

Chapter 2

Plant Tissue Extraction for Metabolomics

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Abstract

Plants are not only important producers of foods and energy storages (e.g., sugars, carbohydrates, proteins, and fats) in the form of grains, fruits, and vegetables, they also provide many valuable products to human existence including wood, fibers, oils, resins, pigments, antioxidants, and sources of medicine. Most importantly in light of this book, plants have been a source of therapeutic and health promoting compounds throughout history. This chapter describes several essential considerations for the extraction process when aiming to study plant metabolism or to characterize the chemical composition of plant originated samples using metabolomics technologies.

Key words Plants, Metabolomics, Tissue extraction, Metabolites, GC-MS, LC-MS

1 Introduction

Plants play an important role as the primary producers of food and the source of metabolic energy of which all other life forms sustain off. Photosynthesis in green plant tissues captures sunlight energy, water, and carbon dioxide to build the primary building blocks including, sugars, carbohydrates, proteins, and fats. Plants provide foods (e.g., grains, fruits, and vegetables) and there are many plant products which are fundamental to the sustainability to all life on earth. These include wood and wood products, vitamins, antioxidants, fibers, drugs, oils, latex, pigments, and resins. In addition, plants have played an important role in medicinal applications throughout generations. Most medicinal drugs (about 80 %) are originated from the plant kingdom. However to date, only 2 % of all species in the plant kingdom have been explored for their therapeutic potential which provides us endless opportunities to discover novel bioactive compounds with wide medicinal or health supporting applications.

1.1 Plant-Specific Features to Be Considered for Metabolomics

The major differences between plants and many other organisms are that they contain chlorophyll, are immobile, and have no sensory organs or nervous system. Most plant cells contain plastids and large vacuoles and most importantly are surrounded by often rigid cell walls. Seed-producing plants are divided into three organs: leaves, stems, and roots. Leaves are the main photosynthesizing organs, therefore critical for the survival of the rest of the plant as well as for providing sufficient energy for reproduction. The roots are usually grown underground and have two main functions. Firstly, to anchor the plant in the soil and secondly to absorb water and essential nutrients (both macro- and micro-nutrients) from the soil to be transported to the upper sections of the plant system. Roots of some plant species (e.g., legumes) develop symbiotic relationships with nitrogen fixing bacteria to enhance their own amounts of nitrogen fixated for protein storage. The main function of the stems is to transport water, soluble carbon sources, nutrients, and hormones between roots, leaves, and the reproductive organs.

Apart from considerations about the complex plant tissue anatomy and physiology, there are specific challenges to be considered when aiming to do conduct a metabolomics or comprehensive chemical characterization of any plant tissue. Plant metabolism is highly light dependent and many metabolite levels are altered during the course of a day depending on photosynthetic or respiratory activities. During light conditions many synthesizing and storage processes are functional, whereas during dark processes storage products may be degraded to provide the plant cells with energy through respiration. This consideration has to be reflected when harvesting samples for a metabolomics experiment which needs to be at very similar time points of a day (*see Note 1*). Another important point to consider is the strength of light which leaves are exposed to, e.g., leaves in bright light and leaves in the shade will have different metabolite profiles (*see Note 2*).

1.2 Extraction of Plant Metabolites

A prerequisite for an efficient extraction process of plant tissues is that the often rigid cell walls are broken to release the metabolites in the extraction solvent. Therefore it is important to investigate an appropriate homogenization process. There are many techniques of homogenizing plant sections and mechanical shear seems to be the most efficient. This can be achieved via mortar and pestle but many laboratories are now using automatic grinders such as ball or cryomills. It may be important to observe under a microscope how well the material was ground and if all macromolecular structures were spatially homogenized to a fine powder. Especially important if working with fresh and frozen plant tissues is to make sure that the homogenization process is carried out under freezing conditions, ideally under liquid nitrogen. Since this is not always achievable, plant material can be freeze-dried prior to homogenization and extraction; however the grinding process needs to be carried out under very dry conditions (*see Note 3*).

The next step is to optimize and validate an appropriate extraction method for the plant tissue as well as for the metabolites of interest. If an untargeted metabolomics approach is utilized, the extraction process should be as crude and extract as many metabolites as possible from the tissues. In our laboratory, we use a two-step procedure using methanol in the first instance and water in the second since this covers both polar and apolar compounds simultaneously. Both supernatants after removing all insoluble components such as protein, starch, cell wall, and other high-molecular weight carbohydrates are then combined to allow analysis of a large coverage of the metabolome (*see* **Note 4**).

When extracting plant metabolites, one has to consider that the plant metabolome is very dynamic and concentrations of metabolites may range over several magnitudes. Some compounds may be found in molar ranges such as sucrose or glucose and others, often the ones of interest (secondary metabolites), are found only at very low abundances. Therefore, if metabolites of interest only exist in low concentrations, specific approaches may need to be employed removing high abundant compounds therefore enriching and concentrating low abundant metabolites. However, care has to be taken when removing certain compound classes with similar physiochemical properties since it may lead to unwanted losses of the metabolite(s) of interest.

This chapter aims to emphasize general points of consideration when aiming to extract plant metabolites (primary and secondary). Methods for crude tissue extractions to capture as many metabolites as possible allowing to analyze a “snap shot” of a large portion of the metabolome are presented within. In light of the topic of this book, these approaches also allow to detect and particularly identify as many metabolites as possible for a discovery strategy for novel natural products with a desired activity. For a detailed description of the challenges and approaches in plant-based metabolomics, the reader is referred to Villas-Boas et al. [1], Beckles and Roessner [2], and Roessner and Beckles [3].

2 Materials

There are a wide range of methodologies published for plant tissue extractions. It is important that for every tissue type, multiple extraction procedures should be optimized and validated before settling on the most appropriate method would then be carried out throughout an experiment. Important parameters for consideration for instance are how easy is it to obtain/harvest the tissue? (e.g., in glass house or on the field or if tissue of interest requires dissection from surrounding tissue types); sample size (is there sufficient material available or are samples sizes minimal?); tissue homogenization (availability of appropriate instrumentation?);

storage of frozen tissue or freeze dry tissue for ease of storage; solvent extraction (availability of clean solvents and incubators?); removal of insoluble material from the metabolite extract (availability of appropriate centrifuges); reproducibility of extraction, the stability of metabolites of interest throughout the extraction procedure; recoveries of metabolites throughout extraction process and how to best store extract prior to analysis are several factors to consider. This chapter describes two extraction procedure methods commonly used in our laboratory for untargeted profiling of plant metabolites using GC-MS (modified from ref. [4]) and LC-MS (modified from ref. [5]). The methods are applicable to all plant tissues for untargeted profiling since they are aimed to obtain a crude extract of the tissue without minimal losses of metabolites. It is important to mention that for any targeted approach for selected classes of metabolites, a specific extraction procedure may need to be employed to enhance enrichment of low abundant secondary metabolites.

2.1 Tissue Harvesting and Quenching of Metabolism

1. Reaction tubes (e.g., 2 mL safe lock Eppendorf tubes) labeled with identifiable names for each sample to be harvested.
2. Liquid nitrogen in an appropriate storage container.

2.2 Storage of Samples

1. -80°C freezer if frozen.
2. Freeze drier.
3. Dark, dry, and cool storage if material is freeze dried.

2.3 Homogenization of Tissue Using Mortar and Pestle or Automatic Grinders

1. Mortar and pestle.
2. Automatic grinders such as ball mills, ultra turrax, or cryomills.

2.4 Weighing of Tissue

1. New labeled reaction tubes (e.g., 2 mL safe lock Eppendorf tubes).
2. Analytical balance (to at least four decimal places).
3. Liquid nitrogen if working with frozen fresh tissue.
4. Spatula.

2.5 Metabolite Extraction for GC-MS Profiling

1. Solvents: 100 % (v/v) methanol and distilled water.
2. Internal standards (e.g., $^{13}\text{C}_6$ -Sorbitol and $^{13}\text{C}^{15}\text{N}$ -Valine).
3. Vortexer.
4. Thermoshaker for reaction tubes (e.g., Eppendorf).
5. Bench top centrifuge for reaction tubes (up to 11, 337×g).
6. Speed vacuum.
7. Silica beads.

2.6 Metabolite Extraction for LC-MS Profiling

1. Solvents: 2:3:3 (v/v/v) mixture of water:acetonitrile:isopropanol.
2. Internal standards (e.g., $^{13}\text{C}_6$ -Sorbitol and $^{13}\text{C}^{15}\text{N}$ -Valine).
3. Vortexer.
4. Sonicator.
5. Bench top centrifuge for reaction tubes (up to 11, 337×g).

3 Methods

3.1 Tissue Harvesting and Quenching of Metabolism

Since metabolic reactions are extremely fast it is crucial to harvest tissue and quench metabolism as quickly as possible. This has to be considered when aiming to determine a “snap shot” of the steady-state level of the metabolome. Equally important is to avoid metabolic changes due to wounding of the tissue. Ideally, samples are frozen in liquid nitrogen as quickly as possible which is relatively easy when working with whole leaves or other easily accessible plant tissue types. However, if a particular tissue of interest requires to be dissected from surrounding tissues prior to freezing, it is important to keep the time frame for this process as similar as possible for all samples harvested. Below the protocols are described when working with easily accessible tissues such as leaves or roots.

1. Prepare liquid nitrogen in appropriate storage container.
2. Harvest leaves or roots, put in reaction tube.
3. Freeze in liquid nitrogen (*see Note 5*).

3.2 Storage of Samples

1. Place reaction tubes with frozen tissue in $-80\text{ }^{\circ}\text{C}$ freezer.
2. If no freezer available, dry tissue in freeze drier under freezing conditions.
3. Store freeze dried samples in a dark, dry, and cool environment (*see Note 6*).

3.3 Homogenization of Tissue

1. If working with frozen tissue, homogenize tissue using mortar and pestle under freezing conditions (*see Note 5*), if tissue is freeze dried, there is no need for freezing conditions as long the process is kept under dry conditions.
2. If automatic grinder devices are available such as ball or cryo mills, use as instructed by the manufacturer.

3.4 Weighing of Tissue

1. If working with frozen tissue, prepare liquid nitrogen in appropriate storage containers.
2. Prepare new labeled reaction tubes.
3. Pre-cool reaction tubes in liquid nitrogen.
4. Place tubes on balance and set balance to zero (tare).

5. Place tissue powder in cooled tubes using a pre-cooled spatula or spoon (*see Note 5*).
6. Read weigh and record (*see Note 7*).
7. Place tube with weighed tissue back into liquid nitrogen.
8. Proceed with extraction or store weighed tissue at -80°C .
9. If working with freeze dried tissue the need of pre-cooling and keeping tissue frozen is not required.

3.5 Sample Preparation for GC-MS

1. Weigh approximately 30 mg homogenized tissue powder in a 2 mL Eppendorf tube (round bottom-shaped).
2. Add 500 μL 100 % (v/v) methanol, vortex (Enzymatic activity stops here).
3. Add 20 μL stock $^{13}\text{C}_6$ -Sorbitol and $^{13}\text{C}^{15}\text{N}$ -Valine (1 mg/mL stock solution) as an internal quantitative standard for the polar phase (*see Note 8*).
4. Vortex.
5. Shake 15 min at 70°C in a Thermoshaker.
6. Centrifuge for 15 min at 11, 337 $\times g$.
7. Transfer supernatant to a new reaction tube.
8. Add 500 μL H_2O to the pellet.
9. Vortex.
10. Centrifuge for 15 min at 11, 337 $\times g$.
11. Combine both supernatants.
12. Vortex.
13. Take aliquots for derivatization into an insert for the instrument autosampler vial (*see Notes 9 and 10*).
14. Dry aliquots under *vacuo* without heating by placing the insert with the extract into a reaction tube. Once dry, it can be placed into the autosampler vial prior to analysis. If these need to be stored then store in bag with Silica gel at room temperature (*see Note 11*).

3.6 Sample Preparation for LC-MS

1. Weigh approximately 50 mg of ground tissue into 2 mL reaction tubes (e.g., Eppendorf).
2. Add 500 μL of a 2:3:3 (v/v/v) mixture of water:acetonitrile:isopropanol.
3. Sonicate for 15 min at room temperature.
4. Centrifuge at 11, 337 $\times g$.
5. Transfer supernatant to a new reaction tube.
6. Re-extract pellet with the addition of another 500 μL of a 2:3:3 v/v/v mixture of water:acetonitrile:isopropanol.

7. Sonicate for 15 min at room temperature.
8. Centrifuge at 11, 337 $\times g$.
9. Combine both supernatants.
10. Transfer to autosampler vial for analysis. If these need to be stored, freeze extract at $-20\text{ }^{\circ}\text{C}$ (*see Note 12*).

4 Notes

1. To avoid any position or time of harvest effects on the variability of the data set always randomize samples when harvesting.
2. Always select a leaf at the same position (e.g., always the upper or a lower leaf), if not use randomized positions of leaves to be harvest.
3. If working with freeze-dried material avoid any water and moisture. As soon as tissue is in contact with moisture enzymes will become active.
4. For many separation and detection techniques the pigments contained in plant tissues, such as chlorophyll and carotenoids, disturb the analysis and may be removed from the extract.
5. It is important to ensure that tissue is *always frozen* throughout any processes such as weighing or transfer between reaction tubes. At warmer temperatures, enzymatic activity can occur and cause spurious changes in metabolites that are unrelated to the treatment. If larger tissue pieces are to be harvested (e.g., fruits), cut into smaller pieces prior to shock freezing so the freezing process is continuous and quick throughout the tissue and any enzymatic reactions are avoided in the middle of the tissue piece.
6. Some metabolites are sensitive and may alter their structure under light exposure. Any water and moisture, as mentioned in **Note 3**, will activate enzymes.
7. In order to relate metabolite levels between samples, the weight of each sample has to be exactly recorded. These weights (either frozen or freeze-dried samples) are then used for subsequent normalization of metabolite levels per gram of fresh or dry weight.
8. Internal standards are ideally compounds not present in the biological extract and are included prior to or during metabolite extraction. They encounter for any analytical error during extraction, derivatization, separation, and detection processes. For instance, stable isotope-labeled internal standards having identical chemical properties as the metabolites under analysis are ideal.

9. The volume for each aliquot of extract depends on the tissue type, the amount of tissue extracted and volume of extraction solvents used. This needs to be optimized for every tissue type under analysis to have best separation and detection within the linear range of the instrument of choice.
10. It is suggested to always prepare more than one aliquot of each sample for backup. Once the samples have been derivatized for GC-MS, they cannot be stored. Therefore, if there is equipment failure during a run; the experiment can be saved if identical samples are available.
11. If long-term storage of dried extracts is required, they need to be kept under argon to avoid oxidation and degradation of metabolites. Dried extracts need to be kept in the dark and under dry conditions for the same reasons as mentioned in **Note 6**.
12. Liquid extracts for LC-MS analysis are ideally analyzed straight away; however if necessary can be stored at -20°C or lower. However this should be avoided since there may be metabolite alterations during the thawing process.

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