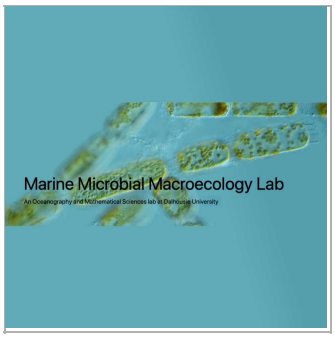

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We use this protocol and it's working

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Measurement of dissolved carbohydrate

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ABSTRACT

Here we describe a protocol to measure the dissolved carbohydrate, including total dissolved monosaccharides and total dissolved polysaccharides.
For total dissolved carbohydrate measurement, freeze-dried dissolved 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 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.
For total dissolved monosaccharide measurement, freeze-dried dissolved carbohydrate samples are alkalinized by 12 M NaOH and then 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 low limit of detection is 0.024 µg C/mL.

SAFETY WARNINGS

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

12h

Sample collection

12h

1 GFF filter is combusted for 04:00:00 at 450 °C

Glass filter holder is combusted for 02:00:00 at 500 °C
Glass filter funnel, flask and 10 mL centrifuge tubes are combusted for 06:00:00 at 500 °C

Equipment

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

Tube caps are acid-washed.

Equipment

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

2 Filter microalgae sample and collect the filtrate, using gentle vacuum pressure (130 mm Hg).

3 Transfer 5 mL filtrate into centrifuge tube and flash freeze.

Note

Three tubes for total dissolved monosaccharide and three tubes for total dissolved carbohydrate measurement.

4 Freeze dry samples before measurement.

Glucose standards

5 Primary standard solution

5.1 In a 2 mL microtube, weigh 1 ~ 2 mg D-glucose D-glucose **Sigma Aldrich Catalog #G8270-100G**

5.2 Add Milli-Q for a final concentration of 1 mg/mL (>600 µL).

6 Secondary standard for total dissolved carbohydrate

6.1 Add 45 µL primary solution into a 2 mL microtube

6.2 Add 955 µL Milli-Q and then vortex for a good mix

6.3 In 10 mL centrifuge tubes, prepare the following standard solutions:

SD	Secondary solution (uL)	Milli-Q (uL)
TCHO-SD1	0	100
TCHO-SD2	20	80
TCHO-SD3	40	60
TCHO-SD4	60	40
TCHO-SD5	80	20
TCHO-SD6	100	0

7 Secondary standard for total dissolved monosaccharide

7.1 Add 10 µL primary solution into a 2 mL microtube

7.2 Add 990 µL Milli-Q and then vortex for a good mix

7.3 In 12 mL amber vials, prepare the following standard solutions:

SD	Secondary solution (uL)	Milli-Q (uL)	12 M NaOH (uL)
MCHO-SD1	0	984	16
MCHO-SD2	10	974	16
MCHO-SD3	20	964	16
MCHO-SD4	50	934	16
MCHO-SD5	100	884	16
MCHO-SD6	150	834	16

Equipment

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

20s

Hydrolysis of total dissolved carbohydrate

8 Prepare water bath 95 °C

9 Add 100 µL Milli-Q to each tube with freeze-dried sample.

10 Use reverse pipetting technique, add 100 µL 18 M H_2SO_4 to standard solution/sample, immediately vortex for 00:00:15 (monitored by timer or stopwatch) 15s

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

11 Add 900 µL Milli-Q, tightly cap the centrifuge tube, and vortex for 00:00:05 . 5s

12 Place tube into water bath, log the time.

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

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

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

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

15 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.

16 Keep all hydrolysate in a dark cabinet at Room temperature .

Prepare TPTZ reagents

17 12 M NaOH

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

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

17.3 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.

18 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**

19 Sodium acetate solution

19.1 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**

19.2 Store at room temperature.

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

20 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.

40m

TPTZ method

21 Prepare boiling bath

22 TPTZ reagents

22.1 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

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

22.2 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.

22.3 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.

23 Total dissolved carbohydrate samples

23.1 Use reverse pipetting technique, transfer 750 µL hydrolysate of standard/sample to amber vial.

23.2 Add 250 µL 12 M NaOH and vortex.

24 Total dissolved monosaccharide samples

24.1 Add 1200 µL Milli-Q into the tube with freeze-dried sample

24.2 Use reverse pipetting technique, transfer 984 µL solution to amber vial.

24.3 Add 16 µL 12 M NaOH and vortex.

25 In a room with dim light, add 1 mL Reagent A into each amber vial.

26 Tightly cap the vial and vortex.

10m

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

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

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

30m

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

31 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	MCHO-SD1	MCHO-SD1										
B	MCHO-SD2	MCHO-SD2										
C	MCHO-SD3	MCHO-SD3										
D	MCHO-SD4	MCHO-SD4										
E	MCHO-SD5	MCHO-SD5										
F	MCHO-SD6	MCHO-SD6										
G												
H												

Microplate layout for dissolved monosaccharide samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	TCHO-SD1	TCHO-SD1										
B	TCHO-SD2	TCHO-SD2										
C	TCHO-SD3	TCHO-SD3										
D	TCHO-SD4	TCHO-SD4										
E	TCHO-SD5	TCHO-SD5										
F	TCHO-SD6	TCHO-SD6										
G												

	1	2	3	4	5	6	7	8	9	10	11	12
H												

Microplate layout for total dissolved carbohydrate samples

32 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

UV/VIS spectra (optional)

33 Hydrolysate

33.1 Load 200 µL hydrolysate into microplate.

33.2 Blank:
Milli-Q : H_2SO_4 = 10:1

34 Monosaccharide solutions

34.1 Load 200 µL solution into microplate.

34.2 Blank: Milli-Q

35 Scan UV/VIS spectra from 200 to 400 nm at a step of 1 nm.

Calculation

36 Total dissolved carbohydrate

36.1 Subtract the average absorbance of blank (0 ug glucose) from the absorbance of each standard for total dissolved carbohydrate.

36.2 Obtain standard curve by plotting blank subtracted absorbance (Abs') versus carbon (uM C)

$$Abs' = a * C_{(uM)} + b$$

36.3 Subtract the average absorbance of blank (0 ug glucose) from the absorbance of each sample

36.4

$$C_{(uM)} = (Abs' - b)/a$$
$$TCHO_{(uMC)} = C * (1.1/5)/0.75$$

37 Total dissolved monosaccharide

37.1 Subtract the average absorbance of blank (0 ug glucose) from the absorbance of each standard for total dissolved monosaccharide.

37.2 Obtain standard curve by plotting blank subtracted absorbance (Abs') versus carbon (uM C)

$$Abs' = a * C_{(uM)} + b$$

37.3 Subtract the average absorbance of blank (0 ug glucose) from the absorbance of each sample

37.4

$$C_{(uM)} = (Abs' - b)/a$$
$$MCHO_{(uMC)} = C * (1.2/5)/0.984$$

38 Total dissolved polysaccharide

$$PCHO_{(uMC)} = TCHO_{(uMC)} - MCHO_{(uMC)}$$

Waste disposal

39 All hydrolysate and TPTZ reagent C need to be neutralized by soda before disposed into the sink.

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