

Chapter 20. Disruption-free Solid Phase Extraction of Surface Metabolites from Macroalgae

Emilio Cirri and Georg Pohnert, Institute for Inorganic and Analytical Chemistry,
Bioorganic Analytics, Friedrich Schiller University, Germany

Abstract

The surface chemistry of aquatic organisms is decisive for their biotic interactions. Metabolites in the spatially limited laminar boundary layer mediate processes, such as fouling, allelopathy and chemical defense against herbivores. However, very few methods are available for the investigation of such surface metabolites. Here we give a detailed protocol in which surfaces are extracted by means of C18 solid phase material, elution of the solid phase extraction material with solvent and analysis via liquid chromatography / mass spectrometry (LC/MS) and/or gas chromatography / mass spectrometry (GC/MS). The protocol introduced here is based on a previous publication (Cirri et al. 2016) where validation is described. The method is robust, picks up metabolites of a broad polarity range and is easy to handle. It was developed for the macroalgae *Fucus vesiculosus*, *Caulerpa taxifolia* and *Gracilaria vermiculophylla*, but can be easily transferred to other algae and to other aquatic organisms in general.

Key words

Surface chemistry, extraction protocol, macroalgae, natural products chemistry, non-disruptive, chemical ecology

1. Introduction

Surface metabolites play a fundamental role in the mediation of interactions on biotic surfaces of e.g., macroalgae, corals or sponges. Such compounds control settling processes, regulate predator / prey relationships and mediate infection processes (da Gama et al. 2014, Dobretsov et al. 2013, Wahl 2009). A hallmark of such interactions is the locally much focused action of the compounds in question (Dworjanyn et al. 1999, Dworjanyn et al. 2006). The surface concentration of such metabolites often exceeds the concentration in the medium, but it can even exceed concentrations within the tissue since exuded compounds can accumulate in a diffusion limited laminar boundary layer. Despite their ecological importance, until now, only a few methods allow the estimation of local concentrations of surface metabolites. As a consequence, many investigations on the effect of surface metabolites were based on bioassays with extracts of whole organisms (see, e.g., Hellio et al. 2000). Such experiments do not reflect the real ecological relevance of surface active substances because only metabolites at the surface or near a producer should be considered (Nylund et al. 2007). The determination of metabolites within the laminar boundary layer around an aquatic organism, a thin film of about 100-200 μm that determines the transition between the surface and the surrounding water, is thus crucial for experiment planning and evaluation. So far the laminar boundary layer has been studied to determine uptake rates of nutrients (Wheeler 1980) or to model them (Hadley 2014), or it has been investigated in correlation with climate changes and oceans' acidification (Cornwall 2014), but the study of metabolites identity and concentration at macroalgal surface is crucial both from an ecological and an industrial point of view, especially in the framework of antifouling substances research (Bhadury 2004, Rajan 2016).

2. State of the Art

Several methods to study the laminar boundary layer have been established during the last 20 years: these include Raman micro spectroscopy (Grosser, et al. 2012), mass spectrometry imaging, like MALDI and DESi (Slaveykova 2009, Andras 2012) and the widely used dipping methods (de Nys et al. 1998, Lachnit et al. 2010). For extraction, algae are immersed in a solvent for a short period, during which the metabolites are partially extracted from the surface. Care has to be taken that the solvent does not damage the cells of the extracted organism. After concentration in vacuum, the extracts can be submitted to analytical methods, such as GC-MS and LC-MS. Dipping methods are really easy to handle and can be used with a lot of different species of macroalgae. By optimizing the solvent (or mixtures of solvents) for extractions, as well as the solvent's volume, these technique allows extracting very different kind of metabolites, proving to be flexible and adaptable to specific questions (see Chapter 19 by Weinberger). Although useful, dipping methods could also be problematic since solvent exposure can cause cell lysis and thereby contamination of the surface extract with intracellular metabolites; these problems depend both on the algal species and on the physiological conditions, as well as on the part of the alga that needs to be extracted. Some algae only tolerate exposure to rather nonpolar solvents such as hexane for few seconds. However, these solvents only cover a very limited range of unpolar metabolites and do not penetrate surface associated water. If solvent mixtures containing methanol are employed, massive damage of the algae could be observed, thereby questioning the validity of results. To overcome these limitations, we developed a new, non-destructive solvent-free and universal method for extracting secondary metabolites from marine macroorganisms (Cirri et al. 2016). The method is based on the adsorption of organic metabolites onto C18 extraction sorbent and has proved to be not harmful to algal surface (Figure 20.1)

Figure 20.1: Evaluation of surface damage by different extraction methods. (a) Photographs of *F. vesiculosus* surfaces (scale bars 100 μm). Top row: control after removal from water for 5, 60 and 600 s. Middle row: algae after C18 extraction. Bottom row: after

hexane/methanol dipping for the same time spans. (b) Evaluation of cell damage after Evans blue staining by red/green ratio analysis at 5, 30, 60, 120, 300, 600 s exposure to C18 material (gray), hexane/methanol dipping (white) and control (black), ($n = 5 \pm \text{SD}$).

The technique has been optimized regarding recovery, reproducibility, and ease of use with the brown macro alga *Fucus vesiculosus* as a model organism. *F. vesiculosus* is a common, well studied brown alga that can be found on the coasts of the North Sea, the western Baltic Sea, and the Atlantic and Pacific Oceans. However, also the green alga *Caulerpa taxifolia* and the red alga *Gracilaria vermiculophylla* were extracted for proof of concept, demonstrating the universality of the method that can be potentially used for all aquatic macroscopic organisms. Here a detailed commented protocol is given, based on our publication Cirri et al. 2016.

3. Materials

3.1. *Fucus vesiculosus*

1. *Fucus vesiculosus* samples can be collected independently of the season. The alga can be extracted directly after collection in the field or can be kept in aquaria as described below.
2. Artificial sea water (ASW): 33 g of Instant Ocean™ (Aquarium Systems, France) per liter of deionized water is stirred at least for 12 h to accomplish a complete dissolution. In our experiment, ASW was diluted with deionized water to half of the initial concentration to reproduce the salinity of the Baltic Sea.
3. Nutrient solutions: 0.22 ml of a K_2HPO_4 solution (1.79 g K_2HPO_4 / 200 mL water) and 0.59 ml of a $NaNO_3$ solution (17.99 g $NaNO_3$ /200 ml water) were added to 1 L of ASW.
4. Glass Aquaria (7 L or more).
5. Controlled climate chamber (15 °C) under 12h light /12h darkness regime (light intensity of $65 \mu\text{mol}\cdot\text{m}^2/\text{s}$).
6. Air pump for constant ventilation of aquaria.

3.2 Solid phase surface extraction method

1. Fully end-capped SPE material 90 C18 material (pore size 90 Å, particle dimensions 40–63 μm , Sigma-Aldrich, Deisenhofen, Germany).
2. Forceps.
3. Empty 6 ml polypropylene columns with PE (polyethylene) frits (Macherey-Nagel, Düren, Germany).
4. Artificial sea water (as described in 2.1.2).
5. 500 ml spray bottle.
6. Deionized water.
7. Methanol (HPLC Grade, Sigma-Aldrich, Germany).
8. Plastic Petri dishes (92 mm $\times$ 10 mm).
9. Glass or plastic funnels (100 mm diameter).
10. Silicone adaptors for Büchner flask.
11. Büchner flask (500 mL).

12. A vacuum pump (ca. 550 mbar) (not essential, **Note 10**).
13. 4 ml glass vials with a black cap.
14. 1.5 ml glass vials with a blue cap.
15. 200 μ l glass inserts and metal springs.
16. Analytical balance.
17. Digital camera.
18. ImageJ (Rasband 1997) or another image processing software.

4. Experimental procedures

The following operations (Figure 20.2) are suitable for different species of macroalgae when they are in healthy condition (not wounded or damaged). The method can be easily adapted to other aquatic organisms by changing handling parameters.

Figure 20.2: Schematic workflow of the C18 method. (1) Algal fronds are removed from the water and left for 2 min to remove excess water by dripping; (2) fronds are transferred to Petri dishes and covered with absorption material; (3) the C18 material is washed off with excess seawater and collected in an empty solid phase extraction cartridge equipped with a frit; (4) the material is washed with deionized water to remove salts; (5) elution with organic solvents finalizes sample preparation.

4.1. Extraction procedure

1. Spread C18 SPE material (**Notes 1, 2**) on the small thecae of the petri dish and weight it. For a Petri dish with a diameter of 9.2cm (92 mm $\times$ 10 mm), an amount of 0.5 g of SPE material is sufficient to reach uniform covering of the plate (**Note 3**).
2. Weight the empty polypropylene cartridge (**Note 2**).
3. Take the piece of alga that you desire to extract out of the aquarium (in this case, the fronds of *F. vesiculosus*). Pieces of around 40 cm² of this alga were

small enough to fit in a 9.2 cm diameter Petri dish and sufficient for generating surface extracts that can be investigated in GC/MS and LC/MS.

4. Hold the alga for 2 minutes so that excess of water can drop off (**Note 4**).
5. Place the alga inside the dish, close it and shake it manually for ca 10 s, taking care to obtain a homogenous cover of the surface with SPE material.
6. Leave the alga in the Petri dish for 60 s (**Note 5**).
7. Take the alga out of the Petri dish using forceps, shaking it gently to remove excess SPE material.
8. Hold the alga with forceps over the funnel connected with the empty polypropylene column and with the Büchner flask (Figure 20.3).

Figure 20.3: Vacuum setup for solid phase extraction.

9. Wash the algal surface with ASW from a spray bottle to remove the most of SPE material. The SPE material is collected in an empty 6 ml polypropylene column (**Note 6**).
10. Wash the funnel and the cartridge with the SPE material with excess (ca. 20 ml) MilliQ water to remove salt from the seawater/medium, taking care that all SPE material is going into the cartridge. While washing, you can apply a gentle vacuum (≈ 550 mbar) to make the powder settle in the cartridge (0.5-1 mL bed volume) (**Note 7**).
11. Add an appropriate internal standard for recovery calculations
12. Elute the compounds with methanol (3 times 0.5 mL) into a four mL glass vial under ambient pressure. Optimal flow rate should be maximum one drop/second (**Note 8**).
13. Remove the solvent under a stream of nitrogen and re-dissolve the sample in 100 μ L of methanol (**Notes 9, 10**). Transfer the sample into a 250 μ L glass insert placed in a 1.5 mL glass vial (or other vials suitable for the autosampler of your own GC or HPLC).

14. Store the sample at -20 °C or proceed to measure it with the desired technique (GC-MS or LC-MS, as in, e.g., Cirri et al. 2016, Vidoudez and Pohnert 2011) (see also Chapter 18 by Kuhlisch et al.).
15. Place the extracted alga on a white, plane surface equipped with a scale bar and take a picture. Calculate the surface as seen in Chapter 19 by Weinberger of the alga with an image processing software (in our case, we used the open source software ImageJ, <http://imagej.nih.gov/ij/>.) (**Note 11**). For thalli with flat morphology, surface areas (SA) can be calculated as

$$SA = 2 \times TP$$
 where TP is the thallus projection.
 In contrast, filaments can be regarded as cylinders, therefore

$$SA = \pi \times \text{thallus diameter} \times \text{thallus length}$$
 and, because $TP = \text{thallus diameter} \times \text{thallus length}$,

$$SA = \pi \times TP.$$
16. After the C18 material is completely dry, weight the cartridge to calculate the amount of extraction powder used for the experiment.

4.2. Data evaluation

17. After LC-MS or GC-MS measurements, integrate both the chromatographic peak area of the internal standard and the substance(s) of interest with the appropriate software associated with your analytical instrument (e.g., Waters[®] Masslynx or Thermo[®] Xcalibur).
18. Calculate the ratio between the chromatographic peak area of the internal standard and the substance(s) of interest for a relative quantification. For absolute quantification, the ratio should be compared to an external calibration curve.
19. Normalize the quantity (relative or absolute) to the surface area previously calculated with ImageJ.

20. For recovery calculation, normalize the chromatographic peak area of the internal standard (in our experiment, canthaxanthin) to the weight of the C18 material effectively extracted.

5. Notes

SECURITY ADVICE: Care should be taken that the dust of the SPE material is not inhaled since aerosols are problematic under prolonged exposure. MeOH is flammable and toxic, thus handling under a fume hood or a good ventilated area is advised.

Note 1. Take care of where you are cutting your alga: during the following step of the method, you have to avoid washing and removing the SPE material which is in contact with these edges. Otherwise, you would risk extracting internal metabolites.

Note 2. Our method development was limited to test a different type of C18 SPE material, but the flexibility of this approach allows extending the range of absorbing material that can be used. For the example material contained in prepackaged SPE cartridges (HLB, StrataX, Ionic Exchanger) can be used as well. The choice depends on the polarity of the metabolites under consideration. It is not necessary to purchase new empty cartridges: you can use old used cartridges, empty them and clean them before reusing them.

Note 3. The Petri dish dimensions can be changed as desired, and smaller or bigger samples can be used depending on the availability of biological material and the concentration of surface metabolites. The “Petri dish” method was preferred to other methods because it is fast and assures a uniform and simultaneous covering of the algal surface. A drawback of the method is the loss of a huge percentage of SPE material since only ca. a fifth of the powder spread in the Petri dish attach to the algal surface. However, this powder can be reused since it does not get contaminated by exometabolites of the alga. An alternative to the Petri dish method could be to use a small mesh sieve to spread the SPE material over the algal surface. Either plastic or glass funnels can be used. Glass guarantees less chemical contaminations. However, the plastic material allows for stirring and

collecting the C18-powder easily, as the silica material tends to stick to the glass surfaces.

Note 4. It is essential that the surface is not getting completely dry: the thin layer of water that remains on the surface (laminar boundary layer) is the environment from which metabolites shall be determined.

Note 5. Different extraction times were tested, and 60s was chosen as the best compromise between a good interaction and absorption of metabolites with C18 material and an easy recovery of the powder from the alga.

Note 6. The amount of water used for the washing steps is not a crucial parameter, but for more precise absolute quantification of metabolites, this step can also be easily standardized, always using the same amount of water.

Note 7. Different vacuum setups could be used, as a vacuum manifold (Visiprep™, Supelco, USA) for multiple, simultaneous extractions. Anyway, the use of a vacuum pump is not necessary. In case the vacuum is not applicable, the settlement of the SPE material will require longer time and a larger amount of water to make sure that the absorbing material is properly packed.

Note 8. Our method was optimized for 1.5 mL of methanol as an extraction solvent, but as in all SPE methods, the amount and the polarity of the solvent (or mixture solvents) can be adapted. Other solvents can be applied depending on the used solid phase and the nature of the metabolites which should be extracted. C18 material does not need to be dry before elution; however, each material has its specific treatment to be fully effective. For all these information, also check the recommendations of the manufacturer.

Note 9. If needed or desired, the sample can be stored at this point at -20 °C, and the drying step could be done afterward

Note 10. The volume of re-dissolution should be adapted to some surface metabolites present and the sensitivity of the analytical instrument to obtain a response in the linear range. For GC-MS analysis of polar compounds, a derivatization protocol can be used starting from this step. For more information about derivatization, see chapter 18 by Kuhlisch et al.) and Vidoudez and Pohnert (2011).

Note 11. The measurement of the surface is an essential step of this method, but sometimes difficult to achieve, because of the texture and morphology of the alga. Try to get your sample as flat as possible, using, e.g., a glass slide to cover your alga and some weights to keep the alga plane.

6. Acknowledgement

The authors acknowledge funding the Jena School for Microbial Communication and the International Max Planck Research School on the Exploration of Ecological Interactions with Molecular and Chemical Techniques and the EU Marie Skłodowska-Curie Initial Training Network (ITN) program Algal Microbiome: Friends or Foes (ALFF) for funding.

7. References

- Andras, T.D., Alexander, T.S., Gahlén, A., Parry, R.M., Fernandez, F.M., Kubanek, J., Wang, M.D., and Hay, M.E. 2012. Seaweed Allelopathy Against Coral: Surface Distribution of a Seaweed Secondary Metabolite by Imaging Mass Spectrometry. *J. Chem. Ecol.* 38:1203-1214
- Bhadury, P. and Wright, P.C. 2004. Exploitation of marine algae: biogenic compounds for potential antifouling applications. *Planta* 219 (4): 561-578.
- Cirri, E. 2014, Development and optimization of an extraction method for the analysis of algal, surface metabolites, Master Thesis, University of Florence
- Cirri, E., Grosser, K., and Pohnert, G. 2016. A solid phase extraction based non-disruptive sampling technique to investigate the surface chemistry of macroalgae. *Biofouling* 32(2):145-53
- Cornwall, E.C., Boyd, P., McGraw, C.M., Hepburn, C.D., Pilditch, C.A., Morris, J.N., Smith A.M., and Hurd, C.L. 2014. Diffusion Boundary Layers Ameliorate the Negative Effects of Ocean Acidification on the Temperate Coralline Macroalga *Arthrocardia corymbosa*. *PLoS One* 9 (9): e109468

- Dobretsov, S., Abed, R.M.M. and Teplitski, M. 2013. Mini-review: Inhibition of biofouling by marine microorganisms. *Biofouling* 29:423–441.
- Dworjanyn, S.A De Nys R, and Steinberg PD. 1999. Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*. *Mar. Biol.* 133:727–736.
- Dworjanyn, S.A., Wright, J.T., Paul, N.A., de Nys, R., and Steinberg, PD. 2006. Cost of chemical defence in the red alga *Delisea pulchra*. *Oikos* 113:13–22.
- da Gama, BAP., Plouguerne, E., and Pereira, R.C. 2014. The antifouling defence mechanisms of marine macroalgae. In: *Advances in botanical research – Sea plants*. eds. Bourgonon, N., Jacquoud, JP., Gadal, P. p. 413-440. Amsterdam: Elsevier.
- Grosser, K., Zedler, L., Schmitt, M., Dietzek, B., Popp, J., and Pohnert, G. 2012. Disruption-free imaging by Raman spectroscopy reveals a chemical sphere with antifouling metabolites around macroalgae. *Biofouling* 28:687–696.
- Hall, S., Wild-Allen K., Johnson, C., Macleod C. 2015. Modeling macroalgae growth and nutrient dynamics for integrated multi-trophic aquaculture. *J. Appl. Phycol.* 27 (2): 901-916.
- Hellio, C., Bremer, G., Pons, A.M., and Le Gal, Y., Bourgonon, N. 2000. Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany, France. *Appl. Microbiol. Biotechnol.* 54:543–549.
- Kuhlisch, C., G. Califano, T. Wichard, G. Pohnert. 2017. Metabolomics of intra- and extracellular metabolites from micro- and macroalgae using GC-MS and LC-MS. In *Biotechnology Protocols for Macroalgae Research*, eds. B. Charrier, T. Wichard, C.R.K. Reddy, Chapter 18. Boca Raton: CRC Press, Francis & Taylor Group.
- Lachnit, T., Fischer, M., Kuenzel, S., Baines, J.F., and Harder, T. 2013. Compounds associated with algal surfaces mediate epiphytic colonization of the marine macroalga *Fucus vesiculosus*. *FEMS Microbiol. Ecol.* 84:411–420.

Lachnit, T., Wahl, M., and Harder, T. 2010. Isolated thallus-associated compounds from the macroalga *Fucus vesiculosus* mediate bacterial surface colonization in the field similar to that on the natural alga. *Biofouling* 26:247–255.

Nylund, G.M., Gribben, P.E., de Nys, R., Steinberg, P.D., and Pavia, H. 2007. Surface chemistry versus whole-cell extracts: antifouling tests with seaweed metabolites. *Mar. Ecol. Prog. Ser.* 329:73–84.

Nylund, G.M., Weinberger, F., Rempt, M., and Pohnert, G. 2011. Metabolomic assessment of induced and activated chemical defence in the invasive red alga *Gracilaria vermiculophylla*. *PLoS One* e29359.

de Nys, R., Dworjanyn, S.A., and Steinberg, P.D. 1998. A new method for determining surface concentrations of marine natural products on seaweeds. *Mar. Ecol. Prog. Ser.* 162:79–87.

Rasband, W. 1994-2014. ImageJ, US National Institutes of Health, Bethesda, MD, USA. Available from: <http://imagej.nih.gov/ij/>

Saha, M., Rempt, M., Gebser, B., Grueneberg, J., Pohnert G, and Weinberger F. 2012. Dimethylsulphopropionate (DMSP) and proline from the surface of the brown alga *Fucus vesiculosus* inhibit bacterial attachment. *Biofouling* 28:593–604.

Saha, M., Rempt, M., Grosser, K., Pohnert, G., Weinberger, F. 2011. Surface-associated fucoxanthin mediates settlement of bacterial epiphytes on the rockweed *Fucus vesiculosus*. *Biofouling* 27:423–433.

Slaveykova, V.I., Guignard, C., Eybe, T., Migeon, H-N., and Hoffmann, L. 2009. Dynamic NanoSIMS ion imaging of unicellular freshwater algae exposed to copper. *Anal. Bioanal. Chem.* 393:583-589.

Vidoudez, C. and Pohnert, G. 2011. Comparative metabolomics of the diatom *Skeletonema marinoi* in different growth phases. *Metabolomics* 8(4), 654-669.

Weinberger, F., 2017. Preparative extraction of exometabolites from seaweed surfaces. In *Biotechnology Protocols for Macroalgae Research*, eds. B. Charrier, T. Wichard, C.R.K. Reddy, Chapter 19. Boca Raton: CRC Press, Francis & Taylor Group.

Wheeler, W.N. 1980. Effect of boundary layer transport on the fixation of carbon by the giant kelp *Macrocystis pyrifera*. *Mar. Bio.* 56(2): 103-110.