6. ANALYTICAL METHODS

The purpose of this chapter is to describe the analytical methods that are available for detecting, and/or measuring, and/or monitoring dinitrophenols, their metabolites, and other biomarkers of exposure and effect to dinitrophenols. 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.

A survey of literature revealed that dinitrophenols (mostly 2,4-DNP) in environmental and biological samples are quantitated following extraction and sample clean-up steps. Acid-base partitioning is generally used for sample clean-up. This clean-up method takes advantage of the fact that DNPs are extractable from an organic phase into aqueous phase at pH 12 or above, and back into the organic phase at a pH of 1 to 2. Extreme care should be taken while concentrating solvent extracts to prevent evaporative loss; propylene glycol may be used as a keeper to reduce evaporative loss (Robert and Hagardom 1985; Schultz 1983).

Both gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) columns are often used as the final step towards resolution of components in a mixture. When GC is used, a much higher resolution of components in the sample can be attained with fused silica capillary columns than with packed columns. Low polarity capillary columns, such as HP-l (Hewlett-Packard) and DB-5 (J & W Scientific), give higher resolution for phenolic compounds than higher polarity columns such as DB-7 and DB-1701 (Williams et al. 1991). A variety of detectors has been used to quantitate dinitrophenols; these include flame ionization (FID), Fourier transformation infrared spectrometric (FTIR), mass spectrometric, ultraviolet (UV), photodiode array, electrochemical and nitrogenphosphorus detectors (NPD) (Alber et al. 1989; Bihm et al. 1989; Borra et al. 1986; EPA 1982a, 1986c; Nick and Schoeler 1992; Robert and Hagardom 1985; Wegman and Wammes 1983; Williams et al. 1991).

Because underivatized nitrophenols are irreversibly sorbed on active sites of GC column materials, it may be necessary to derivatize the compounds to increase their volatility to enhance elution from the GC column (Nojima et al. 1983). HPLC is advantageous for determining dinitrophenols because derivatization is not required, and the detectors used in conjunction with HPLC methods are more sensitive than either flame ionization or mass spectrometric detection used in GC methods (Alber et al. 1989; Bengtsson 1985; Bihm et al. 1989) (see Section 6-2). Gas chromatography-Fourier transformation infrared spectroscopy (GC-FTIR) gives slightly better sensitivity of detection for dinitrophenols than GC-electron impact mass spectrometry (EIMS) (Williams et al. 1991). Mass spectrometry with negative ion chemical ionization (NICI) provides considerably higher sensitivity for determination of nitroaromatics compared to the EI method (Levsen et al. 1993). When HPLC methods are used in combination with pulsed amperometric detection, the detection limit can be at least an order of magnitude lower than photodiode array-UV detection and two orders of magnitude lower than UV detection alone (Alber et al. 1989; Baldwin and Debowski 1988; Bihm et al. 1989). The sensitivity of detection for 2,4-DNP derivatized by diazomethane and quantitated by HRGC/NPD (high resolution gas chromatography-nitrogen phosphorus detection) is comparable to the sensitivity of photodiode array-UV detection (Nick and Schoeler 1992).

Besides the methods given in Table 6-l and Table 6-2, several others are available to determine dinitrophenols in biological and environmental samples. Immunoassay methods with sensitivities comparable to those of the conventional methods given in Tables 6-l and 6-2 are noteworthy (Bush and Rechnitz 1987; Huang et al. 1992; Kusterbeck et al. 1990; Wannlund and DeLuca 1982). However, methods based on antibody sorption have not yet been validated on samples derived from environmental and biological sources, so it is not clear what methods of clean-up are necessary prior to quantitation of dinitrophenols. Some of the other detection methods with superior sensitivities that can determine dinitrophenols are surface-enhanced resonance Raman spectroscopy (Ni et al. 1989), tandem mass spectrometry (McLukey et al. 1985), fiber optic-modified photothermal deflection spectrometry (Bohnert et al. 1992), and combined liquid chromatography/mass spectrometry (Christensen et al. 1980).

6.1 BIOLOGICAL SAMPLES

Analytical methods used to determine dinitrophenols in biological samples are listed in Table 6-l. Whether the sensitivity of the analytical methods given in Table 6-l will permit determination of

Table 6-1. Analytical Methods for Determining Dinitrophenols in Biological Samples

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference	Isomer
Serum	Extract sample in sodium chloride-sodium carbonate with methyl ethyl ketone	Spectrophotometric	≈2 mg/L	No data	Gehring and Buerge 1969a	2,4-DNP
Serum and plasma	Extract acidified sample with chloroform; evaporate and reconstitute in methanol-pyridine	HRGC/MS	<0.05 mg/L	70	Robert and Hagardorn 1985	2,4-DNP
Liver and kidney	Extract homogenized and acidified sample with chloroform; extract organic phase with aqueous sodium carbonate; acidify aqueous phase and extract with diethyl ether; evaporate ether; reconstitute in benzene	HRGC/MS	<0.05 mg/L	70	Robert and Hagardorn 1983	2,4-DNP
Urine	Reflux sample in sulfuric acid; extract with ethyl ether; evaporate organic solvent and dissolve residue in dilute sodium hydroxide	Polarographic	<10 mg/L	No data	Gisclard and Woodward 1946	2,4-DNP

2,4-DNP = 2,4-dinitrophenol; HRGC = high resolution gas chromatography; MS = mass spectrometry

Table 6-2. Analytical Methods for Determining Dinitrophenols in Environmental Samples

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference	Isomer
Air	Extract air particulate collected on glass fiber filter with aqueous sodium hydroxide; extract acidified aqueous fraction with ethyl ether; concentrate; dissolve with ethereal diazomethane	GC/MS	≈0.05 mg/kg in particulate matter	No data	Nojima et al. 1983	2,4- and 2,6 DNP
Waste water	Extract acidified sample with methylene chloride; concentrate and solvent exchange to 2-propanol	GC/FID (method 604)	13.0 µg/L	74	EPA 1982a	2,3-DNP
Water and waste water	Extract acidified sample with methylene chloride; concentrate	GC/MS (method 625)	42.0 µg/L	78 (water) 108 (waste water)	EPA 1982a	2,4-DNP
Water	Extract acidified sample with methylene chloride; concentrate	HPLC/UV	0.025 µg/L	88 (at 0.05 µg/L)	Schultz 1983	2,4-DNP
Water	Concentrate acidified sample on graphitized carbon black; phenol-extract with methylene chloride/ methanol/tetra-methyl-ammonium hydroxide	HPLC/UV	0.005 µg/L	87–94	Borra et al. 1986	2,4-DNP
Water and leachate	Preconcentrate analyte on an HPLC column; elute into analytical column	HPLC/UV HPLC/PAD	2.0 µg/L (UV) 0.01 µg/L (PAD)	No data	Baldwin and Debowski 1988	2,4-DNP
Rain and snow	Extract basic sample with methylene chloride; extract acidified aqueous phase with methylene chloride; concentrate	HPLC/PID	0.4 µg/L (2,4- and 2,6-DNP) 0.8 µg/L (2,5-DNP)	99 (2,4-DNP) 94 (2,5-DNP) 84 (2,6-DNP)	Alber et al. 1989; Bîhm et al. 1989	2,4-, 2,5- and 2,6-DNP
Groundwater	Extract acidified sample with methylene chloride; concentrate	GC/FID (method 8270)	13.0 µg/L	78 at 100 µg/L	EPA 1986c	2,4-DNP

Table 6-2. Analytical Methods for Determining Dinitrophenols in Environmental Samples (continued)

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference	Isomer
Soil and sediment	Extract sample by soxhlet or sonification; shake organic phase in basic solution; acidify and extract aqueous layer with methylene chloride; concentrate	HRGC/MS (method 8270)	3.3 mg/kg	81 at 10,000 µg/kg	EPA 1986c	2,4-DNP
Soil and sediment	Extract sample with acetone concentrate and dilute with water; add cetrimide, sodium chloride, and buffer; extract aqueous phase with methylene chloride; evaporate; dissolve in methanol/water	HPLC/UV	0.05 mg/kg (2,3-, 2,5- and 2,6-DNP) 0.01 mg/kg (2,4- and 3,5-DNP)	68–73 (2,3-) 71–82 (2,4-) 35–60 (2,5-) 60–76 (2,6-) 74–89 (3,5-)	Wegman and Wammes 1983	2,3-, 2,4-, 2,5- 2,6- and 3,5-DNP
Soil	Extract with methanol in rotary shaker; centrifuge	HPLC/UV	≤0.1 mg/kg	93-94	Bouchard 1987	2,4-DNP
Incubated culture medium	Purge and trap	HRGC/FID and HRGC/MS	No data	No data	Boopathy and Kulpa 1993	Phenol and benzene
Sludge	Extract with methylene chloride; clean up by acid-base partitioning and gel-permeation chromatography	HPLC/ELCD	0.18 mg/kg	44	Phillips et al. 1983	2,4-DNP

DNP = dinitrophenol; ELCD = electrochemical detection; FID = flame ionization detection; GC = gas chromatography; HPLC = high pressure liquid chromatography; HRGC = high resolution gas chromatography; MS = mass spectrometry; PAD = pulse amphrometric detection; PID = photodiode array detection; UV = ultraviolet detection

dinitrophenols in body tissues and fluids of the general population cannot be ascertained because information on dinitrophenol levels in the general population is not available. The urine of a man fatally poisoned by 2,4-DNP was found to contain 2-amino-4-nitrophenol, 4-amino-2-nitrophenol and diaminophenol (possibly 2,4-diaminophenol) (NAS/NRC 1982). 2,4-DNP is also excreted as partially unchanged and partially conjugated with glucuronic acid, and the metabolite 2-amino-4-nitrophenol is excreted as partially conjugated with sulfate in mammalian urine (NAS/NRC 1982). The conjugated metabolites are usually hydrolyzed by heating with acid/base to release the parent compounds for quantification.

Very few sensitive methods are available for the determination of dinitrophenol metabolites in biological samples. Most metabolites contain three functional groups (i.e., nitro, amino and phenolic hydroxyl groups). Extraction of these substances from an aqueous phase (e.g., urine) to an organic solvent is expected to give poor recovery (Levsen et a1.1993). The peak tailing observed in most packed GC column separations requires that these compounds be either derivatized or a suitable capillary column be used for the extracts of an underivatized sample. Since the metabolites contain two nitrogen atoms in each molecule, NPD detection after methylation with diazomethane is useful for quantitation of these compounds. Derivatization with heptafluorobutyric anhydride is expected to be more suitable for electron capture detection. The determination of underivatized amino-nitrophenols by HPLC with UV-photodiode array detection may also be possible. Robert and Hagardorn (1985) had used a HRGC/MS method for the determination of 2,4-DNP and the amino-nitrophenol metabolites in serum and plasma (see Table 6-l). However, the values for the recovery and precision of the compounds by this method were not provided. In the absence of experimentally determined values of recovery, precision and detection limit for each of these methods, it is not possible to select a preferred method for the determination of dinitrophenol metabolites in biological samples.

6.2 ENVIRONMENTAL SAMPLES

Analytical methods used to determine dinitrophenols in environmental samples are given in Table 6-2. For aqueous samples, continuous liquid-liquid extraction with organic solvent(s) provided higher recovery of 2,4-DNP in solvent extract than discontinuous (e.g., shaking in separatory funnel) extraction (Levsen et al. 1993). Dinitrophenols can also be extracted from aqueous samples by Solid-phase extraction. The adsorbed 2,4-DNP is eluted from the column by a suitable solvent. The

recovery of 2,4-DNP was >90% with membrane disks impregnated with acetyl-polystyrene-divinyl-benzene (Schmidt et al. 1993) and 89% with a C-18 adsorbent (Levsen et al. 1993).

6.3 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 dinitrophenol is available. Where adequate information is not available, ATSDR, in conjunction with the 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 dinitrophenols.

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.

6.3.1 Identification of Data Needs

Methods for Determining Biomarkers of Exposure and Effect. No biomarker has been identified that can be quantitatively related to dinitrophenol exposure (see Section 2.5.1); however, the presence and the amount of 2,4-DNP and 2-amino-4-nitrophenol, a metabolite in the urine, can be used as rough indicators of the intensity of exposure (see Section 2.5.1). The methods presently available for determining 2,4-DNP and 2-amino-4-nitrophenol (diazotization) in urine are outdated (Gisclard and Woodward 1946). An enzyme-linked immunosorbent assay (ELISA) is available for the quantitation of 2-amino-4-nitrophenol in water samples, but is not effective in urine (Li et al. 1991). It would be useful to develop an updated routine method for determining 2,4-DNP, 2-amino-4-nitrophenol, and 4-amino-2-nitrophenol in urine with well-defined detection limits, precision, and accuracy.

No known effect in humans can be quantitatively related to dinitrophenol exposure.

Methods for Determining Parent Compounds and Degradation Products in Environmental Media. Analytical methods with high sensitivity are available for determining dinitrophenols in environmental samples (Alber et al. 1989; Baldwin and Debowski 1988; Bihm et al. 1989; Borra et al. 1986; Nojima et al. 1983; Wegman and Wammes 1983). Highly sensitive analytical methods are also available for determining the observed mineralization products, carbon dioxide, methane, nitrate, and nitrite (Bartha et al. 1967; Battersby and Wilson 1989; Richartz et al. 1990; Schmidt and Gier 1989).

Analytical methods are also available for the determination of 2-amino-4-nitrophenol, 4-amino-2-nitrophenol, and diaminophenol, which are intermediate products of 2,4-DNP biodegradation (Kohping and Wiegel 1987; Madhosingh 1961). However, the details of the HPLC method used for the determination of a biodegradation product of 2,4-DNP (diaminophenol) was not given (see Kohping and Wiegel 1987). A GC/FID and a GC/MS method used for the determination of biodegradation products (phenol and benzene) of 2,4-DNP (Boopathy and Kulpa 1993) did not provide data on accuracy, precision or sensitivity of the method. This method is presented in Table 6-2.

6.3.2 Ongoing Studies

No ongoing studies were located in Federal Research in Progress (FEDRIP 1993) that would improve the available analytical methods for determining dinitrophenols in biological and environmental samples.