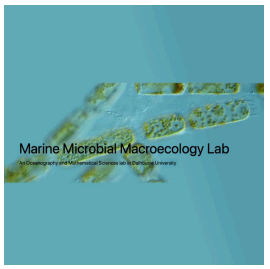
Jan 28, 2025 Version 2

Total particulate carbohydrate from microalgae V.2



DOI
dx.doi.org/10.17504/protocols.io.yxmvmk24ng3p/v2

Ying-Yu Hu[1], Zoe V. Finkel[1]

[1]Dalhousie University

Marine Microbial Macroecology Lab
Tech. support email: **ruby.hu@dal.ca**

Ying-Yu Hu
Dalhousie University

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.yxmvmk24ng3p/v2

Protocol Citation: Ying-Yu Hu, Zoe V. Finkel 2025. Total particulate carbohydrate from microalgae. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.yxmvmk24ng3p/v2**Version created by **Ying-Yu Hu**

Manuscript citation:
Ying-Yu Hu, Andrew J. Irwin and Zoe V. Finkel. 2024. An improved method to quantify bulk carbohydrate in marine planktonic samples. https://doi.org/10.1002/lom3.10614

Protocol status: Working
We use this protocol and it's working

Created: June 30, 2023

Last Modified: January 28, 2025

Protocol Integer ID: 84319

Keywords: TPTZ method, ferricyanide, hydrolysis, Total particulate carbohydrate

Funders Acknowledgements:
Simons Foundation
Grant ID: 723789
Simons Foundation
Grant ID: 549937

Abstract

Here we describe a protocol to estimate the total particulate carbohydrate from microalgae. Carbohydrate samples are initially vortexed in 9 M H_2SO_4 for 15 s. The solution is diluted for a final H_2SO_4 molarity of 1.6 M and hydrolyzed for 3 hours at 90 °C. The hydrolysate is alkalinized by adding 12 M NaOH to the hydrolysate, the ratio of $[H^+]$ from the hydrolysate to $[OH^-]$ from NaOH is 0.82. The alkalinized hydrolysate is oxidized by ferricyanide solution. The absorbance of TPTZ-Fe^{2+} complex is measured in microtiter plate at 595 nm. Our method has shown high reproducibility in aldohexoses, ketohexoses, deoxysugars, aldopentoses, uronic acid and amino sugars. The linear range of response is between 5 ng to 10 µg C/mL.

Protocol materials

- Sodium acetate anhydrous **Fisher Scientific Catalog #**BP333-500 Step 36.1
- Citric acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #** 251275-500G Step 36.1
- Na2CO3 **VWR International (Avantor) Catalog #**97061-972 Step 35
- TPTZ **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T253-5G Step 41
- D-glucose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G8270-100G Step 12.1
- K3[Fe(CN)6] **Fisher Scientific Catalog #**AC424120050 Step 39
- NaOH **Fisher Scientific Catalog #**BP359-500 Step 35
- Acetic acid **Fisher Scientific Catalog #**M1000632500 Step 36.1
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**439142-4L Step 29
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34860-4X2L-R Step 33

Safety warnings

Ferric waste should be disposed into trace metal waste container.
Waste acid should be neutralized before disposed into sink.

Before start

- Combust pasteur pipets at 500 °C 02:00:00
- Combust vials at 500 °C 06:00:00
- Combust glassware to hold 18 M H_2SO_4 at 500 °C 06:00:00
- Combust glass centrifuge tubes at 500 °C 06:00:00
- Centrifuge caps are acid washed and oven-dried.
- If lipids extraction is performed after the carbohydrate hydrolysis:

(1) Centrifuge caps for samples and blanks are 95% ethanol washed and air-dried
(2) 5 mL glass serological pipet for dispensing chloroform and methanol is 95% ethanol washed and air-dried

Sample collection

2h

1 Combust GF/F filter for 04:00:00 at 450 °C

4h

2 Filter microalgae in liquid media onto precombusted GF/F filters, using gentle vacuum pressure (130 mm Hg).

Equipment

Filter forceps	NAME
blunt end, stainless steel	TYPE
Millipore	BRAND
XX6200006P	SKU

3 Rinse filtration funnel with filtered saline to avoid sample loss.

4 Fold the filter with two tweezers:
(1) Fold in half along its diameter, creating a semicircular shape;
(2) Fold once more in the same direction, resulting in a long strip

5 Place sample filters in Cryogenic Vials.

6 Filter same volume of blank media (without cells) through filter as blank.

7 Fold the filter with two tweezers:
(1) Fold in half along its diameter, creating a semicircular shape;
(2) Fold once more in the same direction, resulting in a long strip

8 Place filter in Cryogenic Vials.

9 Flash freeze filters and store at -80 °C

10 Freeze-dry before processed.

Day 1- Preparation

30m

11 Prepare water bath 95 °C

30m

Equipment

Digital General Purpose Water Baths	NAME
20 L	TYPE
VWR®	BRAND
76308-900	SKU

Day 1- Glucose standard solutions

12 Primary standard

12.1 In a 2 mL microtube, weigh ~ 2 mg D-glucose

D-glucose **VWR International Catalog #**G8270-100G

12.2 Add Milli-Q for a final concentration of 1 mg/mL

Note

Volume requirement for preparing standard working solutions: >1400 µL

13 Prepare eight 10 mL precombusted centrifuge tubes, label tubes from SD1 to SD8.
Caps for the standard solutions: acid-washed and dried

Equipment

Disposable Glass Screw-Cap Centrifuge Tubes	NAME
10 mL	TYPE
Corning®	BRAND
99502-10	SKU

Equipment

Polypropylene Screw Caps	NAME
Linerless, 15-415	TYPE
Kimble Chase	BRAND
73805-15415	SKU

14 Follow the sheet to add primary standard and Milli-Q into the tube for working standard solutions.

	Standards	Primary (uL)	MilliQ (uL)
	SD1	0	500
	SD2	25	475
	SD3	50	450
	SD4	100	400
	SD5	150	350
	SD6	250	250
	SD7	350	150
	SD8	450	50

Day 1 - Samples

2h

15 Considering the working hours from 9 am to 4 pm, suggested sample number is:
# of blank + # of samples = 24

16 Label 10 mL centrifuge tubes, log sample information.

Note

Caps for the samples: 95% ethanol rinsed and dried (if lipids are measured from the same filter)

17 Rinse forceps with 95% ethanol and air dry.

Equipment

Filter forceps	NAME
blunt end, stainless steel	TYPE
Millipore	BRAND
XX6200006P	SKU

18 Transfer each filter strip into its centrifuge tube, starting from blank.

19 Add 500 µL Milli-Q directly to the bottom of the tube, , avoiding contact with the filter.

Note

Do not vortex!

GF/F, especially 47 mm, quickly absorbs all Milli-Q after vortexing and leaves no Milli-Q to dilute 18 M H_2SO_4. Besides, only 500 uL 18 M H_2SO_4 is not enough to soak the entire filter, which might result in insufficient pre-treatment.

Day 1- Hydrolysis

3m

20 Transfer 18 M H_2SO_4 into a precombusted glassware (such as, scintillation vial, beaker... etc)

21 Using the reverse pipetting technique, carefully dispense 500 µL 18 M H_2SO_4 into the MilliQ solution, ensuring it does not touch the filter. Immediately vortex the mixture for 00:00:15 using a timer or stopwatch.

15s

> **Note**
>
> Do not cap the centrifuge tube!

22 Add 4.5 mL MilliQ, **tightly cap the centrifuge tube**, and vortex for 00:00:05 .

5s

23 Place tube into water bath, log the time for each tube.

3h

> **Note**
>
> Three-hour hydrolysis duration for each sample/blank/standard should be accurately monitored.

24 After all samples are placed in the water bath, reduce temperature to 90 °C .

25 Label pre-combusted 12 mL clear vials for supernatant.

# of vials = # of samples + # of blanks

26 Label pre-combusted 12 mL amber vials for TPTZ measurement with white oil based sharpie.

# of vials = # of samples + # of blanks + # of standards

Equipment

Storage Vials and Closures	NAME
12 mL amber	TYPE
Thermo Scientific	BRAND
B7800-12A	SKU
VWR 66030-686	SPECIFICATIONS

27 As soon as hydrolysis duration reaches 3 hours, remove the tubes from water bath, cool in the tap water bath with ice to quickly stop hydrolysis.

Day 1- Prepare for lipids extraction

3m

28

Note

The carbohydrate hydrolysis procedure breaks the bond between lipids and non-lipid components, releasing bound lipids into an easily extractable form.
1. The acid in lipids can charge phospholipids to optimize extraction.
2. The acid facilitates the separation of the lipid fraction from extraneous material such as protein.
3. Hydrolysis removes most pigments (including chlorophyll and carotenoids), carbohydrates, and proteins from lipids.

29 Use glass serological pipet, add 2 mL chloroform into the hydrolysate of the samples (**not the standard solutions**). Vortex well.

Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**439142-4L

Note

- Glucose is insoluble in chloroform in the presence of water, and the concentration of glucose in the hydrolysate is no higher than 0.5 mM. While phospholipids can induce the migration of glucose into chloroform, this process doesn't occur instantly; the attainment of equilibrium is substantially delayed. Even when glucose reaches about 5 mM in the aqueous layer and phospholipids are present at concentrations of at least 8.5 mM, the molar ratio of solubilized glucose to phospholipid content remains approximately 0.0025. Therefore, under our conditions, glucose is unlikely to migrate into the lipid extract.

CITATION

CHAN Y. JUNG, JAMES E. CHANEY, AND PAUL G. LEFEVRE. Enhanced Migration of Glucose from Water into Chloroform in Presence of Phospholipids. ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS.

LINK

10.1016/0003-9861(68)90454-2

30 Centrifuge 3200 rpm, 00:05:00 5m

31 Use pre-combusted Pasteur pipets, transfer supernatant to 12 mL clear or amber vial by avoiding disturbing organic layer, ***leaving around 1 mL of hydrolysate*** for phase separation in lipids extraction.

Equipment

Disposable Soda-Lime Glass Pasteur Pipets	NAME
5 3/4"	TYPE
Fisherbrand	BRAND
13-678-6A	SKU

Equipment	
Disposable Glass Screw-Cap Centrifuge Tubes	NAME
10 mL	TYPE
Corning®	BRAND
99502-10	SKU

32 Keep all hydrolysate (standards, blank and samples) in a dark cabinet at Room temperature .

33 Use glass serological pipet, add 1 mL methanol into the organic layer, vortex well, freeze at -80 °C until lipids extraction.

Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34860-4X2L-R

Prepare reagents

34 12 M NaOH

34.1 Add 15 mL Milli-Q water into a 50 mL Falcon tube.

34.2 Add 12 g NaOH pellet into the water, swirl and have the pellets completely dissolved, let it cool down to Room temperature .

34.3 Transfer the solution into a 25 mL PP volumetric flask, rinse the tube three times by small amount of Milli-Q and combine the rinsed water into flask, top with Milli-Q water to 25 mL.

35 Alkaline solution for potassium ferricyanide
Dissolve 400 mg NaOH and 20 g Na_2CO_3 in volumetric flask and top to 1 L by Milli-Q. Store at room temperature.

NaOH **VWR International Catalog #**BP359-500

Na2CO3 **VWR International Catalog #**97061-972

36 Sodium acetate solution

36.1 Dissolve 164 g sodium acetate, 42 g citric acid and 300 g acetic acid in a 1 L volumetric flask and top to1 L with Mill-Q water.

Note

1. In this solution, sodium acetate, citric acid and acetic acid is 2 M, 0.2 M and 5 M respectively.
2. Add sodium acetate into the dry volumetric flask first. Sodium acetate is highly hygroscopic, the absorbance of moist hardens the powder into a bulk and clogs the neck of flask.

Sodium acetate anhydrous **VWR International Catalog #**BP333-500

Citric acid **VWR International Catalog #** 251275-500G

Acetic acid **VWR International Catalog #**M1000632500

36.2 Store at room temperature.

36.3 Dispense solution by serological pipet to avoid having salt precipitated around sealing surface of the bottle.

37 3 M acetic acid

Weigh 180 g acetic acid in fumehood, transfer the acid into volumetric flask, top to 1 L with Milli-Q water. Store at room temperature.

Day 2 Preparation

38 Boiling bath

Day 2 TPTZ reagents

39 Potassium ferricyanide (Reagent A)

Weigh 23 mg potassium ferricyanide and transfer into a 100 mL amber reagent bottle.
Add 100 mL alkaline solution, vortex until powder is completely dissolved. It is stable for two weeks at room temperature.
K3[Fe(CN)6] **VWR International Catalog #**AC424120050

Equipment

Reagent bottle	NAME
100 mL, amber	TYPE
VWR	BRAND
14216-240	SKU

40 Ferric chloride (Reagent B)
Ferric chloride hexahydrate is in spherical shape. It is hard to weigh exact 54 mg for a 100 mL solution. Pick a very small ferric chloride ball and log the weight. Transfer the ball into a 100 mL amber reagent bottle. Calculate the acetate solution required.
Add acetate solution into the amber bottle, vortex until the ball is completely dissolved.

V_acetate = 100 X W_actual/54

Note

This reagent needs to be prepared right prior to analysis. It can only be stable for no more than two days.

41 TPTZ (Reagent C)

Estimate the total volume required for the assay: 2 mL X (standard # + blank # + sample #)

For each 100 mL TPTZ reagent, weigh and transfer 78 mg TPTZ into an amber reagent bottle, add 100 mL acetic acid solution, vortex until the powder is completely dissolved.
TPTZ **VWR International Catalog #**T253-5G

Note

This solution is stored at room temperature and stable for one week.

Day 2- Alkalinization of standard solutions

3m

42 Forward pipetting: transfer 270 µL of hydrolysate of standard working solutions to amber vial.

Equipment

Storage Vials and Closures	NAME
12 mL amber	TYPE
Thermo Scientific	BRAND
B7800-12A	SKU
VWR 66030-686	SPECIFICATIONS

43 Add 640 µL Milli-Q and vortex.

44 Add 90 µL 12 M NaOH and vortex.

Note

12 M NaOH: reverse pipetting

Day 2- Alkalinization of samples

3m

45 Transfer 2 mL of hydrolysate into 2 mL microtube, centrifuge 13000 rpm, 00:05:00 5m

46 Use reverse pipetting to transfer 750 µL hydrolysate to a 12 mL amber vial.

> **Note**
>
> If using forward pipetting, the hydrolysate of samples tends to retain a small volume of liquid at the tip, which reduces the chance of error due to incomplete dispensing.

47 Add 250 µL 12 M NaOH , vortex.

> **Note**
>
> 12 M NaOH: reverse pipetting

TPTZ method

10m

48 In a room with dim light, add 1 mL Reagent A into blanks, standards and samples.

49 Tightly cap the vial and vortex.

50 Keep in a boiling water bath for 00:10:00

10m

51 Remove boiling bath from the heat, keep all vials in the hot water and move them into the room with dim light.

52 Add 1 mL Reagent B and 2 mL Reagent C into the vial and vortex.

53 Shake at Room temperature for 00:30:00 .

30m

54 Under dim light, using reverse pipetting, load 250 uL of blanks, standards, and samples into the microplate (duplicate).

Load column by column. After one column has been loaded, immediately cover the column with a lid, which has a black membrane on the top to protect sample from light.

55 Read in microplate reader:

Shake for 5 s at 600 rpm in a continuous and high force mode
Read endpoint 595 nm with a measurement time 100 ms

56 If the absorbance is higher than the absorbance of SD7, calculate the volume of sample to be loaded into the microplate.

$$V_{sample} = 250_{mL} * Abs_{SD7} / Abs_{sample}$$

Where, V_{sample} is the volume of sample with absorbance higher than the absorbance of SD7, Abs_{SD7}

is the absorbance of SD7, Abs_{sample} is the absorbance of sample after TPTZ assay.

57 Transfer V_{sample} of sample into microplate in duplicate

58 Transfer V_{sample} of sample blank into microplate in duplicate

59 Add $V_{milliQ} = 250_{uL} - V_{sample}$ into the sample

60 Read in microplate reader:

Shake for 5 s at 600 rpm in a continuous and high force mode
Read endpoint 595 nm with a measurement time 100 ms

Spectra of hydrolysate (optional step) 10m

61 Load 250 ul hydrolysate into microplate.

62 Scan UV/VIS spectra from 200 to 850 nm at a step of 2 nm.

Waste disposal

63 All hydrolysate and TPTZ reagents need to be neutralized by soda before disposed into the sink.

64 TPTZ reagent B is collected in trace metal waste container.

Citations

Step 29

CHAN Y. JUNG, JAMES E. CHANEY, AND PAUL G. LEFEVRE. Enhanced Migration of Glucose from Water into Chloroform in Presence of Phospholipids
10.1016/0003-9861(68)90454-2