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Methods for the Determination of Chemical Substances in Marine and Estuarine Environmental Matrices - 2nd Edition

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National Exposure Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

DISCLAIMER

This manual has been reviewed by the National Exposure Research Laboratory - Cincinnati, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. The National Exposure Research Laboratory - Cincinnati (NERL-Cincinnati) conducts research to:

- Develop and evaluate analytical methods to identify and measure the concentration of chemical pollutants in marine and estuarine waters, drinking waters, surface waters, ground waters, wastewaters, sediments, sludges, and solid wastes.
- Investigate methods for the identification and measurement of viruses, bacteria, and other microbiological organisms in aqueous samples and to determine the responses of aquatic organisms to water quality.
- Develop and operate a quality assurance program to support the achievement of data quality objectives in measurements of pollutants in marine and estuarine waters, drinking waters, surface waters, ground waters, wastewaters, sediments, and solid wastes.
- Develop methods and models to detect and quantify responses in aquatic and terrestrial organisms exposed to environmental stressors and to correlate the exposure with effects on chemical and biological indicators.

This NERL-Cincinnati publication, "Methods for the Determination of Chemical Substances in Marine and Estuarine Environmental Matrices - 2nd Edition" was prepared as the continuation of an initiative to gather together under a single cover a compendium of standardized laboratory analytical methods for the determination of nutrients, metals, chlorophyll and organics in marine matrices. It is the goal of this initiative that the methods that appear in this manual will be multilaboratory validated. We are pleased to provide this manual and believe that it will be of considerable value to many public and private laboratories involved in marine studies for regulatory or other reasons.

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ABSTRACT

This manual contains eleven methods for determination of nutrients, metals, and chlorophyll. Since Revision 1.0 appeared in 1992, four new methods have been added, one deleted and four have been multilaboratory validated. Methods 440.0, 445.0, 446.0 and 447.0 have been multilaboratory validated, and Method 353.4 has been replaced with an improved method.

The metals methods, Methods 200.10, 200.12 and 200.13 have not changed since the 1992 manual. Method 365.5 has remained the same and Method 440.0, that appeared in 1992, now contains multilaboratory validation data. Two new chlorophyll methods, Methods 446.0 and 447.0, have been added and all three chlorophyll methods have been multilaboratory validated. Since the chlorophyll methods validation study was also a comparison study of the methods, that data has been added to the methods. Anyone interested in obtaining a copy of the full chlorophyll study final report should contact the Chemical Exposure Research Branch office of NERL-Cincinnati.

CONTENTS

	Page
Disclaimer	ii
Foreword	iii
Abstract	iv
Acknowledgments	vii
Introduction	1

Method Number	Title	Revision	Multilab Validation Status
200.10	Determination of Trace Elements in Marine Waters by On-line Chelation Preconcentration and Inductively Coupled Plasma - Mass Spectrometry	1.6	No
200.12	Determination of Trace Elements in Marine Waters by Stabilized Temperature Graphite Furnace Atomic Absorption	1.0	No
200.13	Determination of Trace Elements in Marine Water by Off-Line Chelation Preconcentration with Graphite Furnace Atomic Absorption	1.0	No
349.0	Determination of Ammonia in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis	1.0	No
353.4	Determination of Nitrate and Nitrite in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis	1.0	No
365.5	Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis	1.4	Yes
366.0	Determination of Dissolved Silicate in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis	1.0	No

440.0	Determination of Carbon and Nitrogen in Sediments and Particulates of Estuarine/Coastal Waters Using Elemental Analysis	1.4	Yes
445.0	*In Vitro* Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Phytoplankton by Fluorescence	1.2	Yes
446.0	*In Vitro* Determination of Chlorophylls *a*, *b*, c_1+c_2 and Pheopigments in Marine and Freshwater Algae by Visible Spectrophotometry	1.2	Yes
447.0	Determination of Chlorophylls *a* and *b* and Identification of Other Pigments of Interest in Marine and Freshwater Algae Using High Performance Liquid Chromatography with Visible Wavelength Detection	1.0	Yes

ACKNOWLEDGMENTS

This manual is dedicated to the memory of Dr. Barbara Metzger, late Director of the Environmental Services Division of USEPA Region 2. She was the impetus and driving force for this work.

This manual was prepared by the Chemical Exposure Research Branch of the Microbiological and Chemical Exposure Assessment Research Division, NERL-Cincinnati. The metals and chlorophyll methods were authored by in-house scientists and the nutrient methods were authored under contract by Carl Zimmermann and Carolyn Keefe at the Chesapeake Biological Laboratory, University of Maryland and under an interagency agreement by Dr. Jia-Zhong Zhang, National Oceanic and Atmospheric Administration, Atlantic Oceanographic and Meteorological Laboratory, Ocean Chemistry Division. Dr. Zhang deserves recognition for the outstanding efforts he put into making these methods both informative and practical.

Special thanks go out to Dr. Margo Hunt of USEPA Region 2 for staying so involved in the chlorophyll methods study. The need to standardize analytical methods for use in the marine environment was identified and championed by the USEPA regions. The staff at Regions 2 and 3 were instrumental in identifying resources for this project. They provided insight from the regional perspective and served as technical advisors. Their input has been valuable.

Diane Shirmann and Helen Brock put a tremendous effort into preparing this manuscript and we are extremely thankful for their hard work.

INTRODUCTION

Since the first edition of this manual was published in 1992, the Environmental Monitoring Systems Laboratory (EMSL) has been reorganized and its name changed to the National Exposure Research Laboratory (NERL). The principal aim of this manual is to bring together under one cover a suite of analytical methods specifically adapted or developed for the examination of coastal and estuarine environmental samples. Many of the methods presented here are adaptations of analytical techniques which, for many years, have been used routinely by the marine community. Hallmarks of the methods which appear in this manual, however, are the integrated quality control/quality assurance requirements, the use of standardized terminology and the Environmental Monitoring Management Council (EMMC) format. The mandatory demonstration of laboratory capability and the continuing checks on method performance ensure the quality and comparability of data reported by different laboratories and programs. Another distinction of this manual is the multilaboratory validation data for many of the methods.

Multilaboratory validation studies test the ruggedness of methods, provide single-analyst and multilaboratory precision and accuracy statements, and method detection limits that are "typical" of what most laboratories can achieve. Methods that reach this level of evaluation have been thoroughly investigated to the fullest extent possible by a single laboratory and have usually been informally adopted as standard methods by the analytical community. When a method does not perform as expected in a multilaboratory study, it must be returned to the development phase. For example, although widely accepted and routinely used in the marine community, Method 353.4 (Determination of Nitrite + Nitrate in Estuarine and coastal Waters by Automated Colorimetric Analysis) failed the ruggedness test in 1992 when 50% of the participating laboratories in the multilaboratory study returned unacceptable data. Review of the data suggested that the cadmium reduction column chemistry and maintenance required further investigation. The method was subsequently reevaluated by Dr. Jia-Zhong Zhang, under an Interagency Agreement between the U.S. EPA and NOAA. The new nitrite/nitrate method is improved in technical detail and QA/QC requirements.

We are pleased to present this 2nd Edition manual to the public and to research and monitoring labs in the hope that it contributes to better protection and preservation of our estuarine and coastal ecosystems.

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Method 200.10

Determination of Trace Elements in Marine Waters by On-Line Chelation Preconcentration and Inductively Coupled Plasma - Mass Spectrometry

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Method 200.10

Determination of Trace Elements in Marine Waters by On-Line Chelation Preconcentration and Inductively Coupled Plasma - Mass Spectrometry

1.0 Scope and Application

1.1 This method describes procedures for preconcentration and determination of total recoverable trace elements in marine waters, including estuarine water, seawater, and brines.

1.2 Acid solubilization is required prior to the determination of total recoverable elements to facilitate breakdown of complexes or colloids that might influence trace element recoveries. This method should only be used for preconcentration and determination of trace elements in aqueous samples.

1.3 This method is applicable to the following elements:

Element		Chemical Abstracts Service Registry Numbers (CASRN)
Cadmium	(Cd)	7440-43-9
Cobalt	(Co)	7440-48-4
Copper	(Cu)	7440-50-8
Lead	(Pb)	7439-92-1
Nickel	(Ni)	7440-02-0
Uranium	(U)	7440-61-1
Vanadium	(V)	7440-62-2

1.4 Method detection limits (MDLs) for these elements will be dependent on the specific instrumentation employed and the selected operating conditions. However, the MDLs should be essentially independent of the matrix because elimination of the matrix is a feature of the method. Reagent water MDLs, which were determined using the procedure described in Section 9.2.4, are listed in Table 1.

1.5 A minimum of 6-months experience in the use of commercial instrumentation for inductively coupled plasma mass spectrometry (ICP-MS) is recommended.

2.0 Summary of Method

2.1 This method is used to preconcentrate trace elements using an iminodiacetate functionalized chelating resin.[1,2] Following acid solubilization, the sample is buffered prior to the chelating column using an on-line system. Groups I and II metals, as well as most anions, are selectively separated from the analytes by elution with ammonium acetate at pH 5.5. The analytes are subsequently eluted into a simplified matrix consisting of dilute nitric acid and are determined by ICP-MS using a directly coupled on-line configuration.

2.2 The determinative step in this method is ICP-MS.[3-5] Sample material in solution is introduced by pneumatic nebulization into a radio frequency plasma where energy transfer processes cause desolvation, atomization and ionization. The ions are extracted from the plasma through a differentially pumped vacuum interface and separated on the basis of their mass-to-charge ratio by a quadrupole mass spectrometer having a minimum resolution capability of 1 amu peak width at 5% peak height. The ions transmitted through the quadrupole are registered by a continuous dynode electron multiplier or Faraday detector and the ion information is processed by a data handling system. Interferences relating to the technique (Section 4) must be recognized and corrected. Such corrections must include compensation for isobaric elemental interferences and interferences from polyatomic ions derived from the plasma gas, reagents or sample matrix. Instrumental drift must be corrected for by the use of internal standardization.

3.0 Definitions

3.1 ***Calibration Blank (CB) --*** A volume of reagent water fortified with the same matrix as the calibration standards but without the analytes, internal standards, or surrogate analytes.

3.2 ***Calibration Standard (CAL) --*** A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.3 ***Instrument Detection Limit (IDL) --*** The minimum quantity of analyte or the concentration equivalent that gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time, absorbance line, etc.

3.4 ***Instrument Performance Check Solution (IPC) --*** A solution of one or more method analytes, surrogates, internal standards, or other test substances used to evaluate the performance of the instrument system with respect to a defined set of criteria.

3.5 ***Internal Standard (IS) --*** A pure analyte(s) added to a sample, extract, or standard solution in known amount(s) and used to measure the relative responses

of other method analytes and surrogates that are components of the same sample or solution. The internal standard must be an analyte that is not a sample component.

3.6 Laboratory Fortified Blank (LFB) -- An aliquot of reagent water or other blank matrices to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control and whether the laboratory is capable of making accurate and precise measurements.

3.7 Laboratory Fortified Sample Matrix (LFM) -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.8 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.9 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.10 Material Safety Data Sheet (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.11 Method Detection Limit (MDL) -- The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero.

3.12 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations that is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.13 Stock Standard Solution (SSS) -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

3.14 Total Recoverable Analyte (TRA) -- The concentration of analyte determined to be in either a solid sample or an unfiltered aqueous sample following treatment by refluxing with hot dilute mineral acid(s) as specified in the method.

3.15 Tuning Solution (TS) -- A solution that is used to adjust instrument performance prior to calibration and sample analyses.

4.0 Interferences

4.1 Several interference sources may cause inaccuracies in the determination of trace elements by ICP-MS. These are:

4.1.1 Isobaric elemental interferences -- Are caused by isotopes of different elements that form singly or doubly charged ions of the same nominal mass-to-charge ratio and that cannot be resolved by the mass spectrometer in use. All elements determined by this method have, at a minimum, one isotope free of isobaric elemental interference. The analytical isotopes recommended for use with this method are listed in Table 1.

4.1.2 Abundance sensitivity -- Is a property defining the degree to which the wings of a mass peak contribute to adjacent masses. The abundance sensitivity is affected by ion energy and quadrupole operating pressure. Wing overlap interferences may result when a small ion peak is being measured adjacent to a large one. The potential for these interferences should be recognized and the spectrometer resolution adjusted to minimize them.

4.1.3 Isobaric polyatomic ion interferences -- Are caused by ions consisting of more than one atom that have the same nominal mass-to-charge ratio as the isotope of interest and that cannot be resolved by the mass spectrometer in use. These ions are commonly formed in the plasma or interface system from support gases or sample components. Such interferences must be recognized, and when they cannot be avoided by the selection of alternative analytical isotopes, appropriate corrections must be made to the data. Equations for the correction of data should be established at the time of the analytical run sequence as the polyatomic ion interferences will be highly dependent on the sample matrix and chosen instrument conditions.

4.1.4 Physical interferences -- Are associated with the physical processes that govern the transport of sample into the plasma, sample conversion processes in the plasma, and the transmission of ions through the plasma mass spectrometer interface. These interferences may result in differences between instrument responses for

the sample and the calibration standards. Physical interferences may occur in the transfer of solution to the nebulizer (e.g., viscosity effects), at the point of aerosol formation and transport to the plasma (e.g., surface tension), or during excitation and ionization processes within the plasma itself. Internal standardization may be effectively used to compensate for many physical interference effects.[6] Internal standards ideally should have similar analytical behavior to the elements being determined.

4.1.5 Memory interferences -- Result when isotopes of elements in a previous sample contribute to the signals measured in a new sample. Memory effects can result from sample deposition on the sampler and skimmer cones and from the buildup of sample material in the plasma torch and spray chamber. The site where these effects occur is dependent on the element and can be minimized by flushing the system with a rinse blank between samples. Memory interferences from the chelating system may be encountered especially after analyzing a sample containing high concentrations of the analytes. A thorough column rinsing sequence following elution of the analytes is necessary to minimize such interferences.

4.2 A principal advantage of this method is the selective elimination of species giving rise to polyatomic spectral interferences on certain transition metals (e.g., removal of the chloride interference on vanadium). As the majority of the sample matrix is removed, matrix induced physical interferences are also substantially reduced.

4.3 Low recoveries may be encountered in the preconcentration cycle if the trace elements are complexed by competing chelators in the sample or are present as colloidal material. Acid solubilization pretreatment is employed to improve analyte recovery and to minimize adsorption, hydrolysis, and precipitation effects.

5.0 Safety

5.1 Each chemical reagent used in this method should be regarded as a potential health hazard and exposure to these reagents should be as low as reasonably achievable. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method.[7,8] A reference file of material data handling sheets should also be available to all personnel involved in the chemical analysis.

5.2 Analytical plasma sources emit radio frequency radiation in addition to intense UV radiation. Suitable precautions should be taken to protect personnel from such hazards.

5.3 The acidification of samples containing reactive materials may result in the release of toxic gases, such as cyanides or sulfides. Acidification of samples should be performed in a fume hood.

5.4 All personnel handling environmental samples known to contain or to have been in contact with human waste should be immunized against known disease causative agents.

5.5 It is the responsibility of the user of this method to comply with relevant disposal and waste regulations. For guidance see Sections 14.0 and 15.0.

6.0 Equipment and Supplies

6.1 Preconcentration System -- System containing no metal parts in the analyte flow path, configured as shown in Figure 1.

6.1.1 Column -- Macroporous iminodiacetate chelating resin (Dionex Metpac CC-1 or equivalent).

6.1.2 Sample loop -- 10-mL loop constructed from narrow bore, high-pressure inert tubing, Tefzel ethylene tetra-fluoroethylene (ETFE) or equivalent.

6.1.3 Eluent pumping system (Pl) -- Programmable flow, high pressure pumping system, capable of delivering either one of two eluents at a pressure up to 2000 psi and a flow rate of 1-5 mL/min.

6.1.4 Auxiliary pumps -- On line buffer pump (P2), piston pump (Dionex QIC pump or equivalent) for delivering 2M ammonium acetate buffer solution; *carrier pump (P3),* peristaltic pump (Gilson Minipuls or equivalent) for delivering 1% nitric acid carrier solution; *sample pump (P4),* peristaltic pump for loading sample loop.

6.1.5 Control valves -- Inert double stack, pneumatically operated four-way slider valves with connectors.

6.1.5.1 Argon gas supply regulated at 80-100 psi.

6.1.6 Solution reservoirs -- Inert containers, e.g., high density polyethylene (HDPE), for holding eluent and carrier reagents.

6.1.7 Tubing -- High pressure, narrow bore, inert tubing (e.g., Tefzel ETFE or equivalent) for interconnection of pumps/valve assemblies and a minimum length for connection of the preconcentration system to the ICP-MS instrument.

6.2 Inductively Coupled Plasma - Mass Spectrometer

6.2.1 Instrument capable of scanning the mass range 5-250 amu with a minimum resolution capability of 1 amu peak width at 5% peak height. Instrument may be fitted with a conventional or extended dynamic range detection system.

6.2.2 Argon gas supply (high-purity grade, 99.99%).

6.2.3 A mass-flow controller on the nebulizer gas supply is recommended. A water-cooled spray chamber may be of benefit in reducing some types of interferences (e.g., polyatomic oxide species).

6.2.4 *Operating conditions* -- Because of the diversity of instrument hardware, no detailed instrument operating conditions are provided. The analyst is advised to follow the recommended operating conditions provided by the manufacturer.

6.2.5 If an electron multiplier detector is being used, precautions should be taken, where necessary, to prevent exposure to high ion flux. Otherwise changes in instrument response or damage to the multiplier may result. Samples having high concentrations of elements beyond the linear range of the instrument and with isotopes falling within scanning windows should be di-luted prior to analysis.

6.3 ***Labware --*** For the determination of trace elements, contamination and loss are of **critical** concern. Potential contamination sources include improperly cleaned laboratory apparatus and general contamination within the laboratory environment. A clean laboratory work area, designated for trace element sample handling, must be used. Sample containers can introduce positive and negative errors in the determination of trace elements by (1) contributing contaminants through surface desorption or leaching or (2) depleting element concentrations through adsorption processes. For these reasons, borosilicate glass is *not* recommended for use with this method. All labware in contact with the sample should be cleaned prior to use. Labware may be soaked overnight and thoroughly washed with laboratory-grade detergent and water, rinsed with water, and soaked for 4 hr in a mixture of dilute nitric and hydrochloric acids, followed by rinsing with ASTM type I water and oven drying.

6.3.1 *Griffin beakers,* 250-mL, polytetrafluoroethylene (PTFE) or quartz.

6.3.2 *Storage bottles* -- Narrow mouth bottles, Teflon FEP (fluorinated ethylene propylene), or HDPE, 125-mL and 250-mL capacities.

6.4 ***Sample Processing Equipment***

6.4.1 *Air displacement pipetter* -- Digital pipet system capable of delivering volumes from 10 to 2500 μL with an assortment of metal-free, disposable pipet tips.

6.4.2 *Balances* -- Analytical balance, capable of accurately weighing to ±0.1 mg; top pan balance, accurate to ± 0.01g.

6.4.3 *Hot plate* -- Corning PC100 or equivalent.

6.4.4 *Centrifuge* -- Steel cabinet with guard bowl, electric timer and brake.

6.4.5 *Drying oven* -- Gravity convection oven with thermostatic control capable of maintaining 105°C±5°C.

6.4.6 *pH meter* -- Bench mounted or hand-held electrode system with a resolution of ± 0.1 pH units.

7.0 Reagents and Standards

7.1 ***Water --*** For all sample preparation and dilutions, ASTM type I water (ASTM D1193) is required.

7.2 Reagents may contain elemental impurities that might affect the integrity of analytical data. Because of the high sensitivity of this method, ultra high-purity reagents must be used unless otherwise specified. To minimize contamination, reagents should be prepared directly in their designated containers where possible.

7.2.1 Acetic acid, glacial (sp. gr. 1.05).

7.2.2 Ammonium hydroxide (20%).

7.2.3 *Ammonium acetate buffer 1M, pH 5.5* -- Add 58-mL (60.5 g) of glacial acetic acid to 600-mL of ASTM type water. Add 65 mL (60 g) of 20% ammonium hydroxide and mix. Check the pH of the resulting solution by withdrawing a small aliquot and testing with a calibrated pH meter, adjusting the solution to pH 5.5±0.1 with small volumes of acetic acid or ammonium hydroxide as necessary. Cool and dilute to 1 L with ASTM type I water.

7.2.4 *Ammonium acetate buffer 2M, pH 5.5* -- Prepare as for Section 7.2.3 using 116 mL (121g) glacial acetic acid and 130 mL (120 g) 20% ammonium hydroxide, diluted to 1000 mL with ASTM type I water.

Note: The ammonium acetate buffer solutions may be further purified by passing them through the chelating column at a flow rate of 5.0-mL/min. With reference to Figure 1, pump the buffer solution through the column using pump P1, with valves A and B off and valve C on. Collect the purified solution in a container at the waste outlet. Following this, elute the collected contaminants from the column using 1.25M nitric acid for 5 min at a flow rate of 4.0 mL/min.

7.2.5 Nitric acid, concentrated (sp.gr. 1.41).

7.2.5.1 Nitric acid 1.25M -- Dilute 79 mL (112 g) conc. nitric acid to 1000-mL with ASTM type I water.

7.2.5.2 Nitric acid 1% -- Dilute 10 mL conc. nitric acid to 1000 mL with ASTM type I water.

7.2.5.3 Nitric acid (1+1) -- Dilute 500 mL conc. nitric acid to 1000-mL with ASTM type I water.

7.2.5.4 Nitric acid (1+9) -- Dilute 100 mL conc. nitric acid to 1000-mL with ASTM type I water.

7.2.6 Oxalic acid dihydrate (CASRN 6153-56-6), 0.2M -- Dissolve 25.2 g reagent grade $C_2H_2O_4 \cdot 2H_2O$ in 250-mL ASTM type I water and dilute to 1000 mL with ASTM type I water. **Caution** - Oxalic acid is toxic; handle with care.

7.3 Standard Stock Solutions -- May be purchased from a reputable commercial source or prepared from ultra high-purity grade chemicals or metals (99.99-99.999% pure). All salts should be dried for 1 h at 105°C, unless otherwise specified. (**Caution-** Many metal salts are extremely toxic if inhaled or swallowed. Wash hands thoroughly after handling.) Stock solutions should be stored in plastic bottles. The following procedures may be used for preparing standard stock solutions:

Note: Some metals, particularly those that form surface oxides require cleaning prior to being weighed. This may be achieved by pickling the surface of the metal in acid. An amount in excess of the desired weight should be pickled repeatedly, rinsed with water, dried, and weighed until the desired weight is achieved.

7.3.1 Cadmium solution, stock 1 mL = 1000 μg Cd: Pickle cadmium metal in (1+9) nitric acid to an exact weight of 0.100 g. Dissolve in 5-mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100-mL with ASTM type I water.

7.3.2 Cobalt solution, stock 1 mL = 1000 μg Co: Pickle cobalt metal in (1+9) nitric acid to an exact weight of 0.100 g. Dissolve in 5 mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.3 Copper solution, stock 1 mL = 1000 μg Cu: Pickle copper metal in (1+9) nitric acid to an exact weight 0.100 g. Dissolve in 5 mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.4 Indium solution, stock 1 mL = 1000 μg In: Pickle indium metal in (1+1) nitric acid to an exact weight 0.100 g. Dissolve in 10 mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.5 Lead solution, stock 1 mL = 1000 μg Pb: Dissolve 0.1599 g $PbNO_3$ in 5 mL (1+1) nitric acid. Dilute to 100 mL with ASTM type I water.

7.3.6 Nickel solution, stock 1 mL = 1000 μg Ni: Dissolve 0.100 g nickel powder in 5 mL conc. nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.7 Scandium solution, stock 1 mL = 1000 μg Sc: Dissolve 0.1534 g Sc_2O_3 in 5 mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.8 Terbium solution, stock 1 mL = 1000 μg Tb: Dissolve 0.1176 g Tb_4O_7 in 5 mL conc. nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.9 Uranium solution, stock 1 mL = 1000 μg U: Dissolve 0.2110 g $UO_2(NO_3)_2 \cdot 6H_2O$ (Do Not Dry) in 20 mL ASTM type I water. Add 2-mL (1+1) nitric acid and dilute to 100-mL with ASTM type I water.

7.3.10 Vanadium solution, stock 1 mL = 1000 μg V: Pickle vanadium metal in (1+9) nitric acid to an exact weight of 0.100 g. Dissolve in 5-mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.11 Yttrium solution, stock 1 mL = 1000 μg Y: Dissolve 0.1270 g Y_2O_3 in 5 mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.4 Multielement Stock Standard Solution -- Care must be taken in the preparation of multielement stock standards that the elements are compatible and stable. Originating element stocks should be checked for impurities that might influence the accuracy of the standard. Freshly prepared standards should be transferred to acid cleaned, new FEP or HDPE bottles for storage and monitored periodically for stability. A multielement stock standard solution containing the elements, cadmium, cobalt, copper, lead, nickel, uranium, and vanadium (1 mL = 10 μg) may be prepared by diluting 1 mL of each single element stock in the list to 100 mL with ASTM type I water containing 1% (v/v) nitric acid.

7.4.1 Preparation of calibration standards -- Fresh multielement calibration standards should be prepared weekly. Dilute the stock multielement standard solution in 1% (v/v) nitric acid to levels appropriate to the required operating range. The element concentrations in the standards should be sufficiently high to produce good measurement precision and to accurately define the slope of the response curve. A suggested mid-range concentration is 10 μg /L.

7.5 Blanks -- Four types of blanks are required for this method. A calibration blank is used to establish the analytical calibration curve, and the laboratory reagent blank is used to assess possible contamination from the sample preparation procedure. The laboratory fortified blank is used to assess the recovery of the method

analytes and the rinse blank is used between samples to minimize memory from the nebulizer/spray chamber surfaces.

7.5.1 *Calibration blank* -- Consists of 1% (v/v) nitric acid in ASTM type I water (Section 7.2.5.2).

7.5.2 *Laboratory reagent blank (LRB)* -- Must contain all the reagents in the same volumes as used in processing the samples. The LRB must be carried through the entire sample digestion and preparation scheme.

7.5.3 *Laboratory Fortified Blank (LFB)* -- To an aliquot of LRB, add aliquots from the multielement stock standard (Section 7.4) to produce a final concentration of 10 μg/L for each analyte. The fortified blank must be carried through the entire sample pretreatment and analytical scheme.

7.5.4 *Rinse Blank (RB)* -- Is a 1% (v/v) nitric acid solution that is delivered to the ICP-MS between samples (Section 7.2.5.2).

7.6 ***Tuning Solution --*** This solution is used for instrument tuning and mass calibration prior to analysis (Section 10.2). The solution is prepared by mixing nickel, yttrium, indium, terbium, and lead stock solutions (Section 7.3) in 1% (v/v) nitric acid to produce a concentration of 100 μg/L of each element.

7.7 ***Quality Control Sample (QCS) --*** A quality control sample having certified concentrations of the analytes of interest should be obtained from a source outside the laboratory. Dilute the QCS if necessary with 1% nitric acid, such that the analyte concentrations fall within the proposed instrument calibration range.

7.8 ***Instrument Performance Check (IPC) Solution --*** The IPC solution is used to periodically verify instrument performance during analysis. It should be prepared by combining method analytes at appropriate concentrations to approximate the midpoint of the calibration curve. The IPC solution should be prepared from the same standard stock solutions used to prepare the calibration standards and stored in a FEP bottle. Agency programs may specify or request that additional instrument performance check solutions be prepared at specified concentrations in order to meet particular program needs.

7.9 ***Internal Standards Stock Solution, 1 mL = 100 μg --*** Dilute 10-mL of scandium, yttrium, indium, terbium, and bismuth stock standards (Section 7.3) to 100-mL with ASTM type I water, and store in a Teflon bottle. Use this solution concentrate for addition to blanks, calibration standards and samples (Method A, Section 10.5), or dilute by an appropriate amount using 1% (v/v) nitric acid, if the internal standards are being added by peristaltic pump (Method B, Section 10.5).

Note: Bismuth should not be used as an internal standard using the direct addition method (Method A, Section 10.5) as it is not efficiently concentrated on the iminodiacetate column.

8.0 Sample Collection, Preservation, and Storage

8.1 Prior to the collection of an aqueous sample, consideration should be given to the type of data required, so that appropriate preservation and pretreatment steps can be taken. Acid preservation should be performed at the time of sample collection or as soon thereafter as practically possible. The pH of all aqueous samples must be tested immediately prior to aliquoting for analysis to ensure the sample has been properly preserved. If properly acid preserved, the sample can be held up to 6 months before analysis.

8.2 For the determination of total recoverable elements in aqueous samples, acidify with (1+1) nitric acid (high purity) at the time of collection to pH<2; normally, 3 mL of (1+1) acid per liter of sample is sufficient for most samples. The sample should not be filtered prior to analysis.

Note: Samples that cannot be acid preserved at the time of collection because of sampling limitations or transport restrictions, or are >pH2 because of high alkalinity should be acidified with nitric acid to pH<2 upon receipt in the laboratory. Following acidification, the sample should be held for 16 h and the pH verified to be <2 before withdrawing an aliquot for sample processing.

8.3 For aqueous samples, a field blank should be prepared and analyzed as required by the data user. Use the same container and acid as used in sample collection.

9.0 Quality Control

9.1 Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the periodic analysis of laboratory reagent blanks, fortified blanks and other laboratory solutions as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data generated.

9.2 ***Initial Demonstration of Performance (Mandatory)***

9.2.1 The initial demonstration of performance is used to characterize instrument performance (determination of

linear dynamic ranges and analysis of quality control samples) and laboratory performance (determination of method detection limits) prior to samples being analyzed by this method.

9.2.2 *Linear calibration ranges* -- The upper limit of the linear calibration range should be established for each analyte. Linear calibration ranges should be determined every six months or whenever a significant change in instrument response is expected.

9.2.3 *Quality control sample (QCS)* -- When beginning the use of this method, on a quarterly basis or as required to meet data-quality needs, verify the calibration standards and acceptable instrument performance with the preparation and analyses of a QCS (Section 7.7). If the determined concentrations are not within ± 10% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of method detection limits or continuing with ongoing analyses.

9.2.4 *Method detection limit (MDL)* -- MDLs must be established for all analytes, using reagent water (blank) fortified at a concentration of two to three times the estimated instrument detection limit.[9] To determine MDL values, take seven replicate aliquots of the fortified reagent water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

$$MDL = (t) \times (S)$$

where: t = Student's t value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom [t = 3.14 for seven replicates].

S = standard deviation of the replicate analyses.

Note: If the relative standard deviation (RSD) from the analyses of the seven aliquots is <15%, the concentration used to determine the analyte MDL may have been inappropriately high for the determination. If so, this could result in the calculation of an unrealistically low MDL. If additional confirmation of the MDL is desired, reanalyze the seven replicate aliquots on two more nonconsecutive days and again calculate the MDL values for each day. An average of the three MDL values for each analyte may provide for a more appropriate MDL estimate. Concurrently, determination of MDL in reagent water represents a best case situation and does not reflect possible matrix effects of real world samples. However, successful analyses of LFMs (Section 9.4) can give confidence to the MDL value determined in reagent water. Typical single laboratory MDL values using this method are given in Table 1.

MDLs should be determined every six months, when a new operator begins work or whenever there is a significant change in the background or instrument response.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 *Laboratory reagent blank (LRB)* -- The laboratory must analyze at least one LRB (Section 7.5.2) with each batch of 20 or fewer samples. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the MDL indicate laboratory or reagent contamination should be suspected. Any determined source of contamination must be corrected and the samples reanalyzed for the affected analytes after acceptable LRB values have been obtained.

9.3.2 *Laboratory fortified blank (LFB)* -- The laboratory must analyze at least one LFB (Section 7.5.3) with each batch of samples. Calculate accuracy as percent recovery (Section 9.4.3). If the recovery of any analyte falls outside the required control limits of 85-115%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.

9.3.3 The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 85-115% (Section 9.3.2). When sufficient internal performance data become available (usually a minimum of 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

$$\text{Upper Control Limit} = x + 3S$$

$$\text{Lower Control Limit} = x - 3S$$

The optional control limits must be equal to or better than the required control limits of 85-115%. After each five to ten new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also, the standard deviation (S) data should be used to established an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.3.4 *Instrument performance check (IPC) solution* -- For all determinations the laboratory must analyze the IPC solution (Section 7.8) and a calibration blank immediately following daily calibration, after every tenth sample (or more frequently, if required) and at the end of the sample run. Analysis of the IPC solution and calibration blank immediately following calibration must verify that the instrument is within ±10% of calibration. Subse-

quent analyses of the IPC solution must verify the calibration within ±15%. If the calibration cannot be verified within the specified limits, reanalyze the IPC solution. If the second analysis of the IPC solution confirms calibration to be outside the limits, sample analysis must be discontinued, the cause determined and/or in the case of drift the instrument recalibrated. All samples following the last acceptable IPC solution must be reanalyzed. The analysis data of the calibration blank and IPC solution must be kept on file with the sample analyses data.

9.3.5 The overall sensitivity and precision of this method are strongly influenced by a laboratory's ability to control the method blank. Therefore, it is recommended that the calibration blank response be recorded for each set of samples. This record will aid the laboratory in assessing both its long- and short-term ability to control the method blank.

9.4 Assessing Analyte Recovery and Data Quality

9.4.1 Sample homogeneity and the chemical nature of the sample matrix can affect analyte recovery and the quality of the data. Taking separate aliquots from the sample for replicate and fortified analyses can in some cases assess these effects. Unless otherwise specified by the data user, laboratory or program, the following laboratory fortified matrix (LFM) procedure (Section 9.4.2) is required.

9.4.2 The laboratory must add a known amount of each analyte to a minimum of 10% of the routine samples. In each case the LFM aliquot must be a duplicate of the aliquot used for sample analysis and for total recoverable determinations added prior to sample preparation. For water samples, the added analyte concentration must be the same as that used in the laboratory fortified blank (Section 9.3.2).

9.4.3 Calculate the percent recovery for each analyte, corrected for concentrations measured in the unfortified sample, and compare these values to the designated LFM recovery range of 75-125%. Recovery calculations are not required if the concentration added is less than 25% of the unfortified sample concentration. Percent recovery may be calculated in units appropriate to the matrix, using the following equation:

$$R = \frac{(C_s - C)}{S} x\ 100$$

where, R = percent recovery.
C_S = fortified sample concentration.
C = sample background concentration.
S = concentration equivalent of analyte added to sample.

9.4.4 If the recovery of any analyte falls outside the designated LFM recovery range and the laboratory performance for that analyte is shown to be in control (Section 9.3), the recovery problem encountered with the LFM is judged to be either matrix or solution related, not system related.

9.4.5 If analysis of LFM sample(s) and the test routines above indicate an operative interference and the LFMs are typical of the other samples in the batch, those samples that are similar must be analyzed in the same manner as the LFMs. Also, the data user must be informed when a matrix interference is so severe that it prevents the successful analysis of the analyte or when the heterogeneous nature of the sample precludes the use of duplicate analyses.

9.4.6 Where reference materials are available, they should be analyzed to provide additional performance data. The analysis of reference samples is a valuable tool for demonstrating the ability to perform the method acceptably.

10.0 Calibration and Standardization

10.1 Initiate proper operating configuration of ICP-MS instrument and data system. Allow a period of not less than 30 min for the instrument to warm up. During this process conduct mass calibration and resolution checks using the tuning solution. Resolution at low mass is indicated by nickel isotopes 60, 61, 62. Resolution at high mass is indicated by lead isotopes 206, 207, 208. For good performance adjust spectrometer resolution to produce a peak width of approximately 0.75 amu at 5% peak height. Adjust mass calibration if it has shifted by more than 0.1 amu from unit mass.

10.2 Instrument stability must be demonstrated by analyzing the tuning solution (Section 7.6) a minimum of five times with resulting relative standard deviations of absolute signals for all analytes of less than 5%.

10.3 Prior to initial calibration, setup proper instrument software routines for quantitative analysis and connect the ICP-MS instrument to the preconcentration apparatus. The instrument must be calibrated for the analytes of interest using the calibration blank (Section 7.5.1) and calibration standard (Section 7.4.1) prepared at one or more concentration levels. The calibration solutions should be processed through the preconcentration system using the procedures described in Section 11.

10.4 Demonstration and documentation of acceptable initial calibration is required before any samples are analyzed. After initial calibration is successful, a calibration check is required at the beginning and end of each period during which analyses are performed and at requisite intervals.

10.4.1 After the calibration has been established, it must be initially verified for all analytes by analyzing the IPC (Section 7.8). If the initial calibration verification exceeds ±10% of the established IPC value, the analysis should be terminated, the source of the problem identified and corrected, the instrument recalibrated, and the new calibration verified before continuing analyses.

10.4.2 To verify that the instrument is properly calibrated on a continuing basis, analyze the calibration blank (Section 7.5.1) and IPC (Section 7.8) after every 10 analyses. The results of the analyses of the standards will indicate whether the calibration remains valid. If the indicated concentration of any analyte deviates from the true concentration by more than 15%, reanalyze the standard. If the analyte is again outside the 15% limit, the instrument must be recalibrated and the previous 10 samples reanalyzed. The instrument responses from the calibration check may be used for recalibration purposes.

10.5 Internal Standardization -- Internal standardization must be used in all analyses to correct for instrument drift and physical interferences. For full mass range scans, a minimum of three internal standards must be used. Internal standards must be present in all samples, standards and blanks at identical levels. This may be achieved by directly adding an aliquot of the internal standards to the CAL standard, blank or sample solution (Method A), or alternatively by mixing with the solution prior to nebulization using a second channel of the peristaltic pump and a mixing coil (Method B). The concentration of the internal standard should be sufficiently high that good precision is obtained in the measurement of the isotope used for data correction and to minimize the possibility of correction errors if the internal standard is naturally present in the sample. Internal standards should be added to blanks, samples and standards in a like manner, so that dilution effects resulting from the addition may be disregarded.

Note: Bismuth should not be used as an internal standard using the direct addition method (Method A, Section 10.5) because it is not efficiently concentrated on the iminodiacetate column.

11.0 Procedure

11.1 Sample Preparation -- Total Recoverable Elements

11.1.1 Add 2-mL(1+1) nitric acid to the beaker containing 100-mL of sample. Place the beaker on the hot plate for solution evaporation. The hot plate should be located in a fume hood and previously adjusted to provide evaporation at a temperature of approximately but no higher than 85°C. (See the following note.) The beaker should be covered with an elevated watch glass or other necessary steps should be taken to prevent sample contamination from the fume hood environment.

Note: For proper heating, adjust the temperature control of the hot plate such that an uncovered Griffin beaker containing 50 mL of water placed in the center of the hot plate can be maintained at a temperature approximately but no higher than 85°C. (Once the beaker is covered with a watch glass the temperature of the water will rise to approximately 95°C.)

11.1.2 Reduce the volume of the sample aliquot to about 20-mL by gentle heating at 85°C. **Do Not Boil.** This step takes about 2 h for a 100-mL aliquot with the rate of evaporation rapidly increasing as the sample volume approaches 20 mL. (A spare beaker containing 20-mL of water can be used as a gauge.)

11.1.3 Cover the lip of the beaker with a watch glass to reduce additional evaporation and gently reflux the sample for 30 min. (Slight boiling may occur, but vigorous boiling must be avoided.)

11.1.4 Allow the beaker to cool. Quantitatively transfer the sample solution to a 100-mL volumetric flask, dilute to volume with reagent water, stopper and mix.

11.1.5 Allow any undissolved material to settle overnight, or centrifuge a portion of the prepared sample until clear. (If after centrifuging or standing overnight, the sample contains suspended solids, a portion of the sample may be filtered prior to analysis. However, care should be exercised to avoid potential contamination from filtration.) The sample is now ready for analysis. Because the effects of various matrices on the stability of diluted samples cannot be characterized, all analyses should be performed as soon as possible after the completed preparation.

11.2 Prior to first use, the preconcentration system should be thoroughly cleaned and decontaminated using 0.2M oxalic acid.

11.2.1 Place approximately 500-mL 0.2M oxalic acid in all the eluent/solution containers and fill the sample loop with 0.2M oxalic acid using the sample pump (P4) at a flow rate of 3-5 mL/min. With the preconcentration system disconnected from the ICP-MS instrument, use the pump program sequence listed in Table 2 to flush the complete system with oxalic acid. Repeat the flush sequence three times.

11.2.2 Repeat the sequence described in Section 11.2.1 using 1.25M nitric acid and again using ASTM type I water in place of the 0.2M oxalic acid.

11.2.3 Rinse the containers thoroughly with ASTM type I water, fill them with their designated reagents (see Figure 1) and run through the sequence in Table 2 once to prime the pump and all eluent lines with the correct reagents.

11.3 Initiate ICP-MS instrument operating configuration. Tune the instrument for the analytes of interest (Section 10).

11.4 Establish instrument software run procedures for quantitative analysis. Because the analytes are eluted from the preconcentration column in a transient manner, it is recommended that the instrument software is configured in a rapid scan/peak hopping mode. The instrument is now ready to be calibrated.

11.5 Reconnect the preconcentration system to the ICP-MS instrument. With valves A and B in the off position and valve C in the on position, load sample through the sample loop to waste using pump P4 for 4 min at 4 mL/min. Switch on the carrier pump (P3) and pump 1% nitric acid to the nebulizer of the ICP-MS instrument at a flow rate of 0.8-1.0-mL/min.

11.6 Switch on the buffer pump (P2), and pump 2M ammonium acetate at a flow rate of 1.0 mL/min.

11.7 Preconcentration of the sample may be achieved by running through an eluent pump program (P1) sequence similar to that illustrated in Table 2. The exact timing of this sequence should be modified according to the internal volume of the connecting tubing and the specific hardware configuration used.

11.7.1 Inject sample -- With valves A, B, and C on, load sample from the loop onto the column using 1M ammonium acetate for 4.5 min at 4.0 mL/min. The analytes are retained on the column, while the majority of the matrix is passed through to waste.

11.7.2 Elute analytes -- Turn off valves A and B and begin eluting the analytes by pumping 1.25M nitric acid through the column at 4.0 mL/min, then turn off valve C and pump the eluted analytes into the ICP-MS instrument at 1.0 mL/min. Initiate ICP-MS software data acquisition and integrate the eluted analyte profiles.

11.7.3 Column Reconditioning -- Turn on valve C to direct column effluent to waste, and pump 1.25M nitric acid, 1M ammonium acetate, 1.25M nitric acid and 1M ammonium acetate alternately through the column at 4.0 mL/min. During this process, the next sample can be loaded into the sample loop using the sample pump (P4).

11.8 Repeat the sequence described in Section 11.7 for each sample to be analyzed. At the end of the analytical run leave the column filled with 1M ammonium acetate buffer until it is next used.

11.9 Samples having concentrations higher than the established linear dynamic range should be diluted into range with 1% HNO_3 (v/v) and reanalyzed.

12.0 Data Analysis and Calculations

12.1 Analytical isotopes and elemental equations recommended for sample data calculations are listed in Table 3. Sample data should be reported in units of µg/L. Do not report element concentrations below the determined MDL.

12.2 For data values less than 10, two significant figures should be used for reporting element concentrations. For data values greater than or equal to 10, three significant figures should be used.

12.3 Reported values should be calibration blank subtracted. If additional dilutions were made to any samples, the appropriate factor should be applied to the calculated sample concentrations.

12.4 Data values should be corrected for instrument drift by the application of internal standardization. Corrections for characterized spectral interferences should be applied to the data.

12.5 The QC data obtained during the analyses provide an indication of the quality of the sample data and should be provided with the sample results.

13.0 Method Performance

13.1 Experimental conditions used for single laboratory testing of the method are summarized in Table 4.

13.2 Data obtained from single laboratory testing of the method are summarized in Tables 5 and 6 for two reference water samples consisting of National Research Council Canada (NRCC) Estuarine Water (SLEW-1) and Seawater (NASS-2). The samples were prepared using the procedure described in Section 11.1.1. For each matrix, three replicates were analyzed and the average of the replicates was used to determine the sample concentration for each analyte. Two further sets of three replicates were fortified at different concentration levels, one set at 0.5 µg/L, the other at 10 µg/L. The sample concentration, mean percent recovery, and the relative standard deviation of the fortified replicates are listed for each method analyte. The reference material certificate values are also listed for comparison.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that place pollution pre-

vention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation (e.g., Section 7.8). When wastes cannot be feasibly reduced at the source, the Agency recommends recy--cling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction,* available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036, (202)872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel,* available from the American Chemical Society at the address listed in Section 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Total Recoverable Method Detection Limits for Reagent Water

Element	Recommended Analytical Mass	MDL[1] μg/L
Cadmium	111	0.041
Cobalt	59	0.021
Copper	63	0.023
Lead	206, 207,208	0.074
Nickel	60	0.081
Uranium	238	0.031
Vanadium	51	0.014

[1] Determined using 10-mL sample loop.

Table 2. Eluent Pump Programming Sequence for Preconcentration of Trace Elements

Time (min)	Flow (mL/min)	Eluent	Valve A,B	Valve C
0.0	4.0	1M ammonium acetate	ON	ON
4.5	4.0	1.25M nitric acid	ON	ON
5.1	1.0	1.25M nitric acid	OFF	ON
5.5	1.0	1.25M nitric acid	OFF	OFF
7.5	4.0	1.25M nitric acid	OFF	ON
8.0	4.0	1M ammonium acetate	OFF	ON
10.0	4.0	1.25M nitric acid	OFF	ON
11.0	4.0	1M ammonium acetate	OFF	ON
12.5	0.0		OFF	ON

Table 3. Recommended Analytical Isotopes and Elemental Equations for Data Calculations

Element	Isotope	Elemental Equation	Note
Cd	106, 108, *111,* 114	$(1.000)(^{111}C)-(1.073)[(^{108}C)-(0.712)(^{106}C)]$	(1)
Co	59	$(1.000)(^{59}C)$	
Cu	*63,* 65	$(1.000)(^{63}C)$	
Pb	*206, 207, 208*	$(1.000)(^{206}C)+(1.000)(^{207}C)+(1.000)(^{208}C)$	(2)
Ni	60	$(1.000)(^{60}C)$	
U	238	$(1.000)(^{238}C)$	
V	51	$(1.000)(^{51}C)$	

C - calibration blank subtracted counts at specified mass.
(1) - correction for MoO interference. An additional isobaric elemental correction should be made if palladium is present.
(2) - allowance for isotopic variability of lead isotopes.
NOTE: As a minimum, all isotopes listed should be monitored. Isotopes recommended for analytical determination are italized.

Table 4. Experimental Conditions for Single Laboratory Validation

Chromatography	
Instrument	Dionex chelation system
Preconcentration column	Dionex MetPac CC-1
ICP-MS Instrument Conditions	
Instrument	VG PlasmaQuad Type I
Plasma forward power	1.35 kW
Coolant flow rate	13.5 L/min
Auxiliary flow rate	0.6 L/min
Nebulizer flow rate	0.78 L/min
Internal standards	Sc, Y, In, Tb
Data Acquisition	
Detector mode	Pulse counting
Mass range	45-240 amu
Dwell time	160 μs
Number of MCA channels	2048
Number of scan sweeps	250

Table 5. Precision and Recovery Data for Estuarine Water (SLEW-1)

Analyte	Certificate (μg/L)	Sample Conc. (μg/L)	Spike Addition (μg/L)	Average Recovery (%)	RSD (%)	Spike Addition (μg/L)	Average Recovery (%)	RSD (%)
Cd	0.018	<0.041	0.5	94.8	9.8	10	99.6	1.1
Co	0.046	0.078	0.5	102.8	4.0	10	96.6	1.4
Cu	1.76	1.6	0.5	106.0	2.7	10	96.0	4.8
Pb	0.028	<0.074	0.5	100.2	4.0	10	106.9	5.8
Ni	0.743	0.83	0.5	100.0	1.5	10	102.0	2.1
U	--	1.1	0.5	96.7	7.4	10	98.1	3.6
V	--	1.4	0.5	100.0	3.2	10	97.0	4.5

-- No certificate value

Table 6. Precision and Recovery Data for Seawater (NASS-2)

Analyte	Certificate (μg/L)	Sample Conc. (μg/L)	Spike Addition (μg/L)	Average Recovery (%)	RSD (%)	Spike Addition (μg/L)	Average Recovery (%)	RSD (%)
Cd	0.029	<0.041	0.5	101.8	1.0	10	96.4	3.7
Co	0.004	<0.021	0.5	98.9	3.0	10	99.2	1.7
Cu	0.109	0.12	0.5	95.8	2.3	10	93.1	0.9
Pb	0.039	<0.074	0.5	100.6	8.5	10	92.1	2.6
Ni	0.257	0.23	0.5	102.2	2.3	10	98.2	1.2
U	3.00	3.0	0.5	94.0	0.7	10	98.4	1.7
V	--	1.7	0.5	104.0	3.4	10	109.2	3.7

--No certificate value

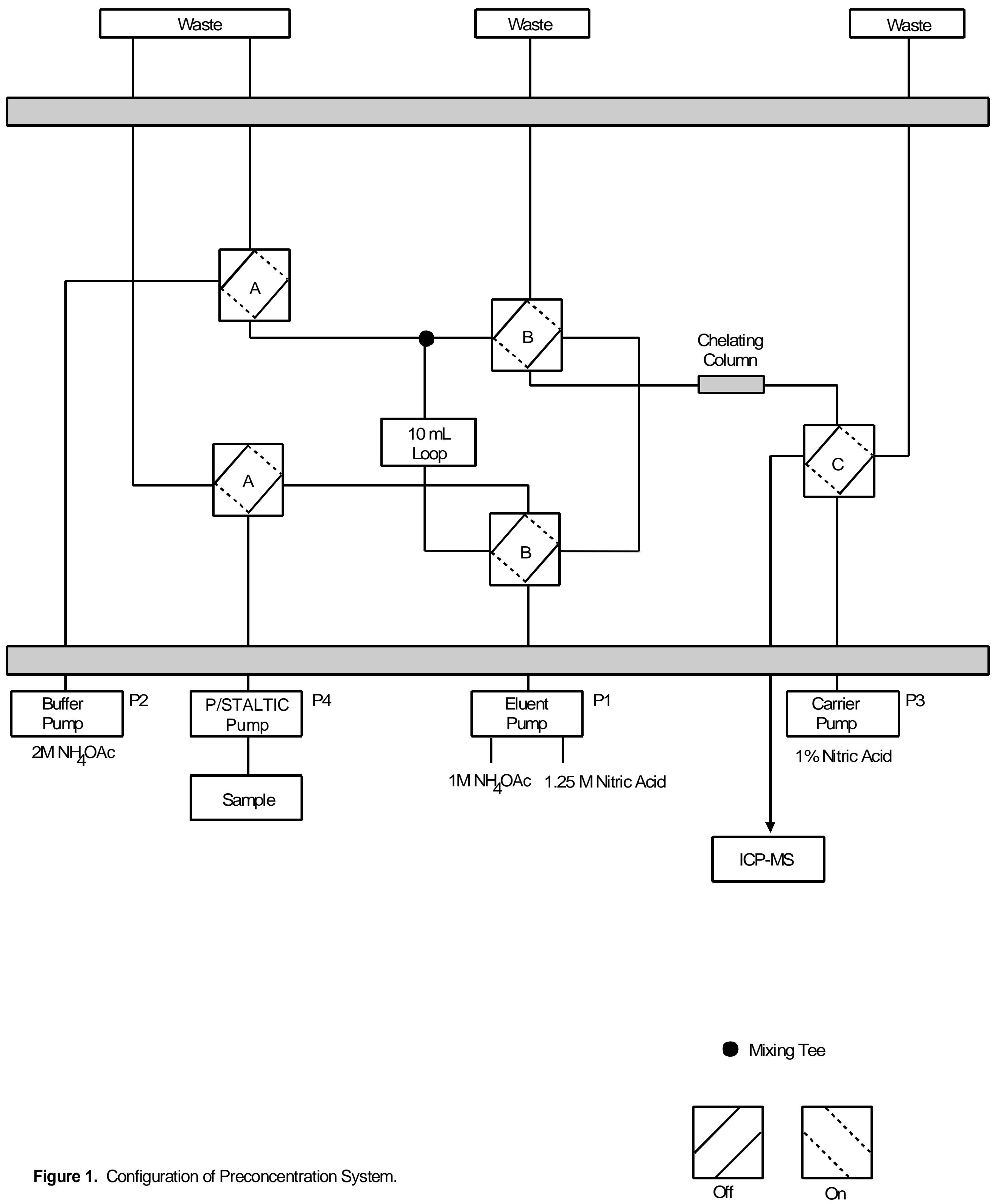

Figure 1. Configuration of Preconcentration System.

Method 200.12

Determination of Trace Elements in Marine Waters by Stabilized Temperature Graphite Furnace Atomic Absorption

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Revision 1.0
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Method 200.12
Determination of Trace Elements in Marine Waters by Stabilized Temperature Graphite Furnace Atomic Absorption

1.0 Scope and Application

1.1 This method provides procedures for the determination of total recoverable elements by graphite furnace atomic absorption (GFAA) in marine waters, including estuarine, ocean and brines with salinities of up to 35 ppt. This method is applicable to the following analytes:

Analyte		Chemical Abstracts Service Registry Numbers (CASRN)
Arsenic	(As)	7440-38-2
Cadmium	(Cd)	7440-43-9
Chromium	(Cr)	7440-47-3
Copper	(Cu)	7440-50-8
Lead	(Pb)	7439-92-1
Nickel	(Ni)	7440-02-0
Selenium	(Se)	7782-49-2

1.2 For determination of total recoverable analytes in marine waters, a digestion/extraction is required prior to analysis.

1.3 Method detection limit and instrumental operating conditions for the applicable elements are listed in Tables 1 and 2. These are intended as a guide and are typical of a commercial instrument optimized for the element. However, actual method detection limits and linear working ranges will be dependent on the sample matrix, instrumentation and selected operating conditions.

1.4 Users of the method data should state the data quality objectives prior to analysis. The ultra-trace metal concentrations typically associated with marine water may preclude the use of this method based on its sensitivity. Users of the method must document and have on file the required initial demonstration performance data described in Section 9.2 prior to using the method for analysis.

2.0 Summary of Method

2.1 Nitric acid is dispensed into a beaker containing an accurately weighed or measured, well-mixed, homogeneous aqueous sample. Then, for samples with undissolved material, the beaker is covered with a watch glass and heated, made up to volume, centrifuged or allowed to settle, and the sample is then analyzed.

2.2 The analytes listed in this method are determined by stabilized temperature platform graphite furnace atomic absorption (STPGFAA). In STPGFAA, the sample and the matrix modifier are first pipetted onto the platform or a device which provides delayed atomization.

The furnace chamber is then purged with a continuous flow of a premixed gas (95% argon - 5% hydrogen) and the sample is dried at a relatively low temperature (about 120°C) to avoid spattering. Once dried, the sample is pretreated in a char or ashing step which is designed to minimize the interference effects caused by the concomitant sample matrix. After the char step, the furnace is allowed to cool prior to atomization. The atomization cycle is characterized by rapid heating of the furnace to a temperature where the metal (analyte) is atomized from the pyrolytic graphite surface into a stopped gas flow atmosphere of argon containing 5% hydrogen. (Only selenium is determined in an atmosphere of high purity argon.) The resulting atomic cloud absorbs the element-specific atomic emission produced by a hollow cathode lamp (HCL) or an electrodeless discharge lamp (EDL). Following analysis, the furnace is subjected to a cleanout period of high temperature and continuous argon flow. Because the resulting absorbance usually has a nonspecific component associated with the actual analyte absorbance, Zeeman background correction is required to subtract from the total signal the component which is nonspecific to the analyte. In the absence of interferences, the background-corrected, absorbance is directly related to the concentration of the analyte. Interferences relating to STPGFAA (Section 4.0) must be recognized and corrected. Suppressions or enhancements of instrument response caused by the sample matrix must be corrected for by the method of standard addition (Section 11.3).

3.0 Definitions

3.1 Calibration Blank (CB) -- A volume of reagent water fortified with the same matrix as the calibration standards, but without the analytes, internal standards, or surrogate analytes.

3.2 Calibration Standard (CAL) -- A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.3 Field Reagent Blank (FRB) -- An aliquot of reagent water or other blank matrix that is placed in a sample container in the laboratory and treated as a sample in all respects, including shipment to the sampling site, exposure to sampling site conditions, storage, preservation, and all analytical procedures. The purpose of the FRB is to determine it method analytes or other interferences are present in the field environment.

3.4 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent which gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time, absorbance line, etc.

3.5 Instrument Performance Check Solution (IPC) -- A solution of one or more method analytes, surrogates, internal standards, or other test substances used to evaluate the performance of the instrument system with respect to a defined set of criteria.

3.6 Laboratory Duplicates (LD1 and LD2) -- Two aliquots of the same sample taken in the laboratory and analyzed separately with identical procedures. Analyses of LD1 and LD2 indicate precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

3.7 Laboratory Fortified Blank (LFB) -- An aliquot of reagent water or other blank matrices to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

3.8 Laboratory Fortified Sample Matrix (LFM) -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.9 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.10 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.11 Material Safety Data Sheet (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.12 Matrix Modifier (MM) -- A substance added to the instrument along with the sample in order to minimize the interference effects by selective volatilization of either analyte or matrix components.

3.13 Matrix Performance Check (MPC) -- A solution of method analytes used to evaluate the laboratory's ongoing capabilities in analyzing high salinity samples. The reference material NASS-3 or its equivalent is fortified with the same analytes at the same concentration as the LFB. This provides an ongoing check of furnace operating conditions to assure the analyte false positives are not being introduced via elevated backgrounds.

3.14 Method Detection Limit (MDL) -- The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.

3.15 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and

different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.16 Standard Addition -- The addition of a known amount of analyte to the sample in order to determine the relative response of the detector to an analyte within the sample matrix. The relative response is then used to assess either an operative matrix effect or the sample analyte concentration.

3.17 Stock Standard Solution (SSS) -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

3.18 Total Recoverable Analyte (TRA) -- The concentration of analyte determined to be in either a solid sample or an unfiltered aqueous sample following treatment by refluxing with hot dilute mineral acid(s) as specified in the method.

4.0 Interferences

4.1 Several interference sources may cause inaccuracies in the determination of trace elements by GFAA. These interferences can be classified into three major subdivisions: spectral, matrix, and memory.

4.2 Spectral interferences are caused by absorbance of light by a molecule or atom which is not the analyte of interest or emission from black body radiation.

4.2.1 Spectral interferences caused by an element only occur if there is a spectral overlap between the wavelength of the interfering element and the analyte of interest. Fortunately, this type of interference is relatively uncommon in STPGFAA because of the narrow atomic line widths associated with STPGFAA. In addition, the use of appropriate furnace temperature programs and high spectral purity lamps as light sources can minimize the possibility of this type of interference. However, molecular absorbances can span several hundred nanometers producing broadband spectral interferences. This type of interference is far more common in STPGFAA. The use of matrix modifiers, selective volatilization, and background correctors are all attempts to eliminate unwanted nonspecific absorbance. Table 2 contains typical background absorbances associated with the analysis of the MPC solution (NASS-3) which has a salinity of 35 ppt. These background absorbances were obtained using the suggested matrix modifiers and the appropriate furnace charring conditions. Figure 1 is a plot of integrated background absorbance vs. char temperature for Ni, Cd, Pb, and Se. Figure 1 indicates that the background absorbance in a saline matrix is strongly affected by the char temperature. Therefore, char temperature optimization is a critical part of the successful analysis of metals in saline water by GFAA. The elevated backgrounds associated with ocean water can produce false positives. For this reason, the char temperature profiles shown in Figure 1 should be constructed for each analyte prior to using this method for saline water analysis.

Note: False analyte positives can be generated by large backgrounds. Figure 2 is an atomization profile for Pb using a 800°C char temperature. The background shown in the figure has exceeded the capabilities of the Zeeman corrector. This profile can be used as a guide in screening other analyses which may have background absorbances which exceed the Zeeman capability. The background profile is characterized by a smooth baseline in the beginning of the atomization cycle followed by a sharp increase. During this sharp increase the background peak profile may remain relatively smooth, but when the background exceeds the Zeeman correction capability, the background profile will appear extremely erratic. The atomic profile is also erratic during this part of the atomization cycle. These types of background/atomic profiles obtained during atomization result in false positives.

Since the nonspecific component of the total absorbance can vary considerably from sample type to sample type, to provide effective background correction and eliminate the elemental spectral interference of palladium on copper and iron on selenium, the exclusive use of Zeeman background correction is specified in this method.

4.2.2 Spectral interferences are also caused by black body radiation produced during the atomization furnace cycle. This black body emission reaches the photomultiplier tube, producing erroneous results. The magnitude of this interference can be minimized by proper furnace tube alignment and monochromator design. In addition, atomization temperatures which adequately volatilize the analyte of interest without producing unnecessary black body radiation can help reduce unwanted background emission produced during atomization.

4.3 Matrix interferences are caused by sample components which inhibit the formation of free atomic analyte atoms during atomization. In this method the use of a delayed atomization device which provides a warmer gas

phase environment during atomization is required. These devices provide an environment which is more conducive to the formation of free analyte atoms and thereby minimize this type of interference. This type of interference can be detected by analyzing the sample plus a sample aliquot fortified with a known concentration of the analyte. If the determined concentration of the analyte addition is outside a designated range (Section 9.4.3), a possible matrix effect should be suspected. In addition, the matrix can produce analyte complexes which are lost via volatilization during the char. These losses will result in poor recovery of the analyte within the matrix and should be corrected by adjusting the char temperature.

4.4 Memory interferences result from analyzing a sample containing a high concentration of an element (typically a high atomization temperature element) which cannot be removed quantitatively in one complete set of furnace steps. The analyte which remains in the furnace can produce false positive signals on subsequent sample(s). Therefore, the analyst should establish the analyte concentration which can be injected into the furnace and adequately removed in one complete set of furnace cycles. If this concentration is exceeded, the sample should be diluted and a blank analyzed to assure the memory effect has been eliminated before reanalyzing the diluted sample.

4.5 ***Specific Element Interferences. --*** The matrix effects caused by the saline water can be severe. In order to evaluate the extent of the matrix suppression as a function of increasing salinity a plot of normalized integrated absorbance vs. microliters NASS-3 (Reference Material from the National Research Council of Canada) is constructed. Figure 3 is a plot of relative response of As, Se, Cd, Ni, Cu, and Pb in waters containing salinity of 3.5 ppt (1 µL NASS-3) to 35 ppt (10 µL NASS-3). Figure 3 indicates that the matrix effects caused by the increasing salinity are minor for Pb, Cu, and Ni. The relative responses of Pb, Ni, and Cu shown in Figure 3 are within ± 5% of the 1% HNO_3 standard or zero µL of matrix. Figure 3 indicates that the increasing salinity does cause a substantial matrix interference for Se and Cd. This suppression must be compensated for by methods of standard addition or the use of matrix matched standards where applicable.

4.5.1 Cadmium: The background level associated with the direct determination of Cd in NASS-3 exceeds the Zeeman background correction. Therefore, NH_4NO_3 is used as a matrix removing modifier in addition to the Pd/ $Mg(NO_3)_2$.[1] Figure 4 is a plot of the relative Cd response vs. the amount of seawater on the platform. A similar response profile is observed in a solution containing 10,000 ppm NaCl. Therefore, in well-characterized samples of known salinity it is possible to effectively matrix match the standards with NaCl and perform the analysis directly using matrix matched standards, thereby avoiding the time consuming method of standard additions. If the matrix matched standards are going to be used, it is necessary to document that the use of NaCl is indeed compensating for the suppression. This documentation should include a response plot of increasing matrix vs. relative response similar to Figure 4.

4.5.2 Selenium: The background level associated with the direct determination of Se in NASS-3 exceeds the Zeeman correction capability. Therefore, HNO_3 is used as a matrix removing modifier in addition to the Pd/ $Mg(NO_3)_2$ for the determination of Se in saline waters. Figure 5 is a plot of relative response vs. the amount of seawater on the platform. A similar suppression is observed in a solution containing 10,000 ppm NaCl. Therefore, in well-characterized samples of known salinity it is possible to effectively matrix match the standards with NaCl and perform the analysis directly using matrix matched standards, thereby avoiding the time consuming method of standard additions. If the matrix matched standards are going to be used, it is necessary to document that the use of NaCl is indeed compensating for the suppression. This documentation should include a response plot of increasing matrix vs. relative response similar to Figure 5.

4.5.3 Arsenic: The elevated char temperatures possible with the determination of As minimize the interferences produced by the marine water background levels. Figure 3 is a plot of relative response vs. the amount of seawater on the platform. Figure 3 indicates a matrix suppression on As caused by the seawater. Although this suppression does cause a slight bias as shown in the recovery data in Table 3, the suppression does not warrant the method of standard additions (MSA) given the recovery criteria of 75-125% for LFMS.

5.0 Safety

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure to these compounds should be as low as reasonably achievable. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method.[2-5] A reference file of material data handling sheets should also be made available to all personnel

involved in the chemical analysis. Specifically, concentrated nitric and hydrochloric acids present various hazards and are moderately toxic and extremely irritating to skin and mucus membranes. Use these reagents in a fume hood whenever possible and if eye or skin contact occurs, flush with large volumes of water. Always wear safety glasses or a shield for eye protection, protective clothing, and observe proper mixing when working with these reagents.

5.2 The acidification of samples containing reactive materials may result in the release of toxic gases, such as cyanides or sulfides. Acidification of samples should be done in a fume hood.

5.3 All personnel handling environmental samples known to contain or to have been in contact with human waste should be immunized against known disease causative agents.

5.4 The graphite tube during atomization emits intense UV radiation. Suitable precautions should be taken to protect personnel from such a hazard.

5.5 The use of the argon/hydrogen gas mixture during the dry and char steps may evolve a considerable amount of HCl gas. Therefore, adequate ventilation is required.

5.6 It is the responsibility of the user of this method to comply with relevant disposal and waste regulations. For guidance see Sections 14.0 and 15.0.

6.0 Equipment and Supplies

6.1 Graphite Furnace Atomic Absorption Spectrometer

6.1.1 The GFAA spectrometer must be capable of programmed heating of the graphite tube and the associated delayed atomization device. The instrument must be equipped with Zeeman background correction and the furnace device must be capable of utilizing an alternate gas supply during specified cycles of the analysis. The capability to record relatively fast (< 1 s) transient signals and evaluate data on a peak area basis is preferred. In addition, a recirculating refrigeration unit is recommended for improved reproducibility of furnace temperatures.

6.1.2 Single element hollow cathode lamps or single element electrodeless discharge lamps along with the associated power supplies.

6.1.3 Argon gas supply (high-purity grade, 99.99%) for use during the atomization of selenium, for sheathing the furnace tube when in operation, and during furnace cleanout.

6.1.4 Alternate gas mixture (hydrogen 5%-argon 95%) for use as a continuous gas flow environment during the dry and char furnace cycles.

6.1.5 Autosampler capable of adding matrix modifier solutions to the furnace, a single addition of analyte, and completing methods of standard additions when required.

6.2 Analytical balance, with capability to measure to 0.1 mg, for preparing standards, and for determining dissolved solids in digests or extracts.

6.3 A temperature adjustable hot plate capable of maintaining a temperature of 95°C.

6.4 An air displacement pipetter capable of delivering volumes ranging from 100 to 2500 µL with an assortment of high quality disposable pipet tips.

6.5 ***Labware --*** All reusable labware (glass, quartz, polyethylene, PTFE, FEP, etc.) should be sufficiently clean for the task objectives. Several procedures found to provide clean labware include washing with a detergent solution, rinsing with tap water, soaking for 4 h or more in 20% (v/v) nitric acid or a mixture of HCl and HNO_3, rinsing with reagent water and storing clean. Chromic acid cleaning solutions must be avoided because chromium is an analyte.

Note: Glassware having ground glass stoppers, etc. should be avoided because the ground glass surface is difficult to clean properly and can contain active sites which adsorb metals.

6.5.1 *Glassware* -- Volumetric flasks, graduated cylinders, funnels and centrifuge tubes (glass and/or metal-free plastic).

6.5.2 Assorted calibrated pipettes.

6.5.3 Griffin beakers, 250-mL with 75-mm watch glasses and (optional) 75-mm ribbed watch glasses.

6.5.4 Narrow-mouth storage bottles, FEP (fluorinated ethylene propylene) with screw closure, 125-mL to 1-L capacities.

6.5.5 One-piece stem FEP wash bottle with screw closure, 125-mL capacity.

7.0 Reagents and Standards

7.1 Reagents may contain elemental impurities which might affect analytical data. Only high-purity reagents that conform to the American Chemical Society specifications[6] should be used whenever possible. If the purity of a reagent is in question, analyze for contamination. All acids used for this method must be of ultra high-purity grade or equivalent. Suitable acids are available from a number of manufacturers. Redistilled acids prepared by sub-boiling distillation are acceptable.

7.2 Nitric acid, concentrated (sp.gr. 1.41) HNO_3.

7.2.1 Nitric acid (1+1) -- Add 500 mL concentrated HNO_3 to 400 mL reagent water and dilute to 1 L.

7.2.2 Nitric acid (1+5) -- Add 50 mL concentrated HNO_3 to 250 mL reagent water.

7.2.3 Nitric acid (1+9) -- Add 10 mL concentrated HNO_3 to 90 mL reagent water.

7.3 Reagent water. All references to water in this method refer to ASTM Type I grade water.[7]

7.4 Ammonium hydroxide, concentrated (sp.gr.0.902).

7.5 Matrix Modifier, dissolve 300 mg palladium (Pd) powder in concentrated HNO_3 (1 mL of HNO_3, adding 10 µL of concentrated HCl if necessary). Dissolve 200 mg of $Mg(NO_3)_2 \cdot 6H_2O$ in ASTM Type I water. Pour the two solutions together and dilute to 100 mL with ASTM Type I water.

Note: It is recommended that the matrix modifier be analyzed separately in order to assess the contribution of the modifier to the absorbance of calibration and reagent blank solutions.

7.6 Standard stock solutions may be purchased or prepared from ultra-high purity grade chemicals (99.99 to 99.999% pure). All compounds must be dried for 1 h at 105°C, unless otherwise specified. It is recommended that stock solutions be stored in FEP bottles. Replace stock standards when succeeding dilutions for preparation of calibration standards cannot be verified.

Caution: Many of these chemicals are extremely toxic if inhaled or swallowed (Section 5.1). Wash hands thoroughly after handling.

Typical stock solution preparation procedures follow for 1-L quantities, but for the purpose of pollution prevention, the analyst is encouraged to prepare smaller quantities when possible. Concentrations are calculated based upon the weight of the pure element or upon the weight of the compound multiplied by the fraction of the analyte in the compound.

From pure element,

$$\text{Concentration} = \frac{\text{weight (mg)}}{\text{volume (L)}}$$

From pure compound,

$$\text{Concentration} = \frac{\text{weight (mg) x gravimetric factor}}{\text{volume (L)}}$$

where:

gravimetric factor = the weight fraction of the analyte in the compound.

7.6.1 Arsenic solution, stock, 1 mL = 1000 µg As: Dissolve 1.320 g of As_2O_3 (As fraction = 0.7574), weighed accurately to at least four significant figures, in 100 mL of reagent water containing 10.0 mL concentrated NH_4OH. Warm in solution gently to effect dissolution. Acidify the solution with 20.0 mL concentrated HNO_3 and dilute to volume in a 1-L volumetric flask with reagent water.

7.6.2 Cadmium solution, stock, 1 mL = 1000 µg Cd: Dissolve 1.000 g Cd metal, acid cleaned with (1+9) HNO_3, weighed accurately to at least four significant figures, in 50 mL (1+1) HNO_3 with heating to effect dissolution. Let solution cool and dilute with reagent water in a 1-L volumetric flask.

7.6.3 Chromium solution, stock, 1 mL = 1000 µg Cr: Dissolve 1.923 g CrO_3 (Cr fraction = 0.5200), weighed accurately to at least four significant figures, in 120 mL (1 +5) HNO_3. When solution is complete, dilute to volume in a 1-L volumetric flask with reagent water.

7.6.4 Copper solution, stock, 1 mL = 1000 µg Cu: Dissolve 1.000 g Cu metal, acid cleaned with (1+9) HNO_3,

weighed accurately to at least four significant figures, in 50.0 mL (1+1) HNO_3 with heating to effect dissolution. Let solution cool and dilute in a 1-L volumetric flask with reagent water.

7.6.5 Lead solution, stock, 1 mL = 1000 µg Pb: Dissolve 1.599 g $Pb(NO_3)_2$ (Pb fraction = 0.6256), weighed accurately to at least four significant figures, in a minimum amount of (1+1) HNO_3. Add 20.0 mL (1+1) HNO_3 and dilute to volume in a 1-L volumetric flask with reagent water.

7.6.6 Nickel solution, stock, 1 mL = 1000 µg Ni: Dissolve 1.000 g of nickel metal, weighed accurately to at least four significant figures, in 20.0 mL hot concentrated HNO_3, cool, and dilute to volume in a 1-L volumetric flask with reagent water.

7.6.7 Selenium solution, stock, 1 mL = 1000 µg Se: Dissolve 1.405 g SeO_2 (Se fraction = 0.7116), weighed accurately to at least four significant figures, in 200 mL reagent water and dilute to volume in a 1-L volumetric flask with reagent water.

7.7 Preparation of Calibration Standards -- Fresh calibration standards (CAL Solution) should be prepared weekly, or as needed. Dilute each of the stock standard solutions to levels appropriate to the operating range of the instrument using the appropriate acid diluent. The element concentrations in each CAL solution should be sufficiently high to produce good measurement precision and to accurately define the slope of the response curve. The instrument calibration should be initially verified using a IPC sample (Section 7.9).

7.8 Blanks -- Four types of blanks are required for this method. A calibration blank is used to establish the analytical calibration curve, the laboratory reagent blank (LRB) is used to assess possible contamination from the sample preparation procedure and to assess spectral background, the laboratory fortified blank is used to assess routine laboratory performance, and a rinse blank is used to flush the instrument autosampler uptake system. All diluent acids should be made from concentrated acids (Section 7.2) and ASTM Type I water.

7.8.1 The calibration blank consists of the appropriate acid diluent in ASTM Type I water. The calibration blank should be stored in a FEP bottle.

7.8.2 The laboratory reagent blanks must contain all the reagents in the same volumes as used in processing the samples. The preparation blank must be carried through the entire sample digestion and preparation scheme.

7.8.3 The laboratory fortified blank (LFB) is prepared by fortifying an aliquot of the laboratory reagent blank with all analytes to provide a final concentration which will produce an absorbance of approximately 0.1 for each analyte. The LFB must be carried through the complete procedure as used for the samples.

7.8.4 The rinse blank is a 0.1% HCl and 0.1% HNO_3 solution used to flush the autosampler tip and is stored in the appropriate plastic containers.

7.9 Instrument Performance Check (IPC) Solution -- The IPC solution is used to periodically verify instrument performance during analysis. It should be prepared in the same acid mixture as the calibration standards by combining method analytes at appropriate concentrations to approximate the midpoint of the calibration curve. The IPC solution should be prepared from the same standard stock solutions used to prepare the calibration standards and stored in a FEP bottle. Agency programs may specify or request that additional instrument performance check solutions be prepared at specified concentrations in order to meet particular program needs.

7.10 Quality Control Sample (QCS) -- For initial and periodic verification of calibration standards and instrument performance, analysis of a QCS is required. The QCS must be obtained from an outside source different from the standard stock solutions and prepared in the same acid mixture as the calibration standards. The concentration of the analytes in the QCS solution should be such that the resulting solution will provide an absorbance reading of approximately 0.1. The QCS solution should be stored in a FEP bottle and analyzed as needed to meet data-quality needs. A fresh solution should be prepared quarterly or as needed.

7.11 Matrix Performance Check (MPC) -- The MPC solution is used to periodically evaluate the laboratory/ instrument performance in saline samples. It should be prepared in the same acid mixture as the calibration standards by combining method analytes at appropriate concentrations in a seawater matrix (NASS-3, or its equivalent) to produce an absorbance of 0.1. The MPC solution should be prepared from the same standard stock solutions used to prepare the calibration standards and stored in a FEP bottle. The MPC sample should be analyzed after every 10 samples to assure saline matrix is not producing false positives.

8.0 Sample Collection, Preservation and Storage

8.1 Prior to collection of an aqueous sample, consideration should be given to the type of data required. Acid preservation should be performed at the time of sample collection or as soon thereafter as practically possible. The pH of all aqueous samples must be tested immediately prior to aliquoting for analysis to ensure the sample has been properly preserved. If properly acid-preserved, the sample can be held up to 6 months before analysis.

8.2 For determination of total recoverable elements in aqueous samples, acidify with (1+1) nitric acid at the time of collection to pH<2. Normally, 3 mL of (1+1) nitric acid (ultra high purity) per liter of sample is sufficient for most ambient water samples. The sample should not be filtered prior to analysis.

Note: Samples that cannot be acid-preserved at the time of collection because of sampling limitations or transport restrictions, or are > pH 2 because of high alkalinity should be acidified with nitric acid to pH < 2 upon receipt in the laboratory. Following acidification, the sample should be held for 16 h and the pH verified to be <2 before withdrawing an aliquot for sample processing.

8.3 For aqueous samples, a field blank should be prepared and analyzed as required by the data user. Use the same container and acid as used in sample collection.

9.0 Quality Control

9.1 Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, and the periodic analysis of laboratory reagent blanks, fortified blanks and other laboratory solutions as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data thus generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (determination of linear dynamic ranges and analysis of quality control samples) and laboratory performance (determination of method detection limits) prior to samples being analyzed by this method.

9.2.2 *Linear dynamic range (LDR)* -- The upper limit of the LDR must be established for the wavelength utilized for each analyte by determining the signal responses from a minimum of six different concentration standards across the range, two of which are close to the upper limit of the LDR. Determined LDRs must be documented and kept on file. The linear calibration range which may be used for the analysis of samples should be judged by the analyst from the resulting data. The upper LDR limit should be an observed signal no more than 10% below the level extrapolated from the four lower standards. New LDRs should be determined whenever there is a significant change in instrument response, a change in instrument analytical hardware or operating conditions.

Note: Multiple cleanout furnace cycles may be necessary in order to fully define or utilize the LDR for certain elements such as chromium. For this reason, the upper limit of the linear calibration range may not correspond to the upper operational LDR limit.

Measured sample analyte concentrations that exceed the upper limit of the linear calibration range must either be diluted and reanalyzed (with concern for memory effects Section 4.4) or analyzed by another approved method.

9.2.3 *Quality control sample (QCS)* -- When beginning the use of this method, on a quarterly basis or as required to meet data-quality needs, verify the calibration standards and acceptable instrument performance with the preparation and analyses of a QCS (Section 7.10). If the determined concentrations are not within ± 10% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding on with the initial determination of method detection limits or continuing with ongoing analyses.

9.2.4 *Method detection limit (MDL)* -- MDLs must be established for all analytes, using reagent water (blank) fortified at a concentration of two to three times the estimated instrument detection limit.[8] To determine MDL values, take seven replicate aliquots of the fortified reagent water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

$$MDL = (t) \times (S)$$

where, t = Student's t value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom [t = 3.14 for seven replicates].

S = standard deviation of the replicate analyses.

Note: If the percent relative standard deviation (% RSD) from the analyses of the seven aliquots is < 15%, the concentration used to determine the analyte MDL may have been inappropriately high for the determination. If so, this could result in calculation of an unrealistically low MDL. If additional confirmation of the MDL is desired, reanalyze the seven replicate aliquots on two more nonconsecutive days and again calculate the MDL values for each day. An average of the three MDL values for each analyte may provide a more appropriate MDL estimate. Concurrently, determination of MDL in reagent water represents a best case situation and does not reflect possible matrix effects of real world samples. However, successful analyses of LFMs (Section 9.4) and the analyte addition test described in Section 9.5.1 can give confidence to the MDL value determined in reagent water. Typical single laboratory MDL values using this method are given in Table 2.

MDLs should be determined every six months, when a new operator begins work or whenever there is a significant change in the background or instrument response.

The MDLs reported in Table 2 were determined in fortified NASS-3 samples. It is recommended that a certified saline matrix such as NASS-3 be used to determine MDLS.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory reagent blank (LRB) -- The laboratory must analyze at least one LRB (Section 7.8.2) with each batch of 20 or fewer samples. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the MDL indicate laboratory or reagent contamination should be suspected. Any determined source of contamination must be corrected and the samples reanalyzed for the affected analytes after acceptable LRB values have been obtained.

9.3.2 Laboratory fortified blank (LFB) -- The laboratory must analyze at least one LFB (Section 7.8.3) with each batch of samples. Calculate accuracy as percent recovery (Section 9.4.3). If the recovery of any analyte falls outside the required control limits of 85-115%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.

9.3.3 The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 85-115%. When sufficient internal performance data become available (usually a minimum of 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

$$\text{Upper Control Limit} = x + 3S$$

$$\text{Lower Control Limit} = x - 3S$$

The optional control limits must be equal to or better than the required control limits of 85-115%. After each five to ten new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also, the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.3.4 Instrument performance check (IPC) solution -- For all determinations the laboratory must analyze the IPC solution (Section 7.9) and a calibration blank immediately following daily calibration, after every tenth sample (or more frequently, if required) and after the last sample in the batch is analyzed. Analysis of the IPC solution and calibration blank immediately following calibration must verify that the instrument is within ± 5% of calibration. Subsequent analyses of the IPC solution must verify the calibration within ± 10%. If the calibration cannot be verified within the specified limits, reanalyze the IPC solution. If the second analysis of the IPC solution confirms calibration to be outside the limits, sample analysis must be discontinued, the cause determined and/or, in the case of drift, the instrument recalibrated. All samples following the last acceptable IPC solution must be reanalyzed. Data for the calibration blank and IPC solution must be kept on file with associated sample data.

9.3.5 Matrix performance check (MPC) solution -- For all determinations, the laboratory must analyze the MPC solution (Section 7.11) immediately following daily calibration, after every tenth sample (or more frequently, if required) and after the last sample in the batch is analyzed. Analysis of the MPC must verify that the instrument

is within ± 15% of calibration and confirm that the matrix is not causing matrix/background interferences. If the MPC is not within ± 15%, reanalyze the MPC solution. If the second analysis of the MPC solution is outside the limits, sample analysis must be discontinued the cause determined and/or, in the case of drift, the instrument recalibrated. All samples following the last acceptable MPC solution must be reanalyzed. The analysis data for the calibration blank and MPC solution must be kept on file with the sample analyses data.

9.4 Assessing Analyte Recovery and Data Quality

9.4.1 Sample homogeneity and the chemical nature of the sample matrix can affect analyte recovery and the data quality. Taking separate aliquots from the sample for replicate and fortified analyses can in some cases assess these effects. Unless otherwise specified by the data user, laboratory or program, the following laboratory fortified matrix (LFM) procedure (Section 9.4.2) is required. Also, the analyte addition test (Section 9.5.1) can aid in identifying matrix interferences. However, all samples must have a background absorbance < 1.0 before the test results obtained can be considered reliable.

9.4.2 The laboratory must add a known amount of each analyte to a minimum of 10% of the routine samples. In each case the LFM aliquot must be a duplicate of the aliquot used for sample analysis and for total recoverable determinations added prior to sample preparation. For water samples, the added analyte concentration must be the same as that used in the laboratory fortified blank (Section 9.3.2).

9.4.3 Calculate the percent recovery for each analyte, corrected for concentrations measured in the unfortified sample, and compare these values to the designated LFM recovery range of 75-125%. Recovery calculations are not required if the concentration added is less than 25% of the unfortified sample concentration. Percent recovery may be calculated in units appropriate to the matrix, using the following equation:

$$R = \frac{C_s - C}{s} \times 100$$

where, R = percent recovery.
C_s = fortified sample concentration.
C = sample background concentration.
s = concentration equivalent of analyte added to sample.

9.4.4 If the recovery of any analyte falls outside the designated LFM recovery range (but is still within the range of calibration and the background absorbance is < 1.0 abs.) and the laboratory performance for that analyte is shown to be in control (Section 9.3), the recovery problem encountered with the LFM is judged to be either matrix or solution related, not system related. A flowchart of the remainder of this section can be found in Figure 6. This flowchart may clarify the verbal discussion given below.

If the background absorbance is > 1 abs., the sample and the LFM should be diluted 1:3 and reanalyzed until the background absorbance is < 1, at which point a percent recovery of the LFM should be calculated. If the fortified analyte in the diluted LFM is found to be < 25% of the sample concentration or the diluted LFM produces an atomic signal of <10 times the MDL, the diluted sample should be analyzed by methods of standard addition. If the calculated recovery of the diluted sample is within the designated range, the sample concentration should be calculated from the diluted sample. If the calculated recovery of the diluted sample is outside the designated range, follow the directions given below. If the background is reduced and/or the matrix effect is reduced by dilution, all samples of a similar matrix should be diluted and analyzed in a similar fashion. The result should be flagged indicating the methods sensitivity has been reduced by the dilution. If dilution is unacceptable because of data quality objectives the sample should be flagged indicating the analysis is not possible via this analytical procedure.

If the analyte recovery on the LFM is <75% and the background absorbance is <1, complete the analyte addition test (Section 9.5.1) on the original sample (or its dilution). The results of the test should be evaluated as follows:

1. If recovery of the analyte addition test ($\leq$ 85%) confirms a low recovery for the LFM, a suppressive matrix interference is indicated and the unfortified sample aliquot must be analyzed by method of standard additions (Section 11.3).

2. If the recovery of the analyte addition test is between 85% to 115%, a low recovery of the analyte in the LFM (< 75%) may be related to the heterogeneity of the sample, sample preparation or a poor transfer, etc. Report the sample concentration based on the unfortified sample aliquot.

3. If the recovery of the analyte addition test is less than recovery calculated for the LFM, matrix suppression is confirmed. The unfortified sample should be analyzed by MSA (Section 11.3). Significantly lower recoveries (relative to the LFM) associated with the analyte addition test are unlikely unless the sample is heterogeneous.

4. If the recovery of the analyte addition test is >115%, the dramatic change in analyte response should be verified by fortifying the LFM. The recovery in the sample and the recovery in the LFM should be compared. If the recoveries verify the dramatic response difference, the sample results should be flagged indicating the sample matrix is not homogeneous.

If the analyte recovery in the LFM is > 125% and the background absorbance is < 1, complete the analyte addition test (Section 9.5.1) on the unfortified sample (or its dilution) aliquot.

1. If the percent recovery of the analyte addition test is > 115% and the LFB does not indicate laboratory contamination, an enhancing matrix interference (albeit rare) is indicated, and the unfortified sample aliquot must be analyzed by method of standard additions (Section 11.3).

2. If the percent recovery of the analyte addition test is between 85% to 115%, either random sample contamination of the LFM, an incorrect analyte concentration was added to the LFM prior to sample preparation, or sample heterogeneity should be suspected. Report analyte data determined from the analysis of the unfortified sample aliquot.

3. If the percent recovery of the analyte addition test is < 85%, a heterogeneous sample with matrix interference is suspected. This dramatic change in response should be verified by performing the analyte addition test to the LFM. The recovery in the sample and the recovery in the LFM should be compared. If the recoveries verify the dramatic response difference the sample results should be flagged indicating the sample matrix is not homogeneous.

9.4.5 If the analysis of a LFM sample(s) and the test routines above indicate an operative interference and the LFMs are typical of the other samples in the batch, those samples that are similar must be analyzed in the same manner as the LFMS. Also, the data user must be informed when a matrix interference is so severe that it prevents successful determination of the analyte or when the heterogeneous nature of the sample precludes the use of duplicate analyses.

9.4.6 Where reference materials are available, they should be analyzed to provide additional performance data. Analysis of reference samples is a valuable tool for demonstrating the ability to perform the method acceptably. It is recommended that NASS-3 or its equivalent be fortified and used as an MPC.

9.5 Matrix interference effects and the need for MSA can be assessed by the following test. Directions for using MSA are given in Section 11.3.

9.5.1 Analyte addition test: An analyte standard added to a portion of a prepared sample or its dilution should be recovered to within 85-115% of the known value. The analyte addition should occur directly to sample in the furnace and should produce a minimum absorbance of 0.1. The concentration of the analyte addition plus that in the sample should not exceed the linear calibration range of the analyte. If the analyte is not recovered within the specified limits, a matrix effect should be suspected and the sample must be analyzed by MSA.

10.0 Calibration and Standardization

10.1 Specific wavelengths and instrument operating conditions are listed in Table 1. However, because of differences among makes and models of spectrophotometers and electrothermal furnace devices, the actual instrument conditions selected may vary from those listed.

10.2 Prior to the use of this method, the instrument operating conditions must be optimized. The analyst should follow the instructions provided by the manufacturer while using the conditions listed in Table 1 as a guide. The appropriate charring condition for each of the analytes is a critical part of the metal analysis in saline waters; therefore, the char temperature profiles should be determined in a saline water matrix. The appropriate charring temperature should be chosen so as to minimize background absorbance while providing some furnace temperature variation without the loss of analyte. For analytical operation, the charring temperature is usually set at least 100°C below the point at which analyte begins to be lost during the char. Because the background absorbance can be affected by the atomization temperature, care should be taken in the choice of an appropriate atomization temperature. The optimum conditions se-

lected should provide the lowest reliable MDLs and be similar to those listed in Table 2. Once the optimum operating conditions are determined, they should be recorded and available for daily reference. The effectiveness of these operating conditions are continually evaluated by analyzing the MPC.

10.3 Prior to an initial calibration the linear dynamic range of the analyte must be determined (Sect 9.2.2) using the optimized instrument operating conditions. For all determinations allow an instrument and hollow cathode lamp warm-up period of not less than 15 min. If an EDL is to be used, allow 30 min for warm-up.

10.4 Before using the procedure (Section 11.0) to analyze samples, there must be data available documenting initial demonstration of performance. The required data and procedure are described in Section 9.2. This data must be generated using the same instrument operating conditions and calibration routine to be used for sample analysis. These documented data must be kept on file and be available for review by the data user.

11.0 Procedure

11.1 Aqueous Sample Preparation -- Total Recoverable Analytes

11.1.1 Add 2 mL (1+1) nitric acid to the beaker containing 100 mL of sample. Place the beaker on a hot plate for solution evaporation. The hot plate should be located in a fume hood and previously adjusted to provide evaporation at a temperature of approximately but no higher than 85°C. (See the following note.) The beaker should be covered with an elevated watch glass or other necessary steps should be taken to prevent sample contamination from the fume hood environment.

Note: For proper heating adjust the temperature control of the hot plate such that an uncovered Griffin beaker containing 50 mL of water placed in the center of the hot plate can be maintained at a temperature approximately but no higher than 85°C. (Once the beaker is covered with a watch glass the temperature of the water will rise to approximately 95°C.)

11.1.2 Reduce the volume of the sample aliquot to about 20 mL by gentle heating at 85°C. DO NOT BOIL. This step takes about 2 h for a 100-mL aliquot with the rate of evaporation rapidly increasing as the sample volume approaches 20 mL. (A spare beaker containing 20 mL of water can be used as a gauge.)

11.1.3 Cover the lip of the beaker with a watch glass to reduce additional evaporation and gently reflux the sample for 30 min.

11.1.4 Allow the beaker to cool. Quantitatively transfer the sample solution to a 100-mL volumetric flask, dilute to volume with reagent water, stopper and mix.

11.1.5 Allow any undissolved material to settle overnight, or centrifuge a portion of the prepared sample until clear. (If after centrifuging or standing overnight the sample contains suspended solids, a portion of the sample may be filtered prior to analysis. However, care should be exercised to avoid potential contamination from filtration.) The sample is now ready for analysis. Because the effects of various matrices on the stability of diluted samples cannot be characterized, all analyses should be performed as soon as possible after the completed preparation.

11.2 Sample Analysis

11.2.1 Prior to daily calibration of the instrument, inspect the graphite tube and contact rings for salt buildup, etc. Generally, it will be necessary to clean the contact rings and replace the graphite tube daily. The contact rings are a cooler environment in which salts can deposit after atomization. A cotton swab dipped in a 50/50 mixture of isopropyl alcohol (IPA) and H_2O (such that it is damp but not dripping) can be used to remove the majority of the salt buildup. A second cotton swab is dipped in IPA and the contact rings are wiped down to assure they are clean. The rings are then allowed to thoroughly dry and then a new tube is placed in the furnace and conditioned according to instrument manufacturer's specifications.

11.2.2 Configure the instrument system to the selected optimized operating conditions as determined in Sections 10.1 and 10.2.

11.2.3 Before beginning daily calibration the instrument should be reconfigured to the optimized conditions. Initiate the data system and allow a period of not less than 15 min for instrument and hollow cathode lamp warm up. If an EDL is to be used, allow 30 min for warm up.

11.2.4 After the warm up period but before calibration, instrument stability must be demonstrated by analyzing a standard solution with a concentration 20 times the IDL a minimum of five times. The resulting relative standard deviation of absorbance signals must be $\leq$ 5%. If the

relative standard deviation is > 5%, determine and correct the cause before calibrating the instrument.

11.2.5 For initial and daily operation, calibrate the instrument according to the instrument manufacturer's recommended procedures using the calibration blank (Section 7.8.1) and calibration standards (Section 7.7) prepared at three or more concentrations within the usable linear dynamic range of the analyte (Sections 4.4 and 9.2.2).

11.2.6 An autosampler must be used to introduce all solutions into the graphite furnace. Once the sample and the matrix modifier are injected, the furnace controller completes a set of furnace cycles and a cleanout period as programmed. Analyte signals must be reported on an integrated absorbance basis. Background absorbances, background heights and the corresponding peak profiles should be displayed to the CRT for review by the analyst and be available as hard copy for documentation to be kept on file. Flush the autosampler solution uptake system with the rinse blank (Section 7.8.4) between each solution injected.

11.2.7 After completion of the initial requirements of this method (Section 9.2), samples should be analyzed in the same operational manner used in the calibration routine.

11.2.8 During sample analyses, the laboratory must comply with the required quality control described in Sections 9.3 and 9.4.

11.2.9 For every new or unusual matrix, when practical, it is highly recommended that an inductively coupled plasma atomic emission spectrometer be used to screen for high element concentration. Information gained from this may be used to prevent potential damage to the instrument and to better estimate which elements may require analysis by graphite furnace.

11.2.10 Determined sample analyte concentrations that are $\geq$ 90% of the upper limit of calibration must either be diluted with acidified reagent water and reanalyzed with concern for memory effects (Section 4.4), or determined by another approved but less sensitive procedure. Samples with background absorbances > 1 must be diluted with appropriate acidified reagent water such that the background absorbance is < 1 (Section 9.4.4). If the method of standard additions is required, follow the instructions described in Section 11.3.

11.2.11 When it is necessary to assess an operative matrix interference (e.g., signal reduction due to high dissolved solids), the test described in Section 9.5 is recommended.

11.2.12 Report data as directed in Section 12.

11.3 Standard Additions -- If the method of standard addition is required, the following procedure is recommended:

11.3.1 The standard addition technique[9] involves preparing new standards in the sample matrix by adding known amounts of standard to one or more aliquots of the processed sample solution. This technique compensates for a sample constituent that enhances or depresses the analyte signal, thus producing a different slope from that of the calibration standards. It will not correct for additive interference, which causes a baseline shift. The simplest version of this technique is the single-addition method. The procedure is as follows: Two identical aliquots of the sample solution, each of volume V_x, are taken. To the first (labeled A) is added a small volume V_S of a standard analyte solution of concentration C_S. To the second (labeled B) is added the same volume V_S of the solvent. The analytical signals of A and B are measured and corrected for nonanalyte signals. The unknown sample concentration C_X is calculated:

$$C_x = \frac{S_B V_S C_S}{(S_A - S_B) V_X}$$

where, S_A and S_B are the analytical signals (corrected for the blank) of solutions A and B, respectively. V_S and C_S should be chosen so that S_A is roughly twice S_B on the average. It is best if V_S is made much less than V_X, and thus C_S is much greater than C_X, to avoid excess dilution of the sample matrix. If a separation or concentration step is used, the additions are best made first and carried through the entire procedure. For the results from this technique to be valid, the following limitations must be taken into consideration:

1. The analytical curve must be linear.

2. The chemical form of the analyte added must respond in the same manner as the analyte in the sample.

3. The interference effect must be constant over the working range of concern.

4. The signal must be corrected for any additive interference.

12.0 Data Analysis and Calculations

12.1 Sample data should be reported in units of μg/L for aqueous samples.

12.2 For total recoverable aqueous analytes (Section 11.1), when 100-mL aliquot is used to produce the 100 mL final solution, round the data to the tenths place and report the data in μg/L up to three significant figures. If a different aliquot volume other than 100 mL is used for sample preparation, adjust the dilution factor accordingly. Also, account for any additional dilution of the prepared sample solution needed to complete the determination of analytes exceeding the upper limit of the calibration curve. Do not report data below the determined analyte MDL concentration or below an adjusted detection limit reflecting smaller sample aliquots used in processing or additional dilutions required to complete the analysis.

12.3 The QC data obtained during the analyses provide an indication of the quality of the sample data and should be provided with the sample results.

13.0 Method Performance

13.1 Instrument operating conditions used for single laboratory testing of the method and MDLs are listed in Tables 1 & 2.

13.2 Table 3 contains precision and recovery data obtained from a single laboratory analysis of four fortified sample replicates of NASS-3. Five unfortified replicates were analyzed, and their average concentration was used to determine the sample concentration. Samples were prepared using the procedure described in Section 11.1. Four samples were fortified at the levels reported in Table 3. Average percent recovery and percent relative standard deviation are reported in Table 3 for the fortified samples.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult Less is Better: Laboratory Chemical Management for Waste Reduction, available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202)872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel,* available from the American Chemical Society at the address listed in the Section 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Furnace Conditions for Determination of Metals in Seawater [1]

Element	Wavelength (nm) Slit Width (nm)	Method of Analysis	Modifier [2,3]	Furnaces[5] Cycle	Temp °C	Temp Ramp	Hold Time (sec)
As	193.7	Direct	Pd/Mg	Dry	130	1	60
	0.7			Char	1400[4]	10	60
				Atomization	2200	0	5
Cd	228.8	Matrix Match	Pd/Mg	Dry	130	1	60
	0.7	Standard	+	Char 1	350	45	30
		or	600 µg	Char 2	850	1	30
		Std. Addition	NH_4NO_3	Atomization	1500	0	5
Cr	357.9	Direct	Pd/Mg	Dry	130	1	60
	0.7			Char	1500	5	30
				Atomization	2600	0	5
Cu	324.8	Direct	Pd/Mg	Dry	130	1	60
	0.7			Char	1300	10	30
				Atomization	2600	0	5
Ni	232.4	Direct	Pd/Mg	Dry	130	1	60
	0.2			Char	1400[4]	10	30
				Atomization	2600	0	7
Pb	283.3	Direct	Pd/Mg	Dry	130	1	60
	0.7			Char	1200	10	45
				Atomization	2200	0	5
Se	196.0	Matrix Match	Pd/Mg	Dry	130	1	60
	2.0	Standard	9% HNO_3 on	Char	1000	5	60
		or	Platform	Atomization	2100	0	5
		Std. Addition					

[1] 10-µL sample size.
[2] 5µL of (30 mg Pd Powder and 20 mg $Mg(NO_3)_2 \cdot 6H_2O$ to 10 mL).
[3] A gas mixture of 5% H_2 in 95% Ar is used during the dry and char.
[4] Sodium emission is visibly exiting from the sample inlet port.
[5] The furnace program has a cool down step of 20° between char and atomization and a clean out step of 2600° C after atomization.

Table 2. MDLs and Background Absorbances Associated with a Fortified NASS-3[1-3]

Element	MDL[5] µg/L	Typical Integrated Background Absorbances[6]
Cd	0.1	1.2
Cr	-	0.2
Cu	2.8	0.2
Ni	1.8	0.1
Pb	2.4	0.4
Se[4]	9.5	1.4
As[4]	2.6	0.3

[1] Matrix Modifier = 0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$.
[2] A 5% H_2 in Ar gas mix is used during the dry and char steps at 300 mL/min for all elements.
[3] 10-µL sample size.
[4] An electrodeless discharge lamp was used for this element.
[5] MDL calculated based on fortifying NASS-3 with metal analytes.
[6] Background absorbances are affected by the atomization temperature for analysis, therefore, lowering atomization temperatures may be advantageous if large backgrounds are observed.
- Not Determined.

Table 3. Precision and Recovery Data for Fortified NASS-3

Element	Certified Value µg/L	Observed Value µg/L	Fortified Conc. µg/L[2]	Avg. Recovery, %	%RSD	Fortified Conc. µg/L	Avg. Recovery, %	% RSD
As	1.65 ± 0.19	< MDL	15	89	3.6	37.5	85	1.6
Cd[1]	0.029 ± 0.004	< MDL	1.0	107	4.5	2.5	104	3.8
Cr	0.175 ± 0.010	< MDL	5	88	0.7	12.5	85	1.6
Cu	0.109 ± 0.011	< MDL	15	95	4.4	37.5	91	0.9
Pb	0.039 ± 0.006	< MDL	15	103	2.3	37.5	99	3.4
Ni	0.257 ± 0.027	< MDL	15	92	10.1	37.5	93	7.1
Se[1]	0.024 ± 0.004	< MDL	25	101	2.9	62.5	99	3.9

[1] Standards were made in 10,000 ppm NaCl for this analysis.
[2] Determined from four sample replicates.

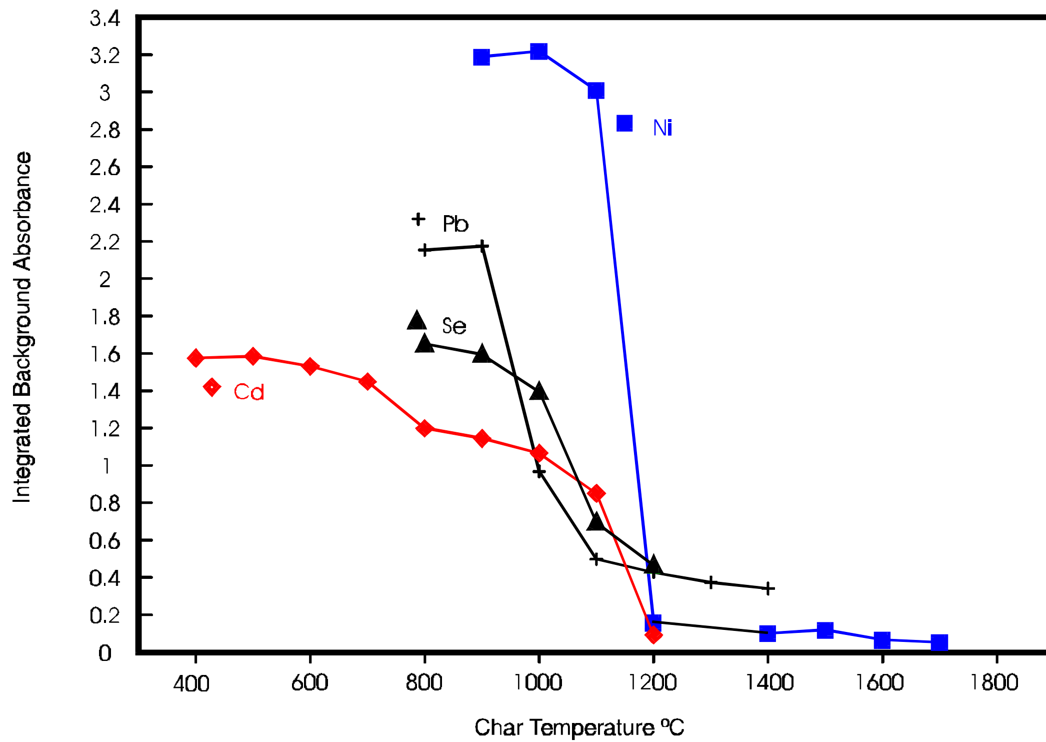

Figure 1. Integrated Background Absorbance vs. Char Temperature.

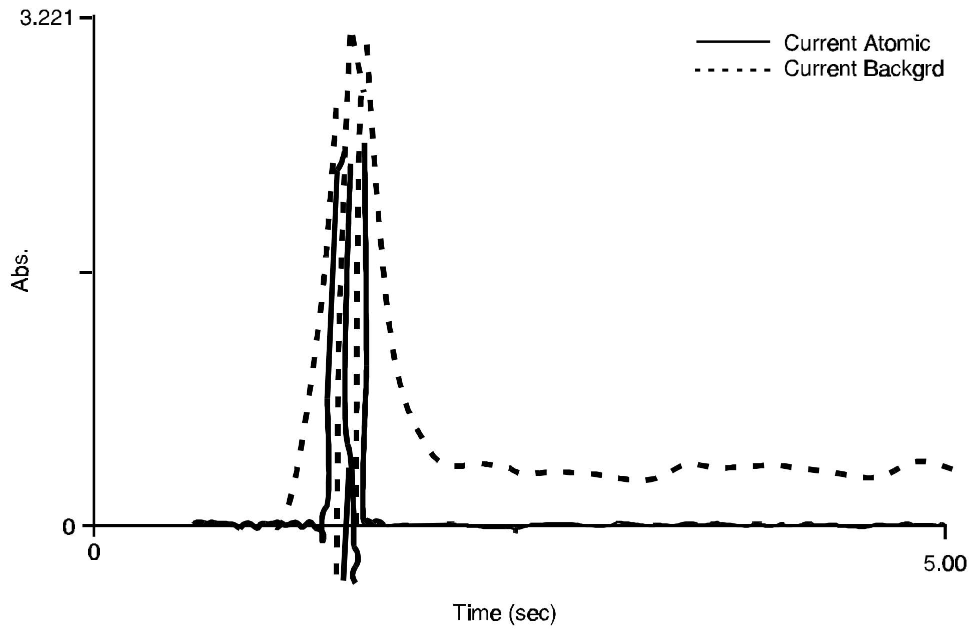

Figure 2. Pb atomization Profile Utilizing a 800° Char.

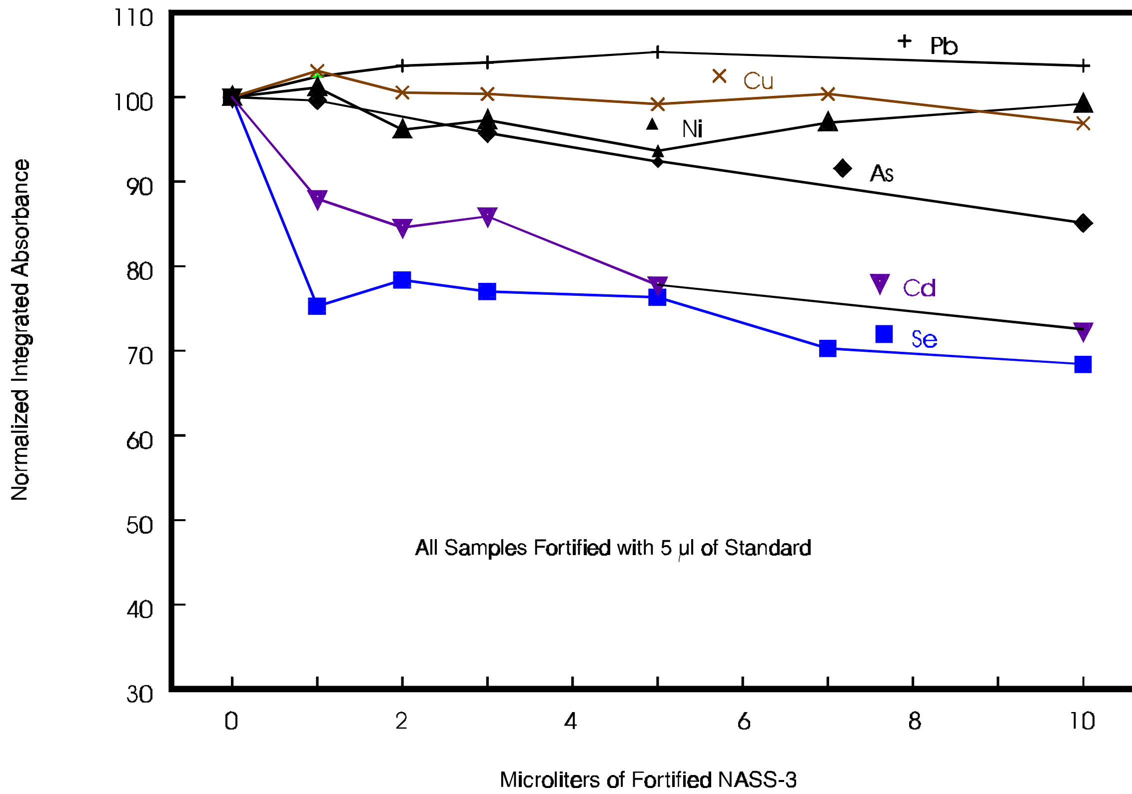

Figure 3. Normalized Integrated Absorbance vs. Microliters of Fortified NASS-3.

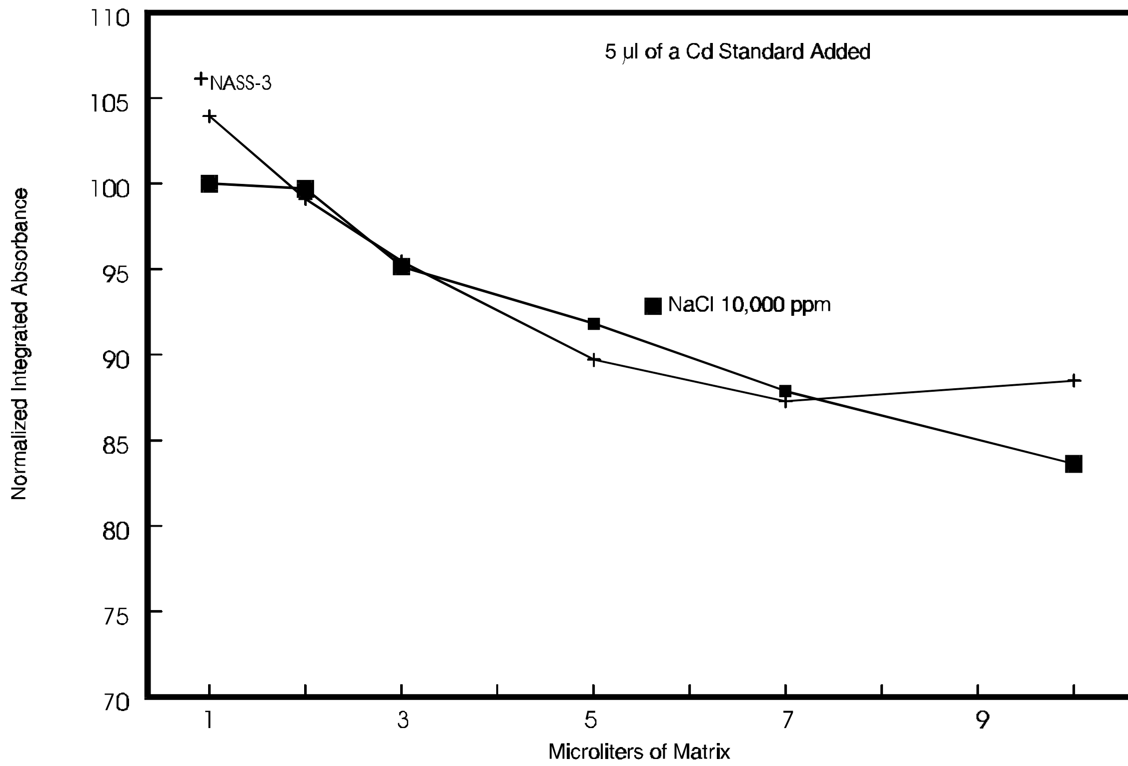

Figure 4. Cd Response in NASS-3 and 10,000 ppm NaCl.

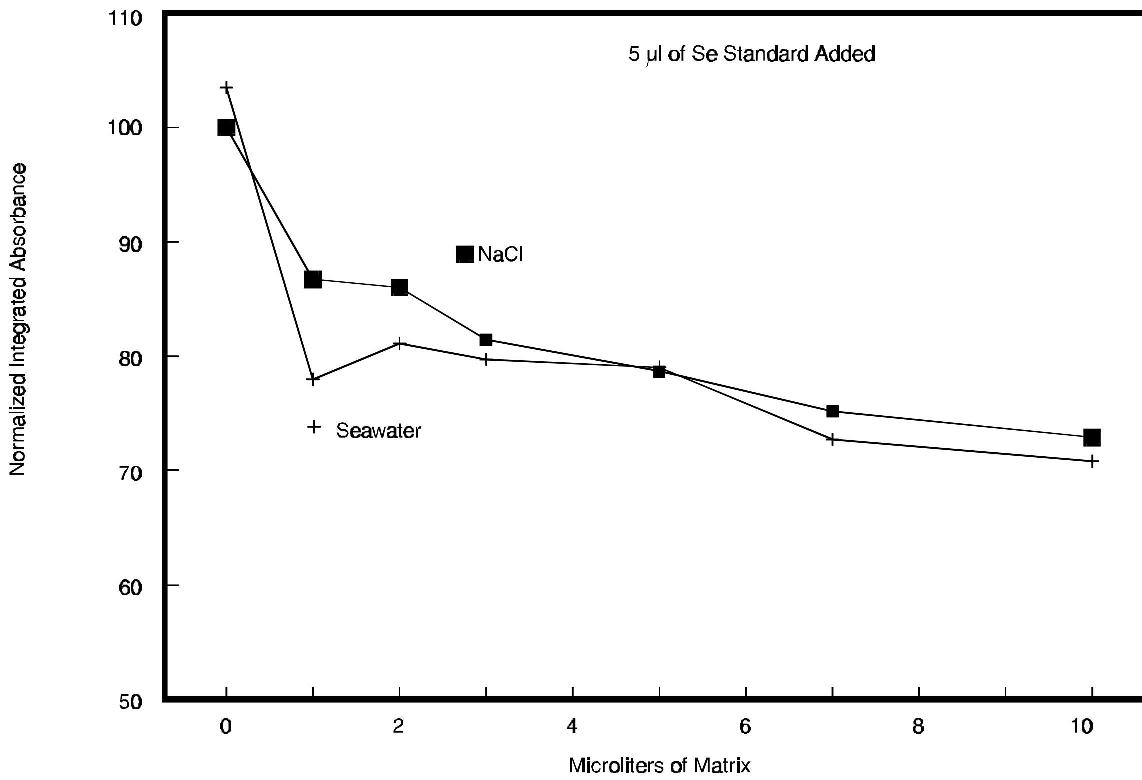

Figure 5. Se Response in Seawater vs 10,000 ppm NaCl

(1) Poor Transfer
(2) Sample Heterogeneity
(3) Digestion/Precipitation
(4) Matrix Suppression/Enhancements
(5) Contamination

IFA = In Furnace Analyte Addition

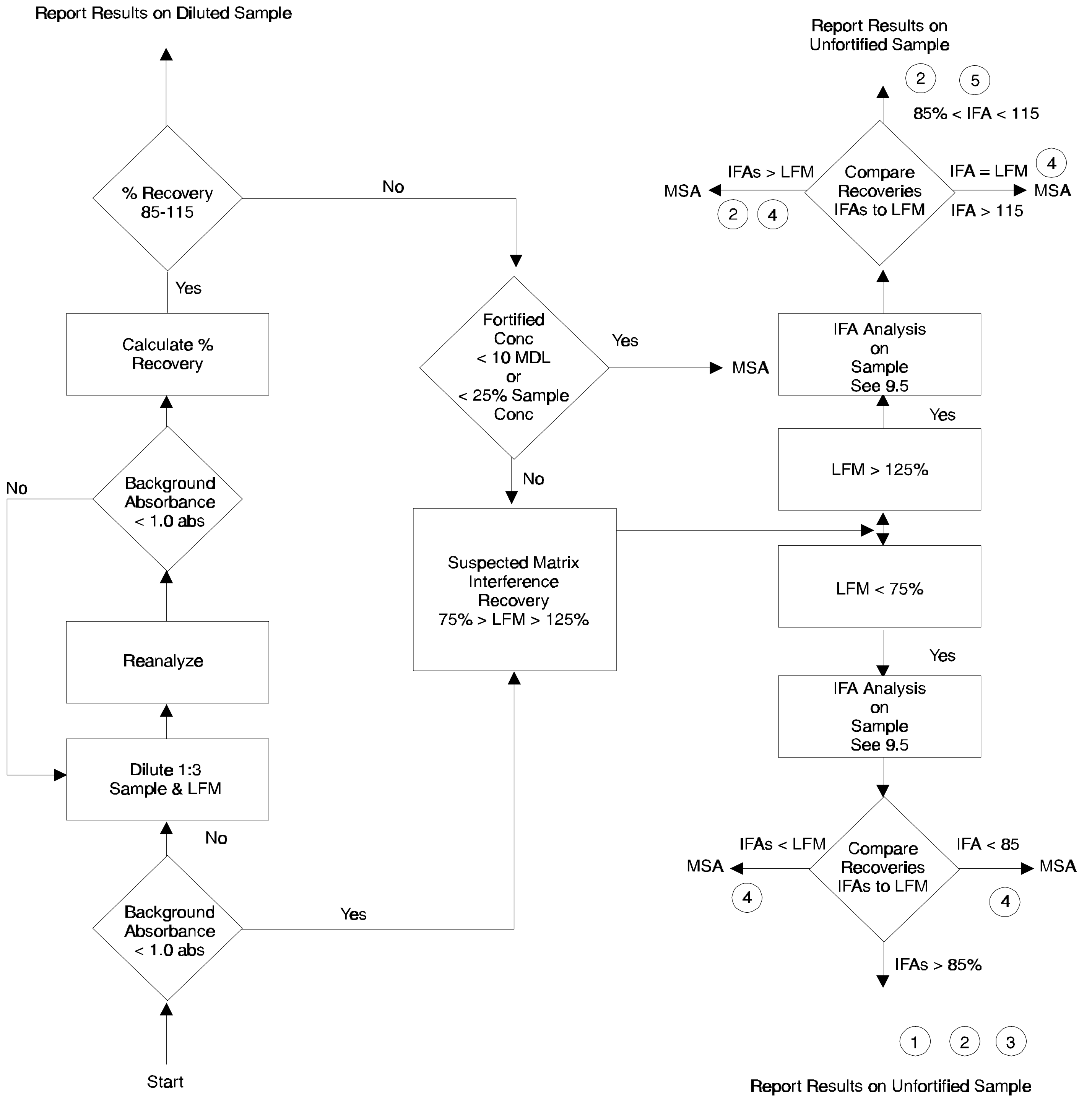

Figure 6. Matrix Interference Flowchart.

Method 200.13

Determination of Trace Elements in Marine Waters by Off-Line Chelation Preconcentration with Graphite Furnace Atomic Absorption

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Revision 1.0
September 1997

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Method 200.13

Determination of Trace Elements in Marine Waters by Off-Line Chelation Preconcentration with Graphite Furnace Atomic Absorption

1.0 Scope and Application

1.1 This method describes procedures for preconcentration and determination of total recoverable trace elements in marine waters, including estuarine water, seawater and brines.

1.2 Acid solubilization is required prior to determination of total recoverable elements to facilitate breakdown of complexes or colloids which might influence trace element recoveries. This method should only be used for preconcentration and determination of trace elements in aqueous samples.

1.3 This method is applicable to the following elements:

Element		Chemical Abstracts Service Registry Numbers (CASRN)
Cadmium	(Cd)	7440-43-9
Cobalt	(Co)	7440-48-4
Copper	(Cu)	7440-50-8
Lead	(Pb)	7439-92-1
Nickel	(Ni)	7440-02-0

1.4 Method detection limits (MDLs) for these elements will be dependent on the specific instrumentation employed and the selected operating conditions. MDLs in NASS-3 (Reference Material, National Research Council of Canada) were determined using the procedure described in Section 9.2.4 and are listed in Table 1.

1.5 A minimum of 6-months experience in graphite furnace atomic absorption (GFAA) is recommended.

2.0 Summary of Method

2.1 Nitric acid is dispensed into a beaker containing an accurately weighed or measured, well-mixed, homogeneous aqueous sample. The sample volume is reduced to approximately 20 mL and then covered and allowed to reflux. The resulting solution is diluted to volume and is ready for analysis.

2.2 This method is used to preconcentrate trace elements using an iminodiacetate functionalized chelating resin.[1,2] Following acid solubilization, the sample is buffered using an on-line system prior to entering the chelating column. Group I and II metals, as well as most anions, are selectively separated from the analytes by elution with ammonium acetate at pH 5.5. The analytes are subsequently eluted into a simplified matrix consisting of 0.75 M nitric acid and are determined by GFAA.

3.0 Definitions

3.1 ***Calibration Blank (CB) --*** A volume of reagent water fortified with the same matrix as the calibration standards, but without the analytes, internal standards, or surrogate analytes.

3.2 ***Calibration Standard (CAL) --*** A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.3 ***Field Reagent Blank (FRB) --*** An aliquot of reagent water or other blank matrix that is placed in a sample container in the laboratory and treated as a sample in all respects, including shipment to the sampling site, exposure to sampling site conditions, storage, preservation, and all analytical procedures. The purpose of the FRB is to determine if method analytes or other interferences are present in the field environment.

3.4 ***Instrument Performance Check Solution (IPC) --*** A solution of one or more method analytes, surrogates, internal standards, or other test substances used to evaluate the performance of the instrument system with respect to a defined set of criteria.

3.5 ***Laboratory Fortified Blank (LFB) --*** An aliquot of reagent water or other blank matrices to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology

is in control, and whether the laboratory is capable of making accurate and precise measurements.

3.6 Laboratory Fortified Sample Matrix (LFM) -- - An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.7 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.8 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.9 Matrix Modifier (MM) -- A substance added to the instrument along with the sample in order to minimize the interference effects by selective volatilization of either analyte or matrix components.

3.10 Method Detection Limit (MDL) -- The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.

3.11 Quality Control Sample -- A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.12 Standard Addition -- The addition of a known amount of analyte to the sample in order to determine the relative response of the detector to an analyte within the sample matrix. The relative response is then used to assess either an operative matrix effect or the sample analyte concentration.

3.13 Stock Standard Solution (SSS) -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

3.14 Total Recoverable Analyte (TRA) -- The concentration of analyte determined to be in either a solid sample or an unfiltered aqueous sample following treatment by refluxing with hot dilute mineral acid(s) as specified in the method.

4.0 Interferences

4.1 Several interference sources may cause inaccuracies in the determination of trace elements by GFAA. These interferences can be classified into three major subdivisions: spectral, matrix, and memory. Some of these interferences can be minimized via the preconcentration step, which reduces the Ca, Mg, Na and Cl concentration in the sample prior to GFAA analysis.

4.2 Spectral interferences are caused by absorbance of light by a molecule or atom which is not the analyte of interest or emission from black body radiation.

4.2.1 Spectral interferences caused by an element only occur if there is a spectral overlap between the wavelength of the interfering element and the analyte of interest. Fortunately, this type of interference is relatively uncommon in STPGFAA (Stabilized Temperature Platform Graphite Furnace Atomic Absorption) because of the narrow atomic line widths associated with STPGFAA.

In addition, the use of appropriate furnace temperature programs and high spectral purity lamps as light sources can minimize the possibility of this type of interference. However, molecular absorbances can span several hundred manometers, producing broadband spectral interferences. This type of interference is far more common in STPGFAA. The use of matrix modifiers, selective volatilization, and background correctors are all attempts to eliminate unwanted nonspecific absorbance. Because the nonspecific component of the total absorbance can vary considerably from sample type to sample type, to provide effective background correction and eliminate the elemental spectral interference of palladium on copper and iron on selenium, the exclusive use of Zeeman background correction is specified in this method.

4.2.2 Spectral interferences are also caused by emissions from black body radiation produced during the atomization furnace cycle. This black body emission

reaches the photomultiplier tube, producing erroneous results. The magnitude of this interference can be minimized by proper furnace tube alignment and monochromator design. In addition, atomization temperatures which adequately volatilize the analyte of interest without producing unnecessary black body radiation can help reduce unwanted background emission produced during atomization.

4.3 Matrix interferences are caused by sample cornponents which inhibit formation of free atomic analyte atoms during the atomization cycle. In this method the use of a delayed atomization device which provides warmer gas phase temperatures is required. These devices provide an environment which is more conducive to the formation of free analyte atoms and thereby minimize this type of interference. This type of interference can be detected by analyzing the sample plus a sample aliquot fortified with a known concentration of the analyte. If the determined concentration of the analyte addition is outside a designated range, a possible matrix effect should be suspected (Section 9.4).

4.4 Memory interferences result from analyzing a sample containing a high concentration of an element (typically a high atomization temperature element) which cannot be removed quantitatively in one complete set of furnace steps. The analyte which remains in the furnace can produce false positive signals on subsequent sample(s). Therefore, the analyst should establish the analyte concentration which can be injected into the furnace and adequately removed in one complete set of furnace cycles. If this concentration is exceeded, the sample should be diluted and a blank analyzed to assure the memory effect has been eliminated before reanalyzing the diluted sample.

4.5 Low recoveries may be encountered in the preconcentration cycle if the trace elements are complexed by competing chelators (humic/fulvic) in the sample or are present as colloidal material. Acid solubilization pretreatment is employed to improve analyte recovery and to minimize adsorption, hydrolysis and precipitation effects.

4.6 Memory interferences from the chelating system may be encountered, especially after analyzing a sample containing high analyte concentrations. A thorough column rinsing sequence following elution of the analytes is necessary to minimize such interferences.

5.0 Safety

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure to these compounds should be as low as reasonably achievable. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method.[3-6] A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Specifically, concentrated nitric and hydrochloric acids present various hazards and are moderately toxic and extremely irritating to skin and mucus membranes. Use these reagents in a fume hood whenever possible and if eye or skin contact occurs, flush with large volumes of water. Always wear safety glasses or a shield for eye protection, protective clothing and observe proper mixing when working with these reagents.

5.2 Acidification of samples containing reactive materials may result in release of toxic gases, such as cyanides or sulfides. Samples should be acidified in a fume hood.

5.3 All personnel handling environmental samples known to contain or to have been in contact with human waste should be immunized against known disease causative agents.

5.4 The graphite tube during atomization emits intense UV radiation. Suitable precautions should be taken to protect personnel from such a hazard.

5.5 The use of the argon/hydrogen gas mixture during the dry and char steps may evolve a considerable amount of HCl gas. Therefore, adequate ventilation is required.

5.6 It is the responsibility of the user of this method to comply with relevant disposal and waste regulations. For guidance see Sections 14.0 and 15.0.

6.0 Equipment and Supplies

6.1 Graphite Furnace Atomic Absorption Spectrometer

6.1.1 The GFAA spectrometer must be capable of programmed heating of the graphite tube and the

associated delayed atomization device. The instrument should be equipped with an adequate background correction device capable of removing undesirable non-specific absorbance over the spectral region of interest. The capability to record relatively fast (< 1 sec) transient signals and evaluate data on a peak area basis is preferred. In addition, a recirculating refrigeration unit is recommended for improved reproducibility of furnace temperatures. The data shown in the tables were obtained using the stabilized temperature platform and Zeeman background correction.

6.1.2 Single element hollow cathode lamps or single element electrodeless discharge lamps along with the associated power supplies.

6.1.3 Argon gas supply (high-purity grade, 99.99%).

6.1.4 A 5% hydrogen in argon gas mix and the necessary hardware to use this gas mixture during specific furnace cycles.

6.1.5 Autosampler-- Although not specifically required, the use of an autosampler is highly recommended.

6.1.6 Graphite Furnace 0perating Conditions -- A guide to experimental conditions for the applicable elements is provided in Table 1.

6.2 Preconcentration System -- System containing no metal parts in the analyte flow path, configured as shown with a sample loop in Figure 1 and without a sample loop in Figure 2.

6.2.1 Column -- Macroporous iminodiacetate chelating resin (Dionex Metpac CC-1 or equivalent).

6.2.2 Control valves -- Inert double stack, pneumatically operated four-way slider valves with connectors.

6.2.2.1 Argon gas supply regulated at 80-100 psi.

6.2.3 Solution reservoirs -- Inert containers, e.g., high density polyethylene (HDPE), for holding eluent and carrier reagents.

6.2.4 Tubing -- High pressure, narrow bore, inert tubing such as Tefzel ETFE (ethylene tetra-fluoro ethylene) or equivalent for interconnection of pumps/ valve assemblies and a minimum length for connection of the preconcentration system with the sample collection vessel.

6.2.5 Eluent pumping system (Gradient Pump) -- Programmable flow, high-pressure pumping system, capable of delivering either one of three eluents at a pressure up to 2000 psi and a flow rate of 1-5 mL/min.

6.2.6 System setup, including sample loop (See Figure 1).

6.2.6.1 Sample loop -- 10-mL loop constructed from narrow bore, high-pressure inert tubing, Tefzel ETFE or equivalent.

6.2.6.2 Auxiliary pumps -- On-line buffer pump, piston pump (Dionex QIC pump or equivalent) for delivering 2M ammonium acetate buffer solution; carrier pump, peristaltic pump (Gilson Minipuls or equivalent) for delivering 1% nitric acid carrier solution; sample pump, peristaltic pump for loading sample loop.

6.2.7 System setup without sample loop (See Figure 2).

6.2.7.1 Auxiliary Pumps - Sample pump (Dionex QIC Pump or equivalent) for loading sample on the column. Carrier pump (Dionex QIC Pump or equivalent) used to flush collection line between samples.

6.3 Labware -- For determination of trace elements, contamination and loss are of **critical** consideration. Potential contamination sources include improperly cleaned laboratory apparatus and general contamination within the laboratory environment. A clean laboratory work area, designated for trace element sample handling must be used. Sample containers can introduce positive and negative errors in determination of trace elements by (1) contributing contaminants through surface desorption or leaching and (2) depleting element concentrations through adsorption processes. For these reasons, borosilicate glass is not recommended for use with this method. All labware in contact with the sample should be cleaned prior to use. Labware may be soaked overnight and thoroughly washed with laboratory-grade detergent and water, rinsed with water, and soaked for 4 h in a mixture of dilute nitric and hydrochloric acids, followed by rinsing with ASTM type I water and oven drying.

6.3.1 Griffin beakers, 250 mL, polytetrafluoroethylene (PTFE) or quartz.

6.3.2 Storage bottles -- Narrow mouth bottles, Teflon FEP (fluorinated ethylene propylene), or HDPE, 125-mL and 250-mL capacities.

6.4 Sample Processing Equipment

6.4.1 Air displacement pipetter -- Digital pipet system capable of delivering volumes from 100 to 2500 µL with an assortment of metal-free, disposable pipet tips.

6.4.2 Balances -- Analytical balance, capable of accurately weighing to ± 0.1 mg; top pan balance, accurate to ± 0.01 g.

6.4.3 Hot plate -- Corning PC100 or equivalent.

6.4.4 Centrifuge -- Steel cabinet with guard bowl, electric timer and brake.

6.4.5 Drying oven -- Gravity convection oven with thermostatic control capable of maintaining 105°C ± 5°C.

6.4.6 pH meter -- Bench mounted or hand-held electrode system with a resolution of ± 0.1 pH units.

6.4.7 Class 100 hoods are recommended for all sample handling.

7.0 Reagents and Standards

7.1 Reagents may contain elemental impurities which might affect analytical data. Only high-purity reagents that conform to the American Chemical Society specifications[7] should be used whenever possible. If the purity of a reagent is in question, analyze for contamination. All acids used for this method must be of ultra high-purity grade or equivalent. Suitable acids are available from a number of manufacturers. Redistilled acids prepared by sub-boiling distillation are acceptable.

7.1.1 Nitric acid, concentrated (sp.gr. 1.41).

7.1.1.1 Nitric acid 0.75M -- Dilute 47.7 mL (67.3g) conc. nitric acid to 1000 mL with ASTM type I water.

7.1.1.2 Nitric acid (1+1) -- Dilute 500 mL conc. nitric acid to 1000 mL with ASTM type I water.

7.1.1.3 Nitric acid (1+9) -- Dilute 100 mL conc. nitric acid to 1000 mL with ASTM type I water.

7.1.2 Matrix Modifier, dissolve 300 mg Palladium (Pd) powder in a minimum amount of concentrated $HN0_3$ (1 mL of HNO_3, adding concentrated HCl only if necessary). Dissolve 200 mg of $Mg(NO_3)_2{\bullet}6H_2O$ in ASTM type I water. Pour the two solutions together and dilute to 100 mL with ASTM type I water.

Note: It is recommended that the matrix modifier be analyzed separately in order to assess the contribution of the modifier to the overall laboratory blank.

7.1.3 Acetic acid, glacial (sp.gr. 1.05). High purity acetic acid is recommended.

7.1.4 Ammonium hydroxide (20%). High purity ammonium hydroxide is recommended.

7.1.5 Ammonium acetate buffer 1M, pH 5.5 -- Add 58 mL (60.5 g) of glacial acetic acid to 600 mL of ASTM type I water. Add 65 mL (60 g) of 20% ammonium hydroxide and mix. Check the pH of the resulting solution by withdrawing a small aliquot and testing with a calibrated pH meter, adjusting the solution to pH 5.5 ± 0.1 with small volumes of acetic acid or ammonium hydroxide as necessary. Cool and dilute to 1 L with ASTM type I water.

7.1.6 Ammonium acetate buffer 2M, pH 5.5 -- Prepare as for Section 7.1.5 using 116 mL (121 g) glacial acetic acid and 130 mL (120 g) 20% ammonium hydroxide, diluted to 1000 mL with ASTM type I water.

Note: If the system is configured as shown in Figure 1, the ammonium acetate buffer solutions may be further purified by passing them through the chelating column at a flow rate of 5.0 mL/min. Collect the purified solution in a container. Following this, elute the collected contaminants from the column using 0.75M nitric acid for 5 min at a flow rate of 4.0 mL/min. If the system is configured as shown in Figure 2, the majority of the buffer is being purified in an on-line configuration via the clean-up column.

7.1.7 Oxalic acid dihydrate (CASRN 6153-56-6), 0.2M -- Dissolve 25.2 g reagent grade $C_2H_2O_4{\bullet}2H_2O$ in 250 mL ASTM type I water and dilute to 1000 mL with ASTM type I water. **CAUTION -** Oxalic acid is toxic; handle with care.

7.2 Water -- For all sample preparation and dilutions, ASTM type I water (ASTM D1193) is required.

7.3 Standard Stock Solutions -- May be purchased from a reputable commercial source or prepared from ultra high-purity grade chemicals or metals (99.99 - 99.999% pure). All salts should be dried for one hour at 105°C, unless otherwise specified. (CAUTION - Many metal salts are extremely toxic if inhaled or swallowed. Wash hands thoroughly after handling.) Stock solutions should be stored in plastic bottles. The following procedures may be used for preparing standard stock solutions:

Note: Some metals, particularly those which form surface oxides require cleaning prior to being weighed. This may be achieved by pickling the surface of the metal in acid. An amount in excess of the desired weight should be pickled repeatedly, rinsed with water, dried and weighed until the desired weight is achieved.

7.3.1 Cadmium solution, stock 1 mL = 1000 µg Cd -- Pickle cadmium metal in (1+9) nitric acid to an exact weight of 0.100 g. Dissolve in 5 mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.2 Cobalt solution, stock 1 mL = 1000 µg Co -- Pickle cobalt metal in (1+9) nitric acid to an exact weight of 0.100 g. Dissolve in 5 mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.3 Copper solution, stock 1 mL = 1000 µg Cu -- Pickle copper metal in (1+9) nitric acid to an exact weight of 0.100 g. Dissolve in 5 mL (1+1) nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.3.4 Lead solution, stock 1 mL = 1000 µg Pb -- Dissolve 0.1599 g $PbNO_3$ in 5 mL (1+1) nitric acid. Dilute to 100 mL with ASTM type I water.

7.3.5 Nickel solution, stock 1 mL = 1000 µg Ni -- Dissolve 0.100 g nickel powder in 5 mL conc. nitric acid, heating to effect solution. Cool and dilute to 100 mL with ASTM type I water.

7.4 Multielement Stock Standard Solution -- Care must be taken in the preparation of multielement stock standards that the elements are compatible and stable. Originating element stocks should be checked for the presence of impurities which might influence the accuracy of the standard. Freshly prepared standards should be transferred to acid cleaned, new FEP or HDPE bottles for storage and monitored periodically for stability. A multielement stock standard solution containing cadmium, cobalt, copper, lead, and nickel may be prepared by diluting an appropriate aliquot of each single element stock in the list to 100 mL with ASTM type I water containing 1% (v/v) nitric acid.

7.4.1 Preparation of calibration standards -- Fresh multielement calibration standards should be prepared weekly. Dilute the stock multielement standard solution in 1% (v/v) nitric acid to levels appropriate to the required operating range. The element concentrations in the standards should be sufficiently high to produce good measurement precision and to accurately define the slope of the response curve.

7.5 Blanks -- Four types of blanks are required for this method. A calibration blank is used to establish the analytical calibration curve, the laboratory reagent blank (LRB) is used to assess possible contamination from the sample preparation procedure and to assess spectral background. The laboratory fortified blank is used to assess routine laboratory performance, and a rinse blank is used to flush the instrument autosampler uptake system. All diluent acids should be made from concentrated acids (Section 7.1) and ASTM type I water.

7.5.1 The calibration blank consists of the appropriate acid diluent in ASTM type I water. The calibration blank should be stored in a FEP bottle.

7.5.2 The laboratory reagent blanks must contain all the reagents in the same volumes as used in processing the samples. The preparation blank must be carried through the entire sample digestion and preparation scheme.

7.5.3 The laboratory fortified blank (LFB) is prepared by fortifying an aliquot of the laboratory reagent blank with all analytes to provide a final concentration which will produce an absorbance of approximately 0.1 for each analyte. The LFB must be carried through the complete procedure as used for the samples.

7.5.4 The rinse blank is prepared as needed by adding 1.0 mL of conc. HNO_3 and 1.0 mL conc. HCl to 1 L of ASTM Type I water and stored in a convenient manner.

7.6 Instrument Performance Check (IPC) Solution -- The IPC solution is used to periodically verify instrument performance during analysis. The IPC solution should be a fortified seawater prepared in the same acid mixture as the calibration standards and should contain method analytes such that the resulting absorbances are near the midpoint of the calibration curve. The IPC solution should be prepared from the same standard stock solutions used to prepare the calibration standards and stored in a FEP bottle. Agency programs may specify or request that additional instrument performance check solutions be prepared at specified concentrations in order to meet particular program needs.

7.7 Quality Control Sample (QCS) -- A quality control sample having certified concentrations of the analytes of interest should be obtained from a source outside the

laboratory. Dilute the QCS if necessary with 1% nitric acid, such that the analyte concentrations fall within the proposed instrument calibration range.

8.0 Sample Collection, Preservation and Storage

8.1 Prior to collection of an aqueous sample, consideration should be given to the type of data required, so that appropriate preservation and pretreatment steps can be taken. Acid preservation, etc., should be performed at the time of sample collection or as soon thereafter as practically possible. The pH of all aqueous samples must be tested immediately prior to aliquoting for analysis to ensure the sample has been properly preserved. If properly acid-preserved, the sample can be held up to 6 months before analysis.

8.2 For determination of total recoverable elements in aqueous samples, acidify with (1+1) nitric acid at the time of collection to pH < 2. Normally 3 mL of (1+1) acid per liter of sample is sufficient. The sample should not be filtered prior to analysis.

Note: Samples that cannot be acid-preserved at the time of collection because of sampling limitations or transport restrictions, or have pH > 2 because of high alkalinity should be acidified with nitric acid to pH < 2 upon receipt in the laboratory. Following acidification, the sample should be held for 16 h and the pH verified to be <2 before withdrawing an aliquot for sample processing.

8.3 For aqueous samples, a field blank should be prepared and analyzed as required by the data user. Use the same container type and acid as used in sample collection.

9.0 Quality Control

9.1 Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and periodic analysis of laboratory reagent blanks, fortified blanks and other laboratory solutions as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (determination of linear dynamic ranges and analysis of quality control samples) and laboratory performance (determination of method detection limits) prior to samples being analyzed by this method.

9.2.2 Linear dynamic range (LDR) -- The upper limit of the LDR. must be established for the wavelength utilized for each analyte by determining the signal responses from a minimum of 6 different concentration standards across the range, two of which are close to the upper limit of the LDR. Determined LDRs must be documented and kept on file. The linear calibration range which may be used for analysis of samples should be judged by the analyst from the resulting data. The upper LDR. limit should be an observed signal no more than 10% below the level extrapolated from the four lower standards. New LDRs should be determined whenever there is a significant change in instrument response, a change in instrument analytical hardware or operating conditions.

Note: Multiple cleanout furnace cycles may be necessary in order to fully define or utilize the LDR. for certain elements such as nickel. For this reason, the upper limit of the linear calibration range may not correspond to the upper LDR limit.

Measured sample analyte concentrations that exceed the upper limit of the linear calibration range must either be diluted and reanalyzed with concern for memory effects (Section 4.4) or analyzed by another approved method.

9.2.3 Quality control sample (QCS) -- When beginning the use of this method, on a quarterly basis or as required to meet data-quality needs, verify the calibration standards and acceptable instrument performance with the preparation and analyses of a QCS (Section 7.7). If the determined concentrations are not within ± 10% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding on with the initial determination of method detection limits or continuing with ongoing analyses.

9.2.4 Method detection limit (MDL) -- MDLs must be established for all analytes, using reagent water (blank) fortified at a concentration of two to three times the estimated instrument detection limit.[8] To determine MDL values, take seven replicate aliquots of the fortified

reagent water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

$$MDL = (t) \times (S)$$

where, t = Student's t value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom [t = 3.14 for seven replicates].

S= standard deviation of the replicate analyses.

Note: If the relative standard deviation (RSD) from the analyses of the seven aliquots is < 15%, the concentration used to determine the analyte MDL may have been in appropriately high for the determination. If so, this could result in the calculation of an unrealistically low MDL. If additional confirmation of the MDL is desired, reanalyze the seven replicate aliquots on two more nonconsecutive days and again calculate the MDL values for each day. An average of the three MDL values for each analyte may provide for a more appropriate MDL estimate. Determination of MDL in reagent water represents a best case situation and does not reflect possible matrix effects of real world samples. However, successful analyses of LFMs (Section 9.4) can give confidence to the MDL value determined in reagent water. Typical single laboratory MDL values using this method are given in Table 1. MDLs should be determined every 6 months, when a new operator begins work, or whenever there is a significant change in the background or instrument response.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory reagent blank (LRB) - The laboratory must analyze at least one LRB (Section 7.5.2) with each batch of 20 or fewer samples. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the MDL indicate laboratory or reagent contamination should be suspected. Any determined source of contamination must be corrected and the samples reanalyzed for the affected analytes after acceptable LRB values have been obtained.

9.3.2 Laboratory fortified blank (LFB) -- The laboratory must analyze at least one LFB (Section 7.5.3) with each batch of samples. Calculate accuracy as percent recovery (Section 9.4.3). If the recovery of any analyte falls outside the required control limits of 85-115%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.

9.3.3 The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 85-115% (Section 9.3.2). When sufficient internal performance data become available (usually a minimum of 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

$$\text{Upper Control Limit} = x + 3S$$

$$\text{Lower Control Limit} = x - 3S$$

The optional control limits must be equal to or better than the required control limits of 85-115%. After each 5-10 new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also, the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.3.4 Instrument Performance Check (IPC) Solution -- For all determinations the laboratory must analyze the IPC solution (Section 7.6) and a calibration blank immediately following each calibration, after every tenth sample (or more frequently, if required) and at the end of the sample run. The IPC solution should be a fortified seawater matrix. Analysis of the IPC solution and calibration blank immediately following calibration must verify that the instrument is within ±10% of calibration. Subsequent analyses of the IPC solution must be within ±10% of calibration. If the calibration cannot be verified within the specified limits, reanalyze the IPC solution. If the second analysis of the IPC solution confirms calibration to be outside the limits, sample analysis must be discontinued, the cause determined and/or in the case of drift the instrument recalibrated. All samples following the last acceptable IPC solution must be reanalyzed. The analysis data of the calibration blank and IPC solution must be kept on file with the sample analyses data.

9.3.5 The overall sensitivity and precision of this method are strongly influenced by a laboratory's ability to control the method blank. Therefore, it is recommended that the calibration blank response be recorded for each set of samples. This record will aid the laboratory in assessing both its long- and short-term ability to control the method blank.

9.4 Assessing Analyte Recovery and Data Quality

9.4.1 Sample homogeneity and the chemical nature of the sample matrix can affect analyte recovery and data quality. Taking separate aliquots from the sample for replicate and fortified analyses can, in some cases, assess these effects. Unless otherwise specified by the data user, laboratory or program, the following laboratory fortified matrix (LFM) procedure (Section 9.4.2) is required.

9.4.2 The laboratory must add a known amount of each analyte to a minimum of 10% of routine samples. In each case, the LFM aliquot must be a duplicate of the aliquot used for sample analysis and for total recoverable determinations added prior to sample preparation. For water samples, the added analyte concentration must be the same as that used in the laboratory fortified blank (Section 7.5.3). Over time, samples from all routine sample sources should be fortified.

9.4.3 Calculate the percent recovery for each analyte, corrected for concentrations measured in the unfortified sample, and compare these values to the designated LFM recovery range of 75-125%. Recovery calculations are not required if the concentration added is <25% of the unfortified sample concentration. Percent recovery may be calculated in units appropriate to the matrix, using the following equation:

$$R = \frac{C_s - C}{s} \times 100$$

where,
- R = percent recovery.
- C_s = fortified sample concentration.
- C = sample background concentration.
- s = concentration equivalent of analyte added to sample.

9.4.4 If the recovery of any analyte falls outside the designated LFM recovery range (but is still within the range of calibration and the background absorbance is < 1 abs.) and the laboratory performance for that analyte is shown to be in control (Section 9.3), the recovery problem encountered with the LFM is judged to be either matrix or solution related, not system related. This situation should be rare given the matrix elimination preconcentration step prior to analysis. If a low recovery is found, check the pH of the sample plus the buffer mixture. The resulting pH should be about 5.5. The pH of the sample strongly influences the column's ability to preconcentrate the metals; therefore, a low recovery may be caused by a low pH. If the pH for the LFM/buffer mixture is about 5.5, the analyst is advised to make an in furnace analyte addition to the LFM using the preconcentrated standard solution. If recovery of the in furnace analyte addition is shown to be out of control, a matrix interference is confirmed and the sample must be analyzed by MSA.

9.5 Utilizing Reference Materials

9.5.1 It is recommended that a reference material such as NASS-3 (from the Research Council of Canada) be fortified and used as an Instrument Performance Check Solution.

10.0 Calibration and Standardization

10.1 The preconcentration system can be configured utilizing a sample loop to define the sample volume (Figure 1) or the system can be configured such that a sample pump rate and a pumping time defines the sample volume (Figure 2). The system illustrated in Figure 1 is recommended for sample sizes of <10 mL. A thorough rinsing of the sample loop between samples with HNO_3 is required. This rinsing will minimize the cross-contamination which may be caused by the sample loop. The system in Figure 2 should be used for sample volumes of >10 mL. The sample pump used in Figure 2 must be calibrated to assure that a reproducible/defined volume is being delivered.

10.2 Specific wavelengths and instrument operating conditions are listed in Table 1. However, because of differences among makes and models of spectrophotometers and electrothermal furnace devices, the actual instrument conditions selected may vary from those listed.

10.3 Prior to the use of this method, instrument operating conditions must be optimized. The analyst should follow the instructions provided by the manufacturer while using the conditions listed in Table 1 as a guide. Of particular importance is the determination of the charring temperature limit for each analyte. This limit is the furnace temperature setting where a loss in analyte will occur prior to atomization. This limit should be determined by conducting char temperature profiles for each analyte and when necessary, in the matrix of question. The charring temperature selected should minimize background absorbance while providing some furnace temperature variation without loss of analyte. For routine analytical operation the charring temperature is usually

set at least 100°C below this limit. The optimum conditions selected should provide the lowest reliable MDLs and be similar to those listed in Table 1. Once the optimum operating conditions are determined, they should be recorded and available for daily reference.

10.4 Prior to an initial calibration, the linear dynamic range of the analyte must be determined (Section 9.2.2) using the optimized instrument operating conditions. For all determinations allow an instrument and hollow cathode lamp warm-up period of not less than 15 min. If an EDL is to be used, allow 30 min for warm-up.

10.5 Before using the procedure (Section 11.0) to analyze samples, data must be available to document initial demonstration of performance. The required data and procedure are described in Section 9.2. This data must be generated using the same instrument operating conditions and calibration routine (Section 11.4) to be used for sample analysis. These documented data must be kept on file and be available for review by the data user.

11.0 Procedure

11.1 Sample Preparation -- Total Recoverable Elements

11.1.1 Add 2 mL (1+1) nitric acid to the beaker containing 100 mL of sample. Place the beaker on the hot plate for solution evaporation. The hot plate should be located in a fume hood and previously adjusted to provide evaporation at a temperature of approximately but no higher than 85°C. (See the following note.) The beaker should be covered with an elevated (ribbed) watch glass or other necessary steps should be taken to prevent sample contamination from the fume hood environment.

Note: For proper heating adjust the temperature control of the hot plate such that an uncovered Griffin beaker containing 50 mL of water placed in the center of the hot plate can be maintained at a temperature approximately but no higher than 85°C. (Once the beaker is covered with a watch glass the temperature of the water will rise to approximately 95°C.)

11.1.2 Reduce the volume of the sample aliquot to about 20 mL by gentle heating at 85°C. DO NOT BOIL. This step takes about 2 hr for a 100-mL aliquot with the rate of evaporation rapidly increasing as the sample volume approaches 20 mL. (A spare beaker containing 20 mL of water can be used as a gauge.)

11.1.3 Cover the lip of the beaker with a watch glass to reduce additional evaporation and gently reflux the sample for 30 min. Slight boiling may occur, but vigorous boiling must be avoided.

11.1.4 Allow the beaker to cool. Quantitatively transfer the sample solution to a 100-mL volumetric flask, dilute to volume with reagent water, stopper and mix.

11.1.5 Allow any undissolved material to settle overnight, or centrifuge a portion of the prepared sample until clear. (If after centrifuging or standing overnight the sample contains suspended solids that would clog or affect the sample introduction system, a portion of the sample may be filtered prior to analysis. However, care should be exercised to avoid potential contamination from filtration.) The sample is now ready for analysis. Because the effects of various matrices on the stability of diluted samples cannot be characterized, all analyses should be performed as soon as possible after the completed preparation.

11.2 Prior to first use, the preconcentration system should be thoroughly cleaned and decontaminated using 0.2M - oxalic acid.

11.2.1 Precleaning the Preconcentration System

11.2.1.1 Place approximately 500 mL 0.2M - oxalic acid in each of the sample/eluent containers. Flush the entire system by running the program used for sample analysis 3 times.

11.2.1.2 Rinse the containers with ASTM type I water and repeat the sequence described in Section 11.2.1.1 using 0.75M nitric acid and again using ASTM type I water in place of the 0.2M - oxalic acid.

11.2.1.3 Rinse the containers thoroughly with ASTM type I water, fill them with their designated reagents and run through the program used for sample analysis in order to prime the pump and all eluent lines with the correct reagents.

11.2.2 Peak Profile Determination

11.2.2.1 The peak elution time or the collection window should be determined using an ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometer) or Flame AA. Figure 3 is a plot of time vs. emission intensity for Cd, Pb, Ni, and Cu. The collection window is marked in Figure 3 and should provide about 30 sec buffer on

either side of the peak. If an ICP-AES is not available, it is recommended that the peak profile be determined by collecting 200-µL samples during the elution part of the preconcentration cycle and then reconstructing the peak profile from the analysis of the 200-µL samples.

11.3 Sample Preconcentration

11.3.1 Preconcentration utilizing a sample loop.

11.3.1.1 Loading Sample Loop -- With valve 1 in the off position and valve 2 in the on position, load sample through the sample loop to waste using the sample pump for 4 min at 4 mL/min. Switch on the carrier pump and pump 1 % nitric acid to flush the sample collection line.

11.3.1.2 Column Loading -- With valve 1 in the on position, load sample from the loop onto the column using 1 M ammonium acetate for 4.5 min at 4.0 mL/min. Switch on the buffer pump, and pump 2M ammonium acetate at a flow rate of 1 mL/min. The analytes are retained on the column, while the majority of the matrix is passed through to waste.

11.3.1.3 Elution Matrix -- With valve 1 in the on position the gradient pump is allowed to elute the matrix using the 1M ammonium acetate. During which time the carrier, buffer and the sample pumps are all off.

11.3.1.4 Elute Analytes -- Turn off valve 1 and begin eluting the analytes by pumping 0.75M nitric acid through the column and turn off valve 2 and pump the eluted analytes into the collection flask. The analytes should be eluted into a 2-mL sample volume.

11.3.1.5 Column Reconditioning -- Turn on valve 2 to direct column effluent to waste, and pump 0.75M nitric acid, 1M ammonium acetate, 0.75M nitric acid and 1M ammonium acetate alternately through the column at 4.0 mL/min. Each solvent should be pumped through the column for 2 min. During this process, the next sample can be loaded into the sample loop using the sample pump.

11.3.1.6 Preconcentration of the sample may be a-chieved by running through an eluent pump program. The exact timing of this sequence should be modified according to the internal volume of the connecting tubing and the specific hardware configuration used.

11.3.2 Preconcentration utilizing an auxiliary pump to determine sample volume.

11.3.2.1 Sample Loading -- With the valves 1 and 2 on and the sample pump on, load the sample on the column buffering the sample utilizing the gradient pump and the 2M buffer. The actual sample volume is determined by knowing the sample pump rate and the time. While the sample is being loaded the carrier pump can be used to flush the collection line.

11.3.2.2 Elution Matrix -- With valve 1 in the off position the gradient pump is allowed to elute the matrix using the 1M ammonium acetate. During which time the carrier, buffer and the sample pumps are all off.

11.3.2.3 Elution of Analytes -- With valves I and 2 in the off position the gradient pump is switched to 0.75M HNO_3 and the analytes are eluted into the collection vessel. The analytes should be eluted into a 2 mL sample volume.

11.3.2.4 Column Reconditioning -- Turn on valve 2 to direct column effluent to waste, and pump 0.75M nitric acid, 1M ammonium acetate, 0.75M nitric acid and 1M ammonium acetate alternately through the column at 4.0 mL/min.

Note: When switching the gradient pump from nitric acid back to the ammonium acetate it is necessary to flush the line connecting the gradient pump to valve 2 with the ammonium acetate prior to switching the valve. If the line contains nitric acid it will elute the metals from the cleanup column.

11.3.2.5 Preconcentration of the sample may be a-chieved by running through an eluent pump program. The exact timing of this sequence should be modified according to the internal volume of the connecting tubing and the specific hardware configuration used.

11.4 Repeat the sequence described in Section 11.3.1 or 11.3.2 for each sample to be analyzed. At the end of the analytical run leave the column filled with 1M ammonium acetate buffer until it is next used.

11.5 Samples having concentrations higher than the established linear dynamic range should be diluted into range and reanalyzed.

11.6 Sample Analysis

11.6.1 Prior to daily instrument calibration, inspect the graphite furnace, the sample uptake system and auto-sampler injector for any change that would affect instrument performance. Clean the system and replace

the graphite tube and/or platform when needed or on a daily basis. A cotton swab dipped in a 50/50 mixture of isopropyl alcohol (IPA) and H_2O (such that it is damp but not dripping) can be used to remove the majority of the salt buildup. A second cotton swab is dipped in IPA and the contact rings are wiped down to assure they are clean. The rings are then allowed to thoroughly dry and then a new tube is placed in the furnace and conditioned according to instrument manufacturers specifications.

11.6.2 Configure the instrument system to the selected optimized operating conditions as determined in Sections 10.1 and 10.2.

11.6.3 Before beginning daily calibration the instrument should be reconfigured to the optimized conditions. Initiate data system and allow a period of not less than 15 min for instrument and hollow cathode lamp warm-up. If an EDL is to be used, allow 30 min for warm-up.

11.6.4 After the warm-up period but before calibration, instrument stability must be demonstrated by analyzing a standard solution with a concentration 20 times the IDL a minimum of five times. The resulting relative standard deviation of absorbance signals must be <5%. If the relative standard deviation is >5%, determine and correct the cause before calibrating the instrument.

11.6.5 For initial and daily operation calibrate the instrument according to the instrument manufacturer's recommended procedures using the calibration blank (Section 7.5.1) and calibration standards (Section 7.4) prepared at three or more concentrations within the usable linear dynamic range of the analyte (Sections 4.4 & 9.2.2).

11.6.6 An autosampler must be used to introduce all solutions into the graphite furnace. Once the standard, sample or QC solution plus the matrix modifier is injected, the furnace controller completes furnace cycles and cleanout period as programmed. Analyte signals must be integrated and collected as peak area measurements. Background absorbances, background corrected analyte signals, and determined analyte concentrations on all solutions must be able to be displayed on a CRT for immediate review by the analyst and be available as hard copy for documentation to be kept on file. Flush the autosampler solution uptake system with the rinse blank (Section 7.5.4) between each solution injected.

11.6.7 After completion of the initial requirements of this method (Section 9.2), samples should be analyzed in the same operational manner used in the calibration routine.

11.6.8 During sample analyses, the laboratory must comply with the required quality control described in Sections 9.3 and 9.4.

11.6.9 Determined sample analyte concentrations that are $\geq$90% of the upper limit of calibration must either be diluted with acidified reagent water and reanalyzed with concern for memory effects (Section 4.4), or determined by another approved test procedure that is less sensitive. Samples with a background absorbance > 1.0 must be appropriately diluted with acidified reagent water and reanalyzed (Section 9.4.6). If the method of standard additions is required, follow the instructions described in Section 11.5.

11.6.10 Report data as directed in Section 12.

11.7 Standard Additions -- If the method of standard addition is required, the following procedure is recommended:

11.7.1 The standard addition technique[9] involves preparing new standards in the sample matrix by adding known amounts of standard to one or more aliquots of the processed sample solution. This technique compensates for a sample constituent that enhances or depresses the analyte signal, thus producing a different slope from that of the calibration standards. It will not correct for additive interference, which causes a baseline shift. The simplest version of this technique is the single addition method. The procedure is as follows: Two identical aliquots of the sample solution, each of volume V_x, are taken. To the first (labeled A) is added a small volume V_S of a standard analyte solution of concentration C_s. To the second (labeled B) is added the same volume V_s of the solvent. The analytical signals of A and B are measured and corrected for nonanalyte signals. The unknown sample concentration C_x is calculated:

$$C_X = \frac{S_B V_S C_S}{(S_A - S_B) V_X}$$

where, S_A and S_B are the analytical signals (corrected for the blank) of solutions A and B, respectively. V_S and C_S should be chosen so that S_A is roughly twice S_B on the average. It is best if V_S is made much less than V_X, and thus C_S is much greater than C_X, to avoid excess dilution of the sample matrix. If a separation or concentration step is used, the additions are best made first and carried through the entire procedure. For the results from this technique to be valid, the following limitations must be taken into consideration:

1. The analytical curve must be linear.

2. The chemical form of the analyte added must respond in the same manner as the analyte in the sample.

3. The interference effect must be constant over the working range of concern.

4. The signal must be corrected for any additive interference.

12.0 Data Analysis and Calculations

12.1 Sample data should be reported in units of µg/L for aqueous samples.

12.2 For total recoverable aqueous analytes (Section 11.1), when 100-mL aliquot is used to produce the 100 mL final solution, round the data to the tenths place and report the data in µg/L up to three significant figures. If an aliquot volume other than 100 mL is used for sample preparation, adjust the dilution factor accordingly. Also, account for any additional dilution of the prepared sample solution needed to complete the determination of analytes exceeding the upper limit of the calibration curve. Do not report data below the determined analyte MDL concentration or below an adjusted detection limit reflecting smaller sample aliquots used in processing or additional dilutions required to complete the analysis.

12.3 The QC data obtained during the analyses provide an indication of the quality of the sample data and should be provided with the sample results.

13.0 Method Performance

13.1 Experimental conditions used for single laboratory testing of the method are summarized in Table 1.

13.2 Table 2 contains precision and recovery data obtained from a single laboratory analysis of a fortified and a non-fortified sample of NASS-3. The samples were prepared using the procedure described in Section 11.1. Four replicates of the non-fortified samples were analyzed and the average of the replicates was used for determining the sample analyte concentration. The fortified samples of NASS-3 were also analyzed and the average percent recovery and the percent relative standard deviation is reported. The reference material certified values are also listed for comparison.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation (e.g., Section 7.8). When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult Less is Better: Laboratory Chemical Management for Waste Reduction, available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202)872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult The Waste Management Manual for Laboratory Personnel, available from the American Chemical Society at the address listed in the Section 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Method Detection Limits for Total Recoverable Analytes in Reagent Water[1]

Element	Slit, nm	Recommended analytical Wavelengths, nm	Char Temp, °C	Atomization Temp, °C	MDL[2], µg/L
Cadmium	0.7	228.8	800	1600	0.016
Cobalt	0.2	242.5	1400	2500	-
Copper	0.7	324.8	1300	2600	0.36
Lead	0.7	283.3	1250	2000	0.28
Nickel	0.2	232.4	1400	2500	*

[1] MDLs were calculated using NASS-3 as the matrix.
[2] MDLs were calculated based on a 10-mL sample loop.
* MDL was not calculated because the concentration in the matrix exceeds the MDL spike level.
- Not Determined.

Table 2. Precision and Recovery Data for NASS-3 Using System Illustrated in Figure 1[1,2]

Analyte	Certified Value, µg/L[3]	Sample Conc., µg/L[3]	Fortified Conc., µg/L	Avg. Recovery, %	% RSD
Cd	0.029 ± 0.004	0.026 ± 0.012	0.25	93	3.3
Co	0.004 ± 0.001	-	-	-	-
Cu	0.109 ± 0.011	<0.36	5.0	87	1.4
Pb	0.039 ± 0.006	<0.28	5.0	90	3.7
Ni	0.257 ± 0.027	0.260 ± 0.04	5.0	117	8.3

[1] Data collected using 10-mL sample loop.
[2] Matrix modifier is $Pd/Mg(NO_3)_2/H_2$.
[3] Uncertainties based on 95% confidence limits.
- Not determined.

	Valves		Buffer	Carrier	Sample
	1	2	Pump	Pump	Pump
Sample Loop Loading	Off	On	Off	On	On
Column Loading	On	On	On	Off	Off
Elution of Matrix	On	On	Off	Off	Off
Elution of Analytes	Off	Off	Off	Off	Off
Column Recondition	Off	On	Off	Off	Off

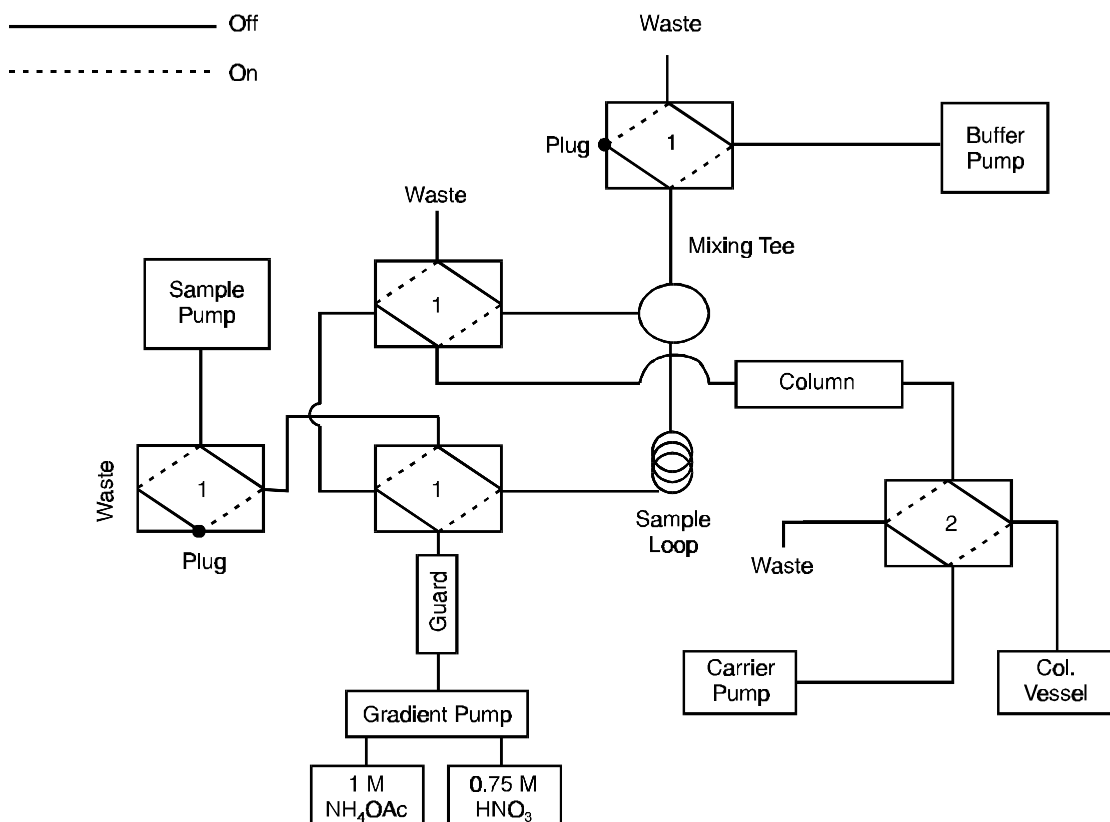

Figure 1. Sample Loop Configuration.

Event	Valves 1	Valves 2	Carrier Pump	Sample Pump
Sample Loading	On	On	On	On
Elution of Matrix	Off	On	Off	Off
Elution of Analytes	Off	Off	Off	Off
Column Recondition	Off	On	Off	On

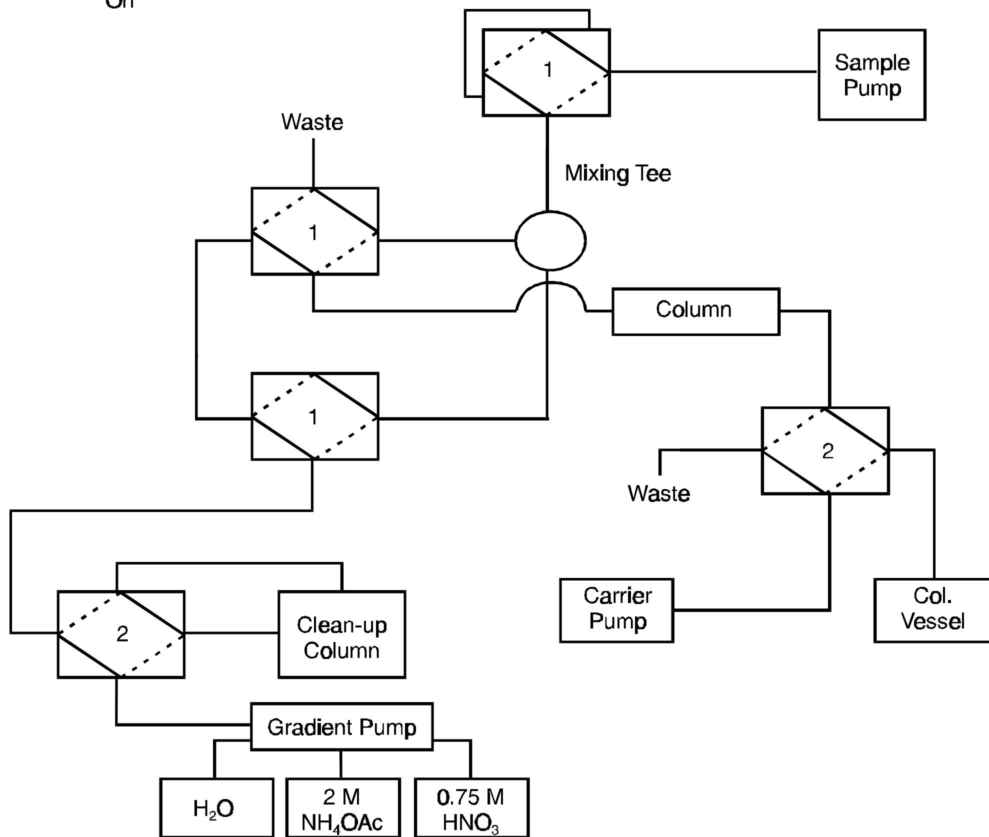

Figure 2. System Diagram without Sample Loop.

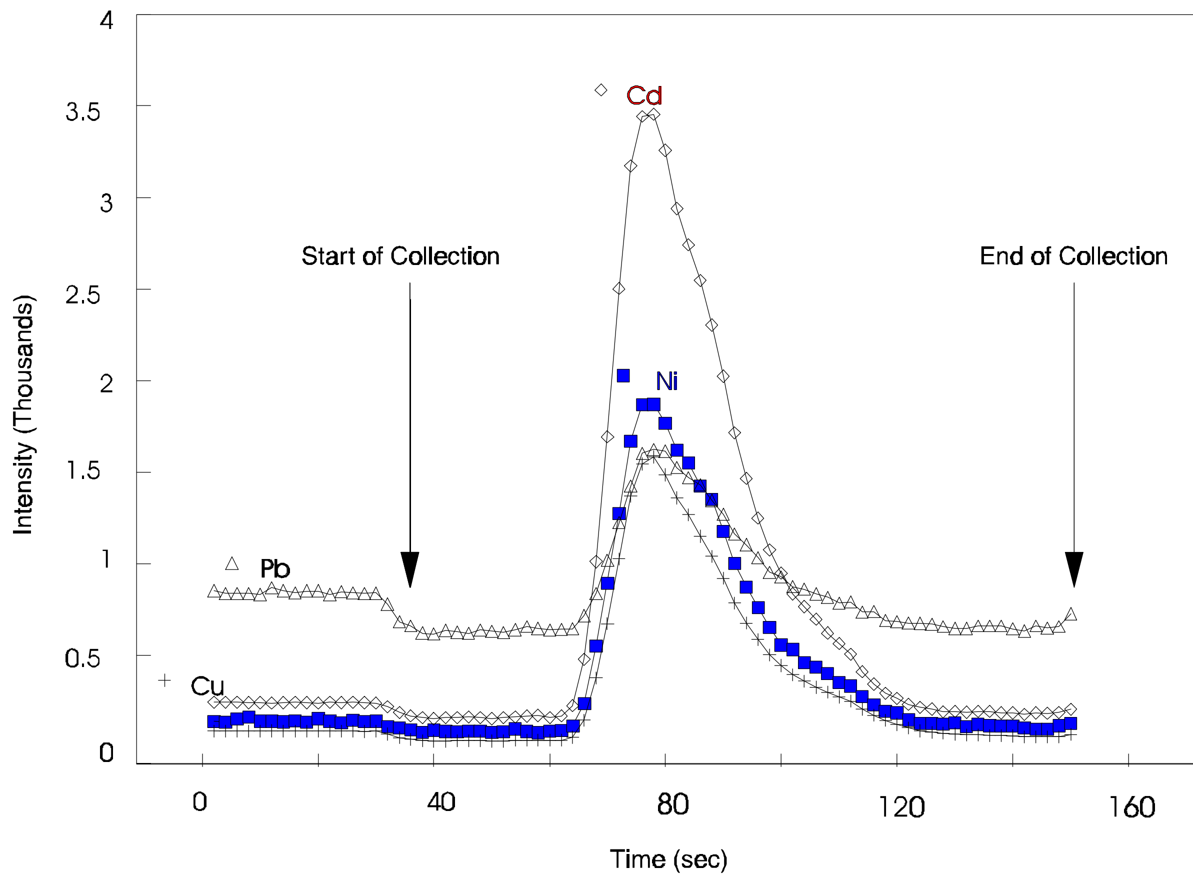

Figure 3. Peak Collection Window from ICP-AES.

Method 349.0

Determination of Ammonia in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

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Version 1.0
September 1997

National Exposure Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

Method 349.0

Determination of Ammonia in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

1.0 Scope and Application

1.1 This method provides a procedure for the determination of ammonia in estuarine and coastal waters. The method is based upon the indophenol reaction,[1-5] here adapted to automated gas-segmented continuous flow analysis.

The term ammonia as used in this method denotes total concentration of ammonia, including both chemical forms, NH_3 and NH_4^+. Because ionization of NH_4^+ has a pK value of about 9.3, NH_4^+ is the dominant chemical form in natural waters. At pH of 8.2 and 25°C only 8.1% is present as NH_3, the form that can be toxic to fish and other aquatic organisms.

The concentration of ammonia in estuarine and coastal water shows considerable temporal and spatial variability. It rarely exceeds 0.005 mg N/L in oxygenated, unpolluted estuarine and coastal water, but in anoxic water, the amount of ammonia can be as high as 0.28 mg N/L.[6]

Although other forms of nitrogen contribute to primary productivity and nutrient cycling in marine and estuarine waters, ammonia is particularly important. Because ammonia represents the most reduced form of inorganic nitrogen available, it is preferentially assimilated by phytoplankton. Whereas nitrate is the source of nitrogen, it must first be reduced to ammonia before it can be assimilated and incorporated into amino acids and other compounds. Ammonia is released during the decomposition of organic nitrogen compounds by proteolytic bacteria, but also excreted directly by invertebrates along with urea and peptides.[7] In regions of coastal upwelling, ammonia released by zooplankton can play a significant role in supplying the nitrogen that supports phytoplankton production.[8]

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Ammonia	7664-41-7

1.2 A statistically determined method detection limit (MDL)[9] of 0.3 µg N/L has been determined by one laboratory from seawaters of four different salinities. The method is linear to 4.0 mg N/L using a Flow Solution System (Alpkem, Wilsonville, Oregon).

1.3 Approximately 60 samples per hour can be analyzed.

1.4 This method should be used by analysts both experienced in the use of automated gas segmented continuous flow colorimetric analyses, and also familiar with matrix interferences and the procedures used in their correction. A minimum of 6-months experience under the close supervision of a qualified analyst is recommended.

2.0 Summary of Method

2.1 The automated gas segmented continuous flow colorimetric method is used for the analysis of ammonia concentration. Ammonia in solution reacts with alkaline phenol and NaDTT (Sect. 7.2.5) at 60°C to form indophenol blue in the presence of sodium nitroferricyanide as a catalyst. The absorbance of indophenol blue at 640 nm is linearly proportional to the concentration of ammonia in the sample. A small systematic negative error caused by differences in the refractive index of seawater and reagent water, and a positive error caused by the matrix effect on the color formation, may be corrected for during data processing.

3.0 Definitions

3.1 ***Calibration Standard (CAL)*** -- A solution prepared from the primary dilution standard solution or stock standard solution containing analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.2 ***Laboratory Fortified Blank (LFB)*** -- An aliquot of reagent water to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed

exactly like a sample, and its purpose is to determine whether method performance is within acceptable control limits, and whether the laboratory is capable of making accurate and precise measurements. This is a standard prepared in reagent water that is analyzed as a sample.

3.3 Laboratory Fortified Sample Matrix (LFM) -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.4 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water that is treated exactly as a sample including exposure to all labware, equipment, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or apparatus.

3.5 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.6 Method Detection Limit (MDL) -- The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.[9]

3.7 Reagent Water (RW) -- Type 1 reagent grade water equal to or exceeding the standards established by the American Society for Testing and Materials (ASTM). Reverse osmosis systems or distilling units followed by Super-Q Plus Water System that produce water with 18 megohm resistance are examples of acceptable water sources. To avoid contamination of ammonia from the air, the reagent water should be stored in a sealed or a collapsible container and used the day of preparation.

3.8 Refractive Index (RI) -- The ratio of the velocity of light in a vacuum to that in a given medium. The relative refractive index is the ratio of the velocity of light in two different media, such as estuarine or sea water versus reagent water. The correction for this difference is referred to as refractive index correction in this method.

3.9 Stock Standard Solution (SSS) -- A concentrated solution of method analyte prepared in the laboratory using assayed reference compounds or purchased from a reputable commercial source.

3.10 Primary Dilution Standard Solution (PDS) -- A solution prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.11 Quality Control Sample (QCS) -- A solution of method analyte of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.12 Synchronization Peak Solution -- A synchronization peak is required by most data acquisition programs to initialize the peak finding parameters. The first cup in every run must always be identified as a SYNC sample. The SYNC sample is usually a high concentration standard, but can be any sample that generates a peak at least 25% of full scale.

3.13 Color SYNC Peak Solution -- A colored solution used to produce a synchronization peak in the refractive index measurement in which no color reagent is pumped through system.

3.14 Sensitivity Drift -- The change in absorbance for a given concentration of analyte due to instrumental or chemical drift during the course of measurement.

3.15 Matrix Effect -- The change of absorbance in different matrices due to the effect of ionic strength and composition on the kinetics of color forming reactions.

4.0 Interferences

4.1 Hydrogen sulfide at concentrations greater than 2 mg S/L can negatively interfere with ammonia analysis. Hydrogen sulfide in samples should be removed by acidification with sulfuric acid to a pH of about 3, then stripping with gaseous nitrogen.

4.2 The addition of sodium citrate and EDTA complexing reagent eliminates the precipitation of calcium and magnesium hydroxides when calcium and

magnesium in seawater samples mix with high pH (about 13) reagent solution.[4]

4.3 Sample turbidity is eliminated by filtration or centrifugation after sample collection.

4.4 As noted in Section 2.1 refractive index and salt error interferences occur when sampler wash solution and calibration standards are not matched with samples in salinity, but are correctable. For low concentration samples (< 20 µg N/L), low nutrient seawater (LNSW) with salinity matched to samples, sampler wash solutions and calibration standards is recommended to eliminate matrix interferences.

5.0 Safety

5.1 Water samples collected from the estuarine and coastal environment are rarely hazardous. However, the individual who collects samples should use proper technique.

5.2 Good laboratory technique should be used when preparing reagents. Laboratory personnel should obtain material safety data sheets (MSDS) for al chemicals used in this method. A lab coat, safety goggles, and gloves should be worn when handling the concentrated acid.

5.3 Chloroform is used as a preservative in this method. Use in a properly ventilated area, such as a fume hood.

6.0 Equipment and Supplies

6.1 Gas Segmented Continuous Flow Autoanalyzer Consisting of:

6.1.1 Automatic sampler.

6.1.2 Analytical cartridge with reaction coils and heater.

6.1.3 Proportioning pump.

6.1.4 Spectrophotometer equipped with a tungsten lamp (380-800 nm) or photometer with a 640 nm interference filter (maximum 2 nm bandwidth).

6.1.5 Strip chart recorder or computer based data acquisition system.

6.1.6 Nitrogen gas (high-purity grade, 99.99%).

6.2 Glassware and Supplies

6.2.1 Gaseous ammonia concentration in the laboratory air should be minimal to avoid sample or reagent contamination. Remove any NH_4OH solution stored in the laboratory. Smoking should be strictly forbidden. An air filtration unit might also be used to obtain ammonia-free lab air.

6.2.2 All labware used in the analysis must be free of residual ammonia to avoid sample or reagent contamination. Soaking with laboratory grade detergent, rinsing with tap water, followed by rinsing with 10% HCl (v/v) and then thoroughly rinsing with reagent water was found to be sufficient when working at moderate and high concentration of ammonia. Ammonia is known for its high surface reactivity.[10] When working at low levels of ammonia (< 20 µg N/L), further cleaning of labware is mandatory. Plastic bottles and glass volumetric flasks should be cleaned in an ultrasonic bath with reagent water for 60 minutes. Bottles and sample tubes made of glass can be easily cleaned by boiling in reagent water. Repeat the cleaning process with fresh reagent water prior to use if necessary.

6.2.3 Automatic pipetters with disposable pipet tips capable of delivering volumes ranging from 100 µL to 1000 µL and 1 mL to 10 mL.

6.2.4 Analytical balance, with accuracy to 0.1 mg, for preparing standards.

6.2.5 60-mL glass or high density polyethylene sample bottles, glass volumetric flasks and glass sample tubes.

6.2.6 Drying oven.

6.2.7 Desiccator.

6.2.8 Membrane filters with 0.45 µm nominal pore size. Plastic syringes with syringe filters.

6.2.9 Centrifuge.

6.2.10 Ultrasonic water bath cleaner.

7.0 Reagents and Standards

Note: All reagents must be of analytical reagent grade.

7.1 Stock Reagent Solutions

7.1.1 Complexing Reagent - Dissolve 140 g of sodium citrate dihydrate($Na_3C_6H_5O_7.2H_2O$, FW 294.11), 5 g of sodium hydroxide (NaOH, FW 40) and 10 g of disodium EDTA ($Na_2C_{10}H_{14}O_8N_2.2H_2O$, FW 372.24), in approximately 800 mL of reagent water, mix and dilute to 1 L with reagent water. The pH of this solution is approximately 13. This solution is stable for 2 months.

7.1.2 Stock Ammonium Sulfate Solution (100 mg N/L) - Quantitatively transfer 0.4721 g of pre-dried (105°C for 2 hours) ammonium sulfate ($(NH_4)_2SO_4$, FW 132.15) to a 1000 mL glass volumetric flask containing approximately 800 mL of reagent water and dissolve the salt. Add a few drops of chloroform as a preservative. Dilute the solution to the mark with reagent water. Store in a glass bottle in the refrigerator at 4°C. It is stable for 2 months.[11]

7.1.3 Low Nutrient Sea Water (LNSW) - Obtain natural low nutrient seawater from surface water of the Gulf Stream or Sargasso Sea (salinity 36 ‰, < 7 µg N/L) and filter it through 0.3 micron pore size glass fiber filters. If this is not available, commercial low nutrient sea water (< 7 µg N/L) with salinity of 35 ‰ (Ocean Scientific International, Wormley, U.K.) can be substituted. **NOTE:** Don't use artificial seawater in this method.

7.2 Working Reagents

7.2.1 Brij-35 Start-up Solution - Add 2 mL of Brij-35 surfactant (ICI Americas, Inc.) to 1000 mL reagent water and mix gently.

Note: Brij-35 is a trade name for polyoxyethylene(23) lauryl ether ($C_{12}H_{25}(OCH_2CH_2)_{23}OH$, FW=1199.57, CASRN 9002-92-0).

7.2.2 Working Complexing Reagent - Add 1 mL Brij-35 to 200 mL of stock complexing reagent, mix gently. Prepare this solution daily. This volume of solution is sufficient for an 8-hour run.

7.2.3 Sodium Nitroferricyanide Solution - Dissolve 0.25 g of sodium nitroferricyanide ($Na_2Fe(CN)_5NO.2H_2O$, FW 297.97) in 400 mL of reagent water, dilute to 500 mL with reagent water. Store in an amber bottle at room temperature.

7.2.4 Phenol Solution - Dissolve 1.8 g of solid phenol (C_6H_5OH, FW 94.11) and 1.5 g of sodium hydroxide (NaOH, FW 40) in 100 mL of reagent water. Prepare this solution fresh daily.

7.2.5 NaDTT Solution - Dissolve 0.5 g of sodium hydroxide (NaOH, FW 40) and 0.2 g dichloroisocyanuric acid sodium salt (NaDTT, $NaC_3Cl_2N_3O_3$, FW 219.95) in 100 mL of reagent water. Prepare this solution fresh daily.

7.2.6 Colored SYNC Peak Solution - Add 50 µL of blue food coloring solution to 1000 mL reagent water and mix thoroughly. Further dilute this solution to obtain a peak of between 25 to 100 percent full scale according to the AUFS setting used for refractive index measurement.

7.2.7 Primary Dilution Standard Solution - Prepare a primary dilution standard solution (5 mg N/L) by diluting 5.0 mL of stock standard solution to 100 mL with reagent water. Prepare this solution daily.

Note: This solution should be prepared to give an intermediate concentration appropriate for further dilution in preparing the calibration solutions. Therefore, the concentration of a primary dilution standard solution must be adjusted according to the desired concentration range of calibration solutions.

7.2.8 Calibration Standards - Prepare a series of calibration standards (CAL) by diluting suitable volumes of a primary dilution standard solution (Section 7.2.7) to 100 mL with reagent water or low nutrient seawater. Prepare these standards daily. The concentration range of calibration standards should bracket the expected concentrations of samples and not span more than two orders of magnitude. At least five calibration standards with equal increments in concentration should be used to construct the calibration curve.

When working with samples of a narrow range of salinities (± 2 ‰) or samples containing low ammonia concentration (< 20 µg N/L), it is recommended that the CAL solutions be prepared in Low Nutrient Seawater (Section 7.1.4) diluted to the salinity of samples, and the Sampler Wash Solution also be Low Nutrient Seawater (Section 7.1.4) diluted to the same salinity. **NOTE:** *If this procedure is employed, it is not necessary to perform the matrix effect and refractive index corrections outlined in Sections 12.2 and 12.3.*

When analyzing samples of moderate and high ammonia concentration (> 20 µg N/L) with varying salinities, calibration standard solutions and sampler wash solutions can be prepared in reagent water. The corrections for matrix effect and refractive index should be subsequently applied (Sections 12.2 and 12.3).

7.2.9 Saline Ammonia Standards - If CAL solutions are not prepared to match sample salinity, then saline ammonia standards must be prepared in a series of salinities in order to quantify the matrix effect (the change in the colorimetric response of ammonia due to the change in the composition of the solution). The following dilution of Primary Dilution Standard Solution (Section 7.2.7) and LNSW with reagent water to 100 mL in volumetric flasks, are suggested.

Salinity (‰)	Volume of LNSW(mL)	Volume of PDS(mL)	Conc. mg N/L
0	0	2	.10
9	25	2	.10
18	50	2	.10
27	75	2	.10
35	98	2	.10

8.0 Sample Collection, Preservation and Storage

8.1 Sample Collection - Samples collected for nutrient analyses from estuarine and coastal waters are normally collected using one of two methods: hydrocast or submersible pump systems.

8.1.1 A hydrocast uses a series of sampling bottles (Niskin, Go-Flo or equivalent) attached at fixed intervals to a hydro wire. These bottles are sent through the water column open and are closed either electronically or via a mechanical messenger when the bottles have reached the desired depth.

8.1.2 In a submersible pump system, a weighted hose is sent to the desired depth in the water column and water is pumped from that depth to the deck of the ship for sample processing.

8.1.3 For collecting surface samples, an acid - cleaned plastic bucket or a large plastic bottle can be used as convenient samplers. Wash the sampler three times with sample water before collecting samples.

8.1.4 Turbid samples must be filtered through a 0.45 µm membrane filter as soon as possible after collection. Wash the filter with reagent water before use. Pass at least 100 mL of sample through the filter and discard before taking the final sample. Care must be taken to avoid the contamination of ammonia especially handling low concentrations of ammonia (< 20 µg N/L) samples.[10] An alternative technique to remove particulate is centrifugation.

8.1.5 60-mL glass or high density polyethylene bottles are used for sample storage. Sample bottles should be rinsed 3 times with about 20 mL of sample, shaking with the cap in place after each rinse. Pour the rinse water into the cap to dissolve and rinse away salt crusts trapped in the threads of the cap. Finally, fill the sample bottle about 3/4 full, and screw the cap on firmly.

8.2 Sample Preservation - After collection and filtration or centrifugation, samples should be analyzed as soon as possible. If samples will be analyzed within 3 hours then keep refrigerated in tightly sealed, glass or high density polyethylene bottles in the dark at 4°C until the analysis can be performed.

8.3 Sample Storage - At low concentrations of ammonia (< 20 µg N/L), no preservation technique is satisfactory. *Samples must be analyzed within 3 hours of collection*. At moderate and high concentrations of ammonia (> 20 µg N/L) samples can be preserved by the addition of 2 mL of chloroform per liter of sample and refrigerated in the dark at 4°C. Samples can be stored in either glass or high density polyethylene bottles. A maximum holding time for preserved estuarine and coastal water samples with moderate to high concentrations of ammonia is two weeks.[12]

9.0 Quality Control

9.1 Each laboratory using this method is required to implement a formal quality control (QC) program. The minimum requirements of this program consists of an initial demonstration of performance, continued analysis of Laboratory Reagent Blanks (LRB), laboratory duplicates and Laboratory Fortified Blanks (LFB) with each set of samples as a continuing check on performance.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance by determining the MDL and LDR and laboratory performance by analyzing quality control samples prior to analysis of samples using this method.

9.2.2 A method detection limit (MDL) should be established for the method analyte, using a low level seawater sample containing, or fortified at, approximately 5 times the estimated detection limit. To determine MDL values, analyze at least seven replicate aliquots of water which have been processed through the entire analytical method. Perform all calculations defined in the method and report concentration in appropriate units. Calculate the MDL as follows:

$$MDL = (t)(S)$$

where, S = the standard deviation of the replicate analyses

t = Student's t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

MDLs should be determined every 6 months or whenever a significant change in background or instrument response occurs or a new matrix is encountered.

9.2.3 The LDR should be determined by analyzing a minimum of eight calibration standards ranging from 0.002 to 2.00 mg N/L across all sensitivity settings (Absorbance Units Full Scale output range setting) of the detector. Standards and sampler wash solutions should be prepared in low nutrient seawater with salinities similar to that of samples to avoid the necessity to correct for salt error, or refractive index. Normalize responses by multiplying the response by the Absorbance Units Full Scale output range setting. Perform the linear regression of normalized response vs. concentration and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response, R, of a standard no longer yields a calculated concentration C_c, that is within 100 ± 10% of known concentration, C, where $C_c = (R-b)/m$. That concentration defines the upper limit of the LDR for the instrument. Should samples be encountered that have a concentration that is ≥ 90% of the upper limit of LDR, then these samples must be diluted and reanalyzed.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) - A laboratory should analyze at least one LRB with each set of samples. LRB data are used to assess contamination from the laboratory environment. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When the LRB value constitutes 10% or more of the analyte concentration determined for a sample, duplicates of the sample must be prepared and analyzed again after the source of contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 Laboratory Fortified Blank (LFB) - A laboratory should analyze at least one LFB with each set of samples. The LFB must be at a concentration within the daily calibration range. The LFB data are used to calculate accuracy as percent recovery. If the recovery of the analyte falls outside the required control limits of 90 -110%, the source of the problem should be identified and resolved before continuing the analyses.

9.3.3 The laboratory must use LFB data to assess laboratory performance against the required control limits of 90 -110%. When sufficient internal performance data become available (usually a minimum of 20 to 30 analyses), optional control limits can be developed from the percent mean recovery (x) and standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

Upper Control Limit = x + 3S
Lower Control Limit = x - 3S

The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5 to 10 new recovery measurements, new control limits can be calculated using only the most recent 20 to 30 data points. Also the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and available for review.

9.4 *Assessing Analyte Recovery - Laboratory Fortified Sample Matrix (LFM)*

9.4.1 A laboratory should add a known amount of analyte to a minimum of 5% of the total number of samples or one LFM per sample set, whichever is greater. The analyte added should be 2-4 times the ambient concentration and should be at least four times greater than the MDL.

9.4.2 Calculate percent recovery of analyte, corrected for background concentration measured in a separate unfortified sample. These values should be compared with the values obtained from the LFBs. Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_S - C)}{S} \times 100$$

where, R = percent recovery

C_S = measured fortified sample addition in mg N/L

C = sample background concentration (mg N/L)

S = concentration in mg N/L added to the environmental sample.

9.4.3 If the recovery of the analyte falls outside the required control limits of 90-110%, but the laboratory performance for that analyte is within the control limits, the fortified sample should be prepared again and analyzed. If the result is the same after reanalysis, the recovery problem encountered with the fortified sample is judged to be matrix related and the sample data should be flagged accordingly.

10.0 Calibration and Standardization

10.1 At least five calibration standards should be prepared fresh daily for system calibration.

10.2 A calibration curve should be constructed for each sample set by analyzing a series of calibration standard solutions. A sample set should contain no more than 60 samples. For a large number of samples make several sample sets with individual calibration curves.

10.3 Analyze the calibration standards, in duplicate, before the actual samples.

10.4 The calibration curve containing five data points or more that bracket the conentrations of samples should have a correlation coefficient, r, of 0.995 or better and the range should not be greater than two orders of magnitude.

10.5 Use a high CAL solution followed by two blank cups to quantify system carryover. The difference in peak heights between two blank cups is due to the carryover from the high CAL solution. The carryover coefficient, k, is calculated as follows:

$$k = \frac{P_{b1} - P_{b2}}{P_{high}}$$

where, P_{high} = the peak height of the high ammonia standard

P_{b1} = the peak height of the first blank sample

P_{b2} = the peak height of the second blank sample

The carryover coefficient, k, should be measured in seven replicates to obtain a statistically significant number. The carryover coefficient should be remeasured with any change in manifold plumbing or upon replacement of pump tubes.

The carryover correction (CO) of a given peak, i, is proportional to the peak height of the preceding sample, P_{i-1}.

$$CO = (k)x(P_{i-1})$$

To correct a given peak height reading, P_i, subtract the carryover correction.[13,14]

$$P_{i,c} = P_i - CO$$

where $P_{i,c}$ is corrected peak height. The correction for carryover should be applied to all the peak heights throughout a run. The carryover coefficient should be less than 5% in this method.

10.6 Place a high standard solution at the end of each sample run to check for sensitivity drift. Apply sensitivity drift correction to all the samples. The sensitivity drift during a run should be less than 5%.

Note: Sensitivity drift correction is available in most data acquisition software supplied with autoanalyzers. It is assumed that the sensitivity drift is linear with time. An interpolated drift correction factor is calculated for each sample according to the sample position during a run. Multiply the sample peak height by the corresponding sensitivity drift correction factor to obtain the corrected peak height for each sample.

11.0 Procedure

11.1 If samples are stored in a refrigerator, remove samples and equilibrate to room temperature prior to analysis.

11.2 Turn on the continuous flow analyzer and data acquisition components and warm up at least 30 minutes.

11.3 Set up cartridge and pump tubes as shown in Figure 1.

11.4 Set spectrophotometer wavelength to 640 nm, and turn on lamp.

11.5 Set the Absorbance Unit Full Scale (AUFS) range on the spectrophotometer at an appropriate setting according to the highest concentration of ammonia in the samples. The highest setting appropriate for this method is 0.2 AUFS for 6 mg N/L.

11.6 Prepare all reagents and standards.

11.7 Choose an appropriate wash solution for sampler wash. For analysis of samples with a narrow range of salinities (± 2 ‰) or for samples containing low ammonia concentrations (< 20 µg N/L), it is recommended that the CAL solutions be prepared in Low Nutrient Seawater (Section 7.1.4) diluted to the salinity of samples, and that the Sampler Wash Solution also be Low Nutrient Seawater diluted to the same salinity. For samples with varying salinities and higher ammonia concentrations (> 20 µg N/L), it is suggested that the reagent water used for the sampler wash solution and for preparing calibration standards and procedures in Section 12.2 and 12.3 be employed.

11.8 Begin pumping the Brij-35 start-up solution (Section 7.2.1) through the system and obtain a steady baseline. Place the reagents on-line. The reagent baseline will be higher than the start-up solution baseline. After the reagent baseline has stabilized, reset the baseline.

Note: To minimize the noise in the reagent baseline, clean the flow system by sequentially pumping the sample line with reagent water, 1 N HCl solution, reagent water, 1 N NaOH solution for few minutes each at tahe end of the daily analysis. Make sure to rinse the system well with reagent water after pumping NaOH solution to prevent precipitation of $Mg(OH)_2$ when seawater is introduced into the system. Keep the reagents and samples free of particulate. Filter the reagents and samples if necessary.

If the baseline drifts upward, pinch the waste line for a few seconds to increase back pressure. If absorbance drops down rapidly when back pressure increases, this indicates that there are air bubbles trapped in the flow cell. Attach a syringe at the waste outlet of the flowcell. Air bubbles in the flowcell can often be eliminated by simply attaching a syringe for a few minutes or, if not, dislodged by pumping the syringe piston. Alternatively, flushing the flowcell with alcohhol was found to be effective in removing air bubbles from the flowcell.

11.9 The sampling rate is approximately 60 samples per hour with 30 seconds of sample time and 30 seconds of wash time.

11.10 Use cleaned sample cups or tubes (follow the procedures outlined in Section 6.2.2). Place CAL solutions and saline standards (optional) in sampler. Complete filling the sampler tray with samples, laboratory reagent blanks, laboratory fortified blanks, laboratory fortified sample matrices, and QC samples. Place a blank after every ten samples.

11.11 Commence analysis.

12.0 Data Analysis and Calculations

12.1 Concentrations of ammonia in samples are calculated from the linear regression, obtained from the standard curve in which the concentrations of the

calibration standards are entered as the independent variable, and their corresponding peak heights are the dependent variable.

12.2 Refractive Index Correction for Estuarine and Coastal Samples

12.2.1 If reagent water is used as the wash solution, the operator has to quantify the refractive index correction due to the difference in salinity between sample and wash solution. The following procedures are used to measure the relationship between the sample salinity and refractive index on a particular detector.

12.2.2 First, analyze a set of ammonia standards in reagent water with color reagent using reagent water as the wash and obtain a linear regression of peak height versus concentration.

12.2.3 Second, replace reagent water wash solution with Low Nutrient Seawater wash solution.

Note: In ammonia analysis absorbance of the reagent water is higher than that of the LNSW. When using reagent water as a wash solution, the change in refractive index causes the absorbance of seawater to become negative. To measure the absorbance due to refractive index change in different salinity samples, Low Nutrient Seawater must be used as the wash solution to bring the baseline down.

12.2.4 Third, replace the phenol solution (Section 7.2.4) and NaDTT solution (Section 7.2.5) with reagent water. All other reagents remain the same. Replace the synchronization sample with the colored SYNC peak solution (Section 7.2.6).

12.2.5 Prepare a series of different salinity samples by diluting the LNSW. Commence analysis and obtain peak heights for different salinity samples. The peak heights for the refractive index correction must be obtained at the same AUFS range setting and on the same spectrophotometer as the corresponding standards (Section 12.2.2).

12.2.6 Using LNSW as the wash water, a maximum absorbance will be observed for reagent water. No change in refractive index will be observed in the seawater sample. Assuming the absolute absorbance for reagent water (relative to the seawater baseline) is equal to the absorbance for seawater (relative to reagent water baseline), subtract the absorbances of samples of various salinities from that of reagent water. The results are the apparent absorbance due to the change in refractive index between samples of various salinities relative to the reagent water baseline.

12.2.7 For each sample of varying salinity, calculate the apparent ammonia concentration due to refractive index from its peak height corrected to reagent water baseline (Section 12.2.5) and the regression equation of ammonia standards obtained with color reagent being pumped through the system (Section 12.2.2). Salinity is entered as the independent variable and the apparent ammonia concentration due to refractive index is entered as the dependent variable. The resulting regression allows the operator to calculate apparent ammonia concentration due to refractive index when the sample salinity is known. Thus, the operator would not be required to obtain refractive index peak heights for all samples.

12.2.8 The magnitude of refractive index correction can be minimized by using a low refractive index flowcell. An example of a typical result using a low refractive index flowcell follows:

Salinity (‰)	Apparent ammonia conc. due to refractive index (µg N/L)
0.0	0.00
4.5	0.18
9.1	0.45
13.9	0.66
17.9	0.86
27.6	1.30
36.2	1.63

Note: You must calculate the refractive index correction for your particular detector. The refractive index must be redetermined whenever a significant change in the design of the flowcell or a new matrix is encountered.

12.2.9 An example of a typical equation is:

Apparent ammonia (µg N/L) = 0.0134 + 0.0457S

where S is sample salinity in parts per thousand. The apparent ammonia concentration due to refractive index so obtained should then be added to samples of

corresponding salinity when reagent water was used as the wash solution for samples analysis.

If a low refractive index flowcell is used and ammonia concentration is greater than 200 µg N/L, the correction for refractive index becomes negligible.

12.3 Correction for Matrix Effect in Estuarine and Coastal Samples

12.3.1 When calculating concentrations of samples of varying salinities from standards and wash solution prepared in reagent water, it is necessary to first correct for refractive index errors, then correct for the change in color development due to the differences in composition between samples and standards (matrix effect). Even where the refractive index correction may be small, the correction for matrix effect can be appreciable.
12.3.2 Plot the salinity of the saline standards (Section 7.2.9) as the independent variable, and the apparent concentration of ammonia (mg N/L) from the peak height (corrected for refractive index) calculated from the regression of standards in reagent water, as the dependent variable for all saline standards. The resulting regression equation allows the operator to correct the concentrations of samples of known salinity for the color enhancement due to matrix effect. An example of a typical result follows:

Salinity (‰)	Peak height of 0.140 mg N/L	Uncorrected NH_3 conc. calculated from standards in reagent water (mg N/L)
0	2420	0.1400
4.5	2856	0.1649
9.1	2852	0.1649
13.9	2823	0.1635
17.9	2887	0.1673
27.6	2861	0.1663
36.2	2801	0.1633

12.3.3 Using the reagent described in Section 7.0, as shown above, matrix effect becomes a single factor independent of sample salinity. An example of a typical equation to correct for matrix effect is:

Corrected concentration (mg N/L)
= Uncorrected concentration /1.17(mg N/L)

12.3.4 Results of sample analyses should be reported in mg N/L or in µg N/L.

mg N/L = ppm (parts per million)
µg N/L = ppb (part per billion)

13.0 Method Performance

13.1 Single Laboratory Validation

13.1.1 Method Detection Limit- A method detection limit (MDL) of 0.3 µg N/L has been determined by one laboratory from spiked LNSW of three different salinities as follows:

Salinity (‰)	$[NH_3]$ (µg N/L)	SD (µg N/L)	Recovery (%)	MDL (µg N/L)
36.2	0.7	0.0252	95.4	0.0792
36.2	0.7	0.0784	100.8	0.2463
36.2	1.4	0.0826	104.7	0.2595
36.2	1.4	0.0966	105.6	0.3035
17.9	0.7	0.0322	106.5	0.1012
17.9	0.7	0.0182	92.2	0.0572
17.9	1.4	0.0938	109.1	0.2947
17.9	1.4	0.0882	100	0.2771
4.5	0.7	0.0672	95.1	0.2111
4.5	1.4	0.1008	94.1	0.3167
4.5	1.4	0.126	106.7	0.3959
0.0	0.7	0.077	98.2	0.2419
0.0	0.7	0.0784	100.8	0.2463
0.0	1.4	0.0854	101.9	0.2683

13.1.2 Single Analyst Precision - A single laboratory analyzed three samples collected from the Miami River and Biscayne Bay, Florida. Seven replicates of each sample were processed and analyzed with salinity ranging from 4.8 to 35.0. The results were as follows:

Sample	Salinity (‰)	Concentration (µg N/L)	RSD (%)
1	35.5	6.3	7.19
2	20.0	72.1	1.57
3	4.8	517.6	0.64

13.1.3 Laboratory Fortified Sample Matrix - Laboratory fortified sample matrices were processed in three different salinities ranging from 4.8 to 35.0 and ambient ammonia concentrations from 0.0 to 72.1 µg N/L. Seven replicates of each sample were analyzed and the results were as follows:

Salinity	Concentration		RSD	Recovery
	ambient	fortified		
(‰)	(µg N/L)		(%)	(%)
35.5	6.3	70	5.01	98.3
20.0	72.1	140	1.71	98.3
4.8	0.0	280	1.81	98.1

13.1.4 Linear Dynamic Range - A linear dynamic range (LDR) of 4.0 mg N/L has been determined by one laboratory from spiked LNSW using a Flow Solution System (Alpkem, Wilsonville, Oregon).

13.1.5 Sample Preservation Study - Natural samples have been preserved by freezing, acidification and addition of chloroform and phenol as preservatives to the samples stored in glass and high density polyethylene bottles. Table 1 summarized the results of preservation study.

There is no significant difference in recovery of ammonia from samples stored in glass and high density polyethylene bottles, suggesting either glass or high density polyethylene bottles can be used for storage of ammonia samples.

For low concentration of ammonia samples (< 20 µg N/L, sample 1 in table 1), no preservation technique is satisfactory. Samples must be analyzed within 3 hours of collection.

Freezing cannot preserve ammonia in samples for more than one week. Acidified samples must be neutralized with NaOH solution prior to analysis. Addition of NaOH to acidified samples induces the precipitation of $Mg(OH)_2$ and $Ca(OH)_2$. Centrifuging the samples cannot completely eliminate the interference, therefore, acidification is not suitable preservation technique. Addition of phenol increases the absorbance of samples. Phenol is not recommended as a suitable preservative although samples preserved with phenol were stable as those preserved by chloroform.[12]

For moderate and high concentrations of ammonia (> 20 µg N/L) samples, it is suggested samples be preserved by the addition of 2 mL of chloroform per liter of sample and refrigerated in the dark at 4°C. A maximum holding time for preserved estuarine and coastal water samples with moderate to high concentrations of ammonia is two weeks.[10]

13.2 Multi-Laboratory Validation

Multi-laboratory data is unavailable at this time.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society, Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The U.S. Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions.

For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in Section 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

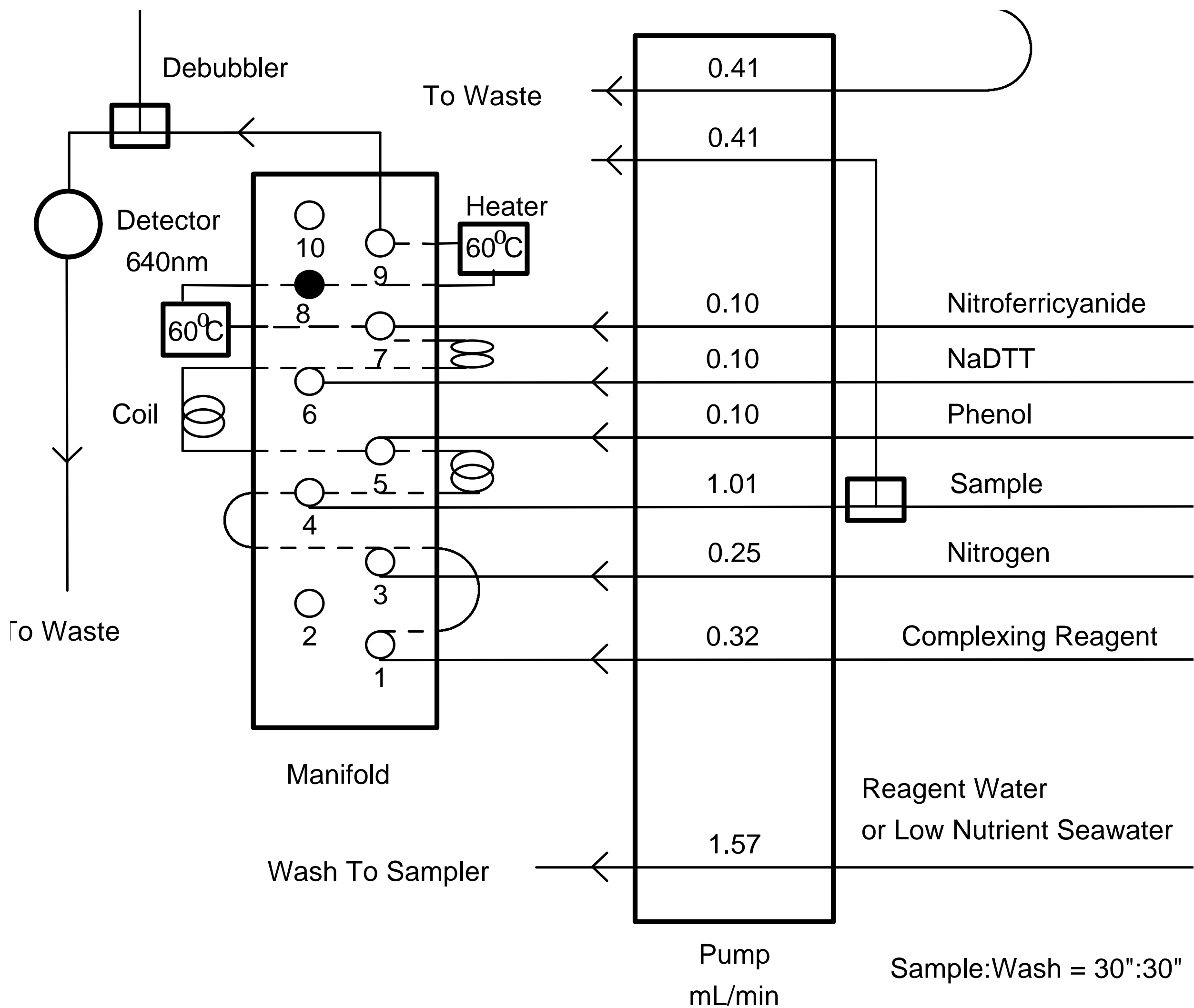

Figure 1. Manifold Configuration for Ammonia Analysis.

Table 1 . Percentage Recovery[A] of Ammonia From Natural Water Samples Preserved by Freezing, Acidification, Addition of Chloroform and Phenol.

sample[B]	method[C]	bottle[D]	time(day) 0	7	14	21	28
1	none	HDPE	100	349	345	18	91
	freezing	glass	100	100	0	0	0
		HDPE	100	102	0	0	0
	H_2SO_4 [E]	glass	200	564	285	73	55
		HDPE	200	113	64	45	36
	$CHCl_3$	glass	193	135	29	47	36
		HDPE	193	193	18	44	36
	phenol [F]	glass	153	36	44	0	0
		HDPE	153	36	0	0	0
1+	freezing	glass	100	101	82	77	102
		HDPE	100	97	76	61	81
	H_2SO_4 [E]	glass	95	105	69	54	37
		HDPE	95	91	91	88	116
	$CHCl_3$	glass	96	105	85	78	89
		HDPE	96	102	85	78	92
	phenol [F]	glass	130	133	110	148	123
		HDPE	130	128	102	103	118
2	none	HDPE	100	32	0	0	0
	freezing	glass	100	109	93	77	88
		HDPE	100	107	82	67	91
	H_2SO_4 [E]	glass	252	162	66	62	50
		HDPE	252	193	45	41	27
	$CHCl_3$	glass	99	114	83	75	96
		HDPE	99	98	80	70	83
	phenol [F]	glass	108	107	88	74	93
		HDPE	108	101	83	74	86
2+	freezing	glass	99	108	109	111	106
		HDPE	99	106	95	78	91
	H_2SO_4 [E]	glass	100	107	51	86	88
		HDPE	100	102	39	98	107
	$CHCl_3$	glass	99	106	116	94	105
		HDPE	99	107	98	95	103
	phenol [F]	glass	117	121	106	105	116
		HDPE	117	124	107	106	117
3	none	HDPE	100	104	14	1	0
	freezing	glass	100	-	116	64	106
		HDPE	100	108	105	65	75
	H_2SO_4 [E]	glass	101	106	44	74	61
		HDPE	101	108	111	106	109
	$CHCl_3$	glass	100	96	98	96	94
		HDPE	100	93	97	95	95
	phenol [F]	glass	112	106	107	112	125
		HDPE	112	112	108	110	112

[A] Recovery is calculated based on the ammonia concentration in non-preserved sample at day 0. Samples with recoveries higher than 100% are subject to interference either from precipitation or phenol.

[B] For salinity and concentration of ammonia in samples 1, 2, 3 see Section 13.1.2.

Sample 1+ and 2+ are the fortified samples 1 and 2 at ammonia concentrations 76.3 and 202.1 µg N/L, respectively.

C Methods of preservation:
None: stored the samples in high density polyethylene carboys at room temperature without any preservative added.
Freezing: Frozen and stored at -20°C.
H_2SO_4 : Acidified to pH 1.8 with H_2SO_4, and stored at 4°C. Neutralized the acid with NaOH solution before analysis.
$CHCl_3$: Added 2 mL chloroform per 1000 mL sample, and stored at 4°C.
Phenol: Added 8 g phenol per 1000 mL sample, and stored at 4°C.

C Glass and high density polyethylene bottles were compared to determine the effect of sample bottle type on the preservation.

E Adding NaOH to neutralize acidified samples induced the precipitation of $Mg(OH)_2$ and $Ca(OH)_2$. Centrifuging the samples can not completely eliminate the interference, therefore, acidification is not suitable preservation technique.

F Although samples preserved with phenol were stable as those preserved by chloroform, an absorbance increase was observed, therefore, this is not recommended as a suitable preservation technique.

Method 353.4

Determination of Nitrate and Nitrite in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

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Method 353.4

Determination of Nitrate and Nitrite in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

1.0 Scope and Application

1.1 This method provides a procedure for determining nitrate and nitrite concentrations in estuarine and coastal waters. Nitrate is reduced to nitrite by cadmium,[1-3] and the resulting nitrite determined by formation of an azo dye.[4-6]

In most estuarine and coastal waters nitrogen is thought to be a limiting nutrient. Nitrate is the final oxidation product of the nitrogen cycle in natural waters and is considered to be the only thermodynamically stable nitrogen compound in aerobic waters.[7] Nitrate in estuarine and coastal water is derived from rock weathering, sewage effluent and fertilizer run-off. The concentration of nitrate usually is high in estuarine waters and lower in surface coastal waters.

Nitrite is an intermediate product in the microbial reduction of nitrate or in the oxidation of ammonia. It may also be excreted by phytoplankton as a result of excess assimilatory reduction. Unlike nitrate, nitrite is usually present at a concentration lower than 0.01mg N/L except in high productivity waters and polluted waters in the vicinity of sewer outfalls.

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Nitrate	14797-55-8
Nitrite	14797-65-0

1.2 A statistically determined method detection limit (MDL)[8] of 0.075 µg N/L has been determined by one laboratory in seawaters of five different salinities. The method is linear to 5.0 mg N/L using a Flow Solution System (Alpkem, Wilsonville, Oregon).

1.3 Approximately 40 samples per hour can be analyzed.

1.4 This method requires experience in the use of automated gas segmented continuous flow colorimetric analyses, and familiarity with the techniques of preparation, activation and maintenance of the cadmium reduction column. A minimum of six-months training is recommended.

2.0 Summary of Method

2.1 An automated gas segmented continuous flow colorimetric method for the analysis of nitrate concentration is described. In the method, samples are passed through a copper-coated cadmium reduction column. Nitrate in the sample is reduced to nitrite in a buffer solution. The nitrite is then determined by diazotizing with sulfanilamide and coupling with N-1-naphthylethylenediamine dihydrochloride to form a color azo dye. The absorbance measured at 540 nm is linearly proportional to the concentration of nitrite + nitrate in the sample. Nitrate concentrations are obtained by subtracting nitrite values, which have been separately determined without the cadmium reduction procedure, from the nitrite + nitrate values. There is no significant salt error in this method. The small negative error caused by differences in the refractive index of seawater and reagent water is readily corrected for during data processing.

3.0 Definitions

3.1 Calibration Standard (CAL) - A solution prepared from the primary dilution standard solution or stock standard solution containing analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.2 Laboratory Fortified Blank (LFB) - An aliquot of reagent water to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether method performance is within acceptable control limits, and whether the laboratory is capable of making accurate and precise measurements. This is a standard prepared in reagent water that is analyzed as a sample.

3.3 Laboratory Fortified Sample Matrix (LFM) - An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.4 Laboratory Reagent Blank (LRB) - An aliquot of reagent water that is treated exactly as a sample including exposure to all labware, equipment, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or apparatus.

3.5 Linear Dynamic Range (LDR) - The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.6 Method Detection Limit (MDL) - The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.[8]

3.7 Reagent Water (RW) - Type 1 reagent grade water equal to or exceeding standards established by American Society for Testing and Materials (ASTM). Reverse osmosis systems or distilling units followed by Super-Q Plus Water System that produce water with 18 megohm resistance are examples of acceptable water sources. To avoid contamination, the reagent water should be used the day of preparation.

3.8 Refractive Index (RI) - The ratio of velocity of light in a vacuum to that in a given medium. The relative refractive index is the ratio of the velocity of light in two different media, such as estuarine or sea water versus reagent water. The correction for this difference is referred to as the refractive index correction in this method.

3.9 Stock Standard Solution (SSS) -A concentrated solution of method analyte prepared in the laboratory using assayed reference compounds or purchased from a reputable commercial source.

3.10 Primary Dilution Standard Solution (PDS) - A solution prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.11 Quality Control Sample (QCS) - A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.12 SYNC Peak Solution - A colored solution used to produce a synchronization peak in the refractive index measurement. A synchronization peak is required by the data acquisition programs to initialize the peak finding parameters. The first cup in every run must always be identified as a SYNC sample. The SYNC sample is usually a high standard, but can be any sample that generates a peak at least 25% of full scale.

4.0 Interferences

4.1 Hydrogen sulfide at concentrations greater than 0.1 mg S/L can interfere with nitrite analysis by precipitating on the cadmium column .[9] Hydrogen sulfide in samples must be removed by precipitation with cadmium or copper salt.

4.2 Iron, copper and other heavy metals at concentrations larger than 1 mg/L alter the reduction efficiency of the cadmium column. The addition of EDTA will complex these metal ions.[10]

4.3 Phosphate at a concentration larger than 0.1 mg/L decreases the reduction efficiency of cadmium[11]. Dilute samples if possible or remove phosphate with ferric hydroxide[12] prior to analysis.

4.4 Particulates inducing turbidity should be removed by filtration after sample collection.

4.5 This method corrects for small refractive index interference which occurs if the calibration standard solution is not matched with samples in salinity.

5.0 Safety

5.1 Water samples collected from the estuarine and coastal environment are generally not hazardous.

However, the individual who collects samples should use proper technique.

5.2 Good laboratory technique should be used when preparing reagents. Laboratory personnel should obtain material safety data sheets (MSDS) for all chemicals used in this method. A lab coat, safety goggles, and gloves should be worn when handling the concentrated acid.

6.0 Equipment and Supplies

6.1 Gas Segmented Continuous Flow Autoanalyzer Consisting of:

6.1.1 Autosampler.

6.1.2 Analytical cartridge with reaction coils for nitrate analysis.

6.1.3 Open Tubular Cadmium Reactor (OTCR, Alpkem, OR) or laboratory prepared packed copper-coated cadmium reduction column (prepared according to procedures in Section 7.4 - 7.5).

6.1.4 Proportioning pump.

6.1.5 Spectrophotometer equipped with a tungsten lamp (380-800 nm) or photometer with a 540 nm interference filter (2 nm bandwidth).

6.1.6 Strip chart recorder or computer based data acquisition system.

6.1.7 Nitrogen gas (high-purity grade, 99.99%).

6.2 Glassware and Supplies

6.2.1 All labware used in the analysis must be low in residual nitrate to avoid sample or reagent contamination. Soaking with lab grade detergent, rinsing with tap water, followed by rinsing with 10% HCl (v/v) and thoroughly rinsing with reagent water is sufficient.

6.2.2 Automatic pipetters capable of delivering volumes ranging from 100 µL to 1000 µL and 1mL to 10 mL with an assortment of high quality disposable pipet tips.

6.2.3 Analytical balance, with capability to measure to 0.1 mg, for preparing standards.

6.2.4 60 mL high density polyethylene sample bottles, glass volumetric flasks and plastic sample tubes.

6.2.5 Drying oven.

6.2.6 Desiccator.

6.2.7 Membrane filters with 0.45 µm nominal pore size. Plastic syringes with syringe filters.

6.2.8 A pH meter with a glass electrode and a reference electrode. A set of standard buffer solutions for calibration of the pH meter.

7.0 Reagents and Standards

7.1 Stock Reagent Solutions

7.1.1 Stock Sulfanilamide Solution - Dissolved 10 g of sulfanilamide ($C_6H_8N_2O_2S$, FW 172.21) in 1 L of 10% HCl.

7.1.2 Stock Nitrate Solution (100 mg-N/L) - Quantitatively transfer 0.7217 g of pre-dried (105°C for 1 hour) potassium nitrate (KNO_3, FW 101.099) to a 1000-mL glass volumetric flask containing approximate 800 mL of reagent water and dissolve the salt. Dilute the solution to the mark with reagent water. Store the stock solution in a polyethylene bottle in refrigerator at 4°C. This solution is stable for six months.

7.1.3 Stock Nitrite Solution (100 mg-N/L) - Quantitatively transfer 0.4928 g of pre-dried (105°C for 1 hour) sodium nitrite ($NaNO_2$, FW 68.99) to a 1000 mL glass volumetric flask containing approximate 800 mL of reagent water and dissolve the salt. Dilute the solution to the mark with reagent water. Store the stock solution in a polyethylene bottle in a refrigerator at 4°C. This solution is stable for three months.

Note: High purity nitrite salts are not available. Assays given by reagent manufacturers are usually in the range of 95-97%. The impurity must be taken into account for calculation of the weight taken.

7.1.4 Low Nutrient Sea Water (LNSW) - Obtain natural low nutrient seawater from surface water of the Gulf Stream or Sargasso Sea (salinity 36 ‰, < 7 µg N/L) and filter it through 0.3 micron pore size glass fiber filters. If this is not available, commercial low nutrient sea water (< 7 µg N/L) with salinity of 35 ‰ (Ocean Scientific International, Wormley, U.K.) can be substituted.

7.2 Working Reagents

7.2.1 Brij-35 Start-up Solution - Add 2 mL of Brij-35 surfactant (ICI Americas, Inc.) to 1000 mL reagent water and mix gently.

Note: Brij-35 is a trade name for polyoxyethylene(23) lauryl ether ($C_{12}H_{25}(OCH_2CH_2)_{23}OH$, FW=1199.57, CASRN 9002-92-0).

7.2.2 Working Sulfanilamide Solution - Add 1 mL of Brij- 35 solution to 200 mL of stock sulfanilamide solution, mix gently.

Note: Adding surfactant Brij-35 to sulfanilamide solution instead of to the buffer solution is to prevent the Brij from being adsorbed on the cadmium surface, which may result in decreasing surface reactivity of the cadmium and reduce the lifetime of the cadmium column.

7.2.3 NED Solution - Dissolve 1 g of NED (N-1-naphthylethylenediamine Dihydrochloride, $C_{12}H_{14}N_2.2HCl$, FW 259.18) in 1 L of reagent water.

7.2.4 Imidazole Buffer Solution - Dissolve 13.6 g of imidazole ($C_3H_4N_2$, FW 68.08) in 4 L of reagent water. Add 2 mL of concentrated HCl. Adjust the pH to 7.8 with diluted HCl while monitoring the pH with a pH meter. Store in a refrigerator.

7.2.5 Copper Sulfate Solution (2%) - Dissolve 20 g of copper sulfate ($CuSO_4.5H_2O$, FW 249.61) in 1 L of reagent water.

7.2.6 Colored SYNC Peak Solution - Add 50 µL of red food coloring solution to 1000 mL reagent water and mix thoroughly. Further dilute this solution to obtain a peak between 25 to 100 percent full scale according to the AUFS setting used for the refractive index measurement.

7.2.7 Primary Dilution Standard Solution - Prepare a primary dilution standard solution (5 mg N/L) by dilution of 5.0 mL of stock standard solutions to 100 mL with reagent water. Prepare this solution daily.

Note: This solution should be prepared to give an appropriate intermediate concentration for further dilution to prepare the calibration solutions. Therefore the concentration of a primary dilution standard solution should be adjusted according to the concentration range of calibration solutions.

7.2.8 Calibration Standards - Prepare a series of calibration standards (CAL) by diluting suitable volumes of a primary dilution standard solution (Section 7.2.7) to 100 mL with reagent water or low nutrient seawater. Prepare these standards daily. The concentration range of calibration standards should bracket the expected concentrations of samples and not exceed two orders of magnitude. At least five calibration standards with equal increments in concentration should be used to construct the calibration curve.

If nitrate + nitrite and nitrite are analyzed simultaneously by splitting a sample into two analytical systems, a nitrate and nitrite mixed standard should be prepared. The total concentration (nitrate+nitrite) must be assigned to the concentrations of calibration standards in the nitrate+nitrite system.

When analyzing samples of varying salinities, it is recommended that the calibration standard solutions and sampler wash solution be prepared in reagent water and corrections for refractive index be made to the sample concentrations determined (Section 12.2).

7.2.9 Saline Nitrate and Nitrite Standards - If CAL solutions will not be prepared to match sample salinity, then saline nitrate and nitrite standards must be prepared in a series of salinities in order to quantify the salt error, the change in the colorimetric response of nitrate due to the change in the composition of the solution. The following dilutions of Primary Dilution Standard Solution (Section 7.2.7) to 100 mL in volumetric flasks with reagent water, are suggested:

Salinity (‰)	Volume of LNSW(mL)	Volume of PDS(mL)	Conc. mg N/L
0	0	2	.10
9	25	2	.10
18	50	2	.10
27	75	2	.10
35	98	2	.10

7.3 Open Tubular Cadmium Reactor

7.3.1 Nitrate in the samples is reduced to nitrite by either a commercial Open Tubular Cadmium Reactor (OTCR, Alpkem, OR) or a laboratory-prepared packed copper-coated cadmium reduction column.

7.3.2 If an OTCR is employed, the following procedures should be used to activate it.[10]

Prepare reagent water, 0.5N HCl solution and 2% $CuSO_4$ solution in three 50 mL beakers. Fit three 10-mL plastic syringes with unions. First flush the OTCR with 10 mL reagent water. Then flush it with 10 mL 0.5N HCl solution in 3 seconds, immediately followed by flushing with a couple of syringe volumes of reagent water. Slowly flush with $CuSO_4$ solution until a large amount of black precipitated copper come out of OTCR, then stop the flushing. Finally flush the OTCR with reagent water. Fill the OTCR with imidazole buffer for short term storage.

7.4 Packed Cadmium Reduction Column

The following procedures are used for preparation of a packed cadmium reduction column.[13]

7.4.1 File a cadmium stick to obtain freshly prepared cadmium filings.

7.4.2 Sieve the filings and retain the fraction between 25 and 60 mesh size (0.25-0.71 mm).

7.4.3 Wash filings two times with 10% HCl followed with reagent water.

7.4.4 Decant the reagent water and add 50 mL of 2% $CuSO_4$ solution. While swirling, brown flakes of colloidal copper will appear and the blue color of the solution will fade. Decant the faded solution and add fresh $CuSO_4$ solution and swirl. Repeat this procedure until the blue color does not fade.

7.4.5 Wash the filings with reagent water until all the blue color is gone and the supernatant is free of fine particles. Keep the filings submersed under reagent water and avoid exposure of the cadmium filings to air.

7.4.6 The column can be prepared in a plastic or aglass tube of 2 mm ID. Plug one end of column with glass wool. Fill the column with water and transfer Cd filings in suspension using a 10 mL pipette tip connected to one end of column. While gently tapping the tube and pipette tip let Cd filings pack tightly and uniformly in the column without trapping air bubbles.

7.4.7 Insert another glass wool plug at the top of the column. If a U- shape tube is used, the pipette tip is connected to the other end and the procedure repeated. Connect both ends of the column using a plastic tube filled with buffer solution to form a closed loop.

7.4.8 If an OTCR or a packed cadmium column has not been used for several days, it should be reactivated prior to sample analysis.

7.5 Stabilization of OTCR and Packed Cadmium Reduction Columns

7.5.1 Pump the buffer and other reagent solutions through the manifold and obtain a stable baseline.

7.5.2 Pump 0.7 mg-N/L nitrite standard solution continuously through the sample line and record the steady state signal.

7.5.3 Stop the pump and install an OTCR or a packed column on the manifold. Ensure no air bubbles have been introduced into the manifold during the installation. Resume the pumping and confirm a stable baseline.

7.5.4 Pump 0.7 mg-N/L nitrate solution continuously through the sample line and record the signal. The signal will increase slowly and reach steady state in about 10-15 minutes. This steady state signal should be close to the signal obtained from the same concentration of a nitrite solution without the OTCR or packed cadmium column on line.

7.5.5 The reduction efficiency of an OTCR or a packed cadmium column can be determined by measuring the absorbance of a nitrate standard solution followed by a nitrite standard solution of the same concentration. Reduction efficiency is calculated as follows:

$$\text{Reduction Efficiency} = \frac{\text{Absorbance of Nitrate}}{\text{Absorbance of Nitrite}}$$

8.0 Sample Collection, Preservation and Storage

8.1 Sample Collection - Samples collected for nutrient analyses from estuarine and coastal waters are normally collected using one of two methods: hydrocast or submersible pump systems.

8.1.1 A hydrocast uses a series of sampling bottles (Niskin, Go-Flo or equivalent) that are attached at fixed intervals to a hydro wire. These bottles are sent through the water column open and are closed either

electronically or via a mechanical messenger when the bottles have reached the desired depth.

8.1.2 In a submersible pump system, a weighted hose is sent to the desired depth in the water column and water is pumped from that depth to the deck of the ship for sample processing.

8.1.3 For collecting surface samples, an acid - cleaned plastic bucket or a large plastic bottle can be used as a convenient sampler. Wash the sampler three times with sample water before collecting samples.

8.1.4 Turbid samples should be filtered as soon as possible after collection.

8.1.5 60-mL high density polyethylene bottles are used for sample storage. Sample bottles should be rinsed 3 times with about 20 mL of sample, shaking with the cap in place after each rinse. Pour the rinse water into the cap to dissolve and rinse away salt crusts trapped in the threads of the cap. Finally, fill the sample bottle about 3/4 full, and screw the cap on firmly.

8.2 Sample Preservation - After collection and filtration, samples should be analyzed as soon as possible. If samples will be analyzed within 3 hours then keep refrigerated in tightly sealed, high density polyethylene bottles in the dark at 4°C until analysis can be performed.

8.3 Sample Storage - Natural samples usually contain low concentrations of nitrite (< 14 g N/L) and no preservation techniques are satisfactory.[14] Samples must be analyzed within 3 hours of collection to obtain reliable nitrite concentrations.[15]

Samples containing high concentrations of ammonia or nitrite may change in nitrate concentration during storage due to microbial oxidation of ammonia and nitrite to nitrate. These samples should be analyzed as soon as possible.

Natural samples containing low concentrations of nitrite and ammonia (< 10% of the nitrate concentration) can be preserved for nitrate analysis by freezing. A maximum holding time for preserved estuarine and coastal water samples for nitrate analysis is one month.[16]

The results of preservation of natural samples are shown in Tables 1 and 2 for nitrate and nitrite, respectively.

9.0 Quality Control

9.1 Each laboratory using this method is required to implement a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of performance, continued analysis of Laboratory Reagent Blanks (LRB), laboratory duplicates and Laboratory Fortified Blanks (LFB) with each set of samples as a continuing check on performance.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance by determining the MDL and LDR and laboratory performance by analyzing quality control samples prior to analysis of samples using this method.

9.2.2 A method detection limit (MDL) should be established for the method analytes using a low level seawater sample containing, or fortified at, approximately 5 times the estimated detection limit. To determine MDL values, analyze at least seven replicate aliquots of water which have been processed through the entire analytical method. Perform all calculations defined in the method and report concentration in appropriate units. Calculate the MDL as follows:

$$MDL = (t)(S)$$

where, S = the standard deviation of the replicate analyses

t = Student's t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

MDLs should be determined every six months or whenever a significant change in background or instrument response occurs or a new matrix is encountered.

9.2.3 The LDR should be determined by analyzing a minimum of eight calibration standards ranging from 0.002 to 2.00 mg N/L across all sensitivity settings (Absorbance Units Full Scale output range setting) of the detector. Standards and sampler wash solutions should be prepared in low nutrient seawater with salinities similar to that of samples, therefore a correction factor for salt error, or refractive index, will not be necessary. Normalize

responses by multiplying the response by the Absorbance Units Full Scale output range setting. Perform the linear regression of normalized response vs. concentration and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response, R, of a standard no longer yields a calculated concentration C_C, that is within 100 ± 10% of known concentration, C, where $C_C = (R-b)/m$. That concentration defines the upper limit of the LDR for the instrument. Should samples be encountered that have a concentration that is ≥ 90% of the upper limit of LDR, then these samples must be diluted and reanalyzed.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) - A laboratory should analyze at least one LRB with each set of samples. LRB data are used to assess contamination from the laboratory environment. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When the LRB value constitutes 10% or more of the analyte concentration determined for a sample, duplicates of the sample must be prepared and analyzed again after the source of contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 Laboratory Fortified Blank (LFB) - A laboratory should analyze at least one LFB with each set of samples. The LFB must be at a concentration that is within the daily calibration range. The LFB data are used to calculate accuracy as percent recovery. If the recovery of the analyte falls outside the required control limits of 90 -110%, the source of the problem should be identified and resolved before continuing the analyses.

9.3.3 The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 90-110%. When sufficient internal performance data become available (usually a minimum of 20 to 30 analyses), optional control limits can be developed from the percent mean recovery (x) and standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

Upper Control Limit = x + 3S
Lower Control Limit = x - 3S

The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5 to 10 new recovery measurements, new control limits can be calculated using only the most recent 20 to 30 data points. Also the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.4 Assessing Analyte Recovery - Laboratory Fortified Sample Matrix (LFM)

9.4.1 A laboratory should add a known amount of analyte to a minimum of 5% of the total number of samples or one sample per sample set, whichever is greater. The analyte added should be 2-4 times the ambient concentration and should be at least four times greater than the MDL.

9.4.2 Calculate percent recovery of analyte, corrected for background concentration measured in a separate unfortified sample. These values should be compared with the values obtained from the LFBs. Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_S - C)}{S} \times 100$$

where,

R = percent recovery

C_S = measured fortified sample concentration (background + addition in mg N/L)

C = sample background concentration (mg N/L)

S = concentration in mg N/L added to the environmental sample.

9.4.3 If the recovery of the analyte falls outside the required control limits of 90-110%, but the laboratory performance for that analyte is within the control limits, the fortified sample should be prepared again and analyzed. If the result is the same after reanalysis, the recovery problem encountered with the fortified sample is judged to be the matrix related and the sample data should be flagged.

10.0 Calibration and Standardization

10.1 At least five calibration standards should be prepared fresh daily for system calibration. The calibration concentrations should bracket the concentrations of samples and the range should not be over two orders of magnitude.

10.2 A calibration curve should be constructed for each sample set by analyzing a series of calibration standard solutions. A sample set should contain no more than 60 samples. For a large number of samples make several sample sets with individual calibration curves.

10.3 Analyze the calibration standards, in duplicate, before actual samples.

10.4 The calibration curve containing five or more data points should have a correlation coefficient, r, of 0.995 or better.

10.5 Place a high CAL solution followed by two blank cups to quantify the carry-over of the system. The difference in peak heights between two blank cups is due to the carry over from the high CAL solution. The carry-over coefficient, k, is calculated as follows:

$$k = \frac{P_{b1} - P_{b2}}{P_{high}}$$

where,

P_{high} = the peak height of the high nitrate standard

P_{b1} = the peak height of the first blank sample

P_{b2} = the peak height of the second blank sample.

The carry over coefficient, k, for a system should be measured in seven replicates to obtain a statistically significant number. k should be remeasured with any change in manifold plumbing or upon replacement of pump tubung.

The carry over correction (CO) on a given peak i is proportional to the peak height of the preceding sample, P_{i-1}.

$$CO = (k)(P_{i-1})$$

To correct a given peak height reading, P_i, subtract the carry over correction,[17,18]

$$P_{i,c} = P_i - CO$$

where $P_{i,c}$ is corrected peak height. The correction for carry over should be applied to all the peak heights throughout a run. The carry over coefficient should be less than 5% in this method.

10.6 Place a high standard nitrate solution followed by a nitrite standard solution of same concentration at the beginning and end of each sample run to check for change in reduction efficiency of OTCR or a packed cadmium column. The decline of reduction efficiency during a run should be less than 5%.

10.7 Place a high standard solution at the end of each sample run (60 samples) to check for sensitivity drift. Apply sensitivity drift correction to all the samples. The sensitivity drift during a run should be less than 5%.

Note: Sensitivity drift correction is available in most data acquisition software supplied with autoanalyzers. It is assumed that the sensitivity drift is linear with time. An interpolated drift correction factor is calculated for each sample according to the sample position during a run. Multiply the sample peak height by the corresponding sensitivity drift correction factor to obtain the corrected peak height for each sample.

11.0 Procedure

11.1 If samples are frozen, thaw the samples at room temperature. If samples are stored in a refrigerator, remove samples and equilibrate to room temperature. Mix samples thoroughly prior to analysis.

11.2 Turn on the continuous flow analyzer and data acquisition components and warm up at least 30 minutes.

11.3 Set up the cartridge according to the type of cadmium reductor used for nitrate + nitrite analysis (configuration for OTCR shown in Figure 1 and packed cadmium column in Figure 2). Configuration for analysis of nitrite alone is shown in Figure 3.

Note: When a gas segmented flow stream passes through the OTCR, particles derived from the OTCR were found to increase baseline noise and to cause

interference at low level analysis. Packed cadmium columns are, therefore, preferred for nitrate analysis at low concentrations.

11.4 Set spectrophotometer wavelength at 540 nm.

11.5 Set the Absorbance Unit Full Scale (AUFS) range on the spectrophotometer at an appropriate setting according to the highest concentration of nitrate in the samples. The appropriate setting for this method is 0.2 AUFS for 0.7 mg N/L.

11.6 Prepare all reagents and standards.

11.7 Begin pumping the Brij-35 start-up solution (Section 7.2.1) through the system and obtain a steady baseline. Place the reagents on-line. The reagent baseline will be higher than the start-up solution baseline. After the reagent baseline has been stabilized, reset the baseline.

NOTE: To minimize the noise in the reagent baseline, clean the flow system by sequentially pumping the sample line with reagent water, 1 N HCl solution, reagent water, 1 N NaOH solution for a few minutes each at the end of the daily analysis. Make sure to rinse the system well with reagent water after pumping NaOH solution to prevent precipitation of $Mg(OH)_2$ when seawater is introduced into the system. Keep the reagents and samples free of particulate. Filter the reagents and samples if necessary.

If the baseline drifts upward, pinch the waste line for a few seconds to increase back pressure. If absorbance drops down rapidly when back pressure increases, this indicates that there are air bubbles trapped in the flow cell. Attach a syringe at the waste outlet of the flowcell. Air bubbles in the flowcell can often be eliminated by simply attaching syringe for a few minutes or, if not, dislodged by pumping the syringe piston. Alternatively, flushing the flowcell with alcohol was found to be effective in removing air bubbles from the flow cell.

For samples of varying salinities, it is suggested that the reagent water used for the sampler wash solution and for preparing calibration standards and procedures in Sections 12.2 and 12.3 be employed.

11.8 Check the reduction efficiency of the OTCR or packed cadmium column following the procedure in Section 7.5.5. If the reduction efficiency is less than 90% follow the procedure in Section 7.5 for activation and stabilization. Ensure reduction efficiencies reach at least 90% before analysis of samples.[19]

11.9 A good sampling rate is approximately 40 samples per hour for 60 second sample times and 30 second wash times.

11.10 Use cleaned sample cups or tubes (follow the procedures outlined in Section 6.2.2). Place CAL solutions and saline standards (optional) in sampler. Complete filling the sampler tray with samples, laboratory reagent blanks, laboratory fortified blanks, laboratory fortified sample matrices, and QC samples. Place a blank after every ten samples.

11.11 Commence analysis.

12.0 Data Analysis and Calculations

12.1 Concentrations of nitrate in samples are calculated from the linear regression, obtained from the standard curve in which the concentrations of the calibration standards are entered as the independent variable, and their corresponding peak heights are the dependent variable.

12.2 Refractive Index Correction for Estuarine and Coastal Samples

12.2.1 If reagent water is used as the wash solution and to prepare the calibration standard solutions, the operator has to quantify the refractive index correction due to the difference in salinity between sample and standard solutions. The following procedures are used to measure the relationship between sample salinity and refractive index for ***a particular detector***.

12.2.2 First, analyze a set of nitrate or nitrite standards in reagent water with color reagent using reagent water as the wash and obtain a linear regression of peak height versus concentration.

Note: The change in absorbance due to refractive index is small, therefore low concentration standards should be used to bracket the expected absorbances due to refractive index.

12.2.3 Second, replace reagent water wash solution with Low Nutrient Seawater wash solution.

Note: In nitrate and nitrite analysis absorbance of the reagent water is higher than that of the LNSW. When using reagent water as a wash solution, the change in refractive index causes the absorbance of seawater to become negative. To measure the absorbance due to refractive index change in different salinity samples, Low Nutrient Seawater must be used as a wash solution to bring the baseline down.

12.2.4 Replace NED solution (Section 7.2.4) with reagent water. All other reagents remain the same. Replace the synchronization sample with the colored SYNC peak solution (Section 7.2.6).

12.2.5 Prepare a set of different salinity samples with LNSW. Commence analysis and obtain peak heights for different salinity samples. The peak heights for the refractive index correction must be obtained at the same AUFS range setting and on the same spectrophotometer as the corresponding standards (Section 12.2.2).

12.2.6 Using Low Nutrient Seawater as the wash water, a maximum absorbance will be observed for reagent water. No change in refractive index will be observed in the seawater sample. Assuming the absolute absorbance for reagent water (relative to the seawater baseline) is equal to the absorbance for seawater (relative to reagent water baseline), subtract the absorbances of samples of various salinities from that of reagent water. The results are the apparent absorbance due to the change in refractive index between samples of various salinities relative to the reagent water baseline.

12.2.7 For each sample of varying salinity, calculate the apparent nitrate or nitrite concentrations due to refractive index from its peak height corrected to reagent water baseline (Section 12.2.5) and the regression equation of nitrate or nitrite standards obtained with color reagent being pumped through the system (12.2.2). Salinity is entered as the independent variable and the apparent nitrate or nitrite concentration due to refractive index is entered as the dependent variable. The resulting regression allows the operator to calculate apparent nitrate or nitrite concentration due to refractive index when sample salinity is known. Thus, the operator would not be required to obtain refractive index peak heights for all samples.

12.2.8 An example of typical results follows:

Salinity ($‰$)	Apparent concentration (µg N/L) Nitrate	Nitrite
0.0	0.000	0.000
3.8	0.026	0.015
9.2	0.096	0.040
13.8	0.142	0.055
18.1	0.190	0.086
26.8	0.297	0.153
36.3	0.370	0.187

Note: You must calculate the refractive index correction for your particular detector. Moreover, the refractive index must be redetermined whenever a significant change in the design of flowcell or a new matrix is encountered.

12.2.9 An example of typical linear equations is:

Apparent nitrate (µg N/L) = 0.01047S

Apparent nitrite (µg N/L) = 0.00513S

where S is sample salinity. The apparent nitrate and nitrite concentration due to refractive index so obtained should be added to samples of corresponding salinity when reagent water is used as wash solution and standard matrix.

If nitrate and nitrite concentrations are greater than 100 and 50 µg N/L respectively, the correction for refractive index is negligible and this procedure can be optional.

12.3 Correction for Salt Error in Estuarine and Coastal Samples

12.3.1 When calculating concentrations of samples of varying salinities from standards and the wash solution prepared in reagent water, it is common to first correct for refractive index errors, and then correct for any change in color development due to the differences in composition between samples and standards (so called salt error).

12.3.2 Plot the salinity of the saline standards (Section 7.2.9) as the independent variable, and the apparent concentration of analyte (mg N/L) from the peak height (corrected for refractive index) calculated from the regression of standards in reagent water, as the dependent variable for all saline standards. The resulting regression equation allows the operator to correct the

concentrations of samples of known salinity for the color enhancement due to matrix effect, e.g., salt error. Following are typical results for the nitrate and nitrite systems:

Salinity (‰)	Apparent concentration (µg N/L) Nitrate	Nitrite
0.0	569.64	558.15
3.8	570.50	565.50
9.2	572.74	563.00
13.8	568.96	564.94
18.1	566.44	563.00
26.8	558.74	559.06
36.3	559.86	554.67

12.3.3 As shown in above results, salinity has no systematic effect on the nitrate and nitrite signal and therefore salt error correction is not recommended.

12.4 Results of sample analyses should be reported in mg N/L or in µg N/L.

mg N/L = ppm (parts per million)
µg N/L = ppb (part per billion)

13.0 Method Performance

13.1 Single Laboratory Validation

13.1.1 Method Detection Limit- A method detection limit (MDL) of 0.075 µg N/L has been determined by one laboratory from LNSW of five different salinities fortified at a nitrate concentration of 0.28 µg N/L.

Salinity (‰)	SD (µg N/L)	Recovery (%)	MDL (µg N/L)
36.5	0.0234	103.5	0.0734
36.5	0.0298	98.9	0.0935
36.5	0.0148	110.3	0.0464
36.5	0.0261	103.6	0.0819
27.5	0.0203	105.4	0.0638
27.5	0.0321	102.3	0.1009
27.5	0.0314	103.8	0.0986
27.5	0.0335	100.1	0.1052
18.6	0.0167	105.8	0.0523
18.6	0.0170	101.6	0.0534
18.6	0.0229	106.4	0.0720
18.6	0.0229	104.5	0.0719
9.4	0.0222	105.3	0.0698
9.4	0.0229	106.4	0.0720
9.4	0.0197	91.5	0.0620
0.0	0.0260	103.9	0.0817
0.0	0.0306	106.9	0.0961
0.0	0.0160	111.0	0.0501
0.0	0.0248	109.5	0.0780

13.1.2 Single analyst precision - A single laboratory analyzed three samples collected from the Miami River and Biscayne Bay, Florida. Seven replicates of each sample were processed and analyzed with salinity ranging from 0.019 to 32.623‰. The results were as follows:

Sample	Salinity (‰)	Concentration (µg N/L)	RSD (%)
		Nitrate	
1	32.623	48.22	2.59
2	13.263	206.41	1.07
3	0.019	276.38	1.99
		Nitrite	
1	32.623	5.21	1.62
2	13.263	31.03	0.58
3	0.019	54.07	0.49

13.1.3 Laboratory fortified sample matrix - Laboratory fortified sample matrices were processed in three different salinities ranging from 0.019 to 32.623 and ambient nitrate concentrations from 48.22 to 276.38 µg N/L. Seven replicates of each sample were analyzed and the results were as follows:

Salinity (‰)	Concentration ambient (µg N/L)	Concentration fortified (µg N/L)	RSD (%)	Recovery (%)
32.623	48.22	139.94	1.50	106.4
13.263	206.41	139.94	1.25	102.6
0.019	276.38	139.94	1.19	102.3

13.2 Multi-Laboratory Validation

Multi-laboratory data is unavailable at this time.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society, Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The U.S. Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in Section 14.2.

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

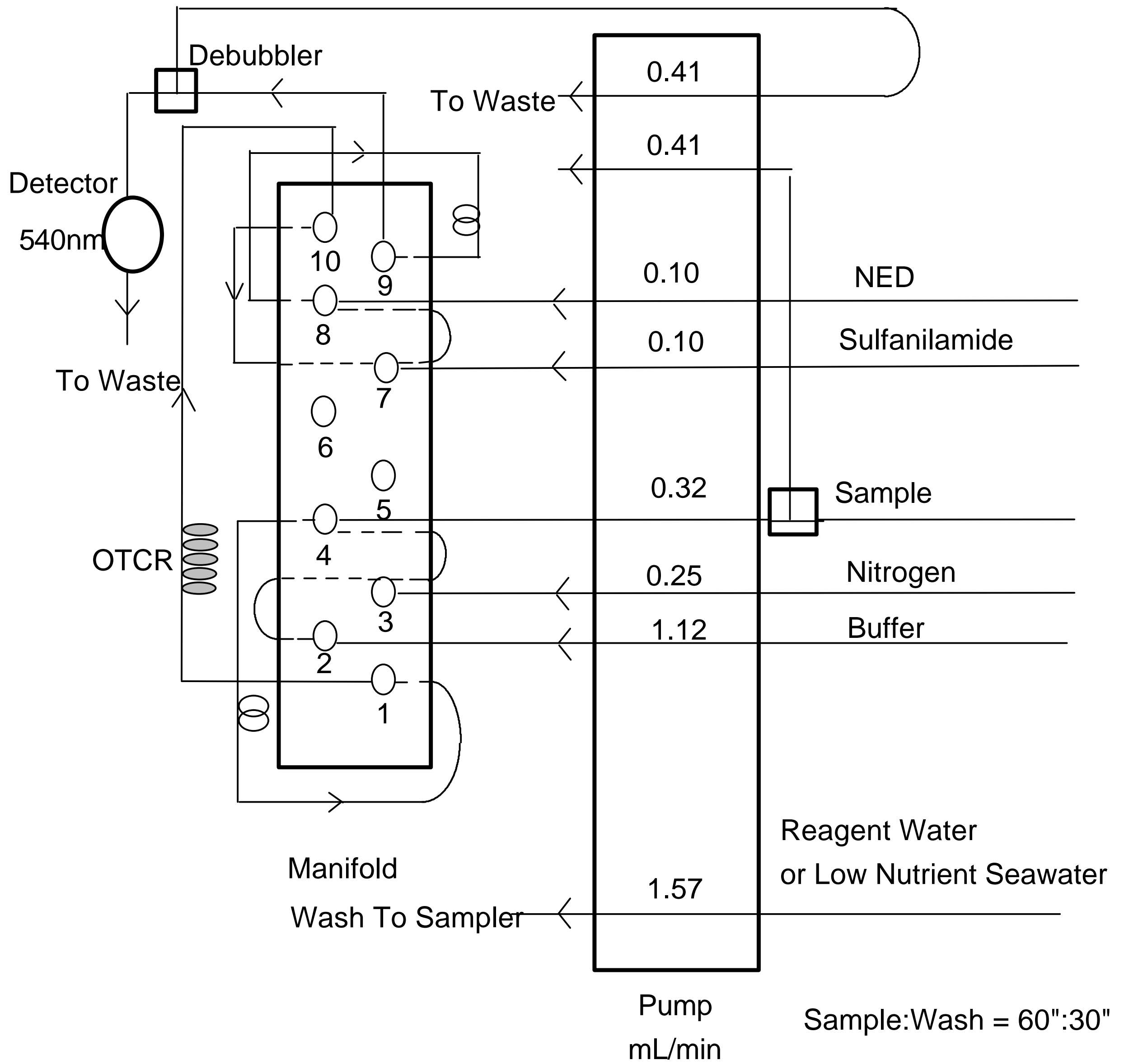

Figure 1. Manifold configuration for nitrate + nitrite analysis using an Open Tubular Cadmium Reactor.

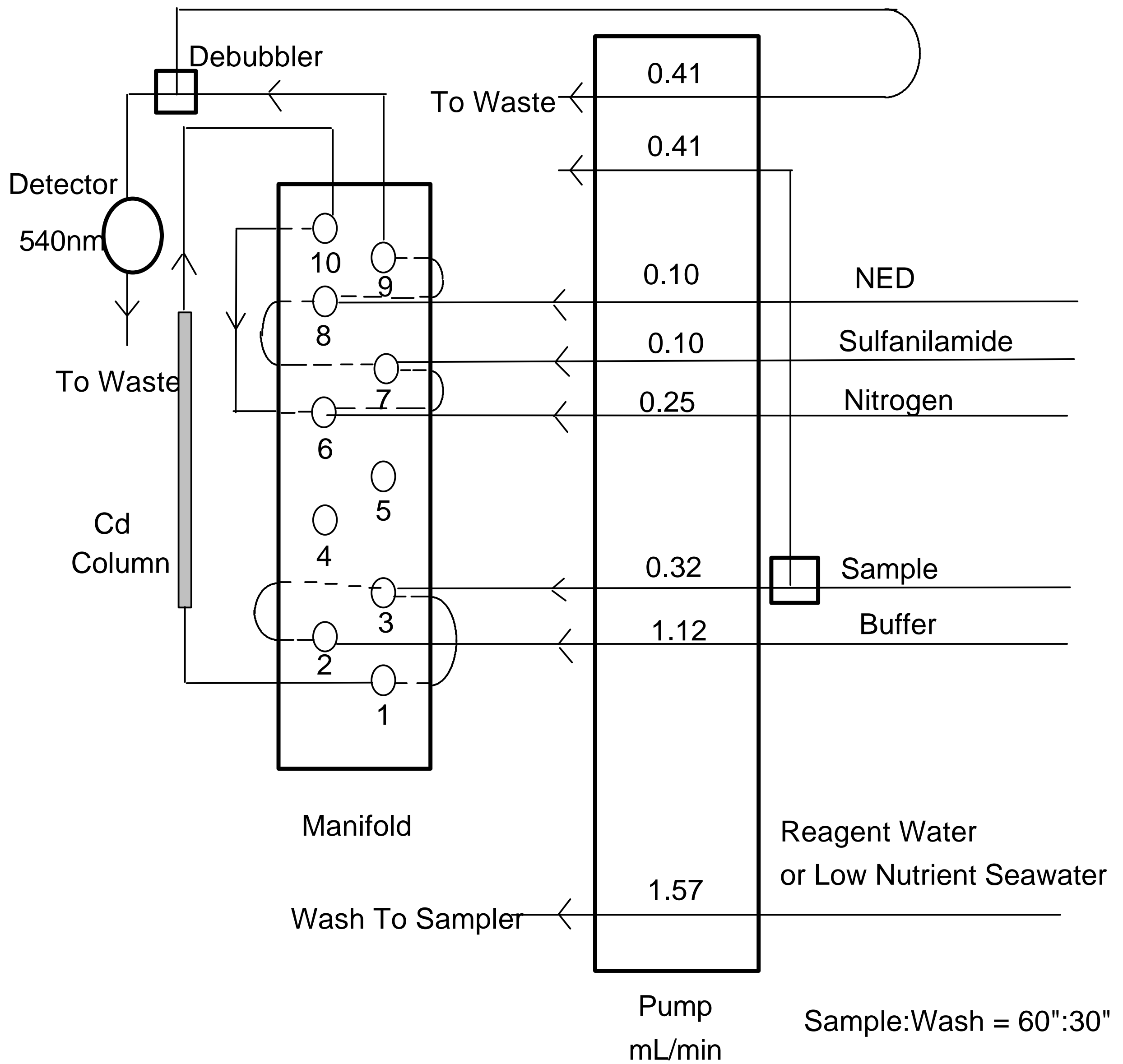

Figure 2. Manifold configuration for nitrate + nitrite analysis using a homemade packed copper-coated cadmium reduction column.

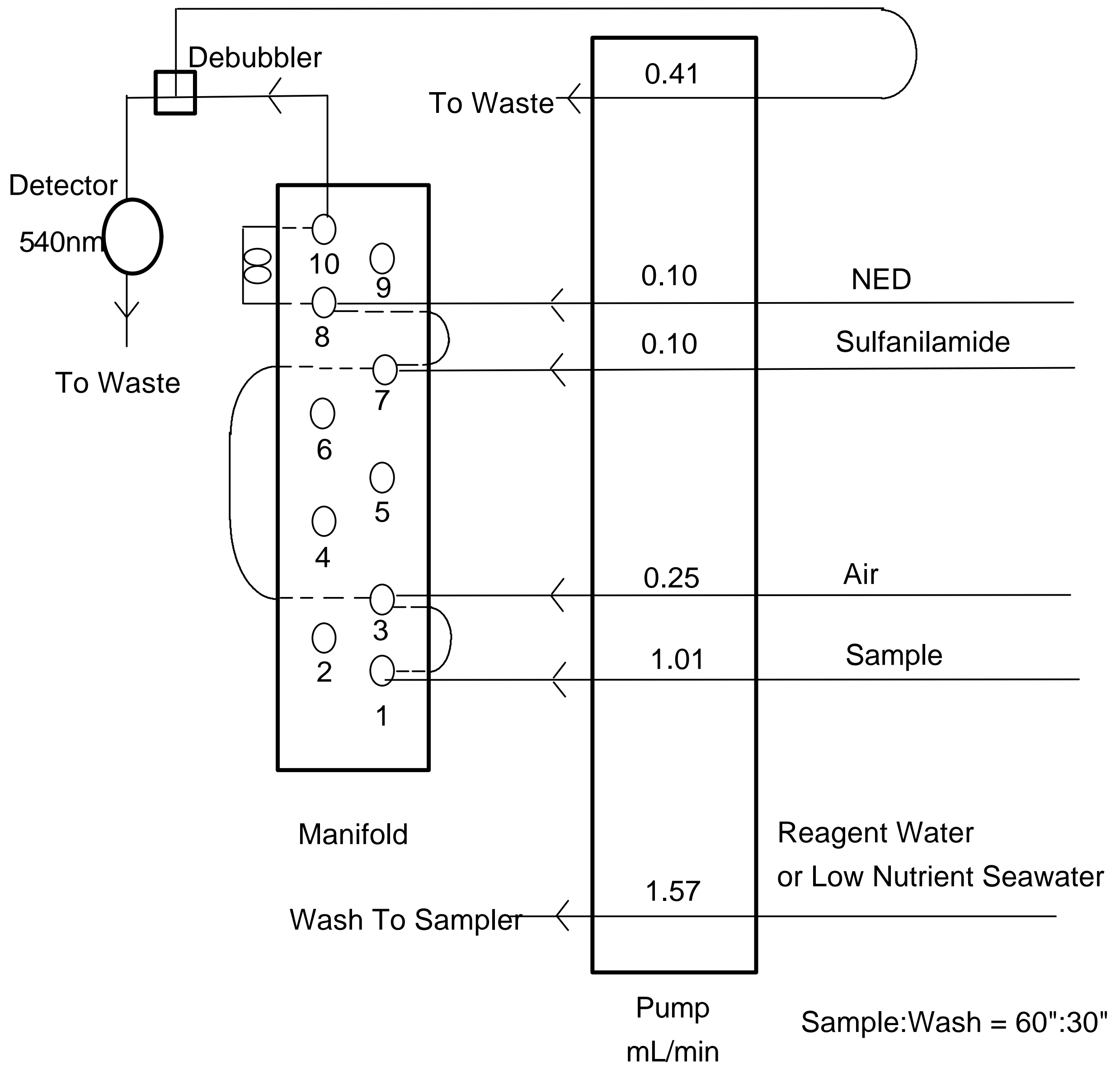

Figure 3. Manifold configuration for nitrite analysis.

Table 1 . Percentage recovery of nitrate from natural water samples preserved by freezing and refrigeration.

Method[A]	Sample[B]	Salinity	Time (Day)								
			0	7	14	21	28	35	46	62	92
25C, P	river	0.019	100	192.5	279	287.3	267.5	262.4	300.7	228.1	260.8
	estuary	13.263	100	108.5	106.2	124	103.9	139.3	258.9	188.5	229.1
	coast	32.623	100	102	128.8	153.8	93.3	89	44.2	72.4	84.9
25C, G	river	0.019	100	257	294.9	316.4	298.2	225.4	135.4	77.6	66.9
	estuary	13.263	100	108.8	108.5	122.5	90.6	79.2	81.5	56.2	128.2
	coast	32.623	100	98	135.2	150.9	98.5	84.3	36.9	56.1	66.6
4C,P	river	0.019	100	105	90	111.6	100.7	82.7	112.2	97.3	104.7
	estuary	13.263	100	104.5	90.4	107.1	102.6	95.9	109	82.4	101.4
	coast	32.623	100	127.6	65.7	149.1	82.3	93.3	43.3	73.5	89.2
4C,G	river	0.019	100	158.2	88.1	108.4	99.4				
	estuary	13.263	100	103.1	84.5	107.4	95.9				
	coast	32.623	100	100.9	54.4	123	68.9				
4C,P,	river+	0.019	100	105.5	99.2	106.1	96.2	91	114.8	98.4	96.9
	estuary+	13.263	100	110.2	116.4	104.8	102.9	93	110.9	85	99.7
	coast+	32.623	100	112.7	112.7	103.8	93.3	90.6	102.4	75.4	98.6
4C,G,	river+	0.019	100			105.7	98.3	101	114.5		
	estuary+	13.263	100			100.1	98	93.3	109.1		
	coast+	32.623	100			104.4	93.6	90.2	99.5		
Fr,P	river	0.019	100	100.5	100.4	103.9	95.8	88.6		85.7	95.9
	estuary	13.263	100	114.1	115.5	105.6	97.9	104.6	98.8	72.8	87.6
	coast	32.623	100	130.5	100.9	128.2	92.7	98.5	42.2	50.9	87.5
Fr,P,	river+	0.019	100	101.9	103.2	103.1	95.4	91.2	82.5	87.4	90.2
	estuary+	13.263	100	102	106.7	102.4	97.4	95	78.5	78	94.7
	coast+	32.623	100	103.2	111.1	101.3	91.5	92.1	104.7	69.6	92.3

Table 2 . Percentage recovery of nitrite from natural water samples preserved by freezing and refrigeration

Method[A]	Sample[B]	Salinity	Time(day)								
			0	7	14	21	28	35	46	62	92
25C, P	river	0.019	100	220	0.3	0	0	0	0	0	0
	estuary	13.263	100	110.6	456.8	920.2	957.8	661.5	58.7	0	0
	coast	32.623	100	104.1	92.2	74.1	89.5	74.1	94.6	72.2	0
25C, G	river	0.019	100	182.8	0.3	0	0	0	0	0	0
	estuary	13.263	100	108.5	519.1	1026.3	1079.1	867.5	843.1	705.7	209.2
	coast	32.623	100	100	87.8	73.8	89.5	73.5	95.9	85.7	66.5
4C,P	river	0.019	100	104.2	88.2	31.8	93.9	0	65	84.1	0
	estuary	13.263	100	102.8	101.8	38.9	0	91	17.8	8.5	0
	coast	32.623	100	68.4	65.7	33.2	70.5	50.5	0	0	0
4C,G	river	0.019	100	104.9	97.8	99.8	96.7				
	estuary	13.263	100	104.4	98.8	100.6	91				
	coast	32.623	100	94.3	87	71.1	97.6				
4C,P	river+	0.019	100	47.6	98.9	98.5	97.2	67.8	0	2.2	75.0
	estuary+	13.263	100	95.4	21.1	0	0	0	2.7	0	0
	coast+	32.263	100	0	0	0	0	0	0	0	0
4C,G	river+	0.019	100			97.9	95.8	84.6	85.9		
	estuary+	13.263	100			100.6	91.6	94.1	100		
	coast+	32.623	100			69.5	97.6	65.9	87.6		
Fr,P	river	0.019	100	70.6	86.2	98	77.3	68.1		74.9	77.3
	estuary	13.263	100	1.3	0.7	0	0	0	96	13.3	57.3
	coast	32.623	100	78.6	4.9	0	0	0	8.6	80	27.8
Fr,P	river+	0.019	100	97	87.2	95.4	75.9	75.9	63.1	75.2	69.2
	estuary+	13.263	100	103.5	98.6	95.9	52	90.5	74.2	0	77.6
	coast+	32.623	100	99.7	95.9	56.5	92.2	67	100.5	80	65.9

Cont'd on next page

Cont'd

[A] Methods of preservation:

25C,P and G: Store the samples in high density polyethylene carboys (P) or glass bottles (G) at room temperature (~25°C).

4C, P and G: Store samples in high density polyethylene bottles (P) or glass bottles (G) in a refrigerator (4°C) in the dark.

Fr,P and Fr,P: Freeze the samples in high density polyethylene bottles (P) and store at -20°C in a freezer in the dark.

Glass and high density polyethylene bottles were used to study the effect of type of sample bottles on the recovery of nitrite and nitrate from refrigeration.

[B] For salinity and concentration of nitrate in river, estuary and coast samples see section 13.1.2. Sample river+, estuary+ and coast+ are the fortified river, estuary and coast samples, respectively, at nitrate concentrations 139.94 µg N/L.

Method 365.5

Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis

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Method 365.5

Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis

1.0 Scope and Application

1.1 This method provides a procedure for the determination of low-level orthophosphate concentrations normally found in estuarine and/or coastal waters. It is based upon the method of Murphy and Riley[1] adapted for automated segmented flow analysis[2] in which the two reagent solutions are added separately for greater reagent stability and facility of sample separation.

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Phosphate	14265-44-2

1.2 A statistically determined method detection limit (MDL) of 0.0007 mg P/L has been determined by one laboratory in 3 parts per thousand (ppt) saline water.[3] The method is linear to 0.39 mg P/L using a Technicon AutoAnalyzer II system (Bran & Luebbe, Buffalo Grove, IL).

1.3 Approximately 40 samples per hour can be analyzed.

1.4 This method should be used by analysts experienced in the use of automated colorimetric analyses, and familiar with matrix interferences and procedures for their correction. A minimum of 6-months experience under experienced supervision is recommended.

2.0 Summary of Method

2.1 An automated colorimetric method for the analysis of low-level orthophosphate concentrations is described. Ammonium molybdate and antimony potassium tartrate react in an acidic medium with dilute solutions of phosphate to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color produced is proportional to the phosphate concentration present in the sample. Positive bias caused by differences in the refractive index of seawater and reagent water is corrected for prior to data reporting.

3.0 Definitions

3.1 ***Calibration Standard (CAL) --*** A solution prepared from the stock standard solution that is used to calibrate the instrument response with respect to analyte concentration. One of the standards in the standard curve.

3.2 ***Dissolved Analyte (DA) --*** The concentration of analyte in an aqueous sample that will pass through a 0.45-μm membrane filter assembly prior to sample acidification or other processing.

3.3 ***Laboratory Fortified Blank (LFB) --*** An aliquot of reagent water to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether method performance is within acceptable control limits. This is basically a standard prepared in reagent water that is analyzed as a sample.

3.4 ***Laboratory Fortified Sample Matrix (LFM) --*** An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.5 ***Laboratory Reagent Blank (LRB) --*** An aliquot of reagent water that is treated exactly as a sample including exposure to all glassware, equipment, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or apparatus.

3.6 ***Linear Dynamic Range (LDR) --*** The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.7 ***Method Detection Limit (MDL) --*** The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero.

3.8 ***Reagent Water (RW) --*** Type 1 reagent grade water equal to or exceeding standards established by American Society of Testing Materials (ASTM). Reverse osmosis systems or distilling units that produce 18 megohm water are two examples of acceptable water sources.

3.9 Refractive Index (RI) -- The ratio of the velocity of light in a vacuum to that in a given medium. The relative refractive index is the ratio of the velocity of light in two different media, such as sea or estuarine water versus reagent water. The correction for this difference is referred to as the refractive index correction in this method.

3.10 Stock Standard Solution (SSS) -- A concentrated solution of method analyte prepared in the laboratory using assayed reference compounds or purchased from a reputable commercial source.

4.0 Interferences

4.1 Interferences caused by copper, arsenate and silicate are minimal relative to the orthophosphate determination because of the extremely low concentrations normally found in estuarine or coastal waters. High iron concentrations can cause precipitation of and subsequent loss of phosphate from the dissolved phase. Hydrogen sulfide effects, such as occur in samples collected from deep anoxic basins, can be treated by simple dilution of the sample since high sulfide concentrations are most often associated with high phosphate values.[4]

4.2 Sample turbidity is removed by filtration prior to analysis.

4.3 Refractive Index interferences are corrected for estuarine/coastal samples (Section 12.2).

5.0 Safety

5.1 Water samples collected from the estuarine and/or ocean environment are generally not hazardous. However, the individual who collects samples should use proper technique.

5.2 Good laboratory technique should be used when preparing reagents. A lab coat, safety goggles, and gloves should be worn when preparing the sulfuric acid reagent.

6.0 Equipment and Supplies

6.1 Continuous Flow Automated Analytical System Consisting of:

6.1.1 Sampler.

6.1.2 Manifold or Analytical Cartridge equipped with 37°C heating bath.

6.1.3 Proportioning pump.

6.1.4 Colorimeter equipped with 1.5 X 50 mm tubular flow cell and a 880 nm filter.

6.1.5 Phototube that can be used for 600-900 nm range.

6.1.6 Strip chart recorder or computer based data system.

6.2 Phosphate-Free Glassware and Polyethylene Bottles

6.2.1 All labware used in the determination must be low in residual phosphate to avoid sample or reagent contamination. Washing with 10% HCl (v/v) and thoroughly rinsing with distilled, deionized water was found to be effective.

6.2.2 Membrane or glass fiber filters, 0.45 μm nominal pore size.

7.0 Reagents and Standards

7.1 Stock Reagent Solutions

7.1.1 Ammonium Molybdate Solution (40 g/L) -- Dissolve 20.0 g of ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}\bullet 4H_2O$, CASRN 12027-67-7) in approximately 400 mL of reagent water and dilute to 500 mL. Store in a plastic bottle out of direct sunlight. This reagent is stable for approximately three months.

7.1.2 Antimony Potassium Tartrate Solution (3.0 g/L) -- Dissolve 0.3 g of antimony potassium tartrate [$(K(SbO)C_4H_4O_6\bullet 1/2H_2O$, CASRN 11071-15-1] in approximately 90 mL of reagent water and dilute to 100 mL. This reagent is stable for approximately three months.

7.1.3 Ascorbic Acid Solution (18.0 g/L) -- Dissolve 18.0 g of ascorbic acid ($C_6H_6O_6$, CASRN 50-81-7) in approximately 800 mL of reagent water and dilute to 1 L. Dispense approximately 75 mL into clean polyethylene bottles and freeze. The stability of the frozen ascorbic acid is approximately three months. Thaw overnight in the refrigerator before use. The stability of the thawed, refrigerated reagent is less than 10 days.

7.1.4 Sodium Lauryl Sulfate Solution (30.0 g/L) -- Sodium dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$, CASRN 151-21-3). Dissolve 3.0 g of sodium lauryl sulfate (SLS) in approximately 80 mL of reagent water and dilute to 100 mL. This solution is the wetting agent and its stability is approximately three weeks.

7.1.5 Sulfuric Acid Solution (4.9 N) -- Slowly add 136 mL of concentrated sulfuric acid (H_2SO_4, CASRN 7664-93-9) to approximately 800 mL of reagent water. After the solution is cooled, dilute to 1 L with reagent water.

7.1.6 Stock Phosphorus Solution -- Dissolve 0.439 g of pre-dried (105°C for 1 hr) monobasic potassium phosphate (KH_2PO_4, CASRN 7778-77-0) in reagent water and

dilute to 1000 mL. (1.0 mL = 0.100 mg P.) The stability of this stock standard is approximately three months when kept refrigerated.

7.1.7 Low Nutrient Seawater -- Obtain natural low nutrient seawater (36 ppt salinity; <0.0003 mg P/L) or dissolve 31 g analytical reagent grade sodium chloride, (NaCl, CASRN 7647-14-5); 10 g analytical grade magnesium sulfate, ($MgSO_4$) CASRN 10034-99-8); and 0.05 g analytical reagent grade sodium bicarbonate, ($NaHCO_3$, CASRN 144-55-8), in 1 L of reagent water.

7.2 Working Reagents

7.2.1 Reagent A -- Mix the following reagents in the following proportions for 142 mL of Reagent A: 100 mL of 4.9 N H_2SO_4 (Section 7.1.5), 30 mL of ammonium molybdate solution (Section 7.1.1), 10 mL of antimony potassium tartrate solution (Section 7.1.2), and 2.0 mL of SLS solution (Section 7.1.4). Prepare fresh daily.

7.2.2 Reagent B -- Add approximately 0.5 mL of the SLS solution (Section 7.1.4) to the 75 mL of ascorbic acid solution (Section 7.1.3). Stability is approximately 10 days when kept refrigerated.

7.2.3 Refractive Reagent A -- Add 50 mL of 4.9 N H_2SO_4 (Section 7.1.5) to 20 mL of reagent water. Add 1 mL of SLS (Section 7.1.4) to this solution. Prepare fresh every few days.

7.2.4 Secondary Phosphorus Solution -- Take 1.0 mL of Stock Phosphorus Solution (Section 7.1.6) and dilute to 100 mL with reagent water. (1.0 mL = 0.0010 mg P.) Refrigerate and prepare fresh every 10 days.

7.2.5 Prepare a series of standards by diluting suitable volumes of standard solutions (Section 7.2.4) to 100 mL with reagent water. Prepare these standards daily. When working with samples of known salinity, it is recommended that the standard curve concentrations be prepared in low-level natural seawater (Section 7.1.7) diluted to match the salinity of the samples. Doing so obviates the need to perform the refractive index correction outlined in Section 12.2. When analyzing samples of varying salinities, it is recommended that the standard curve be prepared in reagent water and refractive index corrections be made to the sample concentrations (Section 12.2). The following dilutions are suggested.

mL of Secondary Phosphorus Solution (7.2.4)	Conc. mg P/L
0.1	0.0010
0.2	0.0020
0.5	0.0050
1.0	0.0100
2.0	0.0200
4.0	0.0400
5.0	0.0500

8.0 Sample Collection, Preservation and Storage

8.1 Sample Collection -- Samples collected for nutrient analyses from estuarine and coastal waters are normally collected using one of two methods: hydrocast or submersible pump systems. Filtration of the sample through a 0.45-μm membrane or glass fiber filter immediately after collection is required.

8.1.1 A hydrocast uses a series of sampling bottles (Niskin, Nansen, Go-Flo or equivalent) that are attached at fixed intervals to a hydro wire. These bottles are sent through the water column open and are closed either electronically or via a mechanical “messenger” when the bottles have reached the desired depth.

8.1.2 When a submersible pump system is used, a weighted hose is sent to the desired depth in the water column and water is pumped from that depth to the deck of the ship for processing.

8.1.3 Another method used to collect surface samples involves the use of a plastic bucket or large plastic bottle. While not the most ideal method, it is commonly used in citizen monitoring programs.

8.2 Sample Preservation -- After collection and filtration, samples should be analyzed as quickly as possible. If the samples are to be analyzed within 24 hr of collection, then refrigeration at 4°C is acceptable.

8.3 Sample Storage -- Long-term storage of frozen samples should be in clearly labeled polyethylene bottles or polystyrene cups compatible with the analytical system’s automatic sampler (Section 6.1.1). If samples cannot be analyzed within 24 hr, then freezing at -20°C for a maximum period of two months is acceptable.[5-8]

9.0 Quality Control

9.1 Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, the continued analysis of LRBs, laboratory duplicates, and LFBs as a continuing check on performance.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (MDLs and linear dynamic range) and laboratory performance (analysis of QC samples) prior to analyses of samples using this method.

9.2.2 MDLs should be established using a low-level estuarine water sample fortified to approximately five

times the estimated detection limit.[3] To determine MDL values, analyze seven replicate aliquots of water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

$$MDL = (t)(S)$$

where, S = the standard deviation of the replicate analyses.

t = the Student's t value for n-1 degrees of freedom at the 99% confidence limit. t = 3.143 for six degrees of freedom.

MDLs should be determined every six months or whenever a significant change in background or instrument response occurs or when a new matrix is encountered.

9.2.3 Linear Dynamic Range (LDR) -- The LDR should be determined by analyzing a minimum of five calibration standards ranging in concentration from 0.001 mg P/L to 0.20, mg P/L across all sensitivity settings of the autoanalyzer. Normalize responses by dividing the response by the sensitivity setting multiplier. Perform the linear regression of normalized response vs. concentration and obtain the constants *m* and *b,* where *m* is the slope and *b* is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response, R, of a standard no longer yields a calculated concentration C_C, that is ± 10% of the known concentration, C, where $C_C = (R - b)/m$. That concentration defines the upper limit of the LDR for your instrument. Should samples be encountered that have a concentration that is ≥90% of the upper limit of the LDR, then these samples must be diluted and reanalyzed.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) -- A laboratory should analyze at least one LRB (Section 3.5) with each set of samples. LRB data are used to assess contamination from the laboratory environment. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples or field duplicates of the samples must be prepared and analyzed again after the source of contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 Laboratory Fortified Blank (LFB) -- A laboratory should analyze at least one LFB (Section 3.3) with each batch of samples. Calculate accuracy as percent recovery. If the recovery of the analyte falls outside the required control limits of 90-110%, the analyte is judged out of control and the source of the problem should be identified and resolved before continuing the analyses.

9.3.3 The laboratory must use LFB data to assess laboratory performance against the required control limits of 90-110% (Section 9.3.2). When sufficient internal performance data become available (usually a minimum of 20 to 30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

$$\text{Upper Control Limit} = x + 3S$$

$$\text{Lower Control Limit} = x - 3S$$

The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5 to 10 new recovery measurements, new control limits can be calculated using only the most recent 20 to 30 data points. Also, the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.4 Assessing Analyte Recovery - Laboratory Fortified Sample Matrix

9.4.1 A laboratory should add a known amount of analyte to a minimum of 5% of the routine samples or one sample per sample set, whichever is greater. The analyte concentration should be two to four times the ambient concentration and should be at least four times the MDL.

9.4.2 Calculate the percent recovery of the analyte, corrected for background concentrations measured in the unfortified sample, and compare these values with the values obtained from the LFBs.

Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_s - C)}{S} x\ 100$$

where,
R = percent recovery
C_S = measured fortified sample concentration (background + concentrated addition in mg P/L)
C = sample background concentration (mg P/L)
S = concentration in mg P/L added to the environmental sample.

9.4.3 If the recovery of the analyte falls outside the designated range of 90-110% recovery, but the laboratory performance for that analyte is in control, the fortified

sample should be prepared again and analyzed. If the result is the same after reanalysis, the recovery problem encountered with the fortified sample is judged to be matrix related, not system related.

10.0 Calibration and Standardization

10.1 Calibration (Refer to Sections 11.5 and 12.0).

10.2 Standardization (Refer to Section 12.2).

11.0 Procedure

11.1 If samples are frozen, thaw the samples to room temperature.

11.2 Set up manifold as shown in Figure 1. The tubing, flow rates, sample:wash ratio, sample rate, etc., are based on a Technicon AutoAnalyzer II system. Specifications for similar segmented flow analyzers vary, so slight adjustments may be necessary.

11.3 Allow both colorimeter and recorder to warm up for 30 min. Obtain a steady baseline with reagent water pumping through the system, add reagents to the sample stream and after the reagent water baseline has equilibrated, note that rise (reagent water baseline), and adjust baseline.

For analysis of samples with a narrow salinity range, it is advisable to use low nutrient seawater matched to sample salinity as wash water in the sampler in place of reagent water. For samples with a large salinity range, it is suggested that reagent wash water and procedure (Section 12.2) be employed.

11.4 A good sampling rate is approximately 40 samples/hr with a 9:1, sample:wash ratio.

11.5 Place standards (Section 7.2.5) in sampler in order of decreasing concentration. Complete filling the sampler tray with samples, LRBs, LFBs, and LFMs.

11.6 Commence analysis.

11.7 Obtain a second set of peak heights for all samples and standards with Refractive Reagent A (Section 7.2.3) being pumped through the system in place of Reagent A (Section 7.2.1). This “apparent” concentration due to coloration of the water should be subtracted from concentrations obtained with Reagent A pumping through the system.

12.0 Data Analysis and Calculations

12.1 Concentrations of orthophosphate are calculated from the linear regression obtained from the standard curve in which the concentrations of the calibration standards are entered as the independent variable and the corresponding peak height is the dependent variable.

12.2 Refractive Index Correction for Estuarine/ Coastal Systems

12.2.1 Obtain a second set of peak heights for all samples and standards with Refractive Reagent A (Section 7.2.3) being pumped through the system in place of Reagent A (Section 7.2.1). Reagent B (Section 7.2.2) remains the same and is also pumped through the system. Peak heights for the refractive index correction must be obtained at the same Standard Calibration Setting and on the same colorimeter as the corresponding samples and standards.[9]

12.2.2 Subtract the refractive index peak heights from the heights obtained for the orthophosphate determination. Calculate the regression equation using the corrected standard peak heights. Calculate the concentration of samples from the regression equation using the corrected sample peak heights.

12.2.3 When a large data set has been amassed in which each sample’s salinity is known, a regression for the refractive index correction on a particular colorimeter can be calculated. For each sample, the apparent orthophosphate concentration due to refractive index is calculated from its peak height obtained with Refractive Reagent A (Section 7.2.3) and Reagent B (Section 7.2.2) and the regression of orthophosphate standards obtained with orthophosphate Reagent A (Section 7.2.1) and Reagent B (Section 7.2.2) for each sample. Its salinity is entered as the independent variable and its apparent orthophosphate concentration due to its refractive index in that colorimeter is entered as the dependent variable. The resulting regression equation allows the operator to subtract an apparent orthophosphate concentration when the salinity is known, as long as other matrix effects are not present. Thus, the operator would not be required to obtain the refractive index peak heights for all samples after a large data set has been found to yield consistent apparent orthophosphate concentrations due to salinity. An example follows:

Salinity (ppt)	Apparent orthophosphate conc. due to refractive index (mg P/L)
1	0.0002
5	0.0006
10	0.0009
20	0.0017

12.2.4 An example of a typical equation is:

mg P/L apparent PO_4^{3-} = 0.000087 X Salinity (ppt) where, 0.000087 is the slope of the line.

where, 0.000087 is the slope of the line.

12.3 Results should be reported in mg PO_4^{3-} - P/L or µg PO_4^{3-} - P/L.

mg PO_4^{3-} - P/L = ppm (parts per million)

µg PO_4^{3-} - P/L = ppb (parts per billion)

13.0 Method Performance

13.1 Single Analyst Precision -- A single laboratory analyzed three samples collected from Chesapeake Bay, Maryland, and East Bay, Florida. Seven replicates of each sample were processed and analyzed randomly throughout a group of 75 samples with salinities ranging from 3 to 36 ppt. The results were as follows:

Sample	Salinity (ppt)	Concentration (mg P/L)	Percent Relative Standard Deviation
1	36	0.0040	6.5
2	18	0.0024	10
3	3	0.0007	24

13.2 Multilaboratory Testing

13.2.1 This method was tested by nine laboratories using reagent water, high salinity seawater from the Sargasso Sea (36 ppt) and three different salinity waters from Chesapeake Bay, Maryland (8.3 ppt, 12.6 ppt, and 19.5 ppt). The reagent water and the Sargasso Seawater were fortified at four Youden pair concentrations ranging from 0.0012 mg P/L to 0.1000 mg P/L.[10] The Chesapeake Bay waters were fortified at three Youden pair concentrations ranging from 0.0050 mg P/L to 0.0959 mg P/L with the highest salinity waters containing the lowest Youden pair and the lowest salinity waters containing the highest Youden pair. Analysis of variance (ANOVA) at the 95% confidence level found no statistical differences between water types indicating that the refractive index correction for different salinity waters is an acceptable procedure. Table 1 contains the linear equations that describe the single-analyst standard deviation, overall standard deviation, and mean recovery of orthophosphate from each water type.

13.2.2 Pooled Method Detection Limit (p-MDL) -- The p-MDL is derived from the pooled precision obtained by single laboratories for the lowest analyte concentration level used in the multilaboratory study. The p-MDLs using reagent water and Sargasso Sea water were 0.00128 and 0.00093 mg P/L, respectively.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better. Laboratory Chemical Management for Waste Reduction,* available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036, (202)872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel,* available from the American Chemical Society at the address listed in Section 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Single-Analyst Precision, Overall Precision and Recovery from Multilaboratory Study

Reagent Water	
(0.0012 - 0.100 mg P/L)	
Mean Recovery	X = 0.972C - 0.000018
Overall Standard Deviation	S_R = 0.033X + 0.000505
Single-Analyst Standard Deviation	S_r = 0.002X + 0.000448
Sargasso Sea Water	
(0.0012 - 0.100 mg P/L)	
Mean Recovery	X = 0.971C - 0.000002
Overall Standard Deviation	S_R = 0.021X + 0.000550
Single-Analyst Standard Deviation	S_r = 0.010X + 0.000249
Chesapeake Bay Water	
(0.005 - 0.100 mg P/L)	
Mean Recovery	
X = 1.019C - 0.000871	
Overall Standard Deviation	S_R = 0.066X + 0.000068
Single-Analyst Standard Deviation	S_r = 0.030X + 0.000165

C True value of spike concentration, mg P/L
X Mean concentration found, mg P/L, exclusive of outliers.
S_R Overall standard deviation, mg P/L, exclusive of outliers.
S_r Single-analyst standard deviation, mg P/L, exclusive of outliers.

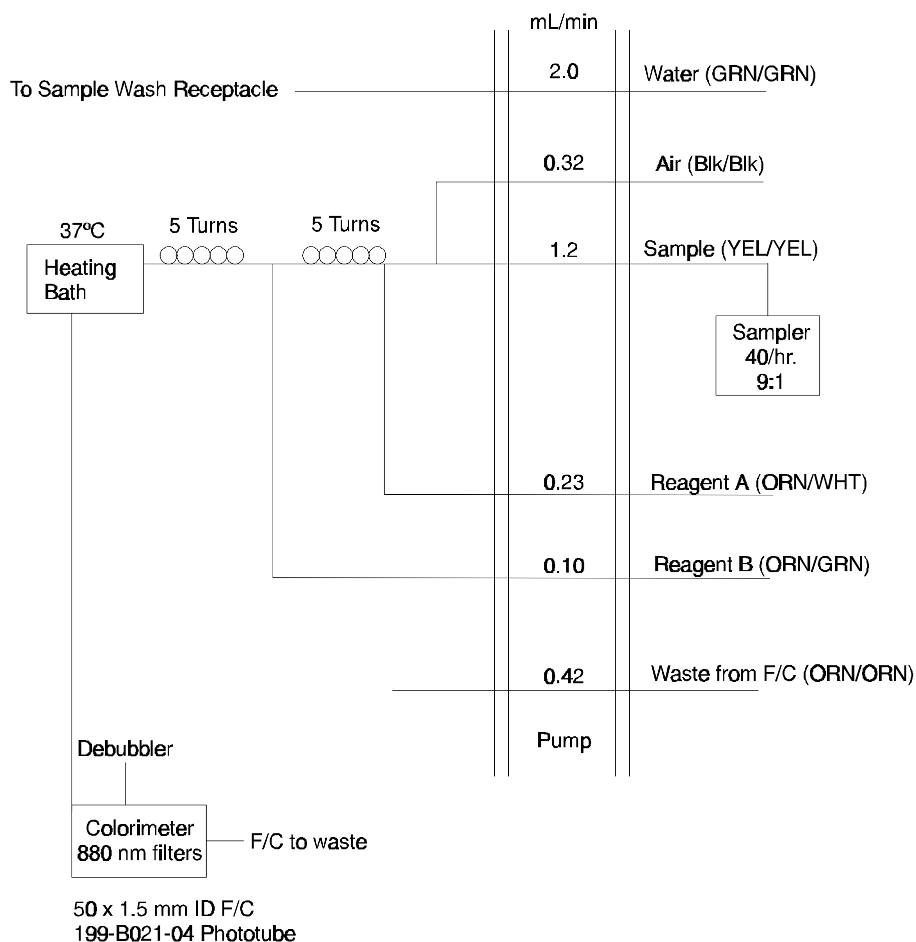

Figure 1. Manifold Configuration for Orthophosphate.

Method 366.0

Determination of Dissolved Silicate in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

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Version 1.0
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Cincinnati, Ohio 45268

Method 366.0

Determination of Dissolved Silicate in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

1.0 Scope and Application

1.1 This method provides a procedure for the determination of dissolved silicate concentration in estuarine and coastal waters. The dissolved silicate is mainly in the form of silicic acid, H_4SiO_4, in estuarine and coastal waters. All soluble silicate, including colloidal silicic acid, can be determined by this method. Long chain polymers containing three or more silicic acid units do not react at any appreciable rate[1], but no significant amount of these large polymers exists in estuarine and coastal waters.[2,3] This method is based upon the method of Koroleff,[4] adapted to automated gas segmented continuous flow analysis.[5-7]

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Silicate	12627-13-3

1.2 A statistically determined method detection limit (MDL) of 0.0012 mg Si/L has been determined by one laboratory in seawaters of three different salinities.[8] The method is linear to 6.0 mg Si/L using a Flow Solution System (Perstorp Analytical Inc., Silver Spring, MD).

1.3 Approximately 60 samples per hour can be analyzed.

1.4 This method should be used by analysts experienced in the use of automated gas segmented continuous flow colorimetric analyses, and familiar with matrix interferences and procedures for their correction. A minimum of 6-months experience under supervision is recommended.

2.0 Summary of Method

2.1 An automated gas segmented continuous flow colorimetric method for the analysis of dissolved silicate concentration is described. In the method, β-molybdosilicic acid is formed by reaction of the silicate contained in the sample with molybdate in acidic solution. The β-molybdosilicic acid is then reduced by ascorbic acid to form molybdenum blue. The absorbance of the molybdenum blue, measured at 660 nm, is linearly proportional to the concentration of silicate in the sample. A small positive error caused by differences in the refractive index of seawater and reagent water, and negative error caused by the effect of salt on the color formation, are corrected prior to data reporting.

3.0 Definitions

3.1 Calibration Standard (CAL) -- A solution prepared from the primary dilution standard solution or stock standard solution containing analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.2 Dissolved Analyte (DA) -- The concentration of analyte in an aqueous sample that will pass through a 0.45 μm membrane filter assembly prior to sample acidification or other processing.

3.3 Laboratory Fortified Blank (LFB) -- An aliquot of reagent water to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether method performance is within acceptable control limits, and whether the laboratory is capable of making accurate and precise measurements.

This is basically a standard prepared in reagent water that is analyzed as a sample.

3.4 Laboratory Fortified Sample Matrix (LFM) -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.5 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water that is treated exactly as a sample including exposure to all labware, equipment, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or apparatus.

3.6 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.7 Method Detection Limit (MDL) -- The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.[8]

3.8 Reagent Water (RW) -- Type 1 reagent grade water equal to or exceeding standards established by American Society for Testing and Materials (ASTM). Reverse osmosis systems or distilling units followed by Super-Q Plus Water System that produce water with 18 megohm resistance are examples of acceptable water sources.

3.9 Refractive Index (RI) -- The ratio of velocity of light in a vacuum to that in a given medium. The relative refractive index is the ratio of the velocity of light in two different media, such as estuarine or sea water versus reagent water. The correction for this difference is referred to as refractive index correction in this method.

3.10 Stock Standard Solution (SSS) -- A concentrated solution of method analyte prepared in the laboratory using assayed reference compounds or purchased from a reputable commercial source.

3.11 Quality Control Sample (QCS) - A solution of method analyte of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.12 SYNC Peak Solution - A colored solution used to produce a synchronization peak in the refractive index measurement. A synchronization peak is required by most data acquisition programs to initialize the peak finding parameters. The first cup in every run must always be identified as a SYNC sample. The SYNC sample is usually a high standard, but can be any sample that generates a peak at least 25% of full scale.

4.0 Interferences

4.1 Interferences caused by hydrogen sulfide, such as occur in samples taken from deep anoxic basins can be eliminated by oxidation with bromine or stripping with nitrogen gas after acidification. Interferences of phosphate at concentrations larger than 0.15 mg P/L is eliminated by the use of oxalic acid in the color development step of this method. Interferences of fluoride at concentrations greater than 50 mg F/L can be reduced by complexing the fluoride with boric acid.[4]

4.2 Glassware made of borosilicate glass should be avoided for use in silicate analysis. Plastic labware such as polyethylene volumetric flasks and plastic sample vials, should be used.

4.3 Sample turbidity and particles are removed by filtration through a 0.45 µm non-glass membrane filters after sample collection.

4.4 This method corrects for refractive index and salt error interferences which occur if sampler wash solution and calibration standards are not matched with samples in salinity.

4.5 Frozen samples should be filled about 3/4 full in the sample bottles. The expansion of water on freezing will squeeze some of the brine out of the bottle if the bottle was overfilled. The overfill of the sample bottle during freezing will drastically alter the nutrient concentrations in the sample that remains.

5.0 Safety

5.1 Water samples collected from the estuarine and coastal environment are generally not hazardous. However, the individual who collects samples should use proper technique.

5.2 Good laboratory technique should be used when preparing reagents. A lab coat, safety goggles, and gloves should be worn when preparing the sulfuric acid reagent.

6.0 Equipment and Supplies

6.1 Gas Segmented Continuous Flow Autoanalyzer Consisting of:

6.1.1 Autosampler.

6.1.2 Analytical cartridge with reaction coils for silicate analysis.

6.1.3 Proportioning pump.

6.1.4 Monochromator or spectrophotometer equipped with a tungsten lamp (380-800 nm) and a low refractive index flowcell.

6.1.5 Strip chart recorder or computer based data acquisition system.

6.2 Glassware and Supplies

6.2.1 All labware used in the analysis must be low in residual silicate to avoid sample or reagent contamination. Soaking with lab grade detergent, rinsing with tap water, followed by rinsing with 10% HCl (v/v) and thoroughly rinsing with reagent water was found to be effective.

6.2.2 Glassware made of borosilicate glass should be avoided for storage of solutions for silicate analysis. Plastic containers are preferable for silicate analysis.

6.2.3 Non-glass membrane filters with 0.45 µm nominal pore size. Plastic syringes with syringe filters, pipets, 60 mL polyethylene bottles, and polyethylene volumetric flasks, plastic sample vials.

6.2.4 Drying oven, desiccator and analytical balance.

7.0 Reagents and Standards

7.1 Stock Reagent Solutions

7.1.1 Sulfuric Acid Solution (0.05 M) - Cautiously add 2.8 mL of concentrated Analytical Reagent Grade sulfuric acid (H_2SO_4) to approximately 800 mL of reagent water, mix then bring up to 1 L with reagent water.

7.1.2 Ammonium Molybdate Solution (10 g/L) - Dissolve 10 g of ammonium molybdate (VI) tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) in approximately 800 mL of 0.05 M sulfuric acid solution and dilute to 1000 mL with 0.05 M sulfuric acid solution. Store in an amber plastic bottle. This solution is stable for one month. Inspect the solution before use. If a white precipitation forms on the wall of container, discard the solution and make a fresh one.

7.1.3 Stock Silicate Solution (100 mg Si/L) - Quantitatively transfer 0.6696 g of pre-dried (105°C for 2 hours) sodium hexafluorosilicate (Na_2SiF_6) to a 1000 mL polypropylene flask containing approximate 800 mL of reagent water, cover with plastic film and dissolve on a stir plate using a Teflon-coated stirring bar. Complete dissolution usually takes 2-24 hours. Dilute the solution to 1000 mL in polyethylene volumetric flask with reagent water. Store the stock solution in a plastic bottle. This solution is stable for one year if care is taken to prevent contamination and evaporation.

7.1.4 Low Nutrient Sea Water (LNSW) - Obtain natural low nutrient seawater from surface seawater in the Gulf Stream or Sargasso Sea (salinity 36 ‰, < 0.03 mg Si/L) and filter through 0.45 µm pore size non-glass membrane filters. In addition, commercially available low nutrient sea water (< 0.03 mg Si/L) with salinity of 35 ‰ (Ocean Scientific International, Wormley, U.K.) can be used.

7.2 Working Reagents

7.2.1 Dowfax Start-up Solution - Add 2 mL of Dowfax 2A1 surfactant (Dow Chemical Company) to 1000 mL reagent water and mix gently.

Note: Dowfax 2A1 contains (w/w) 47% benzene, 1,1-oxybis, tetrapropylene derivatives, sulfonate, sodium salt, 1% sodium sulfate, 3% sodium chloride and 49% water.

7.2.2 Working Molybdate Reagent - Add 0.5 mL Dowfax 2A1 to 250 mL of ammonium molybdate solution, mix gently. Prepare this solution daily. This volume of solution is sufficient for an 8-hour run.

7.2.3 Ascorbic Acid Solution - Dissolve 4.4 g of ascorbic acid ($C_6H_8O_6$) in 200 mL of reagent water and 12.5 mL of acetone(C_3H_6O), dilute to 250 mL with reagent water. Store in a plastic container. This solution is stable for one week if stored at 4°C. Discard the solution if it turns brown.

7.2.4 Oxalic Acid Solution - Dissolve 50 g of oxalic acid ($C_2H_2O_4$) in approximately 800 mL of reagent water and dilute to 1000 mL with reagent water. Store in a plastic container. This solution is stable for approximately 3-months.

7.2.5 Refractive Index Matrix Solution - Add 0.5 mL Dowfax 2A1 to 250 mL of 0.05 M sulfuric acid solution and mix gently.

7.2.6 Colored SYNC Peak Solution - Add 50 µL of blue food coloring solution to 1000 mL reagent water and mix thoroughly. The solution should give a peak of between 25 to 100 percent full scale, otherwise the volume of food coloring added needs to be adjusted.

7.2.7 Calibration Standards - Prepare a series of calibration standards (CAL) by diluting suitable volumes of Stock Silicate Solution (Section 7.1.3) to 100 mL with

reagent water or low nutrient seawater. Prepare these standards daily. The concentration range of calibration standards should bracket the expected concentrations of samples and not exceed two orders of magnitude. At least five calibration standards with equal increments in concentration should be used to construct the calibration curve.

When working with samples of a narrow range of salinities (± 2 ‰), it is recommended that the CAL solutions be prepared in Low Nutrient Seawater (Section 7.1.4) diluted to the salinity of samples, and the Sampler Wash Solution also be Low Nutrient Seawater (Section 7.1.4) diluted to that salinity. If this procedure is performed, it is not necessary to perform the salt error and refractive index corrections outlined in Sections 12.2 and 12.3.

When analyzing samples of varying salinities, it is recommended that the calibration standard solutions and sampler wash solution be prepared in reagent water and corrections for salt error and refractive index be made to the sample concentrations (Section 12.2 and 12.3).

7.2.8 Saline Silicate Standards - If CAL solutions will not be prepared to match sample salinity, then saline silicate standards must be prepared in a series of salinities in order to quantify the salt error, the change in the colorimetric response of silicate due to the change in the ionic strength of the solution. The following dilutions prepared in 100 mL volumetric flasks, diluted to volume with reagent water, are suggested.

Salinity (‰)	Volume of LNSW(mL)	Volume(mL) Si stock std	Conc. mg Si/L
0	0	1.5	1.5
9	25	1.5	1.5
18	50	1.5	1.5
27	75	1.5	1.5
35	98	1.5	1.5

8.0 Sample Collection, Preservation and Storage

8.1 Sample Collection - Samples collected for nutrient analyses from estuarine and coastal waters are normally collected using one of two methods: hydrocast or submersible pump systems.

8.1.1 A hydrocast uses a series of sampling bottles (Niskin, Go-Flo or equivalent) that are attached at fixed intervals to a hydro wire. These bottles are sent through the water column open and are closed either electronically or via a mechanical messenger when the bottles have reached the desired depth.

8.1.2 In a submersible pump system, a weighted hose is sent to the desired depth in the water column and water is pumped from that depth to the deck of the ship for sample processing.

8.1.3 For collecting surface samples, an acid - cleaned plastic bucket or a large plastic bottle can be used as convenient samplers. Wash the sampler three times with sample water before collecting samples.

8.1.4 Samples must be filtered through a 0.45 µm non-glass membrane filters as soon as possible after collection.

8.1.5 60-mL high density polyethylene bottles are used for sample storage. Sample bottles should be rinsed 3 times with about 20 mL of sample, shaking with the cap in place after each rinse. Pour the rinse water into the cap to dissolve and rinse away salt crusts trapped in the threads of the cap. Finally, fill the sample bottle about 3/4 full, and screw the cap on firmly. The expansion of water on freezing will squeeze some of the brine out of the bottle if the bottle was overfilled.

8.2 Sample Preservation - After collection and filtration, samples should be analyzed as soon as possible. If samples will be analyzed within 24 hours then keep refrigerated in tightly sealed, high density polyethylene bottles in the dark at 4°C until analysis can be performed.

8.3 Sample Storage - If samples are to be frozen for long-term storage ensure that none of the sample bottles are filled more than 3/4 full and the cap is firmly screwed on. Place the bottles upright on a rack and store in the freezer (-20°C).

Before analysis, frozen samples must be taken out of the freezer and allowed to thaw in a refrigerator at 4°C in the dark. Thawing times depend upon sample salinities. It was found that the frozen low salinity estuarine water took 4 days to thaw. After completely thawing, take samples out of the refrigerator and mix thoroughly. Keep samples in the dark at room temperature overnight before analysis.

Effects of thawing conditions on the recoveries of frozen samples are more pronounced in low salinity estuarine

waters than high salinity coastal waters as shown in following results:

Day	Recovery (%)			Remark
	S=35.85	S=18.07	S=2.86	
0	100.00	100.00	100.00	
7	102.44	102.65	89.37	a
14	98.59	101.06	86.49	a
21	99.51	99.30	83.49	a
27	98.86			a
		98.86	91.43	b
35	98.70			b
		98.66	92.98	b
42	100.87			b
49		102.44	79.12	c
		103.92	79.10	d
		99.92	89.68	e
56	103.47			c
	104.12			d
	99.35			e
84		100.80	91.71	f
		99.90	93.81	g
91	100.65			f
	100.22			g

S = Salinity
a, overnight thawing at room temperature
b, 20 hours thawing at room temperature
c, 24 hours thawing at room temperature
d, 8 hours thawing at room temperature then heating at 80°C for 16 hours
e, 24 hours thawing at room temperature in the dark
f, 4 days thawing at room temperature in the dark
g, 4 days thawing at 4°C in a refrigerator in the dark

To ensure a slow process of depolymerization of polysilicate to occur, thawing the frozen samples in the dark at 4°C for 4 days is critical condition for obtaining high recoveries of silicate in frozen samples. A maximum holding time for frozen estuarine and coastal waters is two months.[9-11]

9.0 Quality Control

9.1 Each laboratory using this method is required to implement a formal quality control(QC) program. The minimum requirements of this program consists of an initial demonstration of performance, continued analysis of Laboratory Reagent Blanks (LRB), laboratory duplicates and Laboratory Fortified Blanks (LFB) with each set of samples as a continuing check on performance.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The Initial demonstration of performance is used to characterize instrument performance by determining the MDL and LDR and laboratory performance by analyzing quality control samples prior to analysis of samples using this method.

9.2.2 Method Detection Limits (MDLs) should be established using a low level seawater sample containing, or fortified at, approximately 5 times the estimated detection limit. To determine MDL values, analyze at least seven replicate aliquots of water which have been processed through the entire analytical method. Perform all calculations defined in the method and report concentration in appropriate units. Calculate the MDL as follows:

$$MDL = (t)(S)$$

where, S = the standard deviation of the replicate analyses

t = Student's t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

MDLs should be determined every 6-months or whenever a significant change in background or instrument response occurs or a new matrix is encountered.

9.2.3 The LDR should be determined by analyzing a minimum of eight calibration standards ranging from 0.03 to 5.00 mg Si/L across all sensitivity settings (Absorbance Units Full Scale) of the detector. Standards and sampler wash solutions should be prepared in low nutrient seawater with salinities similar to that of samples, therefore a correction factor for salt error, or refractive index, will not be necessary. Normalize responses by multiplying the response by the Absorbance Units Full Scale output range setting. Perform the linear regression of normalized response vs. concentration and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response, R, of a standard no longer yields a calculated concentration C_C, that is within 100 ± 10% of the known concentration, C, where $C_C = (R-b)/m$. That concentration

defines the upper limit of the LDR for the instrument. Should samples be encountered that have a concentration that is ≥ 90% of the upper limit of LDR, then these samples must be diluted and reanalyzed.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 *Laboratory Reagent Blank (LRB)* - A laboratory should analyze at least one LRB with each set of samples. LRB data are used to assess contamination from the laboratory environment. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When the LRB value constitutes 10% or more of the analyte concentration determined for a sample, duplicates of the sample must be prepared and analyzed again after the source of contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 *Laboratory Fortified Blank (LFB)* - A laboratory should analyze at least one LFB with each set of samples. The LFB must be at a concentration that is within the daily calibration range. The LFB data are used to calculate accuracy as percent recovery. If the recovery of the analyte falls outside the required control limits of 90 -110%, the source of the problem should be identified and resolved before continuing the analyses.

9.3.3 The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 90 -110%. When sufficient internal performance data become available (usually a minimum of 20 to 30 analyses), optional control limits can be developed from the percent mean recovery (x) and standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

Upper Control Limit = x + 3S
Lower Control Limit = x - 3S

The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5 to 10 new recovery measurements, new control limits can be calculated using only the most recent 20 to 30 data points. Also the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.4 Assessing Analyte Recovery - Laboratory Fortified Sample Matrix (LFM)

9.4.1 A laboratory should add a known amount of analyte to a minimum of 5% of the total number of samples or one sample per sample set, whichever is greater. The analyte added should be 2-4 times the ambient concentration and should be at least four times greater than the MDL.

9.4.2 Calculate percent recovery of analyte, corrected for background concentration measured in a separate unfortified sample. These values should be compared with the values obtained from the LFBs. Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_S - C)}{S} \times 100$$

where, R = percent recovery

C_S = measured fortified sample concentration (background + addition in mg Si/L)

C = sample background concentration (mg Si/L)

S = concentration in mg Si/L added to the environmental sample.

9.4.3 If the recovery of the analyte falls outside the required control limits of 90-110%, but the laboratory performance for that analyte is within the control limits, the fortified sample should be prepared again and analyzed. If the result is the same after reanalysis, the recovery problem encountered with the fortified sample is judged to be matrix related and the sample data should be flagged.

10.0 Calibration and Standardization

10.1 At least five calibration standards should be prepared daily for system calibration.

10.2 A calibration curve should be constructed for each run by analyzing a set of calibration standard solutions. A run should contain no more than 60 samples.

It is suggested that a large set of samples be analyzed in several sets with individual calibration curves.

10.3 Place the calibration standards before samples for each run. All the calibration solutions should be analyzed in duplicate.

10.4 The calibration curve containing five data points or more should have a correlation coefficient $\geq$ 0.995.

10.5 Place a high standard solution cup and follow by two blank cups to quantify the **carry-over** of the system. The difference in peak heights between two blank cups is due to the carry over from the high standard cup. The carry-over coefficient, k, is calculated as follows:

$$k = \frac{P_{b1} - P_{b2}}{P_{high}} \times 100$$

where, P_{high} = the peak height of the high silicate standard

P_{b1} = the peak height of the first blank sample

P_{b2} = the peak height of the second blank sample.

The carry over coefficient, k, for a system should be measured in seven replicates in order to obtain a statistically significant number. The k should be remeasured when a change in the plumbing of the manifold or replacement of pump tube occur.

The carry over correction (CO) on a given peak, i, is proportional to the peak height of the preceding sample, P_{i-1}.

$$CO = kxP_{i-1}$$

To correct a given peak height reading, P_i, one subtracts the carry over correction.[12,13]

$$P_{i,c} = P_i - CO$$

where $P_{i,c}$ is corrected peak height. The correction for carry over should be applied to all the peak heights throughout a run. The carry over should be less than 2%.

10.6 Place a high standard solution at the end of a run to check sensitivity drift. The sensitivity drift should be ± 5% during the run.

11.0 Procedure

11.1 If samples are frozen, thaw the sample at 4°C in the dark as outlined in Section 8.3. Mix samples thoroughly prior to analyses.

11.2 Turn on the continuous flow analyzer and PC components and warm up at least 30 minutes.

11.3 Set up the cartridge and pump tubes as shown in Figure 1.

Note: Fluctuation of ambient temperature can cause erratic results due to the effect of temperature on kinetics of color development. The laboratory temperature should be maintained as close to a constant temperature as possible. The cartridge should be away from the direct path of air flow from a heater or air conditioner. In cases such as on a ship where the fluctuation of temperature can be extreme, the temperature effect can be minimized by increasing the length of mixing coil 1 (Figure 1) to bring the formation of silicomolybdic acid reaction to completion.

11.4 Set the wavelength at 660 nm on the spectrometer/monochrometer.

Note: The absorption spectra of silicomolybdeum blue complex has two maxima at 820 nm and 660 nm with 820 nm higher than 660 nm. This method measures absorbance at 660 nm because the detector works in the range of 380 to 800 nm. The sensitivity of the method is satisfactory at 660 nm. The sensitivity, however, can be improved by using 820 nm if this wavelength is available on the detector.

11.5 On the monochromator, set the Absorbance Unit Full Scale at an appropriate setting according to the highest concentration of silicate in the samples. The highest setting used in this method was 0.2 for 6 mg Si/L.

11.6 Prepare all reagents and standards.

11.7 Begin pumping the Dowfax start-up solution (Section 7.2.1) through the system and obtain a steady baseline. Place the reagents on-line. The reagent baseline will be higher than the start-up solution baseline. After the reagent baseline has stabilized, reset the baseline.

NOTE: To minimize the noise in the reagent baseline, clean the flow system by sequentially pumping the

sample line with reagent water, 1 N HCl solution, reagent water, 1N NaOH solution for a few minutes each at the end of the daily analysis. Make sure to rinse the system well with reagent water after pumping NaOH solution to prevent precipitation of $Mg(OH)_2$ when seawater is introduced into the system. Keep the reagents and samples free of particulate. Filter the reagents and samples if necessary.

If the baseline drifts upward, pinch the waste line for a few seconds to increase back pressure. If absorbance drops down rapidly when back pressure increases, this indicates that there are air bubbles trapped in the flow cell. Attach a syringe at the waste outlet of the flowcell. Air bubbles in the flowcell can often be eliminated by simply attaching a syringe for a few minutes or, if not, dislodged by pumping the syringe pistion. Alternatively, flushing the flowcell with alcohol was found to be effective in removing air bubbles from the flowcell.

For analysis of samples with a narrow range of salinities (± 2 ‰), it is recommended that the wash water in the sampler be prepared in Low Nutrient Seawater diluted to the salinity of samples in place of reagent water. For samples with varying salinities, it is suggested that reagent waters and procedures in Sections 12.2 and 12.3 be employed.

11.8 A good sampling rate is approximately 60 samples per hour with 40 seconds of sample time and 20 seconds of wash time.

11.9 Use 10% HCl followed by reagent water to rinse sample cups. Place CAL solutions and saline standards (optional) in sampler. Complete filling the sampler tray with samples, laboratory reagent blanks, laboratory fortified blanks, laboratory fortified sample matrices, and QC samples. Place a blank every ten samples and between samples of high and low concentrations.

11.10 Commence analysis.

11.11 If the reagent water is used as wash solution instead of Low Nutrient Seawater and an operator wants to quantify the refractive index correction due to the difference in salinities between sample and wash solution, the following procedures are used. Replace ammonium molybdate solution (Section 7.1.2) with refractive index matrix solution (Section 7.2.5). All other reagents remain the same. Replace the synchronization cup with the colored SYNC peak solution (Section 7.2.6). Commence analysis and obtain a second set of peak heights for all standards and samples. The peak heights obtained from these measurements must be subtracted from the peak heights of samples analyzed with color developing reagent pumping through the system. If a low refractive index flowcell is used, the correction for refractive index is negligible. This procedure is optional.

12.0 Data Analysis and Calculations

12.1 Concentrations of silicate are calculated from the linear regression, obtained from the standard curve in which the concentrations of the calibration standards are entered as the independent variable, and their corresponding peak heights are the dependent variable.

12.2 Refractive Index Correction for Estuarine and Coastal Samples (optional)

12.2.1 Obtain a second set of peak heights for all standards and samples with refractive index matrix solution being pumped through the system in place of color reagent (ammonium molybdate solution). All other reagents remain the same. The peak heights for the refractive index correction must be obtained at the same Absorbance Unit Full Scale range setting and on the same monochromator as the corresponding samples and standards.

12.2.2 Subtract the refractive index peak heights from the peak heights obtained from the silicate determination.

12.2.3 An alternative approach is to measure the relationship between the sample salinity and refractive index on a particular detector.

First analyze a set of silicate standards in reagent water with color reagent and obtain a linear regression from the standard curve.

Prepare a set of different salinity samples with LNSW. Analyze these samples with refractive index matrix solution being pumped through the system in place of color reagent (ammonium molybdate solution). All other reagents remain the same. The peak heights for the refractive index correction must be obtained at the same Absorbance Unit Full Scale setting and on the same monochromator as the corresponding standards.

For each sample, the apparent silicate concentration due to refractive index is then calculated from its peak height obtained with refractive index reagent and the regression of silicate standards obtained with color reagent pumping

through the system. Salinity is entered as the independent variable and the apparent silicate concentration due to refractive index in that detector is entered as the dependent variable. The resulting regression allows the operator to calculate apparent silicate concentration due to refractive index when the salinity is known. Thus, the operator would not be required to obtain refractive index peak heights for all samples.

12.2.4 Refractive index correction can be minimized by using a low refractive index flowcell. An example of typical results using a low refractive index flowcell follows:

Salinity (‰)	Apparent silicate conc. due to refractive index (mg Si/L)
4.5	0.0003
9.0	0.0005
18.0	0.0016
27.0	0.0027
36.0	0.0042

12.2.5 An example of a typical equation is:

Apparent silicate (mg Si/L) = $0.00001953 * S^{1.5}$

where S is sample salinity. The form of fitted equation might vary as the design of flowcell, so the operators are advised to obtain the appropriate equation which has the best fit of their own data with the least fitting coefficients.

12.3 Correction for Salt Error in Estuarine and Coastal Samples

12.3.1 When calculating concentrations of samples of varying salinities from standards and wash solution prepared in reagent water, it is usual to first correct for refractive index errors, then correct for the change in color development due to the differences in ionic strength between samples and standards (salt error). The refractive index correction is negligible, so is optional, but correction for salt error is necessary.

12.3.2 Plot the salinity of the saline standards (Section 7.2.8) as the independent variable, and the apparent concentration of silicate (mg Si/L) from the peak height (corrected for refractive index) calculated from the regression of standards in reagent water, as the dependent variable for all 1.50 mg Si/L standards. The resulting regression equation allows the operator to correct the concentrations of samples of known salinity for the color suppression due to salinity effect, e.g., salt error. An example of typical results follows:

Salinty (‰)	Peak height of 1.50 mg Si/L	Uncorrected Si conc.calculated from standards in reagent water
0	2503	1.50
9	2376	1.32
18	2282	1.27
27	2250	1.25
36	2202	1.23

12.3.3 An example of a typical equation to correct for salt error is:

$$\text{Corrected mg Si/L} = \frac{\text{Uncorrected mg Si/L}}{1 - 0.02186 \times \sqrt{S}}$$

where S is salinity.

12.3.4 Results of sample analyses should be reported in mg Si/L or in µg Si/L.

mg Si/L = ppm (parts per million)
µg Si/L = ppb (part per billion)

13.0 Method Performance

13.1 Single Laboratory Validation

13.1.1 Method Detection Limit - A method detection limit (MDL) of 0.0012 mg Si/L has been determined by one laboratory in seawaters of three different salinities.

Salinity (‰)	SD (µg/L)	Recovery (%)	MDL (µg/L)
36	0.3924	105	1.233
36	0.4980	107	1.565
27	0.2649	104	0.832
27	0.3362	104	1.056
27	0.4671	100	1.468
18	0.3441	101	1.081
18	0.2809	105	0.883
18	0.2432	104	0.764
3	0.3441	101	1.081
3	0.2331	102	0.733
3	0.1963	98	0.617
3	0.2809	99	0.883

13.1.2 Single Analyst Precision - A single laboratory analyzed three samples collected from the Miami River and Biscayne Bay areas of Florida. Seven replicates of

each sample were processed and analyzed with salinities ranging from 2.86 to 35.85. The results were as follows:

Sample	Salinity (‰)	Concentration (mg Si/L)	RSD (%)
1	35.85	0.097	1.2
2	18.07	1.725	1.4
3	2.86	3.322	0.9

13.1.3 Laboratory Fortified Sample Matrix - Laboratory fortified sample matrixes were processed in three different salinities ranging from 2.86 to 35.85 and ambient concentrations from 0.095 to 3.322 mg Si/L with three fortified levels at each salinity. Seven replicates of each sample were analyzed and the results were as follows:

Salinity (‰)	Concentration (mg Si/L)		RSD (%)	Recovery (%)
	Ambient	Fortified		
35.85	0.095	0.1647	0.82	99.37
35.85	0.095	0.2196	1.34	100.61
35.85	0.095	0.2747	1.74	99.62
18.07	1.725	0.5517	1.11	107.18
18.07	1.725	1.1008	0.77	104.69
18.07	1.725	1.6508	0.98	103.62
2.86	3.322	0.5421	0.99	101.03
2.86	3.322	1.0801	1.26	103.22
2.86	3.322	1.6188	0.98	100.59

13.2 Multi-Laboratory Validation

Multi-laboratory validation has not been conducted for this method and, therefore, multi-laboratory data is currently unavailable.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society, Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The U.S. Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in Section 14.2.

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

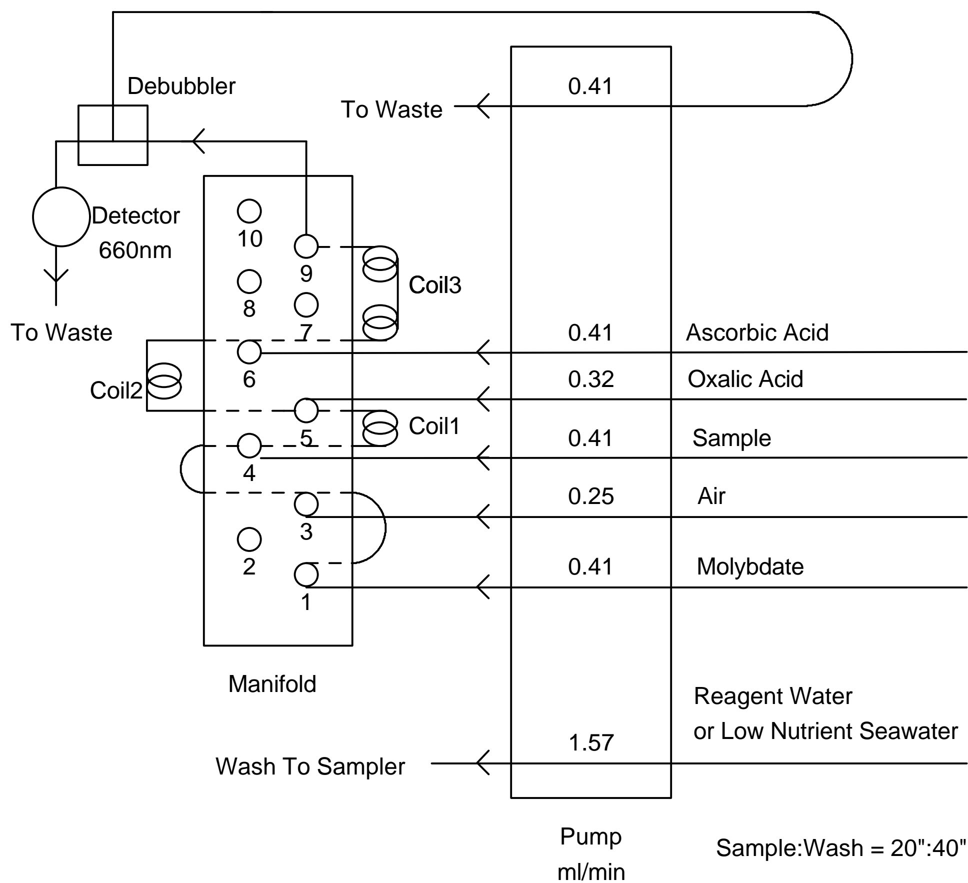

Figure 1. Manifold Configuration for Silicate Analysis.

Method 440.0

Determination of Carbon and Nitrogen in Sediments and Particulates of Estuarine/Coastal Waters Using Elemental Analysis

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Method 440.0

Determination of Carbon and Nitrogen in Sediments and Particulates of Estuarine/Coastal Waters Using Elemental Analysis

1.0 Scope and Application

1.1 Elemental analysis is used to determine particulate carbon (PC) and particulate nitrogen (PN) in estuarine and coastal waters and sediment. The method measures the total carbon and nitrogen irrespective of source (inorganic or organic).

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Carbon	7440-44-0
Nitrogen	1333-74-0

1.2 The need to qualitatively or quantitatively determine the particulate organic fraction from the total particulate carbon and nitrogen depends on the data-quality objectives of the study. Section 11.4 outlines procedures to ascertain the organic/inorganic particulate ratio. The method performance presented in the method was obtained on particulate samples with greater than 80% organic content. Performance on samples with a greater proportion of particulate inorganic versus organic carbon and nitrogen has not been investigated.

1.3 Method detection limits (MDLs)[1] of 10.5 µg/L and 62.3 µg/L for PN and PC, respectively, were obtained for a 200-mL sample volume. Sediment MDLs of PN and PC are 84 mg/kg and 1300 mg/kg, respectively, for a sediment sample weight of 10.00 mg. The method has been determined to be linear to 4800 µg of C and 700 µg of N in a sample. Multilaboratory study validation data are in Section 13.

1.4 This method should be used by analysts experienced in the theory and application of elemental analysis. A minimum of 6 months experience with an elemental analyzer is recommended.

1.5 Users of the method data should set the data-quality objectives prior to analysis. Users of the method must document and have on file the required initial demonstration of performance data described in Section 9.2 prior to using the method for analysis.

2.0 Summary of Method

2.1 An accurately measured amount of particulate matter from an estuarine water sample or an accurately weighed dried sediment sample is combusted at 975°C using an elemental analyzer. The combustion products are passed over a copper reduction tube to convert the oxides of N into molecular N. Carbon dioxide, water vapor and N are homogeneously mixed at a known volume, temperature and pressure. The mixture is released to a series of thermal conductivity detectors/traps, measuring in turn by difference, hydrogen (as water vapor), C (as carbon dioxide) and N (as N_2). Inorganic and organic C may be determined by two methods which are also presented.

3.0 Definitions

3.1 ***Sediment Sample --*** A fluvial, sand, or humic sample matrix exposed to a marine, brackish or fresh water environment. It is limited to that portion which may be passed through a number 10 sieve or a 2-mm mesh sieve.

3.2 ***Material Safety Data Sheet (MSDS) --*** Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.3 ***Instrument Detection Limit (IDL) --*** The minimum quantity of analyte or the concentration equivalent which gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time, absorbance line, etc.

3.4 ***Method Detection Limit (MDL) --*** The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero.

3.5 ***Linear Dynamic Range (LDR) --*** The absolute quantity over which the instrument response to an analyte is linear.

3.6 ***Calibration Standard (CAL) --*** An accurately weighed amount of a certified chemical used to calibrate the instrument response with respect to analyte mass.

3.7 ***Conditioner --*** A standard chemical which is not necessarily accurately weighed that is used to coat the surfaces of the instrument with the analytes (water vapor, carbon dioxide, and nitrogen).

3.8 ***External Standards (ES) --*** A pure analyte(s) that is measured in an experiment separate from the experiment used to measure the analyte(s) in the sample. The signal observed for a known quantity of the pure external standard(s) is used to calibrate the instrument

response for the corresponding analyte(s). The instrument response is used to calculate the concentrations of the analyte(s) in the sample.

3.9 Response Factor (RF) -- The ratio of the response of the instrument to a known amount of analyte.

3.10 Laboratory Reagent Blank (LRB) -- A blank matrix (i.e., a precombusted filter or sediment capsule) that is treated exactly as a sample including exposure to all glassware, equipment, solvents, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.11 Field Reagent Blank (FRB) -- An aliquot of reagent water or other blank matrix that is placed in a sample container in the laboratory and treated as a sample in all respects, including shipment to the sampling site, exposure to sampling site conditions, storage, preservation, and all analytical procedures. The purpose of the FRB is to determine if method analytes or other interferences are present in the field environment.

3.12 Laboratory Duplicates (LD1 and LD2) -- Two aliquots of the same sample taken in the laboratory and analyzed separately with identical procedures. Analyses of LD1 and LD2 indicate precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

3.13 Field Duplicates (FD1 and FD2) -- Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.14 Laboratory Fortified Blank (LFB) -- An aliquot of reagent water or other blank matrices to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the method is in control, and whether the laboratory is capable of making accurate and precise measurements.

3.15 Laboratory Fortified Sample Matrix (LFM) -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.16 Standard Reference Material (SRM) -- Material which has been certified for specific analytes by a variety of analytical techniques and/or by numerous laboratories using similar analytical techniques. These may consist of pure chemicals, buffers or compositional standards. These materials are used as an indication of the accuracy of a specific analytical technique.

3.17 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

4.0 Interferences

4.1 There are no known interferences for estuarine/coastal water or sediment samples. The presence of C and N compounds on laboratory surfaces, on fingers, in detergents and in dust necessitates the utilization of careful techniques (i.e., the use of forceps and gloves) to avoid contamination in every portion of this procedure.

5.0 Safety

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure to these compounds should be as low as reasonably achievable. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method.[2-5] A reference file of material safety data sheets (MSDS) should also be made available to all personnel involved in the chemical analysis.

5.2 The acidification of samples containing reactive materials may result in the release of toxic gases, such as cyanides or sulfides. Acidification of samples should be done in a fume hood.

5.3 All personnel handling environmental samples known to contain or to have been in contact with human waste should be immunized against known disease causative agents.

5.4 Although most instruments are adequately shielded, it should be remembered that the oven temperatures are extremely high and that care should be taken when working near the instrument to prevent possible burns.

5.5 It is the responsibility of the user of this method to comply with relevant disposal and waste regulations. For guidance see Sections 14.0 and 15.0.

6.0 Apparatus and Equipment

6.1 Elemental Analyzer

6.1.1 An elemental analyzer capable of maintaining a combustion temperature of 975°C and analyzing particulate samples and sediment samples for elemental C and N. The Leeman Labs Model 240 XA Elemental Analyzer was used to produce the data presented in this method.

6.2 A gravity convection drying oven. Capable of maintaining 103-105°C for extended periods of time.

6.3 Muffle furnace. Capable of maintaining 875°C ± 15°C.

6.4 Ultra-micro balance. Capable of accurately weighing to 0.1 µg. Desiccant should be kept in the weighing chamber to prevent hygroscopic effects.

6.5 Vacuum pump or source capable of maintaining up to 10 in. Hg of vacuum.

6.6 Mortar and pestle.

6.7 Desiccator, glass.

6.8 Freezer, capable of maintaining -20°C ± 5°C.

6.9 47-mm or 25-mm vacuum filter apparatus made up of a glass filter tower, fritted glass disk base and 2-L vacuum flask.

6.10 13-mm Swinlok filter holder.

6.11 Teflon-tipped, flat blade forceps.

6.12 Labware -- All reusable labware (glass, quartz, polyethylene, PTFE, FEP, etc.) should be sufficiently clean for the task objectives. Several procedures found to provide clean labware include washing with a detergent solution, rinsing with tap water, soaking for 4 hr or more in 20% (v/v) HCl, rinsing with reagent water and storing clean. All traces of organic material must be removed to prevent C-N contamination.

6.12.1 Glassware -- Volumetric flasks, graduated cylinders, vials and beakers.

6.12.2 Vacuum filter flasks -- 250 mL and 2 L, glass.

6.12.3 Funnel, 6.4 mm i.d., polyethylene.

6.12.4 Syringes, 60-mL, glass.

7.0 Reagents and Standards

7.1 Reagents may contain elemental impurities which affect analytical data. High-purity reagents that conform to the American Chemical Society specifications[6] should be used whenever possible. If the purity of a reagent is in question, analyze for contamination. The acid used for this method must be of reagent grade purity or equivalent. A suitable acid is available from a number of manufacturers.

7.2 Hydrochloric acid, concentrated (sp. gr. 1.19)-HCl.

7.3 Acetanilide, 99.9% + purity, $C_8H_{19}NO$ (CASRN 103-84-4).

7.4 Blanks -- Three blanks are used for the analysis. Two blanks are instrument related. The instrument zero response (ZN) is the background response of the instrument without sample holding devices such as capsules and sleeves. The instrument blank response (BN) is the response of the instrument when the sample capsule, sleeve and ladle are inserted for analysis without standard or sample. The BN is also the laboratory reagent blank (LRB) for sediment samples. The LRB for water samples includes the capsule, sleeve, ladle and a precombusted filter without standard or sample. These blanks are subtracted from the uncorrected instrument response used to calculate concentration in Sections 12.3 and 12.4.

7.4.1 Laboratory fortified blank (LFB) -- The third blank is the laboratory fortified blank. For sediment analysis, add a weighed amount of acetanilide in an aluminum capsule and analyze for PC and PN (Section 9.3.2). For aqueous samples, place a weighed amount of acetanilide on a glass fiber filter the same size as used for the sample filtration. Analyze the fortified filter for PC and PN (Section 9.3.2)

7.5 Quality Control Sample (QCS) -- For this method, the QCS can be any assayed and certified sediment or particulate sample which is obtained from an external source. The Canadian Reference Material, BCSS-1, is just such a material and was used in this capacity for the data presented in this method. The percent PC has been certified in this material but percent PN has not.

8.0 Sample Collection, Preservation and Storage

8.1 Water Sample Collection -- Samples collected for PC and PN analyses from estuarine/coastal waters are normally collected from a ship using one of two methods; hydrocast or submersible pump systems. Follow the recommended sampling protocols associated with the method used. Whenever possible, immediately filter the samples as described in Section 11.1.1. Store the filtered sample pads by freezing at -20°C or storing in a desiccator after drying at 103-105° C for 24 hr. No significant difference has been noted in comparing the two storage procedures for a time period of up to 100 days. If storage of the water sample is necessary, place

the sample into a clean amber bottle and store at 4°C until filtration is done.

8.1.1 The volume of water sample collected will vary with the type of sample being analyzed. Table 1 provides a guide for a number of matrices of interest. If the matrix cannot be classified by this guide, collect 2 x 1L of water from each site. A minimum filtration volume of 200 mL is recommended.

8.2 Sediment Sample Collection -- Estuarine/coastal sediment samples are collected with benthic samplers. The type of sampler used will depend on the type of sample needed by the data-quality objectives.[7] Store the wet sediment in a clean jar and freeze at -20°C until ready for analysis.

8.2.1 The amount of sediment collected will depend on the sample matrix and the elemental analyzer used. A minimum of 10 g is recommended.

9.0 Quality Control

9.1 Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory reagent blanks, laboratory duplicates, field duplicates and calibration standards analyzed as samples as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of data thus generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (MDLs, linear dynamic range) and laboratory performance (analysis of QC samples) prior to the analyses conducted by this method.

9.2.2 Linear dynamic range (LDR) -- The upper limit of the LDR must be established by determining the signal responses from a minimum of three different concentration standards across the range, one of which is close to the upper limit of the LDR. Determined LDRs must be documented and kept on file. The LDR which may be used for the analysis of samples should be judged by the analyst from the resulting data. The upper LDR limit should be an observed signal no more than 10% below the level extrapolated from the lower standards. Determined sample analyte concentrations that are 90% and above the upper LDR must be reduced in mass and reanalyzed. New LDRs should be determined whenever there is a significant change in instrument response and for those analytes that periodically approach the upper LDR limit, every 6 months or whenever there is a change in instrument analytical hardware or operating conditions.

9.2.3 Quality control sample (QCS) (Section 7.5) -- When beginning the use of this method, on a quarterly basis or as required to meet data quality needs, verify the calibration standards and acceptable instrument performance with the analyses of a QCS. If the determined concentrations are not within ± 5% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with analyses.

9.2.4 Method detection limits (MDLs) -- MDLs should be established for PC and PN using a low level estuarine water sample, typically three to five times higher than the estimated MDL. The same procedure should be followed for sediments. To determine MDL values, analyze seven replicate aliquots of water or sediment and process through the entire analytical procedure (Section 11). These replicates should be randomly distributed throughout a group of typical analyses. Perform all calculations defined in the method (Section 12) and report the concentration values in the appropriate units. Calculate the MDL as follows:[1]

$$MDL = (t) \times (S)$$

where, S = Standard deviation of the replicate analyses.

t = Student's t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

MDLs should be determined whenever a significant change in instrumental response, change of operator, or a new matrix is encountered.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory reagent blank (LRB) -- The laboratory must analyze at least one LRB (Section 3.10) with each batch of 20 or fewer samples of the same matrix. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the MDL indicate laboratory or reagent contamination. When LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples or field duplicates of the samples must be prepared and analyzed again after the source of contamination has been corrected and acceptable LRB values have been obtained. For aqueous samples the LRB is a precombusted filter of the same type and size used for samples.

9.3.2 Laboratory fortified blank (LFB) -- The laboratory must analyze at least one LFB (Section 7.4.1) with each batch of samples. Calculate accuracy as percent recovery. If the recovery of any analyte falls outside the required control limits of 85-115%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.

9.3.3 The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 85-115% (Section 9.3.2). When sufficient internal performance data become available (usually a minimum of 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

$$\text{Upper Control Limit} = x + 3S$$

$$\text{Lower Control Limit} = x - 3S$$

The optional control limits must be equal to or better than the required control limits of 85-115%. After each five to ten new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.4 Assessing Analyte Recovery and Data Quality

9.4.1 Percent recoveries cannot be readily obtained from particulate samples. Consequently, accuracy can only be assessed by analyzing check standards as samples and quality control samples (QCS). The use of laboratory fortified matrix samples has not been assessed.

10.0 Calibration and Standardization

10.1 Calibration -- After following manufacturer's installation and temperature stabilization procedures, daily calibration procedures must be performed and evaluated before sample analysis may begin. Single point or standard curve calibrations are possible, depending on instrumentation.

10.1.1 Establish single response factors (RF) for each element (C,H, and N) by analyzing three weighed portions of calibration standard (acetanilide). The mass of calibration standard should provide a response within 20% of the response expected for the samples being analyzed. Calculate the (RF) for each element using the following formula:

$$\text{Response factor } (\mu v/\mu g) = \frac{RN - ZN - BN}{WTN}$$

where,
- RN = Average instrument response to standard (µv)
- ZN = Instrument zero response (µv)
- BN = Instrument blank response (µv)

and,

$$WTN = (M)(N_a)(AW/MW)$$

where,
- M = The mass of standard material in µg
- N_a = Number of atoms of C, N or H, in a molecule of standard material
- AW = Atomic weight of C (12.01), N (14.01) or H (1.01)
- MW = Molecular weight of standard material (135.2 for acetanilide)

If instrument response is in units other than µv, then change the formula accordingly.

10.1.2 For standard curve preparation, the range of calibration standard masses used should be such that the low concentration approaches but is above the MDL and the high concentration is above the level of the highest sample, but no more than 90% of the linear dynamic range. A minimum of three concentrations should be used in constructing the curve. Measure response versus mass of element in the standard and perform a regression on the data to obtain the calibration curve.

11.0 Procedure

11.1 Aqueous Sample Preparation

11.1.1 Water Sample Filtration -- Precombust GF/F glass fiber filters at 500°C for 1.5 hr. The diameter of filter used will depend on the sample composition and instrument capabilities (Section 8.1.1). Store filters covered if not immediately used. Place a precombusted filter on fritted filter base of the filtration apparatus and attach the filtration tower. Thoroughly shake the sample container to suspend the particulate matter. Measure and record the required sample volume using a graduated cylinder. Pour the measured sample into the filtration tower, no more than 50 mL at a time. Filter the sample using a vacuum no greater than 10 in. of Hg. Vacuum levels greater than 10 in. of Hg can cause filter rupture. If less than the measured volume of sample can be practically filtered due to clogging, measure and record the actual volume filtered. **Do not** rinse the filter following filtration. It has been demonstrated that sample loss occurs when the filter is rinsed with an isotonic solution or the filtrate.[8] Air dry the filter after the sample has passed through by continuing the vacuum for 30 sec. Using Teflon-coated flat-tipped forceps, fold the filters in half while still on the fritted glass base of the filter apparatus. Store filters as described in Section 8.

11.1.2 If the sample has been stored frozen, place the sample in a drying oven at 103-105° C for 24 hr before analysis and dry to a constant weight. Precombust one nickel sleeve at 875° C for 1 hr for each sample.

11.1.3 Remove the filter pads containing the particulate material from the drying oven and insert into a pre-combusted nickel sleeve using Teflon-coated flat-tipped forceps. Tap the filter pad using a stainless steel rod. The sample is ready for analysis.

11.2 Sediment Samples Preparation

11.2.1 Thaw the frozen sediment sample in a 102-105°C drying oven for at least 24 hr before analysis and dry to a constant weight. After drying, homogenize the dry sediment with a mortar and pestle. Store in a desiccator until analysis. Precombust aluminum capsules at 550°C in a muffle furnace for 1.5 hr for each sediment sample being analyzed. Precombust one nickel sleeve at 875°C for 1 hr for each sediment sample.

11.2.2 Weigh 10 mg of the homogenized sediment to the nearest 0.001 mg with an ultra-micro balance into a precombusted aluminum capsule. Crimp the top of the aluminum capsule with the Teflon-coated flat-tipped forceps and place into a precombusted nickel sleeve. The sample is ready for analysis.

11.3 Sample Analysis

11.3.1 Measure instrument zero response (Section 7.4) and instrument blank response (Section 7.4) and record values. Condition the instrument by analyzing a conditioner. Calibrate the instrument according to Section 10 and analyze all preliminary QC samples as required by Section 9. When satisfactory control has been established, analyze samples according to the instrument manufacturer's recommendations. Record all response data.

11.3.2 Report data as directed in Section 12.

11.4 Determination of Particulate Organic and Inorganic Carbon

11.4.1 Method 1: Thermal Partitioning -- The difference found between replicate samples, one of which has been analyzed for total PC and PN and the other which was muffled at 550°C and analyzed is the particulate organic component of that sample. This method of thermally partitioning organic and inorganic PC may underestimate slightly the carbonate minerals' contribution in the inorganic fraction since some carbonate minerals decompose below 500°C, although $CaCO_3$ does not.[9]

11.4.2 Method 2: Fuming HCl -- Allow samples to dry overnight at 103-105°C and then place in a desiccator containing concentrated HCl, cover and fume for 24 hr in a hood. The fuming HCl converts inorganic carbonate in the samples to water vapor, CO_2 and calcium chloride. Analyze the samples for particulate C. The resultant data are particulate organic carbon.[10]

12.0 Data Analysis and Calculations

12.1 Sample data should be reported in units of µg/L for aqueous samples and mg/kg dry weight for sediment samples.

12.2 Report analyte concentrations up to three significant figures for both aqueous and sediment samples.

12.3 For aqueous samples, calculate the sample concentration using the following formula:

$$\text{Concentration } (\mu g/L) = \frac{\text{Corrected sample response } (\mu v)}{\text{Sample volume (L)} \times \text{RF } (\mu v/\mu g)}$$

where, RF = Response Factor (Section 10.1.1)
Corrected Sample Response (Section 7.4)

12.4 For sediment samples, calculate the sample concentration using the following formula:

$$\text{Concentration } (mg/kg) = \frac{\text{Corrected sample response } (\mu v)}{\text{Sample weight (g)} \times \text{RF } (\mu v/\mu g)}$$

where, RF = Response Factor (Section 10.1.1)
Corrected Sample Response (Section 7.4)

Note: Units of µg/g = mg/kg

12.5 The QC data obtained during the analyses provide an indication of the quality of the sample data and should be provided with the sample results.

13.0 Method Performance

***13.1* Single Laboratory Performance**

13.1.1 Single laboratory performance data for aqueous samples from the Chesapeake Bay are provided in Table 2.

13.1.2 Single-laboratory precision and accuracy data for the marine sediment reference material, BCSS-1, are listed in Table 3.

***13.2* Multilaboratory Performance**

13.2.1 In a multilab study, 13 participants analyzed sediment and filtered estuarine water samples for particulate carbon and nitrogen. The data were analyzed

using the statistical procedures recommended in ASTM D2777-86 for replicate designs. See Table 4 for summary statistics.

13.2.2 Accuracy as mean recovery was estimated from the analyses of the NRC of Canada Marine Sediment Reference Material, BCSS-1. Mean recovery was 98.2% of the certified reference carbon value and 100% of the noncertified nitrogen value.

13.2.3 Overall precision for analyses of carbon and nitrogen in sediments was 1-11% RSD, while the analyses of both particulate carbon and nitrogen in estuarine water samples was 9-14% RSD.

13.2.4 Single analyst precision for carbon and nitrogen in sediment samples was 1-8% RSD and 4-9% for water samples.

13.2.5 Pooled method detection limits (p-MDLs) were calculated using the pooled single analyst standard deviations. The p-MDLs for particulate nitrogen and carbon in estuarine waters were 0.014 mg N/L and 0.064 mg C/L , respectively. The p-MDLs for percent carbon and nitrogen in estuarine sediments were not estimated because the lowest concentration sediment used in the study was still 20 times higher than the estimated MDLs. Estimates of p-MDLs from these data would be unrealistically high.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction,* available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel,* available from the American Chemical Society at the address listed in Section 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Filter Diameter Selection Guide

Sample matrix	Filter diameter 47mm	25mm	13mm
	Sample matrix volume		
Open ocean	2000 mL	500 mL	100 mL
Coastal	1000 mL	400-500 mL	100 mL
Estuarine (low particulate)	500-700 mL	250-400 mL	50 mL
Estuarine (high particulate)	100-400 mL	75-200 mL	25 mL

Table 2. Performance Data--Chesapeake Bay Aqueous Samples

Sample	Measured nitrogen concentration (µg/L)	S.D.[A] (µg/L)	Measured carbon concentration (µg/L)	S.D.[A] (µg/L)
1	147	± 4	1210	± 49
2	148	± 11	1240	± 179
3	379	± 51	3950	± 269
4	122	± 9	1010	± 63

[A] Standard deviation based on 7 replicates.

Table 3. Precision and Accuracy Data - Canadian Sediment Reference Material BCSS-1

Element	T.V.[A]	Mean measured value (%)	%RSD[B]	%Recovery[C]
Carbon	2.19%	2.18	± 3.3	99.5
Nitrogen	0.195%	0.194	± 3.9	99.5

[A] True value. Carbon value is certified; nitrogen value is listed but not certified

[B] Percent relative standard deviation based on 10 replicates.

[C] As calculated from T.V.

Table 4. Overall and Single Analyst Precision Estimates from Collaborative Study

Analyte	Sample	N[1]	Mean[2] Conc.	Overall Std. Dev.	Overall %RSD	Analyst Std. Dev.	Analyst %RSD
Particulate Nitrogen (as N) in Estuarine Waters	A	11	0.0655	0.0081	12.4%	0.0050	7.6%
	B	12	0.0730	0.0076	10.3%	0.0057	7.7%
	C	12	0.0849	0.0110	12.9%	0.0060	7.1%
	D	12	0.126	0.0138	11.0%	0.0071	5.6%
	E	11	0.182	0.0245	13.5%	0.0157	8.6%
Nitrogen (as %N) in Estuarine Water	1	10	0.178	0.0190	10.7%	0.0131	7.3%
	2	10	0.295	0.0114	3.9%	0.0046	1.6%
	3	10	0.436	0.0178	4.1%	0.0104	2.4%
	4	10	0.497	0.0183	3.7%	0.0082	1.6%
	5	10	0.580	0.0207	3.6%	0.0150	2.6%
Particulate Carbon (as C) in Estuarine Waters	B	12	0.369	0.0505	13.7%	0.0222	6.0%
	A	12	0.417	0.0490	11.8%	0.0230	5.5%
	D	12	0.619	0..0707	11.4%	0.0226	3.6%
	C	12	0.710	0.0633	8.9%	0.0367	5.2%
	E	12	0.896	0.1192	13.3%	0.0569	6.4%
Carbon (as %C) in Estuarine Sediments	1	13	1.78	0.1517	8.5%	0.1346	7.6%
	2	13	2.55	0.0372	1.5%	0.0204	0.8%
	3	13	3.18	0.0435	1.4%	0.0348	1.1%
	4	13	4.92	0.1201	2.4%	0.0779	1.6%
	5	13	5.92	0.0621	1.1%	0.0547	0.9%

(1) N = Number of participants whose data was used.

(2) Concentration in mg/L or percent, as indicated.

Method 445.0

In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence

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Method 445.0

In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence

1.0 Scope and Application

1.1 This method provides a procedure for low level determination of chlorophyll *a* (chl *a*) and its magnesium-free derivative, pheophytin *a* (pheo *a*), in marine and freshwater phytoplankton using fluorescence detection.[1,2] Phaeophorbides present in the sample are determined collectively as pheophytin *a*. For users primarily interested in chl *a* there is currently available a set of very narrow bandpass excitation and emission filters (Turner Designs, Sunnyvale, CA) that nearly eliminates the spectral interference caused by the presence of pheo *a* and chlorophyll *b*. The difference between the modified method and the conventional fluorometric method is that the equations used for the determination of chlor *a* without pheo *a* correction (uncorrected chlor *a*), are used instead of the equations for "corrected chlor *a*". This EPA laboratory has evaluated the modified filters and found the technique to be an acceptable alternative to the conventional fluorometric method using pheo *a* correction.[3]

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8

1.2 Instrumental detection limits (IDL) of 0.05 µg chl *a*/L and 0.06 µg pheo *a*/L in a solution of 90% acetone were determined by this laboratory. Method detection limits (MDL) using mixed assemblages of algae provide little information because the fluorescence of other pigments interferes in the fluorescence of chlorophyll *a* and pheophytin *a*.[4] A single lab estimated detection limit for chlorophyll *a* was determined to be 0.11 µg/L in 10 mL of final extraction solution. The upper limit of the linear dynamic range for the instrumentation used in this method evaluation was 250 µg chl *a*/L.

1.3 This method was multilaboratory validated in 1996.[5] Results from that study may be found in Section 13. Additional QC procedures also have been added as a result of that study.

1.4 This method uses 90% acetone as the extraction solvent because of its efficiency for most types of algae. There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol[5-8] or dimethyl sulfoxide.[9] Bowles, et al.[8] found that for chlorophyll *a*, however, 90% acetone was an effective extractant when the extraction period was optimized for the dominant species present in the sample.

1.5 Depending on the type of algae under investigation, this method can have uncorrectable interferences (Sect. 4.0). In cases where taxomonic classification is unavailable, a spectrophotometric or high performance liquid chromatographic (HPLC) method may provide more accurate data for chlorophyll *a* and pheophytin *a*.

1.6 This method is for use by analysts experienced in the handling of photosynthetic pigments and in the operation of fluorescence detectors or by analysts under the close supervision of such qualified persons.

2.0 Summary of Method

2.1 Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated by filtering at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton in 90% acetone with the aid of a mechanical tissue grinder and allowed to steep for a minimum of 2 h, but not to exceed 24 h, to ensure thorough extraction of the chlorophyll *a*. The filter slurry is centrifuged at 675 g for 15 min (or at 1000 g for 5 min) to clarify the solution. An aliquot of the supernatant is transferred to a glass cuvette and fluorescence is measured before and after acidification to 0.003 N HCl with 0.1 N HCl. Sensitivity calibration factors, which have been previously determined on solutions of

pure chlorophyll *a* of known concentration, are used to calculate the concentration of chlorophyll *a* and pheophytin *a* in the sample extract. The concentration in the natural water sample is reported in µg/L.

3.0 Definitions

3.1 Estimated Detection Limit (EDL) -- The minimum concentration of an analyte that yields a fluorescence 3X the fluorescence of blank filters which have been extracted according to this method.

3.2 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.3 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent which gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time, absorbance line, etc. For this method the background is a solution of 90% acetone.

3.4 Stock Standard Solution (SSS) -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

3.5 Primary Dilution Standard Solution (PDS) -- A solution of the analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.6 Calibration Standard (CAL) -- A solution prepared from the primary dilution standard solution or stock standard solutions containing the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.7 Response Factor (RF) -- The ratio of the response of the instrument to a known amount of analyte.

3.8 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or apparatus.

3.9 Field Duplicates (FD1 and FD2) -- Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.10 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. Ideally, the QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.11 Material Safety Data Sheet (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

4.0 Interferences

4.1 Any substance extracted from the filter or acquired from laboratory contamination that fluoresces in the red region of the spectrum may interfere in the accurate measurement of both chlorophyll *a* and pheophytin *a*.

4.2 The relative amounts of chlorophyll *a*, *b* and *c* vary with the taxonomic composition of the phytoplankton. Chlorophylls *b* and *c* may significantly interfere with chlorophyll *a* measurements depending on the amount present. Due to the spectral overlap of chlorophyll *b* with pheophytin *a* and chlorophyll *a*, underestimation of chlorophyll *a* occurs accompanied by overestimation of pheophytin *a* when chlorophyll *b* is present in the sample. The degree of interference depends upon the ratio of *a*:*b*. This laboratory found that at a ratio of 5:1, using the acidification procedure to correct for pheophytin *a*, chlorophyll *a* was underestimated by approximately 5%. Loftis and Carpenter[10] reported an underestimation of 16% when the *a*:*b* ratio was 2.5:1. A ratio of 1:1 is the highest ratio likely to occur in nature. They also reported overestimation of chlorophyll *a* in the presence of chlorophyll *c* of as much as 10% when the *a*:*c* ratio was 1:1 (the theoretical maximum likely to occur in nature). The presence of chlorophyll *c* also causes the under-

estimation of pheophytin *a*. The effect of chlorophyll *c* is not as severe as the effect of chlorophyll *b* on the measurement of chlorophyll *a* and pheophytin *a*. Knowledge of the taxonomy of the algae under consideration will aid in determining if the spectrophotometric method using trichromatic equations to determine chlorophyll *a*, *b*, and *c* or an HPLC method would be more appropriate.[11-16] In the presence of chlorophyll *b* or pheopigments, the modified fluorometric method described here is also appropriate.[5]

4.3 Quenching effects are observed in highly concentrated solutions or in the presence of high concentrations of other chlorophylls or carotenoids. Minimum sensitivity settings on the fluorometer should be avoided; samples should be diluted instead.

4.4 Fluorescence is temperature dependent with higher sensitivity occurring at lower temperatures. Samples, standards, LRBs and QCSs must be at the same temperature to prevent errors and/or low precision. Analyses of samples at ambient temperature is recommended in this method. Ambient temperature should not fluctuate more than ± 3°C between calibrations or recalibration of the fluorometer will be necessary.

4.5 Samples must be clarified by centrifugation prior to analysis.

4.6 All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials and filter samples must be stored in the dark at -20°C or -70°C to prevent degradation.

5.0 Safety

5.1 The toxicity or carcinogenicity of the chemicals used in this method have not been fully established. Each chemical should be regarded as a potential health hazard and handled with caution and respect. Each laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method.[17-20] A file of MSDS should also be made available to all personnel involved in the chemical analysis.

5.2 The grinding of filters during the extraction step of this method should be conducted in a fume hood due to the volatilization of acetone by the tissue grinder.

6.0 Apparatus and Equipment

6.1 Fluorometer -- Equipped with a high intensity F4T.5 blue lamp, red-sensitive photomultiplier, and filters for excitation (CS-5-60) and emission (CS-2-64). A Turner Designs Model 10 Series fluorometer was used in the evaluation of this method. The modified method requires excitation filter (436FS10) and emission filter (680FS10).

6.2 Centrifuge, capable of 675 g.

6.3 Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with 1/4" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity glass grinding tube.

6.4 Filters, glass fiber, 47-mm or 25-mm, nominal pore size of 0.7 µm unless otherwise justified by data quality objectives. Whatman GF/F filters were used in this work.

6.5 Petri dishes, plastic, 50 X 9-mm, or some other solid container for transporting and storing sampled filters.

6.6 Aluminum foil.

6.7 Laboratory tissues.

6.8 Tweezers or flat-tipped forceps.

6.9 Vacuum pump or source capable of maintaining a vacuum up to 6 in. Hg.

6.10 Room thermometer.

6.11 Labware -- All reusable labware (glass, polyethylene, Teflon, etc.) that comes in contact with chlorophyll solutions should be clean and acid free. An acceptable cleaning procedure is soaking for 4 h in laboratory grade detergent and water, rinsing with tap water, distilled deionized water and acetone.

6.11.1 Assorted Class A calibrated pipets.

6.11.2 Graduated cylinders, 500-mL and 1-L.

6.11.3 Volumetric flasks, Class A calibrated, 25-mL, 50-mL, 100-mL and 1-L capacity.

6.11.4 Glass rods.

6.11.5 Pasteur type pipets or medicine droppers.

6.11.6 Disposable glass cuvettes for the fluorometer.

6.11.7 Filtration apparatus consisting of 1 or 2-L filtration flask, 47-mm fritted glass disk base and a glass filter tower.

6.11.8 Centrifuge tubes, polypropylene or glass, 15-mL capacity with nonpigmented screw-caps.

6.11.9 Polyethylene squirt bottles.

7.0 Reagents and Standards

7.1 Acetone, HPLC grade, (CASRN 67-64-1).

7.2 Hydrochloric acid (HCl), concentrated (sp. gr. 1.19), (CASRN 7647-01-0).

7.3 Chlorophyll *a* free of chlorophyll *b*. May be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO). Turner Designs (Sunnyvale, CA) supplies ready-made standards.

7.4 **Water** -- ASTM Type I water (ASTM D1193) is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.

7.5 ***0.1 N HCl Solution*** -- Add 8.5 mL of concentrated HCl to approximately 500 mL water and dilute to 1 L.

7.6 ***Aqueous Acetone Solution*** -- 90% acetone /10% water. Carefully measure 100 mL of water into the 1-L graduated cylinder. Transfer to a 1-L flask or storage bottle. Measure 900 mL of acetone into the graduated cylinder and transfer to the flask or bottle containing the water. Mix, label and store.

7.7 ***Chlorophyll Stock Standard Solution*** (SSS) -- Chlorophyll *a* from a commercial supplier will be shipped in an amber glass ampoule which has been flame sealed. This dry standard should be stored at -20 or -70°C in the dark and the SSS prepared just prior to use. Tap the ampoule until all the dried chlorophyll is in the bottom of the ampoule. In subdued light, carefully break the tip off the ampoule. Transfer the entire contents of the ampoule into a 50-mL volumetric flask. Dilute to volume with 90% acetone, label the flask and wrap with aluminum foil to protect from light. The concentration of the solution must be determined spectrophotometrically using a multiwavelength spectrophotometer.[10] When stored in a light and airtight container at freezer temperatures, the SSS is stable for at least six months. The concentration of all dilutions of the SSS must be determined spectrophotometrically each time they are made.

7.8 ***Laboratory Reagent Blank*** (LRB) -- A blank filter which is extracted and analyzed just as a sample filter. The LRB should be the last filter extracted of a sample set. It is used to assess possible contamination of the reagents or apparatus.

7.9 ***Chlorophyll** a **Primary Dilution Standard Solution*** (PDS) -- Add 1 mL of the SSS (Sect. 7.8) to a clean 100-mL flask and dilute to volume with the aqueous acetone solution (Sect. 7.7). If exactly 1 mg of pure chlorophyll *a* was used to prepare the SSS, the concentration of the PDS is 200 µg/L. Prepare fresh just prior to use.

7.10 ***Quality Control Sample*** (QCS) -- Since there are no commercially available QCSs, dilutions of a stock standard of a different lot number from that used to prepare calibration solutions may be used.

8.0 Sample Collection, Preservation and Storage

8.1 ***Water Sample Collection*** -- Water may be obtained by a pump or grab sampler. Data quality objectives will determine the depth at which samples are taken. Healthy phytoplankton, however, are generally obtained from the photic zone (depth at which the illumination level is 1% of surface illumination). Enough water should be collected to concentrate phytoplankton on at least three filters so that precision can be assessed. Filtration volume size will depend on the particulate load of the water. Four liters may be required for open ocean water where phytoplankton density is usually low, whereas 1 L or less is generally sufficient for lake, bay or estuary water. All apparatus should be clean and acid-free. Filtering should be performed in subdued light as soon as possible after sampling since algal poulations, thus chlorophyll *a* concentration, can change in relatively short periods of time. Aboard ship filtration is highly recommended.

Assemble the filtration apparatus and attach the vacuum source with vacuum gauge and regulator. Vacuum filtration should not exceed 6 in. Hg (20 kPa). Higher

filtration pressures and excessively long filtration times (> 10 min) may damage cells and result in loss of chorophyll.

Prior to drawing a subsample from the water sample container, thoroughly but gently agitate the container to suspend the particulates (stir or invert several times). Pour the subsample into a graduated cylinder and accurately measure the volume. Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum (not to exceed 20 kPa). A sufficient volume has been filtered when a visible green or brown color is apparent on the filter. Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter. Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in the petri dish or other suitable container. If the filter will not be immediately extracted, then wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20 or -70°C. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20 or -70°C as soon as possible.

8.2 ***Preservation*** -- Sampled filters should be stored frozen (-20°C or -70°C) in the dark until extraction.

8.3 ***Holding Time*** -- Filters can be stored frozen at -20 or -70°C for as long as 3½ weeks without significant loss of chlorophyll *a*.[21]

9.0 Quality Control

9.1 Each Laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory reagent blanks, field duplicates and quality control samples as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data thus generated.

9.2 **Initial Demonstration of Performance (Mandatory)**

9.2.1 The initial demonstration of performance is used to characterize instrument performance (instrumental detection limits, linear dynamic range and MDLs) and laboratory performance (analyses of QCSs) prior to sample analyses.

9.2.2 Linear Dynamic Range (LDR) -- The LDR should be determined by analyzing a minimum of 5 calibration standards ranging in concentration from 0.2 µg/L to 200 µg chl *a*/L across all sensitivity settings of the fluorometer. If using an analog fluorometer or a digital fluorometer requiring manual changes in sensitivity settings, normalize responses by dividing the response by the sensitivity setting multiplier. Perform the linear regression of normalized response vs. concentration and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured fluorescence response, R, of a standard no longer yields a calculated concentration, C_c, that is ± 10% of the known concentration, C, where $C_c = (R - b)/m$. That concentration defines the upper limit of the LDR for your instrument. Should samples be encountered that have a concentration which is 90% of the upper limit of the LDR, these samples must be diluted and reanalyzed.

9.2.3 Instrumental Detection Limit (IDL) -- Zero the fluorometer with a solution of 90% acetone on the maximum sensitivity setting. Pure chlorophyll *a* in 90% acetone should be serially diluted until it is no longer detected by the fluorometer on a maximum sensitivity setting.

9.2.4 Estimated Detection Limit (EDL) -- Several blank filters should be extracted according to the procedure in Sect. 11, using clean glassware and apparatus, and the fluorescence measured. A solution of pure chlorophyll *a* in 90% acetone should be serially diluted until it yields a response which is 3X the average response of the blank filters.

9.2.5 Quality Control Sample (QCS) -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify the calibration standards and acceptable instrument performance with the analysis of a QCS (Sect. 7.10). If the determined value is not within the confidence limits established by project data quality objectives, then the determinative step of this method is unacceptable. The source of the problem must be identified and corrected before continuing analyses.

9.2.6 Extraction Proficiency -- Personnel performing this method for the first time should demonstrate proficiency in the extraction of sampled filters (Sect. 11.1).

Twenty to thirty natural samples should be obtained using the procedure outlined in Sect. 8.1 of this method. Sets of 10 or more samples should be extracted and analyzed according to Sect. 11.2. The percent relative standard deviation (%RSD) of uncorrected values of chlorophyll *a* should not exceed 15% for samples that are approximately 10X the IDL. RSD for pheophytin *a* might typically range from 10 to 50%.

9.2.7 Corrected Chl *a* -- Multilaboratory testing of this method revealed that many analysts do not adequately mix the acidified sample when determining corrected chl *a*. The problem manifests itself by highly erratic pheo-*a* results, high %RSDs for corrected chl *a* and poor agreement between corrected and uncorrected chl *a*. To determine if a new analyst is performing the acidification step properly, perform the following QC procedure:

Prepare 100 mL of a 50 ppb chl *a* solution in 90% acetone. The new analyst should analyze 5-10 separate aliquots, using separate cuvettes, according to instructions in Section 11.2. Process the results according to Section 12 and calculate separate means and %RSDs for corrected and uncorrected chl *a*. If the means differ by more than 10%, then the stock chl *a* has probably degraded and fresh stock should be prepared. The %RSD for corrected chl *a* should not exceed 5%. If the %RSD exceeds 5%, repeat the procedure until the %RSD 5%.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one blank filter with each sample batch. The LRB should be the last filter extracted. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the IDL indicate contamination from the laboratory environment. When LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples or field duplicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

10.0 Calibration and Standardization

10.1 Calibration -- Calibration should be performed bimonthly or when there has been an adjustment made to the instrument, such as replacement of lamp, filters or photomultiplier. Prepare 0.2, 2, 5, 20 and 200 µg chl *a*/L calibration standards from the PDS (Sect. 7.11). Allow the instrument to warm up for at least 15 min. Measure the fluorescence of each standard at sensitivity settings that provide midscale readings. Obtain response factors for chlorophyll *a* for each sensitivity setting as follows:

$$F_s = C_a/R_s$$

where:

F_s = response factor for sensitivity setting, S.

R_s = fluorometer reading for sensitivity setting, S.

C_a = concentration of chlorophyll *a*.

NOTE: If you are using special narrow bandpass filters for chl *a* determination, **DO NOT** acidify. Use the "uncorrected" chl *a* calculation described in Section 12.1.

If pheophytin *a* determinations will be made, it will be necessary to obtain before-to-after acidification response ratios of the chlorophyll *a* calibration standards as follows: (1) measure the fluorescence of the standard, (2) remove the cuvette from the fluorometer, (3) acidify the solution to .003 N HCl[6] with the 0.1 N HCl solution, (4) use a pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample, (5) wait 90 sec and measure the fluorescence of the standard solution again. Addition of the acid may be made using a medicine dropper. It will be necessary to know how many drops are equal to 1 mL of acid. For a cuvette that holds 5 mL of extraction solution, it will be necessary to add 0.15 mL of 0.1 N HCl to reach a final acid concentration of 0.003N in the 5 mL. Calculate the ratio, r, as follows:

$$r = R_b/R_a$$

where:

R_b = fluorescence of pure chlorophyll *a* standard solution before acidification.

R_a = fluorescence of pure chlorophyll *a* standard solution after acidification.

11.0 Procedure

11.1 Extraction of Filter Samples

11.1.1 If sampled filters have been frozen, remove them from the freezer but keep them in the dark. Set up the tissue grinder and have on hand tissues and squirt bottles containing water and acetone. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation. Remove a filter from its container and place it in the glass grinding tube. The filter may be torn into smaller pieces to facilitate extraction. Push it to the bottom of the tube with a glass rod. With a volumetric pipet, add 4 mL of the aqueous acetone solution (Sect. 7.6) to the grinding tube. Grind the filter until it has been converted to a slurry. (**NOTE:** Although grinding is required, care must be taken not to overheat the sample. Good judgement and common sense will help you in deciding when the sample has been sufficiently macerated.) Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 6-mL volumetric pipet, rinse the pestle and the grinding tube with 90% acetone. Add the rinse to the centrifuge tube containing the filter slurry. Cap the tube and shake it vigorously. Place it in the dark before proceeding to the next filter extraction. Before placing another filter in the grinding tube, use the acetone and water squirt bottles to thoroughly rinse the pestle, grinding tube and glass rod. The last rinse should be with acetone. Use a clean tissue to remove any filter residue that adheres to the pestle or to the steel rod of the pestle. Proceed to the next filter and repeat the steps above. The entire extraction with transferring and rinsing steps takes 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.2 Shake each tube vigorously before placing them to steep in the dark at 4ºC. Samples should be allowed to steep for a minimum of 2 h but not to exceed 24 h. The tubes should be shaken at least once during the steeping period.

11.1.3 After steeping is complete, shake the tubes vigorously and centrifuge samples for 15 min at 675 g or for 5 min at 1000 g. Samples should be allowed to come to ambient temperature before analysis. This can be done by placing the tubes in a constant temperature water bath or by letting them stand at room temperature for 30 min. Recalibrate the fluorometer if the room temperature fluctuated ± 3°C from the last calibration date.

11.2 SAMPLE ANALYSIS

11.2.1 After the fluorometer has warmed up for at least 15 min, use the 90% acetone solution to zero the instrument on the sensitivity setting that will be used for sample analysis.

11.2.2 Pour or pipet the supernatant of the extracted sample into a sample cuvette. The volume of sample required in your instrument's cuvette should be known so that the correct amount of acid can be added in the pheophytin *a* determinative step. For a cuvette that holds 5 mL of extraction solution, 0.15 mL of the 0.1 N HCl solution should be used. Choose a sensitivity setting that yields a midscale reading when possible and avoid the minimum sensitivity setting. If the concentration of chlorophyll *a* in the sample is ≥ 90% of the upper limit of the LDR, then dilute the sample with the 90% acetone solution and reanalyze. Record the fluorescence measurement and sensitivity setting used for the sample. Remove the cuvette from the fluorometer and acidify the extract to a final concentration of 0.003 N HCl using the 0.1 N HCl solution. Use a pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample. Wait 90 sec before measuring fluorescence again. NOTE: Proper mixing is critical for precise and accurate results. Twenty-five to thirty-five samples can be extracted and analyzed in one 8 hr day.

NOTE: If you are using special narrow bandpass filters for chl *a* determination, **DO NOT** acidify samples. Use the “uncorrected” chl *a* calculations described in Section 12.1.

12.0 Data Analysis and Calculations

12.1 For “uncorrected chlorophyll *a*,” calculate the chlorophyll *a* concentration in the extract as:

$$C_{E,u} = R_b \times F_s$$

where $C_{E,u}$ = uncorrected chlorophyll *a* concentration (µg/L) in the extract solution analyzed,

R_b = fluoresence response of sample extract before acidification, and

F_s = fluoresence respnse factor for sensitivity setting S.

Calculate the "uncorrected" concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{S,u} = \frac{C_{E,u} \; x \; extract\ volume\ (L) \; X \; DF}{sample\ volume\ (L)}$$

where $C_{S,u}$ **=** uncorrected chlorophyll *a* concentration (µg/L) in the whole water sample,

extract volume = volume (L) of extraction prepared before any dilutions,

DF = dilution factor,

sample volume = volume (L) of whole water sample.

12.2 For "corrected chlorophyll *a*", calculate the chlorophyll *a* concentration in the extract as :

$C_{E,c} = F_s\ (r/r\text{-}1)\ (R_b - R_a)$

where:

$C_{E,c}$ = corrected chlorophyll *a* concentration (µg/L) in the extract solution analyzed,

F_s = response factor for the sensitivity setting S,

r = the before-to-after acidification ratio of a pure chlorophyll *a* solution (Sect. 10.1),

R_b = fluorescence of sample extract before acidification, and

R_a = fluorescence of sample extract after acidification.

Calculate the "corrected" concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{s,c} = \frac{C_{E,u} \; x \; extract\ volume\ (L) \; X \; DF}{sample\ volume\ (L)}$$

where $C_{S,c}$ = corrected chlorophyll *a* concetration (µg/L) in the whole water sample,

extract volume = volume (L) of extract prepared before dilution,

12.3 Calculate the pheophytin *a* concentration as follows:

$P_E = F_s\ (r/r\text{-}1)\ (rR_a - R_b)$

$$P_s = \frac{P_E \; X \; extract\ volume\ (L) \; X \; DF}{sample\ volume\ (L)}$$

where P_E = pheophytin *a* concentration (µg/L) in the sample extract; and

P_s = pheophytin *a* concentration (µg/L) in the whole water sample.

12.4 LRB and QCS data should be reported with each sample data set.

13.0 Method Performance

13.1 The single lab EDL for the instrument used in the evaluation of this method was 0.05 µg/L for chlorophyll *a* and 0.06 µg/L pheophytin *a*.

13.2 The precision (%RSD) for chlorophyll *a* in mostly blue-green and green phytoplankton natural samples which were steeped for 2 h vs 24 h is reported in Table 1. Although the means were the same, precision was better for samples which were allowed to steep for 24 h prior to analysis. Since pheophytin *a* was found in the samples, the chlorophyll *a* values are "corrected" (Sect. 12.2). Table 2 contains precision data for pheophytin *a*. A statistical analysis of the pheophytin *a* data indicated a significant difference in the mean values at the 0.05 significance level. The cause of the lower pheophytin *a* values in samples extracted for 24 h is not known.

13.3 Three QCS ampoules obtained from the USEPA were analyzed and compared to the reported confidence limits in Table 3. **NOTE:** The USEPA no longer provides these QCSs.

13.4 **Multilaboratory Testing** - A multilaboratory validation and comparison study of EPA Methods 445.0, 446.0 and 447.0 for chlorophyll *a* was conducted in 1996 by Research Triangle Institute, Research Triangle Park, N.C. (EPA Contract No. 68-C5-0011). There were 21 volunteer participants in the fluorometric methods

component that returned data; 10 that used the modified fluorometric method and 11 that used the conventional method. The primary goals of the study were to determine estimated detection limits and to assess precision (%RSD) and bias (as percent recovery) for select unialgal species, and natural seawater.

13.4.1 The term, pooled estimated detection limit (p-EDL), is used in this method to distinguish it from the EPA defined method detection limit (MDL). An EPA MDL determination is not possible nor practical for a natural water or pure species sample due to known spectral interferences and to the fact that it is impossible to prepare solutions of known concentrations that incorporate all sources of error (sample collection, filtration, processing). The statistical approach used to determine the p-EDL was an adaptation of the Clayton, et.al.[22] method that does not assume constant error variances across concentration and controls for Type II error. The statistical approach used involved calculating an estimated DL for each lab that had the desired Type I and Type II error rates (0.01 and 0.05, respectively). The median DLs over labs was then determined and is reported in Table 4. It is referred to as pooled-EDL (p-EDL).

Solutions of pure chlorophyll *a* in 90% acetone were prepared at three concentrations (0.11, 0.2 and 1.6 ppm) and shipped with blank glass fiber filters to participating laboratories. Analysts were instructed to spike the filters in duplicate with a given volume of solution and to process the spiked filters according to the method. The results from these data were used to determine a p-EDL for each method. Results (in ppm) are given in Table 4. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs. Due to the large dilutions required to analyze these solutions, the fluorometric p-EDLs are unrealistically high compared to what is achievable by a single lab. Typical single lab EDLs can easily be 1000 fold lower than the p-EDL reported in Table 4.

13.4.2 To address precision and bias in chlorophyll *a* determination for different algal species, three pure unialgal cultures (Amphidinium, Dunaliella and Phaeodactylum) were cultured and grown in the laboratory. Four different “concentrations” of each species were prepared by filtering varying volumes of the algae. The filters were frozen and shipped to participant labs. Analysts were instructed to extract and analyze the filters according to the respective methods. The “true” concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separated from other interfering pigments prior to determination. Pooled precision (as determined by %RSD) data are presented in Tables 5-7 and accuracy data (as percent recovery) are presented in Table 8. No significant differences in precision were observed across concentrations for any of the species. It should be noted that there was considerable lab-to-lab variation (as exhibited by the min and max recoveries in Table 8) and in this case the median is a better measure of central tendency than the mean.

In summary, the mean and median concentrations determined for Amphidinium carterae (class dinophyceae) are similar for all methods. No method consistently exhibited high or low values relative to the other methods. The only concentration trend observed was that the spectrophotometric method-trichromatic equations (SP-T) showed a slight percent increase in recovery with increasing algae filtration volume.

For Dunaliella tertiolecti (class chlorophyceae) and Phaeodactylum tricornutum (class bacillariophyceae) there was generally good agreement between the fluorometric and the spectrophotometric methods, however, the HPLC method yielded lower recoveries with increasing algae filtration volume for both species. No definitive explanation can be offered at this time for this phenomenon. A possible explanation for the Phaeodactylum is that it contained significant amounts of chlorophyllide *a* which is determined as chlorophyll *a* in the fluorometric and spectrophotometric methods. The conventional fluorometric method (FL-STD) showed a slight decrease in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The spectrophotometric-trichromatic equations (SP-T) showed a slight increase in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The fluorometric and the spectrophotometric methods both showed a slight decrease in chlorophyll *a* recovery with increasing Phaeodactylum filtration volume.

Results for the natural seawater sample are presented in Table 9. Only one filtration volume (100 mL) was provided in duplicate to participant labs.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of

environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation (e.g., Sect. 11.1.1). When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202)872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in the Sect. 14.2.

16.0 References

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17.0 TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

TABLE 1. COMPARISON OF PRECISION OF TWO EXTRACTION PERIODS

CORRECTED CHLOROPHYLL *a*

	Sample A[1]		Sample B[2]	
	2 h[3]	24 h[3]	2 h[3]	24 h[3]
Mean Concentration (µg/L)	49.6	52.9	78.6	78.8
Standard Deviation (µg/L)	4.89	2.64	6.21	2.77
Relative Standard Deviation (%)	9.9	5.0	7.9	3.5

[1] Values reported are the mean measured concentrations (n=6) of chlorophyll *a* in the natural water based on a 100-mL filtration volume.

[2] Values reported are the mean measured concentrations (n=9) of the extraction solution. Sample filtration volume was 300 mL.

[3] The length of time that the filters steeped after they were macerated.

TABLE 2. COMPARISON OF PRECISION OF TWO EXTRACTIONS PERIODS FOR Pheophytin *a*

Pheophytin *a*

	Sample A[1]		Sample B[2]	
	2 h[3]	24 h[3]	2 h[3]	24 h[3]
Mean Concentration (µg/L)	9.22	8.19	13.1	10.61
Standard Deviation (µg/L)	2.36	3.55	3.86	2.29
Relative Standard Deviation (%)	25.6	43.2	29.5	21.6

1 Values reported are the mean measured concentrations (n=6) of pheophytin *a* in the natural water based on a 100-mL filtration volume.

2 Values reported are the mean measured concentrations (n=9) of pheophytin *a* the extraction solution. Sample filtration volume was 300 mL.

3 The length of time that the filters steeped after they were macerated.

TABLE 3. ANALYSES OF USEPA QC SAMPLES

ANALYTE	REFERENCE VALUE	CONFIDENCE LIMITS
Chlorophyll *a*	2.1 µg/L	0.5 to 3.7 µg/L
Pheophytin *a*	0.3 µg/L	-0.2 to 0.8 µg/L

ANALYTE	MEAN MEASURED VALUE	% Relative Standard[1] Deviation
Chlorophyll *a*	2.8 µg/L	1.5
Pheophytin *a*	0.3 µg/L	33

[1] N = 3

TABLE 4. POOLED ESTIMATED DETECTION LIMITS FOR CHLOROPHYLL *A* METHODS[1]

Method[2]	N[3]	p-EDL[4] (mg/L)
FL -Mod[5]	8	0.096
FL - Std[5]	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.4.1 for a description of the statistical approach used to determine p-EDLs.

(2) FL-Mod = fluorometric method using special interference filters.

FL-Std = conventional fluorometric method with pheophytin *a* correction.

HPLC = EPA method 447.0

SP-M = EPA method 446.0, monochromatic equation.

SP-T = EPA method 446.0, trichromatic equations.

(3) N = number of labs whose data was used.

(4) The p-EDL was determined with p = 0.01 and q (type II error rate) = 0.05.

(5) Due to the large dilutions required to analyze the solutions by fluorometry, the fluormetric p-EDLs are unrealistically high.

TABLE 5. POOLED PRECISION FOR DUNALIELLA TERTIOLECTI SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.163	0.037	22.8
	10	7	0.298	0.080	26.7
	50	7	1.684	0.385	22.9
	100	7	3.311	0.656	19.8
Fl-Std	5	8	0.185	0.056	30.4
	10	8	0.341	0.083	24.4
	50	8	1.560	0.311	19.9
	100	8	3.171	0.662	20.9

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

TABLE 6. POOLED PRECISION FOR AMPHIDINIUM CARTERAE SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.066	0.010	14.6
	10	7	0.142	0.045	31.5
	50	7	0.757	0.208	27.5
	100	7	1.381	0.347	25.1
Fl-Std	5	8	0.076	0.018	23.2
	10	8	0.165	0.040	24.3
	50	8	0.796	0.140	17.5
	100	8	1.508	0.324	21.5

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

TABLE 7. POOLED PRECISION FOR PHAEODACTYLUM TRICORNUTUM SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.221	0.040	18.0
	10	7	0.462	0.094	20.3
	50	7	2.108	0.491	23.3
	100	7	3.568	1.186	33.2
Fl-Std	5	8	0.214	0.053	24.8
	10	8	0.493	0.091	18.4
	50	8	2.251	0.635	28.2
	100	8	4.173	0.929	22.3

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

NOTE: The phaeodactylum extract contained significant amounts of chlorophyll *c* and chlorophyllide *a* which interferes in chlorophyll *a* measurement in the fluorometric method, therefore, the concentration of chlorophyll *a* is overestimated compared to the HPLC method which separates the three pigments. The FL-Mod interference filters minimize this interference more so than the conventional filters.

TABLE 8. MINIMUM, MEDIAN, AND MAXIMUM PERCENT RECOVERIES BY GENERA, METHOD, AND CONCENTRATION LEVEL

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
Amphidinium	Minimum	FL-MOD	70	73	75	76
		FL-STD	66	91	91	90
		HPLC	82	85	87	88
		SP-M	36	48	68	64
		SP-T	21	63	71	70
	Median	FL-MOD	105	112	105	104
		FL-STD	109	107	111	109
		HPLC	102	106	112	105
		SP-M	99	101	101	101
		SP-T	95	96	106	107
	Maximum	FL-MOD	121	126	143	146
		FL-STD	156	154	148	148
		HPLC	284	210	131	116
		SP-M	141	133	126	125
		SP-T	115	116	119	117
Dunaliella	Minimum	FL-MOD	162	159	157	156
		FL-STD	179	171	165	164
		HPLC	165	109	64	41
		SP-M	120	188	167	164
		SP-T	167	169	166	165
	Median	FL-MOD	206	246	227	223
		FL-STD	250	228	224	210
		HPLC	252	177	89	80

Table 8 cont'd

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		SP-M	240	247	247	243
		SP-T	225	244	256	256
Dunaliella	Maximum	FL-MOD	295	277	287	288
		FL-STD	439	385	276	261
		HPLC	392	273	172	154
		SP-M	342	316	296	293
		SP-T	291	283	283	283
Phaeodactylum	Minimum	FL-MOD	216	183	157	154
		FL-STD	189	220	223	219
		HPLC	150	119	84	75
		SP-M	161	138	156	160
		SP-T	203	195	216	244
	Median	FL-MOD	292	285	250	245
		FL-STD	296	263	254	254
		HPLC	225	203	114	90
		SP-M	287	274	254	253
		SP-T	286	281	277	274
	Maximum	FL-MOD	357	337	320	318
		FL-STD	371	415	415	334
		HPLC	394	289	182	139
		SP-M	446	344	330	328
		SP-T	357	316	318	299

TABLE 9. CHLOROPHYLL *A* CONCENTRATIONS IN MG/L DETERMINED IN FILTERED SEAWATER SAMPLES

Method	Con.[1]	No. Obs.	No. Labs	Mean	Std. Dev.	RSD(%)	Minimum	Median	Maxium
FL-MOD	100	14	7	1.418	0.425	30.0	0.675	1.455	2.060
FL-STD	100	15	8	1.576	0.237	15.0	1.151	1.541	1.977
HPLC	100	10	5	1.384	0.213	15.4	1.080	1.410	1.680
SP-M	100	38	19	1.499	0.219	14.6	0.945	1.533	1.922
SP-T	100	36	18	1.636	0.160	9.8	1.250	1.650	1.948
All Methods	100	113	57	1.533	0.251	16.4	0.657	1.579	2.060

(1) Con = mLs of seawater filtered.

Method 446.0

In Vitro Determination of Chlorophylls *a*, *b*, c_1 + c_2 and Pheopigments in Marine And Freshwater Algae by Visible Spectrophotometry

Adapted by

Elizabeth J. Arar

Revision 1.2
September 1997

National Exposure Research Laboratory
Office of Research and Development
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Cincinnati, Ohio 45268

Method 446.0

In Vitro Determination of Chlorophylls *a*, *b*, $c_1 + c_2$ and Pheopigments in Marine and Freshwater Algae by Visible Spectrophotometry

1.0 Scope and Application

1.1 This method provides a procedure for determination of chlorophylls *a* (chl *a*), *b* (chl *b*), $c_1 + c_2$ (chl $c_1 + c_2$) and pheopigments of chlorophyll *a* (pheo *a*) found in marine and freshwater phytoplankton. Chlorophyllide *a* is determined as chl *a*. Visible wavelength spectrophotometry is used to measure the pigments in sub-parts per million (ppm) concentrations. The trichromatic equations of Jeffrey and Humphrey[1] are used to calculate the concentrations of chl *a*, chl *b*, and chl c_1+c_2. Modified monochromatic equations of Lorenzen[2] are used to calculate pheopigment-corrected chl *a* and pheo *a*.

1.2 This method differs from previous descriptions of the spectrophotometric technique in several important aspects. Quality assurance/quality control measures are described in Sect. 9.0. Detailed sample collection and extraction procedures are described in Sect. 8.0, and most importantly, interference data, heretofore only presented in research journals, is included so the analyst may know the potential limitations of the method. Multilaboratory data is included in Section 13.

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8
Chlorophyll *b*	519-62-0
Chlorophyll c_1	18901-56-9
Chlorophyll c_2	27736-03-4

1.3 Instrumental detection limits (IDLs) of 0.08 mg chl *a*/L, 0.093 mg chl *b*/L and 0.085 mg pheo *a*/L in pure solutions of 90% acetone were determined by this laboratory using a 1-cm glass cell. Lower detection limits can be obtained using 2, 5 or 10-cm cells. An IDL for chlorophylls c_1+c_2 was not determined due to commercial unavailability of the pure pigments. Estimated detection limit (EDL) determinations were made by analyzing seven replicate filtered phytoplankton samples containing the pigments of interest. Single-laboratory EDLs (S-EDL) were as follows: chl *a* - 0.037 mg/L, chl *b* - 0.07 mg/L, chl $c_1 + c_2$ - 0.087 mg/L, pheopigment-corrected chl *a* - 0.053 mg/L, and pheo *a* - 0.076 mg/L. The trichromatic equations lead to inaccuracy in the measurement of chlorophylls *b* and c_1+c_2 at chl *a* concentrations greater than ~5X the concentration of the accessory pigment or in the presence of pheo *a*. The upper limit of the linear dynamic range (LDR) for the instrumentation used in this method evaluation was approximately 2.0 absorbance units (AU) which corresponded to pigment concentrations of 27 mg chl *a*/L, 30 mg chl *b*/L and approximately 45 mg pheo *a*/L. No LDR for chl $c_1 + c_2$ was determined. It is highly unlikely that samples containing chl c_1+c_2 at concentrations approaching the upper limit of the LDR will be encountered in nature.

1.4 Chl c_1+c_2 is not commercially available, therefore, the minimum indicator of laboratory performance for this pigment is precision of chl $c_1 + c_2$ determinations in natural samples known to contain the pigments.

1.5 This method uses 90% acetone as the extraction solvent because of its efficiency for extracting chl *a* from most types of algae. (**NOTE:** There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol[3-5] or dimethyl sulfoxide.[6] Using high performance liquid chromatography (HPLC), Mantoura and Llewellyn[7] found that methanol led to the formation of chl *a* derivative products, whereas 90% acetone did not. Bowles, et al.[5] found that for chl *a* 90% acetone was an effective solvent when the steeping period was optimized for the predominant species present.)

1.6 One of the limitations of absorbance spectrophotometry is low sensitivity. It may be preferable

to use a fluorometric[8-10] or HPLC[11-15] method if high volumes of water (>4 L) must be filtered to obtain detectable quantities of chl *a.* The user should be aware of the inaccuracies of fluorometric methods when chl *b* is also present in the sample.

1.7 This method is for use by analysts experienced in handling photosynthetic pigments and in the operation of visible wavelength spectrophotometers or by analysts under the close supervision of such qualified persons.

2.0 Summary of Method

2.1 Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated by filtration at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton in 90% acetone with the aid of a mechanical tissue grinder and allowed to steep for a minimum of 2 h, but not exceeding 24 h, to ensure thorough extraction of the pigments. The filter slurry is centrifuged at 675 g for 15 min (or at 1000 g for 5 min) to clarify the solution. An aliquot of the supernatant is transferred to a glass cell and absorbance is measured at four wavelengths (750, 664, 647 and 630 nm) to determine turbidity, chlorophylls *a*, *b*, and $c_1 + c_2$, respectively. If pheopigment-corrected chl *a* is desired, the sample's absorbance is measured at 750 and 664 nm before acidification and at 750 and 665 nm after acidification with 0.1 N HCl. Absorbance values are entered into a set of equations that utilize the extinction coefficients of the pure pigments in 90% acetone to simultaneously calculate the concentrations of the pigments in a mixed pigment solution. No calibration of the instrument with standard solutions is required. Concentrations are reported in mg/L (ppm).

3.0 Definitions

3.1 Field Replicates -- Separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of field replicates give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.2 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent that gives an analyte signal equal to three times the standard deviation of a background signal at the selected wavelength, mass, retention time, absorbance line, etc. In this method the instrument is zeroed on a background of 90% acetone resulting in no signal at the measured wavelengths. The IDL is determined instead by serially diluting a solution of known pigment concentration until the signal at the selected wavelength is between .005 and .008 AU.

3.3 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or apparatus. For this method the LRB is a blank filter that has been extracted as a sample.

3.4 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.5 Material Safety Data Sheet (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.6 Estimated Detection Limit (EDL) -- The EDL is determined in a manner similar to an EPA MDL. It is not called an MDL in this method because there are known spectral interferences inherent to this method that make 99% confidence that the chlorophyll concentration is greater than zero impossible.

3.7 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations that is used to fortify an aliquot of LRB or sample matrix. Ideally, the QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials. The USEPA no longer provides QCSs for this method.

4.0 Interferences

4.1 Any compound extracted from the filter or acquired from laboratory contamination that absorbs light between 630 and 665 nm may interfere in the accurate measurement of the method analytes. An absorbance measurement is made at 750 nm to assess turbidity in the

sample. This value is subtracted from the sample's absorbance at 665, 664, 647 and 630 nm. A 750 nm absorbance value that is $\geq$ .005 AU indicates a poorly clarified solution. This is usually remedied by further centrifugation or filtration of the sample prior to analysis.

4.2 The relative amounts of chlorophyll *a*, *b* and c_1 + c_2 vary with the taxonomic composition of the phytoplankton. Due to the spectral overlap of the chlorophylls and pheo *a*, over- or underestimation of the pigments is inevitable in solutions containing all of these pigments.

Chl *a* is overestimated by the trichromatic equation of Jeffrey and Humphrey when pheo *a* is present (Figure 1). Lorenzen's modified monochromatic equation only slightly overestimates chl *a* in the presence of chl *b* (Figure 2). The degree of error in the measurement of any pigment is directly related to the concentration of the interfering pigment. Knowledge of the taxonomic composition of the sample, proper storage and good sample handling technique (to prevent degradation of chl *a*) can aid in determining whether to report trichromatic or pheopigment-corrected chl *a*. If no such knowledge exists, it is advisable to obtain values for all of the pigments and to compare the chl *a* results in light of the apparent concentrations of the other pigments. Obviously, if the chl *a* values vary widely, sound judgement must be used in deciding which pigments, chl *b* and chl c_1 + c_2, or pheo *a*, are in greatest abundance relative to each other and to chl *a*. The method of standard additions, explained in most analytical chemistry textbooks, is recommended when greater accuracy is required.

Accuracy of chl *b* measurements is highly dependent upon the concentration of chl *a* and pheo *a*.[16] In pure solutions of chl *a* and *b*, underestimation of chl *b* is observed with increasing concentrations of chl *a* (Figure 3). Using the method of standard additions, the same phenomenon was confirmed to occur in natural samples. The underestimation of chl *b* is due in part to the spectral component of chl *a* that is subtracted from chl *b* as chl c_1 + c_2 in the trichromatic equation. Chl *a* concentrations that range from 4 to 10 times the concentration of chl *b* lead to 13% to 38% underestimation of chl *b*. The highest chl *b*:chl *a* ratio likely to occur in nature is 1:1.

Pheo *a*:chl *a* ratios rarely exceed 1:1. Pheo *a* is overestimated in the presence of certain carotenoids[16] and when chl *b* is converted to pheo *b* in the acidification step required to determine pheopigment-corrected chl *a* and pheo *a*. The rate of conversion of chl *b* to pheo *b*, however, is slower than that of chl *a* to pheo *a*. It is important, therefore, to allow the minimum time required for conversion of chl *a* to pheo *a* before measuring absorbance at 665 nm. Ninety seconds is recommended by this method.

When a phytoplankton sample's composition is known (i.e., green algae, diatoms, dinoflagellates) Jeffrey and Humphrey's dichromatic equations for chl *a*, *b*, and c_1 + c_2 are more accurate than the trichromatic equations used here.[1]

4.3 Precision and recovery for any of the pigments is related to efficient maceration of the filtered sample and to the steeping period of the macerated filter in the extraction solvent (Table 1). Precision improves with increasing steeping periods. A drawback to prolonged steeping periods, however, is the extraction of interfering pigments. For example, if the primary pigment of interest is chl *a*, extended steeping periods may extract more of the other pigments but not necessarily more chl *a*. Statistical analysis revealed steeping period to be a significant factor in the recovery of chl *b* and pheo *a* from a mixed assemblage containing these pigments in detectable quantities, but not a significant factor in the recovery of chl *a*. Chl *b* and pheo *a* are mutual interferents so that an actual increase in the recovery of chl *b* leads to a slight apparent increase in pheo *a*.

4.4 Sample extracts must be clarified by centrifugation prior to analysis.

4.5 All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials, and filtered samples must be stored in the dark at -20 or -70°C to prevent rapid degradation.

5.0 Safety

5.1 Each chemical used in this method should be regarded as a potential health hazard and handled with caution and respect. Each laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method.[17-20] A file of MSDS also should be made available to all personnel involved in the chemical analysis.

5.2 The grinding of filters during the extraction step of this method should be conducted in a fume hood due to the volatilization of acetone by the tissue grinder.

6.0 Apparatus and Equipment

6.1 Spectrophotometer -- Visible, multiwavelength, with a bandpass (resolution) not to exceed 2 nm.

6.2 Centrifuge, capable of 675 g.

6.3 Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with 1/4" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity round-bottomed, glass grinding tube.

6.4 Filters, glass fiber, 47-mm, or 25-mm, nominal pore size of 0.7 µm unless otherwise justified by data quality objectives. Whatman GF/F filters were used in this work.

6.5 Petri dishes, plastic, 50 X 9-mm, or some other solid container for transporting and storing sampled filters.

6.6 Aluminum foil.

6.7 Laboratory tissues.

6.8 Tweezers or flat-tipped forceps.

6.9 Vacuum pump or source capable of maintaining a vacuum up to 6 in. Hg (20 KPa).

6.10 Labware -- All reusable labware (glass, polyethylene, Teflon, etc.) that comes in contact with chlorophyll solutions should be clean and acid free. An acceptable cleaning procedure is soaking for 4 h in laboratory grade detergent and water, rinsing with tap water, distilled deionized water and acetone.

6.10.1 Assorted Class A calibrated pipets.

6.10.2 Graduated cylinders, 500-mL and 1-L.

6.10.3 Volumetric flasks, Class A calibrated, 25-mL, 50-mL, 100-mL and 1-L capacity.

6.10.4 Glass rods.

6.10.5 Disposable Pasteur type pipets or medicine droppers.

6.10.6 Glass cells for the spectrophotometer, 1, 2, 5 or 10 cms in length. If using multiple cells, they must be matched.

6.10.7 Filtration apparatus consisting of 1 or 2-L filtration flask, 47-mm fritted glass disk base and a glass filter tower.

6.10.8 Centrifuge tubes, polypropylene or glass, 15-mL capacity with nonpigmented screw-caps.

6.10.9 Polyethylene squirt bottles.

7.0 Reagents and Standards

7.1 Acetone, HPLC grade, (CASRN 67-64-1).

7.2 Hydrochloric acid (HCl), concentrated (sp. gr. 1.19), (CASRN 7647-01-0).

7.3 Chl *a* free of chl *b* and chl *b* substantially free of chl *a* may be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO).

7.4 Water -- ASTM Type I water (ASTM D1193) is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.

7.5 0.1 N HCl Solution -- Add 8.5 mL of concentrated HCl to approximately 500 mL water and dilute to 1 L.

7.6 Aqueous Acetone Solution -- 90% acetone/10% ASTM Type I water. Carefully measure 100 mL of the water into the 1-L graduated cylinder. Transfer to a 1-L flask or storage bottle. Measure 900 mL of acetone into the graduated cylinder and transfer to the flask or bottle containing the water. Mix, label and store.

7.7 Chlorophyll Stock Standard Solution (SSS) -- Chl *a* (MW = 893.5) and chl *b* (MW = 907.5) from a commercial supplier is shipped in amber glass ampules that have been flame sealed. The dry standards must be stored at -20°C in the dark. Tap the ampule until all the dried pigment is in the bottom of the ampule. In subdued light, carefully break the tip off the ampule. Transfer the entire contents of the ampule into a 25-mL volumetric

flask. Dilute to volume with 90% acetone, label the flask and wrap with aluminum foil to protect from light. Pheo *a* may be prepared by the mild acidification of chl *a* (to .003 N HCl) followed by a 1:1 molar neutralization with a base such as dilute sodium hydroxide solution. When stored in a light- and air-tight container at -20ºC, the SSS is stable for at least six months. All dilutions of the SSS must be determined spectrophotometrically using the equations in Sect. 12.

7.8 Laboratory Reagent Blank (LRB) -- A blank filter that is extracted and analyzed just as a sample filter. The LRB should be the last filter extracted of a sample set. It is used to assess possible contamination of the reagents or apparatus.

7.9 Quality Control Sample (QCS) -- Since there are no commercially available QCSs, dilutions of a stock standard may be used.

8.0 Sample Collection, Preservation and Storage

8.1 Water Sample Collection -- Water may be obtained by a pump or grab sampler. Data quality objectives will determine the depth and frequency[21] at which samples are taken. Healthy phytoplankton, however, are generally obtained from the photic zone (depth at which the illumination level is 1% of surface illumination). Enough water should be collected to concentrate phytoplankton on at least three filters. Filtration volume size will depend on the particulate load of the water. Four liters may be required for open ocean water where phytoplankton density is usually low, whereas 1 L or less is generally sufficient for lake, bay or estuary water. All apparatus should be clean and acid-free. Filtering should be performed in subdued light as soon as possible after sampling since algal populations, thus chlorophyll *a* concentration, can change in a relatively short period of time. Aboard ship filtration is highly recommended.

Assemble the filtration apparatus and attach the vacuum source with vacuum gauge and regulator. Vacuum filtration should not exceed 6 in. Hg (20 kPa). Higher filtration pressures or excessively long filtration times (>10 min) may damage cells and result in loss of chlorophyll. Care must be taken not to overload the filters. Do not increase the vacuum during filtration.

Prior to drawing a subsample from the water sample container, thoroughly but gently agitate the container to suspend the particulates (stir or invert several times). Pour the subsample into a graduated cylinder and accurately measure the volume. Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum (not to exceed 20 kPa). Typically, a sufficient volume has been filtered when a visible green or brown color is apparent on the filter. Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter. Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in the petri dish or other suitable container. If the filter will not be immediately extracted, wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20ºC or -70ºC. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20°C as soon as possible.

8.2 Preservation -- Sampled filters should be stored frozen (-20ºC or -70ºC) in the dark until extraction.

8.3 Holding Time -- Filters can be stored frozen at -20ºC for as long as 3½ weeks without significant loss of chl *a*.[22]

9.0 Quality Control

9.1 Each Laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory reagent blanks, field replicates and QC samples as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (IDLs and LDRs) and laboratory performance (MDLs and analyses of QCSs) prior to sample analyses.

9.2.2 Standard Reference Material (SRM) 930e (National Institute of Standards and Technology,

Gaithersburg, MD) or other suitable spectrophotometric filter standards that test wavelength accuracy must be analyzed yearly and the results compared to the instrument manufacturer's specifications. If wavelength accuracy is not within manufacturer's specifications, identify and repair the problem.

9.2.3 Linear Dynamic Range (LDR) -- The LDR should be determined by analyzing a minimum of 5 standard solutions ranging in concentration from 1 to 15 mg/L. Perform the linear regression of absorbance response (at pigment's wavelength maximum) vs. concentration and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response, R, of a standard no longer yields a calculated concentration, C_c, that is ± 10% of the known concentration, C, where $C_c = (R - b)/m$. That concentration and absorbance response defines the upper limit of the LDR for your instrument. Absorbance responses for samples should be well below the upper limit of the LDR, ideally between .1 and 1.0 AU.

9.2.4 Instrumental Detection Limit (IDL) -- Zero the spectrophotometer with a solution of 90% acetone. Pure pigment in 90% acetone should be serially diluted until it yields a response at the selected wavelength between .005 and .008 AU.

9.2.5 Estimated Detection Limit (EDL) -- At least seven natural phytoplankton samples known to contain the pigments of interest should be collected, extracted and analyzed according to the procedures in Sects. 8 and 11, using clean glassware and apparatus. The concentration of the pigment of interest should be between 2 and 5 times the IDL. Dilution or spiking of the sample extract solution to the appropriate concentration may be necessary. Inaccuracies occur in the measurement of chlorophylls b and $c_1 + c_2$ when the chl a concentration is greater than ~5X the concentration of the accessory pigment. Perform all calculations to obtain concentration values in mg/L in the extract solution. Calculate the EDL as follows[23]:

$$EDL = (3) \times (S)$$

S = Standard deviation of the replicate analyses.

9.2.6 Quality Control Sample (QCS) -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify instrument performance with the analysis of a QCS (Sect. 7.9). If the determined value is not within the confidence limits established by project data quality objectives, then the determinative step of this method is unacceptable. The source of the problem must be identified and corrected before continuing analyses.

9.2.7 Extraction Proficiency -- Personnel performing this method for the first time should demonstrate proficiency in the extraction of sampled filters (Sect. 11.1). Twenty to thirty natural samples should be obtained using the procedure outlined in Sect. 8.1 of this method. Sets of 10 or more samples should be extracted and analyzed according to Sect. 11.2. The percent relative standard deviation (%RSD) of trichromatic chl a should not exceed 15% for samples that are at least 10X the IDL.

9.2.8 Corrected Chl a -- Multilaboratory testing of this method revealed that many analysts do not adequately mix the acidified sample when determining the corrected chl a. The problem manifests itself by highly erratic pheo a results, high %RSDs for correctetd chl a and poor agreement between corrected and uncorrected chl a. To determine if a new analyst is performing the acidification step properly, perform the following QC procedure:

Prepare 100 mL of a 2.0 ppm chl a solution in 90% acetone. The new analyst should analyze 5-10 separate aliquots, using carefully rinsed cuvettes, according to instructions in Section 11.2. Process the results according to Section 12 and calculate separate means and %RSDs for corrected and uncorrected chl a. If the means differ by more than 10%, then the stock chl a has probably degraded and fresh stock should be prepared. The %RSD for corrected chl a should not exceed 5%. If the %RSD exceeds 5%, repeat the procedure until acceptable results are obtained.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one blank filter with each sample batch. The LRB should be the last filter extracted. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the IDL indicate contamination from the laboratory environment. When LRB values constitute 10% or more of the analyte level determined in a sample, fresh samples or field replicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

10.0 Calibration and Standardization

10.1 Daily calibration of the spectrophotometer is not required when using the equations discussed in this method. It is extremely important, therefore, to perform regular checks on instrument performance. By analyzing a standard reference material such as SRM 930e (National Institute of Standards and Technology, Gaithersburg, MD) at least quarterly, wavelength accuracy can be compared to instrument manufacturer's specifications. Filter kits that allow stray light, bandpass and linearity to be evaluated are also commercially available. Although highly recommended, such kits are not required for this method if the LDR is determined for the pigment of interest and QCSs are routinely analyzed.

10.2 Allow the instrument to warm up for at least 30 min. Use a 90% acetone solution to zero the instrument at all of the selected wavelengths. 750 nm, 664 nm, 647 nm and 630 nm are used for the determination of chl *a*, chl *b* and chl $c_1 + c_2$. 750 nm, 665 nm and 664 nm are used for the determination of pheopigment-corrected chl *a* and pheo *a*. The instrument is now ready to analyze samples.

11.0 Procedure

11.1 Extraction of Filter Samples

11.1.1 For convenience, a 10-mL final extraction volume is described in the following procedure. A larger extraction volume may be necessary if using a low-volume 10-cm cell. On the other hand, a smaller extraction volume can be used to obtain a concentration factor. The filter residue retains 2-3 mL of solution after centrifugation and a 1-cm cell requires approximately 3 mL of solution so that a recommended minimum extraction volume is 6 mL.

11.1.2 If sampled filters have been frozen, remove them from the freezer but keep them in the dark. Set up the tissue grinder and have on hand laboratory tissues and squirt bottles containing water and acetone. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation. Remove a filter from its container and place it in the glass grinding tube. The filter may be torn into smaller pieces to facilitate extraction. Push it to the bottom of the tube with a glass rod. With a volumetric pipet, add 4 mL of the aqueous acetone solution (Sect. 7.6) to the grinding tube. After the filter has been converted to a slurry, grind the filter for approximately 1 min at 500 rpm. (**NOTE:** Although grinding is required, care must be taken not to overheat the sample. Good judgement and common sense will help you in deciding when the sample has been sufficiently macerated.) Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 6-mL volumetric pipet, rinse the pestle and the grinding tube with the aqueous acetone. Add the rinse to the centrifuge tube containing the filter slurry. Cap the tube and shake it vigorously. Place it in the dark before proceeding to the next filter extraction. Before placing another filter in the grinding tube, use the acetone and water squirt bottles to thoroughly rinse the pestle, grinding tube and glass rod. To reduce the volume of reagent grade solvents used for rinsing between extractions, thoroughly rinse the grinding tube and glass rod with tap water prior to a final rinse with ASTM Type I water and acetone. The last rinse should be with acetone. Use a clean tissue to remove any filter residue that adheres to the pestle or to the steel rod of the pestle. Proceed to the next filter and repeat the steps above. The last filter extracted should be a blank. The entire extraction with transferring and rinsing takes approximately 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.3 Shake each tube vigorously again before placing them to steep in the dark at 4ºC. Samples should be allowed to steep for a minimum of 2 h but not to exceed 24 h. Tubes should be shaken at least once, preferably two to three times, during the steeping period to allow the extraction solution to have maximum contact with the filter slurry.

11.1.4 After steeping is complete, centrifuge samples for 15 min at 675 g or for 5 min at 1000 g.

11.2 Sample Analysis

11.2.1 The instrument must be zeroed on a 90% acetone solution as described in Sect. 10.2. In subdued lighting, pour or pipet the supernatant of the extracted sample into the glass spectrophotometer cell. If the absorbance at 750 nm exceeds .005 AU, the sample must be recentrifuged or filtered through a glass fiber filter (syringe filter is recommended). The volume of sample required in the instrument's cell must be known if the pheopigment-corrected chl *a* and pheo *a* will be determined so that acidification to the correct acid concentration can be performed. For example, a cell that

holds 3 mL of extraction solution requires .09 mL of the .1 N HCl solution to obtain an acid concentration of .003 N. Measure the sample's absorbance at the selected wavelengths for chl *a*, chl *b* and chl $c_1 + c_2$. Dilute and reanalyze the sample if the signal at the selected wavelength is $\geq$90% of the signal previously determined as the upper limit of the LDR. If pheopigment-corrected chl *a* and pheo *a* will be determined, acidify the sample in the cell to .003 N HCl using the .1 N HCl solution. Use a disposable Pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample, wait 90 sec and measure the sample's absorbance at 750 and 665 nm.

NOTE: Proper mixing of the acidified sample is critical for accurate and precise results.

12.0 Data Analysis and Calculations

12.1 Jeffrey and Humphrey's Trichromatic Equations -- Subtract the absorbance value at 750 nm from the absorbance values at 664, 647 and 630 nm. Calculate the concentrations (mg/L) of chl *a*, *b*, and $c_1 + c_2$ in the extract solution by inserting the 750 nm-*corrected* absorbance values into the following equations:

$$C_{E,a} = 11.85\ (\text{Abs } 664) - 1.54\ (\text{Abs } 647) - .08\ (\text{Abs } 630)$$

$$C_{E,b} = 21.03\ (\text{Abs } 647) - 5.43\ (\text{Abs } 664) - 2.66\ (\text{Abs } 630)$$

$$C_{E,c} = 24.52\ (\text{Abs } 630) - 7.60\ (\text{Abs } 647) - 1.67\ (\text{Abs } 664)$$

where:

$C_{E,a}$ = concentration (mg/L) of chlorophyll *a* in the extraction solution analyzed,

$C_{E,b}$ = concentration (mg/L) of chlorophyll *b* in the extract solution.

$C_{E,c}$ = concentration (mg/L) of chlorophyll $c_1 + c_2$ in the extract solution analyzed.

12.2 Lorenzen's Pheopigment-corrected Chl *a* and Pheo *a* -- Subtract the absorbance values at 750 nm from the absorbance values at 664 and 665 nm. Calculate the concentrations (mg/L) in the extract solution, C_E, by inserting the 750 nm *corrected* absorbance values into the following equations:

$$C_{E,a} = 26.7(\text{Abs } 664_b - \text{Abs } 665_a)$$

$$P_{E,a} = 26.7\ [1.7 \times (\text{Abs } 665_a) - (\text{Abs } 664_b)]$$

where,

$C_{E,a}$ = concentration (mg/L) of chlorophyll *a* in the extract solution measurted,

$P_{E,a}$ = concentration (mg/L) of pheophytin *a* in the extraction measured.

Abs 664_b = sample absorbance at 664 nm (minus absorbance at 750 nm) measured before acidification, and

Abs 665_a = sample absorbance at 665 nm (minus absorbance at 750 nm) measured after acidification.

12.3 Calculate the conentration of pigment in the whole water sample using the following generalized equation:

$$C_s = \frac{C_E \text{ (a,b, or c)} \times \text{extract volume (L)} \times \text{DF}}{\text{sample volume (L)} \times \text{cell length (cm)}}$$

where:

C_s = concentration (mg/L) of pigment in the whole water sample.

$C_{E(a,b,or\ c)}$ = concentration (mg/l) of pigment in extract measured in the cuvette.

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extract volume = volume (L) of extract (before any dilutions), typically 0.0104).

DF = any dilution factors.

sample volume = volume (L) of whole water sample that was filtered, and

cell length = optical path length (cm) of cuvette used (typically 1 cm).

For example, calculate the conentration of chlorophyll *a* in the whole water sample as:

$$C_{s,b} = \frac{C_{E,a} \ X \ extract \ volume \ (L) \ DF}{sample \ volume \ (L) \ X \ cell \ length \ (cm)}$$

12.4 LRB and QCS data should be reported with each sample data set.

13.0 Method Performance

13.1 Single Laboratory Performance

13.1.1 Replicate analyses were performed on low level dilutions of the pure pigments in 90% acetone. The results, contained in Table 2, give an indication of the variability not attributable to sampling and extraction or pigment interferences.

13.1.2 The IDLs and S-EDLs for the method analytes are reported in Table 3.

13.1.3 Precision (%RSD) for replicate analyses of two distinct mixed assemblages are contained in Table 4.

13.1.4 Three QCS ampules were obtained from the USEPA, analyzed and compared to the reference values in Table 5. (**NOTE:** The USEPA no longer provides pigment QCSs.)

13.2 Multilaboratory Testing - A Multilaboratory validation and comparison study of EPA Methods 445.0, 446.0 and 447.0 for chlorophyll *a* was conducted in 1996 by Research Triangle Institute, Research Triangle park, N.C. (EPA Contract No. 68-C5-0011). There were 24 volunteer participants in the spectrophotometric methods component that returned data. The primary goals of the study were to determine detection limits and to assess precision and bias (as percent recovery) for select unialgal species, and natural seawater.

13.2.1 The term, pooled-estimated detection limit (p-EDL), is used in this method to distinguish it from the EPA defined method detection limit (MDL). An EPA MDL determination is not possible nor practical for a natural water or pure species sample due to known spectral interferences and to the fact that it is impossible to prepare solutions of known concentrations that incorporate all sources of error (sample collection, filtration, processing). The statistical approach used to determine the p-EDL was an adaptation of the Clayton, et. al.[24] method that does not assume error variances across concentration and controls for Type II error. The statistical approach used involved calculating an estimated DL for each lab that had the desired Type I and Type II error rates (0.01 and 0.05, respectively). The median DLs over labs was then determined and is reported in Table 6. It is referred to as the pooled-EDL (p-EDL).

Solutions of pure chlorophyll *a* in 90% acetone were prepared at three concentrations (0.11, 0.2, and 1.6 ppm) and shipped with blank glass fiber filters to participating laboratories. Analysts were instructed to spike the filters in duplicate with a given volume of solution and to process the spiked filters according to the method. The results from these data were used to determine a pooled EDL (p-EDL) for each method. Results (in ppm) are given in Table 6. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs.

13.2.2 To address precision and bias in chlorophyll *a* determination for different algal species three pure uniagal cultures (amphidinium, dunnnaliella and phaeodactylum) were cultured and grown in the laboratory. Four different “concentrations” of each species were prepared by filtering varying volumes of the algae. The filters were frozen and shipped to participant labs. Analysts were instructed to extract and analyze the filters according to the respectiave methods. The “true” concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separatead from other interfereing pigments prior to determination. Pooled precision data (%RSD) are presented in Tables 7-9 and accuracy data (as percent recovery) are presented in Table 10. No significant differences in precision were observed across conentrations for any of the species. It should be noted that there was considerable lab-to-lab variation (as exhibited by the min and max recoveries in Table 10) and in this case the median is a better measurement of central tendency than the mean.

In summary, the mean and median concentrations determined for Amphidinium carterae (class dinophyceae) are similar for all methods. No method consistently exhibited high or low values relative to the other methods. The only concentration trend observed was that the spectrophotometric method-trichromatic

equations (SP-T) showed a slight percent increase in recovery with increasing algae filtration volume.

For Dunaliella tertiolecti (class chlorophyceae) and Phaeodactylum tricornutum (class bacillariophyceae) there was generally good agreement between the fluorometric and the spectrophotometric methods, however, the HPLC method yielded lower recoveries with increasing algae filtration volume for both species. No definitive explanation can be offered at this time for this phenomenon. A possible explanation for the Phaeodactylum is that it contained significant amounts of chlorophylide *a* which is determined as chlorophyll *a* in the fluorometric and spectrophotometric methods. The conventional fluorometric method (FL-STD) showed a slight decrease in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The spectrophotometric-trichromatic equations (SP-T) showed a slight increase in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The fluorometric and tahe spectrophotometric methods both showed a slight decrease in chlorophyll *a* recovery with increasing Phaeodactylum filtration volume.

Results for the natural seawater sample are presented in Table 11. Only one filtration volume (100 mL) was provided in duplicate to partaicpant labs.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation (e.g., Sect. 11.1.1). When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The U.S. Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in the Sect. 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

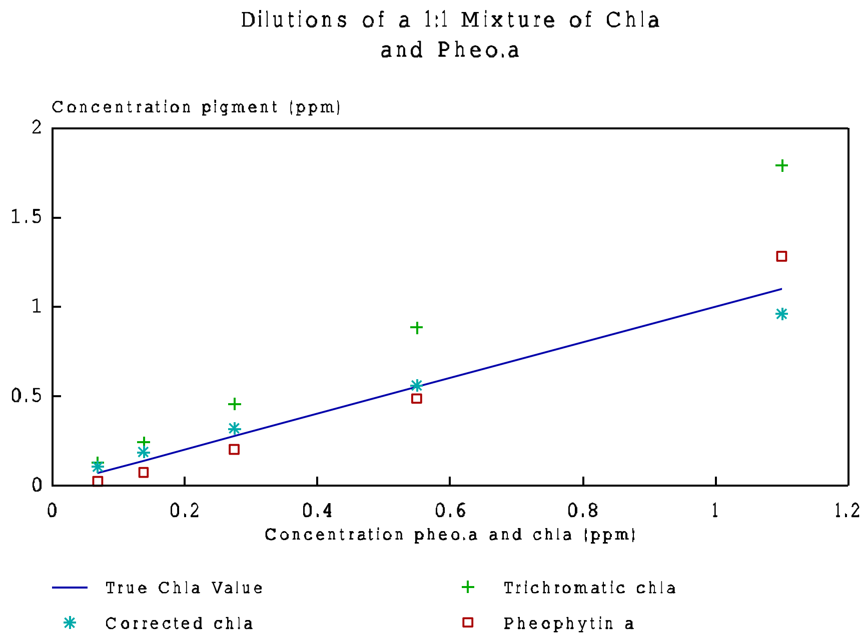

FIGURE 1 - The effect of pheo *a* on calculated pigment concentrations.

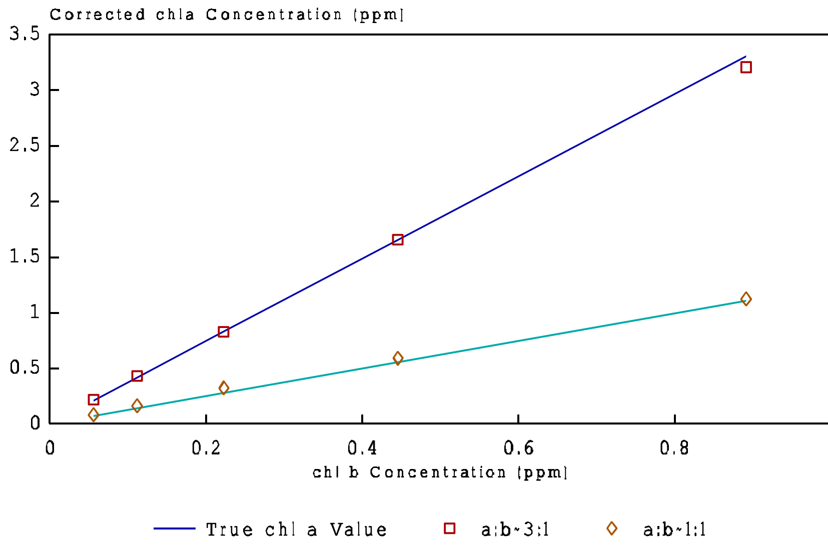

FIGURE 2 - The effect of Chl *b* on pheopigment - corrected Chl *a.*

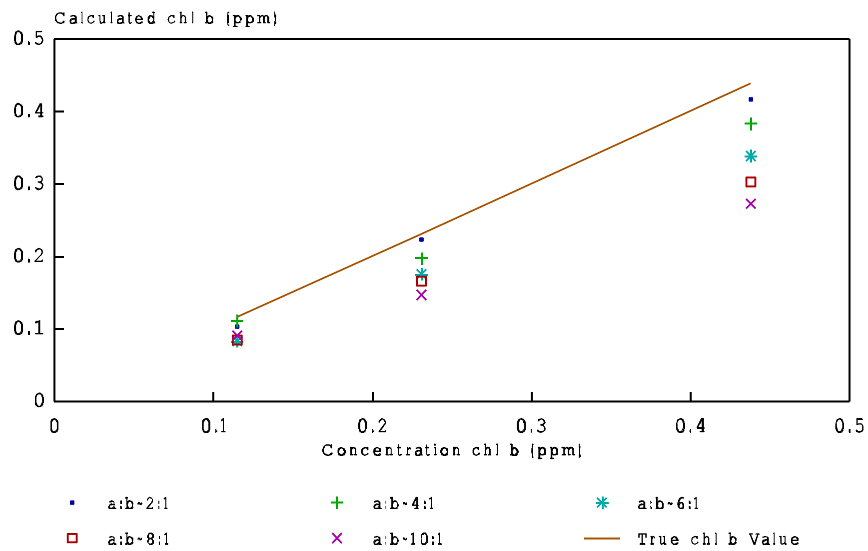

FIGURE 3 - The underestimation of Chl *b* with increasing concentrations of Chl *a.*

TABLE 1. COMPARISON OF PRECISION AND RECOVERY OF PIGMENTS FOR 4 h AND 24 h STEEPING PERIODS

	chl *a*		chl *b*		chl c_1+c_2		pheo *a*		corr *a*	
	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h
N	6	6	6	6	6	6	6	6	6	6
SD	1.22	0.88	0.42	0.21	0.44	0.37	1.08	1.23	1.46	1.04
Mean	26.14	25.73	0.49	1.72	5.87	5.26	1.38	2.88	24.47	23.29
%RSD	24.67	3.40	6.35	12.00	7.43	7.04	78.35	42.62	5.97	4.47

N - Number of samples
SD - Standard deviation
Mean - Concentration in natural water, mg/L
%RSD - Percent relative standard deviation

TABLE 2. REPLICATE ANALYSES OF PURE PIGMENTS AT LOW CONCENTRATIONS

Trichromatic Equations			Modified Monochromatic Equations		
	chl *a*	chl *b*		chl *a*	chl *b*
N	7	7	N	7	6
SD	.000612	.009792	SD	.010091	.011990
Mean	.102 mg/L	.109 mg/L	Mean	.103 mg/L	.171 mg/L
%RSD	.60	8.9	%RSD	9.8	7.0

TABLE 3. INSTRUMENTAL AND METHOD DETECTION LIMITS

INSTRUMENTAL DETECTION LIMITS[1]
(Concentrations in mg/L)

Trichromatic Equations		Modified Monochromatic Equation	
chl *a*	.080	pheo *a*	.085
chl *b*	.093		

S-ESTIMATED DETECTION LIMITS[1]
(Concentrations in mg/L)

Trichromatic Equations		Modified Monochromatic Equation	
chl *a*	.037[2]	chl *a*	.053[2]
chl *b*	.070[2]	pheo *a*	.076[2]
chl $c_1 + c_2$	.087[3]		

[1] Determinations made using a 1-cm path length cell.
[2] Mixed assemblage samples from San Francisco Bay.
[3] Predominantly diatoms from Raritan Bay.

TABLE 4. ANALYSES OF NATURAL SAMPLES

SAN FRANCISCO BAY

	Trichromatic Equations			Modified Monochromatic Equations	
	chl *a*	chl *b*	chl c_1+c_2	pheo *a*	corr *a*
N	7	7	7	7	7
SD	0.0118	0.0062	0.0096	0.0244	0.0168
Mean	0.2097	0.04271	0.03561	0.0806	0.1582
%RSD	5.62	14.50	26.82	30.21	0.64

RARITAN BAY

	Trichromatic Equations			Modified Monochromatic Equations	
	chl *a*	chl *b*	chl c_1+c_2	pheo *a*	corr *a*
N	7	7	7	7	7
SD	0.0732	0.0223	0.0277	0.0697	0.0521
Mean	1.4484	0.0914	0.2867	0.1720	1.3045
%RSD	5.06	24.43	9.65	40.53	3.99

Mean concentrations (mg/L) reported in final extraction volume of 10 mL. Samples were macerated and allowed to steep for approximately 24 h.

N - Number of samples
SD - Standard deviation
Mean - Concentration in natural water
%RSD - Percent relative standard deviation

TABLE 5. ANALYSES OF USEPA QC SAMPLES

Ampule 1 (3 separate ampules, chl *a* only)

	Trichromatic Equations				Modified Monochromatic Equations		
	Mean	Reference	%RSD		Mean	Reference	%RSD
chl *a*	2.54 mg/L	2.59	.61	chl a	2.56 mg/L	2.70	.8
				pheo *a*	ND		

ND - None detected

Ampule 2 (3 separate ampules, all method pigments)

	Trichromatic Equations				Modified Monochromatic Equations		
	Mean	Reference	%RSD		Mean	Reference	%RSD
chl a	4.87 mg/L	4.86	.1	chl *a*	3.70 mg/L	3.76	2.3
chl b	1.12 mg/L	1.02	1.3	pheo a	1.79 mg/L	1.70	4.4
chl c_1 + c_2	.29 mg/L	.37	4.9				

TABLE 6. POOLED ESTIMATED DETECTION LIMITS FOR CHLOROPHYLL *A* METHODS[1]

Method[2]	N[3]	p-EDL[4] (mg/L)
FL -Mod[5]	8	0.096
FL - Std	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.2.1 for a description of the statistical approach used to determine p-EDLs.

(2) FL-Mod = fluorometric method using special interference filters.

FL-Std = conventional fluorometric method with pheophytin *a* correction.

HPLC = EPA method 447.0

SP-M = EPA method 446.0, monochromatic equation.

SP-T = EPA method 446.0, trichromatic equations.

(3) N = number of labs whose data was used.

(4) The p-EDL was determined with p = 0.01 and q (type II error rate) = 0.05.

(5) Due to the large dilutions required to analyze the solutions by fluorometry, the fluormetric p-EDLs are unrealistically high.

TABLE 7. POOLED PRECISION FOR AMPHIDINIUM CARTERAE SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
SP-M	5	17	0.068	0.026	37.8
	10	19	0.139	0.037	26.6
	50	19	0.679	0.150	22.1
	100	19	1.366	0.205	15
SP-T	5	16	0.059	0.021	35.1
	10	18	0.130	0.027	20.8
	50	18	0.720	0.102	14.2
	100	18	1.408	0.175	12.4

(1) SP-M = Pheophytin *a* - corrected chlorophyll *a* method using monochromatic equations.

SP-T = Trichromatic equations method.

(2) N = Number of volunteer labs whose data was used.

TABLE 8. POOLED PRECISION FOR DUNALIELLA TERTIOLECTI SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
SP-M	5	19	0.166	0.043	26.0
	10	19	0.344	0.083	24.0
	50	19	1.709	0.213	12.5
	100	19	3.268	0.631	19.3
SP-T	5	18	0.161	0.030	18.4
	10	18	0.339	0.058	17.1
	50	18	1.809	0.190	10.5
	100	18	3.500	0.524	15.0

(1) SP-M = Pheophytin *a* corrected chlorophyll *a* method using monochromatic equations.

SP-T = Trichromatic equationss method.

(2) N = number of volunteer labs whose data was used.

TABLE 9. POOLED PRECISION FOR PHAEODACTYLUM TRICORNUTUM SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
SP-M	5	19	0.223	0.054	24.1
	10	19	0.456	0.091	19.9
	50	19	2.042	0.454	22.2
	100	19	4.083	0.694	17.0
SP-T	5	18	0.224	0.031	14.0
	10	18	0.465	0.077	16.5
	50	18	2.223	0.217	9.7
	100	18	4.422	0.317	7.2

(1) SP-M = Pheophytin *a* corrected chorophyll *a* method using monochromatic equations.

(2) N = number of volunteer labs whose data was used.

TABLE 10. MINIMUM, MEDIAN, AND MAXIMUM PERCENT RECOVERIES BY GENERA, METHOD, AND CONCENTRATION LEVEL

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
Amphidinium	Minimum	FL-MOD	70	73	75	76
		FL-STD	66	91	91	90
		HPLC	82	85	87	88
		SP-M	36	48	68	64
		SP-T	21	63	71	70
	Median	FL-MOD	105	112	105	104
		FL-STD	109	107	111	109
		HPLC	102	106	112	105
		SP-M	99	101	101	101
		SP-T	95	96	106	107
	Maximum	FL-MOD	121	126	143	146
		FL-STD	156	154	148	148
		HPLC	284	210	131	116
		SP-M	141	133	126	125
		SP-T	115	116	119	117
Dunaliella	Minimum	FL-MOD	162	159	157	156
		FL-STD	179	171	165	164
		HPLC	165	109	64	41
		SP-M	120	188	167	164
		SP-T	167	169	166	165
	Median	FL-MOD	206	246	227	223
		FL-STD	250	228	224	210
		HPLC	252	177	89	80
		SP-M	240	247	247	243

Table 10 cont'd

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		SP-T	225	244	256	256
Dunaliella	Maximum	FL-MOD	295	277	287	288
		FL-STD	439	385	276	261
		HPLC	392	273	172	154
		SP-M	342	316	296	293
		SP-T	291	283	283	283
Phaeodactylum	Minimum	FL-MOD	216	183	157	154
		FL-STD	189	220	223	219
		HPLC	150	119	84	75
		SP-M	161	138	156	160
		SP-T	203	195	216	244
	Median	FL-MOD	292	285	250	245
		FL-STD	296	263	254	254
		HPLC	225	203	114	90
		SP-M	287	274	254	253
		SP-T	286	281	277	274
	Maximum	FL-MOD	357	337	320	318
		FL-STD	371	415	415	334
		HPLC	394	289	182	139
		SP-M	446	344	330	328
		SP-T	357	316	318	299

TABLE 11. CHLOROPHYLL *A* CONCENTRATIONS IN mg/L DETERMINED IN FILTERED SEAWATER SAMPLES

Method	Con.[1]	No. Obs.	No. Labs	Mean	Std. Dev.	RSD(%)	Minimum	Median	Maxium
FL-MOD	100	14	7	1.418	0.425	30.0	0.675	1.455	2.060
FL-STD	100	15	8	1.576	0.237	15.0	1.151	1.541	1.977
HPLC	100	10	5	1.384	0.213	15.4	1.080	1.410	1.680
SP-M	100	38	19	1.499	0.219	14.6	0.945	1.533	1.922
SP-T	100	36	18	1.636	0.160	9.8	1.250	1.650	1.948
All Methods	100	113	57	1.533	0.251	16.4	0.657	1.579	2.060

(1) Con = mLs of seawater filtered.

Method 447.0

Determination of Chlorophylls *a* and *b* and Identification of Other Pigments of Interest in Marine and Freshwater Algae Using High Performance Liquid Chromatography with Visible Wavelength Detection

Elizabeth J. Arar

Version 1.0
September 1997

National Exposure Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

METHOD 447.0

DETERMINATION OF CHLOROPHYLLS *a* AND *b* AND IDENTIFICATION OF OTHER PIGMENTS OF INTEREST IN MARINE AND FRESHWATER ALGAE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH VISIBLE WAVELENGTH DETECTION

1.0 Scope and Application

1.1 This method provides a procedure for determination of chlorophylls *a* (chl *a*) and *b* (chl *b*) found in marine and freshwater phytoplankton. Reversed-phase high performance liquid chromatography (HPLC) with detection at 440 nm is used to separate the pigments from a complex pigment mixture and measure them in the sub-microgram range. For additional reference, other taxonomically important yet commercially unavailable pigments of interest are identified by retention time.

1.2 This method differs from previous descriptions of HPLC methods in several respects. Quality assurance/quality control measures are described in Sect. 9.0, sample collection and extraction procedures are described in Sect. 8.0 and reference chromatograms of pure pigments and reference algae are provided.

This method has also been evaluated in a multilaboratory study along with EPA Methods 445.0 and 446.0. Estimated detection limits, precision and bias are reported in Section 13.

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8
Chlorophyll *b*	519-62-0

1.3 Instrumental detection limits (IDLs) of 0.7 ng chl *a*, and 0.4 ng chl *b* in pure solutions of 90% acetone were determined by this laboratory. Method detection limit (MDL) determinations were made by analyzing seven replicate unialgal samples containing the chl *a* and *b*. Single-laboratory MDLs were chl *a* - 7 ng and chl *b* - 4 ng. A multilaboratory estimated detection limit (EDL) (in mg/L of extract is reported in Section 13.

1.4 Most taxonomically important pigments are not commercially available, therefore, a laboratory must be willing to extract and purify pigments from pure algal cultures to quantify and qualitatively identify these very important pigments. This method contains chromatographic information of select pure pigments found either in marine or freshwater algae. The information is included to aid the analyst in qualitatively identifying individual pigments and possibly algal species in natural samples.

1.5 This method uses 90% acetone as the extraction solvent because of its efficiency for extracting chl *a* from most types of algae. (**NOTE:** There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol[1-3] or dimethyl sulfoxide.)[4] Using high performance liquid chromatography (HPLC), Mantoura and Llewellyn[5] found that methanol led to the formation of chl *a* derivative products, whereas 90% acetone did not. Bowles, et al.[3] found that for chl *a* 90% acetone was an effective solvent when the steeping period was optimized for the predominant species present.)

1.6 One of the limitations of visible wavelength detection is low sensitivity. It may be preferable to use fluorometry[6-8] or HPLC[913] with fluorometric detection if high volumes of water (>4 L) must be filtered to obtain detectable quantities of chl *a* or *b*.

1.7 This method is for use by analysts experienced in handling photosynthetic pigments and in the operation of HPLC or by analysts under the close supervision of such qualified persons.

2.0 Summary of Method

2.1 The HPLC is calibrated with a chl *a* and *b* solution that has been spectrophotometrically quantified

according to EPA Method 446. Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated by filtration at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton into 90% acetone with the aid of a mechanical tissue grinder and allowed to steep for a minimum of 2 h, but not exceeding 24 h, to ensure thorough extraction of the pigments. The filter slurry is centrifuged at 675 g for 15 min (or at 1000 g for 5 min) to clarify the solution. An aliquot of the supernatant is filtered through a 0.45 µm syringe filter and 50 to 200 µL is injected onto a reversed-phase column. Following separation using a ternary gradient, concentrations are reported in µg/L (ppb) or mg/L (ppm) in the whole water sample. This method is based on the HPLC work of Wright, et. al.[9]

3.0 Definitions

3.1 Calibration Standard (CAL) -- A solution prepared from dilution of a stock standard solution. The CAL solution is used to calibrate the instrument response with respect to analyte concentration or mass.

3.2 Calibration Check Standard (CALCHK) -- A mid-point calibration solution that is analyzed periodically in a sample set to verify that the instrument response to the analyte has not changed during the course of analysis.

3.3 Field Replicates -- Separate samples collected at the same time and placed under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of field replicates give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.4 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent that gives an analyte signal equal to three times the standard deviation of a background signal at the selected wavelength, mass, retention time, absorbance line, etc.

3.5 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or apparatus. For this method the LRB is a blank filter that has been extracted as a sample.

3.6 Material Safety Data Sheet (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.7 Method Detection Limit (MDL) -- The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.

3.8 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations that is used to fortify an aliquot of LRB or sample matrix. Ideally, the QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

4.0 Interferences

4.1 Any compound extracted from the filter or acquired from laboratory contamination that absorbs light at 440 nm may interfere in the accurate measurement of the method analytes.

4.2 Proper storage and good sample handling technique are critical in preventing degradation of the pigments.

4.3 Precision and recovery for any of the pigments is related to efficient extraction, i.e. efficient maceration of the filtered sample and to the steeping period of the macerated filter in the extraction solvent. Precision improves with increasing steeping periods, however, a drawback to prolonged steeping periods is the possibility of pigment degradation. The extracted sample must be kept cold and in the dark to minimize degradation.

4.4 Sample extracts must be clarified by filtration through a 0.45 µm filter prior to analysis by HPLC to prevent column fouling.

4.5 All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials, and filtered samples must be stored in the dark at -20°C or -70°C to prevent rapid degradation.

5.0 Safety

5.1 Each chemical used in this method should be regarded as a potential health hazard and handled with caution and respect. Each laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method.[15-18] A file of MSDS also should be made available to all personnel involved in the chemical analysis.

5.2 The grinding of filters during the extraction step of this method should be conducted in a fume hood due to the volatilization of acetone by the tissue grinder.

6.0 Apparatus and Equipment

6.1 Centrifuge, capable of 675 g.

6.2 Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with 1/4" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity round-bottomed, glass grinding tube.

6.3 Filters, glass fiber, 47-mm or 25-mm nominal pore size of 0.7 µm unless otherwise justified by data quality objectives. Whatman GF/F filters were used in this work.

6.4 Petri dishes, plastic, 50 X 9-mm, or some other solid container for transporting and storing sampled filters.

6.5 Aluminum foil.

6.6 Laboratory tissues.

6.7 Tweezers or flat-tipped forceps.

6.8 Vacuum pump or source capable of maintaining a vacuum up to 6 in. Hg (20 KPa).

6.9 **Labware** -- All reusable labware (glass, polyethylene, Teflon, etc.) that comes in contact with chlorophyll solutions should be clean and acid free. An acceptable cleaning procedure is soaking for 4 h in laboratory grade detergent and water, rinsing with tap water, distilled deionized water and acetone.

6.9.1 Assorted Class A calibrated pipets.

6.9.2 Graduated cylinders, 500-mL and 1-L.

6.9.3 Volumetric flasks, Class A calibrated, 10-mL, 25-mL, 50-mL, 100-mL and 1-L capacity.

6.9.4 Glass rods or spatulas.

6.9.5 Pasteur Type pipets or medicine droppers.

6.9.6 Filtration apparatus consisting of 1 or 2-L filtration flask, 47-mm fritted glass disk base and a glass filter tower.

6.9.7 Centrifuge tubes, polypropylene or glass, 15-mL capacity with nonpigmented screw-caps.

6.9.8 Polyethylene squirt bottles.

6.9.9 Amber 2-mL HPLC autosampler vials with screw or clamp caps.

6.9.10 Glass syringe, 1 or 2-mL capacity.

6.9.11 HPLC compatible, low-volume, acetone resistant glass fiber or PTFE syringe filters.

6.10 Liquid Chromatograph

6.10.1 This method uses a ternary gradient thus requiring a programmable gradient pump with at least three pump inlets for the three different mobile phases required. A Dionex Model 4500 chromatograph equipped with a gradient pump, UV/VIS detector (cell path length, 6 mm, volume 9 µL) and PC data analysis (Dionex AI450 software, Version 3.32) system was used to generate data for this method. Tubing was made of polyether ether ketone (PEEK). A Dionex degas module was used to sparge all eluents with helium.

6.10.2 Helium or other inert gas for degassing the mobile phases OR other means of degassing such as sonication under vacuum.

6.10.3 Sample loops of various sizes (50-200 µL).

6.10.4 Guard Column -- A short column containing the same packing material as the analytical column placed before the analytical column to protect it from fouling by small particles. The guard column can be replaced periodically if it is noticed that system back pressure has increased over time.

6.10.5 Analytical Column -- A C_{18} reversed-phase column with end capping. A J.T. Baker 4.6 mm X 250 mm, 5 µm pore size column was used to generate the data in this method.

6.10.6 A visible wavelength detector with a low volume flow-through cell. Detection is at 440 nm.

6.10.7 A recorder, integrator or computer for recording detector response as a function of time.

6.10.8 Although not required, an autosampler (preferably refrigerated) is highly recommended.

7.0 Reagents and Standards

7.1 Acetone, HPLC grade, (CASRN 67-64-1).

7.2 Methanol, HPLC grade, (CASRN 67-56-1). Prepare ELUENT A, 80% (v/v) methanol/20% 0.5 M ammonium acetate, by adding 800 mL of methanol and 200 mL of the 0.5 M ammonium acetate (Sect. 7.5) to an eluent container.

7.3 Acetonitrile, HPLC grade, (CASRN 75-05-8). Prepare ELUENT B, 90% (v/v) acetonitrile/10% water, by adding 900 mL of acetonitrile and 100 mL of water (Sect. 7.7) to an eluent container.

7.4 Ethyl acetate, HPLC grade, (CASRN 141-78-6). ELUENT C, 100% ethyl acetate.

7.5 Ammonium acetate, ACS grade (CASRN 631-61-8). Prepare a 0.5 M solution by dissolving 38.54 g in approximately 600 mL of water in a 1-L volumetric flask. After the ammonium acetate has dissolved, dilute to volume with water.

7.6 Chl *a* free of chl *b* and chl *b* substantially free of chl *a* may be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO).

7.7 **Water** -- ASTM Type I water (ASTM D1193) is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.

7.8 **Aqueous Acetone Solution** -- 90% acetone/10% ASTM Type I water. Carefully measure 100 mL of the water into the 1-L graduated cylinder. Transfer to a 1-L flask or storage bottle. Measure 900 mL of acetone into the graduated cylinder and transfer to the flask or bottle containing the water. Mix, label and store.

7.9 **Chlorophyll Stock Standard Solution (SSS)** -- Chl *a* (MW = 893.5) and chl *b* (MW = 907.5) from a commercial supplier is shipped in amber glass ampules that have been flame sealed. The dry standards must be stored at -20 or -70°C in the dark. Tap the ampule until all the dried pigment is in the bottom of the ampule. In subdued light, carefully break the tip off the ampule. Transfer the entire contents of the ampule into a 25-mL volumetric flask. Dilute to volume with 90% acetone: (1 mg in 25 mL = 40 mg chl *a*/L) and (1 mg in 25 ml = 40 mg chl *b*/L), label the flasks and wrap with aluminum foil to protect from light. When stored in a light- and air-tight container at -20 or -70°C, the SSS is stable for at least six months. Dilutions of the SSS should always be confirmed spectrophotometrically using EPA Method 446.

7.10 **Laboratory Reagent Blank (LRB)** -- A blank filter that is extracted and analyzed just as a sample filter. The LRB should be the last filter extracted of a sample set. It is used to assess possible contamination of the reagents or apparatus.

7.11 **Quality Control Sample (QCS)** -- Since there are no commercially available QCSs, dilutions of a stock standard of a different lot number from that used to prepare calibration solutions may be used.

8.0 Sample Collection, Preservation and Storage

8.1 **Water Sample Collection** -- Water may be obtained by a pump or grab sampler. Data quality objectives will determine the depth and frequency[21] at which samples are taken. Healthy phytoplankton, however, are generally obtained from the photic zone (region in which the illumination level is 1% of surface illumination). Enough water should be collected to concentrate phytoplankton on at least three filters so that precision can be assessed. Filtration volume size will depend on the particulate load of the water. Four liters may be required for open ocean water where phytoplankton density is usually low, whereas 1 L or less is generally sufficient for lake, bay or estuary water. All apparatus should be clean and acid-free. Filtering should be performed in subdued light as soon as possible after sampling since algal populations, thus pigment concentrations, can change in relatively short periods of time. Aboard ship filtration is highly recommended.

Assemble the filtration apparatus and attach the vacuum source with vacuum gauge and regulator. Vacuum filtration should not exceed 6 in. Hg (20 kPa). Higher filtration pressures may damage cells and result in loss of chlorophyll. Care must be taken not to overload the filters. Do not increase the vacuum during filtration.

Prior to drawing a subsample from the water sample container, gently stir or invert the container several times to suspend the particles. Pour the subsample into a graduated cylinder and accurately measure the volume. Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum (not to exceed 20 kPa). Typically, a sufficient volume has been filtered when a visible green or brown color is apparent on the filter. Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter. Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in the petri dish or other suitable container. If the filter will not be immediately extracted, wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20°C or -70°C. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20°C or -70°C as soon as possible.

8.2 Preservation -- Sampled filters should be stored frozen (-20°C or -70°C) in the dark until extraction.

8.3 Holding Time -- Filters can be stored frozen at -20°C for as long as 3½ weeks without significant loss of chl *a*.[20]

9.0 Quality Control

9.1 Each Laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory reagent blanks, field replicates, QCSs, and CALCHKs as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (IDLs) and laboratory performance (MDLs, extraction proficiency, and analyses of QCSs) prior to sample analyses.

9.2.2 Instrumental Detection Limit (IDL) -- After a low level calibration (Sect. 10), prepare a standard solution that upon injection into the chromatograph yields an absorbance of 0.002-0.010. If using an autosampler, variable volumes may be injected and the micrograms (µg) injected calculated by multiplying the known concentration (µg/µL) of the standard by the volume injected (µL). A practical starting point may be to inject 0.05 µg (that would be a 50 µL injection of a 1.0 mg/L standard solution) and reduce or increase the mass injected according to the resulting signal. Avoid injecting really small volumes (< 10 µL). After the quantity of pigment has been selected, make three injections and calculate the IDL by multiplying the standard deviation of the calculated mass by 3.

9.2.3 Method Detection Limit (MDL) -- At least seven natural phytoplankton samples known to contain the pigments of interest should be collected, extracted and analyzed according to the procedures in Sects. 8 and 11, using clean glassware and apparatus. Mass of the pigment injected into the chromatograph should be 2 to 5 times the IDL. Dilution of the sample extract solution to the appropriate concentration or reducing the volume of sample injected may be necessary. Calculate the MDL (in micrograms) as follows.[19]

$$MDL = (t) \times (S)$$

where, t = Student's t-value for n-1 degrees of freedom at the 99% confidence level. t = 3.143 for six degrees of freedom.

S = Standard deviation of the replicate analyses.

9.2.4 Quality Control Sample (QCS) -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify instrument performance with the analysis of a QCS (Sect. 7.11). If the determined value is not within $\pm$10% of the spectrophotometrically determined value, then the instrument should be recalibrated with fresh stock standard and the QCS reanalyzed. If the redetermined value is still unacceptable then the source of the problem must be identified and corrected before continuing analyses.

9.2.5 Extraction Proficiency -- Personnel performing this method for the first time should demonstrate proficiency in the extraction of sampled filters (Sect. 11.1). Fifteen to twenty natural samples should be obtained using the procedure outlined in Sect. 8.1 of this method. Sets of 10 samples or more should be extracted and analyzed according to Sect. 11. The percent relative standard deviation (%RSD) should not exceed 15% for samples that are at least 10X the IDL.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one blank filter with each sample batch. The LRB should be the last filter extracted. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the IDL indicate contamination from the laboratory environment. If the LRB value constitutes 10% or more of the analyte level determined in a sample, fresh samples or field replicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 Calibration Check Standard (CALCHK) -- The laboratory must analyze one CALCHK for every ten samples to verify calibration. If the CALCHK is not ±10% of the spectrophotometrically determined concentration, then the instrument must be recalibrated.

10.0 Calibration and Standardization

10.1 Allow the visible wavelength detector (440 nm) to warm up for at least 15 min before calibration. Prepare ELUENTS A - C and degas by sparging with an inert gas for 10 minutes or sonicating under vacuum for 5 minutes. Prime the pump for each eluent taking care to remove all air that may be in the liquid lines. Equilibrate the column for ten minutes with 100% of ELUENT A.

10.2 Remove the SSS from the freezer and allow it to come to room temperature. Add 1 mL of the SSS to a 10-mL volumetric flask and dilute to 10 mL with 90% acetone. Prepare the chl *a* and *b* separately and determine the concentrations according to EPA Method 446 using the monochromatic equations for chl *a* determination. After the concentration of the SSS is determined, add 1 mL of the chl *a* SSS plus 1 mL of the chl *b* SSS to a separate 10-mL flask and dilute to volume. Store the calibration standard in a light tight glass bottle.

10.3 Program the pump with the following gradient:

Time	Flow	%1	%2	%3	Condition
0.0	1.0	100	0	0	Injection
2.0	1.0	0	100	0	Linear Gradient
2.6	1.0	0	90	10	Linear Gradient
13.6	1.0	0	65	35	Linear Gradient
20.0	1.0	0	31	69	Linear Gradient
22.0	1.0	0	100	0	Linear Gradient
25.0	1.0	100	0	0	Linear Gradient
30.0	1.0	100	0	0	Equilibration

Flow is in mL/min.

10.4 The first analysis is a blank 90% acetone solution followed by calibration. Calibrate with at least three concentrations, covering no more than one order of magnitude, and bracketing the concentrations of samples. If an autosampler is used, variable volumes ranging from 10 - 100% of the sample injection loop volume are injected to give a calibration of detector response versus mass of pigment. If doing manual injections, variable solution concentrations are made and a fixed sample loop volume is injected for standards and samples. Calibration can be either detector response versus mass or detector response versus concentration (mg/L or µg/L). Linearity across sensitivity settings of the detector must be confirmed if samples are analyzed at a different sensitivity settings from that of the calibration.

10.5 Construct a calibration curve of analyte response (area) versus concentration (mg/L in solution) or mass (µg) of pigment and perform a linear regression to determine the slope and y-intercept. A typical coefficient of determination is > 0.99.

10.6 Calibration must be performed at least weekly although it is not necessary to calibrate daily. Daily mid-point CALCHKs must yield calculated concentrations ±10% of the spectrophotometrically determined concentration.

11.0 Procedure

11.1 Extraction of Filter Samples

11.1.1 For convenience, a 10-mL final extraction volume is described in the following procedure. A smaller extraction volume may be used to improve detection limits.

11.1.2 If sampled filters have been frozen, remove them from the freezer but keep them in the dark. Set up the tissue grinder and have on hand laboratory tissues and wash bottles containing water and acetone. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation. Remove a filter from its container and place it in the glass grinding tube. You may also tear the filter into smaller pieces and push them to the bottom of the tube with a glass rod. With a volumetric pipet, add 3 mL of the aqueous acetone solution (Sect. 7.6) to the grinding tube. Grind the filter until it has become a slurry. (**NOTE:** Although grinding is required, care must be taken not to overheat the sample. Good judgement and common sense will help you in deciding when the sample has been sufficiently macerated.) Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 7-mL volumetric pipet, rinse the pestle and the grinding tube with the aqueous acetone. Add the rinse to the centrifuge tube containing the filter slurry. Cap the tube and shake it vigorously. Place it in the dark before proceeding to the next filter extraction. Before placing another filter in the grinding tube, use the acetone and water squirt bottles to thoroughly rinse the pestle, grinding tube and glass rod. To reduce the volume of reagent grade solvents used for rinsing between extractions, thoroughly rinse the grinding tube and glass rod with tap water prior to a final rinse with ASTM Type I water and acetone. The last rinse should be with acetone. Use a clean tissue to remove any filter residue that adheres to the pestle or to the steel rod of the pestle. Proceed to the next filter and repeat the steps above. The last filter extracted should be a blank. The entire extraction with transferring and rinsing takes approximately 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.3 Again, shake each tube vigorously before placing them to steep in the dark at 4°C. Samples should be allowed to steep for a minimum of 2 h but not to exceed 24 h. Tubes should be shaken at least once, preferably two to three times, during the steeping period to allow the extraction solution to have maximum contact with the filter slurry.

11.1.4 After steeping is complete, centrifuge samples for 15 min at 675 g or for 5 min at 1000 g. Draw approximately 1 mL into a glass syringe, attach a 0.45 µm syringe filter, filter the extract into an amber autosampler vial, cap and label the vial. Protect the filtered samples from light and heat. If using a refrigerated autosampler, chill to 10°C.

11.2 Sample Analysis

11.2.1 Draw into a clean syringe 2-3 times the injection loop volume and inject into the chromatograph. If using an autosampler, load the sample tray, prepare a schedule and begin analysis. A typical analyses order might be: (1) blank 90% acetone, (2) CALCHK, (3) 10 samples, (4) CALCHK, (5) QCS.

11.2.2 If the calculated CALCHK is not $\pm$10 of the spectrophotometrically determined concentration then recalibrate with fresh calibration solutions.

12.0 Data Analysis and Calculations

12.1 From the chl *a* or *b* area response of the sample, calculate the mass injected or concentration (C_E) of the solution that was analyzed using the calibration data. Mass injected must be converted to concentration in extract by dividing mass by volume injected (µL) and multiplying by 1000 to give concentration in mg/L (mg/L = µg/mL). Concentration of the natural water sample may be reported in mg/L by the following formula:

$$\frac{C_E \text{ X extract volume (L) X DF}}{\text{sample volume (L)}}$$

where:

C_E = concentration (mg/L) of pigment in extract.
DF = any dilution factors.
L = liters.

12.2 LRB and QCS data should be reported with each data set.

13.0 Method Performance

13.1 Single Laboratory Performance

13.1.1 An IDL was determined by preparing a mixed chl *a* (0.703 ppm) and chl *b* (0.437 ppm) standard. The injected mass yielded 0.004 AU for chl *a* (0.035 µg) and 0.003 AU for chl *b* (0.022 µg). Seven replicate 50 µL injections were made and the standard deviation of the calculated concentration was multiplied by three to determine an IDL. The IDL determined for chl *a* was 0.76 ng and 0.44 ng for chl *b*. The %RSDs for chl *a* and chl *b* was 0.45 and 0.67, respectively.

13.1.2 MDLs for chl *a* and chl *b* were determined by spiking seven replicate filtered samples of Pycnacoccus, extracting and processing according to this method. An

injection volume of 100 µL yielded an MDL for chl *a* of 7.0 ng and 4.0 ng for chl *b*. The RSDs were 5.1% for chl *b* and 4.7 % for chl *a*.

13.1.3 Recoveries of chl *a* and chl *b* from filtered samples of phaeodactylum were determined by spiking three filters with known amounts of the pigments, extracting, processing and analyzing the extraction solution according to the method, along with three unspiked filtered samples (to determine the native levels in the algae). The spiked levels were 1.1 ppm chl *a* and 0.53 ppm chl *b* in the 10 mL extraction volume. Chl *a* was 87% recovered and chl *b* was 94% recovered.

13.1.4 Figures 1-7 are chromatograms of seven reference unialga cultures processed according to this method.

13.1.5 Table 1 is a list of pure pigments with retention times obtained using this method. Purified pigments were prepared under contract to EPA by Moss Landing Marine Laboratory, Moss Landing, CA.

13.1.6 Table 2 contains single lab precision data for seven reference algal suspensions.

13.2 Multilaboratory Testing - A Multilaboratory validation and comparison study of EPA Methods 445.0, 446.0 and 447.0 for chlorophyll *a* was conducted in 1996 by Research Triangle Institute, Research Triangle Park, N.C. (EPA Contract No. 68-C5-0011). There were 8 volunteer participants in the HPLC methods component that returned data. The primary goals of the study were to determine estimated detection limits and to assess precision and bias (as percent recovery) for select unialgal species, and natural seawater.

13.2.1 The term, pooled estimated detection limit (p-EDL), is used in this method to distinguish it from the EPA defined method detection limit (MDL). The statistical approach used to determine the p-EDL was an adaptation of the Clayton, et. al.[21] method that does not assume constant error variances across concentration and controls for Type II error. The approach used involved calculating an estimated DL for each lab that had the desired Type I and Type II error rates (0.01 and 0.05, respectively). The median DLs over labs was then determined and is reported in Table 3. It is referred to as Pooled-EDL (p-EDL).

The p-EDL was determined in the following manner. Solutions of pure chlorophyll *a* in 90% acetone were prepared at three concentrations (0.11, 0.2 and 1.6 ppm) and shipped with blank glass fiber filters to participating laboratories. Analysts were instructed to spike the filters in duplicate with a given volume of solution and to process the spiked filters according to the method. The results from these data were used to determine a p-EDL for each method. Results (in ppm) are given in Table 3. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs.

13.2.2 To address precision and bias in chlorophyll *a* determination for different algal species, three pure unialgal cultures (Amphidinium, Dunaliella and Phaeodactylum) were cultured and grown in the laboratory. Four different "concentrations" of each species were prepared by filtering varying volumes of the algae. The filters were frozen and shipped to participant labs. Analysts were instructed to extract and analyze the filters according to the respective methods. The "true" concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separated from other interfering pigments prior to determination . Pooled precision data are presented in Tables 4-6 and accuracy data (as percent recovery) are presented in Table 7. No significant differences in precision (%RSD) were observed across concentrations for any of the methods or species. It should be noted that there was considerable lab-to-lab variation (as exhibited by the min and max recoveries in Table 7) and in this case the median is a better measure of central tendency than the mean.

In summary, the mean and median concentrations determined for Amphidinium carterae (class dinophyceae) are similar for all methods. No method consistently exhibited high or low values relative to the other methods. The only concentration trend observed was that the spectrophotometric method-trichromatic equations (SP-T) showed a slight percent increase in recovery with increasing algae filtration volume.

For Dunaliella tertiolecti (class chlorophyceae) and Phaeodactylum tricornutum (class bacillariophyceae) there was generally good agreement between the fluorometric and the spectrophotometric methods, however, the HPLC method yielded lower recoveries with increasing algae filtration volume for both species. No definitive explanation can be offered at this time for this phenomenon. A possible explanation for the Phaeodactylum is that it contained significant amounts of chlorophyllide *a* which is determined as chlorophyll *a* in the fluorometric and spectrophotometric methods. The conventional fluorometric method (FL-STD) showed a slight decrease in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The spectrophotometric-

trichromatic equations (SP-T) showed a slight increase in chlorophyll *a* recovery with incresing dunaliella filtration volume. The fluorometric and athe spectrophotometric methods both showed a slight decrease in chlorophyll *a* recovery with increasing Phaeodactylum filtration volume.

Results for the natural seawater sample are presented in Table 8. Only one filtration volume (100 mL) was provided in duplicate to Participant labs.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation (e.g., Sect. 11.1.2). When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The U.S. Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in the Sect. 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Pure Pigments and Retention Times

PIGMENT	RETENTION TIME
19' butanoyloxyfucoxanthin	8.13
2,4-divinylpheoporphrin a_5	8.60
Peridinin	8.69
Fucoxanthin	8.75
19' hexanoyloxyfucoxanthin	8.90
Neoxanthin	10.07
Chlorophyll C_3	10.27
Chlorophyll C_2	10.40
Prasinoxanthin	11.20
Violaxanthin	12.00
Diadinoxanthin	15.20
Chlorophyll *b*	15.60
Myxoxanthophyll	17.00
Aphanaxanthin	17.20
Chlorophyll *a*	17.80
Monadoxanthin	17.93
Lutein	18.00
Alloxanthin	18.07
Nostaxanthin	18.70
Diatoxanthin	19.07
Zeaxanthin	19.40

Table 2. Single Lab Precision for Seven Pure Unialgal Cultures

Algae		Chlorophyll *a*	Chlorophyll *b*
Pycnacoccus provasolii	N[1]	3	3
	Mean (mg/L)[2]	2.15	1.47
	STD DEV	0.114	0.065
	% RSD	5.31	4.45
Rhodomonas salina	N[1]	3	3
	Mean (mg/L)	4.0	ND[3]
	STD DEV	0.014	ND
	% RSD	0.28	ND
Selenastrum capricornitum	N[1]	3	3
	Mean (mg/L)	4.25	0.483
	STD DEV	0.199	0.058
	% RSD	4.68	12.01
Amphidinium carterae	N[1]	3	3
	Mean (mg/L)	2.38	ND
	STD DEV	0.176	ND
	% RSD	7.40	ND
Dunaliella tertiolecti	N[1]	3	3
	Mean (mg/L)	6.68	1.42
	STD DEV	0.635	0.0412
	% RSD	9.51	2.90
Emiliania huxleyi	N[1]	3	ND
	Mean (mg/L)	1.03	ND
	STD DEV	0.008	ND
	% RSD	0.79	ND
Phaeodactylum tricornutum	N[1]	3	ND
	Mean (mg/L)	1.09	ND
	STD DEV	0.072	ND
	% RSD	7.07	ND

(1) N = Number of filtered samples.
(2) Mean concentration in extract solution.
(3) ND = none detected.

TABLE 3. POOLED ESTIMATED DETECTION LIMITS FOR CHLOROPHYLL *A* METHODS[1]

Method[2]	N[3]	p-EDL[4] (mg/L)
FL -Mod[5]	8	0.096
FL - Std	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.2.1 for a description of the statistical approach used to determine p-EDLs.

(2) FL-Mod = fluorometric method using special interference filters.

FL-Std = conventional fluorometric method with pheophytin *a* correction.

HPLC = EPA method 447.0

SP-M = EPA method 446.0, monochromatic equation.

SP-T = EPA method 446.0, trichromatic equations.

(3) N = number of labs whose data was used.

(4) The p-EDL was determined with p = 0.01 and q (type II error rate) = 0.05.

(5) Due to the large dilutions required to analyze the solutions by fluorometry, the fluormetric p-EDLs are unrealistically high.

TABLE 4. Measured Chlorophyll *a* (mg/L) in Dunaliella Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.172	0.064	36.8
	10	5	0.276	0.074	26.8
	50	5	0.757	0.344	45.4
	100	5	1.420	0.672	47.3

(1) Not all participants labs followed the EPA method exactly.

(2) N = Number of volunteer labs whose data was used.

TABLE 5. Measured Chlorophyll *a* (mg/L) in Amphidinium Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.104	0.043	56.8
	10	5	0.172	0.083	37.5
	50	5	0.743	0.213	17.4
	100	5	1.394	0.631	14.5

(1) Not all participants labs followed the EPA method exactly.
(2) N = number of volunteer labs whose data was used.

TABLE 6. Measured Chlorophyll *a* in Phaeodactylum Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.193	0.074	38.4
	10	5	0.317	0.114	36.1
	50	5	1.024	0.340	33.2
	100	5	1.525	0.487	29.9

(1) Not all participants labs followed the EPA method exactly.
(2) N = number of volunteer labs whose data was used.

TABLE 7. Minimum, Median, and Maximum Percent Recoveries by Genera, Method, and Concentration Level

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
Amphidinium	Minimum	FL-MOD	70	73	75	76
		FL-STD	66	91	91	90
		HPLC	82	85	87	88
		SP-M	36	48	68	64
		SP-T	21	63	71	70
	Median	FL-MOD	105	112	105	104
		FL-STD	109	107	111	109
		HPLC	102	106	112	105
		SP-M	99	101	101	101
		SP-T	95	96	106	107
	Maximum	FL-MOD	121	126	143	146
		FL-STD	156	154	148	148
		HPLC	284	210	131	116
		SP-M	141	133	126	125
		SP-T	115	116	119	117
Dunaliella	Minimum	FL-MOD	162	159	157	156
		FL-STD	179	171	165	164
		HPLC	165	109	64	41
		SP-M	120	188	167	164
		SP-T	167	169	166	165
	Median	FL-MOD	206	246	227	223
		FL-STD	250	228	224	210
		HPLC	252	177	89	80
		SP-M	240	247	247	243

Table 7. Cont'd.

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		SP-T	225	244	256	256
Dunaliella	Maximum	FL-MOD	295	277	287	288
		FL-STD	439	385	276	261
		HPLC	392	273	172	154
		SP-M	342	316	296	293
		SP-T	291	283	283	283
Phaeodactylum	Minimum	FL-MOD	216	183	157	154
		FL-STD	189	220	223	219
		HPLC	150	119	84	75
		SP-M	161	138	156	160
		SP-T	203	195	216	244
	Median	FL-MOD	292	285	250	245
		FL-STD	296	263	254	254
		HPLC	225	203	114	90
		SP-M	287	274	254	253
		SP-T	286	281	277	274
	Maximum	FL-MOD	357	337	320	318

Table 8. Chlorophyll *a* Concentrations in mg/L Determined in Filtered Seawater Samples

Method	Con.[1]	No. Obs	No. Labs	Mean	Std. Dev	RSD (%)	Minimum	Median	Maximum
FL-MOD	100	14	7	1.418	0.425	30.0	0.675	1.455	2.060
FL-STD	100	15	8	1.576	0.237	15.0	1.151	1.541	1.977
HPLC	100	10	5	1.384	0.213	15.4	1.080	1.410	1.680
SP-M	100	38	19	1.499	0.219	14.6	0.945	1.533	1.922
SP-T	100	36	18	1.636	0.160	9.8	1.250	1.650	1.948
All Methods	100	113	57	1.533	0.251	16.4	0.657	1.579	2.060

(1) Con = mLs of seawater filtered.

Figure 1 - Amphidinium carterae

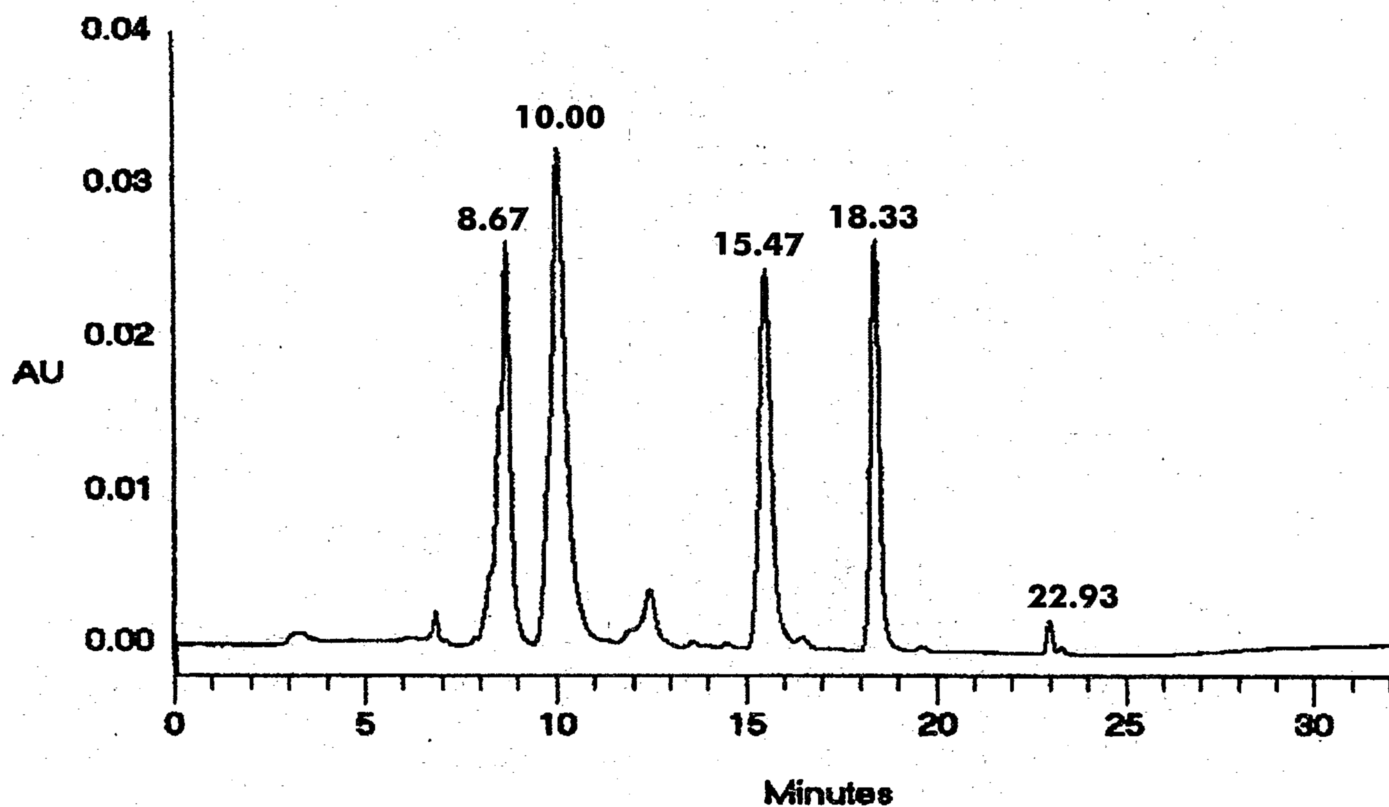


Figure 2 - Dunaliella tertiolecti

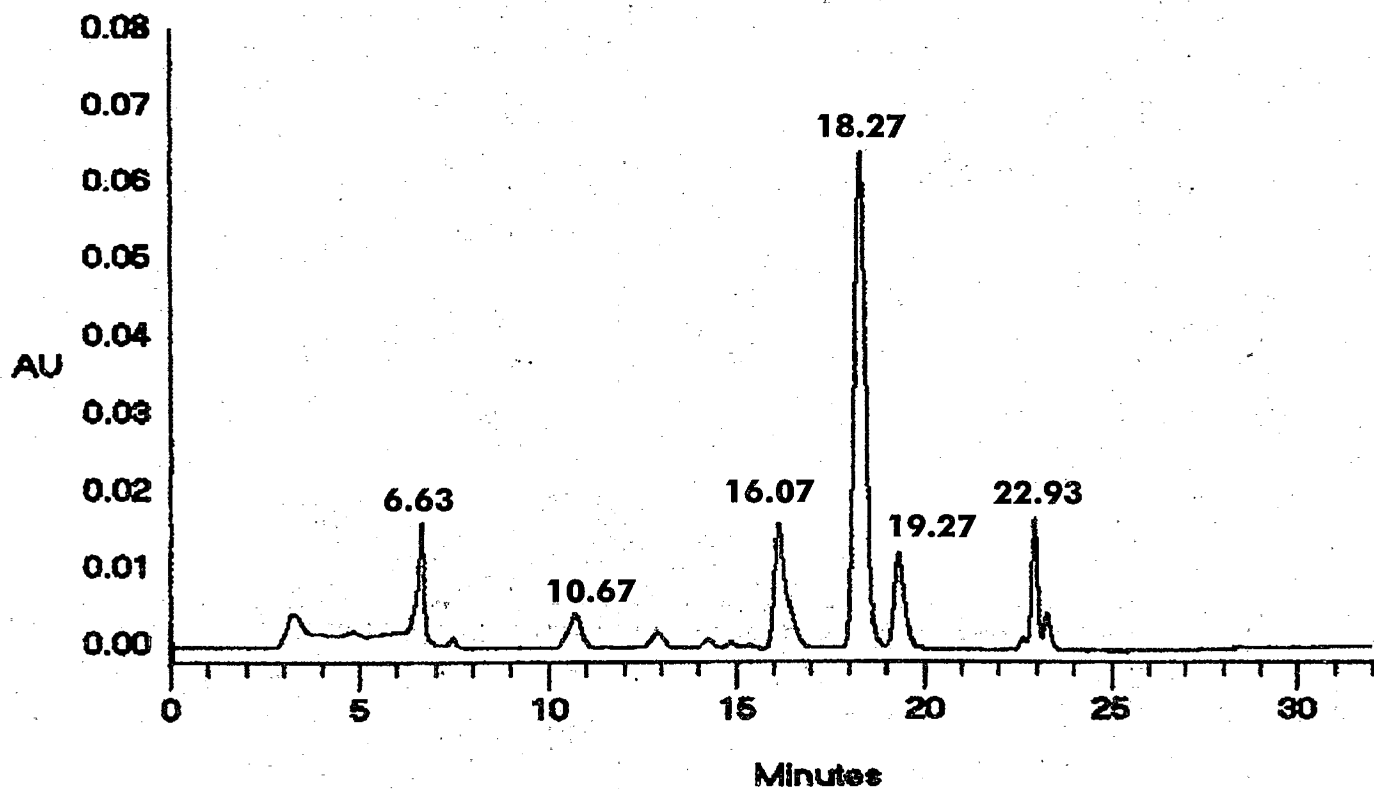


Figure 3 - Pycnococcus provasolii

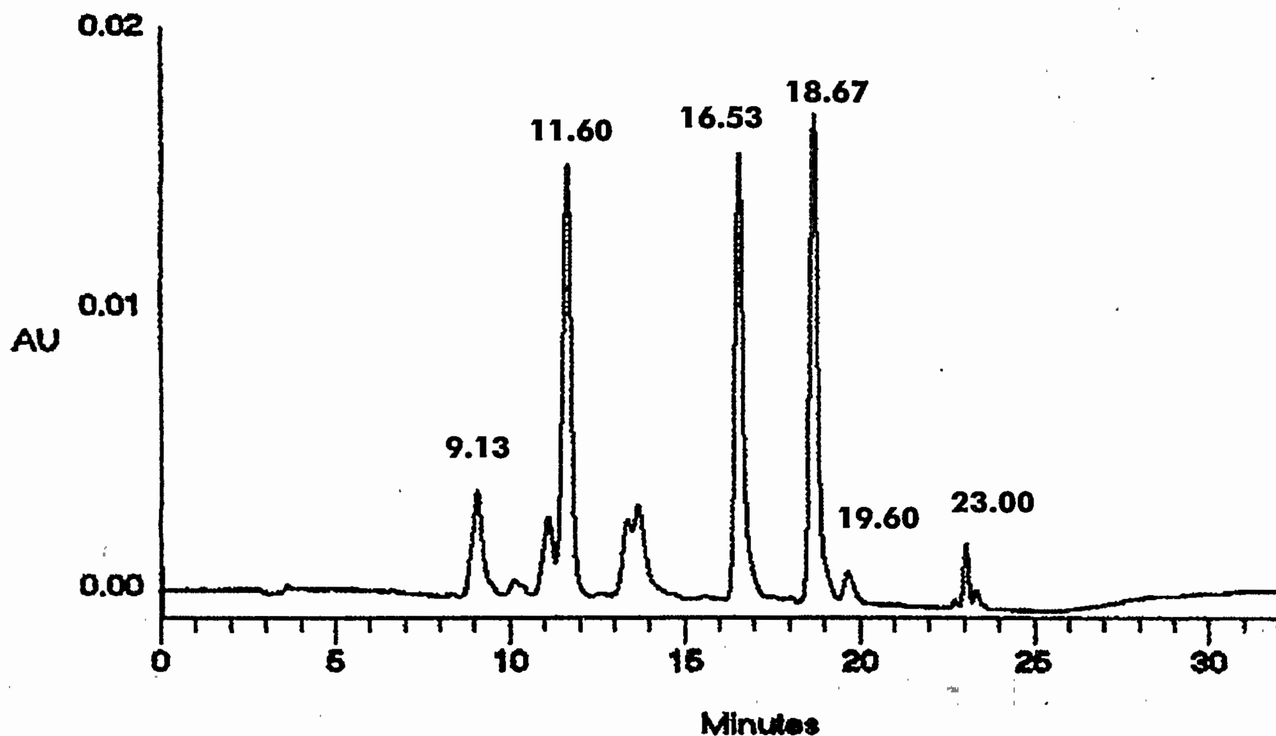


Figure 4 - Selenastrum capricornitum

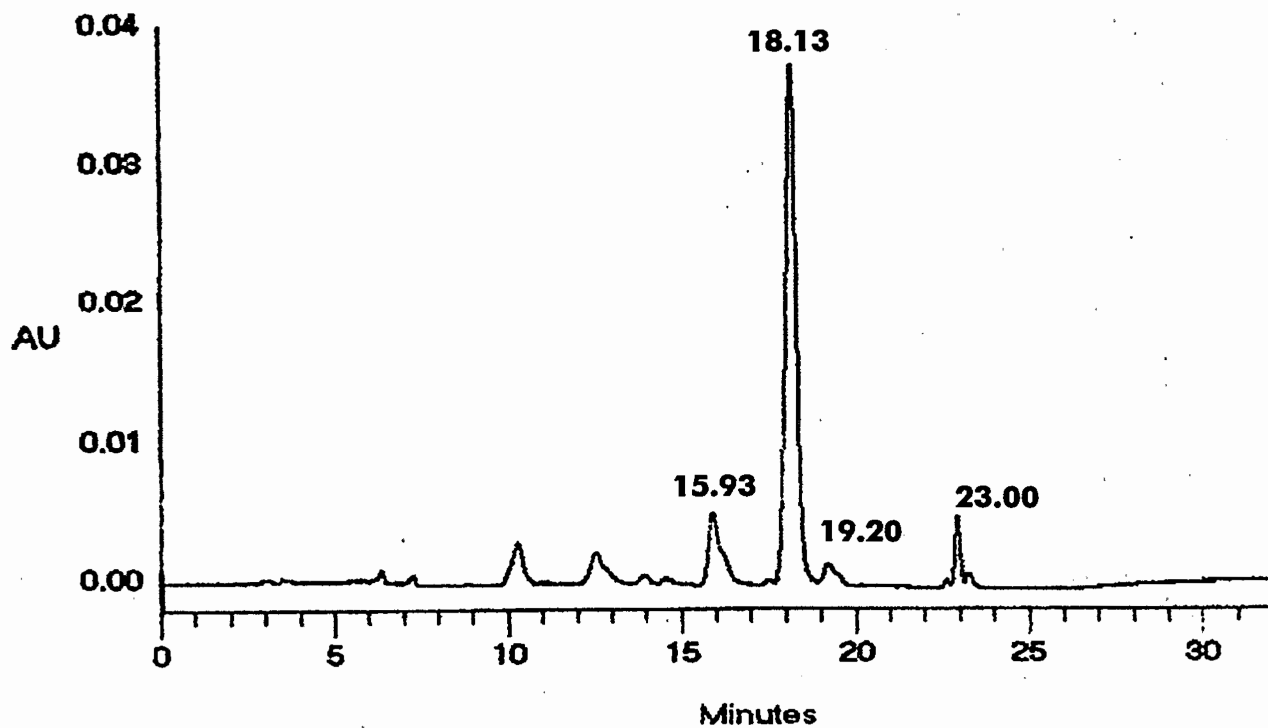


Figure 5 - Emilania huxleyi (slightly senescent)

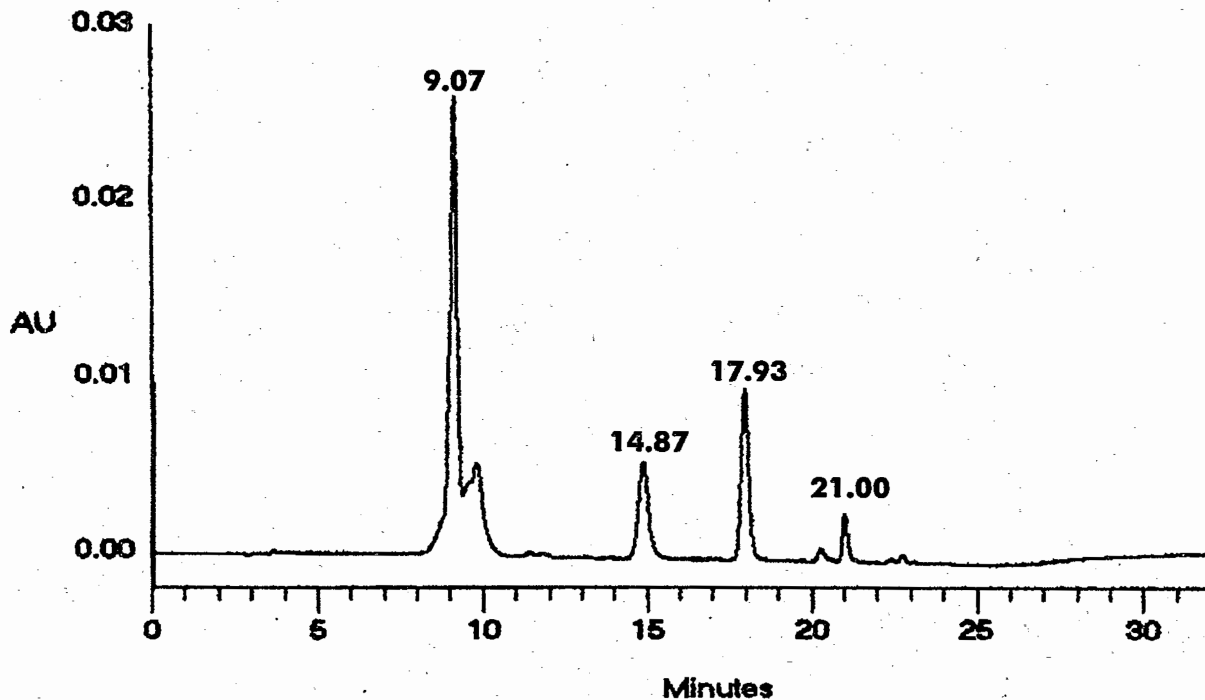
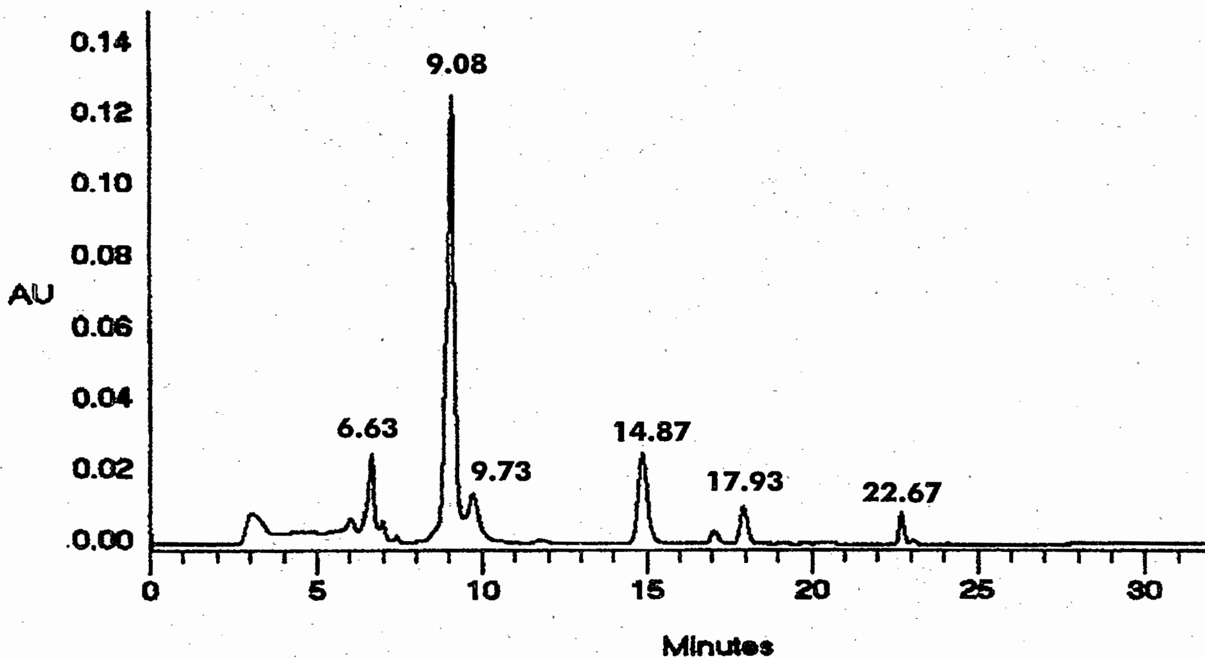


Figure 6 - Phaeodactylum tricornutum



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