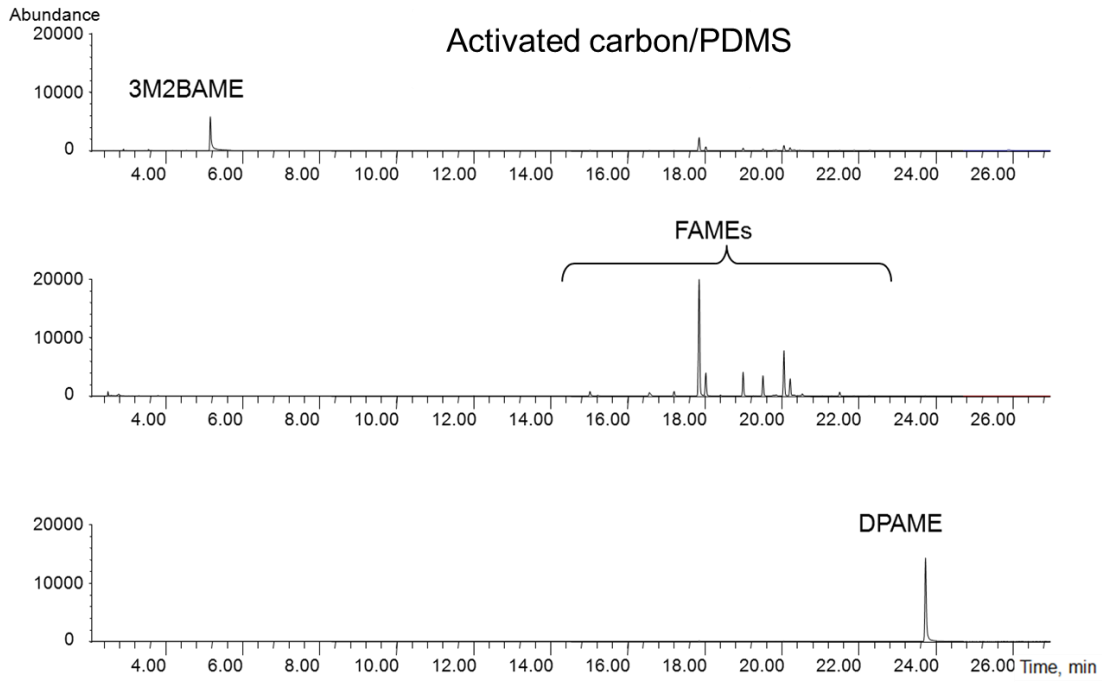

Figure 6.29. Extracted ion chromatogram of 3M2BAME (m/z 83), FAMEs (m/z 74), and DPAME (m/z 137) for 10^7 LD BA spores. Extraction conditions: C/PDMS CNE, liquid extraction, 1 min (6 cycles), 50ºC, and 0.5 mL sample. GC conditions: FFAP, 30 m x 0.25 mm i.d. x 0.25 µm film thickness column, temperature programmed from 50ºC to 180ºC at 8ºC/min, from 180ºC to 250 ºC (hold 16 min) at 10ºC/min, 12 psi He inlet pressure, injection mode 1, 2 min injection time, 250ºC injector temperature, 2 min splitless time.

and 6.31 indicate the high abundance of DPAME and FAMEs obtained using C/PDMS in the CNE device, while higher extraction efficiency of 3M2BAME was obtained using DVB/CAR/PDMS SPME in both headspace and liquid. However, there were no significant differences in peak intensities of the three main biomarkers between the CNE extractions of the two phases. This is different from using conventional SPME; 3M2BAME and DPAME were higher in liquid extraction and lower in headspace extraction, while FAMEs showed the opposite

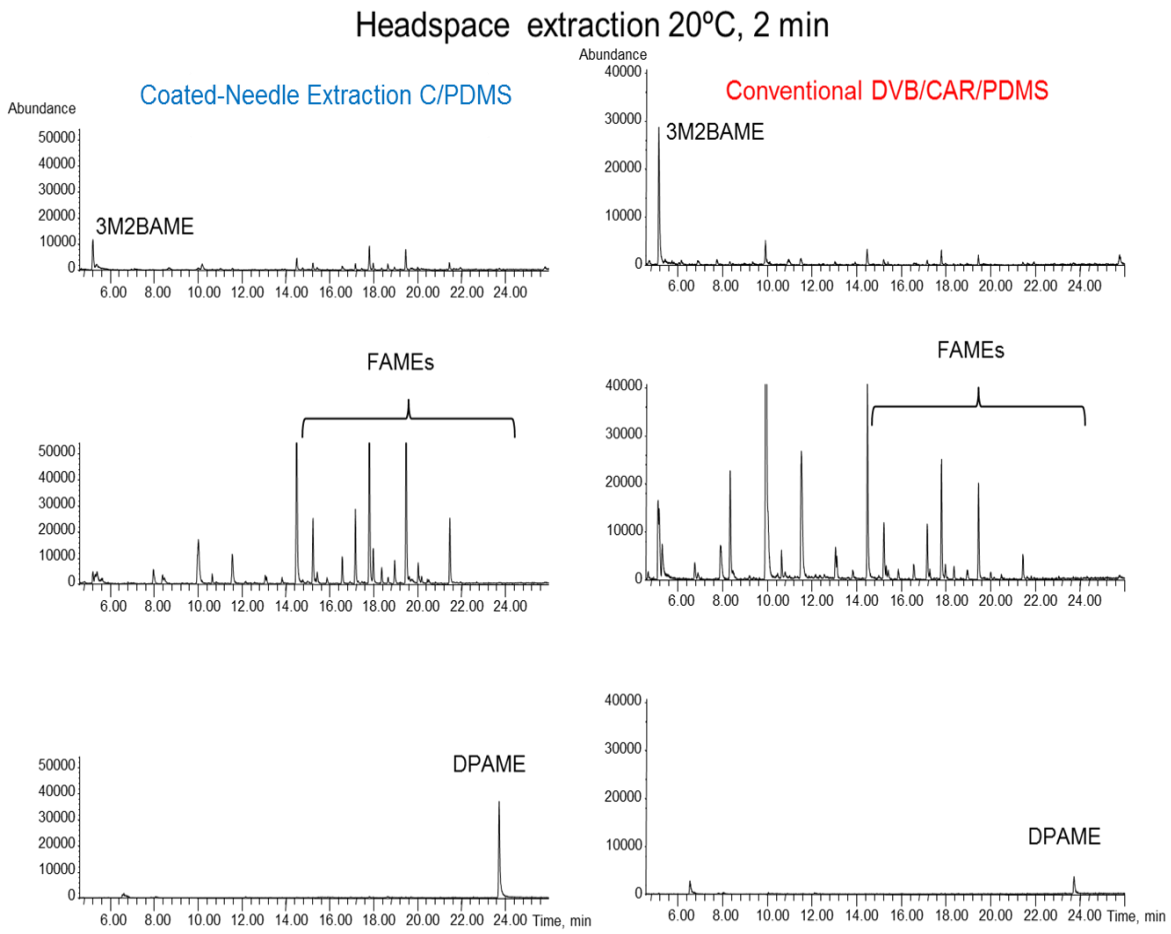

Figure 6.30. Extracted ion chromatogram of 3M2BAME (m/z 83), FAMEs (m/z 74), and DPAME (m/z 137) for 10^7 LD BA spores. Extraction conditions: C/PDMS CNE, headspace extraction, 2 min (12 cycles), 20ºC; DVB/CAR/PDMS, 2 cm long fiber, 500 rpm stir-bar stirring; 2 min, 20ºC, 0.5 mL sample. GC conditions: FFAP, 30 m x 0.25 mm i.d. x 0.25 µm film thickness column, temperature programmed from 50ºC to 180ºC at 8ºC/min, from 180ºC to 250ºC (hold 16 min) at 10ºC/min, 12 psi He inlet pressure, injection mode 1, 2 min injection time, 260ºC (SPME) and 280ºC (CNE) injector temperature, 2 min splitless time.

behavior. These results can be explained by the vacuum created during withdrawal the syringe plunger; medium volatility components can be distributed better in the headspace under reduced pressure. The lower intensity of 3M2BAME in CNE compared to conventional SPME can be explained by its high volatility under in ambient conditions. The sample flow in the CNE provide vapor phase volume for partitioning.

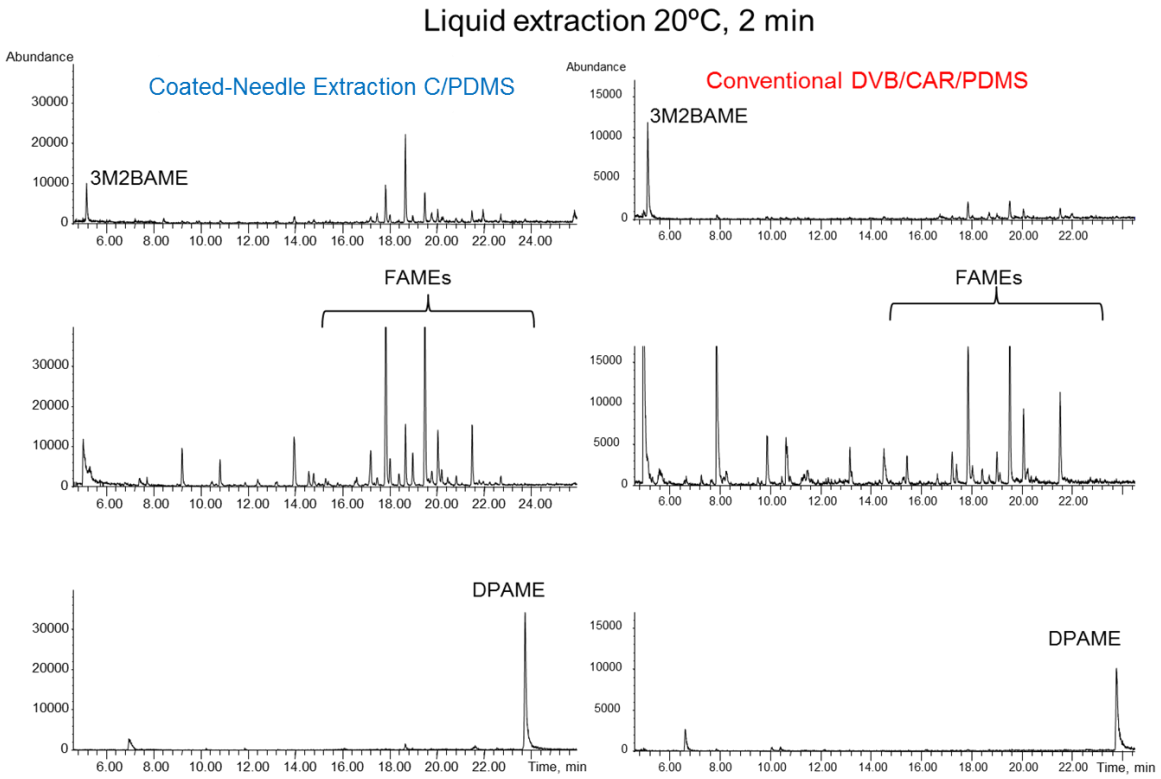

Figure 6.31. Extracted ion chromatogram of 3M2BAME (m/z 83), FAMEs (m/z 74), and DPAME (m/z 137) for 10^7 LD BA spores. Extraction conditions: C/PDMS CNE, liquid extraction, 2 min (12 cycles), 20ºC; DVB/CAR/PDMS, 2 cm long fiber, 500 rpm stir-bar stirring; 2 min, 20ºC, 0.5 mL sample. GC conditions: FFAP, 30 m x 0.25 mm i.d. x 0.25 µm film thickness column, temperature programmed from 50ºC to 180ºC at 8ºC/min, from 180ºC to 250ºC (hold 16 min) at 10ºC/min, 12 psi He inlet pressure, injection mode 1, 2 min injection time, 260ºC (SPME) and 280ºC (CNE) injector temperature, 2 min splitless time.

6.3.8 Advantages of CNE

The experimental results described indicate that CNE has the following advantages: (1) It is suitable for small volume samples that conventional SPME cannot extract without adding more solvent (i.e., diluting the sample); (2) it is very easy and convenient for large volume extraction when using vacuum or pressure to force a large sample through the needle; (3) fast equilibrium can be established without using stirring equipment because pressing and depressing

the syringe plunger helps to increase extraction speed; (4) the stationary phase is protected from damage caused by touching the GC liner wall or other accidental contacts that may degrade or destroy the coating; (5) accumulation of sample residue in the liner is minimized because sample residues remain inside the needle (can be removed by solvent cleaning); and (6) it can be used in field applications.

6.3.9 Disadvantages of CNE

CNE has several disadvantages that are so far not resolved: (1) the coating is hidden inside the needle and unable to be seen; (2) the coating is physically fixed on the needle surface instead of chemically bonded; (3) the needle and syringe barrel must be cleaned after injection to eliminate carryover and sample residues, especially when the samples contain a large amount of nonvolatile compounds (high molecular weight compounds, salts, etc.), (4) headspace extractions give low efficiency for volatile compounds with high sample flow rate extraction, and (5) without purging, carryover is higher than for conventional SPME.

6.4 CONCLUSIONS

CNE provides simple, fast, and sensitive sample preparation and injection for GC. PDMS, activated carbon/PDMS and other coatings provide promising application for field analyses, including environmental and, biological samples.

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7 CONCLUSIONS AND FUTURE WORK

7.1 CONCLUSIONS

In order to achieve the goal of fast, simple, fieldable detection and differentiation of *Bacillus anthracis* (BA) from closely-related species and threat microorganism such as *Francisella tularencis* (FT) and *Yersinia pestis* (YP) using GC-MS, we investigated many biomarkers produced by thermochemolysis procedures. Since conventional sample preparation methods as well as traditional sample introduction (syringe injection) for GC and GC-MS are not amenable to field operations, I was left to look for new, non-traditional technique for both sample preparation and introduction. As a result, thermochemolysis methylation (TCM), coiled wire filament (CWF) solid phase sample introduction, coated-nedle extraction (CNE), and half-half solid phase micro extraction (SPME) were developed. Different biomarkers, including dipicolinic acid (DPA), unique fatty acids (FAs), anthrose TCM by-product 3-methyl-3-hydroxy butyric acid (3M3HBA), 3-hydroxy butyric acid (3HBA), and certain sugars were identified which were effective in detecting *Bacillus* species endospores from other micro-organisms.

7.1.1 Coiled wire filament

The CWF was developed as a simple device for field sampling and concentration of analytes for subsequent introduction into the injection port for GC-MS analysis. The CWF was used effectively to produce methylated biomarkers from TCM directly in the heated injection port. Another major advantage of this sampling device is that nonvolatile sample matrix residues remain on the wire coil, reducing the required injection port and liner cleaning frequency, contamination of the head of the chromatographic column, and sample preparation time, which in most cases was less than 5 min. The peak shapes (asymmetry factors) produced by the CWF

method were also better than those obtained using the splitless and on-column injection techniques. Extraction efficiencies of SPME using polymer (PDMS) coated CWFs were also improved compared to commercial SPME fibers.

7.1.2 CNE device for sample introduction

The polymer coated CWF and conventional SPME sample introduction methods offer many advantages over other methods for sample introduction. By eliminating the solvent, a narrow sample band can be introduced at the column inlet, improving resolution and sensitivity, plus allowing for a higher initial oven temperature than when using traditional solvent injection methods. However, some attention must be given to protect both from damage during use. CNE was developed to overcome this problem.

Generally speakingCNE provides simple, fast, sensitive sample preparation and injection for GC and GC-MS. Although few polymer coatings, such as PDMS and C/PDMS were investigated, this technique has great potential.

7.1.3 Detection of biomarkers for differentiation of *Bacillus* species endospores

The uses of TCM, CWF, SPME and GC-MS allowed for the detection of specific biomarkers for differentiation of biological agents, especially BA. In this work, discriminating biomarkers for closely related *Bacillus* species [i.e., *B. anthracis* (BA), *B. thuringiensis* (BT), *B. cereus* (BC), and *B. atrophaeus* (BG)] were found by studying the large number of mass spectra produced by GC-MS analysis of the TCM products of these bacteria. The biomarkers and their respective m/z values were fatty acid methyl esters (FAMEs) (m/z 74), dipicolinic acid methyl ester (DPAME) (m/z 137), 3-methyl-2-butenoic acid methyl ester (3M2BAME) (m/z 83), 2-butenoic acid methyl ester (2BAME) (m/z 69), and various methylated sugars (different m/z

values). The discrimination of the *Bacillus* endospores as well as other organisms was based on a combination of the different biomarkers.

Differentiation based on DPAME and specific FAMEs. Differentiation of BA, BT, BG, and BC grown at different temperatures and in different media was performed by detecting DPAME and specific saturated and unsaturated C15, C16, and C17 FAMEs produced from the TCM-CWF method. Sampling was accomplished by dipping the CWF in an endospore sample suspension, evaporating the suspension liquid, and introducing the CWF into the GC injection port. While DPAME can be used for general detection of endospores, the specific fatty acid methyl esters provided additional information for differentiating various *Bacillus* species grown at different temperatures and in different media. DPAME could be detected in samples containing as few as 6,000 endospores, and the GC-MS peak area percent reproducibility for FAMEs varied from 3 to 13% (RSD). Better than 97% correct predictability of the identities of the *Bacillus* species was obtained.

Differentiation based diverse biomarker derivatives. Detection of the sugar, anthrose, in addition to DPA, specific FAs, and 3HBA using both TCM-CWF and TCM-SPME improved the identification and differentiation of BA from other *Bacillus* species and micro-organisms in terms of both sensitivity and accuracy (i.e., few false positives). Treatment with H_2SO_4 at controlled temperature was important for the production of biomarkers. Using the TCM-CWF method, DPAME, FAMEs, 2BAME, and sugars were detected faster and with better sensitivities than when using the TCM-SPME method. However, anthrose could not be easily detected until TCM was combined with SPME (DVB/CAR/PDMS fiber). The spider charts shown in Figure 7.1 present a qualitative comparison of the important detection characteristics of the two methods.

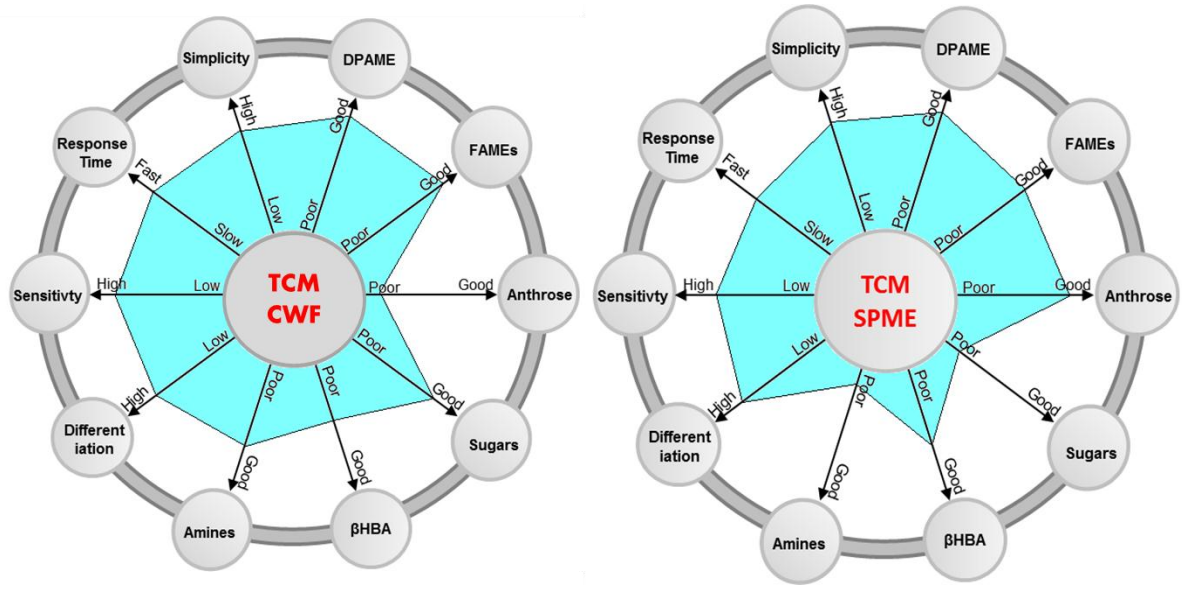

Figure 7.1. Spider charts comparing the detection characteristics of the TCM-CWF and TCM-SPME methods.

The detection of DPAME and FAMEs is not significantly different using the two techniques; however, they are better analyzed using TCM-CWF. The amines, 3HBA (or β-HBA), and sugars were detected better using TCM-CWF, and anthrose was detected at much lower detection limits in TCM-SPME.

7.2 RECOMMENDATIONS FOR FUTURE RESEARCH

Although a number of new biomarkers have been identified in this work, there still remain issues that must be resolved to further reduce the analysis time and improve the detection limits. Furthermore, not all biomarkers used for differentiation can be detected using a single method, and the feasibility of using hand-portable GC-MS with these methods has not yet been confirmed. Therefore, I recommend the following future work.

7.2.1 Optimization of sugar derivative detection using TCM-SPME

It would be very useful if both sugars and the anthrose by-product could be detected with greater sensitivity using the TCM-SPME method. Formation of sugar derivatives from bacteria using TCM-CWF require hydrolysis and methylation in low H_2SO_4 concentration followed by further methylation with TMAH inside the GC inlet. In comparison, H_2SO_4 was used in higher concentration (5-10% vs. 0.1-0.5% v/v) for TCM-SPME, while the temperature and time for hydrolysis in the two methods were almost the same (from 120-140ºC for several min) when the procedure was optimized for anthrose detetction. There is the possibility than an average concentration of H_2SO_4 (2-5% v/v) and suitable time exist that would produce simultaneously increased levels of sugars and 3M3HBA. After hydrolysis, TMAH could be added to the sample to methylate (off-line) the biomarkers. SPME could then be applied after the sample is neutralized by a suitable reagent. Detection of sugars and anthrose at the same time would significantly enhance identification and differentiation of BA from other species. Additional sugar biomarkers could simplify the differentation of bacteria. Figure 7.2 shows the different sugar derivatives detected using the TCM-CWF method among the four *Bacillus* species (BA, BC, BG, and BT). Methylated sugars can be extracted well by SPME using a low polarity coating, such as PDMS, DVP/PDMS, and DVB/CAR/PDMS fibers if a small amount of low polar organic solvent is added to the sample solution. Ciucanu et al.[1] indicated that addition of an immiscible organic solvent such as benzene or chloroform would generate a three-phase extraction system that would increase the extraction efficiency of per-*O*-methylated carbohydrates by more than 50 times, which otherwise would have a low solubility in the PDMS sorbent. This is because the organic solvents improve the diffusion coefficient of the

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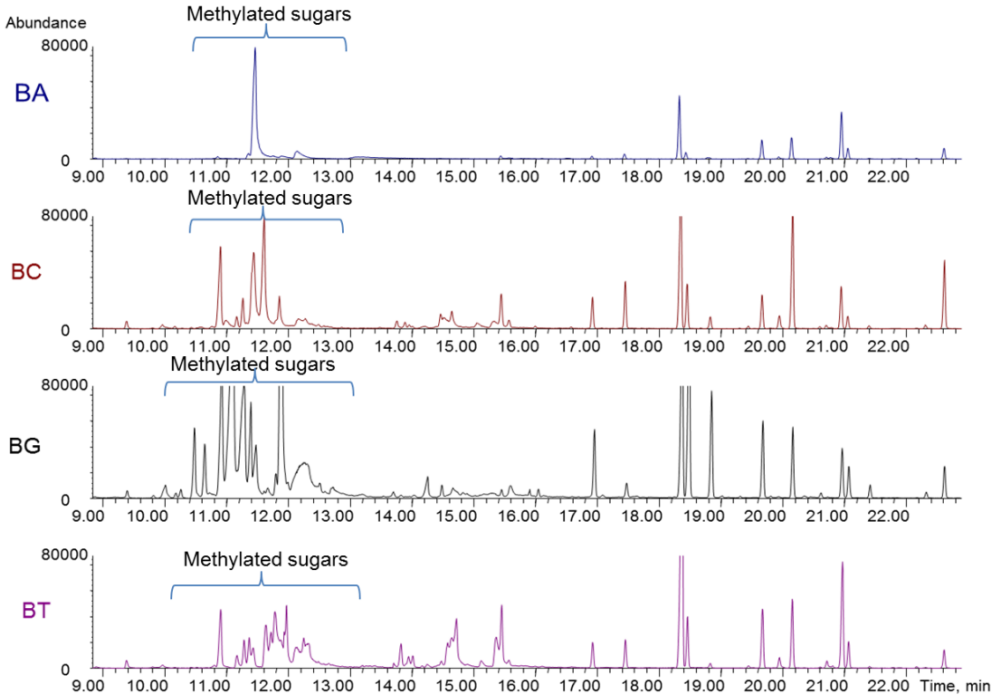

Figure 7.2. Extracted ion chromatograms (m/z 74) of methylated sugars produced using the TCM-CWF method, indicating the significant difference in sugars among BA, BC, BG, and BT.

per-*O*-methylated carbohydrate in the PDMS sorbent. The organic solvent retained by PDMS may also increase the volume of the extraction phase.[1]

7.2.2 Detection of methylated polysaccharides

Polysaccharides in bacteria may contribute additional useful information for differentiation. Fortunately, they can be detected by TCM followed by GC-MS. Shadkami[2] indicated that when arabinogalactan was heated in the presence of TMAH (at 350°C), a methylated oligosaccharide (a high molecular weight compound containing two hexopyranosyl rings attached to a hexose, whose structure is shown in Figure 7.3) was found by mass spectrometric detection. This was the first time an oligosaccharide product was observed after thermochemolysis.[2] Interestingly, this study showed no low molecular weight carbohydrates such

as produced in a previous study.[3] Because of a lower thermochemolysis temperature (350°C *vs.* 700°C), low molecular weight compounds were not observed.

In our research, a CWF was used for on-line TCM of BG endospores at low temperature (290-300°C) after hydrolysis with concentrated H_2SO_4 (~20% v/v at 120ºC, 4 min) and addition of TMAH to create a final pH of ~8.5-9. These experimental conditions structures of compounds found in the BG endospore sample.

These results indicate that intact methylated oligosaccharides that are not observed using other thermochemolysis approaches, likely due to a high molecular weight discrimination using traditional pyrolyzers, can be detected using a simple, fast TCM-CWF method. The detection of these biomarkers may provide valuable information for detection and differentiation of microorganisms.

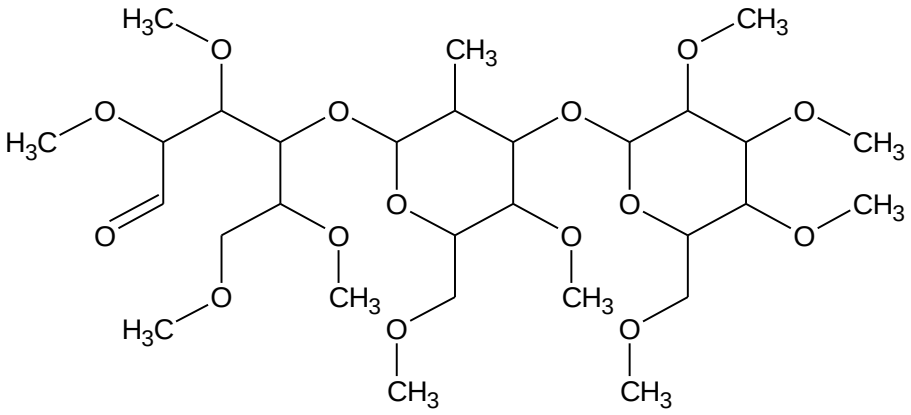

Figure 7.3. Structure of methylated oligosaccharides found by MA after TCM using TMAH at 350ºC.[2]

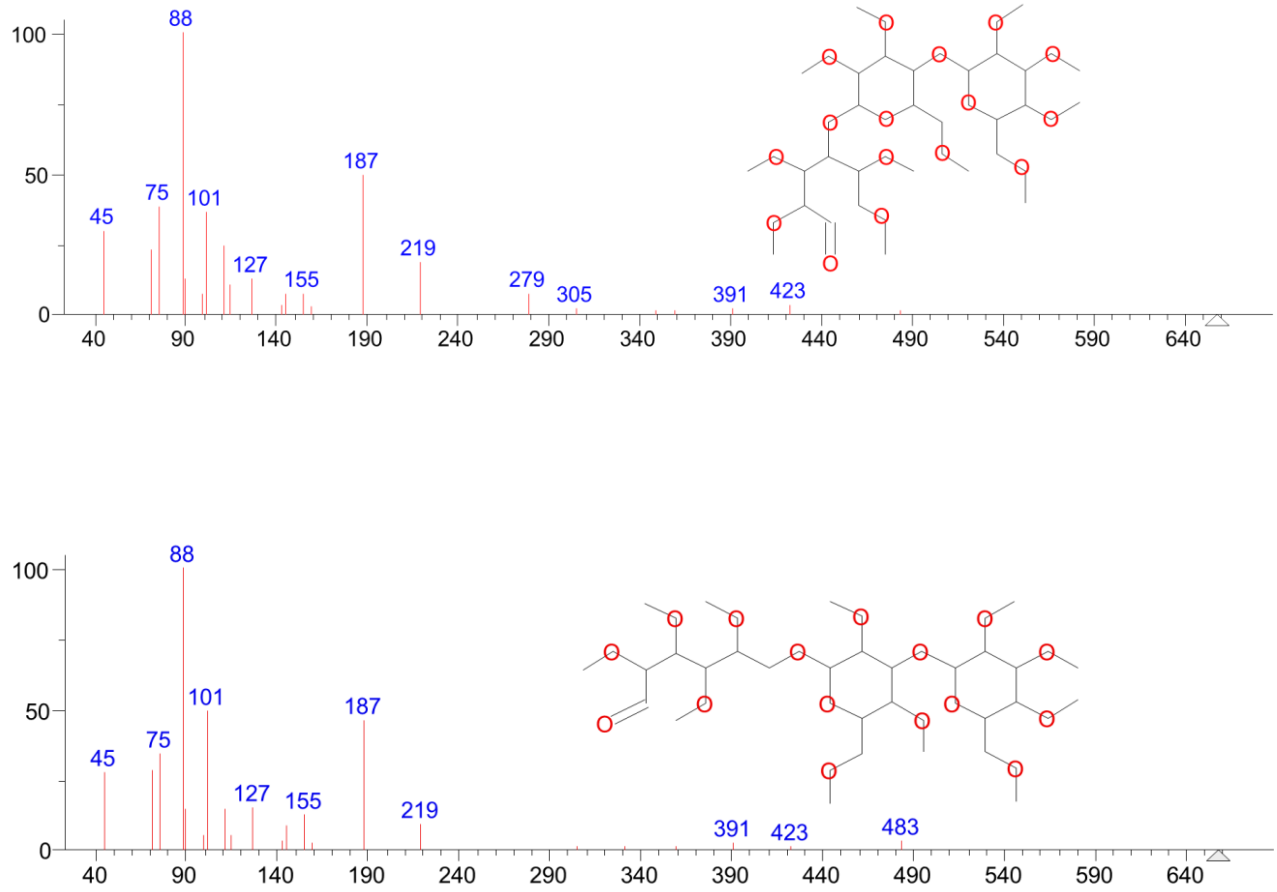

Figure 7.4. Mass spectra and structures of methylated oligosaccharides found in BG endospores using the TCM-CWF method.

7.2.3 Use of a microwave to reduce TCM time

As presented in Chapter 4, the use of a microwave for both TCM-CWF and TCM-SPME methods to reduce the heating time was studied. However, this was not considered to be practical for field applications. In reality, the method of heat treatment can significantly reduce the time for TCM. It normally requires only seconds (2,450-MHz microwave oven at high intensity) for approximately 50-100 µL sample suspension compared to minutes with a heating block. Woo et

al.[4] indicated that microwave radiation applied to *Escherichia coli* and *Bacillus subtilis* cell suspensions resulted in a dramatic reduction in viable counts as well as enhancement in the amounts of DNA and protein released from the cells according to the final temperature of the cell suspensions. Liebeke[5] reported that microwave-assisted methoxylation and trimethylsilylation of metabolome samples, compared to the commonly used method, significantly reduced the sample preparation time prior to GC-MS analysis without loss of experimental quality. More recently, as presented in Chapter 4, many authors have used household microwave ovens as a useful treatment tool for chemical analysis of proteins, lipids, fatty acids, phospholipids, and sugar structures in different sample types including bacteria.[6-14]

In my research, the presence of H_2SO_4 or TMAH speeds up the disruption of biological materials caused by the microwave process. I believe that rapid microwave-assisted derivatization can produce the same results as heating block treatment, but reduce significantly the sample preparation time for detection of *Bacillus* endospore biomarkers. Optimization of the microwave procedure is needed to achieve the best information for detection and differentiation of *Bacillus* species and other bacterial agents.

7.2.4 Development of polymer-coated CNE and CWF for biomarker extraction and injection

The sensitivity of SPME can be improved by using polymer coated CWF or CNE compared to conventional SPME due to larger surface area and volume of the stationary phase that can be deposited on the coiled wire or inside the needle. For biomarker detection, non-polar or medium-polar stationary phases are desirable, such as cross-linked PDMS or combined of sorbents (i.e., DVB/PDMS, CAR/PDMS, DVB/CAR/PDMS, C/PDMS, and DVB/C/PDMS).

For polymer-coated CWF sampling, the coating can be prepared as a layer on the surface of the wire used for making the coil, which facilitate fast extraction (see Figure 7.5), or it can be located inside the coil as a solid rod, which provides large sample capacity (see Figure 7.6). PDMS and C/PDMS, as well as other stationary phases, have been used in CNE (see details in Chapter 6) but not yet for CWF sampling for biomarkers.

An advantage of the CWF and CNE devices is that they can be used for methylated sugar extraction, with the addition of a tiny amount of organic solvent as mentioned before. Non-bonded PDMS is normally not stable in the presence of organic solvents and the reproducibility is poor. The extraction capacity can also be reduced significantly (e.g., ~50% after three consecutive extractions in the presence of chloroform) while the extraction with a cross-linked PDMS sorbent can be repeated more than 100 times without loss of extraction capacity.[1] The

Figure 7.5. CWF coated with Apiezon N using low concentration solution (2.5% in CH_2Cl_2).

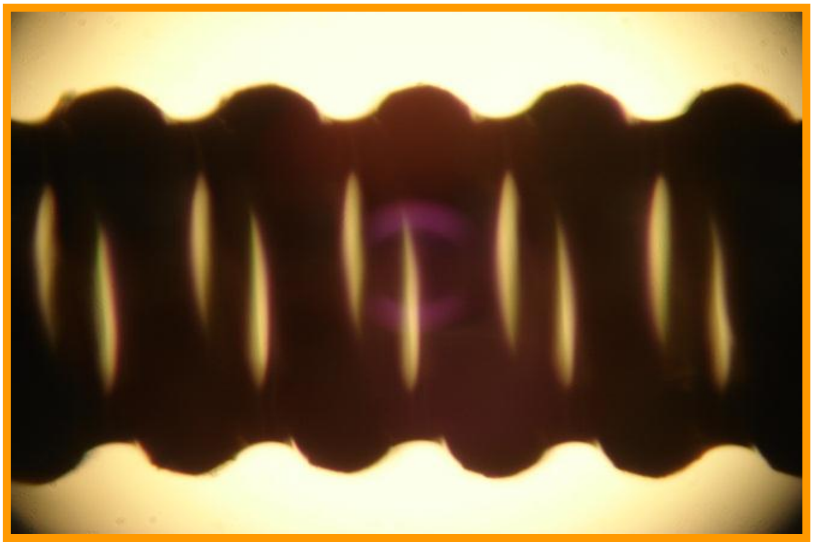

Figure 7.6. CWF coated with Apiezon N using high concentration (30% in CH_2Cl_2).

equilibrium times for CNE are normally shorter than when using in conventional SPME fiber (minutes vs. hours).[15]

7.2.5 Detection of biomarkers using a portable sample preparation system and handheld GC-MS

The detection of *Bacillus* endospores and other microorganism biomarkers using a hand-portable GC-MS is work planned for the near future. For hand-portable GC-MS, TCM-SPME is the most suitable method for detecting the main biomarkers, including DPA, 3M3HBA, 3HBA, and FAs. Furthermore, a short (e.g., 5 m), small diameter (e.g., 100 µm) column would provide fast analysis with minimal usage of carrier gas and electrical power. A preliminary analysis of FAMEs and other biomarkers in various *Bacillus* endospores using a 5 m x 0.1 mm i.d. x 0.4 µm film thickness metal column that allows for high heating rate is shown in Figure 7.7. While loss in resolution is observed compared to using conventional columns (i.e., 10 m x 0.1 mm x 0.4 µm

film thickness or 30 m x 0.25 mm x 0.25 µm film thickness) the analyses were completed in a little over 5 min, and the critical discriminating biomarkers were detected, such as iso and anteiso FAMEs of C13:0, C15:0, and C17:0, n16:1(Δ9) (green strip) , n C17:1(Δ7) (red strip), iso C16:1 (blue strip), and DPAME and other biomarkers (not shown). Further optimization to improve the analysis using short columns is obviously necessary.

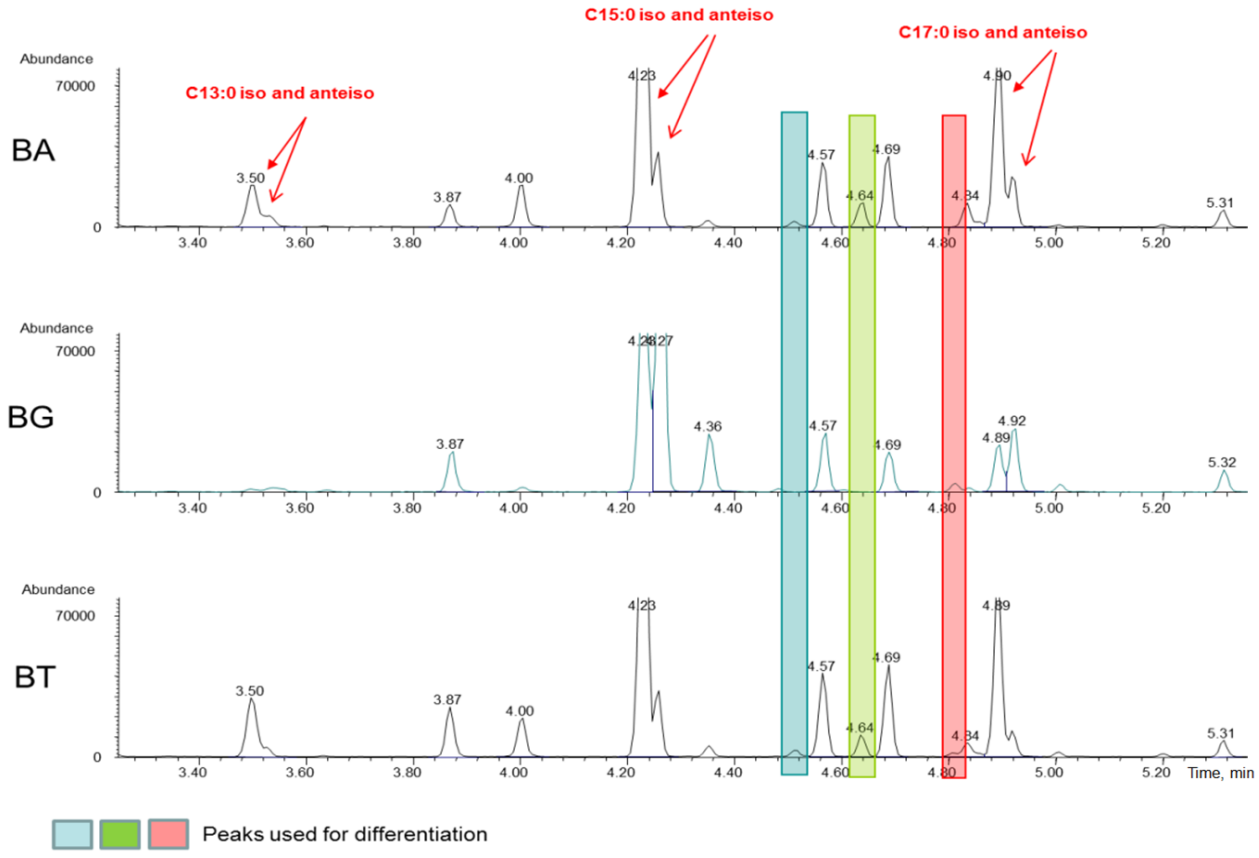

Figure 7.7. Typical FAMEs from BA, BG, and BT endospores separated using a short column. Conditions: 40ºC initial temp., 32°C/min rate, 280ºC final temp, MTX 5, 5 m x 0.1 mm i.d. x 0.4 µm film thickness, 7 psi He.

7.2.6 Detection of *Bacillus anthracis* endospores in complex matrices

Field analysis is often difficult due to the present of interfering compounds in sample matrices. The anthrose byproduct, DPA, and specific fatty acids (iso and anteiso C15:0 and C17:0) are key biomarkers that can be initially used to detect the possible presence of BA. Sample preparation and introduction depends on the site where the experiment is performed and the matrix properties. For mainly inorganic matrices, such as soil, dust, and inorganic salts, sample preparation may be carried out by following the methods developed in this dissertation, for which TCM-SPME should be the first choice for the target biomarkers due to its higher selectivity compared to TCM-CWF.

For other matrices, such as food and water supply systems, which could be deliberately contaminated since they are two of the most viable targets for bioterrorism,[16] sample preparation can be more difficult. For example, milk, which is consumed every day, especially by children, poses a high probability/high severity risk potential if *Bacillus anthracis* spores were purposely added to it at the farm, truck delivery, or processor level.[17] Although most milk products in the United States (raw materials, semi-products, and final products) are pasteurized to eliminate bacterial pathogens, a number of potential bioterrorism agents, including *Bacillus anthracis* spores can survive this heat treatment.[18] Most cases of intestinal anthrax from other food stuffs have resulted from eating insufficiently cooked, for example, meat from *B. anthracis*-infected animals in the Middle East, Africa, and Central and Southeastern Asia.[19]

The isolation of *Bacillus* endospores or micro-organisms from complex matrices must often be carried out with extraction and centrifugation. Extraction methods are based on the hydrophobic properties of bacterial cell surfaces that have been demonstrated to be important for understanding interactions between bacteria and their environment. Hydrocarbon solvents can be

used for the extraction followed by filtration before further treatment to isolate the micro-organisms. Leishman[18] reported the extraction of milk and orange juice in which 20% of *B. anthracis* was recovered by using *n*-hexadecane as solvent.

Centrifugation is recognized to be more effective than extraction to remove endospores from aqueous samples. Leishman[18] used centrifugation at 1500 x g for 10 min to recover *B. anthracis* from skim and whole milk. Relatively high recovery rates (80 to 100%) of *B. anthracis* spores from commercial whole milk were obtained by centrifugation after addition of silicone oil. The recoveries increased with centrifugation temperature and speed.[18]

The solvent plays an important role in any spore isolation method. It is desirable to have an appropriate solvent that only dissolves the matrix, but not endospores. For field application, where use of a centrifuge is impossible, it is possible to use a syringe and membrane filter to wash away the matrix and collect the endospores onto the membrane after suspending the biological agent and its matrix in a suitable solvent.

Generally, detection of endospores or cells in complex matrices require flexible sample treatment methods that are compatible with each specific sample matrix. After isolation, TCM-SPME or TCM-CWF can be applied in the same way as for clean micro-organism samples.

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