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Review

Plant Sample Preparation for Metabolomics, Lipidomics, Ionomics, Fluxomics, and Peptidomics

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Abstract: Plant metabolomics, lipidomics, ionomics, fluxomics, and peptidomics are essential approaches for exploring how plants respond to epigenetic, pathological, and environmental stimuli through comprehensive chemical profiling. Over the past decades, significant progress has been made in protocols and methodologies to address the challenges in sample collection and extraction. Despite these advancements, sample preparation remains intricate, with ongoing debates about the most effective strategies. This review emphasizes the importance of clear research questions and well-designed experiments to minimize complexity, save time, and enhance reproducibility. It provides an overview of the key steps in these fields, including harvesting, drying, extraction, and data pre-acquisition for major analytical platforms. By discussing best practices and common challenges, this review aims to streamline methods and promote more consistent and reliable research outcomes.

Keywords: plants; sample preparation; metabolomics; lipidomics; ionomics; fluxomics; peptidomics

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1. Introduction

In recent years, advancements in omics technologies have greatly enhanced our understanding of the biochemical and physiological processes in plants. Approaches such as metabolomics [1–5], lipidomics [1,4,5], ionomics [6–8], peptidomics [9,10], fluxomics [11], and the more recent single-cell metabolomics [12,13] have collectively provided a more comprehensive overview of the molecular mechanisms that drive plant growth, adaptation, variation in genetic expression, disease resistance, phenotypic traits, and environmental interactions. While each of these omics approaches offers unique insights, they also present significant challenges, particularly in terms of sample preparation.

To address these challenges, various global initiatives have been established to develop standardized protocols for sample handling and analysis. Organizations like the Metabolomics Standards Initiative (MSI) [14], COordination of Standards in MetabOlomicS (COSMOS) [15], and platforms like Metabolomics Workbench [16] have been instrumental in developing guidelines that promote reproducibility and consistency across laboratories. Quality assurance (QA) and quality control (QC) protocols remain pivotal for reliable and replicable metabolomics data [17]. Supporting this effort, the Metabolomics Quality Assurance and Quality Control Consortium (mQACC) provides comprehensive guidelines to enhance data reliability, and is accessible at https://www.mqacc.org/outputs.

The choice of sample preparation protocols is closely linked to the analytical approach—whether targeted, untargeted, fingerprinting, or profiling—and the specific omics layer being studied, such as the complete metabolome or specific classes of compounds [5,18–24]. Each method requires different levels of precision, accuracy, and chemical coverage. For instance, untargeted methods aim to capture as many metabolites as possible, providing a comprehensive snapshot of the plant's biochemical state. In contrast, targeted approaches focus on the precise quantification of specific metabolites.

Sample preparation presents several challenges, mainly due to the chemical diversity and concentration range of metabolites, the influence of the plant's ontogenetic stage, and the specific edaphoclimatic conditions in which the plants are grown/cultivated [1,5,21,23–26]. These factors vary between controlled environments, such as growth chambers, natural, and open-field settings. Furthermore, constraints related to data acquisition and instrument settings also play a role. No single analytical technique can comprehensively analyze the full range of metabolites—from highly polar to non-polar—in a qualitative or quantitative study [27–29]. As a result, combining complementary orthogonal approaches is often necessary. For instance, liquid chromatography–mass spectrometry (LC-MS) is widely used to cover a broad spectrum of semi-polar compounds, while nuclear magnetic resonance (NMR) excels in quantifying a wider range of metabolites.

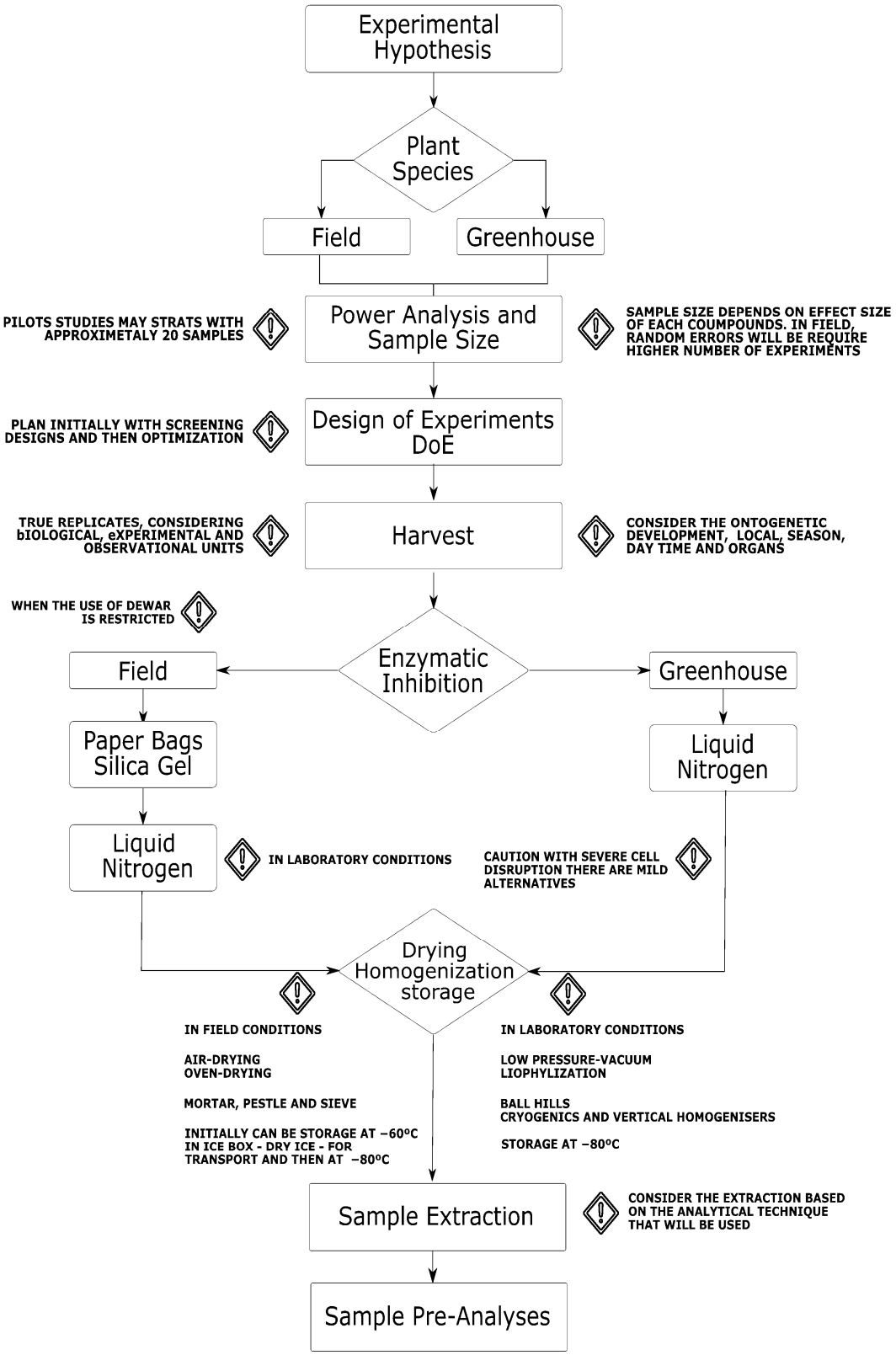
In lipidomics, efficiently extracting a range of lipids—from membrane lipids to signaling molecules—while preventing degradation during extraction is a core challenge. Techniques like LC-MS and GC-MS are commonly used for these analyses due to their sensitivity and precision [1,4,5]. Ionomics refers to the study of minerals and trace elements in plant and animal systems according to an external change, it is an approach that performs genomic correlations to understand how changes in gene expression influence ionic profiles in organisms [30,31]. Avoiding contamination is critical, as even trace elements can distort the ionic profile. ICP-MS and ICP-OES are frequently employed, providing the necessary specificity and sensitivity to accurate quantification of elemental composition [6,7].

Fluxomics refers to the quantification of the flow of metabolites through their pathways in living organisms and presents the challenge of capturing the real-time movement of these metabolites through biochemical pathways. This omics approach combines experimental techniques in order to simulate metabolic fluxes under stress or normal conditions by using isotopic labeling with mathematical models [32,33]. This requires rapid quenching to halt metabolic activity to preserve the integrity of metabolic fluxes. LC-MS and GC-MS, especially when combined with isotope-labeling techniques, enable the tracking of dynamic metabolic flow [11,34,35]. In peptidomics, which focuses on the profiling of small peptides (<10 kDa), LC-MS/MS is typically used to identify peptides and their post-translational modifications. Ensuring peptide integrity during sample preparation is essential, often requiring protease inhibitors or rapid freezing to prevent degradation [9,10]. Single-cell metabolomics faces the unique challenge of isolating individual cells without contamination from surrounding tissues [36,37].

Overall, selecting an analytical technique depends primarily on the type of sample, but also on factors like metabolite concentration, chemical properties, and the sample volume available. To illustrate the complexities involved, Table 1 compares the main techniques based on sensitivity, reproducibility, resolution, quantification capabilities, and the difficulty of metabolite identification.

With the main challenges in sample preparation across omics fields—metabolomics, lipidomics, ionomics, peptidomics, fluxomics, and single-cell metabolomics—we can delve into experimental design and the specific steps involved in optimizing sample preparation for plant-based studies. Scheme 1 outlines the key steps hierarchically, from defining the research hypothesis to power analysis, the design of experiments (DOE), and sample

collection, storage, and transport. This scheme highlights the essential precautions and conditions for each step, allowing adaptations based on the unique requirements of each omics layer.



Scheme 1. A representation of the different critical steps from experimental hypothesis, power analysis, DOE, harvest, sample treatment, storage, and the transport of plant samples.

Table 1. A comparison of the analytical techniques in metabolomic and related areas. For metabolites we used the symbols + for low levels and +++ for the maximal contribution.

Feature	Analytical Technique					
	LC-MS	**GC-MS**	**CE-MS**	**ICP-MS**	**MALDI-MS**	**NMR**
Amount of sample preparation	++	+++	++	+++	+++	+
Volume for injection	++	++	+	+++	++	+++
Range of metabolites	RP: non-polar HILIC: polar	Volatile and thermostable	Polar	Elements	Polar and non-polar	Polar and non-polar
Sensitivity	+++	+++	+	+++	+++	+
Resolution	+++	+++	+	+++	+++	+
Quantification	IS needed		IS	IS	IS	IS
Reproducibility	<NMR		<NMR	High	<NMR	High
Identification of metabolites	Difficult (few or no standard libraries)	Easy (large spectral libraries)	Difficult (databases with few data)	Easy	Difficult (few or no standard libraries)	Easy

2. Experimental Hypothesis and Statistical Power Analysis

A strong research hypothesis (RH) is the foundation of a well-designed experiment. It should be directly linked to the metabolic pathways and metabolites of interest, guiding the choice of the most appropriate analytical tools [2].

When designing the experiment, it is important to consider the biological levels involved. For example, metabolite concentrations can vary significantly between leaves on the same branch, different branches, or even between individual plants. Consistent sampling across growth stages and environmental conditions is vital to maintain data integrity and ensure reliable results. In this context, replication is a key factor for boosting an experiment's statistical power. However, it is critical to employ true replication [22]. Sampling different parts of the same plant or multiple samples from a single source leads to pseudo-replication, which fails to provide independent data points. True replication uses independent experimental units, such as different plants, to capture genuine biological variation. Clearly defining biological units (BUs), experimental units (EUs), and observational units (OUs) within the experimental design ensures accurate data interpretation and avoids replication errors [38].

Randomization is another key factor for controlling potential biases. By randomizing the order of sample collection or treatment application, systematic effects are evenly distributed, minimizing bias in the results. However, randomization is more effective when the sample size is large enough. Statistical validity in experimental studies requires careful power analysis and proper sample size determination. Hypothesis testing can be affected by false positives (type I) and false negatives (type II), which can lead to misleading conclusions. Power analysis helps to identify the minimum sample size needed to achieve the desired effect and level of significance, reducing the likelihood of such errors [22].

In metabolomics, determining the right sample size is particularly challenging due to the high dimensionality in data and the multicollinearity between variables. Optimizing workflows and ensuring consistency in sample collection, extraction, and data analysis are critical to managing these complexities. Moreover, employing quality control (QC) samples at regular intervals helps monitor drift and variability in data, which is essential for maintaining the robustness of experimental results. This aspect is particularly relevant when determining the power analysis and sample size required for untargeted metabolomics

studies, where sample heterogeneity is often high [22,39–41]. Tools like MetSizeR [42] and MetaboAnalyst [43,44] offer practical methods for calculating sample size and power analysis, addressing the high-dimensional data challenges common in metabolomics.

As in metabolomics, the experimental design for lipidomics studies must consider the complexity of the samples and biological variability. Lipidomics faces challenges due to the variety of lipids, their differences in polarity, size, and solubility. Extraction methods must be standardized. Statistical power analysis is sensitive to the quality of analytical methods and technical variability. The use of quality control samples is crucial. Tools such as LipidQC and MS-DIAL can help in normalization and statistical power analysis, ensuring greater reliability in studies [45–47].

In the statistical power analysis of fluxomics, it is challenging to integrate metabolic and isotopic data from stable isotope experiments. It is important to have clear hypotheses about metabolic fluxes and to standardize the collection time to minimize variations. Replication is essential, as small variations in isotope incorporation can introduce errors. Using tools such as 13CFlux and INCA helps to calculate statistical power, ensuring adequate sample sizes to detect changes in fluxes [48,49]. Peptidomics requires careful sample collection and preparation, as peptides degrade easily. Randomization and replication are essential to avoid bias and capture biological variation. Assessing statistical power is challenging due to instrument sensitivity and data complexity. Multicollinearity between peptides can complicate interpretation. The use of QC and tools such as Skyline and MaxQuant helps monitor analytical accuracy and optimize sample sizes in research [50,51].

In ionomic analysis, it is important to consider the variety of ion concentrations in different parts of the plant, such as roots, leaves, and fruits. This diversity is influenced by genotype, evolutionary stage, and environment. Standardizing cultivation and ion extraction is essential for consistent results. Randomization and replication help reduce bias and detect biological variations. The use of tools such as ionomicQC and MetaboAnalyst is crucial to manage high-dimensional data and minimize type I and II errors [52,53].

3. Design of Experiments

The design of experiments (DOE) is essential for ensuring accuracy and reproducibility in metabolomics by systematically identifying and minimizing errors throughout the experimental process [2,54–56]. It focuses on key variables and responses relevant to the research hypothesis. Screening designs, such as Fractional, Factorial Designs (FDs) or Plackett–Burman Designs (PBDs) helps pinpoint significant variables with fewer experiments, while optimization designs like Box–Behnken (BB) or Central Composite Design (CCD) refine experimental conditions and create response surfaces to predict optimal setups [56]. DOE has been widely applied across various omics fields, enhancing sample preparation, method development, and data processing. Table 2 illustrates several examples of DOE application in metabolomics, lipidomics, ionomics, peptidomics, and other omics studies.

In metabolomics, DOE frequently optimizes extraction protocols. For example, a Box–Behnken Design in a GC-MS analysis of apple fruit improved the extraction and derivatization of polar compounds, enhancing quantification accuracy [57]. In lipidomics, a Headspace Solid–Phase Microextraction (HS-SPME)-GC-MS study on grape berries used DOE to optimize fiber type, extraction time, and temperature, achieving comprehensive lipid recovery [58]. Ionomics leverages DOE to refine digestion methods for dried fruits using ICP-OES, comparing wet, dry, and microwave digestion techniques to minimize contamination and enhance trace element extraction [59]. Similarly, peptidomics benefits from DOE when optimizing solvent ratios for small peptide extraction, as demonstrated by a Taguchi-based design in an LC-MS study on lichens, which improved peptide recovery [60].

In fluxomics, DOE was used for refining quenching techniques to preserve real-time metabolic fluxes. A Plackett–Burman design applied to a GC-MS study of olive oil optimized temperature and column flow, enabling accurate measurement of dynamic metabolic processes [61]. For single-cell metabolomics, DOE helps refine micro-level extraction and preparation. For example, a fractional factorial design was used to optimize extraction parameters in studies of individual pancreatic progenitor cells, enhancing metabolite recovery [62].

Table 2. Metabolomics studies using DOE for sample preparation, method development and validation, and data processing in omics studies.

DOE Approach	Analytical Technique	Optimization	Plant	Ref
Sample Preparation				
Box–Behnken design	GC-MS	Extraction and derivatization of polar compounds	Apple fruit	[57]
Central composite design	LC-UV	Extraction (solvent, time, and temperature)	Saffron	[63]
D-optimal	UPLC-MS	Accelerated solvent extractions	*Camellia sinensis*	[64]
	GC-MS	Extraction and derivatization of compounds	*Arabidopsis thaliana*	[65]
	HS-SPME-GC-MS	Type of fiber, extraction time, equilibration time, and temperature	Grape berry	[66]
	ICP-OES	Digestion method (wet, dry, microwave)	Dried fruits	[59]
Full factorial and Box–Behnken designs	NMR	Extraction (solvent, time, power, and solvent/material ratio)	Apricots	[67]
Simplex centroid	LC-DAD	Mixture of solvents of extraction	*Jatropha* species	[68]
Taguchi-based designs	LC-MS	Extraction (gridding solvent/material ratio, and stirring)	Lichens	[60]
Method Development and Validation				
Box–Behnken Design	LC-MS	Funnel technology and ion-source parameters	Standards	[69]
Box–Behnken Design	LC-MS	Four parameters for LC and six for ESI-MS	*Meconopsi* species	[70]
Central composite and factorial design	LC-DAD	LC solvent, injection volume, temperature	*Jatropha* species	[68]
Plackett–Burman and Factorial Design	GC-MS	Temperature, ramp rate, split, column flow	Olive oils	[60]
Data processing				
Plackett–Burman and Central Composite Design	LC-MS	XCMS parameters	Standards	[71]
Modified full factorial	LC-MS	XCMS parameters	Poplar	[72]

4. Plant Ontology, Harvesting, and Sampling

Plant metabolomics, lipidomics, peptidomics, and others involve a wide range of environmental variations, genetic expression changes, and ontological factors, all of which

can influence study results and conclusions [19,73]. Before any harvesting or study design, it is paramount that the plant species under investigation be unequivocally identified, as improper identification can undermine all subsequent results [74].

In studies where plants are collected from different locations, regions, or biomes, considering the plant's ontogenetic stage—whether flowering or at another developmental phase—is crucial [75–77]. Additionally, with the rise of single-cell metabolomics, accurately selecting and sectioning tissues demands a strong understanding of plant anatomy [76]. The Plant Ontology Consortium [76] offers a structured vocabulary covering aspects like plant taxa, development, anatomy, and genetic data.

Harvesting plays a significant role in determining the results of plant omics studies. Factors such as circadian and seasonal cycles can dramatically influence a plant's chemical profile. It is crucial to schedule multiple harvests at consistent times of the day or season to capture representative metabolite profiles. Additionally, the specific plant organ or tissue harvested and its position—whether exposed to full sunlight or shade—can affect metabolite levels [19,78]. For example, research on *Arabidopsis thaliana* under ultraviolet light stress revealed higher levels of primary metabolites, such as ascorbate derivatives, and increased production of flavonoids and phenolics [69].

It is also important to consider whether plant material is sourced from greenhouses or natural environments. In controlled greenhouse settings, even small variations in factors like light intensity, irrigation, temperature, and CO_2 gradients can affect the metabolic profiles of genetically identical plants [19,23,73]. For studies targeting specific metabolic pathways, these environmental differences can contribute to data variability. To minimize such "edge effects" in greenhouses, redistributing plants between the center and peripheral locations regularly can help maintain uniform conditions [23,79].

In natural settings, where eco-metabolomic research is often conducted, controlling environmental conditions is much more challenging. The use of global positioning systems (GPSs) or tools like Google Maps helps accurately delineate plant locations. However, understanding the exact environmental conditions—such as soil type, herbivory frequency, or the ontogenetic stage of the plant—can be difficult, adding complexity to the interpretation of results. Ideally, sampling regions should be chosen where factors such as precipitation, soil characteristics, altitude, ultraviolet radiation, and diurnal temperature variations are well documented [75,79].

5. Enzymatic Inhibition

After plant material is collected, enzymatic activity must be immediately inhibited to preserve metabolic integrity, also commonly referred to as quenching. This is particularly important for preventing the degradation of sensitive metabolites like ATP and NADH, which can rapidly break down when exposed to heat and light [80–82].

The quenching process is vital for ensuring accurate results. For example, Moreira and coworkers [83] demonstrated that the accumulation of chlorogenic acid in certain plants used in traditional Chinese medicine was influenced not only by anabolic processes but also by catabolism and turnover. This highlights how postharvest treatments can shape metabolic profiles.

When performing enzymatic denaturation, a few key principles must be followed: (i) minimal leakage: even as proteins are denaturated, cell structures should remain intact to prevent the release of oxidative species that could degrade metabolites during the extraction process; (ii) energy charge: monitoring the balance between ATP, ADP, and AMP can provide insight into the energy state of the cell, serving as an indicator of metabolic inactivation; (iii) residues from quenching: any solvents, temperature conditions,

or equipment used for quenching must not interfere with later steps like lyophilization or extraction [80].

There are several quenching methods available, including the use of hot or cold solvents, acidic or basic conditions, or by freeze-drying. Liquid nitrogen at −180 °C is a widely used method due to its rapid thermal shock, which effectively halts enzymatic activity. Being inert, nitrogen can be easily removed during freeze-drying or storage. However, extreme freezing can sometimes lead to the formation of ice crystals, which might cause cell lysis and the mixing of endogenous and exogenous compounds, including oxidative species [75,82].

Milder quenching methods involve using hot or cold organic solvents like ethanol and methanol, typically at controlled pH values (3–8). However, hot conditions may not completely inhibit enzymatic activity and can potentially degrade sensitive compounds or create artifacts. Cold solvents, such as methanol at −20 to −40 °C or dry ice (−78 °C), are effective for both plant and microbial metabolomics [19,80,82]. For instance, Faijes and colleagues [84] compared different quenching strategies and found that a mixture of ammonium carbonate–methanol provided efficient inactivation with minimal cell lysis (under 10%), and minimal interference with downstream analysis.

In peptidomics, the rapid inhibition of protease activity is crucial, as proteases can degrade endogenous peptides, leading to the loss of valuable information. Common methods to preserve peptide integrity include heating, such as microwave heating [85], as well as freezing [86], and the use of protease inhibitors [87]. These approaches help to ensure that peptides remain intact for subsequent analysis.

Despite its importance, systematic investigations of enzymatic inhibition and quenching procedures in plant omics are still limited. In contrast, extended research has been conducted for other biological matrices. For example, Yang and colleagues [88] tested different quenching and extraction methods in terms of cell membrane integrity and the metabolite abundance of intracellular metabolites, and concluded that for their matrix, mild quenching methods (20% MeOH at −4 °C) wielded better results than harsher conditions. Wang et al. [89] contributed with a systematic evaluation of cell quenching and metabolite extraction of the HeLa carcinoma cell line was performed by combining the GC-MS and LC-MS analytical tools for a wide range of substances, reporting the optimal overall coverage of intracellular metabolites to be a combination of liquid nitrogen and 50% acetonitrile. Canelas et al. [90] evaluated protocols for yeasts, and reported better results with comparatively more abrasive methods, utilizing boiling methanol and chloroform–methanol, and did not discriminate in terms of metabolite classes.

Given the variety of strategies available for selecting a quenching method in plant-based matrices, it is not always clear which is best suited to a researcher's objectives. When prior knowledge about the plant species being studied is limited, smaller-scale pilot studies are recommended to assess the impact on various substances. However, due to the practical challenges of field sample collection, such studies are not always feasible. In such cases, researchers should choose field-friendly quenching methods, mindful that the appropriate solvent conditions (in terms of temperature and composition) may not be achievable in the field. The selected method should be aligned with the study's goals, as it may affect data acquisition in terms of detection sensitivity for the targeted substances.

6. Drying, Homogenization, and Storage

The presence of water in plant matrices can significantly impact various stages of plant metabolomics studies. Water not only affects the efficiency of organic solvent extraction but also influences sample storage and can interfere with subsequent instrumental analyses [19,75,79]. Moreover, residual water content can prolong enzymatic activity and

promote the growth of bacteria and fungi, further altering the chemical profile [19,91,92]. This is particularly problematic in ^{1}H NMR experiments, where broad water signals can distort signals and complicate data interpretation [19,79].

To mitigate these effects, various drying methods are commonly used, including oven drying with circulating air at temperatures of 40 °C, air drying at room temperature, and vacuum drying, which may involve speed-vacuum systems or be combined with sublimation processes like freeze-drying [19,21,24,79,90,93]. Table 3 expands each method's advantages and limitations. In the field or in eco-metabolomics studies, where infrastructure may not be accessible, alternative drying methods like using silica gel in paper bags can suffice until samples can be transported to a laboratory for proper drying [94].

Table 3. Advantages and limitations of drying methods.

Drying Method	Advantages	Limitations	Ref.
Oven Drying	Efficient and cost-effective; suitable for large sample volumes.	Prolonged exposure to heat may degrade heat-sensitive metabolites; risk of inconsistent drying if temperature fluctuates.	[19,75,79,93]
Air Drying	Low-cost; no specialized equipment needed.	Longer drying times; potential for microbial contamination in humid conditions.	[19,21,79,94]
Vacuum Drying	Minimizes oxidation and thermal degradation; effective for heat-sensitive compounds.	Equipment cost; potential loss of volatile compounds.	[19,24,79,85]
Freeze-Drying	Preserves heat-sensitive metabolites; produces highly reproducible results.	Expensive; loss of volatiles; thermal shock may affect metabolite adherence.	[23,24,79,95]
Drying with Silica Gel	Portable; suitable for fieldwork.	Limited capacity; inconsistent drying efficiency compared to lab-based methods.	[19,21,94]

In controlled laboratory settings, freeze-drying is the most used technique, particularly for samples that are sensitive to heat [23,24,95]. This process involves two stages: (i) cooling the sample, followed by (ii) sublimation at reduced pressure and around −40 °C. While freeze-drying produces highly reproducible results, it has drawbacks. Rapid sublimation or excessive thermal shock can cause metabolites to adhere to cell walls, affecting extraction quality and yield. Additionally, freeze-drying under reduced pressure can lead to the loss of volatile compounds, altering the sample's chemical profile. Air drying and oven drying are more common in phytochemistry, but they must be used cautiously in metabolomics to avoid errors related to water content or inconsistent exposure to light or heat, which can result in chemical imbalances.

Homogenization is another critical step to ensure sample consistency and comparability [19,21,79,91]. Crushing or grinding reduces tissue size, which enhances uniformity and solvent contact. Standardizing this process, known as granulometric control, is essential. Mortar and pestle are commonly employed, but for larger sample sets, equipment like ball mills, cryogenics, or vertical homogenizers provides rapid and uniform sample comminution [18,25]. For example, research by Zheng et al. (2020) [96] demonstrated how thermal processing, such as the baking and frying of blueberry-filled bakery products, impacted their phytochemical profile, especially anthocyanins. This highlights the importance of controlling sample processing steps that involve heat, as new compounds can form, affecting results.

Ideally, samples should be processed through grinding, drying, and extraction soon after harvesting. If immediate processing is not possible, storage at −80 °C is recommended.

Lower temperatures like −20 °C or 4 °C are often insufficient, as biochemical reactions may continue, especially in samples with salts or organic solvents. Proper storage helps prevent unwanted chemical changes and preserve the sample's metabolic profile [85,97].

For transport, especially from field sites, using dry ice or liquid nitrogen in well-insulated containers helps maintain low temperatures and prevents sample degradation. Packaging should be designed to sustain −60 °C for at least a week, and freeze-dried samples should be sealed in plastic bags with desiccants like silica gel to prevent moisture absorption. It is best to avoid storage temperatures between 0 and 40 °C, as metabolites may concentrate in residual aqueous phases, altering their properties [23].

7. Extraction Process

Until this point, the steps discussed are relevant to various analytical platforms, regardless of whether the compounds are volatile, non-volatile, polar, or non-polar. However, with the extraction process, the choice of analytical techniques becomes crucial. The extraction method used determines chemical coverage, polarity, and solubility, as well as the suitability for specific analytical platforms. It is crucial to keep extractions as simple, rapid, and minimal as possible, as metabolomics typically requires highly reproducible protocols for large-scale experiments.

A range of extraction techniques are available, including solvent extraction (maceration), steam distillation, supercritical fluid extraction, ionic liquids, and solvent extraction under high temperatures and pressures [18,20,75,85,98]. Device-assisted extractions like ultrasound-assisted extraction offer high efficiency [99]. Unlike microwave-assisted extraction, ultrasound minimizes the overheating or degradation of volatile compounds, making it a preferred method due to its simplicity, speed, and solvent flexibility. That said, longer extraction times and greater temperature fluctuations may yield more compounds but also increase the risk of reactions and degradation [85].

Liquid pressurization extraction (LPE) uses high temperatures and pressures to increase extraction efficiency, reducing solvent consumption in closed systems. Enzymatic methods use enzymes to release bioactive compounds from complex matrices without the need for aggressive solvents and stand out for their specificity. Adsorption on activated carbon is widely used due to its selective adsorption. Membrane extraction uses selective barriers to separate compounds based on physicochemical characteristics, while cold plasma-assisted extraction appears to be a promising alternative, disrupting cellular structures quickly and efficiently. Hyperbaric extraction, in turn, uses extreme pressures to access compounds that are difficult to extract, increasing the recovery of bioactive compounds [100–105].

Organic solvents combined with ultrasound-assisted extractions are frequently employed due to their compatibility with LC-MS, GC-MS, and NMR. Environmental concerns have spurred the development of more sustainable extraction techniques, like Natural Deep Eutectic Solvents (NADESs), supercritical CO_2, and greener organic solvents [98,106,107]. While optimizing the extraction step is important, certain methods can hinder comparisons with other studies or databases due to differences in solubilizing power, toxicity, and selectivity.

Methanol–water mixtures (50–90% methanol) are commonly used for broad metabolic coverage, extracting sugars, organic acids, alkaloids, and phenolic compounds [85,87,108]. Ethanol is also a greener alternative. Controlling pH (around 6–7) during extraction helps prevent artifact formation from oxidative reactions. In NMR, pH, water, and other solvents can shift chemical signals and affect retention times in HPLC and ionization in mass spectrometry, particularly with electrospray ionization. Buffers help minimize these effects

in NMR, while in LC-MS, it is important to avoid metal salt precipitation in the ionization source [109].

Methanol, water, and chloroform (2:2:1) effectively extract both polar and non-polar compounds. For example, Lisec et al. (2006) [110] developed a protocol for GC-MS plant metabolomics, optimized to minimize enzyme activity. This protocol has been effective in extracting a wide range of metabolites, including primary metabolites and specialized compounds like glucosinolates, alkaloids, and polyamines [111]. LC-MS is particularly well-suited to semi-polar metabolite profiling, as shown by De Vos et al. (2007) [112], who used acidity methanol (formic acid) and sonication to extract plant tissues like *A. thaliana*, tomato, and strawberry [113,114].

The Lipidomics Standards Initiative (LSI) was stablished to allow for the standardization and comparison of results across different lipidomic studies (https://lipidomicstandards.org) (accessed on 20 October 2024). Popular lipid extraction protocols include Folch [115], Bligh and Dyer [116], Matyash [117], and the BUME method [118,119]. These methods, mainly developed for animal tissues, involve varying solvent ratios. For instance, some protocols use methanol followed by MTBE, adding water to induce phase separation. This chloroform-free method offers high recovery rates across lipid types. The BUME method, suited for high throughput extractions, uses smaller solvent volumes. Since these protocols were developed for animal matrices, it is recommended to perform pilot studies on plant organs, as results may vary for leaves, bark, seed, or fruits. Creydt and colleagues (2018) [120] found that the Folch protocol was optimal for their matrix, while Romsdahl et al. (2022) [121] and Hu et al. (2021) [122] identified Matyash as most suitable for their lipid analyses of seeds and oilseeds. Table 4 shows some analytical methods applied to different type of compounds.

Table 4. Extraction methods for different types of compounds.

Characteristics	Analytical Method	Ref.
Volatile	- Steam distillation- Microwave-assisted extraction- Cold plasma-assisted extraction- Liquid pressurization extraction (LPE)- Hyperbaric extraction	[18,85,99,109]
Non-Volatile	- Solvent extraction (maceration)- Supercritical fluid extraction (e.g., CO_2)- Membrane extraction- Ultrasound-assisted extraction	[18,85,98,106,107]
Polar	- Methanol–water mixtures (50–90% methanol)- Methanol, water, and chloroform (2:2:1)- Buffers for NMR to control pH	[85,87,108–110]
Non-Polar	- Chloroform-free methods (e.g., MTBE-based protocols)- Lipid extraction protocols (e.g., Folch, Bligh and Dyer, Matyash, BUME) [108–110]- Supercritical CO_2	[115–119]

Kim et al. (2010) [19] focused on the ^{1}H NMR analysis of primary and secondary metabolites, utilizing labeled solvents like deuterated methanol and water, to enhance signal clarity. Advances now allow for single-cell mass spectrometry for rapid metabolite detection, enabling the real-time analysis of individual plant cells. Fujii et al. (2015) [99] developed a protocol involving nanospray microcapillary tips and optical microscopy for single-cell metabolomics, allowing for precise detection from plant cells with minimal manipulation. Table 5 shows some protocols commonly used in plant omics analysis.

Table 5. Plant metabolomics protocols for CG-MS, LC-MS, NMR, ICP-MS or OES, and MALDI-MS.

Analytical Method	Characteristics	Ref.
GC-ToF-MS	Established protocol with a large database for compound identification. Difficult to detect secondary metabolites.	[110]
GC-MS and LC-MS	Focused on pre- and post-treatment data.	[13]
LC-QToF-MS	Established protocol for analysis of semi-polar metabolites, mainly secondary metabolites.	[112]
UHPLC-MS	Protocol for analysis of large data sets using molecular networking (identifying tool).	[123]
LC-MS	Optimization of extraction steps evaluating efficiency, repeatability, and ionization efficiency	[124–126]
MALDI-ToF MS	Protocol for MALDI-TOF-MS with multivariate analysis and taxonomic approach.	[127]
NMR	Use perchloric acid as extraction solvent. Used mainly for primary metabolism of plants.	[128]
NMR	Relatively simple protocol including different groups of primary/secondary metabolites.	[19]
NMR	Quality for extraction of tissues for NMR. Also applied for quantitative metabolomics.	[129]
LC-MS	Used for live single-cell metabolomics.	[87]
ICP-OES and ICP-MS	Used for elemental analysis of plant material.	[130–132]
ICP-MS	Analysis of metals in nanoparticles	[133]

8. Pre-Processing to Acquisition

With extracts prepared, it is essential to address potential interferences and residues that could affect data acquisition. The pre-processing steps vary depending on the analytical platform and the metabolites being analyzed. For example, when examining polar compounds from aqueous alcohol extracts with GC-MS, an additional methoxylation step is often required. This step involves the cyclization of sugars and protection of carbonyl groups, followed by silylation to enhance compound volatility. BSTFA and MSTFA are common silylating agents, with BSTFA derivatives typically resulting in longer retention times.

A factor to be considered in LC-MS analysis is the matrix effect, which is caused by competition between the sample constituents with the analyte(s) of interest; this affects the ionization efficiency, which means that the generated signal can be increased due to ion enrichment or decreased due to ion suppression. Especially at low concentrations, the matrix effect can cause a reduction in the accuracy and precision of results. Considering the complexity of the sample, some procedures can be included to eliminate or at least minimize the matrix effect. This can be achieved by changing the ionization source usually used, improving the chromatographic conditions with the use of corrective calibration methods, optimizing the extraction method and sample dilution [134,135].

For LC-MS analysis, extracts from chloroform–methanol mixtures frequently need further cleanup. Since many secondary metabolites are analyzed in reversed-phase mode, it is helpful to remove non-polar compounds like fatty acids using reversed-phase cartridges (C8, C18, or similar), preventing column clogging, and enhancing efficiency and reproducibility. Filtering before injections is crucial, with 0.45 µm and 0.22 µm PTFE filters being widely used to eliminate particulates and prevent system blockages.

In fluxomics analysis, sample preparation requires careful use of isotopic markers, such as (13C), (15N), or (2H), to monitor metabolic flux. Pre-processing involves eliminating cellular debris. Filtration and centrifugation help reduce metabolite losses. It is important to separate non-reactive tracers prior to analysis [136,137]. Preprocessing for peptidomics analysis often includes enzymatic digestion to convert proteins into peptides, with trypsin

being widely used for specific cuts. After digestion, cleanup is performed using techniques such as solid-phase extraction or protein precipitation. Complex samples may require fractionation by high-performance liquid chromatography. Desalination is important prior to LC-MS/MS analysis to remove salts that may affect ionization and mass spectra [138,139]. Lipidomics requires the careful extraction and storage of samples to avoid lipid oxidation. Solvents such as methanol–chloroform–water are used to separate phases. The removal of unwanted lipids and the addition of antioxidants are important. Modifications in ESI ionization are necessary to improve analysis [140,141].

The goal of preprocessing for ionomics is to eliminate inorganic pollutants that could impair trace element detection by ICP-MS or ICP-OES. Plant samples are digested with potent acids to release ions of interest [142]. In NMR or ICP (OES or MS) analysis, removing particulates is equally important to ensure shimming quality and clear chemical shift resolution. Centrifugation at 13,000$\times$ g or filtration aid in this, while cartridge filters or suppression pulse sequences address interferences from high concentrations of sugars, fatty acids, and phthalates, helping maintain data integrity.

9. Minimum Reports and Best Practices in mQACC

Standardization is critical for ensuring comparability and reliability in metabolomics research. While targeted metabolomics benefits from established guidelines by organizations like COSMOS, Metabolomics Workbench and MSI for metabolite quantification, untargeted metabolomics faces challenges due to its exploratory nature. The mQACC consortium addresses these gaps by promoting QA/QC practices, aiming to harmonize quality standards and enhance reproducibility and confidence in results [17].

Key recommendations include minimum reporting standards that ensure basic but essential information, such as instrument details, sample preparation methods, and quality control criteria. Best practices build on this foundation, encouraging the comprehensive documentation of experimental parameters and, where feasible, making raw and processed data available. These practices strengthen methodological integrity and align with mQACC's broader goal of advancing metabolomics research through transparent and reproducible methods [143].

Quality control (QC) samples play a vital role in maintaining analytical reliability. These include pooled QC samples for monitoring system stability and correcting drift, internal standards for addressing fluctuations during analysis, and long-term reference materials for evaluating instrument stability across runs. Blanks and system suitability samples further ensure data quality by identifying contaminants and verifying system performance [143,144].

Together, these practices provide a structured framework that integrates QC measures with robust reporting standards, enabling researchers to navigate methodological challenges and deliver reliable metabolomics data. Table 6 provides a simplified overview of the concepts of best practices and minimum reports.

Quality assurance and control should include clear acceptance criteria, such as analysis frequency, calculation of Relative Standard Deviation (RSD), and intra- and inter-batch variabilities for reported compounds. Whenever possible, both raw and processed data should be made available, along with batch drift corrections, including details on the software and parameters used. QC performance can be monitored manually, but automated scripts are recommended for system suitability checks, evaluating parameters like baseline stability [17,143,144].

Table 6. Minimum Reports and Best Practices Based on https://www.mqacc.org/outputs (accessed on 6 November 2024).

Quality Assurance and Control	Minimum Reports	Best Practices
QC Sample types	Specify the types of QC samples (e.g., pooled samples).	Use multiple QC types (e.g., intra- and inter-batch) and ensure consistency.
Blanks and System Suitability	Identify blank types used to detect contamination.	Differentiate between true blanks and process blanks; provide details of composition, suppliers, and preparation methods.
Internal Standards	Report concentration and source of internal standards used.	Include internal standard normalization details, signal tracking over time, and exact time of addition in analysis.
Preparation and Storage	Report preparation and storage conditions, including freeze–thaw cycles.	Include detailed aliquoting information and monitor freeze–thaw stability with evidence of sample integrity.
Data Normalization and Scaling	Provide basic normalization details, e.g., total area or selected features.	Report all normalization, scaling, and transformation steps applied, with justification for each.

Sample preparation is a critical process that includes metabolism quenching to preserve metabolite integrity, along with strict control over storage conditions like temperature and aliquot size. Standardized thawing and aliquoting schemes ensure consistent handling of both experimental and quality control (QC) samples. Key steps such as solvent selection, extraction, derivatization, centrifugation, and filtration must follow rigorous protocols to maintain data quality [144,145].

Different analytical technologies, including LC-MS, GC-MS, and NMR, have distinct requirements. For LC-MS, careful parameter documentation, ionization settings, and the use of standards are vital for data integrity. GC-MS requires compound derivatization and precise temperature control, while NMR relies on chemical shift calibration and stringent control of magnetic field strength. Despite these variations, robust QC protocols, including the use of QC samples and adherence to standardized operating procedures (SOPs), are fundamental across all platforms to ensure reproducibility and reliability [120].

The control of systematic block effects, arising from experimental or environmental variations, is another essential aspect. Using control samples helps monitor accuracy and identify anomalies. Statistical tools such as Principal Component Analysis (PCA) assist in isolating patterns and managing batch effects, enhancing data quality. Uniform adoption of SOPs further strengthens validation and replication efforts, ensuring reliable and interpretable analytical results [17,145].

10. Concluding Remarks

Despite the rapid advances in chromatography, mass spectrometry (MS), and NMR technologies, plant metabolomics and their related fields still face challenges, especially in harvesting, storage, and the extracting the wide range of metabolites in plant matrices. For a successful study, it is critical to focus on sample preparation by: (i) developing a clear and focused research hypothesis that considers the study's objectives and all potential confounding factors; (ii) implementing an experimental design aligned with the study's aims; and (iii) minimizing random errors, from enzymatic activity to drying, storage, and extraction.

Emerging technologies, like the miniaturization of live single-cell analysis and ion mobility for MS separation, offer new ways to explore the biochemical mechanisms in plant metabolomics. The expansion of metabolomic databases and advanced analytical tools

also promises to streamline the identification of the vast array of signals and molecules encountered in these studies, making the process more accurate and efficient.

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