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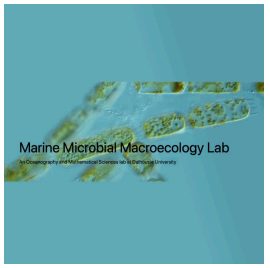
Estimate phospholipids from microalgae V.2



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We use this protocol and it's working

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Abstract

Here we describe a protocol to estimate phospholipids from microalgae.

After extracting and measuring the total lipids from microalgae, the remaining lipid extract is dried using a nitrogen flow, followed by drying with magnesium sulfate at 90°C. However, it has been observed that traditional dry combustion at 500°C only decomposes approximately 50% of phospholipids (Hu et al., 2022). To achieve complete conversion of phospholipids to pyrophosphate, a temperature of around 800°C is required, but such high temperatures cannot be used with glassware. As the acid digestion method involves using only 500 µL of 0.2 M HCl, which must be placed in tightly capped glass vials to prevent concentration changes due to evaporation, combustion must be carried out using glassware instead of crucibles. It should be noted that the recovery rate of phospholipids is around 80% when combusted at 650°C, but this recovery rate is consistent, making the use of glass vials applicable. Therefore, we recommend using 650°C to combust phospholipids and using 80% to correct the final results.

The resulting ash is digested using 0.5 mL of 0.2 M HCl for 30 minutes at 90°C. After digestion, the resulting orthophosphate is detected by mixing the sample with a combination of molybdate and ascorbic acid to produce molybdenum blue, as described in Chen's work (1956).

Citation

P.S. Chen, T.Y. Toribara and Huber Warner. Microdetermination of Phosphorus. Anal. Chem..

https://doi.org/10.1021/ac60119a033 LINK

Citation

Ying-Yu Hu, Andrew J. Irwin, Zoe V. Finkel (2022). Improving quantification of particulate phosphorus. Limnology and Oceanography: Methods.

https://doi.org/10.1002/lom3.10517 LINK

Protocol materials

- Magnesium sulfate anhydrous **Fisher Scientific** **Catalog #**M65500
- 12 N Hydrochloric acid
- 18M sulfuric acid
- Ammonium molybdate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**09878-100G
- Ascorbic acid **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A5960-100G
- Potassium dihydrogen orthophosphate **ACP Chemicals** **Catalog #**P-4550

Troubleshooting

Prepare phospholipids sample

1 Dry remaining organic phase extract of total lipids at 37 °C under a stream of N_2 gas (<2 psi)

Phosphate primary standard

2h

2 KH_2PO_4 primary standard stock solution (≈ 1 mM)

Potassium dihydrogen orthophosphate **ACP Chemicals Catalog #**P-4550

2.1 Transfer about 1 g KH_2PO_4 into a beaker, cover the beaker with foil

2.2 Place the beaker into an oven, dry KH_2PO_4 at 110 °C for at least 02:00:00 2h

2.3 Move KH_2PO_4 into a vacuum desiccator, allow KH_2PO_4 to cool to room temperature

2.4 Dissolve around 0.136 g dried KH_2PO_4 in 1 L MilliQ water.

- Use 1 L volumetric flask
- Take notes of the actual weight of KH_2PO_4 for final concentration of standard stock solution

2.5 Transfer standard stock solution into a 1 L bottle and store in the fridge.

Note

This stock solution lasts quite a long time, unless there is evidence for growth of algae or other extraneous biotic material.

High temperature dry combustion

9h

3 Use diamond pen to engrave the sample vials with numbers. Log number and sample code.

4 [M] 0.17 M $MgSO_4$ reagent:

Dissolve 1.023 g $MgSO_4$ in 50 mL MilliQ water

Magnesium sulfate anhydrous **Fisher Scientific Catalog #**M65500

5 Add 200 µL [M] 0.17 M $MgSO_4$ to the dry extract.

Note

Sing-use pipet tip to avoid cross-contamination.

6 Cover the uncapped vials with foil and place in the oven at 90 °C until samples are completely dry.

Equipment

Forced air oven	NAME
VWR	BRAND
89511-410	SKU

Note

Remove samples out of the oven as soon as they are dried. If muffle furnace is not available, keep samples in vacuum desiccator.

7 Combust dried samples at 650 °C for 09:00:00 9h

Equipment

NAME	TYPE	BRAND	SKU
Muffle furnace	F30428C	Thermo	10-505-13

Note

Only place glass vials in the muffle furnace. 650 °C turns foil into ash.

8 Allow samples to gradually cool down in the muffle furnace.

Digestion

9 [M] 0.2 M HCl reagent:

In a reagent bottle, dissolve one part of [M] 12 N HCl in 59 parts of MilliQ water

12 N Hydrochloric acid

Note

Volume of HCl_0.2M_mL = (0.5_mL) X (#Sample + #Blank)

10 Preheat oven to 90 °C

11 Add 0.5 mL [M] 0.2 M HCl to each vial.

12 Tightly cap the vial and vortex.

13 Place vials in the oven for 00:30:00 30m

14 Cool samples down to Room temperature

Preparing standard working solutions 2h

15 Preheat shaker/incubator to 37 °C

Equipment

SHAKING INCUBATOR	NAME
71L	TYPE
Corning® LSE™	BRAND
6753	SKU

16 Standard working solutions and reagents can be prepared during sample digestion.

17 Standard working solution

	Standard	Primary (uL)	MilliQ (uL)
	S1	0	1000
	S2	5	995

	Standard	Primary (uL)	MilliQ (uL)
	S3	10	990
	S4	20	980
	S5	50	950
	S6	100	900
	S7	150	850
	S8	200	800

18 Transfer 500 µL of each standard working solution to 2 mL microtube.

Preparing working reagents

19 All reagents are freshly prepared before colorimetric measurement.

20 [M] 2.5 % ammonium molybdate reagent:

Weigh 0.25 g ammonium molybdate in a Falcon tube and top to 10 g with MilliQ water.
Cap and shake until totally dissolved.

Ammonium molybdate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**09878-100G

21 [M] 10 % ascorbic acid reagent (avoid light exposure):

Weigh 1 g ascorbic acid in a Falcon tube and top to 10 g with MilliQ water;
Cap and shake until all dissolved.

Ascorbic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A5960-100G

22 [M] 6 N (3 M) sulfuric acid reagent:

Carefully add 1 part [M] 18 M concentrated sulfuric acid into 5 part MilliQ water
18M sulfuric acid

23 Calculate the volume of molybdate-ascorbic reagent:
Total volume of reagent_mL = (0.5 mL) X (#standard working solution + #samples + #blanks)

24 Mix the reagents into Falcon tube:

	Reagent	Parts as in volume
	MilliQ	2
	6N sulphuric acid	1
	2.5% ammonium molybdate	1
	10% ascorbic acid	1

Colorimetric measurement

3h

25 Add 500 µL reagent to each standard, sample (in the vial) and blank, starting from blanks, including blank for standards and blank for samples.

Equipment

Finntip Stepper Tips	NAME
5 mL	TYPE
Thermo Scientific	BRAND
9404200	SKU

Note

Before dispensing the reagent, wipe or dab the liquid drop on the outside of the tip, avoid wiping the open tip.

26 Vortex.

27 Incubate at 37 °C for 03:00:00 while shaking at 150 rpm 3h

28 Load microplate with 250 ul reactant from each tube, duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	Samples and sample blanks: 40 with duplicate									
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	S8	S8										

Example of loading the microplate

29 Read plate in microplate reader

	A	B
	Shake duration	00:00:05
	Shaking type	Continuous
	Shaking force	High
	Shaking speed [rpm]	600
	Wavelength [nm]	820
	Use transmittance	No
	Pathlength correction	No
	Measurement Time [ms]	100

Equipment

Varioskan LUX Multimode Microplate Reader	NAME
Thermo Fisher	BRAND
VL0L00D0	SKU

Calculation

3h

30 Subtract the average absorbance at 820 nm of the blank standard replicates from the absorbance at 820 nm of all other standard working solutions.

31 Subtract the average absorbance at 820 nm of the blank sample (i.e. blank filter) replicates from the absorbance at 820 nm of all other individual samples.

32 Prepare a standard curve by plotting the average blank-corrected 820 nm absorbance for each standard working solution versus its concentration in uM.
Molar Mass of KH2PO4: 136.086 g/mol

33 Use the standard curve to determine the orthophosphate concentration of each unknown sample by using its blank-corrected 820 nm absorbance.

34 $(P_{measured})$_umol/sample = (orthophosphate)_uM X (V_HCl)_mL X (0.001)
$(P_{corrected})$_umol/sample = $(P_{measured})$ / 0.8
Where, 0.8 is the average recovery of phospholipids after a high temperature dry combustion at 650 °C .

35 (Phospholipids)_ug/sample = $(P_{corrected})$X30.97/(0.01X4.3)

Citations

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https://doi.org/10.1021/ac60119a033

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