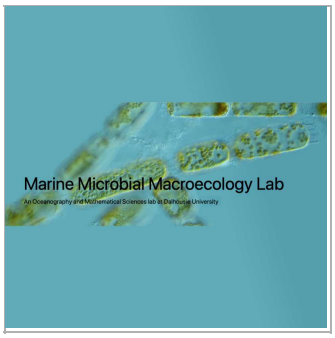

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We use this protocol and it's working

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Total particulate carbohydrate from microalgae

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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 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 0.18 to 10 µg C/mL.

SAFETY WARNINGS

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

Sample collection

2h

4h

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).

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

30m

8 Prepare water bath 95 °C

Day 1- Glucose standard solutions

9 Primary standard

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

6h

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

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

2h

Day 1 - Samples

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.

3m

Day 1- Hydrolysis

17 Transfer 18 M H_2SO_4 into a 30 mL precombusted glassware (scint vial, beaker... etc)

18 Vortex sample.

15s

19 Use reverse pipetting technique, add 500 µL 18 M H_2SO_4 into the suspension instead of onto the filter, immediately vortex for 00:00:15 (Critical step: monitored by timer or stopwatch)

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

5s

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

3h

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

> **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	NAME
12 mL amber	TYPE
Thermo Scientific	BRAND
B7800-12A	SKU
VWR 66030-686	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.

3m

Day 1- Prepare for lipids extraction

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 faciliate 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.

LINK

10.1016/0003-9861(68)90454-2

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.

1d

Estimation

31 Estimate carbohydrate content on the filter for each sample:

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

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

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**
Na2CO3 **VWR international Ltd 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
>
> 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 **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_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 **Sigma Aldrich Catalog #T253-5G**

Note

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

3m

Day 2- Alkalinization of standards

42 Transfer 270 µL 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 µL Milli-Q and vortex.

44 Add 90 µL 12 M NaOH and vortex.

Note

12 M NaOH: reverse pipetting

3m

Day 2- Alkalinization of samples

45 Based on the estimation at go to step #33, transfer a certain volume of hydrolysate to a 12 mL amber vial.

46 Add MilliQ and 12 M NaOH based on the sheet go to step #33, vortex.

Note

12 M NaOH: reverse pipetting

10m

TPTZ method

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

48 Tightly cap the vial and vortex.

10m

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

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.

30m

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

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	SD2	SD2										
C	SD3	SD3										
D	SD4	SD4										
E	SD5	SD5										

	1	2	3	4	5	6	7	8	9	10	11	12
F	SD6	SD6										
G	SD7	SD7										
H	SD8	SD8										

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

10m

Spectra of hydrolysate (optional step)

55 Load 250 ul 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.