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BC derivatization for dissolved marine metabolites

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Brittany Widner¹, Melissa Soule², Frank Ferrer-Gonzalez³, Mary Ann Moran³, Elizabeth Kujawinski¹

¹Woods Hole Oceanographic Institution; ²WOODS HOLE OCEANOGRAPHIC INSTITUTION;

³Department of Marine Sciences, University of Georgia

Kujawinski Lab



Gretchen Swarr

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We use this protocol and it's working

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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 standards prepared from an isotopically labeled reagent. Samples are filtered (0.2 μ m), 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.

This version corrects typographical errors and a bug at protocols.io that caused videos to be lost, and updated SIL-IS and other protocols to reflect the most up-to-date methods.

Materials

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)
- 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.

Prepare Stock Standards and standard curves for 5 mL and 25 mL methods

- 1 Standards for  5 mL culture samples and  25 mL field samples.
 - 1.1 **1^ostocks:** [M] 10–20 ug/mL mixes 1, BC2, 3, 4, 5/6*, BC7*, and 8* (*denotes 20 ug/mL mix, mix8 should be made fresh)
 - 1.2 **2^ostock:** [M] 100 ng/mL mix of all metabolites.  50 µL each of the 10 ug/mL mixes and  25 µL each of the 20 ug/mL mixes in to  5 mL total volume in seawater matrix.
 - 1.3 **3^ostock:** [M] 5 ng/mL mix of all metabolites.  250 µL of secondary stock into  5 mL total volume in matrix.

Prepare Stock Standards and standard curves for 5 mL and 25 mL methods

- 1.4 Volume additions for  5 mL standard curves. Note, for  25 mL samples,  20 mL of matrix should be added to the volumes indicated in Column B so that your total volume in your standards always equates to the volume in your samples.

	A	B	C	D
	[Standard] (ng)	Volume matrix (uL)	Vol 2o stock (uL)	Vol 3o stock (uL)
	0	5000	-	0
	0.05	4990	-	10
	0.1	4980	-	20
	0.5	4900	-	100
	1	4800	-	200
	5	4950	50	-

	A	B	C	D
	10	4900	100	-
	50	4500	500	-
	100	4000	1000	-
	250	2500	2500	-

Prepare Stock Standards and standard curves for 5 mL and 25 mL methods

2 Prepare SIL-IS for  5 mL culture samples and  25 mL field samples.

2.1 Select concentration for Stable Isotope Labeled Internal Standards (SIL-ISs).

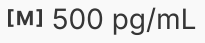
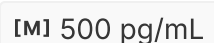
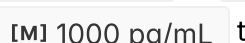
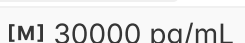
Concentrations (and sensitivities) range over orders of magnitude, and you want to add a spike of the SIL-ISs that is detectable but not crazy high for every compound.

The options are as follows:

A) Recommended / routine protocol : Make two SIL-IS mixes, one derivatized with ^{13}C benzoyl chloride and the other with d_5 benzoyl chloride. We recommend an SIL-IS spike of 5 ng added and 25 ng added, respectively. See steps 2.2 - 2.3.

Additional options include:

B) Make a standard mix with different concentrations for different metabolites based on their LODs. This is very cumbersome.

C) Run each sample twice – once with a spike of  500 pg/mL and once with  10000 pg/mL . This necessitates the preparation of two standard curves: one from  1 pg/mL to  3500 pg/mL (with  500 pg/mL spike) and one from  1000 pg/mL to  30000 pg/mL (with  10000 pg/mL spike).

D) 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 B or C, 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.

- 2.2 **¹³C SIL-IS:** Prepare a [M] 200 ng/mL (C_i) standard mix made in a aged filtered seawater matrix (or other appropriate matrix for your samples). Derivatize this with ¹³C₆-benzoyl chloride using the same protocol as for samples and spike into all samples and standards after they have been derivatized. Adding  20 μ L (Vol_{sp}) will result in a final amount of [M] 5 ng SIL-IS added. You can change these concentrations and volumes as necessary, but you should not derivatize less than 1ml of SIL-IS solution. See note for more details and example

Note

If you have 55 samples (including standards and blanks) and want to spike in 20 uL in to each, you would need 1.1 mL of SIL-IS solution.

- $N_s \times \text{Vol}_{\text{sp}} / 1000 = \text{Vol}_d$
- If you need 1.1 mL of SIL-IS solution, you will need to derivatize more than 0.883 mL of SW (Vol_i) so that when you add the reagents in the derivatization process (24.5% of the initial volume) the final derivatized volume is greater than 1.1 mL. You want to round this number up, here we have chosen to round up to 2 (Vol_i), but this is a little excessive, but you do want to be sure you have adequate amounts. The total volume post-derivatization will be 2.490 mL (Vol_t).
- $\text{Vol}_i = \text{Vol}_d \times 0.803$ (required initial volume) **0.803 accounts for the % volume without reagents*
- $\text{Vol}_i = 2$ mL (selected actual initial volume)
- $\text{Vol}_t = \text{Vol}_i / 0.803$

Suppose you want to spike in 20 uL SIL-IS mix to equate to a total of 5 ng SIL-IS in each sample, and the total volume of SIL-IS you are making (including reagents) is 2490 uL. In that case, your concentrated spike solution needs to have 622.5 ng total standard in the 2 mL SW/standard mix solution we plan to derivatize. This equates to 311.25 ng/mL. Thus, the stock solution you pull from must be greater than 200 ng/mL concentration to remain within your 2 mL volume constraint. If you can do this from your secondary or tertiary stocks you can, but in this case our secondary and tertiary stocks are not adequate to make this solution.

- $5 \text{ ng} / \text{Vol}_{\text{sp}} \times \text{Vol}_t = \text{ng total}$
- $\text{ng total} / \text{Vol}_i = C_i$

In order to get 622.5 ng from our 10 ug/mL and 20 ug/mL primary mix stocks we will need 62.3 uL from each of the 10 ug/mL mixes and 31.1 uL from each of the 20 ug/mL mixes. This volume of standard added equates to 342.5 uL so you will need an additional 1,657.5 uL of seawater matrix to get to your 2 mL total volume that you will derivatize.

- $\text{ng total} / C_{\text{st}} \times 1000 = \text{Vol}_{\text{st}}$
- $\text{Vol}_i = (\Sigma \text{Vol}_{\text{st}} / 1000) + \text{Vol}_m$

N_s - number of samples including blanks and standards

Vol_{sp} - volume of SIL-IS spike to add to each samples (uL)

Vol_d - minimum volume needed of SIL-IS solution (mL)

Vol_i - total volume you will derivatize to make your SIL-IS (mL)

0.803 - constant needed to equate for reagents added during derivatization

Vol_t - total volume of SIL-IS after derivatization (mL)

C_i - initial concentration of your solution to derivatize to make your SIL-IS (ng/mL)

C_{st} - concentration of the stock solution diluted to make your SIL-IS (ng/mL)

Vol_{st} - volume of stock solution added (uL)

$\Sigma \text{Vol}_{\text{st}}$ - sum of the volumes of all stock solutions added if multiple (uL)

Vol_m - volume of seawater matrix (mL)



- 2.3 **d₅ SIL-IS**: Prepare a [M] 1000 ng/mL (C_i) standard mix made in a aged filtered seawater matrix (or other appropriate matrix for your samples). Derivatize this with d₅-benzoyl chloride using the same protocol as for samples and spike into all samples and standards after they have been derivatized. Adding  50 µL (Vol_{sp}) will result in a final amount of [M] 25 ng SIL-IS added. See note in 2.2.

Prepare Reagents

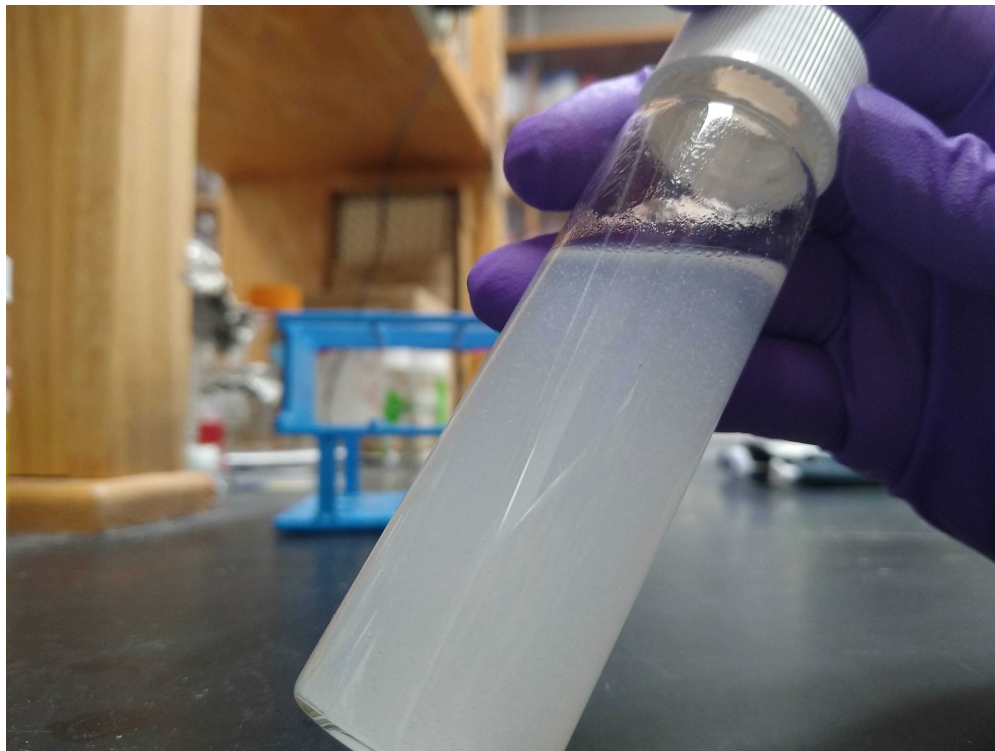
- 3 Prepare reagents. Unless otherwise stated plastic pipette tips are appropriate for all steps.
- 3.1 Prepare working reagent ([M] 5 % volume benzoyl chloride in acetone). Avoid plastic when measuring acetone, use glass (e.g. glass Hamilton syringes with metal needle instead of pipettes). Benzoyl chloride can be transferred with plastic pipette tips. Prepare fresh daily.
- ### Safety information
- Benzoyl chloride is toxic, flammable, and corrosive. Use under hood with proper PPE. All BC-related waste (liquid/solid) should be disposed of in the hood and collected using appropriate hazardous waste protocols.
- 3.2 Prepare [M] 8 Mass Percent NaOH. Store indefinitely at room temperature.
- 3.3 Prepare [M] 0.01 Mass Percent HCl. Store indefinitely 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 ¹³C₆ benzoyl chloride or d₆ benzoyl chloride for the ¹²C benzoyl chloride. Prior to this method, samples have been filtered (0.2 µm, teflon (OmniPore)). Samples should be derivatized as quickly as possible after collection and stored at  -20 °C if they cannot be processed right away. This recipe is for a  5 mL sample. Scale volumes accordingly for the sample size.

5 Add  150 μL NaOH ( 8 Mass Percent) and invert 5 times.

6 Add  1 mL working reagent. Avoid plastic, use glass or Teflon. We recommend a glass bottle dispenser pump with Teflon tubing. 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 or manually inverting sample rack.

8 Add  75 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 (as determined in Step 2). Samples can now be stored at -20 °C .

Post-Derivatization Cleanup

- 10 Remove at least 95% of the acetone (by weight) from the samples using either the Vacufuge (00:30:00 to start) or a stream of nitrogen gas (TurboVac -

1h

00:30:00 at 30 °C and 7-8 psi to start)

Before removing weigh each sample (with cap on) and record the mass so that you know how much acetone you need to remove by weight. The volume of acetone that needs to be removed includes both the acetone added in derivatizing the samples, as well as the small amount added through SIL-IS addition.

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

- 11.1 Separate precipitate (as best you can) from liquid either by centrifuging 5 mL samples ( 1000 x g, 22°C for  00:15:00 , this may not be necessary if they have just been in the vacufuge) or by waiting for the precipitate to naturally separate in the fridge overnight for 25 mL samples. 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 unnecessary plastics for fear of plasticizer contamination.
- 11.2 Condition PPL cartridge with  6 mL (one cartridge volume) methanol (Optima). Don't let the cartridge dry out. Methanol waste should be collected in glass test tube and disposed of as hazardous waste.
- 11.3 Rinse PPL cartridge 4 times (4 cartridge volumes) with  0.01 Mass Percent HCl. Don't let the cartridge dry out. HCl can be collected in the glass SPE manifold and neutralized with baking soda in a connected vacuum carboy.
- 11.4 Carefully remove the supernatant (same volume as initial sample volume, e.g. ( 5 mL or  25 mL) from your sample and load onto the PPL cartridge via a glass Pasteur pipette. It is difficult to avoid transferring some precipitate along with the liquid, but try to minimize this. It is more important to get all the sample volume than to avoid some of the precipitate. Let the sample drip through the cartridge by gravity or very gentle pressure (< 5 " Hg) for larger volumes.
- 11.5 Rinse the PPL cartridge 4 times (4 cartridge volumes) with  .01 Mass Percent HCl and then dry (pull air through at ~15" Hg) for 5 minutes.
- 11.6 Using a Kimtech wipe, remove any drops of HCl from the edges of each PPL cartridge and from the extraction manifold needle tips prior to elution to avoid excess HCl from eluting into your final sample.
- 11.7 Slowly elute with  6 mL (one cartridge volume) methanol (Optima grade) into combusted test tubes. The slower the elution speed the better, generally 1mL/minute is an acceptable elution speed. Slight intermittent use of vacuum (< 5 " Hg) may be necessary to get the elution started.
- 11.8 Once all visible MeOH has been eluted slowly, use the vacuum at 5-10 " Hg, pull all remaining methanol from the cartridge into your elution vial.



- 11.9 Transfer eluent to  8 mL combusted amber vials using glass pipette. Label caps as well as vials (we recommend with label tape as Sharpie tends to fade in downstream vacufuge steps). Store in flammable freezer or proceed to next step.
- 11.10 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, it is better to have an over dry sample than a sample that is too wet. 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, see note in Step 12.3.
- 12.1 "Wash" the benzoic acid precipitate out of the samples. Add  500 μ L  5 % volume acetonitrile/H₂O (MQ) 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 centrifuge tube. It is more important to get all the liquid then to avoid transferring some precipitate.

Note

For  25 mL samples continue to step 12.4, for  5 mL samples skip to step 12.7 or 12.8.

- 12.4 "Wash" the benzoic acid precipitate out of the remaining pellet from Step 12.3. Add  500 μ L  5 % volume acetonitrile/H₂O (MQ) to each sample and vortex thoroughly. The precipitate will mostly not dissolve.
- 12.5 Centrifuge  1000 x g, 22°C for  00:15:00 to separate ppt and liquid.

15m

- 12.6 Transfer supernatant from step 12.5 into the same Eppendorf tube from step 12.3 and vortex. You should have a combined volume of approximately  1 mL .
- 12.7 Optional: Centrifuge at  20000 x g, 22°C for  00:15:00 . This step can help facilitate faster drying especially for  25 mL samples by pelleting any floating precipitate that may impede the drying process. 15m
- 12.8 Dry down (vacufuge) on aqueous setting with  30 °C heat. Samples that are too wet will cause deviations in results. Samples should then be stored at  -20 °C and don't proceed to step 12.9 until you are ready to run them on the mass spectrometer.
- 12.9 Add  100 µL  5 % volume acetonitrile/H₂O (MQ) with injection standards (we use our typical  50-300 ng/mL deuterated injection mix used for our RP method), vortex thoroughly, and transfer to centrifuge tubes.
- 12.10 Centrifuge at  20000 x g, 22°C for  00:15:00 .
- 12.11 Add  5 µL acetonitrile to an LC vial with appropriate insert.
- 12.12 Transfer a set volume of liquid (e.g. 80 uL for all samples) from step 12.10 to vial insert from step 12.11 and vortex.
- 13 Create a pooled sample by combining equal volumes (e.g., 10 uL) of each sample into a single LC-vial with insert. Use a fresh pipet tip for each individual sample to avoid cross-contamination of samples.

Calculate the required volume based on the number of samples and required number of injections. For example, if you have 50 samples and want to inject 5 uL of pool every 10 samples, you will need at least 50 uL of pool per ionization mode ~ 100 uL for positive / negative mode. So taking between 5-10 uL of each sample will provide excess pool for this purpose.
- 14 Store at  4 °C . Samples are stored above freezing to minimize precipitation.

UPLC-MSMS

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



Quantify Metabolites

- 16 Use Skyline.
- 17 Use SkyMat: <https://github.com/KujawinskiLaboratory/SkyMat>