7. ANALYTICAL METHODS

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

Several methods are available for the analysis of nitrate and nitrite in biological media; details of selected methods are provided in Table 7-1.

Following the ingestion of nitrate and nitrite, they are readily absorbed from the upper gastrointestinal tract into the blood and readily excreted in human urine as nitrate. This process is essentially complete at 18 hours following ingestion; minor urinary products of nitrate and nitrite metabolism include ammonia and urea (Gangolli et al. 1994). Portions of blood nitrate are transported to human saliva where it is mostly metabolized to nitrite. In human blood and tissues, nitrite is typically oxidized to nitrate. Concentrations of nitrate in urine and saliva fluctuate; therefore, in order to evaluate exposure more precisely, a 24-hour collection of urine is recommended. Analysis is achieved via hydrazine reduction (IARC 2010; Levallois et al. 2000).

Levels of nitrate and nitrite in plasma, urine, and saliva can be measured by gas chromatography/mass spectrometry (GC/MS) (Bondonno et al. 2012; Tsikas 2005). Frozen samples are treated with tetraoctylammonium bromide and derivatizing reagent pentafluorobenzyl bromide in acetone solutions at elevated temperature. Acetone is removed by evaporation under a nitrogen atmosphere and the remaining aqueous phase is extracted with an isooctane/toluene solution and analyzed by GC/MS (m/z = 62 for nitrate and 46 for nitrite). Sample procedures must involve precautionary steps to minimize the

Table 7-1. Analytical Methods for Determining Nitrate and Nitrite in Biological Materials

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Urine	Hydrazine reduction	GC-MS	5 ng/L	Not reported	Levallois et al. 2000
Plasma, urine, or saliva	Sample derivitization with tetraoctyl-ammonium bromide and pentafluorobenzyl bromide	GC-MS	Not reported	Not reported	Bondonna et al. 2012
Plasma (serum)	Deproteinization; Griess reaction	HPLC-UV spectrometry; GC-MS	0.3–20 µM (nitrite) 4–81 µM (nitrate)	Not reported	Hibbs et al. 1992; Sun et al. 2003; Tsikas 2005
Urine	Griess	GC-MS	690 µmol/ 24 hours	Not reported	Hibbs et al. 1992
Urine	International standard dilution	IC-MS/MS	500 µg/L (nitrate)	95	Valentín-Blasini et al. 2007
Urine (metabolite-ammonia)	24-Hour specimen collected, preserve with HCl and refrigerate	Colorimetric (Berthelot reaction)	Not reported	Not reported	Tietz 1970
Urine (metabolite-ammonia)	24-Hour specimen analyzed immediately, or stored up to 8 weeks at -20 °C	Indophenol reaction	Not reported	Not reported	Huizenga et al. 1994)
Whole blood	Deprotonized using acetonitrile followed by purification	HPLC/direct conductivity detection	0.4 µmol/L (nitrite)		Yan et al. 2016

GC-MS = gas chromatography-mass spectrometry; HPLC = high-performance liquid chromatography; IC-MS/MS = ion chromatography-mass spectrometry/mass spectrometry; UV = ultraviolet

endogenous contribution of analytes in the laboratory chemicals and materials (glassware, filtration equipment, etc.) used for sample collection and work up (Tietz 1970; Tsikas 2005). Chemical interferences in samples resulting in reduction of nitrate to nitrite, or conversely, the oxidation of nitrite to nitrate during analysis should be evaluated and accounted for. Microbial conversion via hydrolysis may cause an increase in values (Tietz 1970). Preparation of blood samples must involve procedures that limit the oxidation of nitrite by oxyhaemoglobin and loss due to methods requiring acidification or derivatization. Possible interferences in ammonia quanitification and to the Griess assay, such as anticoagulants, must also be factored (Huizenga 1994; Tsikas 2005).

The Griess assay is one of the first methods used to measure levels of nitrate and nitrite in biological and environmental samples. The method involves reduction of nitrate to nitrite followed by a diazotization reaction and then measuring the absorbance of the diazo chromophore in the visible spectrum. To determine the levels of nitrate and nitrite separately, the procedure is first carried out without the preliminary reduction step in order to quantify the level of nitrite solely. This assay was originally performed using sulfanilic acid, which forms a diazonium cation with nitrite under acidic conditions followed by coupling with α-naphthylamine to form a diazo compound, which contains a strong absorption band at about 540 nm (Tsikas 2005). Other methods include diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form the diazo compound (EPA 1993). GC/MS methods were shown to provide superior quantification of nitrate and nitrite in human plasma and urine samples when compared to the Griess assays (Tsikas 2005)

Reverse-phase high performance liquid chromatography (HPLC) by means of ion pairing in the mobile phase without derivitization followed by ultraviolet (UV) detection around 210 nm has also been used to detect nitrate in urine samples (Tsikas 2005). Urinary nitrate levels can be measure using ion chromatography-tandem mass spectrometry (IC-MS/MS) by means of internal standard dilution (Valentín-Blasini et al. 2007).

The level of methemoglobin in the blood is often the biomarker for assessing nitrate exposure (Manassaram et al. 2010). Methemoglobin can be measured in blood collected via finger stick samples. Samples are analyzed with portable AVOXimeter 4000 whole-blood oximeter devices. The device measures total hemoglobin, and further characterizes percentages of oxyhemoglobin, carboxyhemoglobin, and methemoglobin. The accuracy and precision of the method were reported as ±0.5 and ±0.7%, respectively. Refer to ATSDR (Agency for Toxic Substance and Disease Registry 2013b) for discussion of other nitrate and nitrite laboratory tests.

Yan et al. (2016) developed a simple method for the quantitative determination of nitrite in whole blood samples employing ion chromatography and electrochemical detection. The blood sample is prepared by adding acetonitrile followed by purification using mini-cartridges to remove interfering compounds. The detection limit for the method is reported as 0.4 μmol/L.

7.2 ENVIRONMENTAL SAMPLES

Methods are available for determining the level of nitrate and nitrite in a variety of environmental matrices. A summary of representative methods is shown in Table 7-2.

Ion chromatography and spectrometry methods are the most common analytical techniques employed for the detection and quantification of nitrate and nitrite in environmental samples; detection limits range from 0.01 to 1 mg/L (ppm) (IARC 2010; WHO 2011b). Samples must be analyzed as soon as is reasonably possible in order to minimize any changes in the sample due to microbial transformations. Sample preservation using chemicals and or deep freezing methods have been reported; however, interference with the analysis can occur in certain methods (Mulvaney 1996).

Methods based on the Griess assay are available for the determination of nitrate and nitrite in potable water, raw water and wastewater (EPA 1993; WHO 2011b). The limit of detection for the International Organization for Standardization ISO method 6777/1 lies within the range of 0.005–0.01 mg/L (ppm) (WHO 2011b). A continuous-flow spectrometric method (ISO method 7890-1) for the determination of nitrite, nitrate or the sum of both in various types of water is suitable at concentrations ranging from 0.05 to 5 mg/L (ppm) for nitrite and from 1 to 100 mg/L (ppm) for nitrite and nitrate, both in the undiluted sample (WHO 2011b).

NIOSH method 7903 employs ion chromatography for the determination of nitric acid in air (NIOSH 1994a). Method 7903 is an analytical technique for determining inorganic acids by measuring the total concentration of airborne anions. Particulate nitrate has been successfully detected and quantified in atmospheric samples via ion chromatographic techniques and NO_x chemiluminescent analyzers (Small et al. 1975; Yoshizumi et al. 1985)

Table 7-2. Analytical Methods for Determining Nitrate and Nitrite in Environmental Samples

Sample matrix[a]	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Air/water (nitrate, nitrite)	Nitrite prepared using Griess-Ilosvay reaction; nitrate prepared using hydrazine reduction; aqueous extracts from aerosol filters are analyzed without pretreatment	UV spectrometry	0.07 ppm (nitrite); 0.2 ppm (nitrate)	Not reported	Oms et al. 1995
Air (nitrate)	Personal air sampled at 0.2–0.5 L/minute for total sample size of 3–100 L using silica gel sample tube; boil sorbent from sample tube in bicarbonate/ carbonate buffer for 10 minutes	Ion chromatography/ conductivity detector; NIOSH 7903	0.7 µg/sample	Not reported	NIOSH 1994a
Air (nitrite)	Ambient air is sampled at 0.025 L/minute for 3-L air sample using glass sorbent tubes with glass wool retainers; add adsorbing solution; add solution of hydrogen peroxide, sulfanilamide and NEDA to extracted sample, set for 10 minutes	Visible absorption spectro-photometry; NIOSH 6014	1 µg/sample	Not reported	NIOSH 1994b
Air (nitrite)	Ambient air is sampled at 0.025 L/minute for 3-L air sample using diffusive sampler tubes with three triethanolamine screens; add adsorbing solution; add solution of hydrogen peroxide, sulfanilamide and NEDA to extracted sample, set for 10 minutes	Visible absorption spectro-photometry; NIOSH 6700	0.01 µg/sample	Not reported	NIOSH 1998
Water (nitrate)	Drinking water or river water samples are prepared with lanthanum (III) chloride and placed into the cell	Voltammetry/ static mercury drop electrode	20 µg/L	Not reported	Markusova et al. 1996

Table 7-2. Analytical Methods for Determining Nitrate and Nitrite in Environmental Samples

Sample matrix[a]	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Drinking water	Clean samples can be used directly	IC/CD	Not reported	93–114 (nitrite-N) 83–113 (nitrate-N)	IARC 2010
Soil (nitrate)	Soil samples are added to Gohler solutions followed by neutralization before analysis	Voltammetry/ static mercury drop electrode	20 µg/L	Not reported	Markusova et al. 1996
Soil (nitrite, nitrate)	Extraction of field samples with 2 M KCL; nitrate reduction; diazoitization and coupling with resulting dye formation	UV-Vis (λ=540 nm)	Not reported	Not reported	Mulvaney 1996
Foods and juices (nitrate, nitrite)	Blended/pureed food samples are mixed with water and filtered	Capillary ion electrophoresis	Not reported	>73 (nitrate); >88 (nitrite)	Marshall and Trenerry 1996
Food (nitrate, nitrite)	Direct injection	HePI-MS	Not reported	Not reported	Pavlov and Attygalle 2013
Fish and water (nitrate, nitrite)	Fish muscle homogenized, digested with perchloric acid and centrifuged; nitrate reduction to nitrite using copperised cadmium redactor; reaction with phosphomolybdenum blue complex and ammonium chloride	FIA spectrometry	0.01 µg/mL (nitrite); 0.025 µg/mL (nitrate)	97.8–102.1 (nitrite); 98.5–101.6 (nitrate)	Monser et al. 2002
Water and vegetables (nitrate)	Food: homogenization with deionized water and heated at 80°C; cooled and diluted with deionized water Water: direct analysis	Potentiometry with solid contact ISE	0.0037 µ/mL (nitrate)	98.9–105.9 (nitrate)	Wardak and Grabarczky 2016
Cured meat (nitrite)	Reaction with sulfanilamide followed by reaction with NEDA	Colorimetry; absorbance 540 nm	Not reported	Not reported	IARC 2010
Milk and milk products (nitrate, nitrite)	Suspension in buffer solution; centrifuge and reduce with cadmium; react with sulfanilamide followed by reaction with NEDA	FIA spectrometry; absorbance 540 nm	0.5 mg/kg (nitrate) 1.0 mg/kg (nitrite)	Not reported	IARC 2010

Table 7-2. Analytical Methods for Determining Nitrate and Nitrite in Environmental Samples

Sample matrix[a]	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Dairy products and cheese (nitrate, nitrite)	Extract cheese slurry using $ZnSO_4$ and NaOH; reduce in Jones reductor using zinc and $CdSO_4$	Spectro-photometry; absorbance at 522 nm	≥1 µg/g nitrate	Not reported	IARC 2010
Fried bacon (N-nitros-amines)	Grind frozen sample; vacuum distill with NaOH and mineral oil; extract and dry with DCM and anhydrous $NaSO_4$; concentrate	GC	Not reported	Not reported	IARC 2010

DCM = dichloromethane; FIA = flow injection analysis; GC = gas chromatography; HePI-MS = helium-plasma ionization-mass spectrometry; IC/CD = ion chromatography/conductivity detector; ISE = ion-selective electrodes; NEDA = N-1-naphthylethylenediamine dihydrochloride; NIOSH = National Institute for Occupational Safety and Health; UV = ultraviolet absorbance detection

A sequential injection method coupled with spectrophotometry has been developed for the detection of nitrate and nitrite in environmental samples such as atmospheric aerosol filter extracts and waste water samples (Oms et al. 1995). The method is advantageous due to the small volumes of sample and reagents required for analysis. The detection limits were reported as 0.07 ppm for nitrite and 0.2 ppm for nitrate. Nitrite is analyzed using the Griess-Ilosvay reaction; nitrate is reduced to nitrite using hydrazine sulphate.

Markusova et al. (1996) developed a sensitive voltammetric method that can determine nitrate levels in drinking water, river water, or soil extracts three orders of magnitude lower than the allowed levels of nitrate in drinking water. The method employs a multi-purpose electrochemical analyzer and a voltammetric cell. Water samples are prepared with lanthanum (III) chloride and placed into the cell; soil samples are added to Gohler solutions followed by neutralization before analysis. The reported limit of detection is 20 µg nitrate/L (20 ppb) (5 µg NO_3^-N/L).

The most commonly used method for soil and soil extract analysis of nitrite is a modified Griess-Ilosvay colorimetric method using a continuous flow analyzer. Nitrites react with primary aromatic amines to form a diazonium salt which is then coupled with an aromatic compound; the resulting complex has a characteristic absorbance band in the UV-Vis spectrum. The concentration of nitrite is proportional to the color intensity of the resulting azo compound measured using a spectrophotometer or colorimeter. This technique is also used for sensitive analysis of nitrate following reduction to nitrite. Cadmium reduction to nitrite is achieved in a column of copperized cadmium with an ammonium chloride (NH4Cl) matrix at pH between 5 and 10. Other various reducing agents have been reported. Analysis for nitrate must account for initial concentrations of nitrite in the sample prior to reduction. Maximum accuracy is seen when absorbance is measured at wavelengths of 540 nm; however, wavelengths between 510 and 550 nm are acceptable (Mulvaney 1996).

Pavlov and Attygalle (2013) developed an analytical method with minimal sample preparation employing helium-plasma ionization-mass spectrometry. Nitrate was successfully identified and quantified using this solvent-less ambient pressure mass spectrometry technique in various foodstuffs. Samples of fruit juice and meat pieces (i.e., tomato and celery juice, hot dog and beef) can be placed onto glass slides and analyzed directly without any modification. Quantification of nitrate in such complex matrices is suggested to be determined with accuracy by spiking with known quantities of radiolabeled nitrate. The method detection limit for determining the nitrate concentration is in the range of 20 ng/sample and depends on the specific sample matrix.

Capillary ion electrophoresis has been successfully employed for the determination of nitrite and nitrate in foods and juices (Marshall and Trenerry 1996). The authors tested the procedure using cheese, cabbage, fruit juices, and meats. Percent recovery for three processed meat samples ranged from 88 to 118% for nitrite and from 73 to 106% for nitrate.

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 nitrate and nitrite is available. Where adequate information is not available, ATSDR, in conjunction with 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 nitrate and nitrite.

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. Nitrate and nitrite may be converted to many other compounds in the body, such as N-nitroso compounds, including nitrosamines. Approximately 25% of absorbed nitrate is secreted to saliva and about 20% of this is reduced to nitrite. Nitrite is converted to nitric oxide by the acidic environment on the stomach. Methods exist for the measurement of nitroso compounds and nitrite in plasma and salivary nitrite (Bondonno et al. 2012). Nitrate in the diet may contribute to nitric oxide levels in the body, and increases in these levels can be a biomarker of exposure. Ammonia is a minor urinary product of nitrite and nitrate in which analytical methods are available (Huizenga et al. 1994; Tietz 1970). N-Methyl-nicotinamide has also been shown to be a potential biomarker of exposure to nitrate and nitrite and there are methods to measure this (Jansen et al. 1995). No data needs were identified.

Effect. Methemoglobinemia caused by the presence of higher-than-normal levels of methemoglobin is a biomarker of effect for exposure to high levels of nitrate; however, this effect is not unique for nitrate and nitrite since other substances may also cause this condition (Bruning-Fann and Kaneene 1993). Methods are available to measure methemoglobin in the blood (Manassaram et al. 2010).

Methods for Determining Parent Compounds and Degradation Products in Environmental Media. Methods are available for determining nitrate and nitrite levels in environmental samples such as air (NIOSH 1994a; Small et al. 1975; Yoshizumi et al. 1985) and water (EPA 1993; Markusova et al. 1996; WHO 2011b).

7.3.2 Ongoing Studies

No ongoing analytical methodologies for nitrate or nitrite were identified using the NIH RePORTER version 6.1.0 or the DTIC online database.