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Measuring Glycine Betaine concentrations in *Typha/Spartina*

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We use this protocol and it's working

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Abstract

Glycine betaine (GB) is a compatible osmolyte that many plants produce in response to various types of stress, particularly drought and salinity (Ashraf & Foolad, 2007; Jarin *et al.*, 2024). GB accumulation is an important aspect of salt tolerance for many tidal marsh plant species (Adrian-Romero *et al.*, 1998), as well as being a substrate for methanogenesis by microbes in salt marsh and marine sediments (Boysen *et al.*, 2022; Bueno De Mesquita *et al.*). We measured glycine betaine content in both the leaves and roots of *Typha angustifolia* grown under different salinities, using the method of Grieve and Grattan (1983), as modified by Valdez-Bustos *et al.* (2016). We made a further modification, using a plate reader instead of a spectrophotometer, which makes it possible to analyze samples more quickly.

Image Attribution

All images taken by Suzanne Thomas

Materials

-  Betaine, anhydrous **Spectrum Chemical MFG Corp Catalog #B1572**
-  Sulfuric acid **Fisher Scientific Catalog #A300C-212**
-  1,2-dichloroethane **Thermo Fisher Scientific Catalog #032462.K2**
-  potassium iodide **Thermo Fisher Scientific Catalog #A12704.18**
-  Iodine flakes **Fisher Scientific Catalog #137-100**

Equipment:

Equipment	
MM200	NAME
Grinding mill	TYPE
Retsch	BRAND
MM200	SKU
	

Equipment

Synergy H1	NAME
Plate Reader	TYPE
BioTek	BRAND
H1M	SKU



Equipment

96 well plate	NAME
acrylic UV transparent plate	TYPE
Corning	BRAND
3635	SKU



Equipment

block heater	NAME
module block heater	TYPE
American Scientific	BRAND
2025-1	SKU



Equipment

Neoprene	NAME
lab gloves	TYPE
MicroFlex	BRAND
NPG-888	SKU



Equipment

Eppendorf 5415R Refrigerated Centrifuge	NAME
Refrigerated Centrifuge	TYPE
Eppendorf	BRAND
EP-5415R	SKU



Equipment

Eppendorf tubes	NAME
2mL clear	TYPE
USA Scientific	BRAND
1620-2700	SKU



Equipment

Eppendorf tubes

NAME

2mL amber

TYPE

Eppendorf

BRAND

022363379

SKU

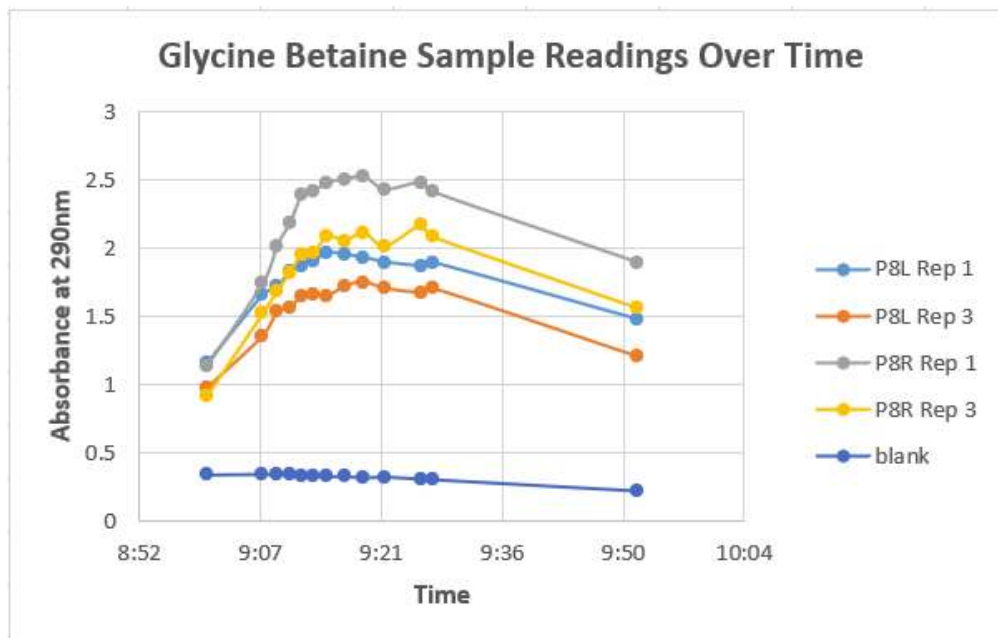


Safety warnings

-  1,2-dichloroethane (also known as ethylene dichloride) is toxic, corrosive, and carcinogenic. It is also highly volatile. This method calls for using undiluted 1,2-dichloroethane. It is imperative to use neoprene gloves, safety glasses, lab coat, and work in a fume hood at all times.

Before start

Iodine is light sensitive. Excess iodine will be removed in Step #20, but iodine will also be incorporated into the betaine-iodide crystals. All steps where the iodine reagent is involved (Steps 14-27) were done under low light. The graph below shows how quickly iodine reacts with light. A black cover was used to protect the samples from light as they were being pipetted into the 96 well plate to read the absorbance.



Repeated absorbance readings on samples (plate removed from plate reader between readings- volatile dichlorethane could damage the plate reader). The iodine reacts with light and quickly changes the absorbance.



96 well plate lid covered with black duct tape. This helps to decrease the reaction of iodine with light while samples are being pipetted to read absorbance in the plate reader.

It is critical to remove the iodine reagent when it is in excess. In the photo below, I ran samples in quadruplicate, intentionally leaving a small drop of iodine solution in the vial with the betaine iodide crystals. This shows that it is critical to remove the iodine reagent in Step #20.

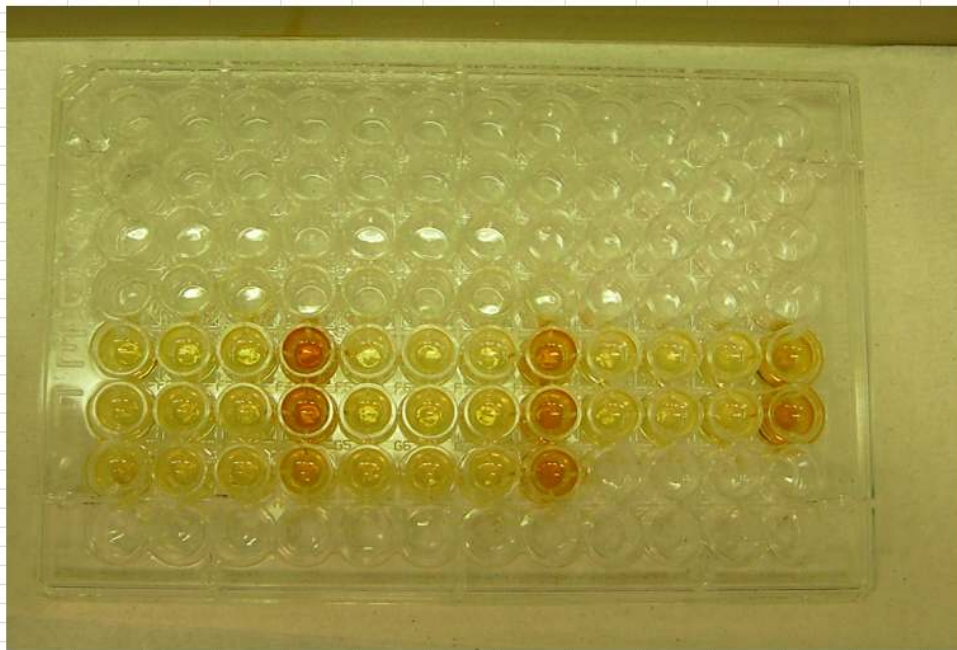


Photo of 96 well plate.

For the 4th rep of each set, I intentionally left one small drop of iodine solution to see what effect that would have on the final value.

96 well plate showing samples run in quadruplicate, where the 4th sample of each set was intentionally contaminated with a small drop of iodine solution from Step # 20.

Collect and prepare plant material

12h 10m

- 1 snip ~20cm of a leaf (we are currently looking at glycine betaine in saltmarsh and brackish marsh plants (*Sporobolus* and *Typha*)) and place in a labeled paper bag
- 2 Place sample + bag in a 60°C drying oven overnight or longer
- 3 Grind sample into a homogenized powder

12h



10m



Retsch mill grinder and stainless steel grinding vessels

- 4 Store ground sample in a closed vial

Make Reagents and Standards

4h 45m

5 2N Sulfuric Acid

1h

Add 28 mL 36 Molarity (M) H₂SO₄ (sulfuric acid) to ~300mL deionized water in a 500mL volumetric flask. Swirl to mix. When cool, fill volumetric flask to 500mL. Invert to mix 10x. Transfer to a glass bottle for storage.



Safety information

- Work in a fume hood when handling full-strength sulfuric acid.
- Use nitrile or latex gloves and remove gloves immediately if you come into contact with any concentrated acid.
- Wear eye protection.
- Wear a lab coat.

**6 Potassium Iodide - Iodine Solution**

2h

🧪 1.57 g I₂ (iodine flakes/chunks) + 🧪 2 g KI (potassium iodide) +

🧪 10 mL deionized water in a dark bottle. Swirl from time to time to mix. It will take some time for the KI-I₂ to dissolve.

Safety information

Use appropriate PPE when handling these chemicals- lab coat, safety glasses, latex or nitrile gloves.

**7 Prepare 1,2-dichloroethane (also known as ethylene dichloride)**

15m

Pour 1,2-dichloroethane into a smaller glass bottle (50 or 100mL) as a working reagent (full strength)

Safety information

This chemical is very hazardous! It is toxic, corrosive, carcinogenic, and flammable.

You must:

- Work in a fume hood
- Wear special lab gloves- **neoprene**
- Wear safety glasses and a lab coat
- Always use and store this chemical in a fume hood. It is very volatile

**8 Make Standard Stock Solution (200mg/mL)**

1h 30m

🧪 4 g betaine + 🧪 20 mL 2N H₂SO₄

From this solution, I made standards of 0, 1, 2, 5, 10, 20, 50, 100, 200, and 500 µg/mL

**Extract Glycine Betaine from Ground Plant Material (timing estimates for ~15 samples)**

2d 19h 45m

9 Weigh out ~ 🧪 0.02 g ground material and place in a 2mL Eppendorf tube (can be clear)

30m



- 10 Add 1.5mL 2N H₂SO₄ to tube with ground plant material

10m

**Safety information**

You should be wearing gloves and safety glasses

- 11 Vortex closed tube to mix

- 12 Heat samples to 60 °C for 10 minutes in a heating block (or similar)

10m

- 13 Centrifuge sample tubes at ~ 14,000 rpm, 22°C (our centrifuge only goes to 13,200rpm and that has been fine) for 10-25 minutes at room temperature. The centrifugation time depends on how much plant material is in the vial and how dense it is.

25m



- 14 Transfer 125 µL sample supernatant to an amber 2mL Eppendorf tube and add 50 µL cold KI-I₂ solution

20m

**Note**

The iodine solution is said to be light sensitive, so I do this step with the overhead lab lights off and a small light on to see by.

- 15 Cap tube and place mixture on ice immediately. Ice should be kept in a cooler with a lid to keep the samples dark.



- 16 Place cooler with ice and samples in a refrigerator overnight. Samples should remain at 0–4°C for 16 hrs.

16h



- 17 Cool Centrifuge to 0°C. Ours take 30–60 minutes to cool down.

1h

Note

It is crucial that everything stays cold during these next steps! The betaine iodide complexes that form can dissolve at temps > 0°C.



- 18 Centrifuge samples at 0°C and ~  14,000 rpm for 30 minutes.

30m

- 19 Remove samples from centrifuge and return to ice.

5m

- 20 Remove supernatant from sample tube. Only the crystals of betaine iodide should remain. Do a quick visual inspection to ensure that all liquid iodine reagent has been removed. Supernatant should be disposed of in a labeled hazardous waste container.

15m

Note

Again working with the overhead lab lights off and a small light on to see by.

- 21 Once the supernatant has been removed, the sample tube may be placed in a holder at room temperature.



- 22 Move samples to a fume hood and change gloves to neoprene gloves. Double check that you have your safety glasses on.

10m

**Note**

Continue to work in low light, but dichloroethane is very nasty, so we want to make sure we can see well. Our lab has two banks of lights. We work with our fume hood light off, but with one bank of lab lights on.

- 23 Add  1.4 mL 1,2- dichloroethane to sample vial with crystals. Cap.

10m

**Safety information**

Collect pipette tips in a glass beaker to evaporate in the fume hood. Then dispose of as hazardous waste when dry.

- 24 Let crystals dissolve for 48 hrs. I agitate the vial holder several times over this period to encourage dissolution. We place an opaque plastic box over the samples to ensure darkness.

2d

**Safety information**

These vials need to stay in the fume hood for this step! The dichloroethane is very volatile.

Determine Concentration of Glycine Betaine in Samples on a Plate Reader

1d 0h 22m 30s

- 25 Agitate samples in holder to mix. Prepare plate reader and 96 well plate. We have found that reading one row of 12 samples at a time works well for us, as long as each well in the row is covered as it is filled. The plate reader should be set to read absorbance at 290nm (and 365nm, although we're not sure this wavelength is necessary).

10m

Note

This step can also be done on a spectrophotometer in a fume hood. You will have to use a removable glass/quartz cuvette (as opposed to pump tubing and a sipper unit. The dichlorethane would dissolve pump tubing). **Being in a fume hood is critical to working with 1,2-dichloroethane.**



96 well plate lid covered with black duct tape. When 100 μ L of sample is pipetted into a well, the plate cover is repositioned to cover the well and block out most of the light. The 96 well plate itself is clear, so some light gets in through the bottom plate.

Note

I am again working with only half the lab lights on and am in the fume hood. It is crucial to cover each well after it is filled with sample. The iodine that is released from the betaine iodide crystal is very light sensitive. I use a 96 well plate lid covered in black duct tape to cover each well as I fill a row. Then I place the covered 96 well plate on the plate reader arm and remove the cover as the plate is being pulled into the plate reader.



- 26 Add 100 μ L of sample to a well and read a row of 12 samples at a time in the plate reader at 290nm

2m 30s

Safety information

Working in a fume hood, move quickly (light sensitive extract) but carefully with these samples. The dichloroethane is toxic, corrosive, carcinogenic, and volatile. Do NOT leave the plate in the plate reader for any longer than is necessary. The fumes will degrade the instrument. All pipette tips should be ejected into a glass beaker. Once dry (overnight), dispose of as hazardous waste.

- 27 Repeat until all samples have been read.

10m

- 28 Allow samples to evaporate from the wells. Yes, you are still in a fume hood! The well will have been distorted and stained from the sample. The plates are single-use for this experiment.

1d



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