



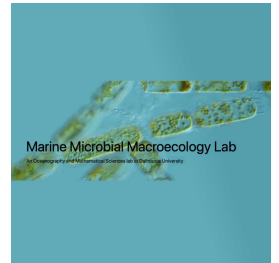
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Total particulate carbohydrate from microalgae V.2

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Ying-Yu Hu¹, Zoe V. Finkel¹

¹Dalhousie University

Marine Microbial Macroecology Lab

Tech. support email: ruby.hu@dal.ca



Ying-Yu Hu

Dalhousie University

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We use this protocol and it's working

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Abstract

Here we describe a protocol to estimate the total particulate carbohydrate from microalgae. Carbohydrate samples are initially vortexed in 9 M H₂SO₄ for 15 s. The solution is diluted for a final H₂SO₄ molarity of 1.6 M and hydrolyzed for 3 hours at 90 °C. The hydrolysate is alkalized by adding 12 M NaOH to the hydrolysate, the ratio of [H⁺] from the hydrolysate to [OH⁻] from NaOH is 0.82. The alkalized hydrolysate is oxidized by ferricyanide solution. The absorbance of TPTZ-Fe²⁺ 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

⊗ Na₂CO₃ VWR International (Avantor) Catalog #97061-972

⊗ NaOH Fisher Scientific Catalog #BP359-500

⊗ TPTZ Merck MilliporeSigma (Sigma-Aldrich) Catalog #T253-5G

⊗ D-glucose Merck MilliporeSigma (Sigma-Aldrich) Catalog #G8270-100G

⊗ K₃[Fe(CN)₆] Fisher Scientific Catalog #AC424120050

⊗ Sodium acetate anhydrous Fisher Scientific Catalog #BP333-500

⊗ Citric acid Merck MilliporeSigma (Sigma-Aldrich) Catalog # 251275-500G

⊗ Acetic acid Fisher Scientific Catalog #M1000632500

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

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

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 H₂SO₄ 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
- 2 Filter microalgae in liquid media onto precombusted GF/F filters, using gentle vacuum pressure (130 mm Hg).

4h

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 **Merck MilliporeSigma (Sigma-Aldrich) 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



	Standards	Primary (uL)	MilliQ (uL)
	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 H₂SO₄. Besides, only 500 µL 18 M H₂SO₄ is not enough to soak the entire filter, which might result in insufficient pre-treatment.

Day 1- Hydrolysis**3m**

- 20 Transfer 18 M H₂SO₄ into a precombusted glassware (such as, scintillation vial, beaker... etc)

- 21 Using the reverse pipetting technique, carefully dispense  500 µL 18 M H₂SO₄ 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 (2026)
. Enhanced Migration of Glucose from Water into Chloroform in Presence of Phospholipids.
ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS.

[10.1016/0003-9861\(68\)90454-2](https://doi.org/10.1016/0003-9861(68)90454-2)

[LINK](#)

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 Fisher Scientific Catalog #BP359-500

 Na_2CO_3 VWR International (Avantor) 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 to 1 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 Fisher Scientific Catalog #BP333-500

 Citric acid Merck MilliporeSigma (Sigma-Aldrich) Catalog # 251275-500G

 Acetic acid Fisher Scientific 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] Fisher Scientific 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.

$$V_{\text{acetate}} = 100 \times W_{\text{actual}} / 54$$

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 Merck MilliporeSigma (Sigma-Aldrich) 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 μ L 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](https://doi.org/10.1016/0003-9861(68)90454-2)