

SECTION VI

HOMELAND SECURITY

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METHODS OF MASS SPECTROMETRY IN HOMELAND SECURITY APPLICATIONS

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19.1 INTRODUCTION

The volatile international relationships of the twentieth century culminated in wars with never-before-seen casualties and financial losses. More importantly, the global unease that swept through most nations in the past decades resulted in emergence of terrorist groups and even government-funded militias in every corner of the world. As a response, member nations of the North Atlantic Treaty Organization (NATO) launched the Chemical, Biological, Radiological, and Nuclear (CBRN) Task Force with the purpose of joint prevention of creation and deployment of weapons of mass destruction.

Mass spectrometry, with its inherent sensitivity, selectivity, high throughput, and enhanced speed of analysis has proven to be an attractive study platform for chemical and biological warfare agents. One can find studies involving use of mass spectrometers for homeland security purposes as early as the 1970s, when electron and chemical ionization (EI and CI) were used in sector-type instruments. The development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) techniques has provided the foundation for an explosive increase of homeland security studies in a variety of more modern instruments. We can find mass spectrometers used for homeland security purposes in research laboratories studying different stages and aspects of a potential biological or chemical attack. Mass spectrometers are widely used in the determination of bacterial proteomic profiles or protein fin-

gerprints for database construction; in the discovery of biomolecules secreted by potentially harmful bacteria; in determination of the effects of a potential infection or contamination in living organisms; and even in the process of development of antibodies and vaccines for certain high-risk pathogens. Extensive studies are focusing on the portability and miniaturization of mass spectrometers. These pilot instruments are in focus of the military with the purpose of fast detection of chemical and biological threats. Such instruments are crucial in a combat scenario as well as for the potential necessity of timely population evacuation from an affected area.

In this chapter, the reader will find the most commonly applied approaches for application of mass spectrometry in homeland security-related research; relevant protocols will be detailed when applicable.

19.1.1 Biological Agents

Analysis of bacteria, viruses, fungi, and biological toxins constitutes a large majority of current research efforts toward establishing the role of mass spectrometry in homeland security applications. Deliberate dissemination of a highly virulent organism in a given region may have consequences of unimaginable proportions. Regardless whether the attack originates from a single person, a terrorist organization, or from a seemingly legitimate military group, the ultimate target is always the human population. Biological agents that cause debilitating or deadly diseases to humans are just one of the many avenues by which the population can be

affected. In addition, infection may be targeted toward farm animals, fungi may be introduced to destroy crops, or toxins can render the water supply unusable. Table 19.1 contains a list of microorganisms and toxins that are designated by the National Select Agent Registry as potential bioterrorism agents [1].

For method development, attenuated virus strains devoid of virulence plasmids, genetically modified bacteria, or simulants are used instead of the actual select agent. This reduces the chance for accidental infections, precludes the need for special handling conditions, and minimizes the possibility of handling hazardous strains by a large number of researchers.

19.1.2 Chemical Warfare Agents

Timely detection of compounds deployed in a combat situation is crucial in evacuation efforts by minimizing individual exposure levels, reducing the number of individuals exposed, and identifying the correct decontamination and treatment methods. In the event of a terrorist attack, a thorough analysis can even lead to the discovery of the geographical origin of the toxins. Table 19.2 summarizes the most commonly tested chemical warfare agent (CWA) classes, their corresponding health effects, and their toxicity levels [2].

The following toxicity levels are represented in Table 19.2:

- IC_{50} : median incapacitating dosage of a chemical agent vapor or aerosol
- ID_{50} : median incapacitating dosage of a liquid chemical agent
- LC_{50} : median lethal dosage of a chemical agent vapor or aerosol
- LD_{50} : median lethal dosage of a liquid chemical agent

Depending on the nature and amount of sample contaminated with CWAs, different handling protocols are required prior to analysis. Most commonly the following types of samples are collected after a CWA deployment: munition (neat liquid, artillery shell casing, etc.), environmental (soil, water, vegetation, or air), man-made materials (swabs, clothing, polymers), and biological matrices (blood, urine, etc.) [3]. Neat liquid samples generally require a simple dilution with a solvent appropriate for the compound in question. Aqueous samples and biological fluids require an extraction and preconcentration and, depending on the type of agent present, derivatization is performed. Analytes present in soil samples or other solids are usually extracted with dichloromethane, hexane, or water using an ultrasonication

of ~10 min in closed vials [3]. More novel extraction methods currently under investigation are solid-phase microextraction (SPME) [4], single-drop microextraction [5], as well as supercritical fluid extraction [6].

19.2 PROTEOMICS-BASED DETECTION METHODS

19.2.1 Whole-Cell Mass Spectrometry

Whole-organism mass spectrometry is an emerging approach toward the identification and differentiation of bacteria based on the biomarker signature across a wide mass-to-charge ratio (m/z) range. The protein biomarkers that are measured in mass spectrometry of prokaryotic microorganisms are highly expressed proteins that are responsible mostly for housekeeping functions and include ribosomal, chaperone, and transcription/translation factor proteins [7–9]. Because the goal is to identify and classify the type of organism present in the sample based on its chemical “signature,” these studies are commonly referred to as chemotaxonomy approaches.

One of the main tools to determine the chemotaxonomy of an organism is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). This instrument configuration is ideal for the determination of the chemical signature for several reasons. First, because MALDI is a “soft” method, all ions in the spectrum are assumed to be singly charged precursor ions with little or no in-source fragmentation. Second, time-of-flight (TOF) analyzers are fast and offer a good resolution over a wide mass range; generally m/z is measured between 4 and 20 kDa if a reflectron is used. Mass spectrometry for chemotaxonomy is highly favorable in that it is broadband (able to measure multiple analytes simultaneously), does not require a priori knowledge of the organism, and is both fast and sensitive by not requiring a prefractionation step [10]. A typical experiment consists of outgrowth of bacteria, colony selection, and placement on a target. The addition of a matrix, a chemical that will absorb the laser energy and allow for ionization of the analyte, is necessary. Details of MALDI ionization and the types of matrices most commonly employed are described in earlier chapters. The sample spot is analyzed with MALDI-TOF-MS as illustrated in Figure 19.1 [8].

Analysis of whole cells was first proposed in 1975 to study the biomarker profile of various bacterial species using pyrolysis-mass spectrometry (Py-MS) for low molecular weight, nonvolatile species [11]. However, it was not until 1996 that the first MALDI-TOF-MS experiment was successful for identifying bacteria

TABLE 19.1 Select Biological Agents and Toxins on the National Select Agent Registry [1]

Health and Human Services (HHS) Select Agents and Toxins		U.S. Department of Agriculture (USDA) Select Agents and Toxins	
Type	Name	Type	Name
Bacteria	<i>Coxiella burnetii</i>	Bacteria	<i>Ehrlichia ruminantium</i> (heartwater)
	<i>Francisella tularensis</i>		<i>Mycoplasma capricolum</i> subspecies <i>capripneumoniae</i>
	<i>Rickettsia prowazekii</i>		<i>Mycoplasma mycoides</i> subspecies <i>mycoides</i> small colony
	<i>Rickettsia rickettsii</i>	Prion	Bovine spongiform encephalopathy agent
	<i>Yersinia pestis</i>	Virus	African horse sickness virus
Fungus	<i>Coccidioides posadasii</i> / <i>Coccidioides immitis</i>		African swine fever virus
Toxin	Botulinum neurotoxins		Akabane virus
	Botulinum neurotoxin-producing species of <i>Clostridium</i>		Avian influenza virus (highly pathogenic)
	<i>Clostridium perfringens</i> epsilon toxin		Bluetongue virus (exotic)
	Conotoxins		Camel pox virus
	Diacetoxyscirpenol		Classical swine fever virus
	Ricin		Foot-and-mouth disease virus
	Saxitoxin		Goat pox virus/sheep pox virus
	Shiga-like ribosome inactivating proteins		Japanese encephalitis virus
	Shigatoxin		Lumpy skin disease virus
	Staphylococcal enterotoxins		Malignant catarrhal fever virus
	T-2 toxin		Menangle virus
	Tetrodotoxin		<i>Peste des petits ruminants</i> virus
	Abrin		Rinderpest virus
	Cercopithecine herpesvirus 1 (herpes B virus)		Swine vesicular disease virus
	Crimean-Congo hemorrhagic fever virus		Vesicular stomatitis virus (exotic): VSV-IN2, VSV-IN3
Virus	EEE virus		Virulent Newcastle disease virus
	Ebola virus	USDA Plant Protection and Quarantine Select Agents and Toxins	
	Lassa fever virus	Bacteria	<i>Ralstonia solanacearum</i> race 3, biovar 2
	Marburg virus		<i>Rathayibacter toxicus</i>
	Monkeypox virus		<i>Xanthomonas oryzae</i>
	Reconstructed 1918 influenza virus		<i>Xylella fastidiosa</i> (citrus variegated chlorosis strain)
	South American hemorrhagic fever viruses	Fungus	<i>Peronosclerospora philippinensis</i> (<i>Peronosclerospora sacchari</i>)
	Tick-borne encephalitis complex (flavi) viruses		<i>Phoma glycinicola</i> (formerly <i>Pyrenochaeta glycines</i>)
	Variola major virus (smallpox virus)		<i>Sclerophthora rayssiae</i> var <i>zeae</i>
	Variola minor virus (Alastrim)		<i>Synchytrium endobioticum</i>
Overlap Select Agents and Toxins		Name	
Type			
Bacteria		<i>Bacillus anthracis</i>	
		<i>Brucella abortus</i>	
		<i>Brucella melitensis</i>	
		<i>Brucella suis</i>	
		<i>Burkholderia mallei</i> (formerly <i>Pseudomonas mallei</i>)	
		<i>Burkholderia pseudomallei</i> (formerly <i>Pseudomonas pseudomallei</i>)	
		Hendra virus	
		Nipah virus	
		Rift Valley fever virus	
		VEE virus	
Virus			

TABLE 19.2 CWAs, Health Effects, and Relevant Toxicity Levels [2]

Classification	Health Effect	Example	Toxicity
Blister/vesicant agent	Lesions on skin, damage the eyes, mucous membranes, respiratory tract, internal organs. Usually persistent or highly persistent agents. Most agents approved for U.S. military operations.	Lewisite (L-1) Arsenicals: Ethyldichloroarsine (ED); methylchloroarsine (MD); phenyldichloroarsine (PD) Nitrogen mustards (HN-1, HN-2, HN-3)	LD ₅₀ (skin) 8 mg/kg ED: LCt ₅₀ (respiratory) 3,000–5,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 5–10 mg-min/m ³ HN-3: LD ₅₀ (skin) 0.7 gm/person; LCt ₅₀ (respiratory) 1,500 mg-min/m ³ ; LCt ₅₀ (percutaneous) 10,000 mg-min/m ³ ; ICt ₅₀ (percutaneous) 2,500 mg-min/m ³ LCt ₅₀ (percutaneous) 3,200 mg-min/m ³ H: LD ₅₀ (skin) 100 mg/kg; LCt ₅₀ (respiratory) 1,000–1,500 mg-min/m ³ ; LCt ₅₀ (percutaneous) 10,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 1,500 mg-min/m ³ ; ICt ₅₀ (percutaneous) 2,000 mg-min/m ³ LCt ₅₀ (respiratory) 11,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 7,000 mg-min/m ³ LD ₅₀ (skin) 100 mg/kg (liquid); LCt ₅₀ 2,000 mg-min/m ³ ; LCt ₅₀ 20,600 mg-min/m ³ LCt ₅₀ (respiratory) 5,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 2,500 mg-min/m ³ LCt ₅₀ (respiratory) 200,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 101 mg-min/m ³
Blood agent	Blocking of cytochrome oxidase enzyme, leading to seizures, respiratory failure, cardiac arrest. Highly volatile, nonpersistent. Used in U.S. military operations.	Phosgene oxime (CX) Sulfur mustards (H, HD, HT) Cyanogen chloride (CK) Hydrogen cyanide (AC) Arsine (SA)	
Incapacitating agent	Temporarily incapacitates by interfering with the proper functioning of nervous system, by blocking or overstimulating the central nervous system. Highly persistent agents, approved for U.S. military operations.	3-Quinuclidinyl benzilate (BZ)	

Nerve agent	Viscous liquids that reversibly or irreversibly disable proper functioning of enzymes and nerve impulse transmission by inhibiting acetylcholinesterase. Exposure symptoms include difficulty in breathing, nausea, vomiting, cramps, loss of bladder/bowel control, convulsion, coma; prolonged exposure may lead to death. G agents are volatile, nonpersistent, while V agents are highly persistent. Approved for U.S. military operations.	Dimethylphosphoramidocyanidic acid, ethyl ester (Tabun or GA) Isopropyl methylphosphonofluoridate (Sarin or GB) Pinacolyl methylphosphonofluoridate (Soman or GD) Cyclohexyl methylphosphonofluoridate (GF) Methylphosphonothioic acid S-(2-bis(1-methylethyl)amino)ethyl) O-ethyl ester (VX) Phosphonofluoridic acid, ethyl-, isopropyl ester (GE) Phosphonothioic acid, ethyl-, S-(2-(diethylamino)ethyl) O-ethyl ester (VE) Amiton (VG) Edemo: Phosphonothioic acid, methyl-, S-(2-(diethylamino)ethyl) O-ethyl ester (VM)	LD ₅₀ (skin) 1–1.5 mg/person; LCt ₅₀ (respiratory) 135–400 mg-min/m ³ ; ICt ₅₀ (respiratory) 300 mg-min/m ³ LD ₅₀ (skin) 24 mg/kg; LCt ₅₀ (respiratory) 70–100 mg-min/m ³ ; LCt ₅₀ (percutaneous); ICt ₅₀ (respiratory) 35–75 mg-min/m ³ LCt ₅₀ (respiratory) 70–400 mg-min/m ³ ; LCt ₅₀ (percutaneous) 10,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 35–75 mg-min/m ³ LD ₅₀ (skin) 16–400 µg/kg (mice); LCt ₅₀ (respiratory) 35 mg-min/m ³ N/A; isomer developed by Russians (VR) LD ₅₀ (skin) 11.3 µg/kg Similar to GB N/A N/A N/A
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(Continued)

TABLE 19.2 (Continued)

Classification	Health Effect	Example	Toxicity
Pulmonary agent	Irritation of mucous membrane of respiratory tract that may lead to swelling, respiratory failure, pulmonary edema, and ultimately, death via asphyxiation. Nonpersistent agents. Most agents approved for U.S. military operations.	Chlorine Chloropicrin (PS) Diphosgene (DP) Perfluoroisobutylene (PFIB) Phosgene (CG)	LD ₅₀ (skin) <50 mg/kg N/A LCt ₅₀ (respiratory) 3,000–3,200 mg-min/m ³ ; ICt ₅₀ (respiratory) 1,600 mg-min/m ³ Extremely toxic, 10× more toxic than CG LCt ₅₀ (respiratory) 3,200 mg-min/m ³ ; ICt ₅₀ (respiratory) 1,600 mg-min/m ³
Tear agent	Irritation of the eye, severe tearing. Mostly used in situations where less severe and transient incapacitation is sought, such as in training scenarios and riot control.	Bromobenzylcyanide (CA) Chloroacetophenone (CN) Chloropicrin (PS) Chloroacetophenone; carbon tetrachloride; and benzene (CNB) Dibenz-(b,f)-1,4-oxazepine (CR) o-Chlorobenzylidene malononitrile (CS)	LCt ₅₀ (respiratory) 8,000–11,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 30 mg-min/m ³ LCt ₅₀ (respiratory) 7,000–14,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 80 mg-min/m ³ LCt ₅₀ (respiratory) 2,000 mg-min/m ³ LCt ₅₀ (respiratory) 11,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 80 mg-min/m ³ ICt ₅₀ (percutaneous) 0.15 mg/m ³ LCt ₅₀ (respiratory) 61,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 10–20 mg-min/m ³ LCt ₅₀ (respiratory) 15,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 12 mg-min/m ³
Vomit agent	Strong irritation of upper respiratory tract, nausea, vomiting. Nonpersistent agents.	Adamsite or DM (diphenylaminochloroarsine) DC (diphenilcyanoarsine)	LCt ₅₀ (respiratory) 11,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 22–150 mg-min/m ³ LCt ₅₀ (respiratory) 10,000 mg-min/m ³ ; ICt ₅₀ (respiratory) 30 mg-min/m ³

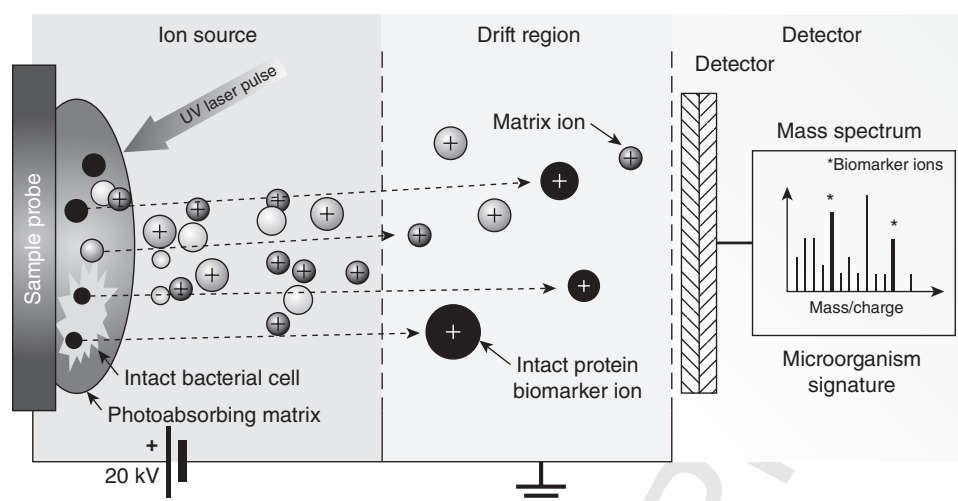


FIGURE 19.1 Schematic illustrating whole-cell mass spectrometry. The intact bacterial cell is crystallized within the matrix, and intact biomarker proteins are ionized during the MALDI process, and are detected by TOF-MS [8].

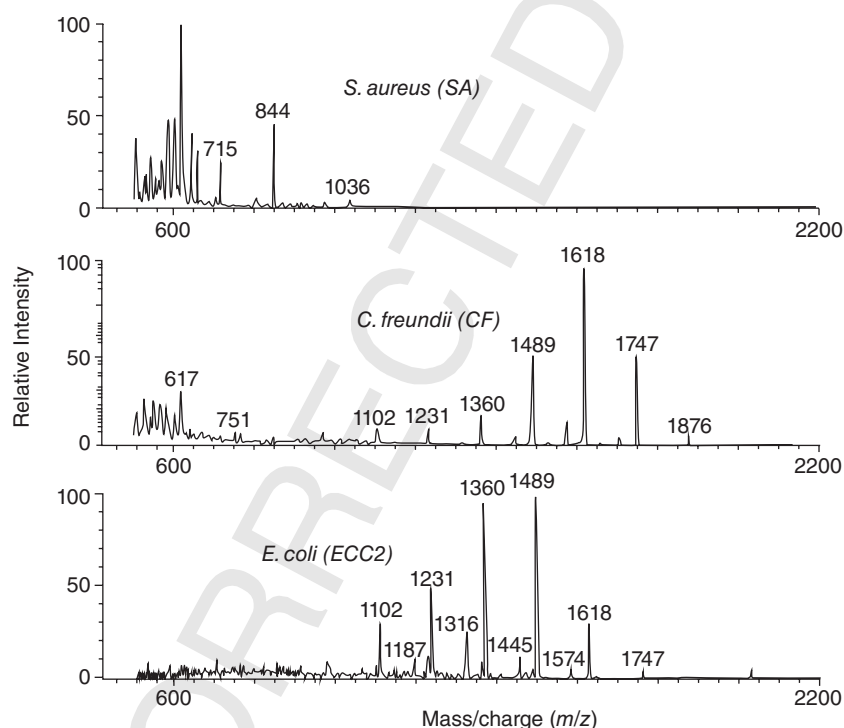


FIGURE 19.2 Partial, positive ion mass spectra (m/z range = 550–2200 Da) of SA, CF, and ECC2 obtained with MALDI-TOF-MS [12].

directly from whole colonies based on protein biomarkers [12,13]. In the Holland study [13], five different strains of bacteria were distinguished in a blind experiment by visually comparing spectra from unknown bacteria to spectra collected from reference standards, demonstrating the ability to distinguish bacterial strains by the mass spectral fingerprint. At nearly the same time, Claydon et al. also reported the use of MALDI-TOF-MS to identify intact microorganisms, using 10 dif-

ferent strains to demonstrate the obvious differences between the spectra collected [12]. Figure 19.2 illustrates the diversity of spectra observed when comparing *Staphylococcus aureus*, *Citrobacter freundii*, and *Escherichia coli* CJ532NCTC50167. Table 19.3 details the unique patterns of m/z values observed with different species in this MALDI-TOF-MS experiment. It can be noted that while there is some conservation of biomarker m/z values between several organisms as seen

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TABLE 19.3 Unique Patterns of m/z Values from Desorbed Cell Wall Moieties Obtained by MALDI-TOF-MS

<i>E. coli</i> (m/z)				Other Genera (m/z)			<i>Staphylococcus</i> (m/z)		
ECN	ECC1	ECC2	ECK	CF	KA	MS	SS	SA	SE
				617	617		602	602	602
						618	618	618	618
							624	624	624
							642	642	642
						656	656	656	656
								715	715
				751	750				
							756		756
							800	800	800
							844	844	844
							888		
						1021			989
								1036	
		1058	1058						
		1093							
1102	1102	1102	1102	1102	1102				
						1158			
						1172			
						1186			
		1187	1187						
						1200			
		1222				1214			
						1228			
1231	1231	1231	1231	1231	1231				
						1242			
						1256			
						1270			
						1284			
						1298			
					1314				
1316	1316	1316	1316						
	1351	1351							
1360	1360	1360	1360	1360	1360				
		1445				1445			
		1480				1480			
1489	1489	1489	1489	1489	1489				
							1532		1532
				1534	1534				
		1574							
				1575					
1618	1618	1618	1618	1618	1618				
		1747				1747			
1852			1852			1852			
				1876					

Bold print indicates similarities between strains [12].

in bold in the table, in all cases there are spectral features that distinguish one organism from the rest. It is this difference in the mass spectra of individual species that allows for rapid identification of bacteria, which is especially important in the clinical setting, as well as in biological warfare agent detection.

Over the last decade, significant developments have been made toward the application of whole-organism MALDI-TOF-MS; however, significant questions remain to be solved before achieving real-time, high-throughput homeland security applications. One of the greatest obstacles toward large-scale implementation of intact organism mass spectrometry is reproducibility. Instrument variability due to different calibration and operation settings is one source identified as crucial for interlaboratory reproducibility. In addition, protein expression levels may vary due to various growth conditions, also causing spectral variance. A mass spectral fingerprint of a particular organism can be heavily impacted by culture broth composition and environmental conditions, which in turn may affect the expression level of proteins [14–18].

In order to have a standardized approach, several researchers are developing a protocol to compare obtained mass spectral data to spectral libraries [19–22]. This standardization is especially important when the goal is to differentiate between closely related species or strains. Experimental parameters such as incubation time [19], matrix type, and concentration [20] have been studied extensively. The conclusion of these studies is unanimous: experimental spectra should be collected under the same conditions as reference spectra to ensure consistent reproducibility.

The most obvious approach to ensure reproducibility of mass spectra is to standardize culturing conditions for each individual organism. To illustrate that the variability of spectra collected from different laboratories can be minimized by ensuring identical experimental conditions, a study was performed using three different mass spectrometers in three different locations [23]. Bacterial cultures of *E. coli*, matrix, solvents, and calibration standards were aliquots from the same preparation. Using automated data acquisition and processing algorithms, as well as a library containing model tri-laboratory fingerprint spectra, the researchers found that interlaboratory variability can be minimized and a reproducibility of 100% can be achieved.

Although successful, this standardization somewhat diminishes the advantages of the mass spectrometer itself; reproducibility comes with the price of losing flexibility. With such stringent conditions, the application of whole-cell mass spectrometry and proteomic fingerprinting could become inadequate when analyzing a sample that poses a threat to homeland security.

19.2.1.1 Pattern Matching of Biomarkers for Bacterial Identification In an ideal situation where the genome of the microorganism is fully deciphered, proteomic databases can be used to identify the major peaks in a bacterial fingerprinting experiment. In this case, instead of comparing the experimental spectrum to a library of mass spectra, the peaks present in the mass spectrum would be correlated back to molecular mass of the proteins present in each species in part. It is evident that the certainty of an identification will always depend on a multitude of factors: first, the capabilities of the instrument such as sensitivity, mass accuracy, and resolution; second, the quality and size of the database; and third, the database-searching algorithm itself, with its inherent scoring system and assignment thresholds.

Pattern-matching analysis of mass spectra has been demonstrated using conserved biomarker proteins that are present in the spectrum of a particular organism regardless of culturing conditions, experimental parameters, or instrument used. In this type of investigation, biomarker peaks consistently present are used for comparison to a proteomic database. This approach is similar to peptide mass fingerprinting techniques in proteomics, where peptides produced from tryptic digests are compared to in silico digestion of a known proteome. Jarman and coworkers designed an algorithm to determine the relevant biomarkers that are conserved at various conditions and uses these to construct a reference spectrum for comparison to blind bacterial samples [24,25]. This algorithm determines the frequency of occurrence for each peak as a function of peak location, standard deviation of location, normalized peak height, standard deviation of peak height, and the fraction of replicates in which a particular peak is seen, using 70% as the threshold to ensure only highly occurring peaks are used. Experimental spectra were compared to the reference spectrum and the probability of a match was calculated. To determine the ability of the algorithm to correctly identify the organism in a mixture, real-world experiments were simulated by using blind samples. These consisted of an uncharacterized bacterium or a mixture of two or more characterized bacteria. With this approach a 0% false-positive identification and 75% correct identification rate was observed, demonstrating the ability of this technique to rapidly identify bacteria from a mixture of organisms.

In order to evaluate the proteomic profile of whole cells and to correctly identify proteins based solely on the intact mass, advanced instrumentation offering high mass accuracy is necessary. The application of Fourier transform mass spectrometry (FTMS) to intact cell analysis allowed for unambiguous identification of biomarker proteins due to the high resolution and mass

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accuracy of FTMS. In a study by Jones et al., Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry was used to identify ribosomal proteins in *E. coli* based on accurate mass and isotope abundance values [26]. The accurate mass and isotope patterns were used to identify the chemical composition of a protein, which can then be correlated to database entries of a given proteome to more confidently characterize bacteria. To further increase the confidence of identifications, fatty acid and lipid profiles were also included in the characterization. This was not feasible with traditional matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) due to the poorer resolving power in the low m/z region, causing matrix peak overlap with potential fatty acid and lipid signatures. A drawback to the FTMS approach is the requirement of high cost and large size instrumentation that is not readily available to many routine laboratories.

19.2.1.2 Bruker BioTyper MALDI-TOF-MS Analysis

Recent studies on intact organism mass spectrometry have been performed using the commercially available Bruker BioTyper (Bruker Daltonik GmbH, Bremen, Germany) platform. The development of a dedicated spectral matching software and a rigorously composed reference database constructed from culture collection strains has increased the reliability of MALDI-TOF-MS pattern matching. A specific reason for the enhanced performance is the type of proteins composing the reference database. Because the mass range is higher, analyzing data from 2 to 20 kDa, this allows for the detection of mostly ribosomal proteins rather than tracing metabolic pathways [27,28]. These proteins are abundant in cell lysates and their expression levels do not vary dramatically under various growth conditions, therefore greatly enhancing the reproducibility. Pattern matching is performed with a built-in recalibration algorithm, allowing for the use of measurements conducted at suboptimal mass accuracy.

The ability of this enhanced MALDI-TOF-MS approach to identify microorganisms has been validated in a variety of studies [27–30]. Initial experiments were conducted using nonfermenting bacteria; the goal of these studies was to determine the effect of various media types and growth conditions on the ability to identify the organism [27]. The reproducibility was measured on a subset of organisms grown on Columbia blood agar, chocolate agar, Mueller-Hinton agar, and tryptic soy agar. Additionally, the strains grown on Columbia blood agar were evaluated after 2, 5, and 7 days after storage at room temperature. The authors report that correct identifications were made regardless of growth conditions.

Interlaboratory reproducibility of this technique was evaluated using nonfermenting bacteria such as from *Pseudomonas*, *Comamonas*, *Burkholderia*, and *Stenotrophomonas* genera [30]. Sixty blind-coded samples were sent to eight different laboratories; 30 samples were pure cultures from culture collection strains and the remaining 30 were cell lysates prepared prior to sending. All analyses were performed using the Bruker Biotyper software and reference database and used a variety of commercially available Bruker instruments. For all analyses, a standardized sample cultivation and preparation guide was used to ensure reproducibility.

A recommended protocol uses blood agar plates for overnight growth [27]. A single colony is selected for sample preparation. This colony is resuspended in 300 μL ddH₂O; 900 μL ethanol is added to the suspension followed by mixing. The cells are pelleted by centrifugation, the supernatant is removed, and the pellet is air-dried. The dried pellet is resuspended in 50 μL formic acid : water (70:30 v/v), and 50 μL acetonitrile is added and mixed. This mixture is centrifuged for 2 min at $13,000 \times g$ and 1 μL is added to a MALDI plate. Two microliters (2 μL) saturated solution of α -cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid solution is spotted on the bacterial extract and dried. The sample is now ready to be analyzed by MALDI-TOF-MS from 2 to 20 kDa. The spectra obtained are imported into the Bruker BioTyper software and analyzed against the reference database.

To interpret data, a log score is given from 0 to 3 that indicates the strength of the pattern match, and results were ranked with the highest score being the most confident. Evaluation of 480 samples (60 samples at eight laboratories) resulted in a 98.75% species identification using the highest score assigned. When a log score ≥ 2.0 was used as the threshold, 97.29% of samples were correctly identified on the species level. Results typical to this type of experiment are shown in Figure 19.3. These results demonstrated a consistent reproducibility across a variety of laboratories across a variety of instrument types, indicating the strength of this technique for adequate microbial identification.

It is important to note some of the current limitations associated with MALDI-TOF-MS. The need for outgrowth of organisms from potentially contaminated material is still required in order to obtain isolated colonies of organisms, as the ability to resolve mixtures with this technique is deficient. Additionally, a high number of bacterial cells are required for identification; typically, a whole intact colony is used for analysis. This limits the ability to rapidly identify microorganisms directly from biological fluids or environmental samples where the bacterial count is expected to be relatively

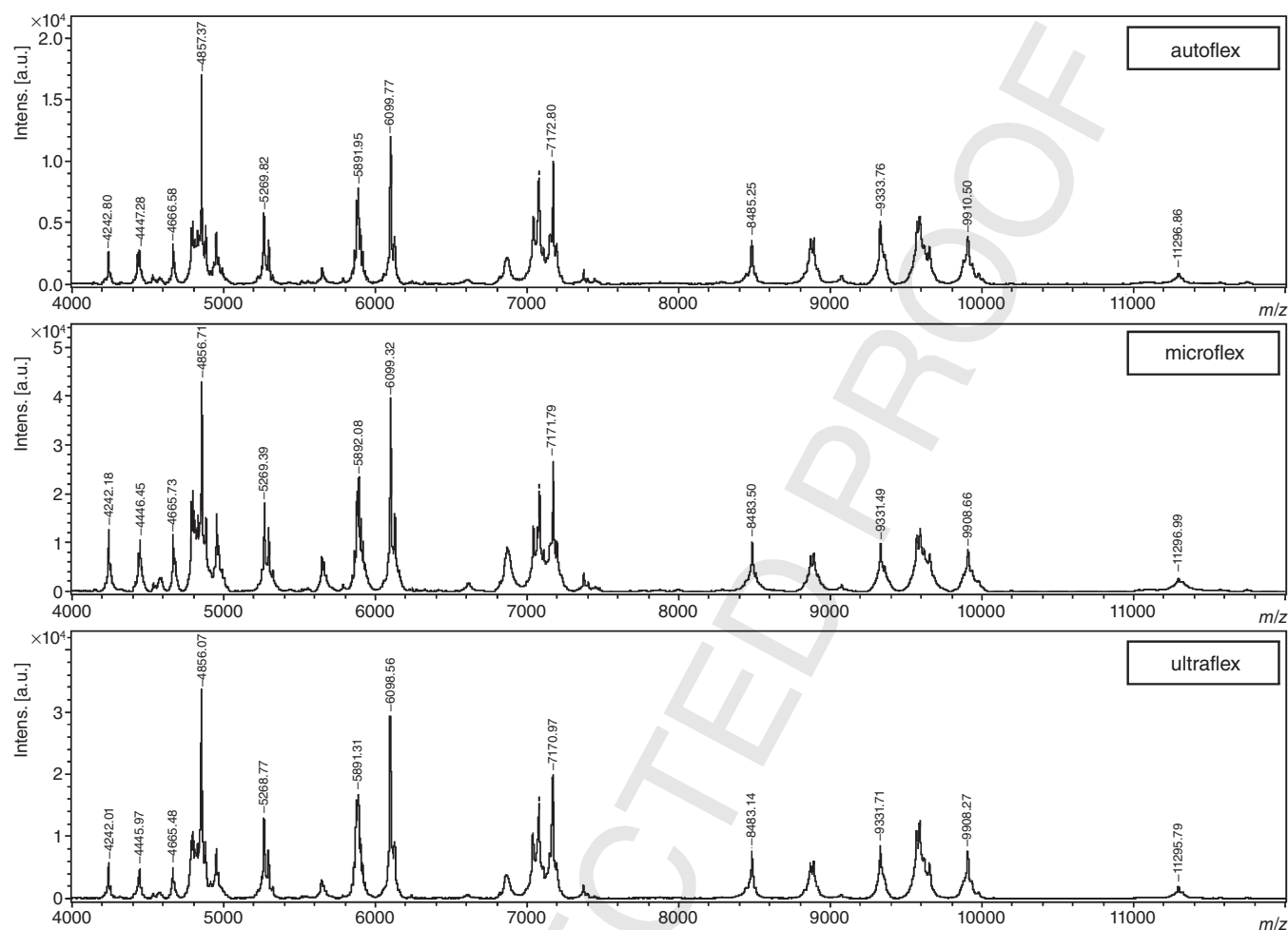


FIGURE 19.3 Comparison of mass spectra for one exemplary nonfermenting strain (strain Galv12) generated on three different MALDI-TOF-MS instruments (autoflex, microflex, and ultraflex). Exemplary masses (in daltons) are depicted. Intens. [a.u.], intensity (in arbitrary units) [27].

low. Current research efforts are focused toward mitigating some of these shortcomings via algorithm manipulation to reduce the complexity of mixed and low titer samples and bringing the mass spectrometry-based identification of microorganisms from complex matrices closer to feasibility. An alternative to outgrowth of bacteria is to analyze DNA, allowing for polymerase chain reaction (PCR) amplification to be used, as discussed in Section 19.3.

19.2.1.3 Aerosol Time-of-Flight Mass Spectrometer

An instrument that combines the advantages of MALDI ionization, TOF mass analyzer, and aerosol sampling was developed by van Wuijckhuijse and coworkers in The Netherlands [31]. Standard samples were prepared by mixing insulin, cytochrome-c, or myoglobin in dis-

tilled water : acetonitrile (70:30 v/v) solution to a concentration of 1 mg/mL and adding up to 10 mg/mL ferulic acid matrix. Trifluoroacetic acid was added to acidify the solution and aid proton transfer (0.1%). Alternatively, in-flight matrix application was achieved by introducing the sample particles into a heated chamber where matrix vapors were allowed to interact with the particles for 30 s. Ionization was achieved with a 308 nm laser, 5 mJ per pulse with a pulse width of 3 ns. Analysis of positive ions up to 20 kDa was performed in the TOF analyzer. These proof-of-principle experiments have shown a promising step toward detection of aerosolized small proteins, albeit extensive work is required toward optimization of the sensitivity and speed of analysis for real-time detection of biological species.

To

19.3 POLYMERASE CHAIN REACTION–MASS SPECTROMETRY

In order to effectively assess a sample in the eventuality of a biological threat, an ideal detection technique should be sensitive, high throughput, and have an unambiguous result output. Mass spectrometers are capable of detecting components of a mixture and can provide spectral data that can be deconvoluted to identify mixture components in one single measurement, without the need for prior knowledge about the sample components. Both whole-cell MALDI-TOF-MS, discussed in the previous section, and mass spectrometry coupled to the PCR (polymerase chain reaction/electrospray ionization mass spectrometry, PCR/ESI-MS), are ideally suited for the detection of relevant biodefense-related organisms because of this broadband characteristic.

Traditional means for detecting the presence of bacteria pertinent to homeland security as well as clinical applications often involve phenotypic evaluation based on the bacterial biochemical properties. Targeted experiments are performed with the PCR using single or multiplexed primers and specifically designed molecular probes. Regions of interest are amplified and detected by fluorescence or by gel electrophoresis. While this is an effective strategy, it has one major caveat: the presence of a particular organism in the sample can only be confirmed or refuted. In contrast, PCR coupled with mass spectrometry (PCR-MS) has the advantage of providing direct information about the type of organisms that are in a sample by allowing for determination of base composition from the measured m/z , as opposed to a merely confirmatory response that traditional PCR assays can provide.

The PCR/ESI-MS method relies on the principle that most organisms share common features in their genomes. This technique takes advantage of these common features by use of broad-range primers. Highly variable regions in the DNA are flanked by conserved regions; these can be highly conserved among species or selective to a specific class of microorganism. Figure 19.4 illustrates the workflow of the PCR/ESI-MS process, from DNA extraction and amplification through mass spectrometric analysis and determination of base composition and organism identification using bioinformatics. Broad-range primers bind to these conserved regions in the DNA. PCR amplification of these regions and the adjacent nonconserved region that shows species variability results in a target DNA amplicon that has a particular m/z value measured with high resolution by a TOF mass spectrometer.

An algorithm is used to predict the number of adenine, guanine, cytosine, and thymine bases present in the amplicon using the accurate molecular weight. The

reverse complementarity of DNA is used to narrow down the candidate list, resulting in an unambiguous composition for each amplicon. This base count is compared to a standardized database of genes from a great range of microorganisms, containing strain variants as well as a wide variety of different genera. The use of similar information from multiple primer pairs allows for the triangulation to a particular organism that is capable of producing this genetic fingerprint, and organism identification is made. This method has the potential to identify not only bacteria, but also fungi and viruses, demonstrating its great potential for biodefense applications.

The first reported use of this technique was in 2005 by Hofstadler et al., and was then called “Triangulation Identification for the Genetic Evaluation of Risks” or TIGER [32]. In this work, three organisms were used to demonstrate the potential of the technique. First, *Bacillus anthracis* was analyzed to illustrate the ability to detect an amplicon and derive base composition by comparing the results from FT-ICR to those obtained by a TOF mass analyzer. TOF analyzers have the advantage of a faster analysis time, therefore a higher throughput; the operation of these two analyzers is detailed in different chapters of this book. The authors concluded that, due to the detection of the reverse complement of each amplicon, only 20 ppm mass accuracy is required to derive a single putative base composition from a measured molecular weight. The mass accuracy of a typical TOF experiment using postcalibration is about 5–10 ppm, indicating that time-of-flight mass spectrometry (TOF-MS) is sufficient for mass analysis.

The second example from the TIGER work focused on virus identification, which is less straightforward than bacterial identification. Virus genomes do not possess the same highly conserved regions across all classes as bacteria do; therefore, more virus-family-targeted primers need to be used. To demonstrate the successful detection of viruses, the *Orthopoxvirus* genus was used, which contains organisms such as smallpox, monkeypox, rabbitpox, and cowpox, all of which are viruses that may potentially be used as biological weapons. Primers were derived to amplify the most conserved regions and the flanking nonconserved regions that will show species and strain variability in the DNA for this genus, and expected base compositions were calculated using GenBank. Five different *Orthopoxvirus* species were analyzed with PCR/ESI-MS following amplification and desalting. The resulting mass spectra were deconvoluted and the expected base composition was calculated. Figure 19.5 illustrates the results obtained, demonstrating that the PCR/ESI-MS results are in agreement with GenBank base compositions for all five organisms. Furthermore, through this process, a new strain of the

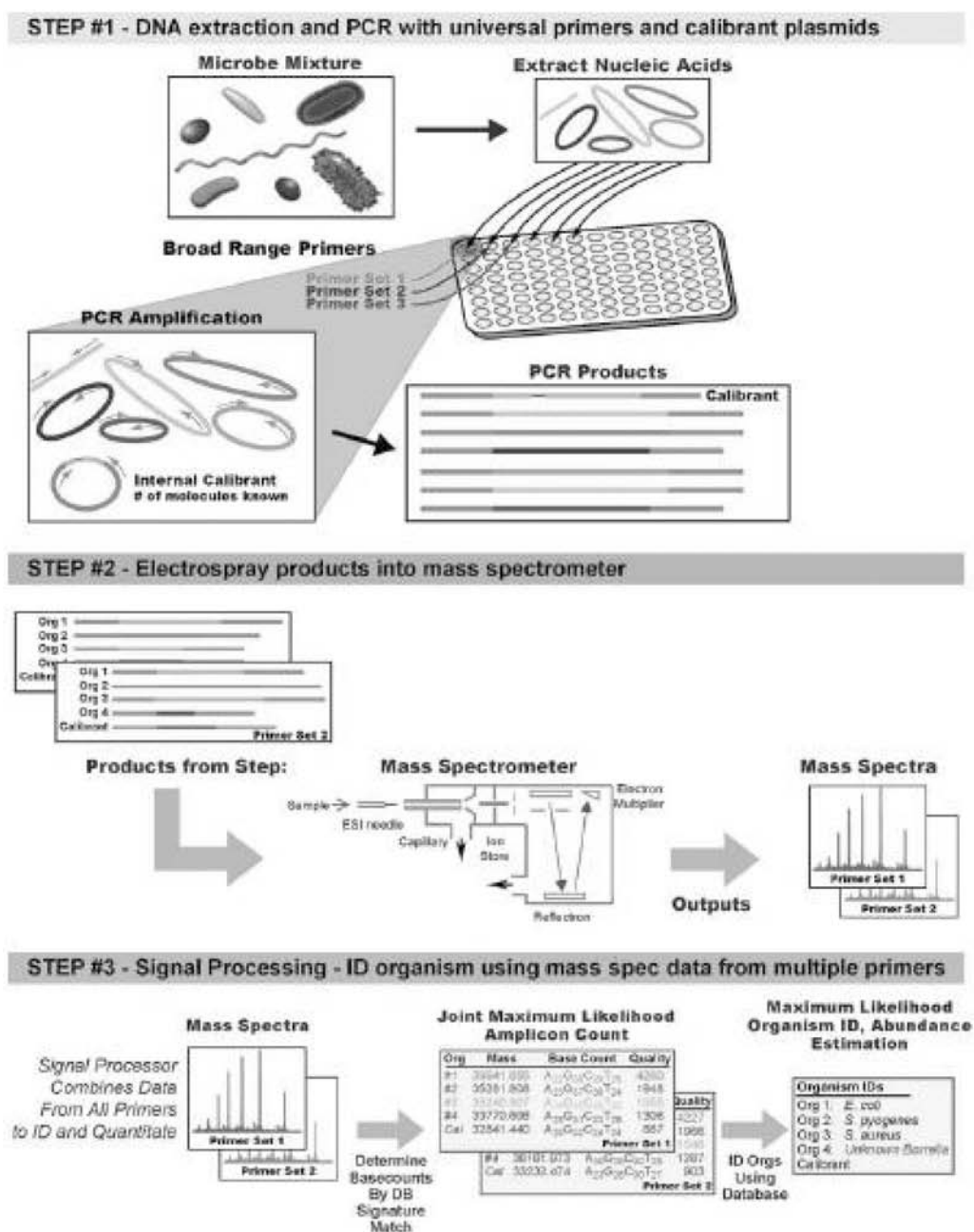


FIGURE 19.4 Workflow of the PCR/ESI-MS process. Step #1: Extraction and amplification of target DNA with coamplification of calibrant DNA for quality control and semiquantitative analysis of PCR products. Step #2: Mass spectrometric analysis is performed separately on each well in a TOF mass spectrometer with ESI. Step #3: Data processing to determine base composition from mass spectrometric signal and correlation of that base composition to a database of known base compositions for each primer pair to determine organism identification [32].

monkeypox virus was identified and confirmed through full genome sequencing, further demonstrating the power of PCR/ESI-MS.

The third and final example used to illustrate the capabilities of PCR/ESI-MS in the TIGER study was using alphaviruses. Members of the genus *Alphavirus*,

such as Venezuelan equine encephalitis (VEE) and Eastern equine encephalitis (EEE) viruses, both of which are relevant select agents, are particularly challenging to detect using traditional methods because of their high variability on the nucleotide and protein level. To overcome this shortcoming, primers were

To

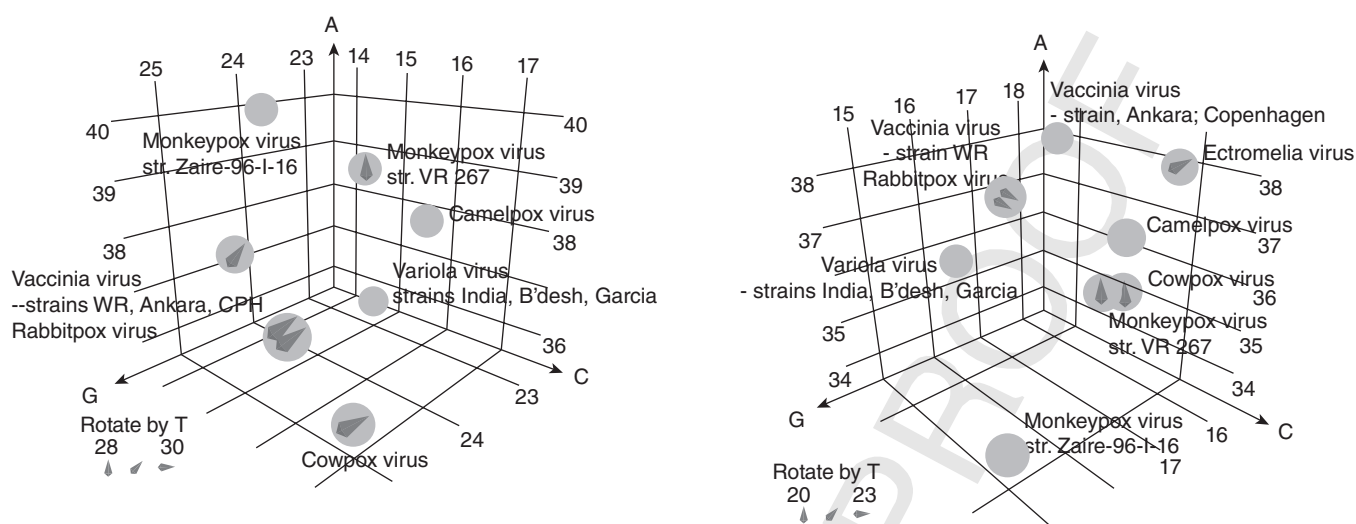


FIGURE 19.5 Four-dimensional plot showing the base composition of amplicon for several respiratory viruses, where each dimension represents one nucleotide. This illustrates that each species occupies a particular space and can allow for correlation of unknown data to predicted base compositions [32].

designed that target all alphaviruses, and the ability to resolve the members of this genus were evaluated with GenBank. Four VEEs, one EEE, and four Western equine encephalitis (WEE) were successfully resolved and correctly identified.

19.3.1 Applications of PCR-MS

19.3.1.1 Viruses Typically, viruses are identified via culture-based methods and microscopy; these methods are extremely slow with respect to the rate of infectivity of many viruses. Therefore, using a molecular-based technique with a rapid analysis time is imperative for biological defense applications where rapid identification of the microorganism is vital. The ability of PCR/ESI-MS to characterize viruses allows for the immediate serotyping of the virus and identification of new strains. In addition, it may predict an emerging outbreak and therefore provide information to make decisions about quarantine, and allow medical personnel to provide appropriate supportive care.

One of the first published reports of PCR/ESI-MS aimed toward identification of *Coronaviruses*, the organism that was responsible for causing the severe acute respiratory syndrome (SARS) epidemic in recent years [33]. In this work, Sampath and coworkers used an RNA-targeting approach. RNA is subjected to reverse-transcription (rt) and amplified with PCR using two primer pairs targeting conserved regions predicted by aligning GenBank genomes. Samples were desalted

and analyzed with an FT-ICR mass spectrometer. Fourteen different coronavirus species were identified in these experiments. Most interestingly, the ability to successfully resolve mixtures was demonstrated using a mixture of three species. The sensitivity of this assay was also evaluated using dilutions of spiked virus in human serum, demonstrating detectable signal down to 1.7 plaque forming units (PFU) per milliliter of serum, which was estimated to be ~300 rt viral genomes/PFU, which is consistent with standard PCR detection limits for coronaviruses.

Additional research has been conducted on a variety of other viruses, including adenoviruses, which cause several types of respiratory infections [34], and influenza [35]. The PCR/ESI-MS technology was at the forefront as a diagnostic method during the recent H1N1 epidemic due to its ability to objectively assess strain variation during detection [36].

19.3.1.2 Bacteria Bacteria are of major concern when developing detection methods for a possible biological attack because of their relative ease of production and maintenance. In addition, bacteria can be easily disseminated to a large group of people simultaneously as aerosols, either in the water or food supplies. Traditional methods for bacterial detection, similar to those employed for viruses, revolve around culture-based and phenotypic methods that can require long growth time and tedious isolation. PCR/ESI-MS evaluates genomic base compositions, requires very little amount of material, and is therefore ideal for the detec-

tion of low-level bacteria in bioterrorism situations. Extensive studies have focused on detection of bacteria, including common respiratory pathogens [37], the environmental and nosocomial pathogen *Acinetobacter baumannii* [38], the genetic diversity studies involving methicillin-sensitive and -resistant *S. aureus* [39,40], tick-borne organisms [41], and bloodstream infections [42]. All of these studies have demonstrated that PCR/ESI-MS is capable of rapidly identifying bacteria in a variety of biologically relevant media and is an effective means of screening and assessing biological warfare attacks.

The field of PCR/ESI-MS is currently dominated by Abbott Molecular (Abbott Park, IL); their PLEX-ID platform contains an in-house database that is crucial for data analysis. As a recommended protocol for this type of work, the first step is extraction of DNA from the mixture to be analyzed. Many different types of extraction kits exist and are suitable for PCR-MS analysis; however, most published studies have used the Qiagen DNA extraction kits (Qiagen, Valencia, CA) and KingFisher extraction (Thermo Scientific, Waltham, MA). The resulting DNA is then aliquoted into a 96-well plate for PCR, where each well contains a single set of broad-range primers to amplify the DNA present.

A typical experiment for PCR/ESI-MS uses a mixture of PCR reagents: four units AmpliTaq Gold (Applied Biosystems, Warrington, UK), 1.5 mM MgCl₂, 0.4 M betaine to reduce secondary structure in gas chromatography (GC)-rich regions, 800 μM dNTPs, and 250 nM of each primer. PCR-MS amplification requires a two-step PCR protocol to allow for a great extent of nonspecific primer annealing, a parameter that enables the use of broad-range primers. The protocol used is: 95°C for 10 min; eight cycles of 95°C 30 s, 48°C 30 s, and 72°C 30 s, increasing the annealing temperature by 0.9°C each cycle; and 37 cycles of 95°C 15 s, 56°C 20 s, and 72°C 20 s. The plate containing amplified DNA is then desalted and analyzed via ESI using the Abbott Molecular PLEX-ID PCR/ESI-MS platform.

19.4 SMALL MOLECULE-BASED MASS SPECTROMETRY METHODS

Analysis of chemical samples and biological substances of nonproteomic origin requires a rather different approach than that implemented for the study of proteins and nucleic acids. Sample collection and introduction may be achieved via aerosol particle collection for volatile compounds and air-borne pathogens or via a direct insertion probe for solid, nonvolatile samples. Alternatively, SPME may be used to extract and pre-concentrate volatile compounds present in air, water, or

soil samples for subsequent gas chromatography–mass spectrometry (GC-MS) analysis.

19.4.1 Fatty Acid Detection

It has been found that the fatty acid profile of bacteria is relevant to early detection of potential bioterrorism agents. For these studies two approaches have been most often employed. In the first approach, the fatty acids are a priori derivatized and the resulting methyl esters are separated with GC or with an ion mobility stage. In this type of analysis, the molecules are ionized either via EI, or via reaction with ionized ethanol or other protonating reagent (CI). To minimize sample handling and processing time, a second approach has been developed under the name of Chemical Biological Mass Spectrometer (CBMS). This field-deployable instrument is currently the only platform to offer simultaneous detection of select chemical and biological threats and will be discussed in more detail in a subsequent section.

19.4.1.1 Dipicolinic Acid (DPA) Detection *Bacillus* and *Clostridia* species have been found to contain 5%–14% dipicolinic acid (DPA; dry weight); this compound has been found to be characteristic to sporulated bacterial cultures. Beverly and coworkers have reported a 10 min detection protocol from *B. anthracis* cultures using in situ derivatization with Py-MS [43].

Bacterial Culture *Bacillus anthracis* is a highly pathogenic bacterium causative of pulmonary infection that has an extremely large fatality rate. Its use as a possible biological weapon arises from this innate pathogenicity, and only laboratories with Biosafety Level (BSL) 4 may handle these colonies. In their work, Beverly et al. used a casein acid digest broth at 37°C. The culture was harvested after 10 days, centrifuged at 7000 rpm, and frozen at –70°C and killed with a Co gamma source [43].

In Situ Methylation and Py-MS The sample (standards of known concentration or the cells to be analyzed) and 5 μL of 0.1 M tetramethylammonium hydroxide (TMAH) is added to the Curie-point wire and dried in hot air. The resulting dimethyl–DPA-coated wire is introduced in a quadrupole ion trap equipped with microtube furnace pyrolyzer capabilities, using a 200–460°C temperature ramping program. Alternatively, a triple quadrupole mass spectrometer may be employed using a Curie-point pyrolysis inlet with a 70 eV EI source. Figure 19.6 shows the pyrolysis mass spectra of *B. anthracis* with and without in situ methylation obtained after a brief 10 min sample preparation and analysis from a 2.2×10^7 CFU sample.

19.4.1.2 Fatty Acid Methyl Ester (FAME) Detection

The unique fatty acid composition and variability of the relative abundances of these species among microorganisms from different genera have been summarized as early as half a century ago by O'Leary (1962). Subsequent developments in increasing sensitivity of mass spectrometers obviated the need for analysis of these nonvolatile, highly abundant compounds for biodefense approaches. Laboratory protocols have been developed to optimize the esterification procedure to increase the yield and minimize the required reaction time.

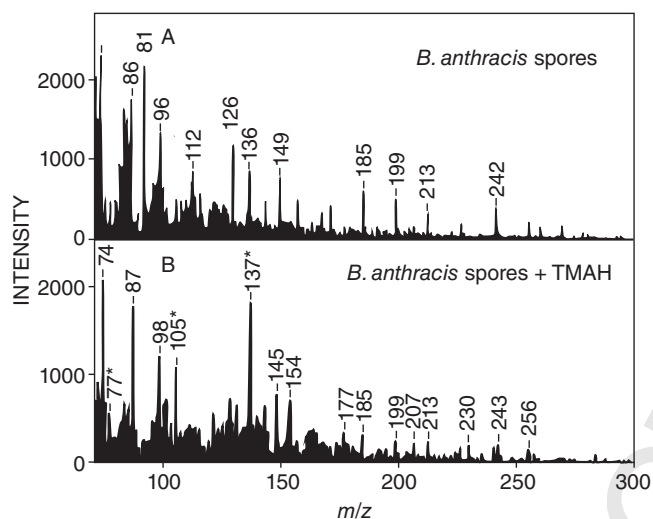


FIGURE 19.6 (A) Py-mass spectrum of *B. anthracis* sporulated whole cells. (B) Py-mass spectrum showing the in situ methylation of the whole cells. The asterisked masses in (B) are the fragment ions of dimethylated dipicolinic acid (mDPA) [43].

Fatty Acid Esterification The most cited fatty acid esterification protocol for GC separation was developed by Sasser, published in a technical note in 1990, and revised in 2001 [44]. Four reagents are used for this procedure:

Reagent 1: 45 g NaOH, 150 methanol, 150 mL H₂O.

Reagent 2: 325 mL 6.0 N HCl and 275 mL methanol.

Reagent 3: 200 mL hexane and 200 mL methyl *tert*-butyl-ether.

Reagent 4: 10.8 g NaOH in 800 mL H₂O.

Cells are harvested using a 4 mm loop (40 mg) and saponified using 1 mL of reagent 1 for 5 min on boiling water bath. Samples are cooled and methylated with 2 mL reagent 2 at 80°C for 10 min. Extraction of FAMES is performed with 1.25 mL of reagent 3 by gentle tumbling for 10 min. To clean the sample and minimize instrument contamination, a base wash is performed using 3 mL of reagent 4.

Early FAME studies of microorganisms involved a GC separation step. Table 19.4 summarizes the typical GC columns and conditions used for FAME detection.

It was soon postulated that in a real homeland security threat scenario this adds to the total analysis time and reduces the number of samples analyzed per time unit. To overcome this shortcoming, several research groups developed fast and ultrafast GC methods, reducing the separation step by orders of magnitude [45, 46]. With the recognition of the benefits of pyrolysis for volatilizing fatty acids and their corresponding FAMES, Voorhees and coworkers reported the successful discrimination between bacterial species [47,48]. This method greatly simplified and shortened the sample

TABLE 19.4 GC Columns Generally Used for Detection of FAMES

Column	Conditions
Supelco/Sigma Aldrich (Bellefonte, PA): bonded; polyethylene glycol 30 m, 0.25 μ m	50–280°C
Agilent J&W (Palo Alto, CA): DB-225	40–220/240°C, medium to high polarity
Perkin-Elmer (Waltham, MA): PE-225	70°C 1 min, 70–180°C at 20°C/min, 180–220°C at 3°C/min, hold 220°C for 15 min.
Alltech Associates (Deerfield, IL):	
1. AT-225 (25% phenyl, 25% cyanopropyl-methyl silicone)	1. Up to C22:1, 200°C
2. DB-5 (5% phenyl, 95% methyl silicone)	2. Hold 150°C 4 min, ramp to 250°C at 4°C/min up to C20:0
3. Heliflex AT-1 (100% methyl silicone)	3. 40–100°C (5°C/min), up to C:6
Agilent J&W DB-23	90°C for 6 min, 90–210°C at 10°C/min
A. Polar example 68% bixcyanopropyl-32%dimethylsiloxane, 50 m	A. 90°C 1 min, 30°C/min to 160°C, 15°C/min to 200°C, slower ramps to 225°C. Separate C10–C24 less than 12 min at 2 mL/min.
B. Intermediate example: wax, 15 m	B. 160°C 1 min, 5°C/min. to 185°C, 8°C/min to 240, at 50 cm/s.

Based on Reference [57].

analysis time albeit at the price of complex mass spectra. In addition, culturing conditions such as temperature, growth period, and medium have been found to be crucially responsible for the reproducibility of fatty acid fingerprinting studies. The need for more complex chemometric deconvolution algorithms resulted in significant progress in the field of applied mathematics in pattern recognition and multivariate analysis [49,50].

19.4.2 CBMS

To combine the benefits of portability, speed, and sensitivity of mass spectrometry, an ion trap mass analyzer was designed to simultaneously detect select chemical and biological agents from air and soil. In 2001, researchers from the Oak Ridge National Laboratory reported the successful implementation of Block II CBMS, an instrument built to U.S. Army standards for field deployment [51]. The sample collection is achieved via a “bio-concentrator,” a pump that draws air for 2 min at a rate of 330 L/min. Using an air jet of opposing direction at a set rate, the particle size trapped can be tuned; the exiting sample jet flow is reduced to 1 L/min as it enters the quartz pyrolysis tube. Fatty acid components of potentially present bacterial membranes are methylated with 1–2 μ L of 0.1 M TMAH in situ in the course of 1 min. The pyrolysis tube is heated from 100 to 550°C in 16 s. In this time period bacterial cells are lysed and the esterification reaction proceeds. The FAMES are transmitted to the ion trap mass spectrometer via a heated 1/16 inch silica capillary and undergo CI with ethanol reagent. Mass analysis is achieved in full-scan mode and a FAME fingerprint is obtained as early as 4 min after sampling.

Although relatively heavy (130 lb.) and energy inefficient (500 W), initial field studies of the Block II CBMS proved that field-deployable mass spectrometers are instruments of the future battlefield. The new generation Block III CBMS is a commercial instrument manufactured by Bruker Daltonics (Billerica, CT). Currently, the instrument is capable of detection of select chemical and biological warfare agents included on the U.S. Army military standard (MIL-STD) list in fully automated fashion. The figures of merit are summarized in Table 19.5.

19.4.3 Aerosol Mass Spectrometry

The bioaerosol mass spectrometer (BAMS) is an instrument designed specifically for real-time, automated detection of select airborne bioagents without the need for sample pretreatment, cleaning, or chemical derivatization. The development of the BAMS is the result of the collaborative effort of a research division

TABLE 19.5 Figures of Merit of the Block III CBMS Commercial Instrument Manufactured by Bruker-Daltonics

Dimensions	Mass Spectrometer Figures of Merit	Detection Limits
Height: 650 mm	Mass range 45–250 amu	CWAs (mg/m ³)
Width: 470 mm	Automated mode	GB 0.04, GD 0.04
Depth: 350 mm	10–450 amu	VX 0.02, HD 0.07
Weight: approximately 65 kg	Manual mode	L 1.4
Power consumption	Scan speed 28,000 amu/s	BWAs (bacterial spores BG) (pyrolyzer load)
Max. 480 W	Ion getter pump 20 L/s	100 ng manual mode
Norm. 360 W	Pyrolyzer temperature 600°C	1 μ g automated mode

in Lawrence Livermore National Laboratory with the supervision of Eric E. Gard, and Carlito B. Lebrilla’s group at University of California, Davis. In a 2004 communication, Fergenson and coworkers reported the results of the first BAMS, a mass spectrometry-based technique capable of distinguishing between different *Bacillus* species from single-cell analysis. In this setup, aerosol particles were sampled from ambient air and analyzed with an aerosol TOF mass spectrometer (TSI, Inc., St. Paul, MN) modified in house. The particle passes through three stages of differential pumping while crossing the path of two continuous-wave laser beams. Since the acceleration of the particle is dependent on its size, the time between the two scattering events is indicative of particle size. The particle is ionized with a 266 nm pulsed laser; positive and negative ion spectra are recorded simultaneously by separate TOF analyzers. At this wavelength, DPA, which is highly prevalent in most living cells, absorbs the laser energy, behaving as matrix in the ionization process. With this first attempt to analyze single aerosolized biological particles, the authors were able to distinguish between *Bacillus atrophaeus*, *Bacillus globigii*, and *Bacillus thuringiensis* based on their mass spectral fingerprint even when different growth conditions were employed. Although these results proved to be encouraging, sensitivity at higher mass range left room for improvement [52]. A year later, the same collaboration yielded a breakthrough in taking BAMS closer to the goal; Russell and Czerwieniec and their coworkers reported on a new design that led to a great increase in sensitivity at m/z range up to ~1200 Th [53,54]. Using a linear electrostatic

ion guide and delayed extraction before the flight tube, the sensitivity for gramicidin S standard was reduced to 14 zmol (corresponding to ~8400 molecules). Although nonproteomics-based fingerprinting of aerosolized biological species has drawn less interest in the scientific community than protein or peptide mapping, BAMS would be a suitable approach for detecting and typing biological aerosol particles in scenarios where high throughput is crucial.

The aerosol mass spectrometer described above is capable of analyzing particles of nonbiological origin. When used for analysis of CWAs, the BAMS instrument is named single-particle aerosol mass spectrometer (SPAMS). Using this instrument design, Martin and coworkers reported the successful detection of liquid nerve agent simulants from individual aerosol particles [55].

19.4.4 GC-MS Analysis of CWAs

Gas chromatography coupled with mass spectrometry (GC-MS) has proved to be one of the most successful approaches for detection of toxic substances from complex matrices such as environmental samples, body fluids, postblast debris, and so on. In general, fused silica columns coated with moderately polar polysiloxane films (e.g. DB-5, RTX-5MS) are used for separation of CWAs; compounds are ionized with a 70 eV EI source and analyzed with an ion trap mass spectrometer. Alternatively, CI (isobutane, ethane, methane, etc.) can be used in both positive or negative polarity mode to improve detection of longer-chain mustard agents and organophosphorous nerve agents that ordinarily do not provide molecular ion signal under EI [3].

Requiring minimal power consumption and relatively simple to operate and maintain, GC-MS instruments are suitable candidates for miniaturization. A man-portable GC-MS instrument capable of detecting volatile organic compounds (VOCs) has been developed for environmental monitoring by Inficon (Syracuse, NY) and commercialized under the name Hapsite GC-MS. This instrument operates using a rechargeable nickel metal hydride battery, weighs ~16 kg and uses N₂ carrier gas. The quadrupole mass detector has a mass range of 1–300 Da and uses a 70 eV EI source for ionization. Sekiguchi and coworkers evaluated the capabilities of the Hapsite system to detect nerve gases and blistering agents on site. The authors used a vapor mixture containing 1 mg/m³ sarin, 1 mg/m³ soman, 3 mg/m³ tabun, and 0.5 mg/m³ mustard gas and, after a 10-min total analysis time, they obtained detection limits of 0.2, 0.5, 8, and 0.3 µg/m³, respectively [56]. One of the main advantages of this system is portability and ease of deployment; however, it suffers from several shortcomings. First, the low *m/z* range and unit mass resolution

of the ion trap employed may lead to unidentified components and/or misidentifications. Second, due to the direct sampling involved, compounds with low vapor pressure (low volatility) cannot be identified without a priori derivatization. In addition, nerve gases, blistering agents, and other compounds containing functional groups may strongly adsorb to the coating of the GC column, greatly increasing sample carryover and leading to false-positive identifications.

Because much improvement is needed in order to overcome these shortcomings, research efforts in governmental, academic, as well as private laboratories all over the world are focusing on development of novel approaches toward optimization of the current technology.

19.5 CONCLUSIONS

Novel approaches for deciphering the proteomic profile of living systems have opened the avenue toward application of mass spectrometry toward biodefense-related applications. With the increasing sensitivity and resolution of mass analyzers, ever-improving ionization techniques, innovative sampling methods, and development of fast and reliable database-searching algorithms, mass spectrometers have gained important terrain in reaching the goal of unambiguous identification of microorganisms in a biological threat situation. The prospect of utilizing mass spectrometers in the battlefield of the future for detection of hazardous toxins, CWAs, to assess the safety of the environment, and to offer a timely signaling of a potential threat is today well recognized in the scientific community. Instrument development and difficult applications go side by side: while new instrument designs, novel sample preparation techniques, and improved computing hardware and software open up avenues toward homeland security applications, the increasing need for development of more sophisticated detection methods is a driving force for improvement of mass spectrometers.

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