

The evolution of DESI-MSI: A comprehensive look at preprocessing and quantification strategies for metabolomics

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Abstract

Mass spectrometry imaging (MSI) has become an indispensable tool in metabolomics for visualizing the spatial distribution of biomolecules within tissues. Due to its direct, ambient analysis capability, desorption electrospray ionization (DESI-MSI) has evolved from a rapid screening tool into a precise and reliable quantitative platform. We delve into its technical principles and particularly review the criticality of sample preprocessing, highlighting the paradigm shift from a “no-preprocessing” approach to one of “optimized preprocessing.” The paper details how factors like sample preservation, choice of embedding agents, washing, drying, and on-tissue chemical derivatization all impact data quality. We also discuss how the addition of standards and internal standards can mitigate matrix effects and signal variability, enabling accurate quantitative analysis. Finally, this article looks ahead to the future of DESI-MSI, including its combination with emerging technologies such as tissue expansion mass spectrometry imaging to achieve single-cell-level spatial resolution and open up new possibilities for metabolomics research.

Keywords: DESI-MSI, Mass spectrometry imaging, Sample preprocessing

1. Introduction

Mass spectrometry (MS) is a pivotal methodology within the disciplines of pharmaceutical evaluation, chemical profiling, and metabolomic investigations, attributed to its exceptional specificity and enhanced sensitivity. By ionizing analytes and subsequently separating them according to their mass-to-charge ratio (m/z), MS enables both qualitative and quantitative analyses. Advancements in spatial sampling methodologies and instrument engineering have fostered the integration of MS with image coordinates, culminating in the development of mass spectrometry imaging (MSI), which adeptly visualizes the molecular distributions within biological specimens without the necessity for dyes or antibody labels [1,2]. MSI employs a raster-scanning technique on tissue sections, capturing a spectrum at

each pixel; the intensity values at designated m/z points are then depicted as ion images to effectively map the presence of metabolites, lipids, and proteins [1,2].

The field of MSI primarily utilizes several prominent ionization techniques, each offering distinct advantages. The most widely employed methods include matrix-assisted laser desorption/ionization (MALDI-MSI), secondary ion mass spectrometry (SIMS-MSI), and desorption electrospray ionization (DESI-MSI) [2]. These techniques are highly complementary, and the choice of which to use is critically dependent on the specific characteristics of the sample and the overarching analytical objectives. For instance, MALDI-MSI is well-suited for a broad range of biomolecules and provides high mass resolution, while SIMS-MSI excels in achieving superior spatial resolution, making it ideal for subcellular

Received 19 September 2025; accepted 9 December 2025.
Available online 15 June 2026

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<https://doi.org/10.38212/2224-6614.3583>

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analysis. In contrast to MALDI and SIMS, DESI-MSI offers benefits such as reduced sample preparation requirements, functioning in ambient conditions, and compatibility with small molecules that are non-volatile and sensitive to heat, thus enabling in situ and near-real-time chemical imaging. Its gentle ionization technique permits multiple measurements on the same sample under varying conditions, thereby improving high-throughput metabolomics and lipidomics [1]. Although “direct analysis” is a key focus in DESI, pretreatment is still crucial in certain situations to enhance the signal-to-noise ratio, reduce matrix effects, or stabilize the sample. This review emphasizes the core principles of DESI-MSI and showcases the effects of sample pretreatment on signal response, molecular coverage, and quantitative imaging techniques, followed by a discussion of current challenges and prospective directions.

2. Principles of desorption electrospray ionization mass spectrometry imaging (DESI-MSI)

Desorption electrospray ionization mass spectrometry imaging (DESI-MSI) represents a significant advancement in ambient ionization methodologies, facilitating the direct acquisition of mass spectral data from sample surfaces at atmospheric pressure. This intrinsic property negates the necessity for a vacuum environment and eradicates the cumbersome process of matrix deposition, which are conventional prerequisites for established mass spectrometry imaging (MSI) techniques such as matrix-assisted laser desorption/ionization (MALDI) and secondary ion mass spectrometry (SIMS) [2]. Fundamentally, the DESI procedure distinctly amalgamates electrospray ionization (ESI) with a surface desorption mechanism.

The fundamental concept encompasses a charged solvent aerosol, typically constituted of micro-droplets, being propelled at a high velocity towards a specimen affixed to a motorized platform. Upon collision, the primary droplets establish a thin liquid layer on the surface of the sample, thereby facilitating the extraction of analytes into the solvent. Subsequently, through the dynamics of momentum transfer and shear forces, secondary droplets enriched with the extracted analytes are expelled from the surface. These secondary droplets subsequently traverse through the surrounding gas medium, which is generally nitrogen, undergoing an expedited desolvation process via solvent evaporation and coulombic fission. Ultimately, in accordance with established theoretical frameworks

such as the ion-evaporation and charge-residue models, this process yields gas-phase ions possessing single or multiple charges, which are subsequently directed into a mass spectrometer for analytical evaluation [1].

A principal advantage of DESI-MSI lies in its adaptability to integrate with a variety of mass spectrometric analyzers, such as quadrupole, linear ion trap, quadrupole time-of-flight (QTOF), and triple-quadrupole configurations, thereby providing ample flexibility to accommodate diverse analytical requirements [1]. In contrast to vacuum-based methodologies such as MALDI-MSI and SIMS-MSI, DESI offers several significant advantages. These advantages encompass a reduction in the necessity for extensive sample preparation, a gentle ionization process for nonvolatile and thermally sensitive compounds, as well as the capacity to modulate selectivity through the incorporation of acids, bases, metal salts, or on-line derivatization agents within the spray solvent.

3. From “no preprocessing” to “optimized preprocessing”: the inevitable evolution of technological maturity

An overview of DESI-MSI analysis can be found in Fig. 1. Early literature and technological promotion often highlighted a key selling point of DESI-MSI: its “sample preparation-free” nature. This represented a significant advantage over techniques like MALDI-MSI, which require intricate applications and must operate within a vacuum environment. This streamlined workflow capability made DESI-MSI particularly well-suited for rapid on-site screening and real-time analysis. However, as the application of DESI-MSI has deepened into complex biological substrates, the notion of “no sample preparation” has revealed itself to be a relative concept rather than an absolute truth. The inherent complexity of biological tissue matrices, including their native salt content, high lipid abundance, and diverse metabolite composition, can significantly influence mass spectrometry signals through matrix effects, leading to phenomena such as ion suppression or signal interference. These challenges are difficult to fully overcome solely by adjusting instrumental parameters, such as spray nozzle geometry or solvent flow rates.

Consequently, to ensure the accuracy, sensitivity, and reproducibility of data, the purposeful and strategic preprocessing of samples has become the prevailing consensus in current research. This shift in thinking—from “completely unprocessed” to “optimized processing”—signifies the maturation

Desorption electrospray ionization mass spectrometry imaging analysis process

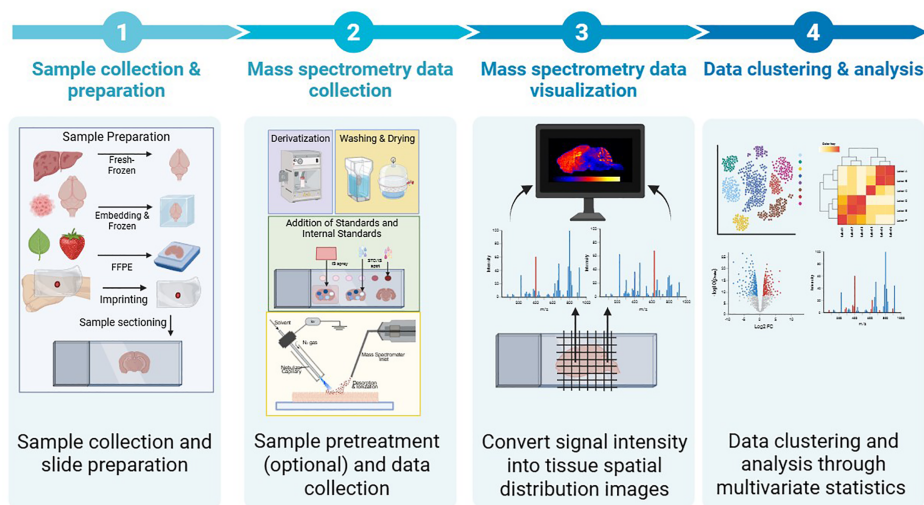


Fig. 1. A comprehensive workflow for desorption electrospray ionization mass spectrometry imaging (DESI-MSI) analysis. This figure provides a detailed, step-by-step overview of the entire DESI-MSI analysis process, from sample collection to data analysis. The workflow is divided into four main stages: 1. Sample Collection & Preparation: Tissues are prepared using various methods, including fresh-frozen, formalin-fixed paraffin-embedded (FFPE), or via imprinting. The prepared tissues are then processed into thin sections and mounted on slides for analysis. 2. Sample Pretreatment & Data Collection: This stage highlights the crucial pre-analysis steps that enhance data quality. These optional but vital processes include on-tissue derivatization, washing and drying to remove interferents, and the addition of internal standards to enable quantification. The prepared sample is then placed in the DESI-MSI system for data collection. 3. Mass Spectrometry Data Visualization: The raw mass spectral data from each pixel on the sample is processed. The signal intensity of specific mass-to-charge (m/z) values is converted into a 2D spatial map, visualizing the distribution of target molecules within the tissue. 4. Data Clustering & Analysis: The final stage involves advanced data analysis. The complex mass spectrometry data is processed using multivariate statistics and clustering algorithms. This allows researchers to identify molecularly distinct regions within the tissue, create heatmaps of correlated features, and perform quantitative comparisons, ultimately revealing deeper biological insights. Created in BioRender. Cheng, M. (2025).

of DESI-MSI technology from a rapid screening tool towards a more rigorous and reliable platform for quantitative and qualitative analysis. The focus of sample preparation has transitioned from absence (“no”) to targeted optimization to overcome these inherent fundamental limitations and maximize the quality and depth of analytical results.

Sample preparation methods, encompassing aspects from sample acquisition, embedding agent selection, section preparation, storage conditions, sample washing and drying, to the addition of internal standards (Fig. 2), have a profound impact on mass spectrometry imaging detection results and subsequent analysis. The following sections will detail the influence of various preprocessing steps, along with their respective advantages and disadvantages.

3.1. The impact of sample preservation: fresh-frozen vs. formalin-fixed paraffin-embedded tissues

Formalin-fixed paraffin-embedded (FFPE) tissues have historically served as the foundational

element of conventional histopathology, esteemed for their remarkable capacity for tissue preservation and their virtually limitless duration of storage. The capacity to maintain FFPE specimens at ambient temperature for prolonged intervals significantly enhances the efficiency of sample collection protocols and mitigates expenditure, thereby facilitating comprehensive research endeavors that utilize clinical biobanks established over decades on a global scale. Nevertheless, the process of formalin fixation induces irreversible modifications to the molecular architecture of specimens, resulting in extensive covalent cross-linking among proteins.

Furthermore, the fixation and dehydration steps are prone to leaching substantial amounts of soluble small molecule metabolites, including highly polar species such as inorganic salts, amino acids, organic acids, and carnitines [3,4]. This process can also distort or lead to the complete loss of metabolite distribution due to uncontrolled diffusion of endogenous small molecules and drugs during fixation. Endogenous lipids are particularly

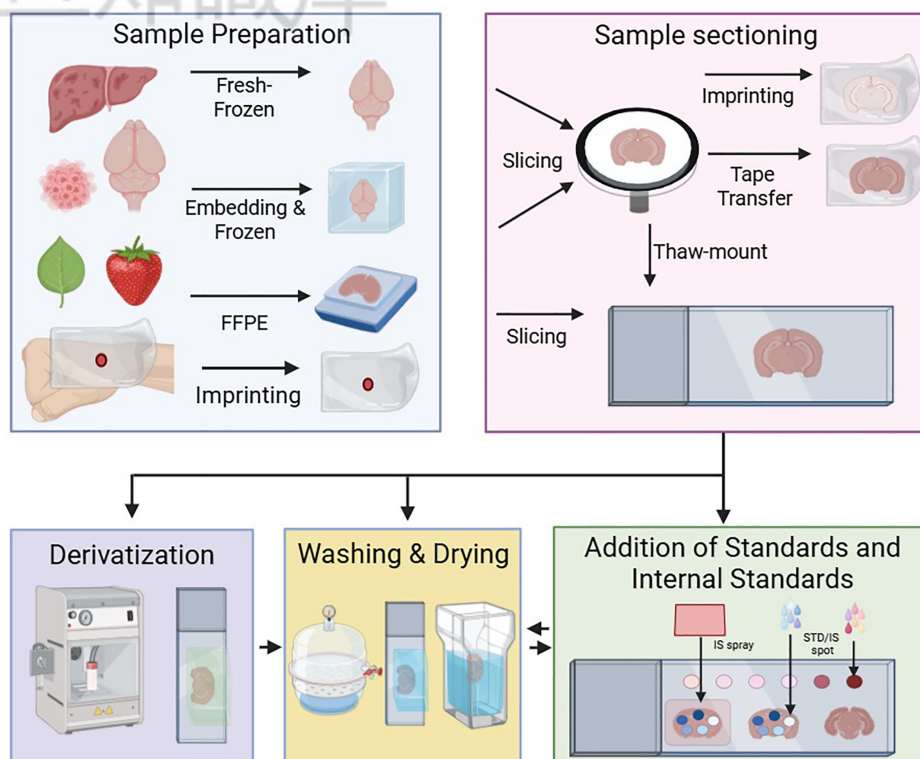


Fig. 2. Comprehensive workflow for desorption electrospray ionization mass spectrometry imaging (DESI-MSI) sample preparation. This figure illustrates the complete workflow for preparing biological samples for DESI-MSI analysis, encompassing a range of critical pretreatment steps. The process begins with Sample Preparation (top left), where tissues are handled based on their preservation method—either as fresh-frozen (FF), formalin-fixed paraffin-embedded (FFPE), or via imprinting for direct surface analysis. Following preparation, the tissues undergo Sample Sectioning (top right) using various techniques: slicing for FFPE tissues or cryosectioning for FF tissues, followed by mounting via thaw-mount, tape transfer, or imprinting. The prepared tissue sections can then proceed through three optional but crucial pre-analysis treatment modules. The Washing & Drying (bottom center) module is designed to remove interfering matrix components and ensure optimal DESI desorption efficiency. The Addition of Standards and Internal Standards (bottom right) module depicts the application of calibrators via spraying or spotting, which is vital for achieving accurate and reproducible quantification. Finally, the Derivatization (bottom left) module shows an optional step to chemically modify analytes on-tissue, enhancing their ionization efficiency and detection sensitivity. This comprehensive workflow highlights the evolution of DESI-MSI from a “no-preprocessing” method to a sophisticated platform that integrates meticulous preparation steps for high-fidelity analysis. Created in BioRender. Cheng, M. (2025).

vulnerable to oxidative alterations, leading to the formation of reaction products between lipids and components of the fixative. For example, amine-containing lipids (e.g., phosphatidylethanolamines (PEs) and phosphatidylserines (PSs)) can undergo N-formylation, while their carboxylic acid moieties may undergo transesterification with methanol [3]. These chemical modifications complicate mass spectrometry analysis and can substantially alter the lipidome. Prior to DESI-MSI analysis of FFPE samples, a deparaffinization procedure is mandatory [3,4]. This adds processing steps that can potentially lead to the diffusion and loss of target compounds, increase the variability of analyte abundance, and diminish lipid signals. The integrity of metabolite information in FFPE samples can be compromised over prolonged storage times. Specifically, the intensity of metabolites in the lower mass range (100–500 m/z) has been observed

to decrease linearly with storage duration [4]. Consequently, despite the advantage of leveraging vast archives, the data quality obtainable from FFPE tissues may not rival that of fresh-frozen (FF) samples.

In contrast, FF tissues are extensively acknowledged as the benchmark for metabolomic mass spectrometry imaging owing to their exceptional preservation of endogenous tissue metabolomes. They most closely represent the *in vivo* molecular arrangement and allow for direct analysis without chemical modification or extensive preprocessing [5,6]. The brief delay between tissue removal and rapid freezing is a critical variable influencing the credibility of MS imaging results. Metabolites are often unstable, highly mobile molecules prone to degradation or delocalization within tissues. During this brief freezing interval, intracellular metabolic activities may not completely stop immediately;

enzymatic reactions can continue, resulting in irreversible modifications to the original composition and concentration of the metabolome. Such alterations exert a direct influence on the biological state represented in the resultant mass spectrometry data, thereby diminishing the precision of research findings. Furthermore, the conditions of cryopreservation and freeze-thaw cycles may lead to the degradation of labile substances, such as lipids, which can compromise data reproducibility [7]. Even when stored at -80°C , exposure to an open environment can result in lipid oxidation. Thus, storage under vacuum packaging [6] or nitrogen protection [7] at -80°C is crucial to maximally prevent lipid degradation. A study utilizing a breast cancer model demonstrated that the duration of the freezing delay significantly influenced the concentrations of various metabolites. Specifically, samples frozen within 30 min post-excision showed no significant alteration in metabolite levels. However, beyond this timeframe, choline levels within the tissue markedly increased, while the levels of ascorbate, creatine, and glutathione significantly decreased [8]. This finding highlights that even seemingly trivial discrepancies in collection and processing durations can profoundly affect the metabolic phenotype of tissues. Therefore, rigorous control over the time elapsed from collection to rapid freezing is paramount in clinical or laboratory settings to ensure the biochemical integrity of the samples.

3.2. The impact of sample preparation: embedding agent selection and sectioning techniques

Tissue sectioning is a critical step in mass spectrometry imaging, aiming to transform a three-dimensional tissue sample into a thin slice suitable for two-dimensional analysis. The thickness of the section directly impacts the quality of the final mass spectrometric data. Generally, for MS imaging analysis, a recommended tissue section thickness ranges between 8 and 12 μm [9]. Thinner sections (e.g., within the 3–20 μm range) can often provide better spatial resolution by reducing the “3D convolution” effect caused by sample thickness. However, this may also lead to relatively lower signal intensity due to a reduced total amount of molecules available for desorption. Furthermore, thinner sections are more challenging to prepare and are prone to curling and fragmentation during the sectioning process [9].

After sectioning, samples should be dried with a nitrogen stream and stored under nitrogen or vacuum conditions. This helps to minimize metabolite

diffusion and “halo” effects while preventing analyte degradation [6,7]. Storage at -80°C under a nitrogen or anaerobic atmosphere maximally preserves lipids and limits lipid oxidation. Before analysis, samples removed from the freezer should be immediately placed in a vacuum desiccator for thawing for approximately 15–20 min. This allows the sample temperature to equilibrate while preventing surface condensation or over-drying. Prolonged storage in the desiccator can lead to excessive sample dehydration, thus reducing DESI desorption efficiency [5].

3.2.1. Embedding agent selection

The type of sample and the choice of embedding agent during section preparation can directly influence the difficulty of sample preparation and the outcomes of mass spectrometric imaging detection. For more fragile or high-water-content tissues, embedding agents are commonly used to maintain their integrity and provide a stable cutting surface. In traditional pathology and biomedical research, the most widely used embedding agent is Optimal Cutting Temperature (OCT) compound. However, research has revealed that the chemical components within OCT can have a significant negative impact on mass spectrometric data.

OCT contains high concentrations of polyethylene glycol (PEG) polymers, which are a major cause of ion suppression and signal interference in mass spectrometric analysis. PEG molecules possess repeating $-\text{CH}_2-\text{CH}_2-\text{O}-$ units with a mass of 44 Da. Consequently, OCT contamination manifests as a series of peaks in the mass spectrum with a 44 Da interval, competing for available ions with the target analytes and leading to signal suppression [10]. These regularly distributed peaks can overlap with the signals of many endogenous metabolites or lipids in the tissue, making data analysis extremely difficult and potentially generating inaccurate spatial distribution maps due to the inability to effectively distinguish analytes from contaminants. Although these PEG ion peaks largely disappear after additional washing steps, these extra steps can lead to the loss of analytes and should therefore be avoided whenever possible [9,10]. Several alternative materials to OCT are commonly employed in the literature. These include sodium carboxymethyl cellulose (Na-CMC) [9,11], gelatin [12], and HPMC/PVP (hydroxypropyl methylcellulose/polyvinylpyrrolidone) mixtures.

Sodium Carboxymethyl Cellulose: Na-CMC is a water-soluble cellulose derivative often used as an alternative to traditional OCT for frozen tissue embedding. It offers good operational performance

and histological preservation, avoiding the strong ionic interference from OCT in mass spectrometry. Tissue sections embedded with CMC in DESI-MSI have yielded characteristic ion species and signal intensities comparable to non-embedded fresh frozen tissues [11]. However, increasing the concentration to 10% (wt/vol) Na-CMC can alter the formation of sodium adducts due to high sodium concentration and potentially cause ion suppression effects [6]. If subsequent use of eosin counterstaining is intended, it can lead to a strong background obscuring tissue outlines [13]. Lung perfusion samples with higher CMC content may be fragile during cryosectioning, making it difficult to obtain complete sections [14].

Gelatin: Gelatin, a protein polymer derived from partial hydrolysis of collagen, has been traditionally used as a support medium for frozen tissue sectioning. As a biodegradable material that is less prone to ionization under mass spectrometric conditions, gelatin is considered an embedding medium with minimal interference for MS imaging [14,15]. The Avard group combined gelatin and collagen I to establish a dual-hydrogel system, DISC-3D (Dual-hydrogel *In vitro* Spheroid Cryosectioning of Three-Dimensional samples), suitable for studying multicellular tumor spheroids and cell invasion, enabling spatial lipidomics and metabolomics analysis of invasive cells [16]. This technique leverages the gelling properties of collagen I (gels upon heating) and gelatin (gels upon cooling) to achieve controlled, sequential gelling, forming an interpenetrating dual-hydrogel network. Collagen I forms a microfibrillar network supporting cell invasion, while gelatin forms a nanoporous network providing suitable physical properties for cryosectioning. This allows for the preservation of the original structure of invasive spheroids and their microenvironment without formalin fixation. Samples can be directly cryosectioned after observation with super-resolution microscopy, enabling subsequent DESI-MSI for spatial lipidomics and metabolomics studies, thereby addressing the challenges associated with sectioning solely collagen I. However, embedding with gelatin in its hot solution state can potentially damage tissue morphology due to heat. Commercially available porcine gelatin typically requires melting at 50–60 °C. Using such hot gelatin solutions can cause thermal shock to the tissue, affecting its morphology upon re-freezing. While low-melting-temperature (LM) fish gelatin has been developed to reduce thermal shock, remaining liquid at room temperature (18–22 °C) [17], it still does not allow for cold embedding, thus failing

to minimize the temperature difference between the frozen specimen and the embedding agent [6]. Furthermore, during mass spectrometric detection, background signals from small molecules like choline and arginine may arise. Subsequent staining with eosin counterstain can also produce background signals that interfere with interpretation [13].

Hydroxypropyl methylcellulose/Polyvinylpyrrolidone: A hydrogel formulated from hydroxypropyl methylcellulose (HPMC) and polyvinylpyrrolidone (PVP) has been established [6,13]. This hydrogel serves a critical role in supporting tissues and enhancing adhesion to the integrated tissues, thereby operating as a versatile embedding medium. The application of this hydrogel embedding technique, coupled with rapid freezing protocols, improves the morphology of tissue sections and reduces the displacement of polar analytes. It shows compatibility with subsequent mass spectrometry imaging techniques, including MALDI-MSI, DESI-MSI, and SIMS. Since HPMC and PVP polymers are non-ionic, they do not produce polymeric mass spectral peaks or cause ion suppression in MSI experiments. This method is also suitable for histological staining, immunohistochemistry (IHC), imaging mass cytometry (IMC), fluorescence in situ hybridization (FISH), and RNA in situ hybridization (RNA-ISH), as well as other probe or label-based techniques. Furthermore, preprocessing can be performed on ice during the process, reducing the temperature difference between frozen tissue samples and the embedding medium, minimizing the possibility of thawing during embedding, and reducing tissue damage and analyte delocalization risks. A drawback is that for samples requiring sectioning at very low temperatures (around –30 °C to –35 °C), such as adipose or subcutaneous skin tissue, the HPMC/PVP hydrogel can become quite brittle, and its sectioning performance may decline. Additionally, due to its higher viscosity, it can be more challenging to handle for soft, unfrozen samples.

3.2.2. Beyond traditional thaw-mounting: tape transfer and imprinting for challenging samples

Following sectioning, the traditional thaw-mount method, which involves rapidly adhering frozen tissue sections to a room-temperature glass slide leveraging temperature differentials for instantaneous fixation, proves inadequate for certain sample types. Fragile, hard, or highly aqueous tissues, as well as non-invasive sampling from surfaces like skin, pose significant challenges for this technique.

In such scenarios, tape transfer and imprinting methods offer viable alternatives.

Crucially, DESI-MSI does not necessitate conductive surfaces, which significantly broadens the utility of adhesive films or tapes for sample mounting. These methods enhance the handling of tissue sections, mitigating issues such as deformation, fragmentation, overlapping, and analyte displacement, while simultaneously facilitating efficient transfer and adhesion to the slide. This approach enables the successful preparation and preservation of the original tissue structure, allowing for DESI-MSI analysis of samples that are difficult or impossible to process with conventional cryosectioning.

Tape transfer techniques, exemplified by Kawamoto's Film, were initially developed to preserve the integrity of delicate samples such as bone, whole-body tissues, or high-water-content specimens like fruits [18]. The evolution saw the use of transparent adhesive films for preparing frozen sections, particularly effective for fragile, tough, or high-moisture plant tissues like strawberries, preserving their native structure [19]. Subsequent adaptations involved utilizing tapes, double-sided tapes, or similar materials to transfer taped sections onto glass slides for downstream analysis, including whole-body tissue imaging [20,21]. More recently, in 2025, Xue et al. introduced a novel sample preparation workflow that integrates tape transfer with a custom-designed adsorption platform [22]. In this method, a transparent adhesive tape is applied to the surface of a frozen tissue block. A thin section is then cut and transferred from the bulk tissue onto the tape. To ensure a uniform and flat sample surface, the tape-mounted section is pressed between two smooth polytetrafluoroethylene (PTFE) sheets. The section is then subjected to freeze-drying before being cryo-preserved for long-term storage. Paired with a custom-designed Adsorption Platform, the dried sections are affixed via adsorption-based fixation onto the platform surface, allowing for precise positional adjustments according to scanning coordinates. This technique reportedly simplifies section preparation, reduces pre-analysis handling, ensures a flat sample surface by preventing bubble formation, and enhances signal intensity and signal-to-noise (S/N) ratios during detection.

Imprinting offers a non-invasive strategy that circumvents the effects of the stratum corneum and induces surface homogenization, making it particularly suitable for certain biological surfaces. For instance, plant tissues are often covered by a cuticle that impedes the penetration and imaging of

internal metabolites by soft ionization techniques like MALDI and DESI. By gently pressing a transfer medium—such as porous polytetrafluoroethylene (PTFE), thin-layer chromatography (TLC) plates, paper, or tapes like Sebutape [23,24]—onto the tissue surface, analytes (including metabolites, fatty acids, and lipids) can be transferred to a flat, uniform substrate. This simplifies sample preparation while retaining spatial distribution. The non-invasive nature of imprinting also holds significant clinical potential. It enables the direct collection of molecular information from human skin surfaces without the need for invasive biopsies or complex tissue sectioning, making it a promising approach for the non-invasive detection of skin cancers, such as basal cell carcinoma (BCC), and other dermatological conditions. For example, Meng et al. successfully utilized Sebutape to collect samples non-invasively from human skin, enabling the differentiation between basal cell carcinoma (BCC), cherry angiomas, and normal skin. Comparative analysis with BCC tissue sections revealed highly similar distribution patterns for key metabolites, fatty acids, and lipids [25].

However, careful consideration is warranted when employing tape-based transfer methods to avoid sample contamination, as any chemicals present in the tape could generate background signals or interfere with the detection of target analytes in mass spectrometry. The imprinting process itself can lead to cell squashing and analyte smearing during transfer, inevitably sacrificing some degree of spatial resolution. Furthermore, imprinting is most effective for relatively “fleshy” plant tissues and may not be universally applicable to all plant tissue types [24].

3.3. Pre-analysis treatments: washing, drying, and derivatization

The ability to perform direct analysis on biological samples under ambient conditions is a cornerstone of DESI-MSI. However, the complexity and heterogeneity of biological matrices necessitate strategic pre-analysis treatments to optimize data quality. These treatments, encompassing washing, drying, and derivatization, are crucial for overcoming matrix effects, enhancing ionization efficiency, and preserving spatial information.

3.3.1. Washing and drying: mitigating matrix effects and ensuring desorption efficiency

Washing serves the primary purpose of removing high-abundance endogenous interferents or embedding media, such as OCT compound and

salts, which can significantly suppress the ionization of target analytes. Procedures involving submersion and resubmersion of tissue sections in chilled solutions, for instance, 150 mM ammonium formate [10], have proven effective in removing OCT and interfering K and Na ions. The removal of these interferents can substantially enhance the ionization efficiency of target analytes, thereby boosting their signal intensity and detection sensitivity. However, excessive washing poses a risk of analyte loss or diffusion, particularly for small, polar metabolites, which can compromise the original spatial distribution and degrade spatial resolution.

The desorption mechanism in DESI-MSI relies on a process often described by the “droplet pick-up model.” The efficiency of analyte extraction and subsequent desorption is contingent upon the analyte's solubility in the spray solvent and the formation of a stable liquid film on the sample surface [1,2]. Sample humidity plays a critical role in this process, influencing solvent droplet transport and desorption efficiency [26]. Over-drying can lead to sample fragmentation and hinder the solid-liquid microextraction mechanism, as an insufficient liquid phase environment limits the dissolution of analytes, consequently reducing DESI efficiency [27].

To address the issue of over-drying, Shepard et al. enhanced the conventional DESI platform by incorporating two additional lines. One line introduces unmodified compressed air, while the other utilizes a bubbler system, where gas flows through high-purity water before entering the chamber. Coupled with sensors to adjust ambient conditions, this system allows for humidity control between 15% and 70% relative humidity (RH) by maintaining a carrier gas flow pressure below 4 psi, without compromising sample detection. Their findings indicate that humidity levels significantly influence the detection signal intensity of various compounds [28].

For protein-targeted MSI, washing steps are often employed to remove lipids and salts while simultaneously fixing proteins to enhance their ionization efficiency. This typically involves sequential immersion of tissue sections in varying concentrations of ethanol-water solutions and chloroform, followed by drying in a vacuum desiccator [29]. To further improve protein signal detection and mitigate the impact of washing, Yang et al. developed a two-step strategy termed HydroWash [30]. This method combines conventional tissue washing with the conditioning of a gelatin hydrogel. Cleaning is achieved by gently placing the solid hydrogel on

the tissue surface for a few minutes. This hydrogel treatment effectively regulates residual salt content post-washing without causing significant component displacement, thereby preserving spatial resolution and significantly enhancing ionization efficiency. The HydroWash treatment resulted in a 3.0–3.6-fold increase in the DESI-MS signal for α -globin and a 1.7–2.5-fold increase for β -globin. Moreover, in brain tissue, this method substantially enhanced the signals of five different proteins, demonstrating that meticulous pre-treatment can significantly improve protein detection sensitivity while maintaining adequate spatial resolution.

3.3.2. Derivatization: enhancing sensitivity and selectivity

Derivatization strategies can be broadly categorized into on-tissue chemical derivatization (OTCD) and reactive desorption electrospray ionization. Reactive DESI-MSI involves dissolving appropriate derivatization reagents directly into the spray solvent. This approach enables the simultaneous derivatization and ionization of analytes that are otherwise difficult to analyze via direct DESI-MSI [31]. However, these methods often suffer from relatively low derivatization efficiency, interference from excess derivatization reagents, and potential analyte delocalization during the ionization process.

OTCD is a widely used pre-treatment technique in MALDI-MSI. Yin et al. developed a method integrating OTCD with airflow-assisted DESI-MSI (AFADESI-MSI) for the spatial analysis of carbonyl compounds in tissue sections. This approach significantly enhances detection sensitivity while preserving spatial resolution, as demonstrated in the detection of carbonyl-containing metabolites (CCMs) in diabetic rat cardiac tissue, including fatty aldehydes (FALs), oxidized fatty acids (OFAs), and sterol lipids (STs) [32]. In their study, Girard's reagent T was employed for chemoselective derivatization of carbonyl compounds on tissue sections. Following derivatization, they successfully performed spatial analysis of 369 CCMs, revealing significant spatial metabolic heterogeneity in cardiac tissue and identifying localized metabolic dysregulation associated with diabetic cardiomyopathy.

Application of OTCD in DESI-MSI can be challenging due to the requirement of multiple sample preparation steps, including matrix distribution onto the tissue, which may lead to analyte delocalization, compromise tissue homogeneity, and interfere with the detection of low-molecular-weight analytes [33]. To mitigate the risk of analyte

delocalization, Zang et al. developed the hydrogel-assisted chemical derivatization (HCD) method [34]. HCD involves encapsulating derivatization reagents, such as Girard's P (GP) reagent, within a hydrogel matrix. This provides a favorable liquid microenvironment for derivatization reactions, significantly boosting the detection sensitivity of low-abundance or difficult-to-ionize functional molecules. HCD can be directly applied to the sample surface for derivatization, thereby reducing matrix effects from both the derivatization reagent and the tissue sample. Furthermore, the hydrogel can absorb highly competitive matrix components like ammonium salts and inorganic salts (e.g., sodium and potassium ions) from the tissue, further reducing matrix effects.

In addition to chemical derivatization, other pretreatment methods have shown promise in enhancing ionization efficiency. The application of low-temperature plasma (LTP) pretreatment has been demonstrated to enhance ionization in DESI-MSI. Rahman's group reported that pretreatment of mouse brain sections with LTP for 1 or 2 min could more than double the cholesterol signal [35]. This suggests that LTP pretreatment is a potential strategy for improving the ionization efficiency of molecules that exhibit low ionization efficiency in MSI techniques.

3.4. Pre-analysis preparation: addition of standards and internal standards

The accurate, precise, and reproducible quantification of analytes in mass spectrometry imaging (MSI), particularly in complex biological matrices, necessitates the judicious use of standards and internal standards (ISs). These compounds are introduced during sample preparation or integrated into the analytical workflow to calibrate for variations in extraction efficiency, matrix effects (including ion suppression and enhancement), monitor system stability, enable normalization for relative quantification, and ultimately, establish calibration curves for absolute quantification [23,36].

Biological tissue samples are characterized by their highly complex and heterogeneous chemical composition. This inherent complexity can lead to significant matrix effects, such as ion suppression or enhancement, which cause variations in analyte signal intensity across different regions or samples, thereby compromising quantitative accuracy.

To address these challenges, the use of ISs is essential. Internal standards, particularly stable isotope-labeled (SIL) analogues, are ideal because their chemical properties are similar to the target

analytes. This similarity ensures they experience comparable matrix effects during ionization and detection, allowing them to effectively compensate for these signal variations.

Furthermore, internal standards help normalize for inconsistencies in analyte extraction efficiency, which can vary due to factors like tissue type, section thickness, and solvent properties. By correcting for these extraction differences, ISs ensure that the measured signal intensity more accurately reflects the true analyte concentration within the tissue. The use of ISs also serves to normalize analyte signals, minimizing variability caused by sample preparation, instrument conditions, and environmental factors (e.g., humidity and temperature). This normalization is crucial for ensuring reliable and reproducible comparisons between different sample batches or tissue regions.

In contrast, external standards are used to establish calibration curves, which link mass spectral signal intensity to the actual concentration of the analyte. Through these calibration curves, the signal intensity of each pixel in an ion image can be directly converted into a specific analyte concentration or abundance. By collectively correcting for these various sources of variation, the strategic use of both internal and external standards significantly enhances the accuracy and precision of quantitative mass spectrometry imaging analysis.

Currently, three primary strategies are employed for the addition of standards and internal standards in MSI experiments [37] (Fig. 3):

1. **On-Tissue Method:** This approach involves the direct application of standards onto the sample or onto untreated control tissue sections [38,39]. Alternatively, internal standards can be uniformly sprayed across all analyzed tissue sections. For endogenous analytes, stable isotope-labeled analogs are the ideal choice as calibrators. For exogenous analytes, such as drugs, the unlabeled compound can be used.

Advantages: Calibrating standards directly spotted onto target plates or ITO slides offer the benefits of rapid and simple preparation, making them suitable for high-throughput screening [38].

Disadvantages: This method cannot fully account for tissue-specific ion suppression and extraction efficiency. Additionally, standards dissolved in organic solvents can be challenging to precisely focus onto glass slides. Spotting or spraying standards or internal standards onto the sample can partially correct for matrix ionization effects, ion suppression, and analyte extraction efficiency,

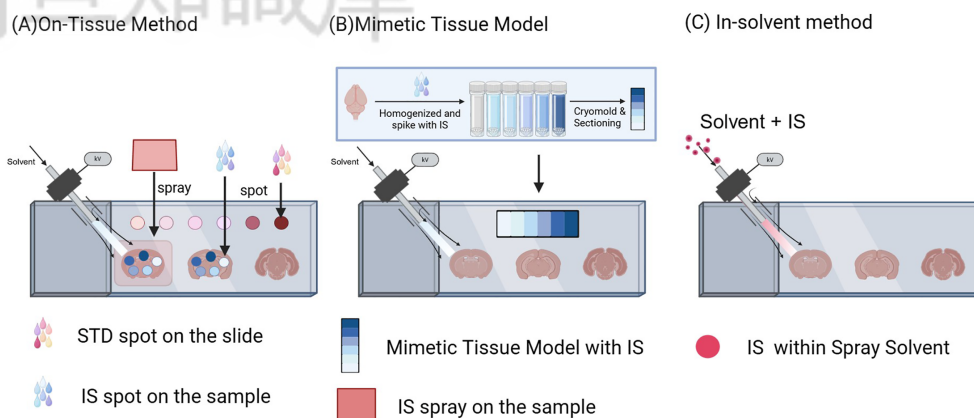


Fig. 3. Common strategies for incorporating standards and internal standards for quantitative mass spectrometry imaging. This figure illustrates three principal methods for introducing standards and internal standards (IS) to enable quantitative mass spectrometry imaging (MSI). (A) The On-Tissue Method involves applying known concentrations of standards (STDs) or ISs directly onto the sample surface. This can be done by spotting discrete droplets onto either a blank slide (STD spot on the slide) or directly onto the tissue section (IS spot on the sample). Alternatively, IS can be uniformly sprayed across the entire sample (IS spray on the sample). (B) Mimetic Tissue Model shows a more sophisticated approach where a tissue homogenate is prepared, spiked with the IS, and then re-solidified into a block using a cryomold. This block can then be sectioned, creating tissue slices with a homogenous distribution of the IS that closely mimics endogenous analytes. (C) The In-Solvent Method depicts the simplest approach, where the IS is added directly into the spray solvent used for the DESI-MSI analysis. This ensures a constant concentration of the IS throughout the entire imaging experiment. Created in BioRender. Cheng, M. (2025).

thereby improving quantitative accuracy [36]. However, the spotting or spraying process may disrupt the tissue's native microenvironment, increasing signal variability. Inconsistent penetration depth within the tissue and droplet overlap of internal standards can alter their surface concentration. Spotting can also lead to a “coffee-ring effect” at the edges of standards, resulting in uneven concentration distribution. If the extraction efficiency of the standard differs from that of the analyte within a heterogeneous tissue matrix, normalization may inadvertently decrease accuracy and reproducibility.

2. **Mimetic Tissue Model:** In this method, analytes are mixed with known concentrations of calibration standards and then prepared as a tissue homogenate. This homogenate is subsequently solidified into a block and sectioned. Because the standards are uniformly integrated into the tissue homogenate, their distribution closely mimics that of endogenous analytes in the actual tissue, thereby providing the most comprehensive correction for matrix effects and extraction efficiencies [40–43]. Studies have demonstrated that this model offers superior correction for tissue-specific ion suppression and extraction efficiencies compared to other methods. A well-prepared mimetic tissue block can yield numerous sections, which is particularly advantageous for large-scale drug discovery in the pharmaceutical industry.

- **Advantages:** Offers the most comprehensive matrix effect and extraction efficiency correction due to uniform integration. This model is highly beneficial for large-scale drug discovery.
- **Disadvantages:** Sample preparation is complex and time-consuming, requiring substantial tissue material. Homogenization destroys tissue morphology, and matrix matching is only feasible for specific tissue types or microenvironments. The fragility of frozen tissue homogenates poses challenges for sectioning.

3. **In-Solution Method:** Internal standards are directly added to the extraction solvent, ensuring a constant IS concentration throughout the imaging experiment. This approach simplifies sample preparation steps and ensures a stable IS concentration. It is particularly effective for targeted quantification of drugs and metabolites, facilitating high-throughput analysis with minimal sample preparation.

- **Advantages:** Simplifies sample preparation and is amenable to high-throughput quantification, especially for targeted analysis of drugs and metabolites.
- **Disadvantages:** Absolute quantification is challenging as it requires additional determination of extraction efficiencies from different tissue regions. Incomplete extraction may occur, and excessively high IS concentrations can suppress the target

analyte signal [37]. This method may not accurately mimic the ionization/desorption process of analytes in typical DESI mechanisms, as the IS is directly charged in solution, whereas analytes are ionized via the “droplet absorption mechanism” [37].

4. Conclusion, challenges, and future prospects

Despite significant advancements, the journey towards fully realizing the potential of DESI-MSI in metabolomics is intrinsically linked to ongoing innovations in sample preprocessing. The field is moving beyond a simple dichotomy of “no preprocessing” versus “basic preprocessing” towards sophisticated, targeted strategies designed to overcome inherent matrix effects, enhance sensitivity, and ensure robust quantitation.

Looking ahead, the future of preprocessing for DESI-MSI lies in several key areas:

- **Advanced Derivatization Techniques for Enhanced Sensitivity and Selectivity:** While OTCD and reactive DESI have shown promise, further development is needed to improve derivatization efficiency, reduce reagent delocalization, and minimize background noise. Future research might explore enzyme-catalyzed derivatization that offers higher specificity and controlled reaction environments. The hydrogel-assisted chemical derivatization (HCD) approach offers a promising avenue for encapsulating reagents and controlling reactions within the tissue microenvironment.
- **Standardization of Internal Standard Spiking and Calibration:** Achieving accurate absolute quantification remains a significant challenge. Future efforts must focus on developing standardized protocols for adding stable isotope-labeled internal standards directly onto or within tissue sections, ensuring their distribution mirrors that of endogenous analytes. Novel methods for creating more realistic mimetic tissue models that better represent complex biological matrices will also be crucial for robust calibration and validation.
- **Exploration of Novel Pretreatment Technologies:** Emerging techniques like low-temperature plasma (LTP) pretreatment and tissue expansion microscopy imaging (TEMI) [44], though nascent in their application to DESI-MSI, hold significant potential. LTP could enhance ionization efficiency for challenging molecules.

TEMI, on the other hand, offers a groundbreaking approach to overcome spatial resolution limitations. Its core principle involves chemically treating biological tissue samples to cause a controlled and isotropic expansion, increasing their linear dimensions several times (e.g., 5 to 10-fold) without using harsh conditions like proteolysis, detergents, or high temperatures. This gentle expansion process preserves the chemical integrity of biomolecules. By physically magnifying the tissue, TEMI can “virtually” increase instrument resolution by an order of magnitude, enabling precise spatial localization of molecules at the single-cell or subcellular level. The combination of this technique could revolutionize how we map molecular distributions and potentially enable single-cell level metabolic profiling with DESI-MSI, providing higher-fidelity data.

The future of DESI-MSI in metabolomics hinges on a paradigm shift where sample preprocessing is no longer viewed as a necessary evil, but as an enabling technology that is meticulously designed, optimized, and integrated into the analytical workflow to unlock the full quantitative and qualitative power of this ambient ionization technique.

Acknowledgments

This work was supported by the National Science and Technology Council (NSTC), Taiwan, under grants NSTC 112-2327-B-001-003 and NSTC 114-2735-B-001-001.

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