

Technical Report Series
Number 75-4

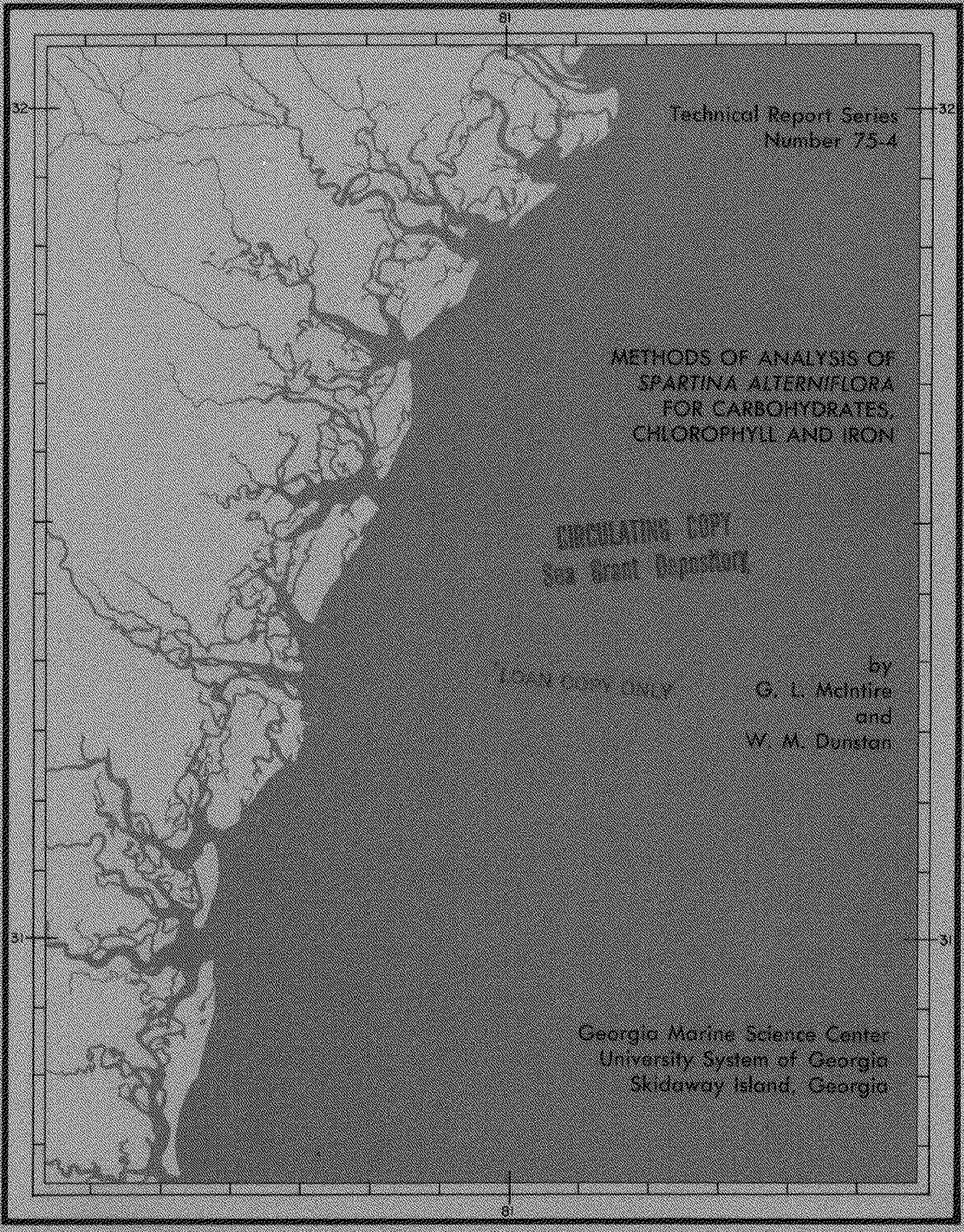
METHODS OF ANALYSIS OF
SPARTINA ALTERNIFLORA
FOR CARBOHYDRATES,
CHLOROPHYLL AND IRON

LIBRATING COPY
SEA GRANT STATION

1975

by
G. L. McIntire
and
W. M. Dunstan

Georgia Marine Science Center
University System of Georgia
Skidaway Island, Georgia



METHODS OF ANALYSIS OF SPARTINA ALTERNIFLORA FOR
CARBOHYDRATES, CHLOROPHYLL AND IRON

G. L. McIntire and W. M. Dunstan

Skidaway Institute of Oceanography
Savannah, Georgia 31406

The Technical Report Series of the Georgia Marine Science Center is issued by the Georgia Sea Grant Program and the Marine Extension Service of the University of Georgia on Skidaway Island (P.O. Box 13687, Savannah, Georgia 31406). It was established to provide dissemination of technical information and progress reports resulting from marine studies and investigations mainly by staff and faculty of the University System of Georgia. In addition, it is intended for the presentation of techniques and methods, reduced data and general information of interest to industry, local, regional, and state governments and the public. Information contained in these reports is in the public domain. If this prepublication copy is cited, it should be cited as an unpublished manuscript.

ABSTRACT

This technical report provides detailed information on sample preparation and analysis for structural and non-structural carbohydrates, iron and chlorophyll in the important marsh plant, Spartina alterniflora.

INTRODUCTION

The marsh grass Spartina alterniflora is the dominant plant on millions of acres of important salt marshes along the East and Gulf coasts of the United States. Other species of Spartina are dominant in marshes throughout the world. In the course of our studies on the ecology, physiology and biochemistry of Spartina, we have employed several techniques for chemical analysis.

The analytical methods have been modified from published procedures, but most of the techniques of field sampling and sample preparation have been devised in our laboratory.

I. TOTAL NONSTRUCTURAL CARBOHYDRATES

Plants in the subfamily Eragrostoideae, which includes the genus Spartina, generally store starch, although most grasses store fructans (Bonner and Varner, 1965). Methods of analysis for non-structural carbohydrates depend upon which type of product is stored. Fructans are readily soluble in water and easily hydrolysed to monosaccharides. Starch is composed largely of amylopectin (approx. 70%) which is insoluble in water and requires enzymatic hydrolysis to monosaccharide moieties (Smith, Dale, 1968). A combination of the two methods results in a measure of Total Non-structural Carbohydrates (TNC). Two steps are necessary: (1) The extraction of TNC, using an enzyme solution, and (2) the actual analysis for sugars (reducing power). The method of analysis of reducing power given herein is adequate for any solution containing less than 2 mg. sugar per 10 ml. Any suitable test for reducing power can be substituted for the one presented here.

Step 1 - The TNC Extraction

A. Sample Preparation

Spartina is extremely variable in nature, and a sampling program should take this into consideration. If, for example, leaves are to be measured for TNC content, leaves from five or more plants scattered over the sample area should be mixed thoroughly and subsamples of the resultant mixture used for the analysis. Plants should be selected and dried as soon as possible at 100°C for one hour to denature the respiratory enzymes and prevent loss of carbohydrates due to respiration. However, drying beyond an hour at 100°C can result in loss of carbohydrates, so it is important that samples be transferred after one hour to an oven at 70°C until dry (generally twenty-four hours). Dried samples should be ground in a Wiley Mill to pass a 20-40 mesh screen and stored in sealed bottles under refrigeration.

B. Reagents

1. Acetate buffer (ph = 4.45). Mix 3 volumes of 0.2N acetic acid with 2 volumes 0.2N sodium acetate. Add a small amount of thymol to prevent microorganism growth and store in a brown glass bottle.
 - a. 0.2N acetic acid. Dilute 11.45 ml glacial acetic acid to 1 liter with distilled water.
 - b. 0.2N sodium acetate. Dissolve 16.4 g anhydrous sodium acetate in distilled water and bring up to 1 liter.
2. 0.5% takadiastase solution. Commercial takadiastase preparations were obtained through Fisher Scientific Co. Add 5 g to 1 liter of distilled water and .5 g thymol. Filter this solution through Whatman No 40 paper and store in a dark bottle under refrigeration. Activity will be limited to approximately six weeks. We have found it convenient to make up the solution fresh before each analysis by adding .5 g of enzyme/100 ml water and using directly.
3. 10% neutral lead acetate. 10 g neutral lead acetate ($\text{PbAc}_2 \cdot 3\text{H}_2\text{O}$)/100 ml distilled water.

C. Extraction

1. Weigh out 200 to 300 mg of dried, ground sample into a clean, dry Erlenmeyer flask.
2. Add 15 ml distilled water and boil on a hot plate for one to two minutes. Include a flask with distilled water only. This flask will be used later as an enzyme blank.
3. Cool to room temperature.
4. Add 10 ml of ph 4.45 buffer and exactly 10 ml of enzyme solution. It is important that the samples be at room temperature as higher temperatures may denature the enzymes.

5. Stopper the flask or cover with parafilm and incubate at 38°C for forty-four hours.
6. Filter samples through Whatman #1 paper into a 100 ml volumetric flask. Rinse the Erlenmeyer flasks several times with distilled water. Add 2 ml of 10% lead acetate to each volumetric flask and dilute to the mark with distilled water.
7. Mix well and decant about 30 ml of the solution into a 50 ml centrifuge tube. Centrifuge at medium speed for five minutes.
8. Decant into a 50 ml Erlenmeyer flask containing approximately 100 mg of powdered potassium oxalate. Cover the flask with parafilm (or stopper) and refrigerate overnight.
9. Filter the solution through Whatman #42 paper into a small Erlenmeyer without washing.
10. Pipette 10 ml of the filtrate into a 25 x 200 mm test tube. Add 1 ml of 1.0 N H₂SO₄ and heat in a boiling water bath for fifteen minutes. Cool the test tube and add 1 ml of 1.0 N NaOH. The sample is now ready for analysis of reducing power. (See reagents for analysis of reducing power for directions for 1.0 N H₂SO₄ and 1.0 N NaOH.)

Step 2 - Analysis of Reducing Power

A. Reagents

1. Reagent "50." Dissolve 25 g of anhydrous sodium carbonate and 25 g of sodium potassium tartrate (Rochelle Salt) in about 600 ml distilled water. Add 75 ml of a 10% copper sulfate (CuSO₄ · 5H₂O) solution (W/V) through a long-stemmed funnel below the surface of the solution. Add 20 g of sodium bicarbonate, 1 g of potassium iodide, and 200 ml of potassium iodate solution (3.567 g pure KIO₃/l). Mix the

solution well and pour into a one-liter volumetric flask. Dilute to the mark with distilled water. Store in dark, glass bottles under refrigeration, and the solution will remain stable for months.

2. Potassium iodide - Potassium oxalate solution. Dissolve 2.5 g of each together in 100 ml distilled water. Make a fresh solution before each analysis.
3. 1.0 N H_2SO_4 . Pour 27 ml of concentrated H_2SO_4 into a quantity of distilled water (approximately 600 ml) and dilute to 1 liter in a volumetric flask.
4. 1.0 N NaOH. Dissolve 40 g of sodium hydroxide in distilled water and make up to a liter.
5. Starch indicator. Stir 1 g of soluble starch into 10 to 15 ml cold distilled water. Heat 100 ml distilled water to boiling and add 1 g boric acid crystals. Add starch solution and allow to continue boiling for one minute. Cool slowly and store in refrigerator.
6. 0.02 N Sodium thiosulfate.
 - a. 0.1 N stock solution. Dissolve 25 g of pure sodium thiosulfate and 1 g of NaOH in distilled water and dilute to 1 liter. This solution should be allowed to stand for two to three hours before making the 0.02 N solution.

The strength of the 0.1 N solution may be checked against 0.1 N potassium dichromate solution. To prepare the dichromate solution, dissolve 4.9033 g of $\text{K}_2\text{Cr}_2\text{O}_7$ in distilled water and make up to 1 liter. Add 25 ml of this solution to a one-liter beaker containing 3 g potassium iodide and dilute to 500 to 600 ml. Add 10 ml of concentrated HCl and titrate immediately with the 0.1 N thiosulfate solution. Add

the starch indicator near the end of the titration. The end point is shown by a color change from dark blue to light green after the starch has been added. If the thiosulfate is exactly 0.1 N, the titration will require 25 ml. If less is used, the solution can be adjusted by diluting with distilled water. For example, if the titration took 24.7 ml, 0.3 ml of water should be added for every 24.7 ml solution remaining; in this case it would require $0.3 \times 975.3/24.7$ ml of water.

- b. Dilute the 0.1 N sodium thiosulfate solution 1 to 5 to make the 0.02 N solution (i.e. 100 ml 0.1 N + 400 ml water).
7. Sugar standard. Dry ASC-grade glucose or fructose in a petri dish over P_2O_5 in a desiccator. Carefully dissolve 1 g of the sugar in a saturated solution of benzoic acid in water. Dilute this with the saturated benzoic acid solution to 1 liter in a volumetric flask. Store in a refrigerator and remake every six months.

B. Procedure

1. Add 10 ml of reagent "50" directly to the neutralized samples in their 25 x 200 ml test tubes. Include an enzyme blank, a reagent "50" blank, and a sugar standard (usually 1 mg glucose).
2. Heat in a gently boiling water bath for fifteen minutes.
3. Cool to less than 30°C in a cool water bath.
4. Add 2 ml of the potassium iodide-potassium oxalate solution to each sample.
5. Add exactly 10 ml of 1.0 N H_2SO_4 to each sample.
6. Rotate the tubes to dissolve the cuprous oxide and titrate with 0.02 N sodium thiosulfate, using about 2 drops of starch indicator per sample.

7. Calculate the mg sugar per sample as follows:

Sample Calculations:

Reagent "50" blank titration	9.70 ml
Enzyme blank titration	4.75 ml
1 mg glucose standard titration	5.35 ml
Unknown TNC titration	3.50 ml

Difference between Reagent "50" and glucose standard - 4.35

Difference between Enzyme and Unknown TNC - 1.25

Amount of sugar in sample = $1.0 \times \frac{1.25}{4.35} = .29$ mg glucose

% TNC in sample = $\frac{.29 \text{ mg}}{\text{Total sample wt in grams}}$

II. STRUCTURAL CARBOHYDRATES

Cellulose is one of the most abundant organic materials in the world. It permeates everything in modern society from wood to fibres for clothing. Spartina maintains a relatively woody stem and underground rhizomes with structural carbohydrate contents of up to 75%. The majority of Spartina photosynthetic production is incorporated directly into structural moieties to be released upon the death of the plant to the detrital food chain (McIntire and Dunstan, 1975). The following procedure yields the amount of holocellulose in the plant. This includes hemi-celluloses, which are sugars combined with uronic acids, and cellulose (Routley and Sullivan, 1958).

A. Sample Method and Preparation

As this is a gravimetric procedure, a large sample would usually result in less error. Samples of 5 to 10 grams seem to be easiest to handle. Sampling should be random and representative of the population. Samples should be dried at 100°C for one hour and then 70° until dry (usually twenty-four hours). After grinding the samples in a Wiley Mill to pass a 20-40 mesh screen, they should be stored in sealed bottles until ready for analysis.

B. Reagents

1. Benzene, alcohol solvent 2.5 parts of Benzene to 1 part ethanol.
2. 0.2 N HCl. Add 8.1 ml of concentrated HCl to about 600 ml distilled water in a liter volumetric flask. Dilute this to 1 liter with distilled water.
3. Glacial acetic acid.
4. Sodium chlorite, technical grade.
5. Pepsin.
6. Ether

C. Analysis

1. Fill a soxhlet thimble with a known weight of sample (5 to 10 g) and place in the extraction apparatus.
2. Extract the sample with a benzene:alcohol mixture (2.5:1) in a soxhlet extraction apparatus for thirty hours.
3. Remove the sample and allow to dry overnight. Spreading on a watch glass facilitates drying.
4. Digest the sample with 250 ml of a 1% pepsin solution in a 0.1 N HCl for thirty hours at 38°C.
5. Filter the mixture through a sintered glass filter and wash with hot distilled water (approximately 80°C).
6. Repeat Step 4.
7. Repeat Step 5.
8. In a one-liter beaker, add to the wet residue in order: 625 ml distilled water, 2 ml glacial acetic acid, and 7.5 grams of sodium chloride. Stir the mixture and cover with a watch glass. Heat in a water bath at 85°C in a well ventilated hood as chlorine gas is released.

9. Make three more additions of acetic acid and sodium chloride at fifteen-minute intervals stirring frequently.
10. Fifteen minutes after the last addition (total time: one hour), cool the mixture to 10°C in an ice bath and filter on a sintered glass filter.
11. Wash at least six times with ice water (i.e. stir on the filter with ice water and no suction).
12. Wash the residue with alcohol and ether and dry in air at room temperature.
13. The weight of the residue is that of holocellulose.

III. IRON

Iron is required in the electron transport system of photosynthesis. Lack of iron results in chlorosis and eventual death of the plant. Some workers have reported that iron may limit Spartina growth (Adams, 1963). In the following procedure, iron is extracted from the ashed plant material with hydrochloric acid and then analyzed spectrophotometrically.

A. Sample Preparation

All samples should be cleaned thoroughly before drying as any soil and other foreign matter present will yield false results. Plants should be thoroughly dried at 110°C for twenty-four hours after sampling. After grinding the dried material in a Wiley Mill to pass a 20-mesh screen, weigh a sample into a clean crucible (usually about 0.50 grams). Ash the samples in a muffle furnace at 550°C overnight. Cool and add 5 ml of 50% HCl to each crucible. Heat gently on a hot plate at fuming for fifteen minutes. Do not heat to dryness. Cool the samples to room temperature and filter into 100 ml volumetric flasks through Whatman #1 filters. Rinse the crucibles and filters five times with warm HCl (1:100 HCl:H₂O) and five times with distilled water. Dilute to 100 ml with distilled

water. The samples are now ready for analysis.

B. Reagents

1. 50% HCl. Add HCl to an equal amount of distilled water.
2. 1% HCl. Dilute 2 ml-50% HCl to 100 ml with distilled water.
3. Ferrozine reagent. Ferrozine was obtained from the Hach Chemical Company. Dissolve 5.14 g of ferrozine and 100 g hydroxylamine hydrochloride in a small amount of water. Add 500 ml of concentrated HCl and cool to 20°C. Dilute to 1 liter with iron-free distilled water.
4. Buffer ph 5.5. Dissolve 400 g of ammonium acetate in water. Add 350 ml of concentrated ammonium hydroxide and dilute to 1 liter.
5. Standard solution. Dissolve 1 gram of electrolytic iron in 50 ml of 10% H₂SO₄, warming if necessary to speed up reaction. Cool and dilute to 1 liter with distilled water. This results in a solution of 1 mg Fe/ml.

C. Procedure

Add 2 ml of the sample solution to a 125 ml Erlenmeyer flask and approximately 45 ml of distilled water. Add 1 ml of the ferrozine reagent and heat at a boil for ten minutes. When cool, transfer the solutions quantitatively to 50 ml volumetric flasks and add 1 ml of ph 5.5 buffer. Dilute to volume with distilled water and read the absorbance at 562 nm. Prepare a standard curve with appropriate standard values from dilutions of the primary standard and calculate the amount of iron in the sample. The amount of iron per gram sample is calculated as follows:

$$\frac{\text{mg/l} \times 2.5}{\text{sample wt in g}} = \text{mg/g}$$

Note on glassware: Results are fairly reproducible if clean glassware is used. All glassware should be soaked in a 10% HCl acid

bath overnight before use.

2° Standard: Add 10 ml of 1° standard to a 500 ml volumetric flask.

= 20 mg Fe/l = .02 mg Fe/ml

1 ml 2°/50 ml sample = .4 mg Fe/l

2 ml 2°/50 ml sample = .8 mg Fe/l

etc.

A good set of standards ranges from .2mgFe/l to 1.2 mg/l.

IV. CHLOROPHYLL

A. Sample Preparation

The extraction of chlorophyll from Spartina may be difficult and must be thorough to yield quantitative results. We have found that shaking in 50 ml stainless steel, capped tubes on a Burrell wrist-type shaker produces the best extraction. Other methods can be used, but complete extraction must be accomplished. Samples may be run on a wet or dry weight basis. Cutting with scissors into fine pieces before extraction helps insure complete extraction of wet chlorophyll samples. We have found that freeze-drying causes little or no loss of chlorophyll. Freeze-dried samples should be ground in a Wiley Mill before extraction in the shaker as the increased surface area will enhance the extraction.

B. Reagents

90% acetone. Add 50 ml distilled water to a 500 ml volumetric flask. Fill to the mark with acetone and mix. Due to molecular interaction, the resulting solution may not be at 500 ml; in which case, fill to the mark again with acetone (probably only 1 or 2 mls).

C. Procedure

1. Place a weighed amount of sample (wet or dry) into the shaker tube along with two stainless steel balls approximately 1/4" in diameter and 10 ml of 90% acetone.
2. Tighten top on tube and shake for five minutes.
3. After shaking, remove the steel balls with tweezers and rinse off into the tube (a squeeze bottle with 90% acetone works well for this purpose).
4. Centrifuge in the steel tubes for five minutes at medium speed.
5. Pour off the supernatant into screw-capped, 50 ml centrifuge tubes and place in the dark with cap on the tube.
6. Add another 10 ml of 90% acetone and the steel balls to the solid residue in the shaker tubes.
7. Shake again for five minutes.
8. Remove steel balls as before and centrifuge again.
9. Pour off supernatant into the same 50 ml centrifuge as before, combining five minute extracts. Make up to a known volume (25 ml) with 90% acetone.
10. Centrifuge the screw-capped tubes for five minutes at medium speed. Check volume and make up to 25 ml if needed.
11. Read the absorption of the solution at 6630 A and again at 6450 A and be certain to zero each time against a blank of 90% acetone. Try to optimize the reading between .1 and 1.0 on the spectrophotometer by diluting the solution with 90% acetone. Generally, if 1 gram of wet Spartina leaf is used, the final solution will have to be diluted ten times to bring the reading into the optimal range.
12. Calculate the concentration in mg/ml chlorophyll a and chlorophyll b from the following equations:

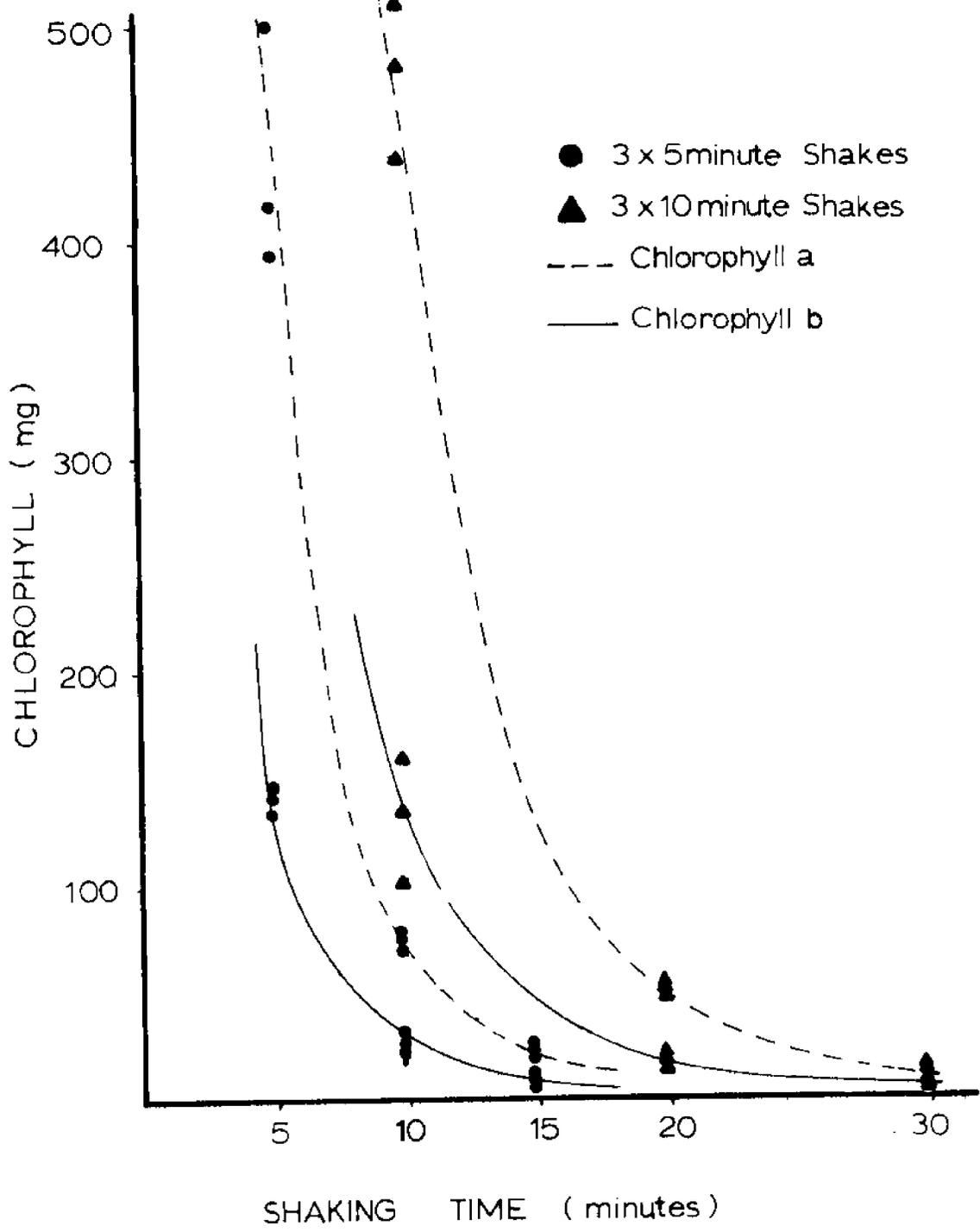
$$\text{CHL a} = 11.65 e_{6630} - 2.14 e_{6450}$$

$$\text{CHL b} = 4.31 e_{6630} + 19.97 e_{6450}$$

Multiply the concentration per milliliter by the total volume and divide by the weight of sample to find the concentration of chlorophyll per gram sample.

Figure 1 illustrates the extraction of both chlorophyll a and chlorophyll b from freeze-dried material. Shaking for five minutes as described above removes over 95% of the total chlorophyll, while little or no advantage is gained from a third shake or by shaking for ten minutes each time rather than five.

Step 10 is unnecessary if care is taken in pouring off the supernatant from the earlier centrifuging. It is necessary to know the total volume of final solution to calculate the chlorophyll concentration/weight of sample.



REFERENCES

- Adams, D.A. 1963. Factors influencing vascular plant zonation in North Carolina salt marshes. Ecology 44:445-456.
- Bonner, J.F. and Varner, J.E., 1965. Plant Biochemistry, Academic Press, 1014 p.
- McIntire, G.L. and Dunstan, W.M., 1974. Nonstructural Carbohydrates in Spartina alterniflora Loisel. In press.
- Nixon, S. and Oviatt, C.A. 1973. Analysis of local variation in the standing crop of Spartina alterniflora. Botanica Marina 16:103-109.
- Routley, D.G. and Sullivan, J.T., 1958. The isolation and analysis of hemicelluloses of Brome Grass. J. Agri. Food Chem. Vol. 6, #9, 687-692.
- Smith, Dale. 1968. Removing and analyzing total nonstructural carbohydrates from plant tissue. Wisconsin Aquaculture Experiment Station, Research Report, No. 41, p. 1-11