UNTARGETED GAS CHROMATOGRAPHY MASS SPECTROMETRY PROTOCOL

(Adapted from Fiehn et al, 2006; Courtesy from Ana Paula Alonso)

1. Introduction

Each cell or tissue features a collection of metabolites (small molecules) which are the end product of cellular processes. Metabolomics is a study of these compounds, providing a unique opportunity to fingerprint physiological activities (Lisec et al. 2006).

2. Methods

2.1. Cold chloroform/methanol/water extraction of intracellular metabolites

2.1.1. Material

- Microcentrifuge pre-chilled at 4 ºC **(*20 minutes before starting experiment*).**
- Vortex at 4 ºC in cold room **(*30 minutes before starting experiment*).**
- Thaw 20 mM ^{13}C-glycine standard solution and put it on ice until use**.**
- 50 mL glass bottle containing a freshly prepared, chilled (-20°C) mixture of chloroform, methanol and water prepared at a ratio of 1:2.5:1 (v/v/v) **(*at least 30 minutes before starting experiment*).**
- 50 mL conical tube containing methanol:water (50:50; v/v).
- Place a 50 mL conical tube containing dd$H_2$0 on ice.
- 5 mm stainless steel beads.
- 2 mL screw capped tubes, 2 mL and 1.5 mL microcentrifuge tubes.
- 3 kDa Amicon filtering devices.
- 2 mL GC-MS vials and 300 µL inserts.
- Liquid nitrogen containers.
- Ice buckets.

2.1.2. Experimental Procedure

1. Add a 5 mm stainless steel bead to each tube containing pre-weighed sugarcane leaves **(*Add the beads gently by inclining the tube at 45° to prevent losing any leaf material. 5 mg of plant tissue is usually enough*).**
2. Place the tubes in the bead beater, and disrupt samples for 3 minutes at a frequency of 30 Hz/s.

3. Transfer tubes on ice and then centrifuge them for 30 seconds at 17,000 *g* at 4 ºC (***Use refrigerated microcentrifuge; this step helps to pull down powder which is trapped in the cap***). Remove tubes from the microcentrifuge and place them immediately on ice.
4. At this point of the experiment, move to the cold room and add 10 µL of 20 mM ^{13}C-glycine standard solution to each biological sample using a P10 pipette (***Open carefully the 2 mL screw capped tube to avoid loss of material***).
5. Add 1 mL of pre-chilled cold chloroform:methanol:water (1:2.5:1; v/v/v) extraction solvent to each sample (***Extraction solvent must be vortexed thoroughly before addition to the tube to avoid phase separation***). Close the tubes.
6. Vortex samples (***on position 6-7***) at 4 ºC for 5 minutes. Place the bottom of the tubes in ice and exit the cold room.
7. Centrifuge the tubes at 17,000 *g* at 4 ºC for 5 minutes (***Wipe tubes before placing them in the refrigerated microcentrifuge, and orientate them in such a way that the white part of the tube is facing you. In the meantime, pre-label 2 mL microcentrifuge tubes and stick them on ice***).
8. **Carefully** remove samples from the microcentrifuge (***Minimize movements of the bead which may disrupt the pellet***).
9. Open the 2 mL screw capped tube containing the sample and carefully collect the supernatant using a P1000 pipettor set to 1000 µL (***Put the tip at liquid level, start to pipet and bring down the tip as liquid goes down. Avoid to create bubbles which will disrupt the pellet***).
10. Transfer supernatant into a new 2-mL tube (***The tube must reside on ice. At this point of the experiment, samples can be stored, deoxygenate with a gentle stream of nitrogen gas for 1 minute. Tubes can be kept in the dark at -20 ºC for about four weeks***).
11. Add 400 µL of <u>cold</u> ddH_2O to each sample and vortex for 10 seconds.
12. Centrifuge samples at 17,000 *g* at 4 ºC for 2 minutes (***you should see separated phases after centrifugation***). Meanwhile, pre-labeled new 1.5 mL microcentrifuge tubes and poke 2 holes through their caps.
13. **Carefully** remove tubes from the microcentrifuge and put them on ice.
14. Collect the <u>upper phase</u> **(water-methanol mixture, 'polar phase')** of the samples with a P1000 pipettor and transfer it into a 1.5 mL microcentrifuge tube (***Be careful with not taking the interphase containing proteins***). The lower phase (a mixture of chloroform and methanol, 'lipophilic phase') can be used for lipid profiling or discarded.

15. Evaporate methanol from 1.5 mL microcentrifuge tube at 35-40 ºC using a speed-vacuum **(*500-600 µL of water should remain after evaporation*).**
16. Remove samples from speed-vacuum and flash-freeze 1.5 mL microcentrifuge tube in liquid nitrogen (In order to prevent the tubes from cracking, ***hold the tubes in liquid nitrogen using forceps while they are open, and then close them after 15-20 seconds***) .
17. Place the frozen tubes in a flask and lyophilize at -83 ºC for 24 hours (***This step should be accomplished within 2 minutes in order to avoid thawing and loss of samples***).
18. Remove samples from the lyophilizer and check that there is no powder on the cap.
19. Resuspend the intracellular metabolites in 250 µL of methanol:water (50:50; v/v), vortex, and centrifuge at 17,000 *g* at 4 ºC for 30 seconds.
20. Transfer samples to 3 kDa Amicon filtering devices (Protein removal) and pre-rinse (***Removal of glycerol***) as follow:
    a. Add 0.5 mL of ddH_2O to 3 kDa Amicon Ultra 0.5 mL centrifugal devices.
    b. Centrifuge at 14,000 *g* for 25 minutes at room temperature.
    c. Spin the filtering device upside down at 1,000 *g* for 2 minutes to remove any residual solution contained on the filter.
    d. Repeat steps a to c two more time. NEVER LET THE FILTER DRY OUT!!!
21. Spin the tubes at 14,000 *g* at room temperature for 30 minutes (***Pre-label 1.5 mL microcentrifuge tubes and poke 2 holes onto their caps***).
22. The filtered samples are processed as follow:

    . 20 µL of sample are transferred to a 1.5 mL microcentrifuge tube and kept in the fridge for Liquid Chromatography-tandem Mass spectrometry (LC-MS/MS) experiment.

    . Remainder of intracellular metabolites is added to a 300 µL GC-MS insert and methanol is evaporated at 35-40 ºC for 60-90 minutes using a speedvacuum (***Insert is placed in a poked 1.5 mL microcentrifuge tube***).
23. Carefully remove 1.5 mL tubes with inserts from speedvacuum and flash-freeze insert in liquid nitrogen for 15-20 seconds using forceps (***Be gentle when holding insert, and avoid to have liquid nitrogen inside the insert***) and transfer it back to its original 1.5 mL microcentrifuge tube. At this point, close 1.5 mL microcentrifuge tube and immerse it in liquid nitrogen.

24. Place frozen tubes with inserts in a flask and lyophilize them at -83 ºC for 24 hours (***This step should be accomplished within 2 minutes in order to avoid thawing and loss of samples. The jar is put inside a box containing dry ice in order to circumvent any thawing of the samples***).

2.1.3. Important note

Remove the beads from the 2 mL screw cap tubes prior to discarding the tubes. The beads will be cleaned as follow: i) Put the beads in a beaker containing ddH_2O and sonicate for 10 minutes, ii) transfer them to a strainer, iii) rinse them heavily with ddH2O, and iiii) dry them with ethanol.

2.2. Untargeted metabolomics using gas chromatography mass spectrometry

2.2.1. Principle

Derivatization for gas chromatography (GC) analysis is the process by which a compound is chemically changed, generating a new compound that has properties more suitable for a particular analytical method. For instance, polar compounds (-OH, -NH groups), which have poor volatility, can be converted into a relatively non-polar compound using non-polar silyl groups. Therefore, the resulting product is more volatile, and can be analyzed by GC. Moreover, the derivatization of a metabolite improves its separation as well as its quantification.

Untargeted metabolomics approaches using GC-MS rely on derivatization with an oximation reagent prior to silylation. The oximation reagent, such as methoxyamine, reacts with carbonyl groups (ketone, carboxylic acid, amide, ester, aldehyde; see step 1 illustrated in Figure 1) to form a methyloxime derivate. This oximation step includes pyridine and HCl as solvent and catalyst, respectively. This oxime derivate is further silylated with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (see step 2 illustrated in Figure 1) which leads to an alkylsilyl derivatives Trimethylchlorosilane (TMCS) reagent is added as a catalyst. The overall derivatization process targets most of the family of metabolites present in the biological sample.

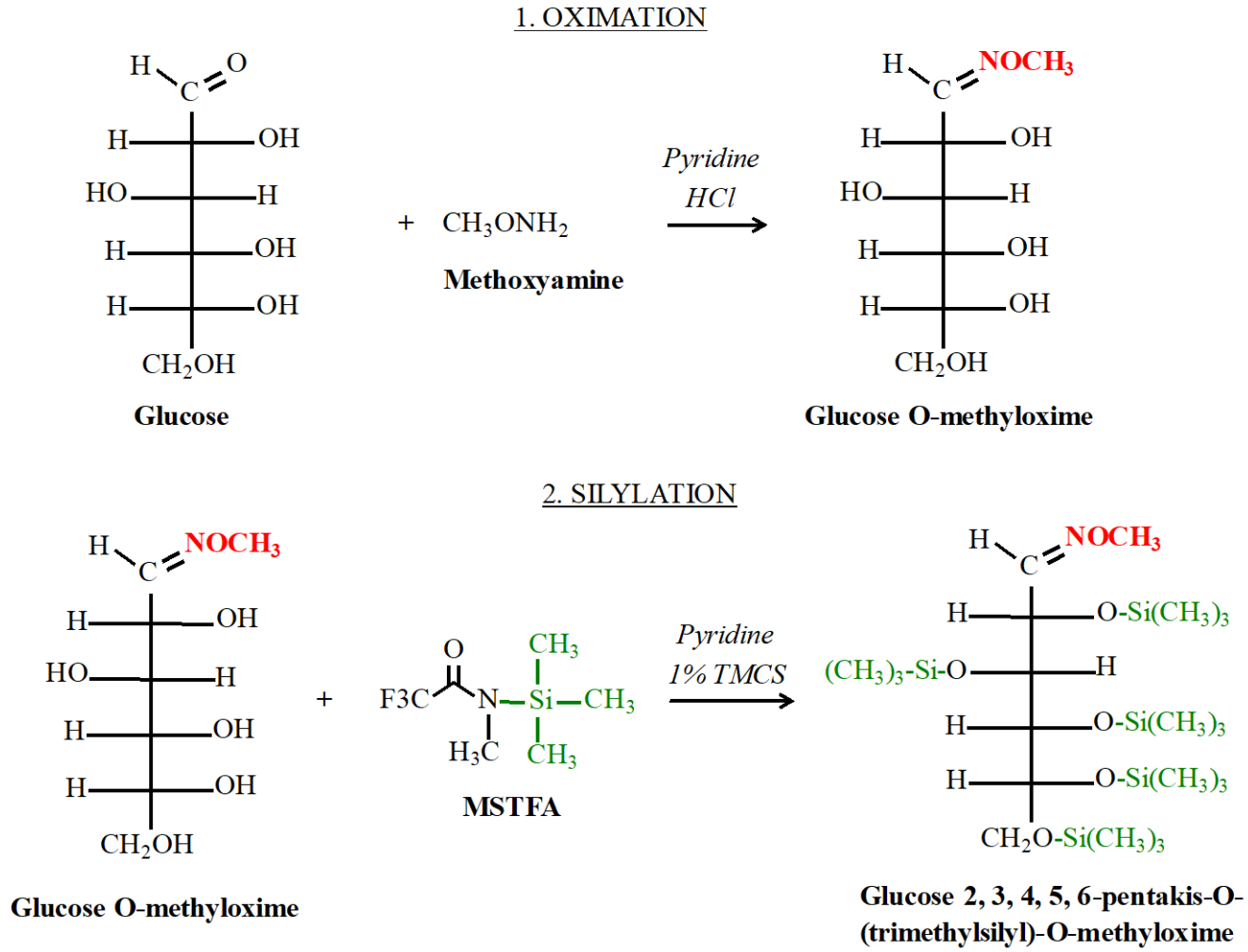

Figure 1. Derivatization steps for GC-MS analysis.

2.2.2. Material

- Set dry bath at 40°C (***20 minutes before starting extraction***).
- Clean nitrogen concentrator needles with 100% ethanol and kimwipes before starting experiment.
- Set Nitrogen pressure delivery between 5-10 psi on the nitrogen concentrator using nitrogen regulator.
- Solution of 20 mg/mL methoxyamine hydrochloride in pyridine (***Warning! This solution is toxic, corrosive and flammable, meaning that it has to be kept under the fume hood at all times! This solution was flushed with nitrogen to minimize oxidation of the solution***).
- Pyridine anhydrous solution of 99.9% (***Warning! This solution is corrosive and flammable meaning that it has to be kept under the fume hood at all times!***).
- Methylene chloride (or dichloromethane; CH_2Cl_2) (***Warning! This solution is toxic flammable, meaning that it***

has to be kept under the fume hood at all times!).

- Two 20 mL beakers: one for methylene chloride and the other for pyridine
- 5 mL syringe as well as needles (***Syringe and needle are used to collect pyridine from the "septum sealed" pyridine stock bottle***).
- Ampoules containing MSTFA + 1% TMCS (***1 mL of solution per ampoule. Warning! This solution is toxic meaning that it has to be kept under the fume hood at all times!***).
- 100 µL Hamilton syringes.
- 500 µL Hamilton syringes.

2.2.3. Experimental procedure

1. Add 200 µL of methylene chloride (CH_2Cl_2) with a 500 µL Hamilton syringe (***previously rinsed twice with methylene chloride***) and flush biological samples under nitrogen at room temperature under the fume hood ***(Vials containing dry samples are placed on holders with non-labeled part facing towards you and a nitrogen flow is supplied by turning the knob (1/8 turn) at the top of the needle. Move the needle closer to the top of the vial and check that liquid is slightly moving and stays at the bottom of the vial. This step removes traces of water that may interfere with the derivatization process).*** Repeat this step two more times. Note: Leave nitrogen flow on and needles open.

2. Add 50 µL of a 20 mg/mL methoxyamine hydrochloride solution in pyridine ***(work under the fume hood)*** with a 100 µL Hamilton syringe to the vials. Flush sample with a stream of nitrogen ***(samples are flushed one at a time for 5-10 seconds using a clean needle placed above the vial, not inside!).*** Cap the vials and vortex them to resuspend your sample ***(The samples are partially resuspended at this time of the experiment. A total resuspension occurs after 90 minutes of incubation; see next step)***. Note that the 100 µL Hamilton syringe has to be cleaned 3 times with methylene chloride after use.

3. Incubate samples at 40°C in a dry bath incubator for 90 minutes. Cover the top of the incubator with aluminum foil. Vortex vials every 20 minutes to help resuspension of samples ***(Forceps are used to remove vials from dry bath incubator).***

4. Remove sample from dry bath and add 50 µL of MSTFA + 1% TMCS derivative reagent ***(kept at 4°C)*** to the vials with a 100 µL Hamilton syringe previously rinsed twice with methylene chloride (***Warning! MSTFA + 1% TBDMCS solution is toxic, meaning that it has to be kept under the fume hood at all times!***). Note that the 100 µL Hamilton syringe has to be cleaned 3 times with water after use.

5. Flush sample with nitrogen for 5-10 seconds ***(samples are flushed one at a time for 5-10 seconds using a clean needle placed above the vial, not inside!)***, cap the vials and vortex them for 10 seconds.

6. Incubate the samples in a dry bath at 40°C for 50 minutes. Do not forget to cover the vials with aluminum foil

7. Derivatized extracts are ready for GC-MS analysis.

2.2.4. GC-MS run

Alkylsilyl derivatives will be analyzed using a Thermo Trace 1310 gas chromatograph coupled to an ISQ single quadrupole mass spectrometer. Alkylsilyl derivatives will be separated using a TG-5MS capillary (30 m x 0.25 mm x 0.50 μm) column from Thermo Scientific at a constant flow rate of 1.4 mL/min. Helium will be used as the carrier gas. The GC conditions will be as follow: initial temperature will be set to 70°C and hold for 5 minutes. The oven temperature will then be raised to 235°C at 3°C/min. A second ramp will be applied at a rate of 6°C/min to reach a final temperature of 320°C which will be held for 5 minutes. The injection temperature will be fixed at 240°C and the injection mode will be set to split with a split ratio of 10. For the MS analysis, the mass spectra will be acquired using electron impact (EI) ionization in positive ion mode. The ion source and the interface temperatures will be respectively set to 300°C and 325°C.

First, GC-MS data will be acquired and processed using Xcalibur 2.2 software. Second, GC-MS chromatograms will be aligned using XCMS Online (Tautenhahn et al. 2012). Third, statistical analysis will be conducted through MetaboAnalyst 3.0 (Chong et al., 2018) to identify “peak/mass” pair(s) or features that are significantly different between experimental conditions. Finally, alkylsilyl derivatives will be identified using the National Institute of Standards and Technology (NIST) 17 library.

3. References

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