Review

Identification of Plant Compounds from Mass Spectrometry Imaging (MSI)

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Identification of Plant Compounds from Mass Spectrometry Imaging (MSI)

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Abstract: The presence and localization of plant metabolites are indicative of physiological processes, e.g., under biotic and abiotic stress conditions. Further, the chemical composition of plant parts is related to their quality as food or for medicinal applications. Mass spectrometry imaging (MSI) has become a popular analytical technique for exploring and visualizing the spatial distribution of plant molecules within a tissue. This review provides a summary of mass spectrometry methods used for mapping and identifying metabolites in plant tissues. We present the benefits and the disadvantages of both vacuum and ambient ionization methods, considering direct and indirect approaches. Finally, we discuss the current limitations in annotating and identifying molecules and perspectives for future investigations.

Keywords: plant metabolomics; mass spectrometry imaging; compound identification

1. Mass Spectrometry Imaging of Plants

Plant metabolites have been extensively studied as a source of bioactive compounds for different industries. Plant biology has sought to elucidate when, where, and which secondary metabolites act as chemical mediators between plants and their surrounding environment [1].

Gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) are routinely used analytical methods for studying plant metabolites. However, the precise location within the tissues remains unknown due to the limitation of sample extraction [2].

Mass spectrometry imaging (MSI) is a technique that generates a snapshot of the distribution of molecules in biological tissue at a specific time. MSI permits the visualization of a diverse range of compounds in a single experiment. MS is a universal method, and, in contrast with other techniques like antibody-based strategies, MS allows the exploration of a more comprehensive chemical profile within the same experiment. Consequently, MSI represents an exciting opportunity to refine our knowledge of plant physiology.

Nevertheless, compound identification from MSI datasets can be challenging because each sampled spot may contain multiple overlapping signals, which are difficult to separate by experimental or data processing methods. Therefore, MSI is more a quantification than an identification technique.

Here, we review the state-of-the-art plant MSI, levels of confidence for metabolite identification, and experimental and computational strategies that can be used at different stages of MSI projects to enrich, separate, and identify metabolites.

MSI is a label-free technique for the untargeted analysis of secondary metabolites without prior knowledge. Independently from the manufacturer, an MSI setup consists of a sampling plate where the tissue is positioned, an ionization/desorption source, a mass analyzer, and a detector.

Over the past two decades, new ionization sources have expanded the range of detectable compounds. The joint goals are to allow direct analyses and enhance sensitivity [3]. Among the numerous ionization sources, we can distinguish two principal categories depending on the pressure conditions of the ionization/desorption source, i.e., analysis in vacuum or ambient conditions.

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Matrix-Assisted Laser Desorption Ionization (MALDI) and Secondary Ion Mass Spectrometry (SIMS) represent the most popular ionization techniques under vacuum conditions [4]. SIMS uses highly energetic primary ions for ionization, resulting in a high degree of fragmentation and making this technique less attractive for identifying unknown species [5]. In MALDI, analytes are co-crystalized with a chemical matrix. The matrix is a small molecule that absorbs the energy from a highly energetic ultraviolet (UV) or infrared (IR) laser. A significant challenge in MALDI imaging experiments is the high number of interference signals from the chemical matrix, which often overlap with the metabolites of interest [6]. Another critical aspect is the sample preparation, which consists of a multi-step process that may result in the delocalization of the analytes and, subsequently, erroneous interpretation of the results. MALDI was used for the first time in plant science in 2005 to detect and image agrochemicals in soybean plants [7].

In 2007, the colloidal graphite-assisted laser desorption ionization (GALDI) technique was introduced as an alternative to the standard MALDI matrixes. Due to its hydrophobic nature, graphite is more compatible with plant material. GALDI was used to image small molecules in strawberry and apple slices. Fatty acids and flavonoids were analyzed in negative mode at the femtomole range. The molecules were detected directly and subsequently identified by comparing the MS and MS/MS spectra with those of standards [8,9].

In 2009, A matrix-free approach was presented to analyze flavonoids in the model plant *Arabidopsis thaliana*. This technique was introduced as laser desorption ionization (LDI) and is ideal for UV-absorbing compounds [10]. In the same year, Harada *et al.* presented a mass-microscopic atmospheric-pressure (AP) LDI [11]. A UV laser under ambient conditions was combined with an optical microscope to image fresh ginger rhizome sections. The imaging system was coupled to a quadrupole ion trap time-of-flight (QIT-TOF) instrument, which facilitated the identification since tandem MS could be compared to the authentic standard. Another approach used an LDI to image trichomes from wild tomatoes (*Solanum habrochaites*). In this report, the authors used a carbon-substrate-based method to transfer the trichomes from the tissue to the carbon slide, resulting in images with high spatial resolution [12]. LC-MS confirmed the identification of metabolites. Postsource decay LDI mass spectra were acquired to verify the metabolite structures further.

In MALDI-2, an additional laser improves the sensitivity and detection of low-abundant compounds by offering a second ionization stage [13]. MALDI-2 was used for imaging apple (*Malus domestica*) sections. Compared with conventional MALDI, sugars and phenolic compounds were detected with an increment of two orders of magnitude.

AP-MALDI was introduced in 2000 to overcome the sublimation of matrixes under high vacuum conditions [14]. In 2007, AP-MALDI was applied for the first time to study plant tissues. Initially, a mid-IR laser was used to visualize sugars and citric acid on strawberry skin [15]. Using an IR laser eliminates the necessity of an external chemical matrix because the water content in the tissue serves as a matrix for absorbing the laser energy.

The AP scanning microprobe (S)MALDI was developed for high lateral resolution [16]. A single laser pulse per pixel was applied for imaging peptides. In 2014, this system was used to image plant tissues; the rhizome of *Glycyrrhiza glabra* was investigated, and two isobaric saponins were mapped with high lateral resolution. Further MS/MS analysis confirmed their identity [17].

A series of ambient ionization mass spectrometry (AIMS) techniques have also been developed. Their popularity relies on their ability to perform direct analysis, reducing or eliminating the sample preparation. AIMS analyses are conducted under natural conditions where plant tissues do not undergo significant changes. The AIMS techniques can be classified based on the desorption/ionization mechanism. This classification includes spray-based, plasma-based, and coupled techniques [18].

Desorption electrospray (DESI) is the most common spray-based technique for MSI under ambient conditions. DESI was presented in 2004 [19] and later coupled to imaging platforms [20]. DESI does not require a matrix application; instead, it relies on an electrically charged solvent that impacts the surface of the tissues. The analyte extraction depends on the solvent used, the sample's complexity,

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and the setup's geometry [21]. DESI has been used for direct and indirect analyses of plant tissues. In the direct mode, biological tissues are mounted on the sampling stage without prior preparation. The first example of direct DESI imaging analysis was presented in 2009, where the macroalga *Callophycus serratus* was studied; bromophycolides were mapped before and after mechanical damage. Identification of the secondary metabolites was corroborated by comparison of the extracts using LC-MS analysis with pure standards [22]. However, direct DESI imaging in plant tissues has a main obstacle: the penetration of the cuticular wax layer. To overcome this problem, Janfelt's group presented an indirect DESI imaging technique in 2011. The imprinting of the sample using a micro-porous Teflon surface extracts the compounds from their natural matrix while maintaining the spatial integrity of the sample. Leaves and petals of *Hypericum perforatum* and *Datura stramonium* leaves were investigated, and compound identification was achieved with tandem MS on the imprints [23]. A couple of months later, Cooks' group presented the same approach to investigate the catabolic products from chlorophyll in *Cercidiphyllum japonicum* leaves. Tandem MS using collision-induced dissociation (CID) verified the identity of the catabolites [24].

Direct real-time analysis (DART) was the first ambient technique based on plasma [25]. DART generates the plasma using a direct current discharge and a helium flow. The temperature of plasma impacting the sample surface ranges between 250 °C and 350 °C. The lack of a defined plasma jet and its high temperature makes DART impractical for imaging applications. 2008 low-temperature plasma (LTP) MS was introduced [26]. An alternating high voltage, high frequency, and low gas flow produces a dielectric barrier discharge. The temperature at the sample surface is maintained around 30 °C. A double dielectric barrier probe was presented in 2013 [27]. This LTP with a defined plasma beam diameter and a controlled temperature was suitable for directly analyzing plant tissues. Soon after, the LTP was used for imaging the distribution of capsaicin and other small metabolites in a cross-section of chili (*Capsicum annum* Jalapeño pepper) fruit [28]. In this example, the spatial resolution was limited to 1 mm; however, it was sufficient to demonstrate that capsaicin is distributed and limited to specific fruit compartments. A 3D-printed version of the LTP probe with about 200 μm plasma jet diameter can be used for LTP MSI with improved lateral resolution and *in vivo* analysis of plants, such as nicotine biosynthesis in tobacco [29,30].

Coupled techniques separate the desorption and the ionization of the analytes. Surface sampling with a laser has several advantages for imaging applications. Lasers can be optically focused to provide high spatial resolution [31]. Laser ablation electrospray ionization (LAESI) was presented in 2007 as a coupled technique for directly analyzing biological samples [32]. Tissue-specific metabolites from *Tagetes patula* seedlings were reported and tentatively identified based on the combination of accurate masses, database, isotopic distribution, and using tandem MS. The year after, an imaging study showed the capabilities of LAESI for plant analysis [33]. Leaves from *Aphelandra squarrosa* were imaged, and characteristic metabolites from green and yellow areas were identified using tandem mass spectrometry. MALDESI was developed as a combined technique, including applying a matrix followed by a laser to ablate the area and a second ionization process using electrospray [34]. The desorption ionization mechanism is similar to LAESI; for IR-MALDESI, applying an ice layer as a matrix showed an increase in the ion intensity by an order of magnitude [35].

In 2020, IR-MALDESI was used to image cherry tomatoes' metabolites [36]. The authors employed discovery-driven and literature-driven methods for identifying compounds in tomato MSI. Discovery-driven involves analyzing the spatial distribution of selected ions and correlating other ions with the same distribution pattern. The authors used the tool MSiCorrelation in the open software MSIreader [37]. After assessing the distribution of the most relevant ions, a literature-driven method was used, and the ions were searched against literature and database resources. For example, thirty-five structural isomers were found for the ion 273,0757 m/z, and from these, naringenin chalcone was selected as the most likely metabolite. Laser ablation atmospheric pressure photoionization (LA-APPI) is another approach presented in 2014 to investigate the distribution of active compounds in sage (*Salvia officinalis*) leaves [38]. The tentative assignment of the primary ions was based on previous reports.

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Plasma-based techniques have also benefited from adding an extra desorption source and have been used for imaging plant tissues. In 2008, an Nd:YAG laser ablation (LA) was coupled to a flowing atmospheric-pressure afterglow (FAPA), demonstrating the capabilities for imaging. In this report, the authors doped celery stock with caffeine and imaged it [39], concluding that adding a laser improves the detection of compounds and makes imaging analysis possible. In 2014, a plasma-assisted laser desorption ionization mass spectrometry (PALDI-MS) was presented using a DART ionization source and a 532 nm laser [40]. The study demonstrates the non-uniform distribution of the active components baicalein and wogonin in the *Radix Scutellariae* root. The accurate mass-to-charge ratio confirmed the identity of these compounds. In 2017, a similar study was presented using a 213 nm Nd:YAG solid-state UV laser and DART; this technique was called laser ablation direct analysis in real-time (LADI). LADI-MS was used to image the spatial distribution of the alkaloid biosynthesis products in the *Datura leichhardtii* seed [41]. MALDI-MS/MS was used to corroborate the identity of the observed masses. In 2019, another plasma-based approach was presented; a UV diode laser was coupled to an LTP to improve the desorption of less volatile compounds and delimitate the area of analysis [42]. This coupled technique LD-LTP was used to image mescaline in the cactus San Pedro (*Echinopsis pachanoi*), nicotine in tobacco (*Nicotiana tabacum*) seedlings, and tropane alkaloids in jimsonweed (*Datura stramonium*) fruits and seeds. This technique demonstrated its flexibility for the analysis of macroscopic and mesoscopic samples. The identification of targeted molecules was conducted using MS/MS. In 2024, a similar concept was put together using a 532 nm laser ablation system followed by a dielectric barrier discharge ionization (LA-DBDI) to study pesticide uptake and translocation in tomato plants [43].

The imaging techniques described here are examples of MSI under vacuum and ambient conditions, as well as coupled techniques. Now, we will review methods for elucidating and validating plant molecules from MSI experiments.

2. Plant Compound Elucidation in Mass Spectrometry Imaging (MSI)

The metabolomics standards initiative defined four plus one) levels of metabolite identification, which reflect their experimental support [44,45]. These levels were revised, and now, using five levels of confidence is recommended for both untargeted metabolomics and MSI experiments [46–48].

We represent them in reverse order with examples of applications from the least to the highest confidence level:

- **Level 5 - Exact mass of interest:** The raw data contain defined *m/z* signals that can be mapped to the sampled surface. With sufficient analytical resolution, it can be assumed that the *m/z* features correspond to unique compounds. Of course, isobaric molecules cannot be distinguished. Features are not identified; however, quantitation and statistical analyses for finding regions of interest (ROIs) or potential biomarkers are possible.
- **Level 4 - Molecular formula:** High-resolution mass spectrometry (HR-MS) data, fragmentation experiments, and isotopic patterns permit calculating the chemical sum formula. The results can be compared with databases to find a possible match.
- **Level 3 - Tentative structure:** Using HR-MS data, tandem MS directly from tissues, in-source decay spectra, isotope distribution, and databases. More than one compound can be explained using the available data. This level requires complementary information, such as multimodal imaging techniques, fluorescence microscopy, IR spectroscopy, immunolocalization, chemical staining for functional groups, tissue extracts, and subsequent analysis using GC-MS and LC-MS.
- **Level 2 - Probable structure:** Further refinement leads to a single structure candidate. The results obtained in level 3 are assessed using expert knowledge, biological context, and bioinformatic analyses. For example, genome analyses and chemoinformatics can reveal theoretically possible metabolites.
- **Level 1 - Confirmed structure:** Unequivocal three-dimensional chemical structure identification. Requiring at least two independent and orthogonal methods should provide different types of

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information and not be affected by the exact source of error. For example, Nuclear Magnetic Resonance (NMR) supports structural studies, and isotopic labeling techniques enable tracing the path of a molecule through a reaction or a metabolic pathway. An authentic standard is required; in MSI, it is a common practice to spike it into a replicated biological tissue.

Figure 1 illustrates the confidence levels for mass spectrometry imaging experiments and suggests methods for compound identifications that can be combined with mass spectrometry imaging (MSI).

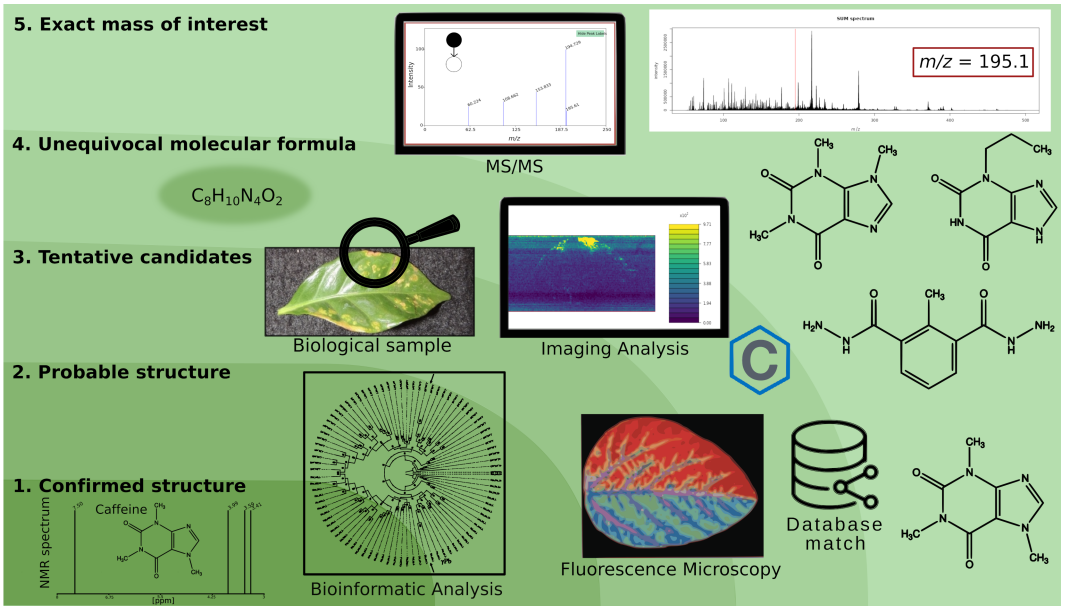

Figure 1. Levels of confidence for the identification of compounds in mass spectrometry imaging.

Table 1. Identification levels for mass spectrometry imaging (MSI) with examples of applicable methods.

ID Level	Requirement	Mass Spectrometry Imaging
1 – Confirmed structure	Unambiguous (3D) structure from at least two independent and orthogonal methods, which refer to methods that provide different types of information and are not affected by the same sources of error **and comparison to an authentic reference sample**.	Recovery of material from regions-of-interest (ROI), which are specific areas selected for detailed analysis; structural studies with orthogonal methods (e.g., NMR and HR-MSn); isotopic label studies, which involve the use of isotopes to trace the path of a molecule through a reaction or a metabolic pathway.
2 – Probable structure (single candidate)	Like Level 3, but with only one candidate left.	Filtering results with expert knowledge and bioinformatic analyses (e.g., theoretically possible metabolites from genome analyses and chemoinformatics).
3 – Tentative structure (multiple candidates)	HR-MS(n) data match with databases and are congruent with additional experiments and the biological context. Still, more than one compound can be explained with the available data.	High-resolution *m/z* data, direct fragmentation from tissues, in-source decay spectra, and isotope distribution data. Matching with databases and comparison with theoretical spectra. Multimodal imaging (e.g., fluorescence and infrared spectroscopy microscopy; immunolocalization); complementary studies with excisions from regions-of-interest (ROIs) or complete extractions, using GC-MS and LC-MS; chemical staining for functional groups.
4 – Molecular formula	HR-MS(n) and isotopic distribution data of *m/z* features that support the elemental composition of compounds	Calculation of theoretical mass spectra and comparison with experimental data; database matches.
5 – Exact mass of interest	*m/z* features are not identified, but unique.	Quantitation and statistical evaluation of *m/z* bins according to their signal intensity.

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Reaching level 1 for proper chemical identification is challenging for several reasons, such as the need for reference standards, low signal intensities and low abundance for the compound of interest, and the overlap of *m/z* features. Therefore, it is crucial to define the biological question and, based on this, identify a suitable experimental strategy. The following section discusses the steps commonly used to perform MSI experiments.

3. Experimental Steps in MSI

Figure 2 presents the four steps of MSI studies:

1. Sample preparation.
2. MSI analysis (data collection).
3. MSI data analysis.
4. Supportive techniques.

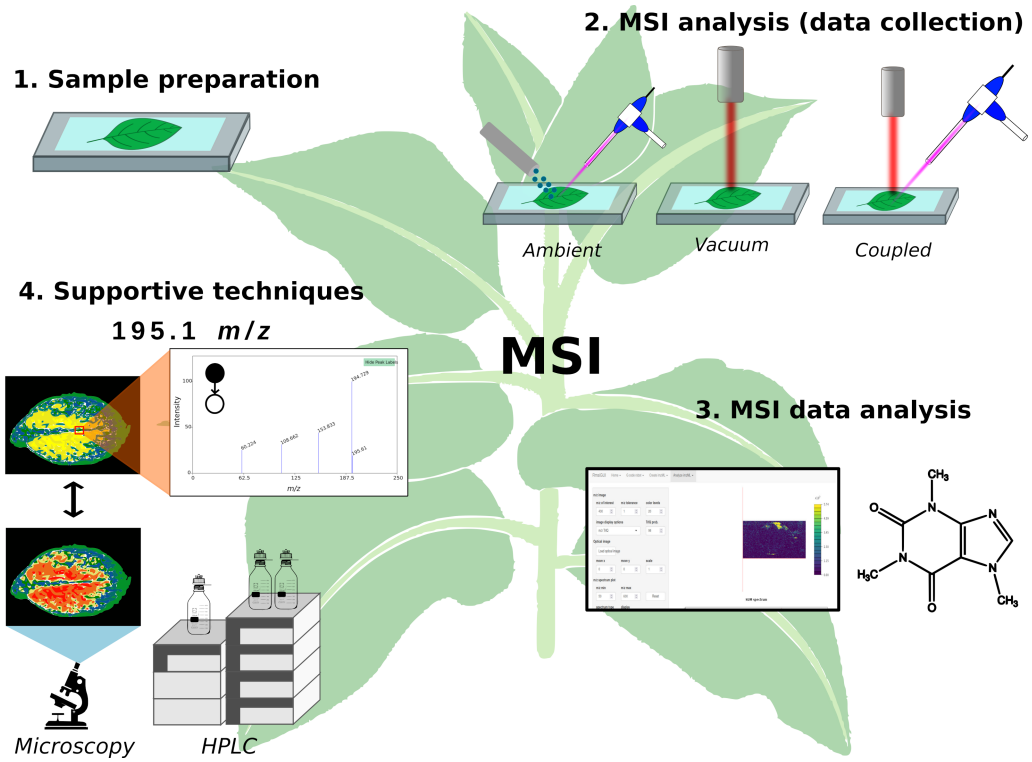

Figure 2. Experimental steps of mass spectrometry imaging (MSI).

3.1. Sample Preparation

MSI analysis typically requires extensive preparation to accurately capture the metabolic status of the biological tissue at a specific point and under defined conditions (e.g., seedlings under drought stress vs control seedlings). Over the past 20 years, the predominant technique for MSI in plant science has been MALDI, followed by the emerging ambient ionization technique DESI. Ambient ionization techniques require much less sample preparation, and ideally, the biological materials can be analyzed 'as is.'

3.1.1. Sample Preservation

It is essential to have appropriate handling and preservation techniques for the samples to ensure the molecules' original distribution, abundance, and identity. Variables such as water content, storage time, and temperature to quench metabolism have to be considered. Plant tissues are commonly freeze-dried (e.g., for SIMS analysis) or frozen, sectioned, and stored at -80 ºC (e.g., for MALDI or DESI analyses) to prevent enzymatic degradation or analyte diffusion [49]. Freezing biological material with a high water content without further precautions is not recommended. Immediate freezing and

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storing plant tissues can alter the sample's initial shape over time due to water sublimation, which causes tissue shrinking and the delocalization of compounds. A strategy to avoid delocalization is to slide tissue sections, mount them in glass slides, vacuum-dry them, and then store samples in falcon tubes with holes vacuum-sealed in plastic bags [3]. Using a method that quenches the metabolism is highly recommended for any biological sample. For example, after being embedded in gelatin and immediately frozen for cryosectioning, maize leaves may be warmed and vacuum-dried [50].

3.1.2. Sectioning

Once the biological tissue has been adequately preserved, the next step is typically the sectioning via a microtome or a cryo-microtome, and the resulting sections are mounted on a flat surface. However, this step depends on the compounds of interest, the selected MSI technique, and the tissue type. For example, for MALDI imaging, conductive glass slides are recommended [4]. The thickness of the tissue sample is also essential. Finer slices improve the conductivity in MALDI experiments. Cutting thin tissue slices with a high water content is technically challenging. Typically, plant tissue slices are in the range of 50 μm [3]. Thin tissue sections are not always necessary, e.g., for tiny seeds (1-3 mm in diameter). There is a limitation due to the thickness of some seeds, leaves, flowers, and even small roots; therefore, it can not be sectioned. Thus, the tissues may be studied intact or imprinted [51,52]. In 2015, transversal cuts of maize leaves with a thickness of 10 μm were obtained [50]. Thin tissue slices were also achieved by placing a root between two polystyrene sections and cutting it tangentially [53].

Histology methods for sectioning have been widely adopted for MSI. However, plant tissue fixation, washing, and staining are incompatible with MS-based techniques. Polyethylene glycol should be avoided because of metabolite diffusion and signal suppression [3]. New ambient imaging techniques presented reduce the sample preservation and sectioning; for instance, with the use of LD-LTP and LADI, sample preservation is not necessary for tissues with low water content, and sectioning of the samples can be accomplished with a scalpel [41,42].

3.1.3. Matrix Application

In MALDI and MALDESI, a chemical matrix is required. Commonly, this matrix is a small organic compound that facilitates the desorption and ionization of the compounds of interest. The matrix choice is analyte-dependent and is crucial for MSI to avoid metabolite diffusion, which is an essential issue in matrix-based techniques. In the case of laser-based methods, there are alternative options to standard MALDI matrixes. For LDI-MSI, a matrix is not necessary [54]. An alternative approach is using nanoparticles, which have the advantage that they do not ionize themselves, thus reducing the matrix interference. In 2020, Shiono *et al.*, demonstrated that the use of nanoparticles for laser desorption ionization improved the detection of phytohormones from 5 to 9 of the nine tested phytohormones, compared with MALDI [55].

3.1.4. Liberation of Plant Cell Compounds

Adequate sample handling avoids diffusion and degradation through enzymatic processes, light, heat, and atmospheric exposure [3]. The preparation and treatments employed before the analysis in plant samples differ from those used in mammalian tissues because of the plant cuticle and the cell wall barriers. Three strategies have been employed to address these challenges.

The first strategy is using an efficient ionization/desorption source according to the plant tissue. Alternatively, coupled ionization/desorption techniques may be employed. In this case, a desorption/ablation source penetrates the tissue, facilitating the release of the molecules. Subsequently or simultaneously, an ionization source is employed to assist the process, for example, a laser, as in LAESI or LD-LTP.

The second strategy involves imprinting a plant tissue section on flat surfaces, such as PTFE or nylon sheets [24,52]. This approach is considered an indirect technique for MSI. This previous process avoids the potential for interferences caused by the cuticle, cellular wall, and water content.

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The third strategy is to remove the plant cuticle, which can be achieved through two methods: a chemical wash-off, which facilitates the removal of the wax layer from tissues and, thereby, boosts detectable compounds that would otherwise be undetectable in a direct technique. The analysis of chloroform-dipped *Arabidopsis* leaves was presented in 2008 by Cha *et al.* [9]. However, this process may also result in the delocalization or washing away of many other compounds, as Tong *et al.* demonstrated in 2022 [51].

3.1.5. Derivatization

In-situ derivatization, also known as *on-tissue* chemical derivatization (OTCD), is a process whereby the molecules are chemically modified directly on the tissue to enhance their ionization efficiency and detection. For instance, such chemical treatment is employed when the compounds of interest have low ionization efficiency or stability. Also, the concentration of the analytes of interest may be low, leading to interference in their detection from other compounds. MSI analysis is expected to detect unwanted molecules, which can give rise to interferences or matrix effects and mask the targeted molecules. Consequently, an *in-situ* derivatization may be employed. Nevertheless, as with any chemical modification, the OTCD is specific for functional groups, and thus, this is used for targeted metabolomics.

A promising OTCD on plant tissue was reported in 2023 by Zemaitis *et al.* to enhance the detection in MSI using MALDI [56]. The authors applied 4-(2-((4-bromophenethyl) dimethylammonio) ethoxy) benzenaminium bromide (4-APEBA) to derivatize molecules from soybean nodules and poplar roots. The application of 4-APEBA increased the detection of various compounds, including amino acids and hormones, reducing sugars, aldehydes, carboxylic acids, and others [57]. This chemical reagent represents a promising tool for MSI as it enables imaging of the distribution of metabolites of opposite polarities and hydrophobicities [56].

3.2. MSI Analysis (Data Collection)

The confidence in assigning molecular identities is reduced In mass spectrometry imaging (MSI) because of the absence of separation methods. Therefore, it is recommended to use high-resolution (HR) or ultra-high-resolution analyzers (UHR) MS analyzers. Furthermore, including other methods, such as ion mobility spectrometry (IMS), mass fragmentation experiments, and utilizing isotopically labeled standard reagents, can increase the confidence level of compound identifications.

This section will discuss these approaches and their respective limitations. Using HR-MS or UHR-MS for imaging can reduce isobaric interferences, improving the identification confidence, but UHR-MS is not a routine approach in MSI of plants [48].

Recently, the combination of MALDI with Fourier-transform ion cyclotron resonance (FT-ICR) for MSI has been reported. As FT-ICR is considered a technique of UHR, combining FT-ICR with MALDI can potentially increase the confidence level in plant MSI. However, higher mass and spatial resolutions require higher measurement times and computational power [58].

Another critical element is the spatial resolution for MSI, crucial to answering a biological question. For example, a high spatial resolution allows for exploring the metabolites at the single-cell and organelles level [10]. In 2009, Holscher *et al.*, reported the distribution of secondary metabolites in *Arabidopsis thaliana* and Hypericum species at the single-cell level, using LDI, achieving a lateral resolution of 10 x 10 μm. This resolution is sufficient for sampling at the plant cell level (10-100 μm) [59]. Harada *et al.* (2009) demonstrated MSI of volatiles at the organelle level of the ginger rhizome by LDI-QIT-TOF [11].

In addition, the precision of the sampling stage and the synchronization with the mass analyzer are crucial for the correct assembly of the MSI datasets.

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3.3. MSI Data Analysis

Mass spectrometry data analysis workflows require the following steps [60]:
1. Raw data import/export and conversion (if necessary). 2. Spectra preprocessing. 3. Features analysis. 4. Statistics and data mining. 5. Integration and interpretation.

MSI data analysis is similar but includes features focused on visualization and quantification. Usually, an MSI analysis contains several hundreds or even thousands of features, and depending on the research question, identifying these ions can be less or more complex. The steps for analyzing MSI data are more directed toward visualizing metabolite distribution and biological interpretation. The first step for analyzing MSI data involves creating the spatial distribution of any given mass-to-charge value or range.

Commercial instruments with MSI setup have vendor-specific software for data processing. In addition, academic instrument developers and programmers are creating in-house software to analyze the data recorded with their own or any given MSI instruments [61]. The vendor's software requires purchasing a license, which can be costly and sometimes lacks the functions needed to answer a bioanalytical question. Therefore, MSI data processing workflows with open-source software are attractive for academic researchers. A file conversion from instrument data to community file formats is usually necessary. The standardized data format for mass spectrometry imaging data imzML has facilitated data management and MSI data exchange [62]. Many vendors' software can now read imzML files (MassLynx, FlexImaging, Imaging For Windows) or provide an option to export imzML files (http://ms-imaging.org/imzml/software-tools/). In addition, several open-source MSI programs provide data conversion [63] and support for imzML files [37,64–67].

PRIDE (https://www.ebi.ac.uk/pride/) [68] and METASPACE (https://metaspace2020.eu/) provide public repositories for uploading MSI files to facilitate collaboration in the research community.

The analysis of MSI data often includes visualization of metabolite distribution, quantitation, or other statistical features, including principal component analysis (PCA). Free and vendor software, such as MSiReader and msiQuant [37,69], is available.

In addition, the open-source program RmsiGUI integrates the control of a robotic imaging platform and the compilation of imzML [61]. Software like MSiReader or RmsiGUI (R package) allows the overlay of optical and ion images [37,61].

In general, MSI software has options for visualization, normalization, or multivariate analysis, but only a few include a molecular annotation/identification function, such as LipostarMSI, METASPACE, Scils Lab, or Cardinal [70–72].

MSI Software offers essential functions for understanding plant metabolism, like color scale intensity-based for ion distribution, to compare the distribution between different single scale colors. Programs like Datacube Explore or Scils Lab offer ROI quantification, which can help understand the state of plant metabolism.

Accurate mass filtering (e.g., HRMS) and a tandem MSI analysis support the identification of compounds from MSI experiments. An R package, rMSIFragment, recently included a function to improve lipidomics annotation considering in-source fragmentation [73]. In 2023, Wadie *et al.* developed METASPACE-ML, a machine-learning model for METASPACE, which includes a false rate discovery (FDR) calculation, to improve the reliability in metabolite annotation [71]. Most data used to train the model were MALDI-based and are still in development. However, tools like METASPACE-ML and rMSIfragment will increase the confidence for future MSI data annotation and identification.

Several free MSI data processing programs are implemented in the free statistical computing and graphics programming language R. R has an active user community and many additional data evaluation and visualization packages. However, R is an interpreter and, therefore, relatively slow. Reading imzML with the Julia language resulted in 100 times faster loading speeds than R, demonstrating the potential for the future data mining of massive MSI datasets [74].

Using dedicated databases for plants (e.g., PlantCyc, https://www.plantcyc.org) increases the confidence in compound identification compared to using generic databases [75].

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Integrating MSI with multi-omics is a promising and valuable approach to understanding the gene-metabolite relationship and discovering novel gene-associated metabolites [76]. In 2020, Dong *et al.*, demonstrated that coupling MSI with RNA interference, gene silencing, agro-infiltration, or samples derived from plant natural variation could spatially map an entire metabolic pathway [76].

Despite the advances in software for MSI data processing, there is still much left to do. Currently, MSI data analysis is mainly manual, which is time-consuming and error-prone. Thus, the development of automated analysis of MSI datasets is required. For a more detailed revision of MSI software, check Weiskirchen *et al.*, 2019 [66].

3.4. Supportive Techniques

Even with HR-MS instruments, identifying and confirming structural isomers is complicated [77]. Therefore, verifying compounds identified in an MSI experiment usually requires supportive techniques. For fragmentation studies on the features of interest, it is recommended to use the same ionization source and references. Fragmentation patterns can be compared with or without spiking the tissue.

ROIs can also be extracted from the tissue and analyzed with LC-MS [51,77]. Recovering the ROI is also handy when reference standards are unavailable [11,52].

Additional techniques are often used to analyze the analytes' chemical and physical properties, e.g., using HPLC coupled to diode array detection (DAD).

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Table 2. Overview of strategies for mass spectrometry imaging (MSI) and supportive techniques used in the identification of plant metabolites. Abbreviations are listed below.

Chemical Class	Analyte	MSI Techn.	Orthol. Methods	Complementary Techn.	ID Level	Refs.
Phenolic compounds	Resveratrol, pterostilbene, stilbene phytoalexins	LDI and MALDI	HPLC-DAD	Fluorescence imaging (macroscopy), confocal fluorescence microscopy	Level 2	[78]
Volatiles and phenolic compounds	Gingerol and terpenoids	AP-LDI	AP LDI MS/MS	Optical microscopy	Level 2	[11]
Flavonoids	Kaempferol, quercetin and isorhamnetin	LDI	AP-MALDI and CID (TOF/TOF)	-	Level 2	[10]
Flavanones	Baicalein, baicalin, wogonin	MALDI	MALDI-Q-TOF-MS	Optical microscopy	Level 2	[79]
Phenolic compounds and carbohydrates	Jasmone, hexose sugars, salvigenin, flavonoids, and fatty acyl glycosides	DESI-MSI	FS FAAS	-	Level, 2, level 3 and level 4	[80]
S-glucosides	Glucosinolates	MALDI, LAESI	ESI (chip-ESI)	-	Level 2	[81]
Phenolic compounds and carbohydrates	Salvianolic acid J	DESI	LC-MS	-	Level 3	[51]
Organic acids, phenolics and oligosaccharides	Ascorbic acid, citric acid, palmitic acid, linoleic acid, linolenic acid, oleic acid, apigenin, kaempferol, ellagic acid, quercetin, apigenin, fructose, glucose, sucrose	MALDI, GALDI	-	-	Level 2	[82]
Amino acids, phenolic compounds, lipids	Indoxyl, clemastanin B, isatindigobisindoloside G, gluconapin, guanine, adenine, adenosine, sucrose, histidine, lysine, arginine, proline, citric acid, malic acid, linolenic acid,	MALDI	DESI-Q-TOF	-	Level 2	[83]
Hydrocarbons and flavonoids	C29 alkane, kaempferol-hexose and quercetin-rhamnose	MALDI	DESI-MS, LAESI-MS, SIMS	-	Level 2	[84]

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Table 2. *Cont.*

Chemical Class	Analyte	MSI Techn.	Orthol. Methods	Complementary Techn.	ID Level	Refs.
Phenolic compounds	Resveratrol, pterostilbene, stilbene phytoalexins	LDI and MALDI	HPLC-DAD	Fluorescence imaging (macroscopy), confocal fluorescence microscopy	Level 2	[78]
Glycoalkaloids and anthocyanins	Tomatidine, α-tomatine, dehydrotomatine	MALDI	LC-MS/GC-MS	Electron microscopy imaging	Level 1	[76]
Fatty acid and amino acids	Palmitic acid, stearic acid, oleic acid, inositol, β-Alanine and tomatidine	MALDI	-	RT-qPCR	Level 2	[85]
Organic acids	Citrate, malate, succinate, fumarate	MALDI	UPLC-HRMS/MS	-	Level 1	[52]
Anthocyanins	Choline, pelargonidin	MALDI, SIMS	MALDI-MS/MS	Optical microscopy	Level 2	[86]
Lipids	Cuticular lipids	MALDI	GC-MS	-	Level 5	[87]
Terpenoids and diterpenoids	Vitexilactone, vietrifolin D, rotundifuran	MALDI	GC-MS	-	Level 3	[88]
Lipid droplet associated protein	Wax ester & Triacylglycerol	MALDI	-	Confocal micrographs of LDAP	Level 4	[89]
Nitrogenated and phenolic compounds	Cocaine, cinnamoylocaine, benzoylecgonine, etc	MALDI, LDI	ESI	-	Level 4	[90]
Organic acids, carbohydrates, flavonoids, lipids	Nobiletin, phenylalanine, trans-Jasmonic Acid, quinic acid, ABA, other	DESI	LC-MS/MS	-	Level 3	[91]
Triacylglycerol and phosphatidylcholines	Palmitic acid, vaccenic, linoleic, and α-linoleic acids	MALDI	NMR, ESI	-	Level 1	[92]
Phytohormones	Abscisic, auxin, cytokinin, jasmonic acid, salicylic acid	PALDI	MALDI	-	Level 2	[55]

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Microscopy can be supported by staining, immunolabelling, and label-free techniques. These labeling methods allow the detection of specific molecules in plant tissues and provide information about the location of enzymes, lipids, carbohydrates, and other molecules.

Dyes like Congo Red or Calcofluor White (CW) stain carbohydrates such as the β-(1→4)-glucans such as cellulose, callose, xyloglucans, and chitin [93,94].

In contrast, label-free imaging techniques use novel imaging technologies that do not require stains or fluorescent markers for visualizing plant cell organelles and structures. Ultraviolet microscopy enables the visualization of lignin and other aromatic compounds within plant tissue.

In 2017, Becker *et al.*, demonstrated the efficacy of combining other imaging techniques with MSI. The researchers employed MALDI and LDI for MSI and confocal fluorescence microscopy (CLSM) to study the distribution of phenolic compounds (stilbene phytoalexins, namely resveratrol, pterostilbene, piceids, and viniferins) in grapevine leaf (*Vitis vinifera*). Moreover, the authors supported their findings in MSI using CLSM and HPLC-DAD techniques to detect, corroborate, and quantify phenolic compounds. CLSM produces high-resolution images without damaging the sample, thus allowing the acquisition of fluorescent images at the organ, tissue, and multicellular levels. Using LDI for MSI (266 nm laser) in positive mode, they could detect, characterize, and localize metabolites such as stilbenes without matrix application. HPLC with a diode array detector (DAD) added quantitative data and validated stilbenes' compound identification.

Dong *et al.* in 2020 not only used MSI but complemented MSI information with different reverse genetics approaches to elucidate gene function [76]. They highlighted the potential of integrating MSI with reverse genetics techniques to elucidate gene functions by analyzing the spatial distribution of metabolites and correlating the MSI results with gene expression. The study employed Virus-Induced Gene Silencing (VIGS), an RNA-mediated reverse genetics technology, to downregulate endogenous genes and analyze their functions and was complemented with liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS). In addition, the study employed scanning electron microscopy (SEM) as a complementary imaging technique.

The continued development of multimodal imaging workflows integrating MSI with super-resolution fluorescence microscopy, electron microscopy, spectroscopic imaging techniques like Raman and FTIR, and other emerging methods holds tremendous potential. Innovative multimodal image acquisition, data fusion algorithms, and open data repositories will be critical for fully exploiting the power of multi-scale, multimodal chemical imaging in both fundamental plant biology research and applied areas like food science, bioenergy, and phytoremediation.

4. Conclusions and Outlook

Mass spectrometry imaging (MSI) of plants has become a routine method for plant research. However, the unequivocal identification (ID level of 1) of chemical structures in MSI data is still challenging. Depending on the bioanalytical question, we suggest a targeted MSI strategy for specific compounds or the statistical analysis and data mining of m/z features with lower ID confidence (level 2-5), with a subsequent identification of features of interest using complementary methods.

High-resolution mass spectrometry (HR-MS) analyzers with ion mobility pre-separation facilitate the discrimination of distinct molecule ions. Nevertheless, in most cases, complementary analytical methods will be necessary for identification. In addition, the comparison with authentic reference standards for level 5 identifications is not trivial in MSI, and new strategies are needed to overcome practical limitations.

In the near future, we expect advances in integrating instrumental methods with chemoinformatic tools that use advanced algorithms such as machine learning and artificial intelligence to detect and identify biologically important features in MSI datasets. A prerequisite for these developments is the adoption of community file formats, FAIR data sharing and algorithms, and software with permissive, open licenses.

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Abbreviations

We followed the "Periodic Table of Mass Spectrometry (MS) Terms" [95]. The following abbreviations are used in this manuscript:

ABA	Abscisic acid
AP	Atmospheric pressure
LDI	Laser desorption ionization
MALDI	Matrix assisted laser desorption ionization
SMALDI	Scanning microprobe matrix assisted laser desorption ionization
CW	Calcofluor-white
CLSM	Confocal laser scanning microscopy
DAD	Diode array detector
DART	Direct analysis in real time
DESI	Desorption electrospray ionization
EIC	Extracted-ion chromatogram
FAPA	Flowing atmospheric-pressure afterglow
FDR	False discovery rate
FS-FAAS	Fast sequential flame atomic-absorption spectrometry
FT-ICR	Fourier-transform ion cyclotron resonance
FT-IR	Fourier-transform infrared spectroscopy
GALDI	Graphite-assisted laser desorption ionization
GC	Gas chromatography
GFP	Green fluorescent protein
HR	High resolution
IR	Infrared
LA	Laser ablation
DBDI	Dielectric barrier discharge ionization
LADI	Laser ablation direct analysis in real time
LAESI	Laser electrospray ionization
LAAPI	Laser ablation atmospheric pressure photoionization
LC	Liquid chromatography
LDAP	Liquid droplet-associated protein
LDI	Laser desorption ionization
LD-LTP	Laser desorption low-temperature plasma
LMD	Laser micro-dissection
LTP	Low-temperature plasma
MALDESI	Matrix-assisted laser desorption electrospray ionization
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
MSI	Mass spectrometry imaging
Nd:YAG	Neodymium-doped yttrium aluminum garnet
NMR	Nuclear magnetic resonance
OTCD	On-tissue chemical derivatization

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PALDI	Plasma assisted laser desorption ionization
QIT	Quadrupole ion trap
ROI	Regions-of-interest
SAMDI	Self-assembled monolayer desorption ionization
SIMS	Secondary-ion mass spectrometry
SEM	Scanning electron microscopy
TOF	Time of flight
UHR	Ultra high resolution
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
VIGS	Virus-induced gene silencing

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