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Sample Preparation and Identification of Biological, Chemical and Mid-Spectrum Agents

A General Survey for the Revised NATO AC/225 (LG/7) AEP-10 Edition 6 Handbook

J.R. Hancock and D.C. Dragon
Defence R&D Canada – Suffield

Technical Memorandum
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Canada

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Abstract

A general survey of sample preparation and identification techniques for biological, chemical and mid-spectrum agents was conducted as part of Canada's contribution to a joint NATO Allied Engineering Publication (AEP) handbook. The handbook integrates, in its various chapters, the diverse aspects of sampling, transport, identification, reporting and training as they relate to biological, chemical and mid-spectrum agents. The handbook provides a common reference to NATO member nations for the development of their own identification capabilities and for the integration of these capabilities into the framework of NATO operations. The handbook could be utilized to direct the development, standardization and integration of member country deployable laboratories, which will support NATO field operations on a rotational basis. This report forms the basis of Canada's contribution to the latest edition of the handbook. In addition to sample preparation and analysis, it discusses; quality assurance, chain of custody and the criteria used for identifying the biological, chemical and mid-spectrum agents.

Résumé

Le Canada a conduit un examen général des techniques de préparation et d'identification d'échantillons des agents biologiques, chimiques et de spectre moyen ; cet examen faisait partie de la contribution du Canada au précis de la Publication interalliée sur l'ingénierie (AEP) de l'OTAN. Ce précis intègre, dans ses chapitres variés, les aspects divers de l'échantillonnage, du transport, de l'identification, de la documentation et de la formation requise concernant les agents biologiques, chimiques et de spectre moyen. Ce précis joue le rôle de référence commune entre les nations membres de l'OTAN en ce qui concerne la mise au point de leurs propres capacités d'identification ainsi que l'intégration de ces capacités dans le cadre des opérations de l'OTAN. Le précis pourrait être utilisé pour diriger la mise au point, la normalisation et l'intégration des laboratoires de secours des pays membres qui soutiendront les opérations sur le terrain de l'OTAN, selon une rotation. Ce rapport constitue le fondement de la contribution du Canada aux éditions les plus récentes du précis. En plus des analyses et des préparations d'échantillons, il discute de l'assurance de la qualité, de la chaîne de possession et des critères utilisés pour identifier les agents biologiques, chimiques et de spectre moyen.

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Executive summary

Introduction: The NATO Allied Engineering Publication 10 (AEP-10) handbook describes procedures and techniques for the field sampling, packaging, transportation, and identification of biological, chemical and mid-spectrum agents. Under NATO land group 7, the Sampling and Identification of Biological, Chemical and Radiological Agents (SIBCRA) subgroup is the custodian of AEP-10. Advances in instrumentation and methods of analysis have made it possible to analyze increasingly complex samples and prompted the SIBCRA subgroup to revise AEP-10 to reflect current techniques and methods. The resulting edition of the handbook for the first time integrates the discussion of biological, chemical and mid-spectrum agents in each chapter. The Handbook provides general guidance to personnel responsible for performing sampling and identification activities in support of NATO operations.

Results: National representatives on SIBCRA were tasked with preparing the individual chapters for the revised AEP-10. Canada accepted to be the lead nation on the chapter dealing with sample preparation and identification of biological, chemical and mid-spectrum agents. The chapter also addresses quality assurance, chain of custody and the criteria used for identifying these agents. This report forms the basis of Canada's contribution to edition 6 of the handbook.

Significance: The alleged use of biological, chemical, and mid-spectrum warfare agents during battlefield or peacekeeping operations requires the ability to collect, transport, process and analyze samples in order that the agent may be identified in a timely and efficient manner. Once the agent has been identified the appropriate protective measures can be adopted and specific treatment for exposed casualties can be initiated. The handbook provides a common reference to NATO member nations for the development of their own identification capabilities and for the integration of these capabilities into the framework of NATO operations.

Future Plans: The latest edition of AEP-10 will be utilized by the SIBCRA subgroup to direct the development, standardization and integration of member country deployable laboratories which will support NATO field operations on a rotational basis.

Hancock, J.R. and Dragon D.C. (2005). Sample Preparation and Identification of Biological, Chemical and Mid-Spectrum Agents. A General Survey for the Revised NATO AC/225 (LG/7) AEP-10 Edition 6 Handbook. (DRDC Suffield TM 2005-135). Defence R&D Canada – Suffield.

Sommaire

Introduction : Le précis 10 de la Publication interalliée sur l'ingénierie de l'OTAN décrit les procédures et les techniques d'échantillonnage sur le terrain, d'emballage, de transport et d'identification des agents biologiques, chimiques et de spectre moyen. Le sous-groupe des Agents biologiques, chimiques et radiologiques (SIBCRA), appartenant au Groupe terrestre des 7 de l'OTAN, est le dépositaire de l'AEP-10. Le progrès concernant l'instrumentation et les méthodes d'analyses permet d'analyser des échantillons de complexité croissante et a incité le sous-groupe SIBCRA à réviser l'AEP-10 pour qu'il reflète les techniques et méthodes actuelles. L'édition du précis qui en a résulté intègre, pour la première fois, la discussion des agents biologiques, chimiques et de spectre moyen, dans chaque chapitre. Le Précis procure une orientation générale au personnel responsable d'effectuer les activités d'échantillonnage et d'identification en soutien aux opérations de l'OTAN.

Résultats : Des représentants nationaux de SIBCRA ont reçu la mission de préparer des chapitres individuels pour la révision de l'AEP-10. Le Canada a accepté d'être la nation chef de file dans le chapitre au sujet de la préparation et de l'identification des agents biologiques, chimiques et de spectre moyen. Ce chapitre traite aussi de l'assurance de la qualité, de la chaîne de possession et des critères utilisés pour l'identification de ces agents. Ce rapport constitue le fondement de la contribution du Canada à l'édition 6 du précis.

La portée : L'utilisation présumée des agents de guerre biologiques, chimiques et de spectre moyen durant les opérations en champ de bataille ou de maintien de la paix requiert la capacité de collecter, transporter, traiter et analyser les échantillons de manière à ce que l'agent puisse être identifié en temps utile et efficacement. Une fois l'agent identifié, les mesures de protection appropriées peuvent être adoptées et un traitement spécifique peut être initié pour les victimes qui ont été exposées. Le précis joue le rôle de référence commune aux nations membres de l'OTAN concernant la mise au point de leurs propres capacités d'identification ainsi que l'intégration de ces capacités dans le cadre des opérations de l'OTAN.

Plans futurs : La dernière édition de l'AEP-10 sera utilisée par le sous-groupe SIBCRA pour diriger le développement, la normalisation et l'intégration des laboratoires de secours des pays membres lesquels soutiendront, selon une rotation, les opérations sur le terrain de l'OTAN.

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Introduction

The alleged use of biological, chemical, and mid-spectrum warfare agents during battlefield or peacekeeping operations requires the ability to collect, transport, prepare, and analyze samples in order that the agent may be identified in a timely and efficient manner. Once the agent has been identified the appropriate protective measures can be adopted and specific treatment for exposed casualties can be initiated. With the development of fieldable analytical instrumentation, it is now theoretically possible to achieve confirmed identification in the field. Unambiguous identification will require the use of instrumentation found in specialized national laboratories.

The NATO Allied Engineering Publication 10 (AEP-10) handbook describes recommended procedures and techniques for the field sampling, packaging, transportation, and identification of biological, chemical and mid-spectrum agents. This edition of the handbook for the first time integrates the discussion of biological, chemical and mid-spectrum agents in each chapter. The Handbook provides general guidance to personnel responsible for performing sampling and identification activities in support of NATO operations.

Under NATO Land Group 7, the Sampling and Identification of Biological, Chemical and Radiological Agents (SIBCRA) subgroup has been the custodian of AEP-10. Advances in instrumentation and methods of analysis have made it possible to analyze increasingly complex samples and prompted the SIBCRA subgroup to revise AEP-10 to reflect current techniques and methods.

National representatives on SIBCRA were tasked with preparing the individual chapters for the revised AEP-10. Canada accepted to be the lead nation on the chapter dealing with sample preparation and identification of biological, chemical and mid-spectrum agents. This report forms the basis of Canada's contribution to edition 6 of the handbook.

Sample Receipt, Storage and Custody

In the interest of clarity the following terms are defined for this section:

(a) **Package.** The sample package is the container and materials used to seal up the sample and transport it from where it was collected to the laboratory for analysis. One package may contain several separate samples and information about the package should remain cross-referenced to the individual samples throughout the analysis.

(b) **Contents.** The contents are the items contained in the sample package and may include not only samples collected at an event but documentation of the samples and field collection efforts. The documentation should remain cross-referenced to the individual samples throughout the analysis.

(c) **Sample.** The environmental or biomedical sample collected at an event and suspected of containing a biological warfare (BW), mid-spectrum (MSA), or chemical warfare (CW) agent. The sample also includes the primary receptacle (i.e., screw-capped tube, "zip-lock" bag) that the environmental or biomedical material was placed in. Any information written on the primary receptacle should be copied into the notes maintained on that sample.

The results of the analysis of samples suspected of containing biological warfare, mid-spectrum or chemical warfare agents may be used for military (battlefield), political (allegations of use) or verification (CWC or BWC) purposes. It is therefore mandatory that the handling and analysis of the samples be performed with the view of maintaining a continuous chain of custody. As a general practice, photographs of the exterior of the sample package and its contents should be obtained for future reference.

When the sample arrives at the laboratory, unless indications are otherwise, it must be assumed that the sample contains a biological agent and the package must be stored in a Biosafety Level 3 (BSL-3) containment suite. The integrity of the sealed sample and its packaging should be confirmed by visual inspection. In cases where samples are collected from the battlefield, comments on the integrity of the package or sample upon arrival at the laboratory should be reported to the tasking authority immediately. Irregardless of its integrity, however, analysis of the sample should be performed. In political or verification cases where there are questions regarding either the integrity of the package or the sample, no analysis should be performed and the sample should be stored pending discussions with the originator.

The responsible scientist should maintain written notes in which they assign the sample a unique identification number and describe the handling and processing of the sample, and the results of all analyses. The package should be opened inside a biosafety cabinet within a BSL-3 containment suite. As the package is opened a real-time chemical agent detector should be used to monitor the contents for chemical agent vapours. If the safety cabinet used draws in new air as it recirculates through the HEPA filters or if the draw of the cabinet is

particularly strong, the scientist may elect to place the sample and the detector in a large plastic bag so that any vapours present are not dispersed by the safety cabinet ventilation before they can be analyzed by the detector. Once the package has been opened, the responsible scientist should assign the content(s) a unique identification number, and date and initial the packaging to show that the sample is now in their custody. Proper and uniform procedures for the labeling, tagging and tracking of samples should be followed. The packaging material should be retained during the analysis of the sample. As far as it is feasible, a representative sample should be retained and archived for possible future examination. The representative sample should also be assigned a unique identification number.

When not being analyzed the sample should be stored in a locked cabinet which is under the sole control of the responsible scientist. If a biological agent is suspected, the sample should be stored within a locked 4°C fridge in order to preserve the viability of any biological agent present. If the sample is transferred to another scientist, a transfer form should be completed which details the transfer. The second scientist should then initial and date the package thus showing when it was placed into their custody.

It is unlikely that battlefield samples arriving at the laboratory would be in a form suitable for direct analysis. In most cases, sample preparation (including extraction, concentration or dilution) will be necessary prior to analysis, especially where unambiguous identification is required.

Quality Assurance and Control

Increasingly, laboratories are being required to provide documented evidence that the results they produce meet a prescribed standard. Terms such as; good laboratory practice (GLP), quality assurance (QA), quality control (QC) and standard operating procedures (SOPs) are used to describe these standards. It remains a national responsibility to ensure that laboratories conform to national and, if appropriate, international standards. In short, it is necessary to demonstrate that accurate, detailed records are maintained by a laboratory, in order that experimental results can be verified by tracing the information back to the raw data. In addition, maintenance and calibration records are also necessary for the wide variety of instrumentation used in the laboratory.

Standard operating procedures apply to both analytical methods and instrument maintenance. Using an analytical method as an illustration, a SOP should contain setup and evaluation procedures for the instrumentation prior to running a sample. It should also contain step-by-step instructions for sample preparation and instrument operation. Instrumental conditions should be detailed and examples of system response should be included as well as the type of quality control samples that should be run with the samples to be tested and the frequency at which the quality control samples should be analysed. A SOP should also contain information on maintaining statistical run charts and troubleshooting instructions in the event the system is out of statistical control or fails to operate correctly.

In order to properly setup and maintain a quality assurance program, it is necessary to establish a quality assurance unit (QAU) which is staffed with personnel responsible for reviewing and auditing the records maintained in laboratories in order to ensure compliance with the QA/QC program. For mobile laboratories between deployments, the ongoing review and auditing of records should be conducted by personnel at their base of operations.

Criteria for Identification

General Considerations

Analysis of samples suspected of containing biological, chemical or mid-spectrum agents, especially when the agent is present in a complex matrix or when the agent is new or unusual must be performed at a properly equipped laboratory experienced in the identification and confirmation of these agents.

Samples should be taken and transported to an identification laboratory according to the procedures described in AEP-10. Whenever possible, samples should be split and sent to at least two laboratories so that the results of their separate analyses can be combined in order to increase the confidence in the final identification of the agent involved or the declaration that no agent was present.

Regardless of the type of agent being investigated, three levels of identification have been defined to indicate the increasing level of certainty associated the agent identification. They are, in increasing order of certainty: provisional, confirmed, and unambiguous. Because of their inherent limitations and restrictions, mobile field laboratories will only be able to establish preliminary and confirmed identifications. Unambiguous identifications can only be provided by national reference and forensic laboratories.

Immunoassay and Polymerase Chain Reaction (PCR) are the techniques of choice for BW agent identification. Gas chromatography-mass spectrometry (GC-MS) is currently the technique of choice for CW agent separation, detection and identification. Liquid chromatography-mass spectrometry (LC-MS) with electrospray ionization is currently the instrumental technique of choice for mid-spectrum agent separation, detection and identification. Immunoassay systems are available for select mid-spectrum agents but their use remains confined to provisional identification of the agent.

Criteria for Identification of Biological Warfare Agents

Provisional Identification: A biological agent may be considered provisionally identified when one of the following criteria has been met:

I The presence of a unique antigen for the biological agent in question is demonstrated by a positive reaction with a specific antibody in an Immunoassay test; or

II The presence of a unique nucleic acid sequence for the biological agent in question is demonstrated by a positive reaction with a specific nucleic acid probe (gene probe) in a Polymerase Chain Reaction assay; or

III A positive response is indicated by *in vitro* culture or multi-metabolic assays.

Confirmed Identification: The identification of a biological agent is confirmed when any two of the three criteria for provisional identification have been met in the presence of authentic reference standards (positive and negative controls) under identical experimental conditions.

Unambiguous Identification: The unambiguous identification of a biological agent provides the highest level of certainty required for the development of strategic and political positions. Confirmed identification becomes unambiguous if the following criteria prove true for the biological agent in question in the presence of authentic reference standards (positive and negative controls) under identical experimental conditions:

I A positive response is obtained by a genetic identification method; and

II A positive response is obtained by an immunological method; and

III A positive match is obtained by *in vitro* culture or multi metabolic assay; and

IV The disease properties of the microbial agent are confirmed in an accepted animal model.

These criteria will apply to all classical microbes with the exception of **unambiguous identification** for:

(a) Biological agents for which there is no suitable culture methods e.g. hepatitis B grows only in humans; and

(b) Biological agents that have been genetically manipulated to change their characteristics; and

(c) Novel agents e.g. microencapsulated organisms, prions, infectious nucleic acid.

It is important to note that in order to cause disease in a living host BW agents must be metabolically active and capable of replication. Until a positive identification is obtained with a culture, multi-metabolic or animal model assay, it is impossible to determine if the biological agent is metabolically active. Until metabolic activity is established, the possibility remains that the incident being investigated was a hoax in which a harmless, killed preparation of the BW agent was used to induce terror. Such a possibility should be kept in mind when evaluating the treatment of exposed individuals and the potential criminal, strategic and political ramifications of the identification results. Furthermore, this underscores the importance of proper collection, transport and storage of a sample prior to analysis so that any BW agent present in the sample remains viable.

Criteria for Identification of Mid-Spectrum Agents

Provisional Identification: A mid-spectrum agent may be considered provisionally identified when one of the following criteria has been met:

I The chromatographic retention data acquired for the mid-spectrum agent under two different experimental conditions matches that of known mid-spectrum data; or

II The molecular mass of the mid-spectrum agent, determined by MS, matches that of known mid-spectrum agent data; or

III A specific immunological assay registers a positive response.

Confirmed Identification: The identification of a mid-spectrum agent is confirmed when any two of the three criteria for provisional identification have been met or:

I In the case of proteinaceous mid-spectrum agents, the molecular mass and corresponding mass map of the enzymatic digestion products (with a minimum of three products) matches that of known mid-spectrum agent data.

Unambiguous Identification: Unambiguous identification provides the highest level of certainty required for the development of strategic and political positions. The identification of a mid-spectrum agent is unambiguous when the following conditions have been met:

For non-proteinaceous mid-spectrum agents:

I The chromatographic retention data acquired for the mid-spectrum agent and spectra acquired using two different spectrometric techniques (MS, NMR or IR) match that for an authentic reference standard acquired under identical experimental conditions. If the molecular ion is not present in the mass spectrum, techniques such as chemical ionization or electrospray mass spectrometry must be performed to confirm the molecular mass.

For proteinaceous mid-spectrum agents:

I The chromatographic retention data acquired for the mid-spectrum agent under two different experimental conditions matches that of an authentic reference standard acquired under identical experimental conditions or a specific immunological assay registers a positive response; and

II The molecular mass and corresponding mass map of the enzymatic digestion products (with a minimum of three products) matches that for an authentic reference standard acquired under identical experimental conditions; and

III Amino acid sequence data for the mid-spectrum agent matches that for an authentic reference standard acquired under identical experimental conditions.

Criteria for Identification of Chemical Warfare Agents

Provisional Identification: A chemical warfare agent may be considered provisionally identified when one of the following criteria has been met:

I The chromatographic retention data acquired for the chemical warfare agent measured under two different experimental conditions matches that of a known chemical warfare agent; or

II The chromatographic retention data acquired for the chemical warfare agent with a specific detector (FPD, TID, AED) matches that of a known chemical warfare agent.

Confirmed Identification: The identification of a chemical warfare agent is confirmed when one of the following criteria has been met:

I A complete spectrum acquired using a single spectrometric technique (MS, NMR or IR) matches the corresponding reference spectra in a database. If the molecular ion is not present in the mass spectrum, techniques such as chemical ionization must be performed to confirm the molecular mass of the compound.

II The chromatographic retention data acquired for the chemical warfare agent during mass spectrometric analysis using selected ion monitoring (minimum of three ions) matches that of a known chemical warfare agent. The ratio of the three ions must fall within 10% of the values of an authentic reference standard. The ions should have coincident maxima, the same peak width at half height, and exhibit a signal to noise ratio greater than three.

Unambiguous Identification: Unambiguous identification provides the highest level of certainty required for the development of strategic positions. The identification of a chemical warfare agent is unambiguous when the following criterion has been met:

I The chromatographic retention data acquired for the chemical warfare agent and spectra acquired using two different spectrometric techniques (MS, NMR or IR) match those obtained for an authentic reference standard under identical experimental conditions. If the molecular ion is not present in the mass spectrum, techniques such as chemical ionization must be performed to confirm the molecular mass of the compound.

Flow Scheme for Sample Preparation and Analysis

Biological Warfare Agents

In general, most laboratories when faced with the problems associated with the sample preparation and analysis of classical biological agents develop a standardized flow scheme to simplify the process. The flow scheme outlines how the samples are to be handled in order to free the substance(s) of interest from the sample matrix followed by a stepwise sequence for the analysis of the preparation obtained.

Given below is an example of a typical flow scheme for the recovery of bacterial agents from a variety of sample matrices.

Sample Preparation for Suspect Bacterial and Viral Agents

Aqueous Samples. Centrifuge at 20,000 G for 10 min and remove and retain **supernatant**. If a pellet is formed, make a **suspension** by adding 1 mL sterile phosphate buffered saline (PBS) to the pellet.

Swab Samples. Remove swab from transport container and place in 10 mL sterile PBS. Form a **suspension** by vigorous agitation using a vortex mixer for 10-15 seconds.

Soil and Other Solid Environmental Samples. To 5 grams of sample add two equivalent volumes of sterile PBS. Form a **suspension** by vigorous agitation on vortex mixer for 10-15 seconds. Filter sample through 2 layers of sterilized gauze to remove large particles. If PBS is absorbed by the sample and no suspension is formed, add another two equivalent volumes of sterile PBS.

Example of Analysis Scheme

Analysis is conducted on a **Suspension,** however, if it is an aqueous sample, the **Supernatant** may also be tested.

↓

Remove Aliquot of **suspension** for ELISA.

↓

Remove Aliquot of **suspension** for Gene Probe Assay.

↓

Remove Aliquot of **suspension** for detection of ATP (bacteria only).

↓

Direct Microscopic examination of **suspension** including Gram stain (bacteria only) and range of immunofluorescent tests if available.

↓

Remove 100 µL of **suspension** and add to 5 mL of blood (bacteria only). Incubate 4 h at 37 °C. Stain film by MacFadyean's stain. Blue bacilli with pink capsule confirm anthrax.

↓

Culture aliquot of **suspension** on suitable growth media (bacteria) or into suitable tissue culture (virus).

Mid-Spectrum Agents

In general, it is assumed that a sample containing a proteinaceous mid-spectrum agent will either be collected as an aqueous sample (from a high volume aerosol sampler) or dissolved in water prior to further handling or analysis by mass spectrometry (ESI-MS).

Step 1: Removal of Particulates/Adjust pH

(a) The removal of particulates from an aqueous sample is necessary prior to analysis in order to minimize damage to analytical instrumentation. Centrifugation appears to be the best approach for the removal of particulates, since low recovery of Ile-Ser-Bradykinin, a bio-active peptide, has been reported even when using low binding filtration membranes.

(b) The pH of the sample is measured and adjusted if necessary, as subsequent sample handling steps, such as reductive alkylation or enzymatic digestion, require that the pH of the sample be compatible with the reaction.

Step 2: Purity and Molecular Mass Determination

(a) Rapid analysis using isocratic LC-ESI-MS conditions with a wide mass range scan under low resolution and low sampling cone voltage conditions may be used to determine the relative purity of the sample and monoisotopic or average molecular mass(es) of the major sample component(s). A wide mass range scan (e.g. 150-1500 Da) is used to ensure that most components from singly charged dipeptides to multiply charged polypeptides are detected. Molecular masses determined under low resolution (e.g. 2000, 10% valley definition) are sufficiently accurate for obtaining matches for target compounds in peptide databases. Low sampling cone voltages (e.g. 50 V) are used to minimize collisionally activated dissociation (CAD) in the interface.

(b) For low molecular mass compounds (<1500 Da), it may be possible to obtain amino acid sequence information by CAD in the ESI interface. Increasing sampling cone voltages generally results in increased fragmentation leading to the formation of product ions that may be used for amino acid sequencing.

Step 3: Separation and Fraction Collection

In cases where the sample contains multiple components, it is recommended that the components be separated by HPLC and collected prior to subsequent sample handling steps.

Step 4: Determining the Presence of Disulphide Bonds

(a) The three dimensional structure of proteins, while critical for biological activity, may restrict access of reagents thereby reducing the effectiveness of digestion and derivatization techniques. One technique for denaturing peptides (i.e. cause the peptide to unfold) is immersion in a boiling water bath. To ensure that the peptide does not re-assume it's original three dimensional shape upon cooling, it

is immediately immersed in an ice bath, after removal from the boiling water bath.

(b) Disulphide bridges between cysteine residues exist either between cysteines in the peptide chain (intramolecular) or between cysteines in different chains (intermolecular). These bridges may be broken using reductive alkylation, which may be performed during peptide denaturing. Sample handling steps such as reductive alkylation require pH 8. Ammonium bicarbonate buffer, a volatile buffer compatible with ESI-MS, can be used to adjust the pH.

(c) Following reductive alkylation, the reaction product(s) may be analysed by ESI-MS. When iodoacetamide is used as the alkylating reagent, the mass increases by 58.03 Da for each cysteine that reacts with the iodoacetamide. The molecular mass of the intact peptide should be compared to that of the reductively alkylated peptide(s). If the molecular mass(es) are greater than that of the intact peptide, this is indicative of intramolecular disulphide bond(s). If the molecular mass calculated from the reductively alkylated peptide is less than the intact peptide this is indicative of intermolecular disulphide bond(s).

Step 5: Enzymatic Digestion and Mass Mapping of Enzymatic Fragments

(a) Enzymatic digestions can be performed either in solution or in a flow-through system. While digestions performed in solution may take 12 hours or more, equivalent flow-through systems digest peptides in minutes. A flow-through system may be assembled from a column packed with trypsin immobilized on a support, a solvent delivery system, an injector and a detector to monitor the elution of the digestion products.

(b) Most enzymes have a pH range within which they will optimally digest peptides. For example, trypsin works best at a pH close to 8 and adjustment of the sample pH is performed by adding a known volume of ammonium bicarbonate buffer solution.

(c) The collection of lower mass peptide fragments may be characterized on the basis of molecular mass by MS. These masses constitute a mass map that may be used to aid in the identification of the mid-spectrum agent that was subjected to enzymatic digestion. This mass map should correspond to the majority of those predicted for the digestion of the peptide. Difficulties arise when there is incomplete cleavage during the digestion.

Step 6: Accessing Amino Acid Primary Sequence Data

The enzymatic digestion products would generally be analysed by LC-ESI-MS under CAD conditions which promote the formation of product ions that provide amino acid sequence data for the tryptic fragments. CAD may be performed either in the ESI interface, or alternatively, in a collisional cell between the first and second mass analyzer. The amount and quality of the CAD data acquired will depend on the amount of sample, the mass of the analyte and its amino acid sequence.

Chemical Warfare Agents

In general, the preparation and analysis of samples suspected of being contaminated with chemical warfare agents initially focuses on the identification of nerve agents and vesicants. If these compounds are not detected in the sample more extensive sample preparation and analysis techniques are employed. This would make it possible to detect either trace levels of the CW agents or CW related compounds (i.e., precursors and degradation products). The following schemes for chemical warfare agents are examples of the steps that could be used for the specific target compounds in solid and liquid samples.

Solid Sample

Target Compounds: GA, GB, GD, GF, H, L and VX

Step 1: If the sample is not homogenous, homogenize the gross sample. Where the amount of sample permits, subdivide gross sample into four or more subsamples and select one subsample for analysis.

Step 2: If the presence of compounds in the vapour above the solid sample is of interest, consider headspace sampling with analysis by GC, MS or FT-IR.

Step 3: On the subsample, perform solid/liquid extraction using one of the following techniques; sonification, soxhlet extraction or supercritical fluid extraction. The following example will use sonification to demonstrate a possible approach for extraction of a soil sample.

Add one milliliter of deuterated chloroform per gram of soil and sonicate for 10 minutes. As nerve agents may react with alcohols, only chloroform that is not stabilized with an alcohol can be used. Remove particulates by either filtering through a 0.45 μm filter or by centrifuging for 10 minutes at 2000 G. Transfer supernatant to clean sample vial. The extract can be then analysed by GC, MS, NMR or FT-IR. If necessary, the extract can be concentrated using solvent evaporation or solid phase extraction and re-analysed.

Step 4: To check for the presence of lewisite by gas chromatography, the extract is derivatized using 3,4 dimercaptotoluene. Add 100 μL of the extract to 100 μL of 3,4-dimercaptotoluene (1 mg/mL in chloroform) and let stand for one minute at 20 °C. Analyze the derivative by either GC-MS or GC-FT-IR. If necessary, the extract can be concentrated using solvent evaporation or solid phase extraction and re-analysed.

Solid Sample

Target Compounds: Phosphonic Acids and Thiodiglycol

Step 1: If the sample is not homogenous, homogenize the gross sample. Where the amount of sample permits, subdivide gross sample into four or more subsamples and select one subsample for analysis.

Step 2: On the subsample, perform solid/liquid extraction using one of the following techniques; sonification, soxhlet extraction or supercritical fluid extraction. The following example will use sonification to demonstrate a possible approach for extraction of a soil sample.

Add one milliliter of water per gram of soil and sonicate for 10 minutes. Remove particulates by either filtering through a 0.45 μm filter or by centrifuging for 10 minutes at 2000 G. Transfer supernatant to clean sample vial. The extract can be then analysed by LC-MS. If necessary, the extract can be concentrated using solvent evaporation and re-analysed. In cases where NMR analysis is warranted, it will be necessary to evaporate the sample to dryness and reconstitute it in deuterated water.

Step 3: Alternatively, the water extract can be passed through an ion exchange cartridge and then evaporated to dryness. To form the trimethylsilyl derivatives of the phosphonic acids and thiodiglycol, 100 μL of dichloromethane, 100 μL of BSTFA and 100 μL of pyridine are added to the dried sample. The solution is heated to 60 °C for 90 minutes. The derivatives can then be analysed by GC, GC-MS or GC-FT-IR.

Liquid Sample (Aqueous)

Target Compounds: Phosphonic Acids and Thiodiglycol

Step 1: Where the amount of sample permits, subdivide gross sample into four or more subsamples and select one subsample for analysis. If necessary, remove particulates from the sample by either filtering through a 0.45 μm filter or by centrifuging for 10 minutes at 2000 G. Transfer supernatant to clean sample vial.

Step 2: The subsample can be analysed in the same way as described above for the water extracts of the solid sample.

Sample Preparation Techniques for Biological Warfare Agents

General Considerations

Samples, prior to analysis, may be subjected to a variety of preparation techniques. Depending on the type of sample and identification technique used, sample preparation can accomplish the following:

(a) Convert the sample into a format compatible with the detection/identification technology employed.

(b) Homogenize the sample so that microbial agents present within are uniformly distributed.

(c) Improve sensitivity through the removal of chemical inhibitor(s) associated with the sample matrix.

(d) Improve selectivity through the removal or inhibition of competing microbial contaminants.

(e) Improve sensitivity through concentration.

Samples may be presented to a laboratory in a number of different forms, for example, air samples captured in liquids or on filters, samples of water, soil, or vegetation, on swabbing material used to wipe contaminated hard surfaces, or biomedical samples from suspect cases. The aim of processing is to recover the agent of interest from the matrix in which it is contained and to eliminate materials that might interfere with the subsequent analysis.

Wetting

The majority of detection and identification techniques usable with BW agents require the sample to be in a liquid phase. Swab samples are often washed and the resultant solution is analyzed. For solid samples, such as soil, a volume of the solid is homogenized in two or more volumes of liquid and the supernatant is tested. The most common wetting agents are water, saline and pH-balanced buffers.

Detergents

Microbes often form adhesive interactions with surfaces, either with the matrix of a solid sample (i.e., humus from soil, plant leaves, rug fibres, etc.) or the solid support of a sampling device (i.e., the cotton of a swab). In order to free microbes from these surfaces so that they enter solution and are loaded into the detection or identification technology non-ionic detergents may be added to the isolation solution to break the adhesive interactions. Some

microbes are more resistant to detergents than others. Bacterial spores and mid-spectrum agents can survive treatment with detergent concentrations of 0.5% or greater, while detergent extraction of vegetative bacterial cells should be limited to 0.1% or less. Viruses are very susceptible to destruction via detergents and samples suspected of containing virus should not be extracted with detergent.

Flotation

Bacterial spores have a high buoyant density and as a result float in solutions of high specific density. A solution of 1.22 g/ml sucrose plus 0.5% Triton X-100 may be used to selectively isolate spores from a sample by breaking the adhesive interactions between the spores and the sample matrix, and floating the freed spores up to the surface of the solution.

Culture Amplification

In some cases it may be worthwhile to pre-incubate a sample in liquid bacterial culture medium in order to amplify the amount of microbe present and thereby increase the sensitivity of the downstream detection technologies. The liquid culture medium and incubation conditions employed depend on the growth requirements of the suspected BW agent. The sample may be cultured for 4 hours to overnight before aliquots of the media are removed for analysis. Note if the liquid medium used is not specific for the suspected BW agents there is a danger that contaminating microbes will grow faster in the medium and will mask the agent of interest resulting in a false negative result. If culture amplification is used, it is strongly suggested that the sample be split and only half of the sample be mixed with liquid medium. The cultured and uncultured portions of the sample can then be processed separately.

Microbial growth in a sample to which liquid medium has been added is an indication that the sample is biologically active but is not conclusive that any BW agent detected in the sample is viable as the growth could have been due to contaminant microbes. To prove a BW agent detected in a culture amplified sample is viable, it is necessary to either subculture a portion on solid agar medium or to detect an increased signal from a genetic- or immunological-based assay in the cultured portion.

If the suspected BW agent is a virus, the virus may be amplified by adding a portion of the sample to a tissue culture containing a cell line that is susceptible to the virus in question. Culture amplification is not possible with mid-spectrum agents as they are not self-replicating.

Centrifugation

Centrifugation is a separation technique in which the rotation of a sample at high speeds generates a centrifugal force which can be used to remove contaminants or selectively isolate microbial populations. Soil particles, fibres, and animal cells collect at the bottom of a centrifuge tube at speeds of 2,000 to 3,000 g while microbial cells remain suspended in the supernatant. Bacterial cells and spores collect at the bottom of a tube at speeds of approximately 10,000 g while ultracentrifuges are required to achieve the speeds in excess of 100,000 g which are necessary to pull virus particles out of solution.

Filtration

With filtration, an aqueous sample is passed through a filter using positive pressure or vacuum. The filter itself can be composed of a variety of materials such as stainless steel, cellulose acetate, teflon, nylon and polyvinylidene difluoride. Depending on the volume to be filtered, the filter unit may attach directly to a low volume syringe or may be part of a larger filtration apparatus. Depending on the pore size of the filter, a BW agent may be purified from larger environmental particles by passing through the filter or it may be concentrated on the surface of the filter if it is larger than the pores. It is also possible to lyse BW agents in a sample and then pass the suspension through a filter to purify components, such as DNA or protein antigens, for subsequent analysis. Low recovery of nucleic acids or proteins due to adsorption onto the filter is a potential problem when attempting to isolate BW agent components as well as mid-spectrum agents. If purifying biomolecules via lysis and filtration, only treat a portion of the sample with the lytic agents so that the remainder of the sample can be analyzed via culture and biochemical methods.

Animal Inoculation

Inoculation of samples into laboratory animals may be necessary if a rapid identification is required for treatment of exposed individuals exhibiting illness, or to resolve tense political or military situations. Animal inoculation may also be used when there is no alternative artificial culture medium available for the BW agent in question. The inner environment of the laboratory animal provides optimal conditions for a pathogenic BW agent to multiply and thrive while non-pathogenic environmental contaminants are unable to survive. Thus animal inoculation may act as both a purification and an amplification step. Death of the animal and subsequent isolation of the suspect BW agent from the tissues is proof that the agent is not only viable but fully virulent.

Sample Preparation Techniques for Mid-Spectrum Agents

General Considerations

Samples, prior to analysis, will be subjected to a variety of preparation techniques. Depending on the type of sample and identification technique used, sample preparation can accomplish some or all of the following objectives:

(a) Improvement in sensitivity through the removal of chemical interference associated with the sample matrix.

(b) Improvement in sensitivity through concentration of the analyte.

(c) Increase the analyte volatility by derivatization.

(d) Removal of sample components that can damage sensitive instrument hardware such as GC columns, HPLC pumps and columns or mass spectrometer sources.

(e) Aids in the structural characterization of peptides and proteins (e.g. enzymatic digestion and derivatization).

Particulate Removal

In general, it is assumed that a sample containing a mid-spectrum agent will either be collected as an aqueous sample (from a high volume aerosol sampler) or dissolved in water prior to further sample handling or analysis by electrospray-mass spectrometry (ESI-MS). The removal of particulates from an aqueous sample is necessary prior to analysis in order to minimize damage to analytical instrumentation. This may be done by filtration or centrifugation.

With filtration, an aqueous sample is passed through a filter using positive pressure or vacuum. The filter itself can be composed of a variety of materials such as stainless steel, cellulose acetate, teflon, nylon, and polyvinylidene difluoride. Depending on the volume to be filtered, the filter unit may attach directly to a low volume syringe or may be part of a larger filtration apparatus. Low recovery of peptides or proteins due to adsorption onto the filter is a potential problem, especially with stainless steel and cellulose acetate filters.

Ultrafiltration is a technique for separating molecules in a solution based on their effective size. This filter is a selective semi-permeable membrane that retains most macro-molecules above a certain size while permitting smaller molecules to pass into the filtrate. These filters are characterized with a molecular weight cut off but do not retain all the molecules larger than the nominal pore size because some molecules deform and pass through the filter. It has been reported that ultrafiltration may lead to significant losses of proteinaceous compounds.

Centrifugation is a separation technique in which the rotation of a sample at high speeds (e.g. 2000-3000 g) generates a centrifugal force, which can be used to remove particulates.

Lyophilization

Lyophilization or freeze drying is a technique for removing moisture from a sample by freezing the sample and subsequently subliming away the moisture. The three basic steps in this technique are: 1) freezing, 2) sublimation and 3) dehydration. This technique is widely used for the preservation of biomolecules. For example, peptides or proteins containing methionine are unstable in aqueous solutions as they are oxidized to the corresponding sulphoxide. For this reason, peptides or proteins are typically purchased and stored as lyophilized powders.

Digestion

Digestion is a chemical or enzymatic process in which the chemical bonds between the amino acids of the proteinaceous mid-spectrum agents are cleaved resulting in a series of smaller peptides. The most frequently employed enzymatic digestion process involves the use of the enzyme trypsin. Trypsin selectively cleaves peptide linkages on the C terminal side of lysine and arginine. The resultant lower mass peptide fragments may be characterized on the basis of molecular mass by mass spectrometry. The masses obtained via digestion constitute a mass map that may aid in the identification of the mid-spectrum agent.

Proteinaceous mid-spectrum agents or their enzymatic fragments may also be subjected to Edmans degradation, a chemical process that results in the sequential cleavage of the peptide linkages. This process uses phenylisothiocyanate which reacts with the amino acid on the N terminal of the peptide. Each amino acid adduct that is cleaved from the peptide is identified by HPLC-UV. Successful application of Edmans degradation will result in the acquisition of the complete amino acid sequence for the mid-spectrum agent or its enzymatic fragments.

Derivatization

Chemical derivatization of a proteinaceous mid-spectrum agent may be used to characterize the amino acids present in the agent. The presence of disulphide bridges can be determined using dithiothreitol, which cleaves the disulphide bond between two cysteine amino acids. The number of basic amino acids can be determined by acetylation using acetic anhydride, which will increase the molecular mass by 63 Da for each basic amino acid. The number of acidic amino acids can be determined by methylation, which will result in an increase in molecular mass of 14 Da for each acidic amino acid.

Sample Preparation Techniques for Chemical Warfare Agents

General Considerations

Samples, prior to analysis, may be subjected to a variety of preparation techniques. Depending on the type of sample and identification technique used, sample preparation can accomplish some or all of the following objectives:

(a) Improvement in sensitivity through the removal of chemical interference(s) associated with the sample matrix.

(b) Improvement in sensitivity through concentration and/or derivatization of the analyte.

(c) Increase the analyte volatility by derivatization.

(d) Removal of sample components that can damage sensitive instrument hardware such as GC columns, HPLC pumps and columns or mass spectrometer sources.

Liquid/Liquid Extraction

Liquid/liquid extractions are primarily used for extracting analytes from an aqueous matrix into a water immiscible organic solvent. Extraction efficiency is a function of the distribution ratio of the analyte between the two phases and the ratio of the volumes of aqueous to organic solvent.

Liquid/liquid extractions can be used to quantitatively pre-concentrate an analyte with concentration factors of about ten-fold being typical. There are numerous water immiscible organic solvents, however in practice only a few, such as hexane and dichloromethane, are routinely used for the LLE of chemical warfare agents. Emulsion formation, especially when using chlorinated solvents, is a common problem. In practice a known volume of the aqueous sample is combined with a smaller volume of organic solvent (ratios of 10:1 are common) and the two phases vigorously mixed, allowed to separate then the organic layer removed. Depending on the extraction efficiency, additional extractions with fresh solvent may be required. Finally the organic layers are combined, dried, concentrated and analyzed.

LLE may result in the cleanup of a sample by selectively extracting the analyte of interest from a "dirty" matrix. Although a solvent may extract an analyte with high efficiency from an aqueous sample, it is likely that the solvent will have co-extracted a range of other compounds. For this reason chromatographic separations will likely be required as part of the analysis.

Solid Phase Extraction

Solid phase extraction (SPE) is a physical extraction technique in which an analyte, usually in aqueous solution, is passed through a solid adsorbent bed. Under proper conditions the analyte interacts with the adsorbent and is selectively extracted from the aqueous solution. SPE is often used as an alternative to liquid/liquid extractions of aqueous samples.

Solid phase adsorbents are typically modified silica, which are packed into cartridges ready for use. The adsorbent is solvated with an appropriate organic solvent and then washed (generally with water for aqueous samples) prior to the application of the sample. The sample is applied to the cartridge, and passed through using either vacuum or positive pressure. The retained analyte is desorbed from the cartridge using a solvent of sufficient strength to interrupt the analyte/adsorbent interaction.

This form of extraction can be used to quantitatively pre-concentrate an analyte with concentration factors of 10-100, or to cleanup a sample by retaining the analyte of interest during removal of matrix components. Typical solvents used in SPE include: hexane, dichloromethane, isopropanol and acetone. Emulsion formation is not a problem as is the case in liquid/liquid extraction. There are documented cases of the irreversible adsorption/ decomposition of chemical warfare agents on solid adsorbents, therefore detailed studies need to be performed with the analyte of interest prior to the use of solid adsorbents. As with liquid/liquid extraction, the extraction process is not entirely specific. It should be expected that a range of other compounds from the sample will be retained and subsequently desorbed from the adsorbent.

Solid Phase Micro-Extraction

Solid phase microextraction (SPME) is a relatively new analytical technique. It can combine sample preparation and GC or LC analysis in one step. Analytes are absorbed or adsorbed on a stationary phase coated onto a polymeric fibre. Stationary phases used in SPME are apolar or moderately polar. Typical phases are: polyacrylate, poly(dimethylsiloxane), poly(dimethylsiloxane) / divinylbenzene and Carbowax / divinylbenzene. The fibre can be either exposed to the headspace above a sample or directly inserted into a liquid sample. Analytes, once collected on the fibre, can be either solvent desorbed and injected into a GC or LC, or thermally desorbed into a GC injection port. This technique complements existing methods and has been recently applied to the analysis of vesicants and nerve agents.

Liquid/Solid Extraction

Liquid/solid extraction is a physical extraction technique used for extracting analytes from solid matrices such as soil, adsorbents or man-made materials. Organic solvents are typically used as the extracting solvent, although in a number of cases water has been used successfully. Due to the volumes of solvents employed, this form of extraction is typically used for sample cleanup rather than concentration.

Extraction is performed by placing a sample of the solid material into a vessel containing an appropriate solvent. For short extraction times (up to 1/2 hour), the use of ultrasonification can enhance the extraction of organics from solids. For longer times, a soxhlet type extractor

is often used. In general a suitable solvent must be chosen that will extract the analyte of interest from the matrix, but leave behind the bulk of the undesired matrix components. It is possible to perform sequential extractions with various solvents or the same solvent at different pH's in order to selectively remove analytes from the matrix. If the analyte is absorbed on a man-made material such as paint, clothing or an aerosol filter, care must be taken in choosing a solvent which will not react with or dissolve the sample material.

Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is a physical extraction technique in which analytes are extracted from either solid or semi-liquid matrices by a fluid maintained at supercritical conditions. Typically the matrix is a solid such as soil and this technique may be used to cleanup a sample or pre-concentrate the analyte of interest.

SFE offers the potential of high concentration factors where the analyte is extracted from the matrix and deposited onto a suitable medium followed by elimination of the supercritical fluid.

In the laboratory, a sample is loaded into an extraction thimble and installed in the extractor. A supercritical fluid is then either allowed to flow through the thimble (dynamic extraction) or to fill the thimble for a preset time (static extraction). The density of the supercritical fluid and the temperature under which the extraction is conducted will influence the extraction efficiency. The supercritical fluid carrying the analyte is then depressurized, vented to atmosphere and the extracted analyte(s) recovered.

Although a relatively new technique, it is clear that no single trapping method has proven to be suitable for all analytes. Trapping methods include; bubbling through a solvent, depressurizing the fluid into a vial (either empty or containing glass beads) and depressurizing the fluid onto a solid phase extraction cartridge. Although carbon dioxide is currently the most commonly used supercritical fluid, it does not possess a very high solvating power and for this reason modifiers such as methanol are added to the fluid.

Headspace

Headspace sampling can be used for the sample cleanup and concentration of analytes from the vapour above either a liquid or solid sample. Although there are a number of variations in how headspace sampling is performed, typically the vapour above a sample is collected and analysed for the presence of volatile compounds.

Concentration factors for these systems are difficult to calculate, but it is clear that when the analyte is trapped onto a solid adsorbent pre-concentration will occur. In practice it is useful to be aware that the sample collected for analysis is only representative of the analytes in the vapour phase. The varying volatilities of analytes will result in an inaccurate reflection of the composition of the bulk sample.

Thermal Desorption

Thermal desorption is not normally considered a sample preparation technique. It is generally regarded as a means of sample introduction into an analytical instrument. However given the range of matrices (solid-gases) that are amenable to thermal desorption, the technique occupies a unique place among sample preparation techniques.

Thermal Desorption, when combined with a chromatographic technique, can be used to analyze volatiles collected using the headspace technique described previously. In some cases, portions of a solid sample may be placed in the thermal desorption apparatus itself and desorbed directly. Care should be taken as thermal desorption may cause decomposition of the sample and the creation of artifacts.

For liquids or extracts in volatile organic solvents, relatively large volumes (e.g., > 100 μL) may be injected onto adsorbent tubes followed by removal of the solvent using a gentle stream of inert gas. The pre-concentration of analytes arises due to differences in "breakthrough volumes" (adsorption affinities) between the solvent and the analytes.

Analysis of the samples is conducted by heating the tube which thermally desorbs the organic components into a gas chromatograph. Proper temperature selection during the thermal desorption step may permit selective desorption of certain materials to the exclusion of others depending, in part, on the relative volatilities and adsorbent affinities exhibited by the adsorbed analytes.

Derivatization

Derivatization of analytes may be necessary in order to improve sensitivity and/or enhance volatility for GC analysis. This technique is most frequently employed during the analysis of samples containing the hydrolysis products of chemical warfare agents.

Some general derivatization methods applicable to chemical warfare agents and their hydrolysis products are:

(a) **Diazoalkylation**. Diazomethane is used to enhance the volatility of chemical warfare agent hydrolysis products. The principal disadvantage of this method is that diazomethane is a toxic, potentially explosive compound.

(b) **Silylation**. Silylation is used to enhance the volatility of chemical warfare agent hydrolysis products. N,O-Bis(trimethylsilyl trifluoroacetamide (BSTFA) and N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) are typically used to produce trimethylsilyl and t-butyldimethylsilyl derivatives of chemical warfare agent hydrolysis products. t-butyldimethylsilyl derivatives have the advantage of being more stable in the presence of small amounts of water.

(c) **Pentafluorobenzylation**. Pentafluorobenzyl bromide is used to enhance the volatility of chemical warfare agent hydrolysis products and has the added

advantage of improving sensitivity during GC with electron capture or negative-ion chemical ionization mass spectrometric detection. The derivatization reaction requires several hours and the use of sodium hydride and 18-crown-6 ether.

(d) **Methylation**. Trimethylphenylammonium hydroxide is used to methylate the hydrolysis products of chemical warfare agents. An advantage of this method is that trimethylphenylammonium hydroxide may be used to elute and derivatize, in one step, the hydrolysis product(s) trapped on an ion exchange column.

(e) **Thioesterification.** GC analysis of lewisite is hampered by either the thermal decomposition or adsorption of lewisite. Derivatizing lewisite with either 3,4-dimercaptotoluene or 1,2-ethanediol significantly enhances sensitivity. The disadvantage of this method is that both lewisite and its major decomposition product, lewisite oxide, form the same derivative, making it impossible to determine whether the original sample contained the chemical warfare agent lewisite, the decomposition product or both.

In general derivatization should be avoided unless absolutely necessary, as it alters the analyte and increases the opportunities for sample contamination and artifact formation.

Solvent Evaporation/Concentration

Many of the sample preparation techniques described above produce sample extracts in organic solvents requiring further concentration prior to instrumental analysis. Concentration by solvent evaporation can be achieved through the use of a Kuderna-Danish evaporator or by solvent blowdown using a gentle stream of nitrogen. The key considerations for concentration by solvent reduction are summarized below:

(a) Highly volatile agents (e.g., GB) may be lost if taken to dryness.

(b) Trace contaminants in the solvent will become concentrated and may interfere with the analysis. Using high purity solvents may minimize this problem.

(c) Some solvents or their stabilizers may react with some chemical warfare agents leading to the formation of artifacts. For example, alcohols react readily with chemical warfare agents and their use during sample preparation should be avoided.

Identification Techniques for Biological Warfare Agents

Introduction

Replicating microbes (such as bacteria, rickettsiae and viruses) and non-replicating toxins (mid-spectrum agents) can be detected by many conventional biological techniques. The agents can be detected directly by their presence in a sample or indirectly, as in the case of an infected individual, by serology based on the presence of elevated levels of specific antibodies against the agent. It should be kept in mind, however, that these antibodies may also result from prior infections or from immunizations. Toxins can be either small molecules (e.g., saxitoxin) or large protein molecules (e.g., botulinum toxin). This section describes the most common analytical biology techniques used to detect and identify biological and mid-spectrum agents. The identification of toxins can also be based on analytical chemical methods such as electrophoresis techniques, HPLC, NMR, or MS (see Identification Techniques for Mid-Spectrum Agents).

There is no standard process for the detection and identification of viruses or bacteria. Although rapid, highly sensitive immunoassay- and genetic-based systems are available and commonly used, one should not forget the classic microbiological techniques such as culture, microscopy and biochemical assays. While by themselves, classical methods may be unable to unambiguously identify a microbe, they can provide preliminary information on the type of microbe involved and direct the identification process. Such classical methods also provide valuable supplemental forensic information once the microbe is isolated. Furthermore culture remains the surest way to demonstrate the suspect BW agent is viable and has a much greater evidentiary impact than the computer printouts and graphs associated with genetic- and immunological-based assays. However, one should keep in mind that the positive identification of a BW agent using multiple technologies improves confidence in the identification and provides the most unambiguous conclusion.

The list of detection assays detailed below is not exhaustive as the field is progressing rapidly. For more detailed information on the techniques, one should consult a recent clinical microbiology text. When employing these techniques, it is helpful to conduct a direct comparison of the properties of the unidentified microbe with those of known type cultures.

Non-specific Bacterial Detection Methods

Although not identification techniques, the following methods can be used to screen samples to determine whether or not they contain particles that are consistent with a viable bacterial agent.

(a) **Measurement of ATP.** All bacteria contain adenosine triphosphate (ATP). This can be detected by the conversion of luciferin to a high-energy derivative by the enzyme luciferase. This unstable derivative (oxyluciferin) decays, releasing its energy as light in a process known as bioluminescence. The light intensity is

measured in a luminator and is proportional to the amount of ATP present. This is extrapolated to give the number of microbes, or the total biological material present. Rapid detection kits based on this principle have been developed during recent years. Results can be obtained within a few minutes. Several manufacturers currently offer hand-held luminators.

(b) **Particle counting.** The presence of bacteria in a sample can be detected by analysis of particle-size distribution with a Coulter counter. Also, bacteria may be collected on a filter, which is then stained, and the bacterial count assessed by the intensity of the staining. Turbidometric and light-scattering techniques, which depend upon but do not measure particle density, form the basis for a number of automated detection systems.

Culture

Microbes need nutrients, a source of energy and certain environmental conditions in order to grow and reproduce. In the environment, microbes have adapted to the habitats most suitable for their needs; in the laboratory, however, these requirements must be met by a culture medium. This is basically an aqueous solution to which all necessary nutrients have been added. Depending on the microbe involved culture may take from 12 hours to two weeks.

(a) **Bacteria**. The mixture of necessary nutrients can be used as a liquid medium, or a solidifying agent can be added. Agar is a natural polysaccharide produced by marine algae and is the most commonly used solidifying agent added to media (end concentration of usually 1.5% w/v). If hydrolysis of the agar is suspected, a silica gel is used as a replacement solidifying agent. A sample can be inoculated with a sterile swab or loop into a suitable liquid medium or streaked out on an agar plate. With the last method, individual colonies can be isolated and identified further. Purity of a colony must be confirmed by replating pure cultures at least once on a streak plate and inspecting the resulting colonies for homogeneity. Samples are usually inoculated onto and into several selective or differential culture media. The first preliminary characterization of an isolated colony is the nature of the medium on which the organism is growing. A bacterium may produce a colony that is indistinguishable from colonies of many other species. Therefore additional confirmatory assays are usually conducted on isolated, pure colonies of the suspect microbe.

(b) **Viruses**. Viruses can only replicate within a living host cell. The growth of viruses, while generally slower than direct detection methods, is currently the standard against which other methods are measured, and is necessary for unambiguous viral identification. There are three major systems for growth of viruses in samples: tissue culture, inoculation of embryonated eggs, and suckling mouse inoculation. Of these, the most widely sensitive and flexible is tissue culture. When a sample suspected of containing a virus arrives in the laboratory, it is logged in and inoculated into several tissue culture tubes or flasks, which are then incubated at 36-37°C either stationary or on a roller drum. The medium is usually changed after an adsorption period of several hours to one day in order to prevent the nonspecific toxicity seen with many clinical specimens. Then,

cultures are periodically examined for signs of developing viral growth. Most viruses produce characteristic morphologic changes in particular cell lines and these so-called cytopathic effects (CPE), which are observable with a light microscope, may aid in identification.

Animal Inoculation

Even though modern identification techniques are getting more important, the use of laboratory animals (especially mice) still plays an important role, in the isolation and identification of potential BW agents. Animal inoculation may be required when there is no alternative artificial culture medium available for the BW agent in question. For some mid-spectrum agents, e.g. botulinium toxins, the use of mice is considered the most sensitive method of detection and also the most reliable method of identification. It is also required by international law.

Light Microscopy

The limited resolving power of a light microscope (0.2 μm) can reveal little detail in bacteria with a diameter of about 1 μm. However, examination of a stained preparation of bacterial colonies under oil immersion (1000x) magnification, with or without phase contrast microscopy, can rapidly provide clues as to possible identity. A light microscope reveals two principle forms of bacteria: more or less spherical organisms known as cocci, and cylindrical ones called bacilli.

Phase contrast microscopy shows more of the internal structure of bacteria than does conventional light microscopy and may be used for unstained preparations. Because of their dehydrated cores, bacterial spores are highly refractive under phase contrast and appear as glowing spheres.

There are numerous staining techniques that may be used with a light microscope to visualize fine details and classify the bacteria to a specific group. One of the most useful stains is the Gram stain. Bacterial cells are first fixed to a slide by heat and stained with a basic dye (e.g., crystal violet), which is taken up in similar amounts by all bacteria. The slides are then treated with an iodine-KI mixture to fix the stain, washed with alcohol, and finally counterstained with a paler dye of different colour (e.g., safranine). Gram-positive organisms retain the violet stain, while Gram-negative organisms are decolourized by the organic solvent and hence show the counterstain. Gram-positive organisms possess a cell wall, which presents a permeability barrier to elution of the dye-iodine complex by alcohol. Most bacteria can be divided into two distinct groups: Gram-positive and Gram-negative organisms, which then aids in the selection of subsequent tests to establish the identity of the bacteria.

Although most viruses are too small to be visualized with a light microscope, the cytopathic effects (CPE) caused by a virus in a cell culture can be observed. Generally each virus group produces a characteristic CPE pattern, which may allow a provisional identification. Typical signs of CPE are: lysis (or necrosis), inclusion formation, cytomegaly, giant-cell (or syncytium) formation, and cytoplasmic vacuolisation. Some of these may be difficult to see in unstained preparations. Specific staining facilitates the evaluation.

Electron Microscopy

In order to visualize viruses an electron microscope must be employed. Large numbers of morphologically distinctive viral particles must be present in a sample in order to be detected (minimum 10^7 per millilitre). Morphology may be sufficiently distinct to allow a precise viral diagnosis. Viruses which grow with difficulty in culture and for which there are no practical immunoassays are often best, if not exclusively, detected by electron microscopy.

Negative staining may be employed with an electron microscope to characterize virus particles with respect to size, shape, surface structure, and often symmetry. The method can be applied to viruses infecting all kinds of hosts, including humans, experimental animals, cell cultures, and arthropods. The technique can also be used to characterize virus particles in unpurified material. Negative staining has facilitated the rapid accumulation of data about the physical properties of many viruses.

Thin-section electron microscopy of virus-infected cell cultures and tissues of infected humans and experimental animals provide complementary data on such features as virion morphology, mode and site of virion morphogenesis, and presence or absence of an envelope. In most cases, viruses can be classified into their appropriate family, and often into their appropriate genus, after visualization and measurement by negative-stain and/or thin-section electron microscopy. Thin-section electron microscopy is especially useful for the detection of virus multiplication in tissues. The technique can also be used to visualize bacteria in environmental and infected biomedical samples.

The visualization of virus-antibody interactions by immunoelectron microscopy adds the dimension of antigenic specificity to the morphological characterization of viruses and the technique is, therefore, more specific than standard electron microscopy. Different types of virus-antibody interactions can be observed by immunoelectron microscopy, and several techniques have been developed to characterize viruses in thin sections by means of labeled antibodies, or in suspensions by simple positive or negative techniques. The technique can also be used to characterize bacteria and mid-spectrum agents *in situ*.

Rapid Bacterial Biochemical Tests

The tests described here can be performed directly with inocula from colonies grown on primary isolation plates. Most of them take only a few minutes perform and they all take less than one hour. There are many more such assays than are described here, with new assays constantly being developed. In general, the rapid biochemical tests do not provide a conclusive identification of an unknown bacterium but they do provide information to assist in the classification of the unknown into a defined group of bacteria.

(a) **Catalase test.** The catalase test is useful for the identification of many bacteria. The enzyme catalase catalyses the liberation of oxygen from hydrogen peroxide, a metabolic end product toxic to bacteria. The test can be performed with a small amount of growth removed from an agar surface. The test is performed on a glass slide. Evolution of bubbles of gas indicates the presence of the enzyme.

(b) **Slide coagulase test (clumping factor test).** The slide coagulase test is used to quickly screen for isolates of *Staphylococcus aureus*. Clumping factor is a cell-associated substance that binds plasma fibrinogen, causing agglutination of the organisms by binding them together with aggregated fibrinogen. The reaction must be read within 10 seconds, and the inclusion of an autoagglutination control will decrease the number of false-positive results.

(c) **Oxidase test.** The oxidase test indicates the presence of the enzyme cytochrome oxidase. This iron-containing porphyrin enzyme participates in the electron transport mechanism and in the nitrate metabolic pathways of some bacteria. If an iron-containing wire is used to transfer growth, a false-positive reaction may result. Plastic sticks are recommended. The test must be read within 10 seconds.

(d) **Spot indole test.** Organisms that produce the enzyme tryptophanase are able to degrade the amino acid tryptophan into pyruvic acid, ammonia and indole. Indole is detected by its combination with the indicator *p*-dimethylaminobenzaldehyde (Kovac's reagent) to form a red coloured end product.

(e) **Bile solubility test.** Some bacteria possess a very active autocatalytic enzyme which lyses the organism's own cell wall during cell division. Under the influence of a bile salt (sodium deoxycholate), the microbes rapidly autolyse. The bile solubility test may not always work as old colonies may have lost their active enzyme.

(f) **Spot urease test.** Several bacteria produce the enzyme urease, which hydrolyses urea into ammonia, water, and carbon dioxide. The alkaline end products can be determined by the indicators phenol red or bromocresol purple.

Metabolic Tests

The tests described here can be performed directly with inocula from colonies grown on primary isolation plates. Most of them take only a few minutes to set up but need to incubate several hours or overnight before the results can be read. In general, a single test does not provide conclusive identification of an unknown bacterium but does provide information to assist in the classification of the unknown into a defined group of bacteria.

(a) **Oxidation and fermentation tests.** The oxidation-fermentation (OF) test is used to distinguish between fermentation, for which oxygen is not necessary, and oxidation, for which it is essential. Acid by-products are formed with these processes. Special media containing low concentrations of the nutrient peptone, together with a specific sugar and a pH indicator which changes colour in presence of acid, have been developed for this assay. Samples of the bacterium are seeded into two tubes containing the medium and the surface of the medium in one tube is covered with a thick layer of soft paraffin. If the bacterium can ferment the sugar, a colour change is observed in both tubes; if the bacterium can only oxidize the sugar, a colour change is only observed in the open tube. OF tests are available with a variety of different sugars.

(b) **Hydrolysis tests.** An enzyme that breaks down a substrate by adding the components of water to key bonds within the substrate molecule is called a hydrolase. Substrates commonly used are sodium hippurate, DNA, urea, esculin, starch, casein, lecithin and polysorbate-80. The ability of an enzyme to hydrolyse a specific substrate may offer an identifying characteristic. The products are visually detected via a pH indicator.

(c) **Amino acid degradation.** Enzymes formed by some organisms may deaminate, dehydrolyse, or decarboxylate amino acids, breaking them down into smaller components. One example of such a test is Moeler's medium. Certain amino acids (lysine, arginine, and ornithine) are incorporated into the culture medium together with an indicator. Along with each amino acid being tested, a control tube without the amino acid serves as a blank.

(d) **Single substrate utilization.** Many organisms can be recognized by their ability to grow in the presence of a single nutrient. Substrates used include citrate, malonate, and acetate.

(e) **Nitrate reactions.** Nitrate serves as the source of nitrogen for many bacteria, but it must be broken down by reduction to nitrite. Certain bacteria further reduce nitrite to nitrogen. Reagents are added to a culture of the unknown bacteria in a tube. The reagents combine with nitrite, if present, to form coloured end products within five minutes. An organism that reduces nitrite to nitrogen yields a negative colourless reaction in the first part of the test.

(f) **Triple sugar iron agar (TSIA) and Kligler's iron agar (KIA) reactions.** These tests can provide information on three primary characteristics of a bacterium: the ability to produce gas from the fermentation of specific sugars, the production of large amounts of hydrogen sulphide gas (by the formation of a black iron-containing precipitate), and the ability to ferment lactose in KIA or lactose and sucrose in TSIA.

Multi Metabolic Tests

Individual metabolic tests do not provide enough information to identify a bacterium, however, dozens of metabolic and enzymatic tests run in parallel and entered into a computer database containing the metabolic profiles of known bacteria can provide an identification. Several multi metabolic test systems are now commercially available, facilitating the identification of several groups of bacteria. The API system is one of the most comprehensive comprised of a series of cupules 'galleries' mounted in plastic strips. The cupules contain dry reagents into which a suspension of the purified test organism is placed. The results of these tests, although generally consistent, do not always conform with those obtained by conventional test methods and thus must be interpreted according to the information provided by the API system. The results can be read after 18-48 hours depending on the test kit used. A rapid detection kit format allows reading in four hours. The interpretation of the results is done with the aid of a computer-assisted database.

Immunological Based Assays

Many methods are currently available which are based on the specific interactions between microbial antigens and monoclonal or polyclonal antibodies. Bacteria, viruses and mid-spectrum agents can be rapidly detected and identified in environmental samples or isolated form with immunochemical methods.

All immunological assays can be designed in either direct or indirect formats.

(a) In the direct format the antigen is detected with a specific antiserum which has been directly conjugated to a reporter molecule (e.g., a fluorchrome for an immunoflurescent test, an enzyme for an ELISA, a radioactive molecule for a radioimmunoassay, etc.).

(b) In the indirect format the antigen is first treated with a specific antiserum of a certain species followed by washing and treatment with a second antibody which recognizes the F_c tail region of antibodies in the first antiserum and is conjugated to a reporter molecule.

The direct test is faster and usually suitable for testing for a single antigen. If a moderate number of different antigens are being investigated, an indirect format is more appropriate. In addition, the indirect format is considered to be much more sensitive than the direct method because the second labeled anti-species antibody can bind to multiple sites on the primary antibody thus amplifying the signal.

In general, all immunological assays share a common procedure and differ only in the specific reagents used.

(a) Antigens from the processed sample are applied to a solid matrix. The antigens may be bound to the solid matrix non-specifically with the remaining non-specific binding sites being blocked by a standard protein (e.g., gelatine, bovine serum albumin, or skimmed milk). Alternately a sandwich format where the antigens are be pulled out of solution by a specific antibody that has previously been annealed to the matrix may be used. In a sandwich format, the capture and detector antibodies must recognize different epitopes of the antigen. However in case of a single multivalent antigen both antibodies may have the same specificity.

(b) Between all steps, the solid matrix is thoroughly washed to ensure that only components of the specific antigen-antibody complex remain attached to the solid matrix.

(c) Once the direct or indirect antigen-antibody complex is formed in the assay, the substrate of the reporter molecule (i.e., the substrate of the enzyme in an ELISA system) is added, and/or the presence of the specific complex is confirmed via a detection system.

Specific immunological based assay systems are:

(a) **Immunofluorescence (IF).** The sample antigen is bound to a microscope slide and the detector antibody is conjugated to a fluorochrome dye. When the slide is loaded into a fluorescent microscope if the fluorochrome remains in the sample due to the antigen-antibody complex and is irradiated with light of a certain wavelength (excitation wavelength), then light is absorbed and promotes the fluorochrome into an excited energy level. This excited energy state t immediately decays and the molecule returns to its ground state, releasing the energy difference between the levels as light. The emitted light is of a longer wavelength (emission wavelength) than that which was used to stimulate the fluorochrome. This longer wavelength emission constitutes fluorescence. By simultaneously using different fluorochromes conjugated to antibodies differing in specificity, screening for antigens from multiple BW agents on one slide is possible.

(b) **Enzyme linked immuno-sorbent assay (ELISA).** In an ELISA the processed sample is adsorbed to the plastic surface of a microtitre plate well and the reporter system is an enzyme conjugated to the reporter antibody. After washing away unbound antibody, the substrate for the enzyme is added and is converted to a coloured product. These assays can be quantitated since the intensity of the colour is proportional to the amount of antibody bound to the antigen. ELISAs are relatively rapid requiring between a few hours and a day to perform. Many samples can be simultaneously measured. In addition, the spectrometer required to quantitate the colour product is relatively inexpensive.

(c) **Inhibition ELISA.** In an inhibition ELISA, the antibody (in a fixed, known concentration) is mixed with varying concentrations of the processed sample. The mixtures are then applied to an ELISA plate that has been coated with a known antigen at a known concentration. Inhibition of the expected signal between the antibody and the known antigen bound to the plate indicates the recognition of antigens in the processed sample by the antibody. Therefore, the processed sample contains the same antigen as is absorbed to the ELISA plate.

(d) **Rapid Chromatographic Immunoassay.** A rapid hand-held test kit, which is sensitive and simple, is based on the sandwich format. A capture antibody is immobilized on a strip of nitrocellulose membrane in the form of a line or a spot. A second antibody is coupled to coloured particles and embedded as a dry reagent on a filtration wick at one end of the membrane strip. The liquid sample containing the antigen is applied onto a second wick contacting the first wick. The antibody-coated particles are rehydrated. The antigen will be bound by the antibody-coated particles and the complex will migrate by capillary action along the nitrocellulose strip until it meets the capture antibody where it is bound and immobilized. A visible coloured line or spot indicates a positive result. The assay only takes approximately 15 minutes to run and is readable with the unaided eye.

(e) **Real Time Biospecific Interaction Analysis (RBIA).** RBIA is a method based on surface plasmon resonance (SPR) detection, but other types of sensors can be

used. SPR is a quantum mechanical phenomenon detecting changes in optical properties near the surface of a sensor chip. These changes are directly related to changes in mass concentration at the sensor chip surface. The biomolecule of choice (antigen or antibody) is coupled to a flexible, uncrosslinked dextran matrix to which samples are subsequently introduced. The use of RBIA offers several important advantages over other immunoassay systems. First, none of the interacting components need to be purified or labeled as the SPR response is obtained from binding of each component to the sensor surface, in proportion to its mass. All stages in the binding process can thus be monitored. Second, each stage of the binding sequence is easily quantitated and the technique allows multi-site specificity tests using a sequence of several antibodies. Finally, large numbers of analyses can be processed automatically.

(f) **Light Addressable Potentiometric Sensor (LAPS).** The LAPS is a solid state semiconductor electrode that detects changes in electropotential which arise from chemical reactions. It employs a pH sensitive insulator surface to specifically measure pH changes in microvolumes. This insulator surface, deposited on a silicon chip, is impervious to aqueous and many organic solutions. Measurements are made using intensity modulated light emitting diodes (LED) which are adjacent to the sensor, but isolated from the electrolytes. An immunoassay using LAPS typically uses two labeled antibodies. The first antibody is used to capture the antigen-antibody complex, the second is used for signal generation. Initially the antigen is incubated with a solution containing the labeled antibodies. The next step involves the filtration-capture of the antigen-antibody complex through interaction of the label on the antibody with a suitably modified membrane. Signal detection can be generated directly by labeling the second antibody with the enzyme urease. Alternatively the second antibody is labeled with fluorescein which in turn reacts with anti-fluorescein urease. This indirect approach is favoured over the direct method as it is easier to label the antibody with fluorescein than labeling the antibody with urease. The membrane is then inserted into the LAPS reader cell (containing urea). Urease catalyses the hydrolysis of urea to ammonia and carbon dioxide and the formation of ammonia increases the pH at the insulator surface. The rate of change in pH is proportional to the number of enzyme molecules at a site on the membrane. As the pH changes so does the magnitude of the photocurrent because of the effect of pH on the sensor's surface potential. The system compensates for this change by adjusting the voltage applied to the LAP sensor. The change in applied voltage required to return the photocurrent to its former value is a direct measure of the change in pH at the insulator surface.

(g) **Radioimmunoassay** (RIA). The principle of a RIA is basically the same as for the ELISA except the reporter antibody is tagged with a radiolabel and the antigen-antibody complex in the plate well is detected with a scintillation counter.

(h) **Western blot.** In the Western blotting technique the antigens in a processed sample are first denatured and separated by gel electrophoresis [usually sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)] and then transferred by blotting to sheets of nitrocellulose membrane before being treated with

antibody. One disadvantage of the technique is that the proteins remain denatured to an unknown degree after separation and blotting. As a result monoclonal antibodies that are specific for the native conformation of an antigen may fail to bind to the antigen fixed to the membrane. For this reason non-SDS-PAGE gels should always be run in parallel. A miniaturized version is commercially available at present. With such a system, prepacked gels (available for both non-denaturing and SDS-PAGE together with gels for isoelectric focusing) and reagents are used, and the separation and staining can be run automatically. The blotting is done by thermal diffusion or, in addition, can also be conducted electrophoretically. Resolution with this system is extremely good. The complete separation and blotting take between two and three hours.

(i) **Agglutination tests.** Agglutination refers to the clumping of cells or beads to which a specific antibody has been conjugated. Upon addition to a processed sample, any specific multivalent antigen present will interact with specific antibody and cause clumping. Agglutination tests are simple, rapid, sensitive and inexpensive but may suffer from nonspecific reactions and prozone effects, where excess antigen inhibits lattice formation. The prozone effect may be overcome by testing the processed sample at multiple dilutions.

(j) **Haemagglutination and Haemagglutination Inhibition (HAI).** Haemagglutination, the clumping and aggregation of red blood cells, is a rapid physical measure of the presence of a virus whose surface proteins cause agglutination of red blood cells. Many viruses possess the property to agglutinate the erythrocytes of certain animal species. The haemagglutination can be inhibited by letting the virions first react with specific antibodies. This is the basis of the haemagglutination inhibition (HAI) tests, which are used either to identify virus isolates by means of specific antisera or to measure antibody levels by means of standard virus suspensions. The HAI test is one of the most widely used procedures for the serological diagnosis of virus infections. Unfortunately, HAI tests are hampered by the fact that many sera contain various nonspecific inhibitors that may block agglutinating sites on the erythrocytes.

(k) **Passive or Indirect Haemagglutination.** Passive agglutination utilizes red blood cells to which either virions or antibodies have been coupled. Agglutination of the coated cells is caused by the addition of the complementary serological reactant. Prior to coupling, the erythrocytes are stabilized with compounds such as tannic acid, glutaraldehyde or sulphosalicylic acid. The passive haemagglutination reaction with virus-coated cells can be inhibited by prior incubation of the antiserum with the homologous virus. This inhibition test lends itself to the measurement of very small quantities of viral antigen.

(l) **Latex test.** In this test either the antigen or the antibody is adsorbed onto polystyrene latex particles. At present, a large variety of latex beads (various sizes and colours) are available, permitting simultaneous identification of various antigens. The principle is basically identical to the passive haemagglutination inhibition. Latex assays have replaced the passive haemagglutination assays since coated latex beads are more stable and better defined. The test is extremely rapid

and should be performed on glass plates as the stickiness of plastic may prevent agglutination. The test requires no instrumentation and can be performed outside the laboratory. Latex tests normally suffer from low sensitivity especially with low concentrations of antigens.

(m) **Immune Adherence Haemagglutination Assay (IAHA).** In this technique, three successive reactions take place. First the antigen and the antibody are allowed to form complexes, next the complement is added and finally type O human erythrocytes are added to agglutinate the complement-bearing complexes. The procedure, which is normally performed in microtitre plates, is more sensitive and easier to perform than classical complement fixation (CF) tests. The IAHA is less affected by variations in test conditions than CF and is also less subject to interference by the anti-complementary activity of certain antisera.

(n) **Complement Fixation (CF).** The complement fixation test is one of the most applicable serological tests. Complement is a multi-component system of enzymes present in an inactive state in any serum. The system becomes activated when a specific site on the F_c portion of antibody molecules becomes exposed as a result of antigen binding. This portion is then able to bind to the C1q component of complement. When the antibody is directed against the membrane antigens of red blood cells, the binding of C1q leads to a cascade of enzymatic reactions, which ends in the lysis of the cells and the release of hemoglobin. When the antibody is directed against a virus particle, complement also becomes irreversibly bound upon formation of the virus-antibody complex and this leads to the effective removal of complement from the antiserum. The CF test is a two-stage procedure involving a viral test system and an indicator system consisting of sheep erythrocytes and their corresponding antibodies. The complement present in both the antiserum sample and the anti-sheep erythrocyte serum is destroyed by heating. A small amount of guinea pig complement, sufficient to cause complete haemolysis of the sheep erythrocytes, is added to the test antiserum.

(o) **Virus neutralisation.** The virus assay is the main culture method for assay detection. It is performed in monolayer cell cultures. The cells are propagated under conditions that will produce a contiguous monolayer after several days of growth. The growth medium is removed and the virus-antibody mixture is added to the surface of the monolayer. The virus should be diluted to yield a suitable number of lesions. The infected monolayer is covered with an overlay medium that nourishes the cells and prevents diffusion of released progeny virus by incorporating a semisolid gelling agent. Lesions are counted and their presence is made more visible by incorporating a vital dye into the overlay medium. The 50% endpoint yields the dilution factor of the antiserum. The neutralization test consists of two phases: (a) reaction of virus with antiserum *in vitro* and (b) assaying the result by infectivity titration. At present, infectivity titrations are performed preferably in cell cultures if possible, or if not, in susceptible animal hosts. The goal is to determine the minimal amount of antiserum that can reduce a small number of infectious units of virus (e.g., 100) to an average of 0.7 infectious units per volume of inoculum. This corresponds to the ID_{50} - the

amount of virus for which there is a 50% probability of causing infection in one test subject. The procedure commonly referred to as constant-virus-varying-serum, entails mixing a constant amount of virus with varying dilutions of antiserum. The concentration of residual infectious virions is assayed by administering each mixture to several test subjects. A modification of the neutralization assay with cells allows quantitative detection. As a result of the cytotoxicity of a virus preparation, cells become detached from the solid phase and the proteins from the remaining living cells, after a wash step, are stained. After removing the excess dye, the remaining dye is dissolved and the absorbance is measured. This procedure makes the assay suitable for measuring a coloured product instead of visual inspection and counting for lesions. More samples can thus be assayed. Such an assay is also suitable for detection of mid-spectrum agents or toxin neutralizing antibodies if the toxin exhibits a cytopathic effect on a cell line.

Serological Tests

Serological tests are immunological based assays where instead of trying to identify an unknown microbe with antibodies of known specificity, known antigenic preparations from pathogenic microbes are used to confirm exposure to an agent or diagnose disease in an individual. Any of the immunological based assay techniques detailed in the last section can be used in an indirect format in a serological test. For a serological test, the known antigenic preparation acts to capture the specific antibodies from the test serum and these are then tagged for detection by a reporter antibody. Most acute primary microbial infections induce a dependable rise in serum antibody. Measurement of antibody levels over the course of disease in comparison with serum collected prior to disease can be used for diagnosis. As it takes time for an immune response to develop, serological tests are generally run one to two weeks after the potential exposure or after the onset of symptoms. It should be kept in mind, however, that these antibodies may also be present as the result of prior infections or immunizations. Serological tests for the diagnosis of bacterial, viral and mid-spectrum exposure have been developed.

Serological tests have several potential advantages over cultural methods for diagnosis. These are particularly evident when the causative organism cannot be cultured in artificial media but a preparation of antigen can be obtained. Cross-reactions between unrelated organisms with antisera occur, however, and results should be interpreted with care. This was especially the case in the past, but with the advent of species-specific monoclonal antibodies, identification can be made more precisely. At present, it has also become common practice to use purified and defined antigens instead of crude extracts from bacteria or whole cell preparations. The main disadvantage of serological tests is the poor detection limit compared with culturing methods.

Because of the time required for an immune response to develop, serological tests would not be helpful in the initial characterization of a BW event. However, the demonstration of an immune response to the agent involved in exposed humans or animals after the event would assist in the confirmation of the event and of the identity of the agent.

Genetic Based Assays

Nucleic acids provide the blueprints for all life on Earth. In bacteria deoxyribonucleic acid (DNA) provides the archival genomic blueprint and is duplicated and passed on to progeny during replication while ribonucleic acid (RNA) is transcribed from the DNA and used to build proteins necessary for the structure and metabolic functions of the cell. Depending on the group they belong to, the genomic blueprint of a virus may be encoded by DNA or RNA. The genetic content of a species is unique to that species although near-neighbour species may possess a high degree of similarity. Several genetic based assay methods are available for the identification of BW agents. The methods can not be used with mid-spectrum agents because these agents do not contain any genetic material.

(a) **Molecular Taxonomy.** Molecular taxonomy is the comparison of the genetic sequences of chromosomal DNA or ribosomal RNA to establish similarity patterns. Although the DNA contents in purine (G, guanine; A, adenine) and pyrimidine (C, cytosine; T, thymine) bases vary from one individual to another, they remain constant within a given species. The G+C content in bacteria and viruses can therefore be used to establish taxonomic relationships. Similarities between the sequences of 16S or 23S ribosomal RNA are also compared in order to classify a bacterium.

(b) **Nucleic Acid Hybridization.** Nucleic acid hybridization relies on the identification of unique sequences in the genome of the BW agent of interest. Single stranded sequences of DNA complementary to the unique sequence are synthesized, labeled with a reporter molecule (i.e., radioactive molecule, fluorochrome dye, or an enzyme) and used to screen a genomic digest of the unknown microbe. Prior to treatment with the labeled DNA sequence, the genomic digest is run on an electrophoretic gel to separate the nucleic acid fragments based on size and then transferred to nitrocellulose. If the technique is used to detect DNA it is called a Southern blot and if it is used to detect RNA it is called a Northern blot. The technique only works if the unique genetic target is present in substantial quantities in the tested sample. If this is not the case, the technique lacks sensitivity, even if the reporter molecule used is a radioactive molecule.

(c) **Polymerase Chain Reaction (PCR).** Like nucleic acid hybridization, PCR also relies on the identification of unique sequences in the genome of the BW agent of interest, however, rather than building a strand of DNA complementary to the complete unique sequence only small tags, called primers, which are complementary to the two ends of the sequence, are constructed. The primers are combined with enzymes and singular nucleotides, added to the sample of interest, and the solution is loaded into an automated PCR machine. If the genetic target sequence is present the primers will bind to each end and the enzymes will use the nucleotides to selectively build from the primers to replicate the entire unique sequence. The replication cycle can be repeated using the original segment and the copies as templates with the unique sequence being amplified exponentially. Because of the exponential amplification the technique is extremely sensitive, theoretically able to detect as low as one copy of the sequence of interest in a

sample. This sensitivity is a both a strong and weak point for the technique. PCR can be used on unpurified samples to give a preliminary detection within hours of receipt, however, the assay can easily be contaminated by previously amplified DNA molecules and therefore stringent sterile techniques must be observed at all times. One must also be careful of inhibitors to the PCR enzymes in soil and other environmental samples. One way to overcome the presence of inhibitors is to split a sample in half, spike one half with a preparation of DNA from the agent of interest, and run both samples in parallel. If the DNA from the spiked sample is successfully amplified then there were no inhibitors present in the sample and one can be confident in the results of the unspiked sample. If neither the spiked or unspiked sample is amplified then there are inhibitors in the sample and the results should be taken as a potentially false negative. There are several ways to detect the amplified product of a PCR reaction:

i. A sample from the reaction tube can be run in a gel electrophoresis, which separates any fragments present on the basis of size. Since the sequence of the genetic target is known, its size can be extrapolated and one need only look for a large band of DNA at that location on the gel. The bands are visualized on the gel either by staining with ethidium bromide for unlabelled PCR products, the addition of a specific substrate for enzymatically labeled products or exposure of the gel to an autoradiograph for radioactively labeled products.

ii. The contents of the reaction tube can be washed of any unused primers and applied to a 96-well plate. If a radioactive reporter was used the samples can then be read with a scintillation counter. If an enzymatic reporter was used, the substrate of the enzyme is added and the samples are read on a spectrometer.

iii. Fluorochrome labeled primers have been designed with inhibitors attached to the primer. The transcription of DNA from the end of the primer either cleaves off or sterically moves the inhibitor so that the fluorochrome is no longer quenched. Several PCR machines have been developed with built-in fluorescent spectrometers which can monitor the development of the fluorescent signal in real-time. This technology is much less laborious and time-consuming that the other PCR visualization techniques and is the focus of future developments for PCR assays.

(d) **Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).** Identical to regular PCR in execution, RT-PCR includes reverse transcriptase; an enzyme that can convert RNA to DNA. The technique can be used to detect and identify viruses which have RNA based genomes.

Microbial Isolate Preservation

Once a suspect microbe has been isolated from a sample through culture it may be stored as follows:

(a) **Refrigeration.** Refrigeration can be used for short-term storage. Cultures streaked on agar slants or stab cultures may be viable over several months when stored at 4°C. Agar plate cultures have to be sealed to prevent drying out. To preserve cultures for longer periods of time, two methods are commonly employed:

(b) **Deep freezing.** A pure culture of bacteria is suspended in a liquid, quick-frozen (often with liquid nitrogen) and subsequently stored at temperatures between -50°C and -95°C. Sensitive microorganisms require the presence of glycerol or extra protein (skimmed milk powder) in a concentration of 15-20%, which act as "anti-freeze" to protect them. Cultures can be thawed and used up to several years later.

(c) **Lyophilization.** A suspension of bacteria is quickly frozen and the water removed by means of a high vacuum. The microbes survive in the powder-like residue for several years and can be revived at any time by rehydration of the culture in a nutrient medium. Bacterial strains ordered from strains collection are usually delivered in this form.

Identification Techniques for Mid-Spectrum Agents

Biological Techniques

The following BW agent identification techniques outlined in the previous section may be used to detect and characterize mid-spectrum agents:

(a) Animal inoculation,

(b) Immunoelectron microscopy,

(c) Immunological based assays particularly ELISA, inhibition ELISA, rapid chromatographic immunoassay and toxin neutralisation formats, and

(d) Serological tests.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a separation technique in which a multi-component sample, in the liquid phase, is resolved into its various components. HPLC is well suited to the separation of thermally labile or low volatility compounds that cannot be easily analysed by GC. The first step in this technique is the introduction of the sample into a chromatographic column where the separation occurs. Sample introduction in HPLC is accomplished with a switching valve equipped with a fixed volume sample loop. The sample is injected as a liquid plug into the flow stream and swept onto the HPLC column for separation.

Separations can be performed under isocratic or gradient elution conditions with reversed phase (e.g., C_8 or C_{18} columns) separations being the most common. Separations are based on the physical interactions of the sample components with the liquid mobile phase and a stationary phase. Under reversed phase gradient elution conditions, the composition of the mobile phase increases in organic content over time. The time that it takes for a component to travel through the column is known as the retention time. For a specific column and mobile phase composition, retention time is a reproducible characteristic of each component. Following chromatographic separation, identification of the sample components is required. Mass spectrometry is the technique of choice for identification of mid-spectrum agents.

Mass Spectrometry

Electrospray ionization (ESI) is the most widely used ionization technique for the LC-MS analysis of mid-spectrum agents. ESI-MS generally produces multiply charged molecular ions of the general form $(M+nH)^{n+}$. These ions may be used to establish the molecular mass of the mid-spectrum agent. Under appropriate ESI conditions, it is possible to promote collisionally activated dissociation in the interface resulting in the formation of product ions that may aid in structural identification. Amino acid sequence information for peptides and

peptide fragments may be determined under these conditions. Tandem mass spectrometry can also be used for this purpose.

Matrix assisted laser desorption ionization (MALDI) represents a complimentary means of ionization for mid-spectrum agents. Analyses are generally performed without chromatographic separation, a disadvantage in the case of multi-component samples. The principal advantage of this technique is it's ability to ionize mid-spectrum agents with molecular masses in excess of 100 kDa.

Gas Chromatography

Gas chromatography (GC) is a technique suited to the separation of gases or volatile compounds. In gas chromatography, a multi-component sample, in the gas phase, is resolved into its various components. The first step in this technique is the introduction of the sample into a chromatographic column where the separation will occur. Liquid samples are usually injected using an on-column injector or a split/splitless injector. Gas samples are usually injected using gas sampling valves, headspace injection or thermal desorption.

The majority of analyses are performed under temperature programming conditions, where the column temperature is increased with time. As the sample moves through the column it partitions between the carrier gas and a stationary liquid phase. Each component of the sample spends a different length of time in the liquid phase. The time that it takes for a component to travel through the column is related to the time spent in the liquid phase and is known as its retention time. The retention time for a component on a specific column is a reproducible characteristic of the component.

In practice, retention time data are difficult to reproduce between different laboratories due to variations in operating conditions and instrumental parameters. These difficulties may be overcome by using one of a variety of retention index systems that relate the retention time of the analyte to the retention times of a series of standards. In the Kovats system (for isothermal analysis) and the McReynold's system (for temperature programmed analysis), the retention time of an analyte is related to that of a series of n-alkanes, while other systems make use of phosphorous and sulphur containing standards. Because it is possible for more than one compound to elute with the same retention time, retention indices for a given analyte should be determined on several columns, each of different stationary phase polarity (e.g., DB-1, DB-5, DB1701, etc.), in order to increase the certainty in the analyte identity.

Detection of the components as they elute from the chromatographic column may be performed by a variety of universal and selective detectors. The most common is the flame ionization detector (FID), which responds to the majority of organic compounds. When dealing with complex matrices, selective detectors such as flame photometric (FPD, which responds to sulphur or phosphorous), thermionic or nitrogen phosphorous (TID or also NPD, which responds to nitrogen or phosphorous) and atomic emission (AED, which responds to the presence of many elements of CWA interest, such as C, N, P, F, S, As, Cl and Br) detectors may provide element-specific information. Although selective detectors provide information on the presence or absence of certain elements they do not provide structural information. Mass spectrometers and infrared spectrometers provide this type of information and for this reason are widely interfaced to chromatographic techniques.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a separation technique in which a multi-component sample, in the liquid phase, is resolved into its various components. HPLC is well suited to the separation of thermally labile or low volatility compounds that cannot be easily analysed by GC. The first step in this technique is the introduction of the sample into a chromatographic column where the separation occurs. Sample introduction in HPLC is accomplished with a switching valve equipped with a fixed volume sample loop. The sample is injected as a liquid plug into the flow stream and swept onto the HPLC column for separation.

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Detection in HPLC is typically performed with an ultraviolet/visible detector. Nerve and blister agents do not generally possess strong chromophores, therefore sensitivity and selectivity remains poor for these compounds. In recent years flame photometric and thermionic detectors have been used for selective identification with microcolumn liquid chromatography. Although selective detectors provide information on the presence or absence of certain elements they do not provide structural information. Mass spectrometers provide this type of information and for this reason are widely interfaced to chromatographic techniques.

Capillary Electrophoresis

Capillary electrophoresis (CE) employs narrow bore (10-200 mm i.d., 370 mm o.d. polyamide coated) capillaries to perform high efficiency separations of both large and small molecules based on their mobilities in an electric field. The instrument consists of two buffer reservoirs connected via a hollow capillary column, a high voltage power supply, and a detection system.

Samples are introduced onto the CE column either electrokinetically (an applied potential) or hydrodynamically (pressure on the sample reservoir forces the sample into the capillary). A potential is then applied across the capillary and the analytes are induced to move (migrate) in this applied field. Because of differences in the effective mobilities (resulting in different migration velocities) of different substances in an electric field, the mixture separates into discrete zones of individual substances. The analytes are detected on-column by ultraviolet (UV) or laser induced fluorescence (LIF) through a small window on the capillary. As was the case with HPLC, CE has been interfaced with mass spectrometry in order to provide molecular mass and structural information.

Different modes of capillary electrophoresis can be performed using a standard CE instrument. These include capillary zone electrophoresis, capillary gel electrophoresis, micellar electrokinetic capillary chromatography, capillary isoelectric focusing and capillary isotachophoresis.

Capillary zone electrophoresis (CZE) is the simplest and most widely used technique in CE. The capillaries used for CZE are bare fused silica containing surface silanol groups. The separation mechanism is mainly based on differences in solute size and charge at a given pH.

As opposed to CZE, capillary gel electrophoresis (CGE) separates species on the differences in solute size as the analytes migrate through the pores of a gel-filled capillary allowing for separation based on "molecular sieving". They also minimize solute diffusion and prevent solute adsorption to the capillary walls.

Micellar electrokinetic capillary chromatography (MEKC) involves a micellar solution of an ionic surfactant to provide a phase for a chromatographic separation and allow for the separation of neutral species.

Isoelectric focusing is a separation method in which analytes are separated on the basis of their isoelectric points or pI values. Capillary isotachophoresis uses a discontinuous buffer system between which sample zones form as the analytes concentrate between the front and end constituents. Where, as in CZE the electropherogram gives distinct peaks like those found in HPLC, in capillary isotachophoresis (CITP) the isotachopherogram gives a series of steps each representing an analyte zone.

Mass Spectrometry

Mass spectrometry (MS) is an identification technique in which sample molecules are ionized, and the ionized molecules and any lower mass fragmentation ions that may have been formed are then separated on the basis of their mass-to-charge ratio. Assignment of the structure of the original molecule is done by either fundamental interpretation of the data or by comparing the acquired data to reference data. In most cases chromatographic separation precedes detection as it allows the individual components of a mixture to be introduced into the MS as relatively pure components. Mass separation can be accomplished using quadrupole, sector (electrostatic and magnetic), time of flight, ion trap or Fourier Transform instruments. High resolution measurements are possible with sector or Fourier Transform instruments. This data can be used to determine the elemental composition.

The most widely used method of ionization for the GC-MS analysis of chemical warfare agents is electron impact (EI) ionization. High energy electrons produced within the mass spectrometer source interact with sample molecules, resulting in the loss of an electron by the molecule. The resulting molecular ion may undergo fragmentation leading to the formation of lower mass fragmentation ions. In some cases the molecular ion, used to determine molecular mass, is not observed. Chemical ionization (CI), a much less energetic ionization technique, has proven very useful for providing complementary molecular ion information. Methane, isobutane and ammonia reagent gases have all been used during the analysis of chemical warfare agents.

In tandem mass spectrometry multiple analyzers (e.g., sector or quadrupole) are combined into a single instrument. Four common modes of operation are: product ion scan, precursor scan, constant neutral loss and reaction ion monitoring. The combination of two or more analyzers may result in the extraction of more structural information and/or greater selectivity than can be achieved with a single analyzer.

Thermospray mass spectrometry and more recently atmospheric pressure ionization (e.g., electrospray, ionspray and atmospheric pressure CI) techniques have enabled the direct mass spectrometric analysis of chemical warfare agent hydrolysis products in aqueous samples. Both techniques may be interfaced to HPLC or CE for component separation. Thermospray has been largely superseded by atmospheric pressure ionization (API) for most applications. API-MS, a relatively gentle ionization technique, generally produces molecular ion information in either the positive or negative ion mode. Under positive ion mode, molecular adducts, dimers and trimers are often observed. Under appropriate API conditions, it is possible to promote collisionally activated dissociation in the interface resulting in the formation of product ions that may aid in structural identification.

Infrared Spectroscopy

Infrared spectroscopy (IR) is an identification technique in which sample molecules absorb radiation (typically in the 4000 to 400 cm^{-1} range). Pure samples may be introduced into the IR spectrometer either as a pellet (typically in a KBr matrix), a thin film between NaCl or KBr plates, gases or solid samples. In most cases chromatographic separation precedes detection as it allows the individual components of a mixture to be introduced into the IR as relatively pure components. Currently two different approaches are used to acquire the infrared spectrum of a component as it elutes from the chromatographic column. The first technique uses a direct deposition mechanism whereby the column effluent is deposited onto a moving ZnSe plate at extremely low temperatures. Condensed phase spectra are collected by either scanning the plate on the fly or in a post chromatographic run mode. The second technique uses a gold plated light pipe through which the column effluent flows. Vapour phase IR spectra are acquired in real time, with higher detection limits than the direct deposition technique. Unlike MS, IR spectra are more difficult to interpret from first principles and identification of a chemical warfare agent is based on the comparison of the acquired spectrum to that obtained for a reference standard.

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is an identification technique in which sample molecules absorb radio frequency radiation (typically in the 100 to 500 MHz range for protons) and the resulting absorbance spectrum is used to uniquely identify the original molecule. Assignment of the structure of the chemical warfare agent is done by either fundamental interpretation or by comparison of the acquired spectrum to that obtained for a reference standard.

In NMR spectroscopy, the sample is analysed as a dilute solution. Deuterated solvents such as: D_2O, DMSO- d_6 and $CDCl_3$ are used to prepare these solutions with tetramethylsilane (TMS) frequently being used as the internal reference. Because NMR does not generally use a separation technique prior to detection (such as GC in MS and FTIR), samples must be in a relatively pure form to avoid spectral interferences.

Instrumentation equipped with multi-nuclear capability (especially ^{13}C, ^{31}P, ^{19}F and ^{1}H) can be useful for identifying chemical agents but detection levels vary depending on the nuclei selected. The use of selective probes such as ^{31}P and ^{19}F provide for selective detection of CW agents in the presence of a complex background. Special NMR techniques or experiments such as multi-dimensional NMR, indirect detection or special probe designs can also increase specificity in complex backgrounds. Where sufficient sample is present, the information obtained by NMR can permit complete structural elucidation of new or previously unreported agents. Two dimensional NMR experiments such as homo- and heteronuclear correlation spectroscopy (COSY, HETCOR, NOESY, ROESY) are now standard methods that aid in the determination of structure.

Preparatory Work in Peacetime

The availability of modern analytical techniques and methods is a prerequisite for the rapid identification of biological, chemical and mid-spectrum warfare agents. However, the presence of instrumentation and methods does not of themselves ensure the unambiguous identification of these agents. Successful identification is dependent on the analysis being performed by a team experienced in the handling, storage, preparation and analysis of BW, CW and mid-spectrum agents. Therefore an analyst team familiar with the identification techniques should periodically participate in inter-laboratory testing, international round-robin exercises and internal quality assurance testing to evaluate their capabilities.

In the event of the actual use of biological, chemical or mid-spectrum agents, or for laboratory training exercises, it is important that the laboratory either stock or be able to rapidly acquire the necessary reference substances and standards. Chemical laboratories require a synthetic capability in order to provide authentic reference standards which can be utilized for unambiguous identification. The spectra acquired during the analyses of these reference standards may be compiled into a computer searchable database. These databases may be used to match the acquired spectra to those obtained for reference standards. It cannot be over-emphasized that proper and careful techniques during sample preparation will greatly reduce the overall analysis time and minimize the potential for the reporting of erroneous results based on the appearance of artifacts, cross-contamination or other interferences.

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A general survey of sample preparation and identification techniques for biological, chemical and mid-spectrum agents was conducted as part of Canada's contribution to a joint NATO Allied Engineering Publication (AEP) handbook. The handbook integrates, in its various chapters, the diverse aspects of sampling, transport, identification, reporting and training as they relate to biological, chemical and mid-spectrum agents. The handbook provides a common reference to NATO member nations for the development of their own identification capabilities and for the integration of these capabilities into the framework of NATO operations. The handbook could be utilized to direct the development, standardization and integration of member country deployable laboratories, which will support NATO field operations on a rotational basis. This report forms the basis of Canada's contribution to the latest edition of the handbook. In addition to sample preparation and analysis, it discusses; quality assurance, chain of custody and the criteria used for identifying the biological, chemical and mid-spectrum agents.

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