

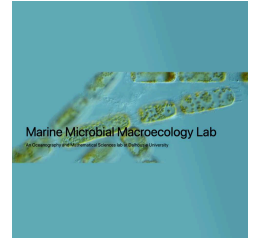
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Version 1

Total particulate carbohydrate from microalgae V.1

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Protocol status: Working

We use this protocol and it's working

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Keywords: TPTZ method, ferricyanide, hydrolysis, Total particulate carbohydrate, total particulate carbohydrate from microalgae, total particulate carbohydrate, carbohydrate sample, alkalized hydrolysate, final h2so4 molarity, hydrolysate, amino sugar, absorbance of tptz, ferricyanide solution, aldopentose, microalgae, high reproducibility in aldohexose

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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 0.18 to 10 µg C/mL.

Protocol materials

- ☒ NaOH Fisher Scientific Catalog #BP359-500
- ☒ Na₂CO₃ VWR International (Avantor) Catalog #97061-972
- ☒ 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
- ☒ D-glucose Merck MilliporeSigma (Sigma-Aldrich) Catalog #G8270-100G
- ☒ TPTZ Merck MilliporeSigma (Sigma-Aldrich) Catalog #T253-5G

Troubleshooting



Safety warnings

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

Sample collection

2h

- 1 Combust GFF filter for ⌚ 04:00:00 at 🌡️ 450 °C
- 2 Filter microalgae in liquid media onto precombusted GFF 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 seawater to avoid sample loss.
- 4 Place sample filters in 2 mL Cryogenic Vials.
- 5 Filter blank media (without cells) through filter as blank.
- 6 Flash freeze filters and store at 🌡️ -80 °C
- 7 Freeze-dry before processed.

Day 1- Preparation

30m

- 8 Prepare water bath 🌡️ 95 °C

30m

Day 1- Glucose standard solutions

9 Primary standard

9.1 In a 2 mL microtube, weigh 1 ~ 2 mg D-glucose

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

9.2 Add Milli-Q for a final concentration of 1 mg/mL (Volume requirement for preparing standard working solutions: >1800 µL).

10 Prepare eight 10 mL precombusted (⌚ 06:00:00 🔥 500 °C) centrifuge tubes, label tubes from SD1 to SD8.

6h

Equipment

Disposable Glass Screw-Cap Centrifuge Tubes^{NAME}

10 mL TYPE

Corning® BRAND

99502-10 SKU

Caps for the standard working solutions are acid-washed.

Equipment

Polypropylene Screw Caps^{NAME}

Linerless, 15-415 TYPE

Kimble Chase BRAND

73805-15415 SKU

11 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

- 12 Considering the working hours from 9 am to 4 pm, suggested sample number is:
blank + # samples = 24
- 13 Label 10 mL centrifuge tubes, log sample information.
- 14 Rinse forceps with 95% ethanol and air dry.

Equipment

Filter forceps

NAME

blunt end, stainless steel


TYPE

Millipore

BRAND

XX6200006P

SKU

- 15 Transfer each filter into its centrifuge tube, starting from blank.
- 16 Add  500 µL Milli-Q into each tube, vortex.





Day 1- Hydrolysis

3m

17 Transfer 18 M H₂SO₄ into a 30 mL precombusted glassware (scint vial, beaker... etc)

18 Vortex sample.

19 Use reverse pipetting technique, add  500 µL 18 M H₂SO₄ into the suspension instead of onto the filter, immediately vortex for  00:00:15 (Critical step: monitored by timer or stopwatch)

15s

Note

Do not cap the centrifuge tube!

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

5s

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

3h

Note

Hydrolysis duration for each sample/blank/standard should be accurately monitored.

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

23 Label pre-combusted 5 mL centrifuge tubes for supernatant.

of vials = # of samples + # of blanks

24 Label amber vials for TPTZ measurement with white oil based sharpie.

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



Equipment

Storage Vials and Closures

12 mL amber

Thermo Scientific

B7800-12A

VWR 66030-686

NAME

TYPE

BRAND

SKU

SPECIFICATIONS

- 25 As soon as hydrolysis duration reaches 3 hours, remove the tube from water bath, let it sit in the tap water bath with ice to quickly stop hydrolysis.

Day 1- Prepare for lipids extraction

3m

26

Note

1. The procedure of carbohydrate hydrolysis can break the bond between lipids and non-lipid component, which releases bound lipids into easily extractable form.
2. The acid in lipids can charge phospholipids to optimize extraction.
3. The acid can facilitate the separation of the lipid fraction from extraneous material such as protein.
4. Hydrolysis helps to remove most of the pigment (including chlorophyll and carotenoids), carbohydrate and protein from lipids.

- 27 Add 2 mL chloroform into hydrolysate. Vortex.

Note

- Glucose is insoluble in chloroform in the presence of water.
- Glucose in hydrolysate is no higher than 0.5 mM.
- Although phospholipids can induce the migration of glucose into chloroform, it doesn't instantly take place. The attainment of equilibrium is substantially delayed.
- The molar ratio of glucose solubilized to the phospholipid content remains approximately 0.0025 when glucose is about 5 mM level in the aqueous layer while phospholipids is up to at least 8.5 mM.
- Therefore, glucose is unlikely to migrate into lipids extract under our condition.



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.

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

LINK

28 Centrifuge  3200 rpm, 00:05:00

5m

29 Transfer supernatant to 12 mLamber vial by avoiding disturbing organic layer. Keep all hydrolysate in a dark cabinet at  Room temperature .


Equipment

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

Note

Precombust pasteur pipets at  500 °C  02:00:00

Precombust vials at  500 °C  06:00:00

30 Add 1 mL MeOH into the organic layer, mix well, freeze at  -80 °C until lipids extraction.

Estimation

1d

31 Estimate carbohydrate content on the filter for each sample:

$$[\text{Carbohydrate}]_{\text{ug/filter}} = [\text{Chl-a}]_{\text{ug/L}} \times (15/1.1) \times \text{Volume}_L$$

32 $C_{\text{assay}} = 0.4 \times \text{Chl} \times (15/1.1) \times V \times (\text{Hy}/1000)/5.5$

Where C_{assay} is Carbon in total particulate carbohydrate (ug/mL) in TPTZ assay, 0.4 is the median content of carbon in carbohydrate, Chl is the concentration of chlorophyll-a (ug/L), 15 and 1.1 are the median content of carbohydrate and chlorophyll-a in microalgae dry mass, V is sampling volume (L), Hy is the volume of hydrolysate (ul), 1000 is the total volume of neutralized hydrolysate, 5.5 is the total volume of MilliQ and H_2SO_4 in hydrolysis (mL).

Linear range in TPTZ assay: 0~10 ug C/mL

LOD in TPTZ assay: 0.02 ug C/mL

33 Use the following sheet to calculate the final concentration of carbon in total particulate carbohydrate, choose the suitable volume of hydrolysate so that the final concentration of estimated carbon of all the samples in TPTZ assay is about

[M] 1 Mass / % volume ug C/mL

Note


Estimated carbon is much lower than actual carbon in microalgae under nutrient stress condition or high light level.



	MilliQ (uL)	H2SO4 (mL)	H2SO4 (M)	MilliQ (uL)	Hydrolysate (uL)	MilliQ (uL)	12 M NaOH (uL)	[H+]/[OH-]
	500	0.5	18.00	4500	90	880	30	0.82
	500	0.5	18.00	4500	180	760	60	0.82
	500	0.5	18.00	4500	270	640	90	0.82
	500	0.5	18.00	4500	360	520	120	0.82
	500	0.5	18.00	4500	450	400	150	0.82
	500	0.5	18.00	4500	540	280	180	0.82
	500	0.5	18.00	4500	630	160	210	0.82
	500	0.5	18.00	4500	720	40	240	0.82
	500	0.5	18.00	4500	750	0	250	0.82

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 Milli-Q water.

Note

In this solution, sodium acetate, citric acid and acetic acid is 2 M, 0.2 M and 5 M respectively.

 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 standards

3m

42 Transfer  270 μ L of hydrolysate of standard working solution 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 μ L Milli-Q and vortex.


44 Add  90 μ L 12 M NaOH and vortex.


Note

12 M NaOH: reverse pipetting

Day 2- Alkalinization of samples

3m

45 Based on the estimation at  , transfer a certain volume of hydrolysate to a 12 mL amber vial.


46 Add MilliQ and 12 M NaOH based on the sheet  , vortex.

Note


12 M NaOH: reverse pipetting

TPTZ method

10m

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



48 Tightly cap the vial and vortex.

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

10m

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

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

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

30m

53 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.

		1	2	3	4	5	6	7	8	9	10	11	12
	A	SD1	SD1										
	B	SD 2	SD 2										
	C	SD 3	SD 3										
	D	SD 4	SD 4										
	E	SD 5	SD 5										



		1	2	3	4	5	6	7	8	9	10	11	12
	F	SD 6	SD 6										
	G	SD7	SD 7										
	H	SD 8	SD 8										

Microplate layout

54 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

55 Load 250 µl hydrolysate into microplate.

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

Waste disposal

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

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

Citations

Step 27

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

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