Chapter 18. Metabolomics of intra- and extracellular metabolites from micro- and macroalgae using GC-MS and LC-MS.

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Abstract

Comparative metabolomics is an emerging technique suitable for the monitoring of metabolic responses of organisms to external stimuli or stress factors like biotic interaction partners, nutrient limitation or adverse environmental conditions. Using data from comparative metabolomics, changes in primary metabolism can be unraveled and connected to the regulation of metabolic pathways. However, the technique is also suitable for an untargeted screening of primary and secondary metabolites. Thereby candidate metabolites can be identified that might play a functional role in the particular stress situation. Especially mass spectrometry-enabled techniques have found their way into many disciplines. Here we describe the extraction, derivatization, gas chromatography-mass spectrometry (GC-MS) as well as liquid chromatography-mass spectrometry (LC-MS) measurement and data analysis for a metabolic profiling of algae. We describe a general protocol for intracellular profiling of microalgae, and germ cells and thalli of macroalgae. The protocol outlines the procedure to extract, derivatize and measure the exo-metabolome of these organisms, i.e., the metabolites exuded into the seawater.

The method was initially developed for metabolomics of the diatom *Skeletonema marinoi* but proved to be suitable for a broad range of micro- and macroalgae after minor adaptations. The entire work-flow can be carried out in laboratories with basic equipment for chemical work and measurements can be recorded on most commercially available GC-MS and LC-MS systems.

Keywords

metabolomics, untargeted analysis, metabolic profiling, exo-metabolome, protocol, solid phase extraction, derivatization, data analysis

1. Introduction

Metabolomics techniques aim to comprehensively extract, quantify and evaluate metabolites from a given organism or community and have developed into an indispensable tool in life sciences (Allwood et al. 2011; Aldridge and Rhee 2014) (for more information refer to a recent special issue in Current Opinion in Chemical Biology (Schroeder and Pohnert 2017). Different approaches have been brought forward that allow to answer a multitude of questions about the physiology of an organism and to generate new hypotheses about its response to stress (Goulitquer, Potin, and Tonon 2012; Krug and Müller 2014). Among others, metabolomics allows us to map changes in primary metabolism that reflect the regulation of biochemical pathways, to categorize samples using metabolic fingerprinting techniques, or to identify metabolites that are regulated in stress or interaction situations by comparative metabolic profiling. Especially comparative metabolomics is suitable for the generation of hypotheses about the role of primary and secondary metabolites (Lee and Fiehn 2008; Kuhlisch and Pohnert 2015). Most commonly, mass spectrometric techniques are used for the recording of metabolomics data. With complex samples, MS can be coupled to efficient separation techniques like ultra performance liquid chromatography (UPLC) and gas chromatography. By using such hyphenated analytical techniques, the profiling of hundreds of metabolites within minutes is feasible. Fundamental pre-requirements for successful metabolomics investigations are the experiment planning, sample preparation, extraction, data recording as well as the statistical evaluation of the results. In this contribution, we describe a very robust and validated protocol for the generation of metabolic data from marine organisms. Initially developed for the investigation of the diatom *Skeletonema marinoi* the protocol was successfully adapted to the investigation of different algal taxa (Nylund et al. 2011; Vidoudez and Pohnert 2012; Mausz and

Pohnert 2015) including flagellated gametes and thalli of the green macroalga *Ulva mutabilis* and water samples for exo-metabolomic profiling (Alsufyani, Weiss, and Wichard 2017). Even profiling of entire plankton communities is feasible without the need for further alterations (Kuhlisch unpublished results), and it can be predicted that only minor adaptations to the protocol are required to make it suitable for a broad range of other (marine) organisms. By introducing a few additional experimental steps, the method that was initially developed for the investigation of the endo-metabolome (i.e., the intracellular metabolites) can also be used to monitor the exo-metabolome (i.e., the metabolites released into the surrounding seawater; see Barofsky, Vidoudez, and Pohnert, 2009 and Alsufyani, Weiss, and Wichard, 2017). For high-quality metabolomics, the sample preparation and handling are decisive, and we thus place here attention to fully document and describe the work-flow. We also introduce one selected work-flow for data analysis of comparative metabolomics data sets.

2. State of the Art

In the past, qualitative and quantitative metabolite investigations were conducted by targeted chemical analyses of certain metabolites or metabolite classes (Gravot et al. 2010; Dittami et al. 2011). However, along with the improvement of hyphenated analytical techniques, the simultaneous and untargeted metabolic profiling of a broader range of substance classes including amino acids, organic acids, sugars and fatty acids became feasible as reviewed in Dunn (2008). Complex biological extracts are thereby separated by, e.g., gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE), followed by their subsequent analysis using mass spectrometry (MS). Variously available mass analyzers like quadrupole, time of flight (TOF), and the Orbitrap provide a range of instruments with variable sensitivity, mass resolution, and mass accuracy. Besides LC-MS, one of the most common analytical

platforms for metabolic profiling is GC-MS due to its high chromatographic resolution, sensitivity, and availability of reference libraries (Dunn 2008). Many experimental steps for GC-MS metabolic profiling were established in terrestrial plants and transferred to mammals and microbes (Fiehn 2008). Thus, the first protocols in plant sciences included plant specific protocols for enzyme quenching by liquid nitrogen, cell homogenization, and metabolite extraction in hot methanol before the separation of polar metabolites, evaporation for derivatization, GC-MS analysis, data processing and statistical analysis. A two-step derivatization with optimized methoxylation conditions and MSTFA as silylation reagent was introduced and DB5-MS columns were recommended for separation (Fiehn et al. 2000a; Fiehn et al. 2000b; Roessner et al. 2000). Isotopically labeled primary metabolites (Fiehn et al. 2000a) and the polyol ribitol (Roessner et al. 2000) were introduced as internal standards for semi-quantitative metabolite analysis. In the following, several steps of the initial protocols were improved (Lisec et al. 2006) or adjusted to the needs of other study systems (Winder et al. 2008), especially for particularly error prone steps such as metabolic quenching (Álvarez-Sánchez, Priego-Capote, and Luque de Castro 2010), or cell extraction, for which a one-phase-solvent mixture was proposed by Gullberg et al. (2004), who also sorted out the effect of oximation time and temperature during derivatization. With the development of mass spectral libraries, GC-TOF-MS systems came in focus, which offer faster scan times compared to quadrupole-MS (Wagner, Sefkow, and Kopka 2003). Furthermore, data processing and analysis strategies were evaluated, e.g., handling of multiple derivatization products (Kanani and Klapa 2007), appropriate data scaling methods (van den Berg et al. 2006), or the choice of uni- and multivariate statistical analyses (Saccenti et al. 2014).

Within the field of marine sciences, metabolomics approaches are still at their advent

(Goulitquer, Potin, and Tonon 2012). Metabolic profiling of micro- and macroalgae was first established for the freshwater microalga *Chlamydomonas reinhardtii* (Bölling and Fiehn 2005), followed by the introduction of protocols for the investigation of the marine diatoms *Phaeodactylum tricornutum (*Allen et al. 2008*)*, *Skeletonema marinoi (*Vidoudez and Pohnert 2012*)*, and *Thalassiosira pseudonana (*Bromke et al. 2013*)*. With *Gracilaria vermiculophylla* and *Gracilaria chilensis* (Nylund et al. 2011; Weinberger et al. 2011) the first marine macroalgae were profiled followed by the brown algae *Ectocarpus siliculosus*, *Laminaria digitata* and *Lessonia spicata* (Ritter et al. 2014; Ritter et al. 2017). Some efforts have already been made to adjust experimental procedures to the needs of macroalgae. Thus, the robust cell wall structures were extracted after flash freezing in liquid nitrogen and thorough grinding with mortar and pestle. Prior to this, epibionts were removed gently if thalli were collected in the field. A challenge that has not been addressed so far is the high morphological and chemical diversity of the diverse macroalgal taxa and life cycle stages.

In contrast to the profiling of endometabolites of marine algae rather few investigations exist that cover exometabolites (Barofsky, Vidoudez, and Pohnert 2009; Gillard et al. 2013; Becker et al. 2014; Longnecker, Soule, and Kujawinski 2015). The methodology of this field is currently developing as reviewed in Minor et al. (2014). Main challenges are the separation of cells and surrounding medium without cell leakage and the extraction of dissolved metabolites. Thus cells should be removed, e.g., by gentle filtration over sand or GF/C filters (Barofsky, Vidoudez, and Pohnert 2009; Alsufyani, Weiss, and Wichard 2017). The most common extraction method for dissolved metabolites in seawater is solid phase extraction (SPE) as it is fast, simple and removes the salt load of marine samples that would otherwise interfere with subsequent analytical processes. Metabolite coverage depends both on the choice of the SPE cartridge adsorbent and

eluting solvents. Styrene divinylbenzene phases showed highest recoveries so far, and we propose CHROMABOND® Easy cartridges due to their high recovery of *m/z* -retention time pairs (Barofsky, Vidoudez, and Pohnert 2009).

In the following protocol, we focus on sample generation for GC-MS analysis in addition to LC-MS and give a brief overview of processes in data treatment. Different sample and extraction strategies for the diverse macroalgal life stages are presented. We also introduce a protocol for profiling the exometabolome of micro- and macroalgae.

3. Materials

3.1. Solvents

- Acetone (certified ACS, Fisher Chemical)
- Chloroform (for HPLC, HiPerSolv CHROMANORM® (≥99.8%, filtered through 0.2 µm, packed under N_2, stabilized with 0.6% ethanol), VWR)
- Ethanol (gradient grade for LC, LiChrosolv® ; ≥ 99.9%, filtered through 0.2 µm, Merck)
- Hexane (for GC, SupraSolv® (≥ 98%), Merck; stored over 4 Å molecular sieve)
- Methanol (for HPLC, Chromasolv®Plus (≥99.9%), Sigma-Aldrich)
- Pyridine (for HPLC, Chromasolv®Plus (≥99.9%), Sigma-Aldrich; stored over 4 Å molecular sieve under argon)
- Tetrahydrofuran (THF; for HPLC, HiPerSolv CHROMANORM® (≥99.7%, filtered through 0.2 µm, packed under N_2, not stabilized), VWR; stored under argon)
- Water (for HPLC, Chromasolv®Plus (filtered through 0.2 µm), Sigma-Aldrich)

- Extraction solution (see Table 18.1 in 3.4 Solution recipes)
- Column elution solution (see Table 18.2 in 3.4 Solution recipes)
- Internal standard (IS) solution (see Table 18.3 in 3.4 Solution recipes)
- Methoxyamine hydrochloride (98%, Sigma-Aldrich; hygroscopic and thus stored in a desiccator under argon and vacuum-dried before use)
- Retention time index (RI) solution (see Table 18.4 in 3.4 Solution recipes)
- *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA; 1 mL vials, Macherey-Nagel)

3.2. Equipment

- Fume hood
- Lab coat
- Safety goggles
- Chemical-resistant gloves (nitril or latex, dependent on solvent)
- Eppendorf® pipettes (1000 µL, 100 µL, 10 µL, recently checked and calibrated)
- Filtration pump system with vacuum control and Nalgene® tubing (**Figure 18.1**)
- DURAN® filtering flasks (1 L, KECK™ assembly, Erlenmeyer shape) with rubber seals (**Figure 18.1**)
- DURAN® dismountable filter holders (ø 50 mm, 250 mL top manifold, sintered filter discs (Porosity 4), FKM seals, PP outlet funnel) (**Figure 18.1a**)
- Tweezers
- 25 mL glass beaker

- TissueLyser II (QIAGEN, max. speed: 30 frequencies sec^{-1})
- Lyophilizer
- PTFE tubing (ø 0.8 mm, each ~30 cm) (**Figure 18.1b**)
- Vortex mixer
- Ultrasonic bath
- Centrifuge (with temperature control, up to 30.000 g)
- Vacuum evaporation facility (desiccator) and vacuum diaphragm pump (down to 7 mbar, with vacuum control, connected to argon)
- Precision balance (± 0.1 mg)
- Hamilton® glass syringes (100 µL)
- Heating block (for 1.5 mL vials)

Figure 18.1. Set-up of filtration system (a) and filtrate extraction system (b).

3.3. Consumables

- Eppendorf® pipette tips (blue, yellow, white)
- Eppendorf® centrifuge tubes (1.5 mL, 2 mL)
- Whatman® glass microfiber filters (ø 47 mm, grade GF/C)
- Solid phase extraction (SPE) columns CHROMABOND® Easy (3 mL, 200 mg, Macherey-Nagel)
- Metal beads (ø 3 mm, stainless steel)

- Centrifuge tubes (15 mL)
- Glass screw neck vials both 1.5 mL and 4 mL, the 1.5 mL vials have to fit GC- or LC-MS auto samplers
- Glass inserts for 1.5 mL vials (200 µL) with metal springs compatible with auto samplers
- Screw caps for 1.5 mL and 4 mL vials with septa (PTFE coated)
- Liquid nitrogen

3.4. Solution recipes

The following solutions have to be prepared in advance (**Tables 18.1-4**):

Table 18.1. Extraction solution

Composition	**Final ratio (v: v: v)**	**Quantity to add (for 10 mL final solution)**
Methanol	1	2 mL
Ethanol	3	6 mL
Chloroform	1	2 mL

Prepare daily and pre-cool at -20°C before use. Store on ice during cell sampling to ensure an ice-cold solution for extraction. The composition is optimized for diatom cells (*S. marinoi*) and was suitable for the extraction of other marine organisms, but can be adjusted to ensure optimal metabolite extraction if using organisms with specific mechanical or chemical properties.

Table 18.2. Column elution solution

Composition	Final ratio (v: v: v)	Quantity to add (for 20 mL final solution)
Methanol	1	10 mL
THF	1	10 mL

Store in inert containers (ideally glass sealed with PTFE caps). The composition can be adjusted to ensure optimal elution of the SPE column.

Table 18.3. Internal standard (IS) solution

Composition	Final concentration	Quantity to add (for 1 mL final solution)
Ribitol or $^{13}C_6$-sorbitol	4 mM	100 µL of stock solution at 40 mM
Water	-	900 µL

Prepare a 40 mM stock solution by dissolving 24.9 mg ribitol (>99%, Sigma-Aldrich) in water. Store stock and working solution at -20°C in inert containers (ideally glass sealed with PTFE caps). Thaw before use. The polyol ribitol serves as IS in this protocol as it has not been reported from marine algae so far. However, it has been shown in bacteria, fungi, plants and green microalgae (Bieleski 1982). Here, isotopically labeled IS are recommended such as $^{13}C_6$-Sorbitol (Bölling and Fiehn 2005). Both the final concentration and composition of the IS can be adjusted with regard to the experiment.

Table 18.4. Retention time index (RI) solution

Composition	Final concentration	Quantity to add (for 1 mL final solution)
Decane, C_{10}	1 mM	10 µL of stock solution at 100 mM
Pentadecane, C_{15}	1 mM	10 µL of stock solution at 100 mM
Nonadecane, C_{19}	1 mM	10 µL of stock solution at 100 mM
Docosane, C_{22}	1 mM	10 µL of stock solution at 100 mM
Octacosane, C_{28}	1 mM	100 µL of stock solution at 10 mM
Dotriacontane, C_{32}	1 mM	100 µL of stock solution at 10 mM
Hexatriacontane, C_{36}	0.5 mM	100 µL of stock solution at 5 mM
Hexane	-	660 µL

Prepare stock solutions for C_{10}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32} and C_{36} (all >99%, Sigma-Aldrich) in hexane. Stock and working solutions are stored at -20°C and thawed before use. The *RI solution* is used to calculate system independent retention times, which is, e.g., necessary for comparison with externally measured reference compounds and thus metabolite identification. Final concentration and composition can be adjusted with regard to the experiment.

3.5. Instrumental Setup

Common GC-MS (and LC-MS) instruments can be used. The described procedure can be easily adjusted to the available instrumentation.

GC-MS instrument: AGILENT 6890N gas chromatograph, equipped with AGILENT 7683B auto

	sampler, coupled to WATERS® Micromass GCT Premier™ mass spectrometer (orthogonal acceleration time-of-flight MS)
Injection parameter:	1 µL injection volume, split/splitless injector at 300°C, splitless to split 10 mode (can be adapted to sample and instrument properties), deactivated AGILENT split liner (4 × 6.3 × 78.5 mm inner ø × outer ø × length) with glass wool
GC parameter:	constant helium (5.0) flow at 1 mL.min^{-1}, AGILENT J&W DB-5ms column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness, 10 m Duraguard pre-column), oven temperature program: 60°C for 1 min, ramp to 310°C at 15°C min^{-1}, 310°C for 10 min
MS parameter:	electron impact source (70 eV) at 300°C, scan rate of 5 scans s^{-1}, dynamic range extension mode, resolution of > 6000 at *m/z* 501.97

4. Experimental procedures

In the following, all steps from sampling to statistical analysis are described in detail (**Figure 18.2**). Preceding steps such as the design of the experimental set-up are not in focus of this protocol. It is however recommended to set up at least 5 biological replicates. Further, the sampling of blanks that should undergo identical treatment as the biological samples is essential for later identification of contaminants.

Figure 18.2. Metabolomics workflow for the analysis of intra- and extracellular metabolites of micro- and macroalgae by GC-MS. All numbers refer to steps within the protocol.

4.1. Sampling and metabolic quenching

The presented protocol was originally developed for metabolite profiling of phytoplankton samples. Therefore, the following paragraph describes several strategies for collecting algal biomass depending on its origin including a sampling of single celled microalgae (4.1.1.), of single-cell stages of macroalgae (4.1.2.), and of whole thalli (4.1.3.). As metabolites underlie diurnal fluctuations the sampling time should be identical for all replicates. Until metabolic "quenching" (arresting the metabolic activity) all steps have to be conducted as rapidly as possible to prevent metabolic alterations.

4.1.1. Filtration of planktonic single celled algae

1. Gently shake the culture to homogeneously distribute the cells.
2. Collect aliquots for cell counting (**Note 1**), bioassays or collection of other metadata if needed.
3. Filter a determined culture volume (**Note 2**) under reduced pressure (~600 mbar) through a GF/C filter. The filter should not run dry. Keep the filtrate at 4°C until solid phase extraction (see 4.2.).
4. Immediately transfer the wet filter to a 25 mL glass beaker.
5. Immediately quench the metabolism by quickly re-suspending the cells in 1 mL cold (-10°C) extraction solution. Therefore rinse cells as far as possible off the filter by repeatedly pipetting the *extraction solution* over the filter. Transfer the suspension into a 1.5 mL centrifuge tube.
6. Add 5 µL *internal standard solution* and vortex 10 sec.
7. Store the sample on ice.
8. Repeat steps 3)-7) for all replicates. Continue with step 15).

4.1.2. Collection of algal gametes (of *Ulva* spp).

9. Once gametes swarm out of the gametangium, they gather towards the light. Collect, count and transfer a volume equivalent to 5×10^6 gametes into 2 mL centrifuge tubes as described in Chapter 9 by Califano and Wichard .
10. *Optional*: Free swimming gametes can be sampled by centrifugation (see **Note 3**).
11. Once the gametes are attached to the 2 mL centrifuge tube wall or are pelleted after centrifugation, remove the growth medium with a pipette and immediately freeze the sample in liquid nitrogen. Due to the low recovery rate of entire cells from surfaces, the tube in which the gametes were grown should also be used for extraction. Continue with step 15).

4.1.3. Collection of algal thalli

12. Gently clean the thalli. First, scrape off epiphytes with a scalpel and then wash three times with autoclaved (filtered) seawater.
13. Collect about 100 mg fresh weight of a specific tissue (e.g., blade tissue or rhizoid, cut with a scalpel) in a 2 mL centrifuge tube and immediately freeze in liquid nitrogen.
14. Remove remaining water by lyophilization at 0.001 mbar at -50°C until completely dry. Continue with step 15).
15. *Optional*: Samples can be stored at -20°C for a few days and -80°C for several weeks.
16. Samples are ready for extraction (see 4.3.).

4.2. Solid phase extraction of extracellular metabolites

The extraction of extracellular metabolites from the filtrates can be done in parallel for all replicates. Therefore randomize the samples.

17. Condition a CHROMABOND® Easy column directly before use. Therefore pipette 4 mL methanol onto the column and let it flow by gravity into a waste vial.
18. Wash the column with 4 mL water. Let it flow by gravity into a waste vial.

19. Take out all filtrates from the fridge (step 4.1.1.3) and place them on ice.
20. Connect the PTFE tube in line with the column. Place the PTFE tube into the filtration flask containing the filtrate and connect the column with the vacuum system.
21. Pass the sample slowly through the column at a flow rate of ~1 L $hour^{-1}$.
22. Disconnect the column from the PTFE tube and wash the column with 4 mL water.
23. Air-dry the column with the vacuum system. Dry column adsorbent is bright red again.
24. Elute the column by gravity with 2 mL methanol into a 4 mL glass vial.
25. Elute in the second step with additional 2 mL column elution solution into the same vial.
26. Add 5 µL IS solution, close the vial and vortex for 10 sec.
27. *Optional:* Samples can be stored at -20°C for a few days and at -80°C for several weeks.
28. Transfer for each sample an aliquot of 1.5 mL into a 1.5 mL glass vial. For LC-MS see **Note 4.**
29. Evaporate to dryness under vacuum using a desiccator (**Note 5**). Reduce the pressure from ambient pressure to 0 mbar considering solvent boiling points (**Note 6**).
30. Vent the desiccator slowly with dry air or argon (**Note 7)** and immediately close all vials.
31. Samples are ready for derivatization (see 4.4.).

4.3. Extraction of intracellular metabolites

The extraction of intracellular metabolites can be carried out in parallel for all replicates. Therefore randomize the samples. Depending on the cell wall morphology of the studied organism different cell disruption methods might be suitable of which two are described below.

32. *Optional*: Thaw samples from step 4.1.15) or directly use samples from step 4.1.16).

4.3.1. Cell disruption by ultrasound-treatment

33. Vortex the samples for 30 sec. Transfer an aliquot equivalent to ~5×10^7 cells into a new

1.5 mL centrifuge tube (**Note 2**).

34. Add extraction solution to reach an adequate cell-to-solvent ratio which is ideally equivalent to a cell density of ~5×10^5 cells μL^{-1} solvent. Continue with step 38).

4.3.2. Cell disruption with a bead mill

35. Place the centrifuge tubes in a pre-cooled (-80°C) TissueLyser II tube support. Add 2 metal beads per tube, close again and disrupt the cells using the TissueLyser II for 30 sec at a frequency of 30 sec^{-1} **(Note 8)**. During this procedure, the sample will remain frozen.
36. Add 5 µL IS solution.
37. Add 1 mL extraction solution to each sample and vortex vigorously in order to homogenize the sample and allow a more uniform extraction.
38. Place the samples for 10 min into an ultrasonic bath.
39. Centrifuge the samples at 30.000 g for 15 min at 4°C.
40. Transfer the debris-free supernatants into 1.5 mL glass vials. For LC-MS analysis see **Note 4**.
41. Evaporate to dryness under vacuum using a desiccator **(Note 5**). Reduce the pressure from ambient pressure to 0 mbar considering solvent boiling points (**Note 6)**.
42. *Optional*: Traces of salt from the medium will precipitate as crystals that restrain water molecules. Thus keep the pressure at 0 mbar for an additional hour to ensure an entirely dry sample.
43. Vent the desiccator slowly with dry air or argon (**Note 7**) and immediately close all vials.
44. Samples are ready for derivatization (see 4.4.).

4.4. Two-Step-Derivatization for GC-MS analysis

To analyze a broad range of metabolites by GC-MS the volatility and thermostability of some

substances classes need to be enhanced by derivatization. In a first step ketones and aldehydes are derivatized to oximes and then functional groups like -OH, $-NH_2$, -SH or -COOH as present in, e.g., sugars, fatty acids or amino acids are chemically derivatized by silylation. Directly use the dried samples from step 4.2.31 or 4.3.44.

45. Prepare the methoxyamine solution immediately before derivatization. Therefore weigh 20 mg dried methoxyamine hydrochloride in a 1.5 mL glass vial.
46. Add 1 mL pyridine, close the vial, and ensure complete dissolution by sonication in an ultrasonic bath for at least 5 min.
47. Pipette 50 µL methoxyamine solution to each sample (a max. of 20 samples is recommended, **Note 9**, and immediately close the vials. Vortex 60 sec to re-dissolve the extract.
48. Incubate at 60°C for 1 hour.
49. Subsequently incubate at room temperature for 9 hours (up to 16 hours, **Note 10**).
50. Prepare the silylation solution. Therefore remove a new vial of MSTFA from the fridge and let it warm up to room temperature. Thaw and vortex the RI solution.
51. Add with a glass syringe 40 µL RI solution to 1 mL MSTFA and vortex the vial. Rinse the syringe with hexane after use.
52. Add with a glass syringe 50 µL silylation solution to each sample. Prevent any cross-contamination between samples. Rinse the syringe with acetone after use.
53. Incubate at 40°C for 1 h (**Note 11**).
54. Let the samples cool down to room temperature.
55. *Optional*: In case of condensation along the glass briefly centrifuge the vials (~5 sec).
56. Transfer each sample into a glass insert and close the vial.

57. Centrifuge all samples at 8.000 g for 5 min using 15 mL centrifuge tubes padded with a cloth as vial mounting. In case of precipitate-formation transfer the supernatant into a new glass insert.
58. Analyze the batch of samples immediately (**Note 9**) by GC-MS (see 4.5.).

4.5. GC-MS analysis

59. Directly use the derivatized samples from step 4.4.58).
60. Analyze each batch of samples in a random order to diminish a potential systematic effect of increasing time delay between silylation and injection. For analysis, details see the *Instrumental setup*.
61. Run air injections before, in between and after each batch to check for contaminations.
62. Use a new glass liner every 21 injections or if air injections indicate contamination.
63. For data analysis see 4.6.

4.6. Data analysis for GC-MS data

In the following protocol, a canonical analysis of principal coordinates (CAP, Anderson and Willis 2003) is used to investigate metabolic alterations. In comparison to other common multivariate statistical analyses, CAP is less sensitive to hidden correlations within the dataset (McCune, Grace, and Urban 2002) which are a direct result from metabolites generating multiple peaks, e.g., due to different levels of derivatization. Alternatively, a range of online platforms is available offering different data processing and analysis strategies. These include MetaboAnalyst (Xia et al. 2009), see **Figure 18.3e**; XCMS Online (Tautenhahn et al. 2012), see **Figure 18.3d**; and Workflow4Metabolomics (Giacomoni et al. 2015). The following workflow is also applicable to LC-MS data after adaptation of the peak detection procedure (Alsufyani, Weiss, and Wichard 2017).

64. Conduct a background-noise correction for each chromatogram using, e.g., the CODA tool of MassLynx 4.1 (Waters, MCQ = 0.8, Smoothing window = 5).
65. Convert the .raw data files to NetCDF (.cdf), e.g., using the Databridge tool (MassLynx 4.1).
66. Deconvolute all chromatograms with AMDIS 2.71 (http://chemdata.nist.gov/, 2012) with the following parameters: minimum match factor = 30, type of analysis = simple, low/high *m*/*z* = auto, instrument type = quadrupole, component width = 32, omitted *m*/*z* = 147, 176, 193, 207, adjacent peak subtraction = 2, resolution = low, sensitivity = medium, shape requirement = low, column bleed = 207. Run one batch job each for extra- and intracellular samples.
67. Integrate the deconvoluted peaks with MET-IDEA 2.08 (http://bioinfo.noble.org, 2012). Select the chromatogram with the highest number of deconvoluted components as ion file and the following parameters for peak integration: chromatography = GC, average peak width = 0.08, minimum peak width = 0.5, maximum peak width = 2, peak start/stop slope = 1.5, adjusted retention time accuracy = 0.25, peak overload factor = 0.9, MS = TOF, mass accuracy = 0.1, mass range = 0.3, lower mass limit = 100, ion per component = 1, exclude ion list = 73, 147, 281, 341, 415. In the output file the peak area of each variable as described by model ion [*m*/*z*] and retention time [min] is listed for each sample.
68. Import the peak area file in Excel (MICROSOFT® Office, 2010). Subtract for each variable the median area of all blanks from each sample. Remove variables with a resulting negative peak area.
69. Normalize the data. Divide each peak area by the peak area of the IS of the same sample. For intracellular metabolites also normalize to extracted biomass (e.g., fresh weight). For extracellular metabolites normalize to the sum of all peak areas within one sample.

70. Export the data set as a .txt file for further statistical analysis. Sample and variable descriptors have to be omitted.

71. Perform a CAP with CAP12 (Anderson and Willis 2003, http://www.esapubs.org/archive/ecol/E084/011/suppl-1.htm) using the following parameters: transformation = none, standardization = none, distance measure = Bray-Curtis dissimilarity, discriminant analysis mode, number of principal coordinates axes chosen by the program, 999 random permutations test.

72. Display the resulting sample coordinates as score plots and the metabolites as loading plot, e.g., with SigmaPlot 13.0 (SYSTAT SOFTWARES). Evaluate the principal coordinate analysis (PCO) score plot (**Figure 18.3a**) to detect sample outliers. Evaluate the CAP score plot (**Figure 18.3b**) for correct sample groups. Use the correlation values of the original variables with the CAP axes to generate a vector plot (**Figure 18.3**) and select highly correlated variables. Describe these variables by their *m*/*z* and t_R values (see 4.6.67).

73. Compare the mass spectra of all highly correlated variables as extracted by AMDIS (see 4.6.66) with spectral libraries using NIST MS Search 2.0 (http://chemdata.nist.gov/, 2005). A common commercial database is the NIST library, other non-commercial alternatives, often focused on specific metabolites, e.g., the Golm-library for plant metabolites, are available. Document the quality of spectral comparison with the R.Match value.

74. Compare the retention times of all highly correlated variables with externally measured reference compounds by calculating non-linear retention indices (van den Dool and Kratz 1963). Follow Fiehn et al. (2008) for MSI-compliant identification (MSI = metabolomics standards initiative).

Figure 18.3. Statistical data analysis approaches for untargeted metabolomics data sets. A combination of unsupervised (a) and supervised (b,c) multivariate analyses with univariate analyses (d) is recommended. Selected metabolites of interest can be further described with heat maps (e) or box plots (f). All plots were generated from a data set that was obtained by GC-MS analysis of the macroalga *Ulva mutabilis* with either two (a-c) or three (d-f) treatment groups (n = 4). Score plots of a) PCO and b) CAP show the separation of sample groups. The vector plot of the CAP (c) and the t-test based cloud plot (d, from XCMS Online) indicate characteristic metabolites. These selected metabolites can be visualized semi-quantitatively with a heat map (e, from MetaboAnalyst) or box plots (f, from XCMS Online).

5. Notes

For a successful untargeted metabolomic approach, the personal security and sample protection against contaminants and chemical reactions with H_2O and O_2 are essential. It is thus recommended to wear personal protective equipment and work under fume hoods whenever necessary. Also, read the material safety data sheets (MSDS) of all reagents and follow the recommendations for correct disposal. As biological samples are rather complex, avoid any contamination by humans or lab equipment (e.g., fatty acids, plasticizers). It is thus advisable to wear gloves, use glass or PTFE whenever possible, and rinse lab equipment with solvents before use. For the same reason, blanks should be carried through the whole procedure. As the derivatization is moisture sensitive all involved chemicals and samples have to be dry, which is especially challenging for salt water samples. Samples should be exposed to air as rarely as possible. Several metabolites are labile. Therefore work should be carried out without longer interruptions.

Note 1: For subsequent data normalization it is important to determine the amount of extracted biomass, e.g., as cell count or fresh weight. For a quantitative comparison or differential screening, it is thus best to collect the same biomass among samples wherever possible.

Note 2: The sample volume should provide ~5×10^7 algal cells of a cell volume of ~100 μm^3 as tested for the diatom *Skeletonema marinoi*. With ~12 pg C $cell^{-1}$ (Menden-Deuer and Lessard, 2000) this cell number equals ~0.6 mg C. For other organisms the required cell numbers have to be determined in test runs or indirectly estimated based on the amount of carbon per cell.

Note 3: Alternatively, algal cells can be sampled by centrifugation. Cold solvent quenching prior to centrifugation is necessary to prevent metabolic alterations during centrifugation (Bölling and Fiehn, 2005).

Note 4: At this stage, the samples can be directly used for LC-MS analysis as described in Barofsky et al. (2009) and Alsufyani, Weiss, and Wichard (2017) for extracellular extracts, and in Barofsky et al. (2010) for cell extracts. Therefore, transfer an aliquot of 100 µL into insert-equipped glass vials and measure in one batch in randomized order.

Note 5: All solvents and water residues have to be evaporated before the derivatization. This can be achieved with a desiccator but, e.g., also under a flow of nitrogen. Any sample contamination should, however, be prevented.

Note 6: Pressure reduction results similarly to heating in solvent boiling. In a desiccator, however, a boiling delay can occur. To avoid any loss of extract due to this uncontrolled boiling reduce the pressure stepwise. Select the pressure steps at the boiling points of the different solvents as listed elsewhere.

Note 7: To vent the desiccator with dry air mount a $CaCO_3$ filled column at the air inflow. Alternatively, argon can be used as shielding gas as it is denser than air and inert.

Note 8: The speed (or the frequency) of the grinding has to guaranty cell wall disruption in a short period of time (before thawing) and has to be optimized for the respective tissue in test runs.

Note 9: The batch size is limited to max. 20 samples due to the instability of the silylated samples. If, e.g., standing in the auto sampler of a GC-MS instrument at room temperature decomposition would be substantial if more than 20 samples in a row would be measured (Kanani et al., 2008). As one GC-MS run lasts about 30 min, 20 samples can be analyzed within ~10 hours. Biological replicates should be randomized within one batch.

Note 10: The oximation depends both on reaction temperature and time. To reduce the decomposition of, e.g., sucrose and increase derivatization efficiency an oximation for 1 hour at 60°C and 16 hours at room temperature were proposed by Gullberg et al. (2004). However, to be able to work up two batches per day the latter time is reduced to 9 hours without significant losses. With the addition of MSTFA, the oximation reaction is quenched due to silylation of the reactive amine group of methoxyamine.

Note 11: MSTFA is a strong trimethylsilyl group (TMS; $Si[CH_3]_3$) donor and thus a common silylation reagent. During the silylation reaction, the active hydrogen of functional groups such as -OH, -NH, $-NH_2$ -SH, and -COOH are replaced by a TMS group. As each functional group has different reaction kinetics a heating time of 1 hour compromises silylation efficiency and required reaction times.

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