# Appendix G

*High Sierra Water Laboratory*
*Standard Operating Procedures and QA/QC*

# HIGH SIERRA WATER LAB

*Specializing in Low Level Nutrient Chemistry*

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| ANALYTE | METHOD | DESCRIPTION | DETECTION LIMIT |
|---|---|---|---|
| 1. Ammonia | EPA 350.1 | Colorimetric, phenate | 1 µg/L |
| 2. Nitrate-Nitrite | EPA 353.1 | Colorimetric / Hydrazine | 1 µg/L |
| 3. Orthophosphate | SM 4500-PE | Colorimetric / Molybdate | 1 µg/L |
| 4. Total Phosphorus | EPA 365.3 | Colorimetric / Two Reagent - Molybdate | 1 µg/L |
| 5. Total Kjeldahl Nitrogen | EPA 351.2 | Colorimetric - Block digestion (phenate) | 35 µg/L |
| 6. Total Suspended Sediment | EPA 160.2 | Gravimetric | 0.1 mg/L |

The samples are delivered to High Sierra Water Lab as soon as possible and chilled to 4°C. They are then filtered immediately with a .45 micron nylon filter for the dissolved portions of the assay ($NH_4$, $NO_3$-$NO_2$, SRP, DP). These samples are then run for the constituents designated within 7-10 days for filtered samples, 28 days for raw samples.

The goal of our lab is to run one spike and one duplicate per 20 samples or one spike and one duplicate for each different project in each assay. A USGS Standard Reference Material (SRM) of known value is also run in each assay.

The wetland efficiency and Village Green Projects are run with many other projects — sich as USGS stream samples, U.S. Forest Service samples, Lahontan Water Board stream samples, Lake Tahoe water to depths of 450 m., precipitation samples, Tahoe City urban runoff and many more.

Our lab also participates biannually in the U.S. Geological Survey, Western Region Round Robin. USGS sends a blind sample which we analyze for nutrients, Kjeldahl Nitrogen, Ammonium, Nitrate-Nitrite, Orthophosphate, and Total Phosphorus. We are then scored along with the other 274 participating labs in the U.S. and other countries. High Sierra Water Lab had the top score in the Spring of 2002 and always has ranked among the highest in previous years.

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# QUALITY CONTROL

**The objective of the QA program is to present a definite QA plan that follows EPA recommendations. The plan includes control measures of the standard operating procedures. Our goal is to produce analytical results with a high level of confidence.**

**Each assay is continually monitored for quality control. Records pertaining to each assay are kept in the laboratory quality assurance notebook.**

## QC CHECKLIST

**1. One important quality control check for all assays is the standard curve slope value. This value should remain consistent from one assay to the next. Slope values are tracked using control charts. An average standard curve slope is plotted on the chart, sandwiched between two levels of limitations. The warning limits are defined as ±1.5sx from the average slope. The control limits are defined as ±3sx from the average slope value. When assay slopes fall outside the control limits, the entire run is automatically done again. After each analysis, the standard curve slope is plotted one appropriate chart. Once every six months, the limits are updated to include data from analyses. of the previous six months. At least 10 new data points must be plotted in the six month period to initiate the update.**

**2. The statistics pertaining to the standard curve of each assay are recorded on a Standard Curve Statistical Summary Form. The structural curve slope, Y-intercept, correlation coefficient, and standard range are noted.**

**3. The concentrations of duplicates are recorded on the Relative Percent Difference Worksheet.**

**4. The reagent blank values for each assay are recorded on the Method Blank Absorbance Summary Form.**

**5. The spike concentrations are noted on the Matrix Recovery Worksheet and the percent recovery from each assay is calculated.**

**6. Calibration verification using either an independent source stock solution or a standard reference material are recorded. The percent difference between the actual value and the assay value is calculated.**

**(continued on page 2)**

For each chemical assay, a spike and a duplicate will be run with each 20 samples. Also, a Standard Reference Material (SRM) will be run with each assay.

The duplicates are evaluated. The goal of our lab is to have the Relative Percent Difference (RPD) fall within 15% of 100%. If the 15% RPD criterion is exceeded in more than two individual runs, checks are made for contamination. The dups will be rerun.

Spike recoveries are used to monitor matrix interferences and method accuracy. The goal for our lab is to have spike recoveries fall within ±15% of 100%. If the criterion is not met, the spike will be rerun.

## PERFORMANCE AND SYSTEM AUDITS

External quality control checks for accuracy provide information for laboratory evaluation. We routinely participate in interlaboratory proficiency checks on replicate samples (splits).

Biannual reference samples are received from the U.S. Geological Survey, Western Region. This Round Robin analysis of reference samples for ammonium, nitrate, orthophosphate, total phosphorus, Kjeldahl nitrogen, and iron test our laboratory's ability to obtain accurate and acceptable results. The concentration of each analyte is unknown to the analyst.

# AMMONIUM ANALYSIS (Indophenol Method) (NH4)
Low level, Colorimetric

## Introduction:

This method is a modification of the method reported by Liddicoat et al. (1975) and Solorzano (1969). A blue indophenol reaction between ammonium, phenol, and hypochlorite takes place using potassium nitroferricyanide as a catalyst. The total ammonium present is then measured spectrophotometrically. The final concentration is reported as µg NH4-N/L. Calcium and magnesium interference is eliminated by complexing with sodium citrate. Other advantages of this method are the eliminations of distillation or solvent extraction steps, improved sensitivity over nesslerization method, speed, stability of blue coloration, and absence of reaction with other forms of nitrogen. Interferences include matrices with high concentrations of Ca+ and Mg+, turbidity, and color in the samples that absorb in the photometric range being used. Mercuric chloride (HgCl2) used as a preservative gives a negative interference by complexing with ammonia.

The method is applicable in the range from 0-500 µg/L NH4-N although it yields best results on samples containing less than 50 µ/L. The method detection limit (MDL) is ± 1-2 µg/L. The precision of the 10 ml method (95% confidence intervals) is ± 2 µg/L.

## Pre-treatment of Water Samples:

Analysis is run on filtered samples. Samples should be filtered on the day of collection with a pre-combusted Whatman GF/C filter. Keep filtered samples in tightly capped bottles, stored at 4°C. Assay should be performed within ten days of sample collection.

## Notes:

1. If the concentration of ammonium is higher than the highest standard, the sample should be diluted and re-analyzed.

2. This assay generates a regulated mixed waste solution. Pour all waste contents into the appropriately labeled waste jug.

## Calculations:

To calculate the ammonium concentration, generate a standard curve by plotting the absorbance versus the concentration of the standards. Absorbance values of samples are then entered to give ammonium concentration in µg NH4/L. If samples have been diluted, multiply the concentration by the appropriate dilution factor to calculate final concentration values.

## QA/QC Procedures:

1. To determine the success and accuracy of each run, it is important to measure the percent recovery of ammonium. Run 5% of the samples as spikes. Use the NH4Cl solution for additions of ammonium. Typical spike concentrations are 10 µg/L. Calculate the percent recovery for each spiked sample.

2. Replicates should be run on 5% of the samples to assess precision of the analytical run. Calculate the relative percent difference (RDP) for each replicate.

3. Calculate the standard curve statistics: r2, Y intercept, and slope.

References:

Liddicoat, M.I., S. Tibbits, and E.I. Butler. 1975. The determination of ammonia in seawater. Limnol. Oceanogr. 20:131-132.

Solorzano, L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. 14:799-801.

Brzezinsik, M.A. 1987. Colorimetric determination of nanomolar concentrations of ammonium in seawater using solvent extraction. Marine Chemistry 20:277-288.

# NITRATE ANALYSIS (Hydrazine Method) ($NO_3$ + $NO_2$)
Low level, colorimetric

## Introduction:

This method utilizes a hydrazine-copper reducing solution which reduces nitrate to nitrite followed by color development using a diazotization-coupling reaction. The method assumes a 1:1 stoichiometric reduction of nitrate to nitrite. The total nitrite present is then measured spectrophotometrically. The resulting concentration values for only nitrate, the nitrite concentration must be analyzed separately from an aliquot of the same sample by omitting the reduction steps. The nitrite concentration is then subtracted from the combined concentration value of [$NO_3$] + [$NO_2$].

Interferences include matrices with high concentrations of $Ca^+$ and $Mg^+$, turbidity, and sample color that absorbs in the photometric range being used. Concentrations of sulfide ion of < 10 mg/L will cause variations of $NO_3$ and $NO_2$ concentrations of ± 10%.

This method is applicable in the range from 0-500 µg/L. The method detection limit (MDL) is 1 µg/L. The precision of the 10ml method (99% confidence level) is ± 0.3 µg/L.

## Pre-treatment of Water Samples:

Analysis is run on filtered samples. Samples should be filtered with precombusted Whatman GF/C filters on the day of collection. Keep samples in tightly capped bottles, stored at 4° C. Assay should be performed within 10 days of sample collection.

## Notes:

1. If the concentration of nitrate is higher than the highest standard, the sample should be diluted and re-analyzed.

2. This assay generates a regulated mixed waste solution. Pour all waste contents into the appropriate waste jug.

## Calculations:

To calculate the nitrate concentration, generate a standard curve by plotting the absorbance versus the concentration of the standards. Absorbance values of samples are then entered to give nitrate concentration in µg $NO_3$ + $NO_2$/L. If samples have been diluted, multiply the concentration by the appropriate dilution factor to calculate final concentration values.

## QA/QC Procedures:

1. To determine the success and accuracy of each run, it is important to measure the percent recovery of nitrate. Run 5% of the samples as spikes. Typical spike concentrations are 50 µg/L. Calculate the percent recovery for each spiked sample.

2. Replicates should be run on 5% of the samples to assess precision of the analytical run.

Calculate the relative percent difference (RDP) for each replicate.

3. Calculate the standard curve statistics: r2, Y intercept, and slope.

References:

Kamphake, L.J., S.A. Hannah, and J.M. Cohen. 1967. Automated analysis for nitrate by nydrazine reduction. Water Research. 1:205-216.

Strickland, J.D.H. and T.R. Parsons. 1972. A practical handbook of seawater analysis. Bulletin 167. Fisheries Research Board of Canada, Ottawa, Ontario, Canada.

# KJELDAHL NITROGEN (TKN)
Total or Dissolved
Low level, Colorimetric

## Introduction:

Organic nitrogen compounds are reduced to the ammonia by digestion with sulfuric acid in the presence of mercuric sulfate (catalyst) and potassium sulfate (increases digestion temperature). The ammonia produced by this digestion, as well as the ammonia originally present, is determined by reaction with phenol, hypochlorite, and potassium nitroferricyanide (catalyst) in an alkaline medium. The resulting blue indophenol color is directly proportional to the concentration of ammonia present and is measured spectrophotometrically. The concentartion is reported as µg Kjeldahl-N/L. Values for organic nitrogen are calculated by subtraction of original nitrate and ammonia concentrations.

Mercuric chloride used as a preservative gives a negative interference by complexing with ammonia. Sulfide ions less than 10mg/L will also interfere with color development. Turbidity may also cause problems during photometric readings.

This method may be used to analyze water and water-suspended sediment with 0 - 500 µg Kjeldahl-N/L. Samples with concentrations greater than 500 µg/L need to be diluted. The method limit of detection (MLD) is approximately 35 µg/L. The precision of the 20 ml method (99% confidence intervals) is ± 16 µg/L.

## Pre-treatment of Water Samples:

Analysis is run on raw samples for total values and filtered samples for dissolved Kjeldahl nitrogen. Samples should be stored in tightly capped bottles at 4° C. Analysis should be run within a month of the collection date or frozen for later analysis.

## Notes:

1. Samples greater than 400 µg/L must be diluted and reanalyzed.

2. This assay generates a regulated mixed waste solution. Pour all waste contents into the appropriately labeled waste jug.

## Calculations:

To calculate the nitrate concentration, generate a standard curve by plotting the absorbance versus the concentration of the standards. Absorbance values of samples are then entered to give TKN concentration in µg TKN-N/L. If samples have been diluted, multiply the concentration by the appropriate dilution factor to calculate final values.

## QA/QC Procedures:

1. To determine the success and accuracy of each run, it is important to measure the percent

recovery of organic nitrogen. Run 5% of the samples as spikes. Typical spike concentrations are 150 µg/L. Calculate the percent recovery for each spiked sample.

2. Replicates should be run on 5% of the samples to assess precision of the analytical run. Calculate the relative percent difference (RDP) for each replicate.

3. Calculate the standard curve statistics: r2, Y intercept, and slope.

References:

Liddicoat, M.I., S. Tibbits, and E.I. Butler. 1975. The determination of ammonia in seawater. Limnol. Oceanogr. 20:131-132.

Methods for the Determination of Inorganic Substances in Water and Fluvial Sediments. M.J. Fishman and L.C. Friedman (eds.) 1985. Open-File Report 85-495. Denver, Colorado.

Solorzano, L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. 14:799-801.

State Water Resource Control Board. Quick Chem Method No. 10-107-06-2-F. Revision: D. Diamond. June 1986.

# ORTHOPHOSPHORUS ANALYSIS (SRP)
Total or Dissolved
Low level, Colorimetric

## Introduction:

Orthophosphorus is converted to a phosphomolybdate complex by acidified ammonium molybdate. When the phosphomolybdate complex is reduced with ascorbic acid in the presence of antimony, an intense blue complex develops. The color intensity is proportional to the orthophosphorus concentration and is measured spectrophotometrically. Concentrations are reported as µg PO4-P/L.

Interferences include arsenates (> 100 µg As/L) which react with the molybdate reagent to produce a blue color similar to that formed with phosphate. Hexavalent chromium and nitrite have a negative interference.

The method is applicable in the range from 0-500 µg/L. The method detection limit (MDL) is 1 µg/L. The precision of the 20 ml method (99% confidence intervals) is ± 0.6 µg/L.

## Pre-treatment of Water Samples:

Analysis is run on raw samples for total values or filtered samples for dissolved orthophosphate commohnly referred to as SRP - soluble reactive phosphorus. For filtered samples, samples should be filtered on the day of collection using precombusted Whatman GF/C filters. Keep filtered samples in tightly capped bottles, stored at 4°C. Assay should be performed within 10 days of collection.

## Notes:

1. If the concentration of orthophosphorus is higher than the highest standard, the sample should be diluted and re-analyzed.

## Calculations:

To calculate the phosphate concentration, generate a standard curve by plotting the absorbance versus the concentration of the standards. Absorbance values of samples are then entered to give phosphate concentration in µg PO4-P/L. If samples have been diluted, multiply the concentration by the appropriate dilution factor to calculate final concentration values.

## QA/QC Procedures:

1. To determine the success and accuracy of each run, it is important to measure the percent recovery of orthophosphate. Run 5% of the samples as spikes. Use the K2HPO4 solution for additions of orthophosphate. Typical spike concentrations are 10 µg/L. Calculate the percent recovery for each spiked sample.

2. Replicates should be run on 5% of the samples to assess precision of the analytical run. Calculate the relative percent difference (RDP) for each replicate.

3. Calculate the standard curve statistics: R2, Y intercept, and slope.

References:

Murphy, J. and Rile, J.P. 1962. A modified single-solution method for the determination of phosphate in natural waters. Analytica Chemica Acta 27:31-36.

# PHOSPHORUS ANALYSIS (Persulfate Digestion) (TP/DP)
Total or Dissolved
Low level, Colorimetric

## Introduction:

All forms of phosphorus, including organic phosphorus, are converted to orthophosphate by an acid-persulfate digestion. The samples are then analyzed for orthophosphate. The sample color intensity is proportional to the orthophosphate concentrations and is measured spectrophotometrically. The values are reported as µg P/L. The acid-persulfate digestion is more rigorous and complete than the sulfuric acid digestion, thus it is the best estimation of "total" phosphorus.

Interferences include arsenates (> 100 µg As/L) which react with the molybdate reagent to produce a blue color similar to that formed with phosphate. Hexavelent chromium and nitrite have a negative interference.

The method is applicable for samples ranging from 0 - 500 µg/L. The method detection limit (MDL) is 2 µg/L. The precision of the 20 ml method (99% confidence intervals) is ± 0.5 µg/L.

## Pre-treatment of Water Samples:

Analysis is run on either raw samples for total values or filtered samples for dissolved "total" phosphorus. If samples are to be filtered, the filtration should occur on the day of collection using a precombusted Whatman GF/C filter. Keep all samples in tightly capped bottles, stored at 4°C. Assay should be performed within one month of collection.

## Notes:

1. If the concentration of phosphorus is higher than the highest standard, the sample should be diluted and re-analyzed.

## Calculations:

To calculate the phosphorus concentration, generate a standard curve by plotting the absorbance versus the concentration of the standards. Absorbance values of samples are then entered to give phosphorus concentration in µg P/L.

## QA/QC Procedures:

1. To determine the success and accuracy of each run, it is important to measure the percent recovery of phosphorus. Run 5% of the samples as spikes. Use the K2HPO4 solution for additions of phosphorus. Typical spke concentrations are 15 µg/L. Calculate the percent recovery for each spiked sample.

2. Replicates should be run on 5% of the samples to assess precision of the analytical run. Calculate the relative percent difference (RDP) for each replicate.

3. Calculate the standard curve statistics: r2, Y intercept, and slope.

References:

Goldman, C. R. 1974. Eutrophication of Lake Tahoe emphasizing water quality. EPA-600/3-74-034. U.S. Govt. Printing Office, Washington, D.C. 408 pp.

Methods for the Determination of Inorganic Substances in Water and Fluvial Sediments. M.J. Fishman and L.C. Friedman (eds.) 1985. Open-File Report 85-495. Denver, Colorado.

Strickland, J.D.H. and T.R. Parson. 1972. A practical handbook of seawater analysis. Bulletin 167. Fisheries Research Board of Canada, Ottawa, Ontario, Canada.

## 10200 H. Chlorophyll

The concentration of photosynthetic pigments is used extensively to estimate phytoplankton biomass.[1,2] All green plants contain chlorophyll *a,* which constitutes approximately 1 to 2% of the dry weight of planktonic algae. Other pigments that occur in phytoplankton include chlorophylls *b* and *c*, xanthophylls, phycobilins, and carotenes. The important chlorophyll degradation products found in the aquatic environment are the chlorophyllides, pheophorbides, and pheophytins. The presence or absence of the various photosynthetic pigments is used, among other features, to separate the major algal groups.

The three methods for determining chlorophyll *a* in phytoplankton are the spectrophotometric,[3-5] the fluorometric,[6-8] and the high-performance liquid chromatographic (HPLC) techniques.[9] Fluorometry is more sensitive than spectrophotometry, requires less sample, and can be used for in-vivo measurements.[10] These optical methods can significantly under- or overestimate chlorophyll *a* concentrations,[11-18] in part because of the overlap of the absorption and fluorescence bands of co-occurring accessory pigments and chlorophyll degradation products.

Pheophorbide *a* and pheophytin *a,* two common degradation products of chlorophyll *a,* can interfere with the determination of chlorophyll *a* because they absorb light and fluoresce in the same region of the spectrum as does chlorophyll *a*. If these pheopigments are present, significant errors in chlorophyll *a* values will result. Pheopigments can be measured either by spectrophotometry or fluorometry, but in marine and freshwater environments the fluorometric method is unreliable when chlorophyll *b* co-occurs. Upon acidification of chlorophyll *b*, the resulting fluorescence emission of pheophytin *b* is coincident with that of pheophytin *a,* thus producing underestimation and overestimation of chlorophyll *a* and pheopigments, respectively.

HPLC is a useful method for quantifying photosynthetic pigments[9,13,15,16,19-21] including chlorophyll *a*, accessory pigments (e.g., chlorophylls *b* and *c*), and chlorophyll degradation products (chlorophyllides, pheophorbides, and pheophytins). Pigment distribution is useful for quantitative assessment of phytoplankton community composition and zooplankton grazing activity.[22]

### 1. Pigment Extraction

Conduct work with chlorophyll extracts in subdued light to avoid degradation. Use opaque containers or wrap with aluminum foil. The pigments are extracted from the plankton concentrate with aqueous acetone and the optical density (absorbance) of the extract is determined with a spectrophotometer. The ease with which the chlorophylls are removed from the cells varies considerably with different algae. To achieve consistent complete extraction of the pigments, disrupt the cells mechanically with a tissue grinder.

Glass fiber filters are preferred for removing algae from water. The glass fibers assist in

breaking the cells during grinding, larger volumes of water can be filtered, and no precipitate forms after acidification. Inert membrane filters such as polyester filters may be used where these factors are irrelevant.

*a. Equipment and reagents:*

1) *Tissue grinder:**#(1) Successfully macerating glass fiber filters in tissue grinders with grinding tube and pestle of conical design may be difficult. Preferably use round-bottom grinding tubes with a matching pestle having grooves in the TFE tip.

2) *Clinical centrifuge.*

3) *Centrifuge tubes,* 15-mL graduated, screw-cap.

4) *Filtration equipment,* filters, glass fiber†#(2) or membrane (0.45-µm porosity, 47-mm diam); vacuum pump; solvent-resistant disposable filter assembly, 1.0-µm pore size;‡#(3) 10-mL solvent-resistant syringe.

5) *Saturated magnesium carbonate solution:* Add 1.0 g finely powdered $MgCO_3$ to 100 mL distilled water.

6) *Aqueous acetone solution:* Mix 90 parts acetone (reagent-grade BP 56°C) with 10 parts saturated magnesium carbonate solution. For HPLC pigment analysis, mix 90 parts HPLC-grade acetone with 10 parts distilled water.

*b. Extraction procedure:*

1) Concentrate sample by centrifuging or filtering as soon as possible after collection. If processing must be delayed, hold samples on ice or at 4°C and protect from exposure to light. Use opaque bottles because even brief exposure to light during storage will alter chlorophyll values. Samples on filters taken from water having pH 7 or higher may be placed in airtight plastic bags and stored frozen for 3 weeks. Process samples from acidic water promptly after filtration to prevent possible chlorophyll degradation from residual acidic water on filter. Use glassware and cuvettes that are clean and acid-free.

2) Place sample in a tissue grinder, cover with the 2 to 3 mL 90% aqueous acetone solution, and macerate at 500 rpm for 1 min. Use TFE/glass grinder for a glass-fiber filter and glass/glass grinder for a membrane filter.

3) Transfer sample to a screw-cap centrifuge tube, rinse grinder with a few milliliters 90% aqueous acetone, and add the rinse to the extraction slurry. Adjust total volume to 10 mL, with 90% aqueous acetone. Use solvent sparingly and avoid excessive dilution of pigments. Steep samples at least 2 h at 4°C in the dark. Glass fiber filters of 25- and 47-mm diam§#(4) have dry displacement volumes of 0.03 and 0.10 mL, respectively, and introduce errors of about 0.3 and 1.0% if a 10-mL extraction volume is used.

4) Clarify by filtering through a solvent-resistant disposable filter (to minimize retention of extract in filter and filter holder, force 1 to 2 mL air through the filter after the extract), or by centrifuging in closed tubes for 20 min at 500 g. Decant clarified extract into a clean, calibrated,

15-mL, screw-cap centrifuge tube and measure total volume. Proceed as in 2, 3, 4, or 5 below.

## 2. Spectrophotometric Determination of Chlorophyll

*a. Equipment and reagents:*

1) *Spectrophotometer,* with a narrow band (pass) width (0.5 to 2.0 nm) because the chlorophyll absorption peak is relatively narrow. At a spectral band width of 20 nm the chlorophyll *a* concentration may be underestimated by as much as 40%.

2) *Cuvettes,* with 1-, 4-, and 10-cm path lengths.

3) *Pipets,* 0.1- and 5.0-mL.

4) *Hydrochloric acid,* HCl, 0.1*N*.

*b. Determination of chlorophyll* a *in the presence of pheophytin* a: Chlorophyll *a* may be overestimated by including pheopigments that absorb near the same wavelength as chlorophyll *a*. Addition of acid to chlorophyll *a* results in loss of the magnesium atom, converting it to pheophytin *a*. Acidify carefully to a final molarity of not more than $3 \times 10^{-3}M$ to prevent certain accessory pigments from changing to absorb at the same wavelength as pheophytin *a*.[13] When a solution of pure chlorophyll *a* is converted to pheophytin *a* by acidification, the absorption-peak-ratio (OD664/OD665) of 1.70 is used in correcting the apparent chlorophyll *a* concentration for pheophytin *a*.

Samples with an OD664 before/OD665 after acidification ratio ($664_b/665_a$) of 1.70 are considered to contain no pheophytin *a* and to be in excellent physiological condition. Solutions of pure pheophytin show no reduction in OD665 upon acidification and have a $664_b/665_a$ ratio of 1.0. Thus, mixtures of chlorophyll *a* and pheophytin *a* have absorption peak ratios ranging between 1.0 and 1.7. These ratios are based on the use of 90% acetone as solvent. Using 100% acetone as solvent results in a chlorophyll *a* before-to-after acidification ratio of about 2.0.[3]

Spectrophotometric procedure—Transfer 3 mL clarified extract to a 1-cm cuvette and read optical density (OD) at 750 and 664 nm. Acidify extract in the cuvette with 0.1 mL 0.1*N* HCl. Gently agitate the acidified extract and read OD at 750 and at 665 nm, 90 s after acidification. The volumes of extract and acid and the time after acidification are critical for accurate, consistent results.

The OD664 before acidification should be between 0.1 and 1.0. For very dilute extracts use cuvettes having a longer path length. If a larger cell is used, add a proportionately larger volume of acid. Correct OD obtained with larger cuvettes to 1 cm before making calculations.

Subtract the 750-nm OD value from the readings before (OD 664 nm) and after acidification (OD 665 nm).

Using the corrected values calculate chlorophyll *a* and pheophytin *a* per cubic meter as follows:

$$\text{Chlorophyll } a, \text{ mg/m}^3 = \frac{26.7\,(664_b - 665_a) \times V_1}{V_2 \times L}$$

$$\text{Pheophytin } a, \text{ mg/m}^3 = \frac{26.7\,[1.7\,(665_a) - 664_b] \times V_1}{V_2 \times L}$$

where:

$V_1$ = volume of extract, L,

$V_2$ = volume of sample, $m^3$,

$L$ = light path length or width of cuvette, cm, and

$664_b$, $665_a$ = optical densities of 90% acetone extract before and after acidification, respectively.

The value 26.7 is the absorbance correction and equals $A \times K$

where:

$A$ = absorbance coefficient for chlorophyll *a* at 664 nm = 11.0, and

$K$ = ratio expressing correction for acidification.

$$= \frac{\left(\frac{664_b}{665_a}\right) \textit{pure chlorophyll } \text{a}}{\left(\frac{664_b}{665_a}\right) \textit{pure chlorophyll } \text{a} - \left(\frac{664_b}{665_a}\right) \textit{pure pheophytin } \text{a}}$$

$$= \frac{1.7}{1.7 - 1.0} = 2.43$$

*c. Determination of chlorophyll* a, b, *and* c *(trichromatic method):* Spectrophotometric procedure—Transfer extract to a 1-cm cuvette and measure optical density (OD) at 750, 664, 647, and 630 nm. Choose a cell path length or dilution to give OD664 between 0.1 and 1.0.

Use the optical density readings at 664, 647, and 630 nm to determine chlorophyll *a, b,* and *c*, respectively. The OD reading at 750 nm is a correction for turbidity. Subtract this reading from each of the pigment OD values of the other wavelengths before using them in the equations below. Because the OD of the extract at 750 nm is very sensitive to changes in the