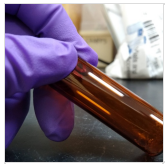

Version 1

Nov 10, 2020

BC derivatization for dissolved marine metabolites V.1

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1 Works for me dx.doi.org/10.17504/protocols.io.biukkeuw

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ABSTRACT

Benzoyl chloride derivatization of dissolved metabolites in seawater and culture medium improves sensitivity, number of quantifiable compounds, and sample throughput. Samples are quantified using a standard curve and stable isotope labeled internal internal standards prepared from an isotopically labeled reagent. Samples are filtered (0.2 um), derivatized in basic solution and extracted from seawater/culture medium using a solid phase extraction resin (PPL, Bond Elut, Agilent). Benzoylated metabolites are detected using UPLC-MSMS with Orbitrap detection. Peaks are analyzed with Skyline.

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MATERIALS TEXT

Supplies and Chemicals for Field (derivatization only)

- Hamilton syringes with needles (250 ul, 1ml, maybe 5ml)
- Pipettes and tips and gloves
- vortex + vortex tube holder (for 1ml method - cultures)

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- Combusted vials (40 ml amber for field samples; 4ml for culture samples) – 1 per sample/standard
- Boxes to hold combusted vials and spare sample racks (for 25ml vials)
- Methanol and acetone for solvent rinsing (optima grade)
- 8M sodium hydroxide (750 ul per 25ml sample)
- Phosphoric acid (concentrated) (375 ul per 25 ml sample)
- Standard mixes
- DI water (18.2 MΩ)
- Benzoyl chloride
- Benzoyl chloride 13C6 – expensive! So always use smallest volume possible
- 8 ml amber vials for making standards

Supplies and Chemicals for Lab (extraction, etc)

- All of the above supplies
- PPL manifold, trap, pump, tubing, baking soda, 2 teflon squirt bottles, etc
- Vacufuge with 8mL, 4mL, and 2mL size rotors
- Centrifuge with rotor like A-4-62 for Eppendorf 5810R.
- PPL – 1g (1 per sample)
- Cuvettes (glass)
- Disposable glass pipettes and suction bulb
- bulb
- Timer
- Optima methanol
- Hydrochloric acid

SAFETY WARNINGS

Benzoyl chloride is toxic, corrosive, and flammable. Derivatization should be done in a hood with proper PPE and frequent glove changes.

DISCLAIMER:

ABSTRACT

Benzoyl chloride derivatization of dissolved metabolites in seawater and culture medium improves sensitivity, number of quantifiable compounds, and sample throughput. Samples are quantified using a standard curve and stable isotope labeled internal internal standards prepared from an isotopically labeled reagent. Samples are filtered (0.2 um), derivatized in basic solution and extracted from seawater/culture medium using a solid phase extraction resin (PPL, Bond Elut, Agilent). Benzoylated metabolites are detected using UPLC-MSMS with Orbitrap detection. Peaks are analyzed with Skyline.

Prepare Standards for 1 mL and 25 mL methods

1 Standards for **1 mL** samples (culture samples)

1.1 **1ºstocks**: [M]**10 ug/mL** mixes 1, 2, 3, 4, 5-6, and thiol* (optional).

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1.2 **2º stock**: [M]**100 ng/mL** mix of all metabolites. 1:100 dilution of primary into matrix.

1.3 **3º stock**: [M]**500 pg/mL** mix of all metabolites. 1:200 dilution of secondary into matrix.

1.4 **Select concentration for Stable Isotope Labeled Internal Standards (SIL-ISs).**
Concentrations (and sensitivities) range over orders of magnitude, and you want to add a spike of the SIL-ISs that is detectable but not crazy high for every compound.
The options are as follows.
A) Make a standard mix with different concentrations for different metabolites based on their LODs. This is very cumbersome.
B) Run each sample twice – once with a spike of [M]**500 pg/mL** and once with [M]**10000 pg/mL** .
This necessitates the preparation of two standard curves: one from [M]**1 pg/mL** to [M]**3500 pg/mL** (with [M]**500 pg/mL** spike) and one from [M]**1000 pg/mL** to [M]**30000 pg/mL** (with [M]**10000 pg/mL** spike).
C) Pre-run the samples (without adding the SIL-ISs) to estimate concentrations and then prepare standard curve and SIL-IS addition appropriately. This will still likely result in the need for either A or B, so I do not recommend this step.
Note: Samples that exceed [M]**30000 pg/mL** will require dilution and reanalysis. Calibration curves for many metabolites are not linear above this concentration.

1.5 Prepare standards and MQ blanks in duplicate. Due to matrix effects, the MQ blank cannot be used quantitatively as a 'blank' but rather is used to identify contamination (e.g. in the reagents or vials) in a particular sample batch.

1.6 Volume additions for standards.

[Standard] (pg/ml)	Volume matrix (uL)	Vol 2o stock (uL)	Vol 3o stock (uL)
0	1000	-	0
5	990	-	10
10	980	-	20
35	930	-	70
70	860	-	140
100	800	-	200
350	300	-	700
700	993	7	-
1000	990	10	-
3500	965	35	-
7000	930	70	-
10000	900	100	-
30000	700	300	-

2 Standards for **25 mL** samples (field samples)

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2.1 **1ºstocks**: [M]**10 ug/mL** mixes 1, 2, 3, 4, 5-6, and thiol* (optional). Thiol mix is prepared fresh, if possible.

2.2 **2ºstock**: [M]**100 ng/mL** mix of all metabolites. **80 µl** each of the 6 mixes above into **8 mL** total volume in matrix

2.3 **3ºstock**: [M]**5 ng/mL** mix of all metabolites. **400 µl** of seconday stock into **8 mL** total volume in matrix.

2.4 **SIL-IS**: Prepare a **pooled sample** by combining filtered, pre-derivatization sub-aliquots of each of the samples. Prepare a [M]**20000 pg/mL** standard mix. Derivatize this with $^{13}C_6$-benzoyl chloride using the same protocol as for samples and spike into all samples and standards after they have been derivativized. Adding **62.5 µl** will result in a final concentration of [M]**200 pg/mL** .

2.5 Prepare standards and MQ blanks in duplicate. Due to matrix effects, the MQ blank cannot be used quantitatively as a 'blank' but rather is used to identify contamination (e.g. in the reagents or vials) in a particular sample batch.

2.6 Volume additions for standards.

[Standard] (pg/ml)	Volume matrix (ml)	Vol 3o stock (uL)
0	25	0
5	25	25
10	24.95	50
30	24.85	150
70	24.65	350
100	24.5	500
300	23.5	1.5
700	21.5	3.5
1000	20	5000

Prepare Reagents

3 Prepare reagents.

3.1 Prepare working reagent ([M]**5 % volume** benzoyl chloride in acetone). Avoid plastic (e.g. use glass Hamilton syringes with metal needle instead of pipettes). Prepare fresh daily.

Benzoyl chloride is toxic, flammable, and corrosive. Use under hood with proper PPE.

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3.2 Prepare [M]**8 Molarity (M)** NaOH. Store indefinitely at room temperature.

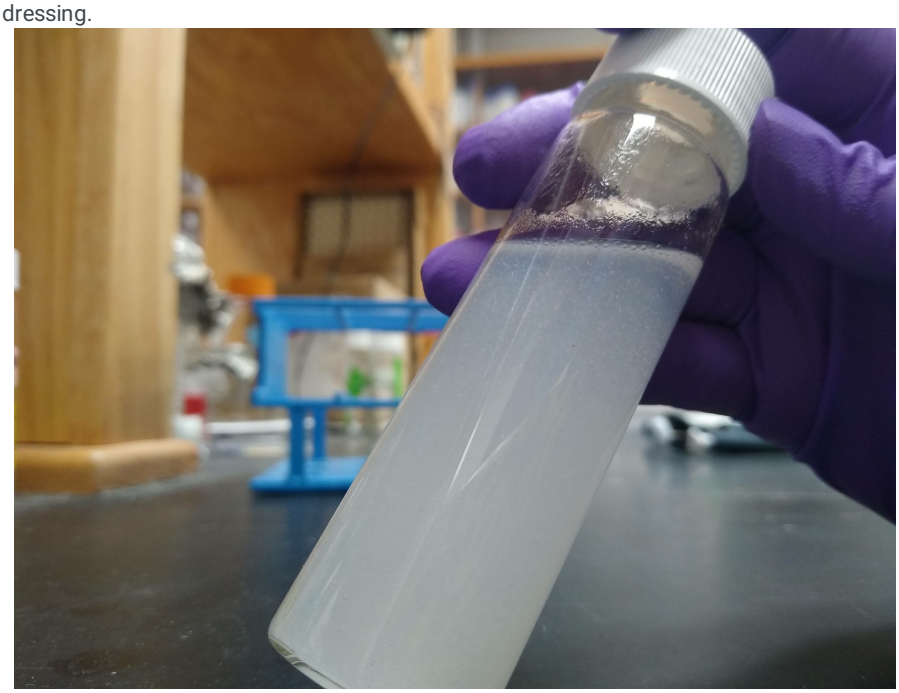
3.3 Prepare [M]**0.01 Molarity (M)** HCl. Store idefinitely at room temperature in teflon or glass.

Derivatize Samples

4 **Derivatization procedure for all samples and standards.** Follow the same procedure to prepare stable isotope labeled internal standards (SIL-IS), substituting $^{13}C_6$ benzoyl chloride for the ^{12}C benzoyl chloride. Prior to this method, samples have been filtered (0.2 um, teflon (Omnipore)). Samples should be derivaitized as quickly as possible after collection and stored at **-20 °C** if they cannot be processed right away. This recipe is for a **1 mL** sample. Scale volumes accordingly for the sample size.

5 Add **30 µl** NaOH ([M]**8 Molarity (M)**) and invert 5 times.

6 Add **200 µl** working reagent. You will observe "beads" of benzoyl chloride that look "oily", like oil in vinegar salad dressing.



Sample after working reagent added. "Beads" of benzoyl chloride can be seen floating throughout the sample and on the top, and there is an oily-looking residue on the sides of the vial.

7 Mix samples for **00:05:00** , either by vortexing (volumes ≤ **5 µl** in 8 mL vials and on land) or manually inverting sample rack (larger volumes and at sea).

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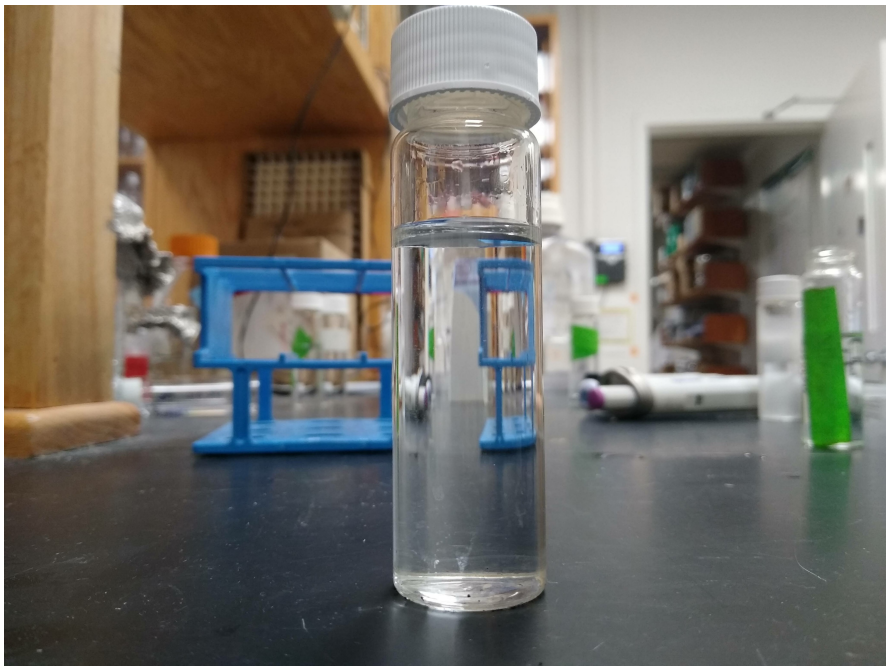
8 Add **15 µl** phosphoric acid. Invert 5 times. The sample will immediately become clear. A white precipitate may form over time .



9 Add spike of SIL-IS mix to all samples/standards. Samples can now be stored at **-20 °C** .

Post-Derivatization Cleanup

10 Remove acetone from the samples using either the Vacufuge or a stream of nitrogen gas.

11 **Setup extraction manifold/vaccuum and extract derivatives (1g/6 mL, Bond Elut PPL, Agilent).**

11.1 Separate precipitate (as best you can) from liquid either by centrifuging (**1000 x g, 22°C** for **00:15:00**) or waiting for the precipitate to naturally separate. Samples are centrifuged at low speed because the glass vials break at higher speeds. Also, the larger glass vials (EPA 40 mL) sometimes break even at this low speed. These methods do not achieve perfect separation of liquid and solid phases. Better separation could be achieved by transferring samples to centrifuge tubes and centrifuging at a higher speed, but this additional work does not seem to be necessary, and we try to avoid using unecessary plastics for fear of plasticizer contamination.

11.2 Condition PPL cartridge with **6 mL** methanol (Optima).

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11.3 Rinse PPL cartridge 4 times with [M]**0.01 Molarity (M)** HCl. Don't let the cartridge dry out.

11.4 Carefully remove the id layer (same volume as initial sample volume, e.g. (**1 mL** or **25 mL**) and load onto the PPL cartridge. It is difficult to avoid transferring some precipitate along with the liquid, but try to minimize this. Let the sample drip through the cartridge by gravity or very gentle pressure (< 5 " Hg) for larger volumes.

11.5 Rinse the PPL cartridge 4x with [M]**.01 Molarity (M)** HCl and then dry (pull air through at ~15" Hg) for 5 minutes.

11.6 Elute with **6 mL** methanol (Optima grade) into combusted cuvettes.

11.7 Transfer eluent to **8 mL** combusted amber vials. Label caps as well as vials. Store in flammable freezer or proceed to next step.

11.8 Dry down samples in vacufuge (Eppendorf). Use the alcohol setting unattended for 3.5 hours, and then switch to the aqueous setting until drying is completed, being careful not to overdry. As the samples dry, a white precipitate may form a film on top of the liquid, which can inhibit drying. Periodically tap the vials against a firm surface to break up this film.

12 **Reconstitute samples and remove precipitate.** This procedure varies based on sample volume. For **25 mL** sample volume, complete all steps. For ≤ **5 mL** , complete steps 12.5 to 12.9.

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12.1 "Wash" the benzoic acid precipitate out of the samples. Add **500 µl** **5 % volume** acetonitrile/DI to each sample and vortex thoroughly. The precipitate will mostly not dissolve.

12.2 Centrifuge **1000 x g, 22°C** for **00:15:00** to separate ppt and liquid.

12.3 Transfer the liquid into a **2 mL** tube. It is more important to get all the liquid then to avoid transferring some precipitate.

12.4 Dry down (vacufuge) on aqueous setting with **30 °C** heat.

12.5 Add **100 µl** **5 % volume** acetonitrile/DI, vortex thoroughly, and transfer to centrifuge tubes.

12.6 Centrifuge at **12000 x g, 22°C** for **00:15:00** .

12.7 Transfer liquid to LC vial with appropriate insert.

12.8 Add **5 µl** acetonitrile.

12.9 Store at **4 °C** . Samples are stored above freezing to minimize precipitation.

UPLC-MSMS

13 Detection with Ultrahigh Performance Liquid Chromatography (UPLC) Tandem Mass Spectrometry.

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Quantify Metabolites

14 Use Skyline.

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