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 DNOC, its metabolites, and other biomarkers of exposure and effect to DNOC. 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.

In the design of a study and the selection of an analytical method, it is very important that adequate attention be paid to the extent of validation and field applicability of a particular method. Not all of the methods have been validated to the same extent. It is the analyst's responsibility to determine the data quality needed before initiating the application of a particular method.

The analytical methods used to quantify dinitrocresols, usually dinitro-*o*-cresol, in biological and environmental samples are summarized below. Table 6-1 lists the applicable analytical methods for determining dinitrocresols in biological fluids and tissues, and Table 6-2 lists the methods used for determining dinitrocresols in environmental samples.

A survey of literature revealed that DNOC in environmental and biological samples can be quantified following several separatory steps to isolate the DNOC from the sample matrix. The separatory steps for biological samples generally use liquid-liquid extraction followed by a spectrophotometric method for quantitation of relatively large concentrations of DNOC.

Extractions are commonly used to recover DNOC from environmental matrices; the exact form depends on the matrix (see below) and could include liquid-liquid extraction, solid phase extraction (SPE), or solid phase microextraction (SPME). In SPME, a small silica fiber coated with an organic layer is equilibrated with the sample, either in solution or in a headspace. The compound, DNOC in this application, is recovered by heating the fiber to desorb the organic compound into a gas

TABLE 6-1. Analytical Methods for Determining Dinitrocresols in Biological Samples

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Animal tissue	Extract sample mixed with methanol, sulfuric acid, and potassium oxalate with petroleum ether; clean up by gel permeation chromatography, methylate, and clean up by Florisil	GC-NPD	No data	45–50	Hopper et al. 1992
Urine, kidney, liver, brain (DNOC and metabolite 4-amino-2-methyl-6-nitrophenol)	Hydrolyze sample directly or after acetone extraction; extract with petroleum ether	Spectrophotometric	No data	No data	Truhaut and De Lavaur 1967
Serum	Dilute with water; add sodium chloride and sodium carbonate and extract with methyl ethyl ketone	Spectrophotometric	<0.5 mg/L	No data	Parker 1949
Tissue	Dilute homogenized tissue with water; add sodium chloride and sodium carbonate; extract with methyl ethyl ketone	Spectrophotometric	No data	No data	Parker 1949
Urine (DNOC and metabolite 4-amino-2-methyl-6-nitrophenol)	Acidify and subject to continuous extraction with diethyl ether	Spectrophotometric	No data	No data	Smith et al. 1953
Urine	Add sodium chloride and sodium carbonate; extract with methyl ethyl ketone	Spectrophotometric	<0.5 mg/L	No data	Parker 1949

GC = gas chromatography; NPD = nitrogen phosphorous detector

TABLE 6-2. Analytical Methods for Determining Dinitrocresols in Environmental Samples

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Technical and formulated products	Dissolve sample in methanol or acetone	HPLC/UV	2 ng[a]	No data	Farrington et al. 1982; Yao et al. 1991
Technical products	Dissolve sample in methanol	HPLC/ELCD	0.1 ng[a] (oxidative) 0.4 ng[a] (reductive)	No data	Yao et al. 1991
Air	Draw air through filter and a midget bubbler in series. DNOC extracted into ethylene glycol and 2-propanol added before analysis	HPLC/UV (Method S166)	0.070 mg/m^3 (8 ppb) for 180 L sample	104 for 0.07 mg loaded onto filter	NIOSH 1984
Water	Sample adjusted to pH 6.1 by buffer	HPLC/AdSV HPLC/DPP	0.1 µg/L (AdSV) 1.5 µg/L (DPP)	No data	Benadikova and Kalvoda 1984
Water	Extract reconstituted in methanol-acetonitrile acetic acid (20:78.5:1.5 v/v)	HPLC/UV	No data	97	Tripathi et al. 1989
Drinking water, atmospheric water	Acidify sample, add salt, and extract continuously with methylene chloride. Dry, reduce volume, and solvent exchange to hexane. Derivatize with acetic anhydride	GC/NPD	0.20 µg/L (0.2 ppm)	102 (5.5% RSD)	Herterich 1991
Drinking water, groundwater	Acidify water, add sodium sulfite, and pass through SPE cartridge of Carbopak. Elute with methanol/ methylene chloride; reduce volume	HPLC/UV	0.009 µg/L (9 ppb)	96	Di Corcia and Marchetti 1992
Drinking water, river water	Acidify sample and pass through SPE disk (Teflon with acetyl-polystyrene-divinylbenzene); elute with three aliquots of tetrahydrofuran	HPLC/UV	No data	95 (2% RSD) at 100 µg/L (0.1 ppm)	Schmidt et al. 1993

TABLE 6-2. Analytical Methods for Determining Dinitrocresols in Environmental Samples (continued)

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Groundwater	Acidify to pH=2, saturate with salt, and extract using SPME	GC/MS	0.070 µg/L (0.07 ppm) (5.6% RSD)	No data	Buchholz and Pawliszyn 1993
Groundwater, sediment	Extract acidified water with methylene chloride, reduce volume and solvent-exchange to 2-propanol	GC/FID (Method 8040)	160 µg/L	0.84C–1.01 where C= true value of concentration in µg/L	EPA 1986a
Groundwater, soil, solid waste	Extract acidified water with methylene chloride, reduce volume and exchange into 2-propanol. For other matrices, mix with anhydrous sodium sulfate and extract (soxhlet or sonication) with methylene chloride. Reduce volume. Clean-up with silica gel or GPC if needed	GC/MS (Method 8270)	50 µg/L (50 ppm water); 3.3 mg/kg (ppm soil/sediment)	1.04C–28.04 where C= true value of concentration in µg/L	EPA 1986b
Waste water	Extract acidified sample with methylene chloride; concentrate and exchange solvent to 2-propanol	GC/FID (Method 604)	16 µg/L (16 ppm)	83 at 100 µg/L	EPA 1984a
Waste water	Extract acidified sample with methylene chloride; concentrate	GC/MS (Method 625)	24 µg/L (24 ppm)	93 at 100 µg/L	EPA 1984b
Waste water	Extract acidified sample with methylene chloride, dry, and reduce volume. Add deuterated standards	GC/MS isotope dilution (Method 1625)	20 µg/L (20 ppm)	77–133 at 100 µg/L	EPA 1984c
Rain and snow	Extract acidified sample with methylene chloride; concentrate	HPLC/PDD	No data	No data	Alber et al. 1989

TABLE 6-2. Analytical Methods for Determining Dinitrocresols in Environmental Samples (continued)

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Soil	Extract with methylene chloride; evaporate to dryness and dissolve residue in alkaline methanol/water	HPLC/UV	0.005 mg/kg (5 ppb)	85–105	Roseboom et al. 1981
Soil	Soxtec extraction of clay loam using hexane:acetone (1:1). Reduce volume	GC/MS	No data	63.4 at 6 mg/kg	Lopez-Avilla et al. 1993
Variety of crops	Extract macerated or homogenized sample with methylene chloride; evaporate to dryness and dissolve in potassium carbonate/methanol mixture	HPLC/UV	0.005 mg/kg (5 ppb)	82–105 at 0.05 mg/kg. %RSD range from 4 to 13%	Roseboom et al. 1981
Various crops	Homogenize sample in blender, adding distilled water as needed. Add Florisil to form free flowing mixture and pack into a column with a sodium sulfate layer at bottom. Elute with methylene chloride-acetone (1:1) or ethyl acetate. Reduce volume	GC/ECD	0.001 mg/kg (1 ppb)	69–79 at 0.01–0.5 mg/kg	Kadenczki et al. 1992
Fatty and nonfat foods	Mix fatty sample with methanol, sulfuric acid, and potassium oxalate and nonfat samples with sulfuric acid and methanol; extract both with petroleum ether or methylene chloride; clean-up by gel permeation chromatography, methylate, and clean-up by Florisil	GC/NPD	No data	45–50 (fatty foods) >80 (nonfat foods)	Hopper et al. 1992

[a] These are absolute detection limits

AdSV = adsorptive stripping voltametric detector; DPP = differential pulse polarographic detector; ELCD = electrochemical detector; FID = flame ionization detection; GC = gas chromatography; HPLC = high performance liquid chromatography; HRGC = high resolution gas chromatography; MS = mass spectrometry; NPD = nitrogen phosphorus detector; PDD = photodiode array detector; RSD = relative standard deviation; SPE = solid phase extraction; SPME = solid phase microextraction; UV = ultraviolet detector; v/v = volume per volume.

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chromatograph. Both high performance liquid chromatographic (HPLC) and gas chromatographic (GC) methods have been used as final separator-y methods for environmental samples. A variety of detection methods, including flame ionization detection (FID), electron capture (ECD), thermal energy analyzer, mass spectrometric, nitrogen-phosphorus (NPD), ultraviolet (UV), photodiode detection, and electrochemical methods have been used to detect and quantify DNOC.

Some GC methods recommend the derivatization of dinitrocresols to increase their volatility to enhance elution from the GC column (Mhalas et al. 1989; Roseboom et al. 1981). Derivatization is not used in other GC methods (Buchholz and Pawliszyn 1993; EPA 1984a, 1984b, 1984c, 1986a, 1986b) and good results are obtained. If used, the relative efficiencies of the various derivatization methods should be evaluated. HPLC is advantageous for DNOC determination because derivatization is not required (Alber et al. 1989). A comprehensive study that compares the sensitivities among the detection methods is not available. However, when HPLC was used, an electrochemical detection method was 20 times more sensitive than the UV method (spectrophotometric) (Yao et al. 1991). When a GC method was used, NPD was more suitable than either ECD or a FID for determining DNOC (EPA 1982a; Hopper et al. 1992). On the other hand, GC/FID was more sensitive than GC/MS (EI mode) and HPLCKJV (at 254 nm) (James et al. 1984). The sensitivity of the overall method can be highly dependent on the extraction and clean-up steps. Exceptional selectivity and recovery during sample preparation can result in a very sensitive method even when the determinative step utilizes a technique not generally considered to provide the highest sensitivity, such as HPLC/UV (Di Corcia and Marchetti 1992).

Although not used in any of the overall methods found, Fourier transform-infrared spectroscopy for detection after GC can supplement MS to verify the presence of DNOC in samples (Budzinski et al. 1992; Gurka et al. 1991; Schneider et al. 1991). Alternative separation methods have also been shown to be applicable to nitrophenols, including DNOC, but have not yet become routine. These methods include supercritical fluid chromatography (Ong et al. 1992; Pospisil et al. 1992), capillary zone electrophoresis (Chao and Whang 1994), and micellar electrokinetic chromatography (Ong et al. 1991).

6.1 BIOLOGICAL SAMPLES

Analytical methods used for determining DNOC in biological samples are listed in Table 6-l. With the exception of the method of Hopper et al. (1982), the methods found rely on spectrophotometry for

identification or quantitation in extracts (Smith et al. 1953) or after paper chromatography (Parker 1949). It should be possible to modify Parker's method (1949) for use with modern thin layer chromatographic techniques (Sherma 1991). A quantitative method for monitoring human exposure to DNOC and other genotoxic agents in urine is also available (San et al. 1989). In this method, urine extracts were assayed for their capacity to induce chromosome and chromatid damage in cultured Chinese hamster ovary fibroblasts. This method is not specific for DNOC and additional analysis is required to verify that the effect was due specifically to DNOC.

6.2 ENVIRONMENTAL SAMPLES

Analytical methods used for determining DNOC in environmental samples are given in Table 6-2. Most of the methods for products, waters, soils, and sludges rely on extraction of DNOC from an acidified matrix; acidification minimizes dissociation of the phenolic hydrogen and thus facilitates extraction into an organic solvent or adsorption onto a solid phase extraction medium. The influence of pH on the adsorption of DNOC to humic materials in coal waste waters has been studied (Pbrschmann and Stottmeister 1993) and significant adsorption was found to occur at pH 7 but not at pH=2. In general, methods utilizing MS or selective detectors were less subject to interferences from complex samples than those methods based on the less selective FID detection. The method for DNOC in air (NIOSH 1984) recovers both DNOC vapor and DNOC adsorbed to particulates. DNOC is present in air both as a vapor and adsorbed to both solid (dust) and liquid (rain, fog) particulate matter (Perez and Soderholm 1991). The distribution between the vapor and particulate phase was not measured.

Dinitrocresols present in water samples, especially when present at low concentrations, can be lost via oxidation by hypochlorite (Di Corcia and Marchetti 1992). This can be eliminated through the addition of sodium sulfite prior to extraction. In the multiresidue method of Chen et al. (1991), Mn^{2+} dissolved in the sample would be oxidized to manganese(III,IV) oxides during base extraction, which in turn would oxidize the phenols during acid extraction. The oxidation of phenols can be eliminated by adding sodium thiosulfate to the sample prior to extraction or extraction at acidic pH as the first step, providing that manganese(III,IV) oxides are not present in the sample before extraction.

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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 dinitrocresols 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 dinitrocresols.

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. In humans, a significant portion of absorbed DNOC appears in the urine as the metabolite 4-amino-2-methyl-6-nitrophenol (WHO 1975). The measurement of this metabolite may be an indicator for DNOC exposure (WHO 1975). Analytical methods for determining DNOC and its urinary metabolite are available (Smith et al. 1953; Truhaut and De Lavaur 1967), although the limits of detection for these methods have not been documented. Harvey et al. (1951) and King and Harvey (1953b) used paper chromatography to study DNOC in blood after exposures of 0.9-1.3 mg/kg/day and the methods were adequate to detect DNOC for many days after exposure. It is not clear, however, if the methods would be adequate to detect DNOC in blood after an exposure at the oral MRL of 0.004 mg/kg/day (calculated in Chapter 2). If a 70 kg person is assumed, an MRL dose of 0.28 mg/day can be calculated. It seems likely that the methods would be sensitive enough to detect DNOC in blood, at least shortly after the exposure, but this has not been shown. The metabolites 4,6-dinitro-2-hydroxymethylphenol and 4,6-diacetamidoo-cresol have also been determined in urine using TLC in conjunction with field desorption mass spectrometry (van der Greel and Leegwater 1983). The limits of detection were not reported for this method either. However, neither blood nor urinary levels of DNOC are reliable indicators for magnitude or the time of exposure to DNOC (Harvey et al. 1951; King and Harvey 1953b). The

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DNOC levels in any other tissue or body fluid of humans have not been correlated with the magnitude and duration of exposure to DNOC (see Section 2.5.1). The identification of a biomarker that can be correlated with the level of exposure to DNOC would be helpful and is needed. The analytical methods should be updated and validated.

Although elevated levels of blood urea nitrogen and blood glucose, and decreased blood pyruvate indicate metabolic disturbances caused by DNOC (Den Tonkelaar et al. 1983; Spencer et al. 1948) (see Section 2.5.2), these effects are not unique to DNOC exposure (see Section 2.5.2). Therefore, it would be useful to identify an effect that may be uniquely and quantitatively associated with exposure to DNOC.

Methods for Determining Parent Compounds and Degradation Products in Environmental Media. The analytical methods presently available are capable of determining DNOC in fatty and nonfat foods at levels well below the tolerance limit (Hopper et al. 1992; Roseboom et al. 1981). Methods for DNOC in water are sufficiently sensitive to monitor concentrations well below the MRL for a 70 kg individual (Buchholz and Pawliszyn 1993; Di Corcia and Marchetti 1992). The method for DNOC in air is sensitive to concentrations below the OSHA standard of 0.2 mg/m^3 (NIOSH 1984) but is inconvenient for personal monitoring because of the liquid contained in the bubbler. Methods are currently available for determining degradation products obtained as a result of DNOC biodegradation by pure cultures of microorganisms (Gundersen and Jensen 1956; McCormick et al. 1976; Tewfik and Evans 1966). The limits of detection have not been established for degradation products. If the degradation products are of interest, methods need to be refined and validated.

6.3.2 Ongoing Studies

The Environmental Health Laboratory Sciences Division of the National Center for Environmental Health, Centers for Disease Control, is developing methods for the analysis of dinitrocresols and other phenolic compounds in urine. These methods use high resolution gas chromatography and magnetic sector mass spectrometry which gives detection limits in the low parts per trillion (ppt) range.

No other ongoing studies that would improve upon the methods to determine the levels of DNOC or its metabolites in biological samples were located. An ongoing study at Rutgers University designed

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to study the degradation of nitroaromatic compounds by microorganisms was found. This work is sponsored by the USDA. It appears that the researchers involved are developing or modifying the analytical methods used to identify metabolic intermediates. The analytical techniques being used include TIC, GC, GC/MS, and infrared absorbance.