7. ANALYTICAL METHODS

The purpose of this chapter is to describe the analytical methods that are available for detecting, measuring, and/or monitoring sulfur mustard, its metabolites, and other biomarkers of exposure and effect to sulfur mustard. The intent is not to provide an exhaustive list of analytical methods. Rather, the intention is to identify well-established methods that are used as the standard methods of analysis. Many of the analytical methods used for environmental samples are the methods approved by federal agencies and organizations such as EPA and the National Institute for Occupational Safety and Health (NIOSH). Other methods presented in this chapter are those that are approved by groups such as the Association of Official Analytical Chemists (AOAC) and the American Public Health Association (APHA). Additionally, analytical methods are included that modify previously used methods to obtain lower detection limits and/or to improve accuracy and precision.

7.1 BIOLOGICAL MATERIALS

The most common currently used method of analyzing for the presence of sulfur mustard and its metabolites in biological and environmental samples is gas chromatography/mass spectrometry (GC/MS). Prior to 1987, however, thin-layer chromatography (TLC) with a colorimetric detection system and gas chromatography with either flame ionization detector (FID), electron capture detector (ECD), or flame photometric detector (FPD) were the most frequently used methods. Sample preparation consists primarily of extraction with an organic solvent. Sodium chloride is sometimes added to improve sample stability and prevent sulfur mustard breakdown to thiodiglycol and other metabolites. Depending on the method used, and the possible interfering compounds present, further cleanup and preparative steps may be included. No specific EPA, NIOSH, or AOAC methods were found for this chemical. Table 7-1 summarizes several representative analytical methods for detecting sulfur mustard and its metabolites in biological samples.

Little information was found on the direct detection of sulfur mustard in biological tissues or fluids. However, in two cases of suspected exposure, sodium chloride was first added to the urine samples to stabilize any sulfur mustard that might be present. A semi-quantitative analysis by GC/MS detected low ppb levels of sulfur mustard in these samples compared to none detected in a control sample of a definitely unexposed person (Vycudilik 1985, 1987). The detection limit of the procedure was in the low

Table 7-1. Analytical Methods for Determining Sulfur Mustard in Biological Samples

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Urine	Saturate with sodium chloride; extract with diethyl ether; centrifuge; isolate organic phase and evaporate; redissolve in methylene chloride; clean up with silica gel; centrifuge; evaporate solvent layer; redissolve in methylene chloride	GC/MS (EI)	10 ng/µL injected	20%	Vycudilik 1985
Urine	Hydrolyze sample with helix pomatia (enzymatic hydrolysis); clean up on carbon column; add concentrated hydrochloric acid to convert thio-diglycol to sulfur mustard; headspace analysis® with collection on Tenax; thermally desorb (Thiodiglycol)	GC/MS	1 µg/L (1 ppb)	75%	Wils et al. 1988
Urine	Treat samples with acidic titanium trichloride; final residue dissolved in acetonitrile and toluene	GC-MS-MS	0.1 µg/L (0.1 ppb)	48–56%	Black and Read 1995b
Human fluids and tissues	Homogenize tissue; extract sample with dichloromethane; centrifuge; remove dichloromethane layer and evaporate; redissolve in hexane; clean up on TLC; remove sample spots and complex with gold; extract with toluene	ET-AAS	1.1 mg/L (ppm, body fluids); 0.1 mg/kg (ppm, body tissues)	No data	Drasch et al. 1987

EI = electron impact; ET-AAS = electrothermal atomic adsorption spectroscopy; GC = gas chromatography; GC-MS-MS = gas chromatography-tandem mass spectroscopy; MS = mass spectroscopy; TLC = thin layer chromatography

ppb range with inadequate recoveries of about 20%. Sulfur mustard has also been detected in body tissues and fluids of an alleged victim (Drasch et al. 1987). In this analysis, abdominal fat samples were first qualitatively analyzed by GC/MS.

Sulfur mustard is generally metabolized rapidly in biological systems. The primary method of analyzing for sulfur mustard exposure is by detecting the presence of its hydrolysis metabolites in biological fluids. GC/MS has been used for this purpose. The procedure involves conversion of the most common hydrolysis metabolite, thiodiglycol, to sulfur mustard by heating with concentrated hydrochloric acid (Wils et al. 1985, 1988). The detection limit for this procedure is in the low ppb range (about 1 μg/L) and with inclusion of deuterated thiodiglycol as an internal standard, recoveries of 75% are obtained (Wils et al. 1988). Unfortunately, thiodiglycol (and thiodiglycol sulphoxide) can exist in the urine of both exposed and nonexposed subjects; detection of thiodiglycol in human urine by this procedure at a concentration level of 10–100 μg/L does not prove sulfur mustard poisoning (Wils et al. 1985). Other methods using GC/MS have determined sulfur mustard in urine of exposed rats and guinea pigs by derivatisation of thiodiglycol with heptafluorobutyric anhydride (Jakubowski et al. 1990). Black and co-workers quantified thiodiglycol (and thiodiglycol sulphoxide) in urine of exposed humans using GC/MS after formation of bis(pentafluorobenzoyl) derivatives (Black and Read 1991, 1995a, 1995b; Black et al. 1992a, 1992b, 1994).

Another recent method for sulfur mustard detection in urine is gas chromatography-tandem mass spectrometry (GC-MS-MS) with selected-reaction monitoring. This method was applied to the analysis of urinary metabolites of sulfur mustard derived from hydrolysis (i.e., thiodiglycol and its sulfoxide) and the glutathione pathway after further metabolism involving the enzyme β-lyase (i.e., 1,1-sulphonylbis-[2-(methylsulphinyl)ethane] and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane). The procedure involves treatment of samples with acidic titanium trichloride to reduce thiodiglycol sulfoxide to thiodiglycol and the two β-lyase metabolites to a single analyte, 1,1-sulphonylbis[2-(2-methylthio)-ethane]. The detection limit for this procedure is in the sub-ppb range (0.1 μg/L) for detection of β-lyase metabolites and in the ppb range (1–12 μg/L) for detection of thiodiglycol. Recoveries, determined in normal urine spiked with 1,1-sulphonylbis[2-(2-methythio)ethane] at a concentration of 1 μg/L, ranged from 48 to 56%. The advantage of this method is that β-lyase metabolites of sulfur mustard have not been observed in normal urine and this method provides an unequivocal biological marker of exposure to sulfur mustard (Black and Read 1995b).

Recently, the detection of DNA adducts formed by the modification of DNA by sulfur mustard in blood offers a promising approach for retrospective detection of exposure. For example, Ludlum et al. (1994) detected an N7-guanine adduct of DNA using high performance liquid chromatography (HPLC) with fluorometric monitoring. In this study, the authors were able to detect one N7-guanine adduct in $3x10^5$ DNA nucleotides. Benschop and co-workers (Benschop et al. 1997; Fidder et al. 1996a) were able to confirm the exposure to sulfur mustard in samples taken in March 1988 from two Iranians. Exposure to sulfur mustard was verified by two independent methods based on immunochemical analysis of the N7-guanine adduct in DNA and GC/MS analysis of the N-terminal valine adduct in globin after a modified Edman degradation. The adduct levels found were considerably higher than the detection limit for the modified Edman procedure (i.e., 0.1 μM sulfur mustard), but just above the detection limit for the immunochemical assay (i.e., 0.07 μM sulfur mustard). In another study, Noort et al. (1996) described the use of liquid chromatography-tandem mass spectrometry (LC-MS-MS) to identify modified sites in human hemoglobin after *in vitro* exposure to sulfur mustard. They note that hemoglobin is efficiently alkylated by sulfur mustard leading to an increase in 104 m/z after hydrolysis. This method is based on cleavage of globin by trypsin and micro-LC-MS analysis of the digests.

7.2 ENVIRONMENTAL SAMPLES

Table 7-2 presents a summary of several common analytical techniques used to analyze for sulfur mustard and its metabolites in environmental samples.

Until recently, GC with FID, ECD, or FPD were the primary methods of analysis for sulfur mustard and its metabolites, with a colorimetric assay utilizing 4-(p-nitrobenzyl) pyridine also frequently used. GC/MS is more commonly used for detecting sulfur mustard and its metabolites in environmental samples. Separation by TLC, followed by detection with a 4-(p-nitrobenzyl) pyridine procedure, has been used qualitatively and quantitatively to detect sulfur mustard in the presence of other vesicant mustards (Sass and Stutz 1981; Stutz and Sass 1969). This technique has proved useful in detecting sulfur mustard in a variety of complex matrices (water, soil, plants) and has a detection limit of 1 μg/sample spot (Sass and Stutz 1981). In addition to being relatively sensitive and selective, it can be scaled up for preparative work and down for small samples. This gives it continued usefulness despite the advent of more sophisticated GC/MS techniques

Table 7-2. Analytical Methods for Determining Sulfur Mustard in Environmental Samples

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Air	Collect in decalin solvent using double trap system	GC/ECD	0.2 ng/µL injected	99.5–101.5%	Casselman et al. 1973
Air	Collect in diethyl succinate using double trap system	GC/FPD	0.2 ng/µL injected	98–101%	Gibson et al. 1974
Air	Collection in Tenax GC in a glass tube; thermal desorption into GC	GC/FPD	10 ng/m^3	No data	Fowler and Smith 1990
Water	Directly inject sample for thiodiglycol detection; extract with hexane and concentrate for detection of other compounds (metabolites)	GC/FID	50 µg/L (ppb)	No data	D'Agostino et al. 1989
		GC/MS (CI)	No data		D'Agostino et al. 1989
		GC/FTIR	No data		D'Agostino et al. 1989
Water or vapor	Extract with hexane	GC/ECD	160 µg/L (water); 1 µg/L (vapor)	No data	Fisher et al. 1969
Standard solutions and vapors	Dissolve standard of known purity in hexane or chloroform (sulfur mustard and metabolites)	GC/ECD GC/FPD	About 160 µg/L (solution); about 1 µg/L (vapor)	No data	Sass and Steger 1982
Soil	Extract with chloroform; sonicate (sulfur mustard and metabolites)	GC/MS (CI)	5–10 ng/ injection	No data	D'Agostino and Provost 1988b
	No data	GC/MS (EI)	No data		Vycudilik 1985
Soil, plants, water	Separation by TLC	4-(p-nitro-benzyl) pyridine procedure	1 µg/sample spot	No data	Sass and Stutz 1981; Stutz and Sass 1969
Sulfur mustard hydrolysate	Extract with hexane; concentrate	GC/FID	No data	No data	D'Agostino and Provost 1988a

CI = chemical ionization; ECD = electron capture detector; EI = electron impact; FID = flame ionization detector; FPD = flame photometric detector; FTIR = Fourier transform infrared spectroscopy; GC = gas chromatography; MS = mass spectrometry

GC with either FID, ECD, or FPD was the most common technique of the 1970s and early 1980s for determining the presence of sulfur mustard and its metabolites, and is still frequently used. It has been used to detect sulfur mustard in air by passing air through a solvent trap. Aliquots of the solvent are directly injected into the gas chromatograph to detect sulfur mustard (Casselman et al. 1973; Gibson et al. 1974). With both ECD and FPD, recoveries were near 100%, and the detection limit was 0.2 ng/μL injected. Advantages of both were speed, simplicity, and reliability. However, the solvent producing the best results with ECD required ice-bath cooling to prevent solvent and sulfur mustard loss (Casselman et al. 1973). The solvent used with FPD had the advantage of allowing room temperature analysis (Gibson et al. 1974).

GC was used to detect sulfur mustard in water (Fisher et al. 1969) and soil (D'Agostino and Provost 1988a). Using GC/ECD, a minimum detection limit (quantifiable) of 160 μg/L (ppb) for aqueous solutions and 1 μg/L for vapor was obtained. The method used was simple, selective, and precise. The authors proposed that with appropriate sample preparation, it could be used for a variety of media, including soil and biological media (Fisher et al. 1969). Sulfur mustard and metabolites were detected in soil by GC/FID by D'Agostino and Provost (1988a), who also analyzed a hydrolysate remaining from the destruction of munitions grade mustard, but no details on accuracy, precision, or sensitivity were given. A comparison of the various detectors used to analyze for sulfur mustard and its metabolites was conducted and showed ECD to be the most sensitive for detecting sulfur mustard in a mixture of mustard compounds, followed by FPD and FID (Sass and Steger 1982). The detection limit using ECD and FPD was in the mid-ppb range (about 160 μg/L) for solutions and in the low ppb range (about 1 μg/L) for vapors. Beck et al. (2001) found that GC-FPD provided a rapid and sensitive method for analysis of thiodiglycol (TDG) in soil extracts with a detection limit of 1.1 μg/g soil. Pressurized liquid extraction (PLE) with methanol-water (9:1) proved to be the most efficient solvent for TDG extraction with recoveries ranging from 12 to 89% of added TDG for various soil types.

GC/MS has been used to analyze for the presence of sulfur mustard and its metabolites (D'Agostino and Provost 1988b; D'Agostino et al. 1989; Munavalli and Jakubowski 1989; Vycudilik 1985). Tests with pure substances have supported the sensitivity, selectivity, and reliability of this technique, and analysis of pure samples has proved its usefulness. Inclusion of deuterated thiodiglycol as an internal standard increases the precision of GC/MS and makes the technique quantitative as opposed to simply semi-quantitative. Both chemical ionization (CI) and electron impact (EI) have been used to detect sulfur mustard and its characteristic metabolites in samples. Detection of specific mustard metabolites is important in determining sulfur mustard exposure since the chemical can degrade rapidly under certain

environmental conditions. Testing of several EI and CI techniques showed that MS was a sensitive, reliable, and precise detection method for sulfur mustard (Ali-Mattila et al. 1983). This was supported in later studies on sample mixtures of vesicant mustards and degradation products, as well as on water and soil samples (D'Agostino and Provost 1988b, 1992; D'Agostino et al. 1989; Munavalli and Jakubowski 1989; Vycudilik 1985). For example, D'Agostino and Provost (1992) used GC/MS for verification of sulfur mustard and its hydrolysis products in soil. They used sequential hexane and dichloromethane extraction followed by trimethylsilyl derivatization and achieved total recoveries in the 50–90% range for most soil types. Wils et al. (1992) used GC/MS to analyze sulfur mustard in rubber and paint samples in combination with diesel fuel and aromatic white spirit as a background. Sulfur mustard was isolated by extraction with methylene chloride or by dynamic headspace analysis at elevated temperatures. Recoveries of sulfur mustard in rubber and paint ranged from 57 to 86%. Black et al. (1993b) analyzed soil, bomb casing, and sheep wool samples associated with a chemical weapons agent (CWA) incident (obtained from a Kurdish village in the northern part of Iraq in 1988) by GC/MS using headspace analysis, solvent extraction, and thermal desorption methods. Using this technique, the presence of sulfur mustard and 21 related compounds were successfully confirmed in these samples.

Sulfur mustard vapor is typically determined in air by bubbling an air sample through a liquid solvent and analyzing the solvent for absorbed mustard by colorimetry or by GC. However, the colorimetric technique lacks specificity and the solvent entrapment sampling technique possesses a number of drawbacks such as limited analyte-trapping efficiency, high detection limits, and degradation of the analyte (Fowler and Smith 1990). Rapid and accurate methodologies for the detection of sulfur mustard have been developed for use during the demilitarization of mustard stockpiles at U.S. storage sites. These procedures are based largely on the Depot Area Air Monitoring System (DAAMS) technology (Smith et al. 1982). DAAMS procedures have undergone extensive Precision and Accuracy studies (Smith and Fowler 1985) and are methods of choice in current and future demilitarization sites. The sampling and analysis process for DAAMS consists of (1) collection of the airborne sample on the sorbent (Tenax GC) in a glass tube, (2) transfer to a glass tube containing smaller amounts of the same sorbent using an external thermal desorber, and (3) thermal desorption in a specially-modified injection port of a gas chromatograph and subsequent analysis using a flame photometric detector (Fowler and Smith 1990; Posner 1991). The detection limit of sulfur mustard vapor in air by these procedures is about 10 ng/m^3 (or 1.5 parts per trillion).

While GC/MS continues to be the definitive method for assessment of sulfur mustard in environmental matrixes, increasing emphasis has been placed on rapid screening procedures such as liquid

chromatography/mass spectrometry (LC/MS). Although LC/MS methods typically have higher detection limits, these techniques allow for more rapid screening of aqueous samples and extracts, with minimum sample pretreatment and no requirement for dehydration or derivatization (Burrows 1998). Electrospray ionization, thermospray ionization (TSP), and atmospheric pressure chemical ionization (APCI) have shown promise as interfaces for the introduction of the liquid solvent stream containing sulfur mustard and metabolites into the mass spectrometer (Munavalli et al. 1995; Smith and Shih 2001). For example, liquid chromatograph/electrospray-mass spectrometry (LC/ESP-MS) was recently recommended as a rapid screening procedure for verification of the presence of traces of the agent in hydrolysis products in water without derivatization (Borrett et al. 1996). It has also been successfully applied for the direct detection of sulfonium ions formed during the storage and hydrolysis of sulfur mustard (Rohrbaugh and Yang 1997). Black and Read (1997) recently demonstrated a rapid screening procedure for sulfur mustard, which involves separation by liquid chromatography and detection by atmospheric pressure chemical ionization-mass spectrometry (HPLC/APCI-MS). Sulfur mustard and its transformation products have been characterized by liquid chromatography/thermospray ionization-mass spectrometry (LC/TSP-MS) in methanol. In both positive and negative modes, a rich complex ion chemistry was observed for the transformation products of sulfur mustard (Munavalli et al. 1995).

Other techniques that have been used to characterize sulfur mustard and its metabolites include capillary electrophoresis (CE) and secondary ion mass spectrometry (SIMS). CE coupled with MS detection is well suited to deal directly with aqueous samples and polar (acidic) degradation products (Hooijschuur et al. 2001). SIMS analysis of solid samples has evidenced the presence of sulfonium ion aggregates resulting from nucleophilic substitution processes (Groenwold et al. 1995). This method also may potentially be able to detect sulfur mustard directly on soil surfaces without the necessity of lengthy extraction procedures (Gresham et al. 2001).

7.3 ADEQUACY OF THE DATABASE

Section 104(i)(5) of CERCLA, as amended, directs the Administrator of ATSDR (in consultation with the Administrator of EPA and agencies and programs of the Public Health Service) to assess whether adequate information on the health effects of sulfur mustard is available. Where adequate information is not available, ATSDR, in conjunction with the National Toxicology Program (NTP), is required to assure the initiation of a program of research designed to determine the health effects (and techniques for developing methods to determine such health effects) of sulfur mustard.

The following categories of possible data needs have been identified by a joint team of scientists from ATSDR, NTP, and EPA. They are defined as substance-specific informational needs that if met would reduce the uncertainties of human health assessment. This definition should not be interpreted to mean that all data needs discussed in this section must be filled. In the future, the identified data needs will be evaluated and prioritized, and a substance-specific research agenda will be proposed.

7.3.1 Identification of Data Needs

Methods for Determining Biomarkers of Exposure and Effect.

Exposure. Available information indicates that sensitive, selective, and reliable methods for determining biomarkers of exposure exist for sulfur mustard and its metabolites (Black and Read 1991, 1995a, 1995b; Black et al. 1992a, 1992b, 1993b, 1994; Kientz 1998; Ludlum et al. 1994). Available studies emphasize detection and quantification of the compound and its metabolites. Further studies that attempt to quantify levels in exposed and unexposed populations are useful in assessing the risk associated with sulfur mustard and its metabolites.

Effect. As discussed, sensitive, selective, and reliable methods exist for detecting sulfur mustard and its metabolites in biological tissues and fluids. Available studies do not emphasize quantifying the levels of these compounds and associating the amounts found with specific biomarkers of effect. Further studies associating specific levels in fluids and tissues with known effects are useful in assessing the risk associated with sulfur mustard and its metabolites.

Methods for Determining Parent Compounds and Degradation Products in Environmental Media. Sensitive, selective, and reliable methods exist for detecting sulfur mustard and its metabolites in air (Casselman et al. 1973; Gibson et al. 1974), water (Fisher et al. 1969; Sass and Stutz 1981; Stutz and Sass 1969), and soil (D'Agostino and Provost 1988a; Sass and Stutz 1981; Stutz and Sass 1969). No information was obtained on the detection of sulfur mustard in other environmental media. The available methods emphasize qualitative and quantitative detection. Further studies to improve the detection of sulfur mustard and its metabolites will aid in assessing the potential risk of sulfur mustard in the environment, especially near hazardous waste facilities and Army storage facilities.

7.3.2 Ongoing Studies

No ongoing studies on the analysis of sulfur mustard in biological and environmental matrixes were located in the Federal Research in Progress (FEDRIP) database.