Video Article

A Simple Fractionated Extraction Method for the Comprehensive Analysis of Metabolites, Lipids, and Proteins from a Single Sample

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Abstract

Understanding of complex biological systems requires the measurement, analysis and integration of multiple compound classes of the living cell, usually determined by transcriptomic, proteomic, metabolomics and lipidomic measurements. In this protocol, we introduce a simple method for the reproducible extraction of metabolites, lipids and proteins from biological tissues using a single aliquot per sample. The extraction method is based on a methyl *tert*-butyl ether: methanol: water system for liquid: liquid partitioning of hydrophobic and polar metabolites into two immiscible phases along with the precipitation of proteins and other macromolecules as a solid pellet. This method, therefore, provides three different fractions of specific molecular composition, which are fully compatible with common high throughput 'omics' technologies such as liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometers. Even though the method was initially developed for the analysis of different plant tissue samples, it has proved to be fully compatible for the extraction and analysis of biological samples from systems as diverse as algae, insects, and mammalian tissues and cell cultures.

Video Link

The video component of this article can be found at https://www.jove.com/video/55802/

Introduction

Systems biology, which emerged in the middle of the last century[1] and was advanced by the large scale analysis of genomic and transcriptomic data sets[2,3], has developed into a new and indispensable approach for the analysis of complex biological systems[4,5]. The main goal of systems biology is to decipher the component interactions and dependencies in biological systems and to bridge the link between genotypes, their realization, molecular transformations and resulting phenotypes. Accordingly, the integration of comprehensive data sets, produced by the various large scale analytical approaches, namely genomics, transcriptomics, metabolomics, lipidomics and proteomics and their computational analysis has become a prerequisite for the description and understanding of complex biological systems.

Based on the huge chemical diversity and complexity of biological components in any living system, the production of large and comprehensive 'omics' data sets strongly relies on the quality of the applied extraction method[9]. In addition to the quality of the extraction method, the economy of the method is important; this means that it would be desirable to obtain as much molecular information from as little sample input as possible. Often sample amounts can be limiting and therefore it is highly desirable to make use of an extraction method, which can derive as many molecular classes from a single extraction of a given sample. This means that instead of using several specialized extraction methods for the extraction of different compound classes from different sample aliquots of the same sample, a sequential extraction method is employed, which fractionates the molecular constituents of a single aliquot into different molecular fractions.

The common method employed for these fractionated extraction methods is based on the two phase lipid extraction method from Folch *et al.* developed in 1957[10]. This method is based on a chloroform: methanol/water partitioning of polar and hydrophobic metabolites and was intended to clean-up and de-complex samples for high quality lipid analysis. With the evolution of multi-omics systems biology, the Folch method was further, stepwise, improved by utilizing it for the sample partitioning of proteins and polar metabolites and lipids for gas- and liquid chromatography-based metabolomics and lipidomics of polar and hydrophobic compounds, in addition to liquid chromatography-based proteomics[11,12,13,14]. Unfortunately, all of these methods rely on a chloroform-based extraction method, which not only leads to the undesired formation of the protein pellet as an interphase between the polar and the lipid phase, but which is also an undesirable solvent from a green chemistry perspective[15,16]. However, the solvent methyl *tert*-butyl ether (MTBE) overcomes both of these aforementioned problems and is a suitable replacement for chloroform. Based on these requirements, we decided to establish a MTBE: methanol : water-based extraction method, which fulfils all of the aforementioned specifications and therefore functions as an ideal starting point for comprehensive multi-omics analysis[16].

This protocol guides the user step-by-step through the simple, fast and reproducible workflow of the sample preparation, including troubleshooting of commonly observed problems. Further, we will briefly introduce exemplary analytical data from ultra-performance liquid

chromatography-mass spectrometry (UPLC-MS)-based lipidomics, metabolomics and proteomics profiling from plant tissue samples. Even though the given examples are derived from 50 mg of an *Arabidopsis thaliana* leaf tissue sample, this protocol has been used for several other biological samples and tissues, including algae[17,18], insects[19] and mammalian cells, organs and tissue[20,21,22]. The scope of the presented extraction protocol is to provide a clear and detailed description of the pre-extraction sample handling and the extraction procedure itself. Even though we provide three brief examples of analytical application, detailed information about the pre- and post-analytical data handling can be obtained from our previous publications [16,23,24,25,26].

Protocol

Caution**:** Methanol (MeOH) and methyl *tert*-butyl ether (MTBE), used during extraction, are flammable, and can have, on prolonged exposure and/or contact, respiratory, eye or skin irritation. Please handle them carefully only in a fume hood and use the appropriate safety procedures during the extraction (lab coat, safety glasses, gloves, *etc.*). Liquid nitrogen and dry ice, used in several steps of this protocol, can cause severe burns by prolonged skin contact. Please handle them carefully by wearing protective gloves and glasses. Users can use different chemicals, reagents or internal standards for sample analysis, some of which may be toxic. Please examine the relevant chemical safety data sheets for all material used.

1. Collection and Harvesting of Biological Samples

1. Prepare labelled harvesting tubes.
   NOTE: Here, harvest biological samples in labelled, 2 mL, round-bottom, safe lock microcentrifuge tubes containing two 5 mm diameter, metal balls for the tissue homogenizer.
2. Prepare a filled liquid nitrogen dewar.
3. Harvest the biological sample and snap freeze the tissue in liquid nitrogen. Perform this step as quickly as possible within few seconds to avoid metabolic changes induced by wounding.
   Note: For demonstration purposes, use rosette leaves from 30 day-old wild-type *Arabidopsis thaliana* (Col-0) grown on soil under long day conditions.
4. Keep the harvested samples on dry ice for short-term breaks or store them at -80 °C for longer periods.

2. Grinding and Tissue Disruption

1. Pre-cool the tube holders of the tissue homogenizer in liquid nitrogen for at least 10 min. If a tissue homogenizer is not available, use clean and pre-cooled mortars and pestles.
2. Take the samples from the liquid nitrogen, dry ice or -80 °C freezer and place them in the pre-cooled tube holders.
3. Quickly put the tube holders in the tissue homogenizer.
4. Grind the biological material into a fine and homogeneous powder. Use 20 Hz for 1 min for leaves.
   Note; Homogenization time and speed may be varied depending on the tissue, make sure that the sample is homogenized into a fine powder and that this powder is kept frozen at every step of the homogenization.
5. Take the biological samples from the tube holders and keep them frozen until further extraction.

3. Weighing of Tissues

1. Use an analytical balance with sufficient precision for the required sample amounts.
2. Prepare a labelled 2 mL round bottom safe-lock microcentrifuge tube.
3. Pre-cool the tubes and spatulas in liquid nitrogen.
4. Aliquot the required amount of tissue powder into the 2 mL safe-lock microcentrifuge tube.
   Caution: Avoid any defrosting of the plant material by minimizing the time taken to weigh the samples.
5. Return the aliquoted samples immediately after weighing to liquid nitrogen.
6. Record for each sample the exact weight. Use 10-50 mg ± 10% for most plant tissues.
7. Store the aliquoted samples at -80 °C until further extraction.

4. Reagent Setup

1. **Use an extraction mixture of methyl *tert*-butyl ether (MTBE)/ methanol (MeOH).**
   1. For the preparation of 100 mL extraction solvent, add 75 mL of MTBE to 25 mL of MeOH to make a mixture of MTBE: MeOH (3:1, vol/vol).
   2. Add internal standards for the post analysis normalization according to analytical needs. Typically, add 50 µL of 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (1 mg/mL in chloroform) as an internal standard for the UPLC-MS-based lipid analysis, while adding 50 µL of ^{13}C sorbitol (1 mg/mL in water) as internal standards for the GC-MS-based analysis of primary metabolites. Internal standards for UPLC-MS-based metabolite analysis are 50 µL of corticosterone (1 mg/mL in methanol) and 25 µL of ampicillin (1 mg/mL in methanol).
   3. Transfer the solvent to a clean glass bottle that was rinsed with MTBE: MeOH mixture.
   4. Store the extraction mixture for up to 1 week at 4 °C.
      NOTE: Do not store the extraction mixture for longer periods to maintain reproducible results.

2. **To induce phase separation, use water (H_2O)/ methanol (MeOH).**
   1. For the preparation of 100-mL H_2O: MeOH, add 75 mL of H_2O to 25 mL of MeOH to make H_2O: MeOH (3:1, vol/vol).

2. Transfer the solvent to a clean glass bottle that was rinsed with H_2O: MeOH mixture. This solvent can be stored for several weeks at room temperature.

5. Extraction of Samples

1. Pre-cool the extraction mixture (MTBE: MeOH, 3:1, vol/vol) to -20 °C using a liquid cooling system or a -20 °C freezer.
2. Take out the aliquoted samples one by one and add 1 mL of the pre-cooled extraction mixture to each sample tube. Caution: Perform this step quickly due to low viscosity of MTBE.
3. Mix immediately on a vortex mixer until the tissue is well homogenized within the extraction mixture.
   NOTE: This step is very important, since it is required to precipitate the proteins and inactivates their enzymatic activities.
4. Incubate all the samples on an orbital shaker at 100 rpm for 45 min at 4°C.
5. Sonicate the samples for 15 min in an ice-cooled sonication bath.

6. Fractionation by Phase Separation

1. Add 650 µL of H_2O: MeOH (3:1, vol/vol) to each sample tube.
2. Mix well by vortexing for 1 min.
3. Centrifuge the samples at a speed of 20,000 x *g* for 5 min at 4 °C.
   NOTE: After this step, there are two immiscible liquid phases with a solid pellet in the bottom of the tube.
   Caution: Handle the tubes with care to avoid mixing of the two liquid phases and avoid disrupting the precipitated pellet.

7. Aliquoting of Polar and Hydrophobic Fractions

1. Transfer 500 µL of the solvent from the upper, lipid-containing, phase into a labelled 1.5 mL microcentrifuge tube.
   NOTE: The aliquoted lipid samples can be directly concentrated for immediate UPLC-MS analysis (step 8.1) or stored for several weeks at -80 °C.
2. Once the 500 µL are removed from the sample, remove the remaining lipid phase using a 200 µL pipette.
3. Transfer 400 µL of the solvent from the lower phase (polar and semi-polar metabolites) into a labelled 1.5 mL microcentrifuge tube. The aliquoted polar samples can be directly concentrated for immediate UPLC-MS analysis (step 8.2) or stored for several weeks at -80 °C.
4. Take an additional aliquot of 200 µL to perform additional analysis, e.g. gas chromatography-based metabolite analysis as described preciously[16].
5. Remove the remainder of the aqueous phase by pipetting off the excess volume.
6. Wash the obtained protein, starch, cell wall pellet with 500 µL methanol by thoroughly vortexing it for 1 min.
7. Centrifuge the samples at a speed of 10,000 x *g* for 5 min at 4°C.
8. Perform protein extraction and digestion (step 11) or starch/cell wall analysis as described previously[16].
   NOTE: If not utilized immediately, these pellets can be stored for several weeks at -80 °C.

8. Concentration and Storage of Fractions

1. Evaporate the solvent from lipid samples (from step 7.1) in either a vacuum concentrator without heating (for 1-2 hours) or preferably use a nitrogen flow evaporator to avoid oxidative modifications of the lipids.
   NOTE: Dried samples should be analyzed immediately. For storage, leave samples in MTBE solution, ideally in glass vials (step 7.1).
2. Evaporate the solvent from the aqueous samples (from step 7.3 or 7.4) overnight in a vacuum concentrator without heating. NOTE: the dried samples can be stored for several weeks at -80 °C before analysis.

9. Analysis of Lipids using UPLC-MS[24]

1. Re-suspend the dried lipid fractions (from step 8.1) in 400 µL of acetonitrile: 2-propanol (7:3, vol/vol).
2. Transfer sufficient liquid to glass vials and cap tightly.
3. Put the glass vials in a cooled autosampler (4 °C).
4. Inject 2 µL per sample and separate the lipids on a Reversed Phase (RP) C_8 column held at 60 °C using a UPLC system running at a flow rate of 400 µL/min.
5. Use the mobile phases described in **Table 1** for the chromatographic separation.
6. Acquire the mass spectra in positive and negative ionization mode using a suitable MS instrument covering the mass range between 150 and 1,500 m/z.

10. Analysis of Polar and Semi-polar Metabolites using UPLC-MS[25].

1. Re-suspend the polar phase (from step 8.2) in 200 µL UPLC-grade methanol: water (1:1, vol/vol).
2. Transfer sufficient liquid to glass vials and cap tightly.
3. Put the glass vials in a cooled autosampler (4 °C).
4. Inject 2 µL from each sample and separate the metabolites on a RP C_{18} column held at 40 °C using a UPLC system running at a flow rate of 400 µL/min.
5. Use the mobile phases for chromatographic separation with the parameters given in **Table 2**.

6. Acquire full scan mass spectra in positive and negative ionization mode using a suitable mass spectrometer covering a mass range between 50 and 1,500 m/z.

11. Protein Extraction, Digestion and Analysis[16]

1. Re-suspend the washed protein/starch/cell wall pellet (from step 7.8) in 200 µL of the protein extraction buffer of choice. Note: We use urea/thiourea buffer (5 M urea, 2 mM thiourea, 15 mM DTT, 2% CHAPS and protease and phosphatase inhibitors)[27].
2. Sonicate the samples for 10 min in an ice-cooled sonic bath.
3. Incubate samples for 30 min on an orbital shaker (100 rpm) at room temperature.
4. Centrifuge the dissolved proteins at 10,000 x *g* for 5 min.
5. Collect the protein supernatant in a new tube.
6. Determine the protein concentration from the collected supernatant[28].
7. Digest 50 µg of protein in-solution with a protocol of choice. Typically, use the Trypsin/Lys-C mix according to the instruction manual.
8. After digestion, perform desalting of the peptides prior to mass spectrometry using C_{18} stage tips and elute the digested peptides [29].
9. Concentrate the samples to near dryness in a vacuum concentrator without heating.
10. Re-suspend the samples in appropriate loading buffer (e.g. 5% acetonitrile, 0.5% formic acid) and analyze the peptide mixtures by LC-MS/MS using a high-resolution mass spectrometer connected to a nano LC system.
    NOTE: In the exemplary proteomics data set presented in this protocol, we used a gradient as described in **Table 3**.
11. Set the mass spectrometer, using a top 15 strategy, where one full scan (FS) was followed by up to 15 data dependent MS/MS scans, to the following parameters: The FS was in the mass range 200 - 2,000 m/z at a resolution of 70,000 with a target value of $3x10^6$ ions. Obtain the data-dependent MS/MS scans by higher-energy collisional dissociation (HCD). Set the target values for the MS/MS to $1e^5$ ions, with a maximal ion fill time of 50 ms, an isolation window of 4.0 m/z, normalized collision energy (NCE) of 30% and an underfill ratio of 1%. Measure the MS/MS ions at a resolution 17.500 and the dynamic exclusion was set to 60 s.

Representative Results

Comprehensive multi-omics data sets are invaluable for understanding of complex biological systems. The strategy for a successful biological experiment usually starts from a meaningful experimental design, experiment set-up and performance, followed by sample-collection, extraction, analytical data acquisition, raw data processing, statistical data analysis, identification of relevant metabolite and biological data interpretation including pathway mapping and visualization (**Figure 1**).

In the extraction protocol introduced here, we focus on the sample-collection, -handling and -extraction steps, which are depicted in the detailed workflow overview in **Figure 2**. For demonstration purposes, 50 mg of Arabidopsis leaf tissue was selected. This material was harvested, ground and extracted before subjecting it to three exemplary analytical UPLC-MS platforms, providing data that can be utilized for targeted and untargeted lipidomic, metabolomic and proteomic analysis. **Figures 2** and **6** additionally include representative pictures of how, under standard conditions, the extraction solvent should look like. Additionally, examples of samples containing excessive amounts of precipitated macromolecules (proteins and starch) and samples with inappropriate sample homogenization are shown (**Figure 3**). The troubleshooting for these two common problems is given in brief in **Figure 3** but it is also discussed in more detail in our previous publication[16].

Figures 4 and **5** outline examples of three analytical chromatograms derived from the lipid, the polar/semi-polar metabolites and protein analyses. Lipids, which were taken from the upper MTBE phase (**Figure 2**), were analyzed by reversed phase (RP) C_8 ultra performance liquid chromatography coupled to high-resolution mass spectrometry. Lipids can be acquired using positive and negative MS ionization modes (**Figure 4**, upper pane)[16,24].

Polar and semi-polar primary and secondary metabolites were analyzed from the polar (water/methanol) phase (**Figure 2**) by reversed phase (RP) C_{18}UPLC-MS[25]. The illustrated method, using the reversed phase chromatography, is highly compatible to the analysis of semi-polar metabolites (namely metabolites from plant secondary metabolism), which can be analyzed using positive and negative ionization modes in the MS (**Figure 4**, lower pane)[16]. More hydrophilic metabolites from this fraction (sugars, polar amino acids etc.), which do not show good retention on the reversed phase material, can be analyzed by other analytical methods such as GC-MS[16]or hydrophilic interaction liquid chromatography[30].

The proteins, which were retrieved from the solid pellet in the bottom of the extraction tube (**Figure 2**), were in-solution digested and analyzed using shot gun LC-MS (**Figure 5**), while the protocol for extraction of starch and cell wall material can be obtained from our previously published protocol[16].

In summary, more than 200 lipid species, 50 annotated semi-polar metabolites and several thousand proteins can be routinely identified from samples of the type used in our example. Additionally, the method showed wide applicability using different tissues, organs and cell culture material (**Figure 6**).

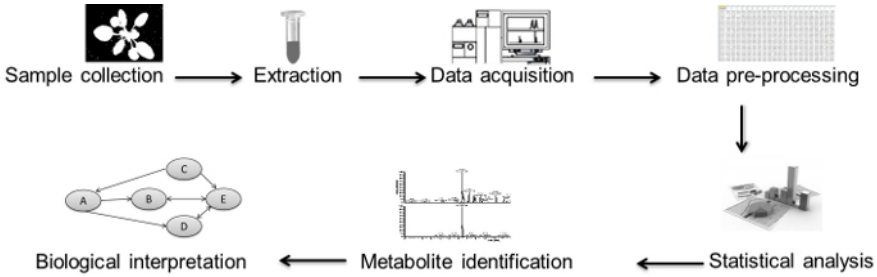

Figure 1: General Workflow for Large Scale Untargeted Omics Analysis. Please click here to view a larger version of this figure.

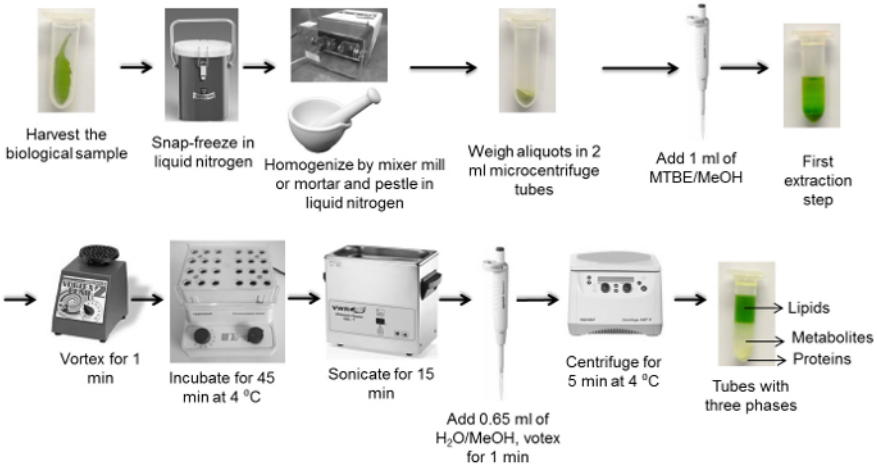

Figure 2: Sample Preparation and Extraction Workflow for the Analysis of Lipids, Metabolites and Proteins from a Single Aliquot of a Biological Sample. Please click here to view a larger version of this figure.

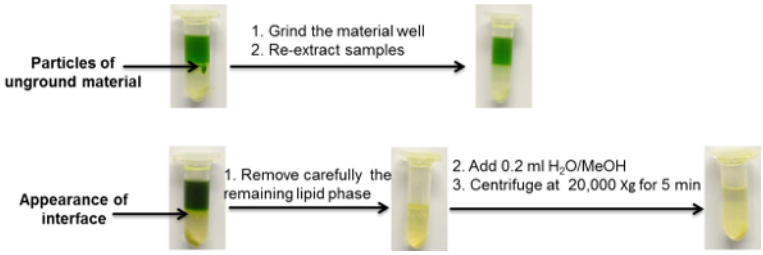

Figure 3: Illustrative Examples of Commonly Observed Problems using Two-phase Partitioning Extraction Protocols. Please click here to view a larger version of this figure.

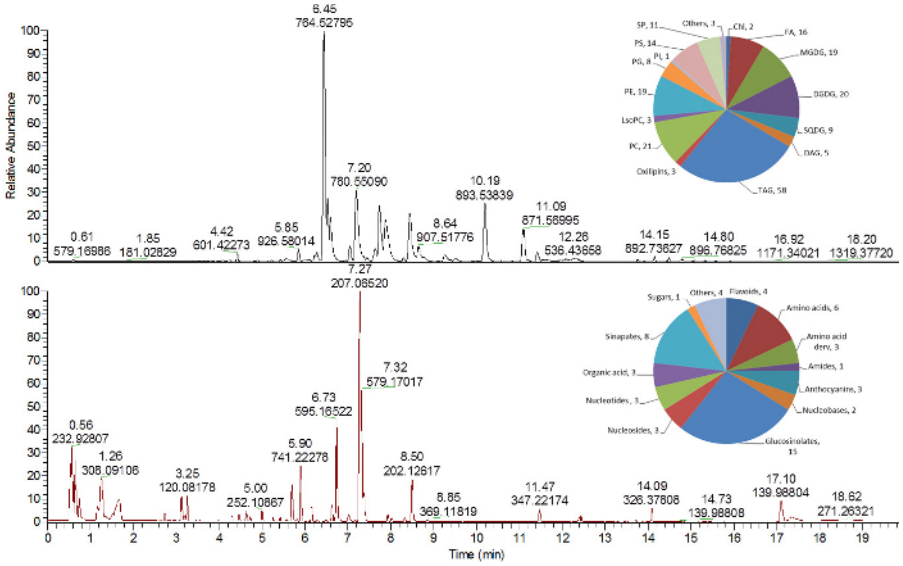

Figure 4: Representative Chromatograms of Lipid and Semi-polar Metabolites from *Arabidopsis thaliana* Leaf Extracts. Base peak chromatograms of lipids (upper pane) and semi-polar metabolites (lower pane) analyzed in positive ionization mode[16]. The pie charts in the upper right corner of each chromatogram shows the number of identified lipids and metabolites assigned to different chemical classes. Chl, chlorophylls; DAG, diacylglyceride; DGDG, digalactosyldiacylglycerol; FA, fatty acid; LysoPC, lysophosphatidylcholine; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingolipid; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglyceride. Please click here to view a larger version of this figure.

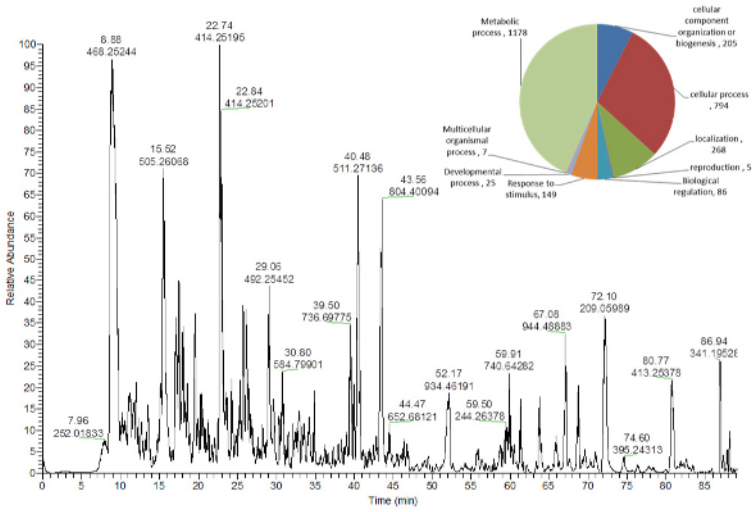

Figure 5: Representative Base Peak Chromatogram of Peptides from *Arabidopsis thaliana* Leaf Extracts.
The pie chart shows in the upper right corner indicates the number of identified proteins assigned to different biological processes[16]. Please click here to view a larger version of this figure.

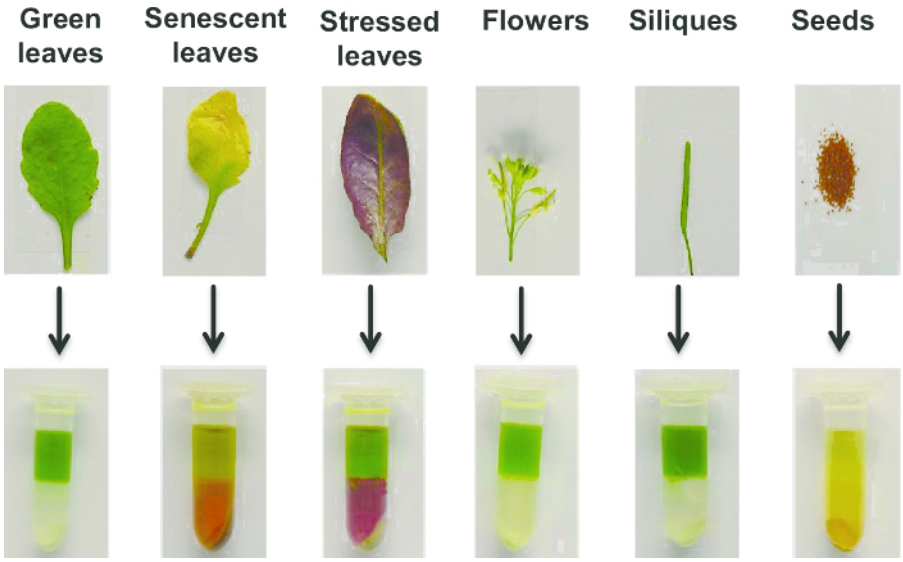

Figure 6: Representative Extraction Examples of Various Tissue Types from Wild-type *Arabidopsis thaliana*.
All the samples were extracted using 50 mg fresh weight from the indicated tissues. Please click here to view a larger version of this figure.

Time (min)	% Buffer A to Buffer B	
0 to 1 min	45% A	Buffer A: 1% 1M NH4-Acetate, 0.1% acetic acid in UPLC MS grade water
1 to 4 min	Linear gradient from 45% A to 25% A	Buffer B: 1% 1M NH4-Acetate, 0.1% acetic acid in acetonitrile/isopropanol 7:3, (v:v)
4 to 12 min	Linear gradient from 25% A to 11% A	Flow rate 400 µL/min
12 to 15 min	Linear gradient from 11% A to 0% A	Injection volume 2 µL
15 to 19.5 min	Wash the column for 4.5 min with 0% A	
19.50 to 19.51 min	Set back to 45% A	
19.51 to 24 min	Equilibrate with 45% A	

Table 1: Gradient Parameters for RP-UPLC Separation of Lipids. RP, reversed phase. UPLC, ultra-performance liquid chromatography.

Time (min)	% Buffer A to Buffer B	
0 to 1 min	99% A	Buffer A: 0.1% formic acid in UPLC grade water
1 to 11 min	Linear gradient from 99% A to 60% A	Buffer B: 0.1% formic acid in UPLC grad acetonitrile
11 to 13 min	Linear gradient from 60% A to 30% A	Flow rate 400 µL/min
13 to 15 min	Linear gradient from 30% A to 1% A	Injection volume 2 µL
15 to 16 min	Wash the column for 1 min with 1% A	
16 to 17 min	Linear gradient from1% A to 99% A	
17 to 20 min	Equilibrate for 3 min at 99% A	

Table 2: Gradient Parameters for RP-UPLC Separation of Polar and Semi-polar Metabolites. RP, reversed phase. UPLC, ultra-performance liquid chromatography.

Time (min)	% Buffer B to Buffer A	
0 to 5 min	Linear gradient from 0 to10%	Buffer A: 0.1% formic acid in UPLC grade water
5 to 80 min	Linear gradient from 10% to 40%	Buffer B: 0.1% formic acid in 60% UPLC grade acetonitrile
80 to 85 min	Linear gradient from 40% to 60%	Flow rate 300 nL/min
85 to 86 min	Linear gradient from 60% to 95%	Injection volume 5 µL
86 to 91 min	Wash column for 5 min with 95%	
91 to 92 min	Linear gradient from 95% to 0%	
93 to 110 min	Equilibrate the column for 17 min at 0%	

Table 3: Gradient Parameters for Nano-LC Separation of Peptides. LC, liquid chromatography.

Discussion

In this article, we describe and illustrate a simple and highly applicable extraction protocol for comprehensive lipidomic, metabolomic and proteomic analysis from a single 50 mg leaf sample. The method has been previously used in several studies, which have been published in diverse articles [17,18,19,20,21,22,23,24,25,26,31,32,33,34,35,36,37] and proved, in addition to its straight-forward workflow and high applicability to be robust and reproducible.

The here provided applications show some routine methods for the initial screening of a complex biological sample. These illustrated large scale metabolomic and lipidomic data sets can provide comprehensive information about the broad or specific changes in the metabolism of the analyzed biological system, while the data obtained from the analysis of proteins, provides insights into the quantitative (abundance) and qualitative (modifications) changes of enzymes, structural proteins or transcription factors (TFs) controlling cellular functions and machinery. Accordingly, integrative omics data has the potential to reveal initial information about possible changes induced by genetic or biotic and/or abiotic perturbations of a biological system, by elucidating molecular changes of diverse molecules associated to specific metabolic pathways or cellular processes.

Of course, in the long term, it is quite essential, for a successful systems biology analysis, to maximize the number of analyzed and annotated molecular entities, allowing the monitoring of cellular functions and activities as completely as possible. For this purpose, the obtained fractions could be applied additionally to diverse analytical methods, targeting further compounds or compound classes (**Figure 4**).

Having said this, it has to be mentioned that the global analysis strategy of the obtained data can be following by two different strategies: on the one hand, we have been emphasizing the elucidation of cellular functions by quantification of known compounds. On the other hand, many of the measured metabolites and lipids are not yet known or annotated. These, yet, un-annotated compound measurements also contain plenty of meaningful information, which, can be utilized by statistical methods for the classification or discrimination between groups or treatments [20,21,22].

Still, these unknown compounds, especially the ones relevant for group classification or serving as biomarkers need to be identified. This identification process is unfortunately quite tedious and cannot be achieved without additional analytical measurements or strategies[38]. As can be seen from **Figure 4**, the number of un-annotated compounds is quite high (actually the vast majority). Nevertheless, as mentioned above, these chromatographic peaks can be handled within the data analysis and therefore significantly affected entities can be elucidated and subjected to further identification strategies.

In summary, we can conclude that the here introduced protocol provides several advantages for experimental systems biology as well as for classical statistical applications.

First, since all fractions are extracted from a single sample, the variation between the different experimental data sets (lipids, metabolites, proteins) is significantly reduced since every data set is derived from the same sample aliquot. This clearly leads to the increased comparability of the obtained results.

Second, the method is easily scalable and makes it therefore highly compatible with small to large sample amounts. We routinely use 10-100 mg of tissue samples, but successful lipidomic studies have also been performed on as little as 20 Arabidopsis seeds[31]. Especially the compatibility with small sample amounts makes this method applicable if limited amounts of biological tissues or samples are available. Still, even if sufficient sample material is available, the method presented here offers the advantage to utilize these samples in a larger number of experimental replicates instead of using them for different extraction procedures. This allows for a better and refined statistical data analysis.

Third, since the method is based on a liquid-liquid fractionation of polar and non-polar molecules, it provides, in contrast to simple one-phase extraction methods (*e.g.* methanol extractions) a significant de-complexing step in the procedure. This efficient sample de-complexing leads to a partial purification of the individual fractions due to the separation of chemically interfering molecules from each other. Accordingly, the chemical partitioning process not only provides a practical advantage for the systematic aliquoting of the extracted samples into different chemical classes but also improves the individual analytical measurements, since it removes contaminating compounds from the different fractions. Clearly, we can observe that especially the lipids, which are partitioned to the organic phase and which usually negatively affect the chromatographic analysis of polar compounds, will be almost completely absent from the polar fraction. The same is true for the analysis of the hydrophobic lipids, which will be depleted of the polar compounds. Besides the purification of polar and nonpolar compounds from each other, we deplete and collect proteins and other macro-molecules from the sample, which not only provides a separate fraction, which can be utilized for protein, starch

and cell wall analysis[16], but also leads to a cleaner sample within the individual fractions. This is especially relevant, since it is known that the presence of large macromolecules, leads to damage or at least shorter lifetime of the analytical columns.

Last but not least, the described MTBE extraction method, which relies on the less hazardous and more favorable chloroform replacement solvent[15], has already been shown by several studies from our group, to be widely applicability for different biological samples from plants[16], algae[17,18], flies[19] but also several mammalian tissues, organs or cells[20,21,22].

Disclosures

The authors have nothing to disclose

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