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Ambient Mass Spectrometry Imaging Using Direct Liquid Extraction Techniques

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Graphical abstract

1. INTRODUCTION

Mass spectrometry imaging (MSI) is a powerful analytical technique that enables label-free spatial localization and identification of molecules in complex samples.^{1–4} MSI applications range from forensics⁵ to clinical research⁶ and from understanding microbial communication^{7,8} to imaging biomolecules in tissues.^{1,9,10} Recently, MSI protocols have been reviewed.¹¹ Ambient ionization techniques enable direct analysis of complex samples under atmospheric pressure without special sample pretreatment.^{3,12–16} In fact, in ambient ionization mass spectrometry, sample processing (e.g., extraction, dilution, preconcentration, or desorption) occurs during the analysis.¹⁷ This substantially speeds up analysis and eliminates any possible effects of sample preparation on the localization of molecules in the sample.^{3,8,12–14,18–20} Venter and co-workers have classified ambient ionization techniques into three major categories based on the sample processing steps involved: 1) liquid extraction techniques, in which analyte molecules are removed from the sample and extracted into a solvent prior to ionization; 2) desorption techniques capable of generating free ions directly from substrates; and 3) desorption techniques that produce larger particles subsequently captured by an electrospray plume and ionized.¹⁷ This review focuses on localized analysis and ambient imaging of complex samples using a subset of ambient ionization methods broadly defined as “liquid extraction techniques” based on the

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classification introduced by Venter and co-workers.¹⁷ Specifically, we include techniques where analyte molecules are desorbed from solid or liquid samples using charged droplet bombardment, liquid extraction, physisorption, chemisorption, mechanical force, laser ablation, or laser capture microdissection. Analyte extraction is followed by soft ionization that generates ions corresponding to intact species. Some of the key advantages of liquid extraction techniques include the ease of operation, ability to analyze samples in their native environments, speed of analysis, and ability to tune the extraction solvent composition to a problem at hand. For example, solvent composition may be optimized for efficient extraction of different classes of analytes from the sample or for quantification or online derivatization through reactive analysis. In this review, we will: 1) introduce individual liquid extraction techniques capable of localized analysis and imaging, 2) describe approaches for quantitative MSI experiments free of matrix effects, 3) discuss advantages of reactive analysis for MSI experiments, and 4) highlight selected applications (published between 2012 and 2015) that focus on imaging and spatial profiling of molecules in complex biological and environmental samples.

Ambient liquid extraction techniques provide unique opportunities for rapid analysis of molecules on solid and liquid substrates.^{17,20,21} The remarkable flexibility in solvent composition is a distinguishing feature of ambient liquid extraction techniques, making them particularly advantageous for selective extraction, online derivatization, and quantification of analytes of interest. For example, the ability to add appropriate reagents to an extraction solvent provides a straightforward pathway for selective modification of specific classes of molecules for improved ionization or structural characterization using tandem mass spectrometry (MS/MS). In addition, the ability to perform quantitative MSI, by adding carefully selected internal standards of known concentration to the extraction solvent, opens up unique opportunities for examining concentration gradients of molecules in complex biological and environmental samples. These distinct capabilities of liquid extraction MSI techniques make them ideally suited for a diversity of applications in biology, clinical studies, environmental sciences, and forensics.

2. AMBIENT LIQUID EXTRACTION TECHNIQUES

Ambient liquid extraction techniques summarized in Table 1 commonly are divided into three major classes:¹⁷ 1) spray-based techniques, in which analyte molecules are desorbed from the sample using droplet bombardment; 2) direct liquid extraction techniques, including liquid capture of desorbed analytes; and 3) substrate spray methods, where electrospray is performed directly from the sample. In the following sections, we briefly introduce these techniques. For detailed descriptions, readers are referred to more comprehensive reviews.^{12–14,18–20,22}

2.1 Spray-based Methods

In spray-based ionization, sample bombardment via a fast stream of charged droplets generates secondary droplets containing analyte molecules desorbed from the sample.²³ Mechanisms of spray-based methods have been previously reviewed.²⁰ Momentum transfer from the primary droplets to a thin liquid film on the surface results in the formation of

secondary droplets that contain desorbed analyte molecules.²⁴ Secondary droplets that reach the mass spectrometer undergo a similar desolvation process as in electrospray ionization (ESI), generating intact ions of analyte molecules. Developed by Cooks and co-workers, desorption electrospray ionization (DESI)^{23,25–27} is the most widely used spray-based technique for spatially resolved analysis of complex samples. In DESI, charged droplets are produced by ESI and accelerated toward the sample surface via a sheath gas flow. A similar desorption process is used in easy ambient sonic-spray ionization (EASI),^{28–30} where charged droplets are produced by sonic spray that does not require high voltage.

In earlier work, analyte desorption mechanisms in DESI were examined using phase Doppler anemometry³¹ and fluid dynamics simulations.²⁴ Both the experiments and simulations support the droplet pick-up mechanism, in which charged droplets colliding with a surface extract the analyte molecules. In a follow-up study, Costa and Cooks demonstrate that primary droplet collisions with a wetted surface in DESI experiments result in ejection of secondary droplets containing analyte molecules.³² The primary droplet size is ~2–4 μm , while the average size of secondary droplets is ~1 μm .^{31,32} In the study, larger secondary droplets were found to be ejected at higher angles relative to the surface. It has been proposed that different types of progeny droplets may be enriched in different types of analyte molecules,³² which rationalizes the experimentally observed mass dependence of ion yields on the collection angle. Simulations further indicate that electrostatic forces do not contribute to droplet production.³² This result is consistent with experimental observations. For example, spray desorption collection experiments^{33,34} have demonstrated that the amount of material collected away from the surface during droplet bombardment is independent of spray voltage, confirming that desorption is unaffected by electrostatic forces. However, transport of progeny droplets away from the surface and droplet charging may be affected by electrostatics. Measurement of the charge distribution on the surface developed during DESI experiments indicated substantial charge buildup over an area of several square centimeters on the surface.³⁵ However, desorption occurs from a much smaller area (~1 mm^2) of highest charge density. Charge distribution on the surface becomes narrower with an increase in sheath gas pressure and a decrease in the distance between the ESI tip and surface.³⁵ These parameters, along with the flow rate and angle between the droplet emitter and surface, control the spatial resolution of DESI experiments. The best spatial resolution of 35 μm has been reported by Campbell *et al.* for imaging of mouse brain tissue sections.³⁶ Similar spatial resolution of 40 μm was reported by Kertesz *et al.* for a printed pattern on a thin-layer chromatography (TLC) plate.³⁷ In that study, the distance between the DESI sprayer and the surface was maintained at 400 μm to reduce the desorption spot size, while analyte redistribution during imaging was minimized by decreasing solvent flow rate to 1.5 $\mu\text{L}/\text{min}$.³⁷

Several DESI variants have been introduced to improve the ionization efficiency of non-polar analyte molecules. For example, enhanced ionization of nonpolar compounds has been achieved by charge exchange with toluene ions in the spray³⁸, or in the presence of metastable atoms in the ionization region.³⁹ Furthermore, improved transfer of secondary droplets into a mass spectrometer inlet may be achieved using air-flow-assisted DESI (AFADESI).⁴⁰ Fernandez and co-workers reported additional improvements in DESI imaging sensitivity by coupling DESI with a differential mobility filter, which resulted in a

10-fold increase in the signal-to-noise ratio.⁴¹ DESI and its variants have been used for imaging tissue sections,^{42,43} imprints of plant tissues⁴⁴ and microbial communities,⁴⁵ fingerprints,⁵ mineral surfaces,⁴⁶ and other.

2.2 Direct Liquid Extraction Methods

Developed by Van Berkel and co-workers, the liquid microjunction surface-sampling probe (LMJ-SSP) is composed of two coaxial tubes.⁴⁷ Solvent delivered to the sample through a gap between the inner and outer tubes is removed through the inner capillary tube and transferred to a mass spectrometer inlet. Analyte molecules extracted into the flowing solvent are ionized by pneumatically assisted electrospray⁴⁷ or atmospheric pressure chemical ionization.⁴⁸ Spatially resolved analysis using LMJ-SSP was first demonstrated for detecting analyte molecules separated using TLC.⁴⁷ A commercial version of LMJ-SSP, called Flowprobe, has been used for analyzing microbial colonies directly from nutrient agar.⁴⁹ The spatial resolution of the LMJ-SSP probe is determined by the diameter of the outer capillary. Early imaging experiments were conducted with a spatial resolution of ~650 μm ,⁵⁰ while a much smaller footprint of the probe on the sample has been obtained using a 330- μm outer diameter (OD) capillary.⁵¹ The LMJ-SSP probe also has been coupled with high-performance liquid chromatography (HPLC) for simultaneous spatially resolved analysis and identification of molecules extracted from tissue sections with a lateral resolution of ~700 μm .^{52,53} The LMJ-SSP probe has been operated both in continuous sampling and plug formation mode.^{51,54} A transparent LMJ-SSP probe enabled visualization of the extraction and transport processes during surface sampling. Experiments with the transparent probe have shown that retraction of the inner capillary relative to the outer capillary results in a transition from the continuous sampling to plug formation mode. Fluid dynamics simulations have revealed that plug formation is caused by eddy currents that develop at the sampling end of the probe when the inner capillary is retracted from the flush position.⁵⁴ Plug formation mode enables analyte preconcentration and reactive analysis.⁵¹ LMJ-SSP has been used for the analysis of molecules separated on TLC plates,⁴⁷ tissue profiling,^{55,56} and spatial mapping of metabolites in microbial⁵⁷ and fungal communities.⁵⁸

Continuous liquid extraction and ionization of analyte molecules from both solid and liquid interfaces has been achieved using nanospray desorption electrospray ionization (nano-DESI).⁵⁹ The nano-DESI probe is composed of two fused silica capillaries, typically positioned at an angle. Analyte molecules are desorbed into a solvent bridge that forms between two capillaries and the analysis surface. The primary capillary supplies solvent to create and maintain the bridge, while the secondary capillary transports the desorbed analyte to a mass spectrometer inlet and generates ions through nanospray at the inlet either by self-aspiration or vacuum-assisted flow. Nano-DESI has been used for sensitive localized analysis and imaging of complex molecules in biological and environmental samples.^{60–62} The spatial resolution in nano-DESI MSI experiments is determined by the size of the capillaries that make up the probe, solvent flow rate, surface properties, and the ability to accurately control the distance between the sample and the probe. Nano-DESI MSI experiments have been performed with moderate spatial resolution of ~100–150 μm ,^{60,63,64} although a spatial resolution of better than 12 μm has been achieved using pulled

capillaries.⁶¹ Nano-DESI applications include imaging of tissue sections,^{61,63} spatially-resolved analysis of microbial communities^{65–67} and environmental samples.^{68,69}

A similar principle, where both the primary and secondary capillary are incorporated into a single device, also can be employed. For example, the swan probe⁷⁰ is a “U”-shaped device fabricated from a single glass capillary with a small sampling hole (produced using sandpaper) at the bottom (~200 μm in length and ~100 μm in width). This device has been used for high-throughput sampling of analytes from multiwell plates and a microfluidic droplet array.⁷⁰ A push-pull electrochemical scanner, used both as an electrochemical device and a liquid extraction probe for mass spectrometry, also has been fabricated by incorporating both channels into a microfluidic chip.⁷¹ This capability has enabled simultaneous imaging of latent fingerprints using scanning electrochemical microscopy (SECM) and mass spectrometry.⁷¹ A different device, the single-probe, has been fabricated by pulling a dual-bore or theta capillary and attaching the primary and secondary capillaries to the two channels.⁷² The primary capillary delivers the extraction solvent to the theta capillary tip, and a liquid bridge forms between the two channels at the pulled tip of the theta capillary, serving as a sampling probe. The secondary capillary, glued into one of the channels, is used for analyte transfer and ionization. This device eliminates the need for independent positioning of the primary and secondary capillaries and enables imaging with high spatial resolution by pulling the capillary tip to 6–10 μm diameter.⁷² Single-probe has been used to analyze individual HeLa cells,⁷² as well as for imaging tissue sections with a spatial resolution of ~8.5 μm .⁷³

In many direct liquid extraction MSI experiments, reproducible extraction requires precise control of the distance between the sample and the probe. Contact profilometry experiments have demonstrated that tissue samples are fairly flat with the root-mean-square (RMS) variation in height of $1.5 \pm 0.6 \mu\text{m}$ observed for 10- μm thick brain tissue sections.⁶⁰ To control the probe's position when imaging thin tissue sections on glass slides, a straightforward method has been introduced that compensates for the sample tilt.⁶⁰ In this approach, a user specifies the plane coordinates where the sample resides. A motorized stage, positioned along the z-axis, is automatically adjusted to maintain constant distance between the sample and probe. This method, which can be readily extended to other direct liquid extraction techniques, has enabled robust imaging of thin samples with a spatial resolution better than 20 μm .⁶⁰ Van Berkel and co-workers have developed a different approach for controlling the distance between the sample and the LMJ-SSP probe.⁵⁰ In these experiments, a working distance of ~500 μm was maintained using real-time imaging and image analysis of the liquid junction between the sample and the probe.⁵⁰ Alternatively, a laser distance sensor may be used to control the distance between the sample and the probe.⁵³

In the past four years, several liquid capture probes have been developed for MSI experiments.^{74–81} In these experiments, analyte molecules are removed from the substrate using laser ablation (LA), either in transmission or reflection mode, and the ablated plume is captured either into a static droplet or continuous liquid flow in a probe similar to LMJ-SSP. The internal energy distributions of ions produced using this approach are similar to ESI, indicating that electrospray of the captured analyte molecules is the dominant ionization

mechanism.⁸² Both infrared^{75,78} and ultraviolet (UV) lasers^{74,77} have been employed in LA-liquid capture experiments. The best spatial resolution of ~50 μm has been achieved using transmission geometry LA with a UV laser.⁷⁷

Liquid extraction surface analysis (LESA) mass spectrometry relies on analyte extraction into a static droplet placed on the sample surface.⁸³ Next, the droplet is withdrawn from the surface and placed in front of a mass spectrometer inlet for nanospray ionization. LESA has been implemented on a commercial Advion NanoMate sampling/chip-based ionization platform.⁸³ In a typical LESA experiment, only the droplet comes in contact with the sample. However, Randall et al. observed efficient protein extraction from living *E. coli* colonies only when the pipet tip was brought in direct contact with the sample.⁸⁴ Such “contact” model LESA may be advantageous for the analysis of samples containing a protective layer on the surface. When installed on a fully automated robotic system, it enables high-throughput, spatially resolved analysis of metabolites,^{85,86} lipids,^{87–90} and proteins^{91,92} in complex samples, including dried blood spots,^{84,88} contact lenses,⁸⁹ tissues,^{55,83,86,91} and microbial colonies.^{84,93} LESA also has been used for analyzing molecules separated using TLC⁸³ and capillary electrophoresis.⁹⁴ Coupling LESA with ion mobility spectrometry has exhibited significant improvement in the sensitivity and speed of LESA experiments.^{93,95} Furthermore, ion mobility separation is advantageous for identifying analyte molecules.⁹³ LESA’s spatial resolution is determined by the size of the pipette tip and the surface properties. Although a spatial resolution of ~350 μm recently was achieved using an electrofocusing LESA probe (EF-LESA),⁹⁶ the typical spatial resolution of LESA MSI, usually ~1 mm, is much lower than the spatial resolution available with many other liquid extraction techniques.⁹⁷ Nevertheless, spatial profiling with LESA is advantageous because of its high sensitivity and long-lasting signals of extracted analytes, which are necessary for their structural characterization using MS/MS.

A modified version of LESA, known as pressurized liquid extraction surface analysis (PLESA), has been developed to improve the extraction efficiency of lipids from tissue samples.⁸⁷ In PLESA experiments, a pipet filled with ~500 nL of organic extraction solvent is pressed into the sample, forming a sealed microextraction volume. Improved extraction efficiency is achieved by applying headspace pressure to the pipet during the ~30 s extraction time. PLESA has been used for spatial profiling of tissue sections with a spatial resolution of ~400 μm and a total footprint of ~800 μm left by the probe on the surface.⁸⁷

A related approach, direct analyte-probe nanoextraction (DAPNe), has been developed to reduce LESA’s footprint on the sample for forensics applications, where minimal sample damage is required.^{98,99} DAPNe uses a small nanospray tip with an inner diameter of ~1 μm , leaving only a ~5- μm footprint on the surface. The tip is accurately positioned on the sample using a nanomanipulator mounted on an optical microscope stage. In a typical experiment, a small water droplet is placed on the sample. The nanospray tip, filled with an appropriate solvent, is positioned inside the water droplet, and the extraction solvent is injected by applying backpressure of ~15 psi to the tip. The droplet subsequently is extracted with a fill pressure of ~35 psi, and the extracted analyte mixture is analyzed using nanospray ionization.⁹⁹ Notably, Huynh et al. have demonstrated the utility of DAPNe for identification of falsified documents with minimal physical damage to the document.

Electrostatic spray ionization directly from the extraction droplet delivered by the primary capillary to the surface also has been reported and used for imaging with a spatial resolution of $\sim 110\text{ }\mu\text{m}$.¹⁰⁰ In this system, ionization is induced by applying a pulsed voltage of up to 9 kV at a frequency of 5 or 40 Hz to a metal microelectrode positioned below the sample and aligned with the heated inlet of a mass spectrometer. Secondary droplets ejected from the extraction droplet then are analyzed by a mass spectrometer. Liquid extraction into a static droplet also has been used in polarization-induced ESI.^{101,102} In this approach, ionization occurs directly from the droplet placed on an electrically insulated substrate by applying a potential to a mass spectrometer inlet.

A related approach, in which the a vibrating capillary probe is operated either in contact or tapping mode is called scanning probe electrospray ionization (SPESI).¹⁰³ In SPESI, solvent is delivered to the surface through the probe capillary, and analyte molecules are extracted into a liquid bridge that forms between the probe capillary and the surface. In contact mode, a Taylor cone, generating charged droplets, forms on the side of the probe capillary that faces the instrument inlet. Vibrating the sample helps to maintain a stable liquid bridge between the sample and the probe capillary, while ionization of the extracted analyte molecules is continuous. In tapping mode (t-SPESI), the probe capillary undergoes stimulated oscillation that brings it either close to the sample surface for liquid extraction or to the instrument inlet for ionization. As a result, the system alternates between extraction and ionization events. An $\sim 60\text{-}\mu\text{m}$ spatial resolution has been achieved using this approach.¹⁰³ Imaging of mouse brain tissue sections using t-SPESI has enabled spatial mapping of lipids and metabolites typically observed in ambient MSI experiments.¹⁰⁴ Lorenz et al. have developed an advanced tapping-mode SPESI system capable of imaging tissue sections with a spatial resolution of $35\text{ }\mu\text{m}$.¹⁰⁵

2.3 Substrate Spray Methods

Probe electrospray ionization (PESI) uses a sharp, solid needle as a sampling probe for localized analysis of liquid and wet solid samples.¹⁰⁶ A similar approach is used in touch spray (TS) ionization, in which analyte is removed using an angled teasing needle.¹⁰⁷ First, the needle is brought in contact with the sample then positioned at the mass spectrometer's inlet. ESI is achieved either directly from the needle or by using an auxiliary sprayer in the presence of high voltage. The latter approach eliminates problems associated with the sample drying on the needle prior to analysis, which inhibits ionization. The solvent also may be delivered to the tip of the extraction needle using sheath flow probe, where the sampling needle is inserted into a concentric insulating capillary that supplies the extraction solvent.¹⁰⁸ In TS ionization, solvent is delivered to the needle either manually using a pipette or continuously via a syringe pump.¹⁰⁷ Tissue sample imaging and depth profiling with a spatial resolution of $60\text{ }\mu\text{m}$ and $20\text{ }\mu\text{m}$, respectively, have been demonstrated using an automated PESI device. In the device, the sampling depth, determined by the distance between the needle and sample, was controlled by measuring the direct current (DC) on the sampling needle.¹⁰⁹ PESI's selectivity and detection limits may be controlled by applying different coatings to the probe. For example, Deng et al. have designed a surface-coated probe for selective detection of perfluorinated compounds in small organisms and individual cells by coating a sharp tungsten needle with an alkylsilane layer.¹¹⁰ Following analyte

extraction from the sample, the needle is inserted into a nanospray tip, and analyte molecules extracted in the appropriate solvent are detected using mass spectrometry. Various surface coatings can be used for selective extraction and enrichment of compounds of interest in complex samples. For example, using tips coated with nano-sized TiO_2 , Zhao et al. have demonstrated selective extraction of phosphopeptides from biological samples.¹¹¹ Substrate spray ionization also has been achieved using porous extraction substrates, such as paper (paperspray)^{112,113} or wooden tips,^{114–116} or non-porous materials, such as bamboo pen nibs¹¹⁷ or sharp objects naturally present in the sample.^{118–120}

Scanning probe mass spectrometry (SMS) uses a short, tapered electrospray capillary (~8–30 mm length; 20–50 μm ID) as a sampling probe directly immersed into a liquid analyte.¹²¹ The probe removes the liquid analyte through self-aspiration or via vacuum-assisted flow. Two SMS operating modes have been introduced. In one, the probe is positioned in the same location while collecting time-dependent mass spectra. This mode may be used for monitoring reaction kinetics. In the second mode, the composition of spatially heterogeneous liquid samples is examined by scanning the sample under the probe while collecting mass spectra. This operating mode has been demonstrated by profiling an interface between two immiscible liquids: water and cyclohexane.¹²¹ A similar principle is used for analyzing intact plants with *vivo* nanospray ionization, where analyte molecules are extracted from the plant tissue by inserting a nanospray capillary into the tissue and applying a high voltage to the plant. Using this approach, Peng et al. were able to detect flavonoids, saccharides, alkaloids, and other bioactive metabolites from different locations on living plants.¹¹⁹ Furthermore, Hu et al. detected secondary metabolites released by living scorpions and toads.¹²⁰

3. MATRIX EFFECTS AND QUANTIFICATION

3.1 Matrix Effects

Ion suppression during ionization of complex mixtures has been extensively discussed in the literature.¹²² It is well established that the complex chemical composition of analyte mixtures extracted from typical biological or environmental samples may significantly impact the detected ion signal of individual compounds.¹²² This phenomenon typically is referred to as matrix effect. Matrix effects originate from competitive ionization and reflect differences in ionization efficiencies of different classes of analytes competing for charge. In the absence of separation prior to mass spectrometry analysis, as in MSI experiments, matrix effects are particularly severe. Using DESI MSI, Vismeh et al. examined the impact of matrix effects on the signal intensity of clozapine deposited onto an Omni-Slide and different regions of rat brain tissue sections.¹²³ As shown in Figure 1a, the clozapine signal on the tissue is ~80% lower than the signal obtained on the Omni-Slide. This decrease in signal has been attributed to ion suppression of analyte molecules in the presence of the complex chemical matrix extracted from the tissue.¹²³ In addition, as shown in Figure 1a, differing clozapine signal intensities has been observed on different tissue types, indicating that the extent of ion suppression depends on the tissue's molecular composition.¹²³ Similarly, a DESI MSI study of fingerprints by Muramoto et al. reported complete suppression of methamphetamine and heroin signals, as well as an order of magnitude

decrease in the cocaine signal, in the chemical matrix of a fingerprint when compared with the signal obtained from a glass slide.¹²⁴ These results clearly demonstrate that the chemical composition of the extracted mixture may have substantial impact on the signal intensity of the compound of interest.

The ionization efficiency of analyte molecules also may be affected by the abundance of cations present during ionization. For example, nano-DESI MSI of brain tissue sections obtained from mice subjected to middle cerebral artery occlusion (MCAO)¹²⁵ shows a clear difference in the abundance of $[M+Na]^+$ and $[M+K]^+$ adducts of the same molecule in the ischemic region. Specifically, it is observed that $[M+Na]^+$ signals increase while $[M+K]^+$ signals decrease in the ischemic region. These trends have been attributed to differences in Na^+ and K^+ concentrations across the tissue. Specifically, ischemia is known to cause a substantial increase in sodium and decrease in potassium concentration, which is reflected in ion images obtained experimentally.¹²⁵ Lanekoff et al. have introduced an approach that efficiently compensates for matrix effects in nano-DESI MSI, which can be readily extended to other liquid extraction techniques.¹²⁵ In this approach, internal standards are added to the extraction solvent, and the analyte signal is normalized to the signal of the standard supplied at a constant rate during the imaging experiment. During nano-DESI MSI of mouse brain tissue, Lanekoff et al. added two phosphatidylcholine (PC) standards, PC 25:0 and PC 43:6, at known concentrations into the extraction solvent. Figure 1b shows the optical image of the analyzed tissue section with pink highlight corresponding to the ischemic region and blue highlight showing the white matter regions. Figure 1c shows ion images of the standard PC 43:6, $[M+Na]^+$ and $[M+K]^+$, normalized to the total ion current (TIC), a procedure often used when processing MSI data. Although the internal standard was supplied at a constant rate, the ion images in Figure 1c show an enhanced signal of the standard in some regions. Specifically, an increased signal of the standard cationized on sodium is observed in the white matter and the ischemic region of the tissue whereas the signal of the standard cationized on potassium is enhanced in the white matter and suppressed in the ischemic region. The increased signal of the standard in the white matter is attributed to matrix effects originating from the differences in the molecular composition of the complex matrix. Meanwhile, signal variations observed for $[M+Na]^+$ and $[M+K]^+$ ions in the ischemic region originate from differences in concentration of alkali metals extracted from these regions. In Figure 1d, the intensity of the sodium and potassium adducts of the standard, PC 43:6, are normalized to the intensity of the corresponding adducts of the second standard, PC 25:0. The ion images in Figure 1d depict a uniform distribution of the signal of the standard, indicating that such normalization efficiently compensates for both types of matrix effects.¹²⁵

For static liquid extraction techniques such as LESA, where molecules on the surface are extracted into a static solvent droplet, internal standards of known concentration can be spiked into the solvent prior to extraction. In this scenario, analyte molecules desorbed into the solvent mix with the internal standard present in the solvent. During mass spectrometry analysis, the internal standard, of known concentration in the extraction solvent, is analyzed simultaneously with compounds extracted from the sample. Assuming the ionization efficiency of the standard and endogenous analyte molecules is equally affected by the complex chemical matrix composition, matrix effects may be eliminated by normalizing the

observed analyte signal to that of the standard. The advantage of this approach is that no additional complexity is introduced into the sample preparation and analysis steps. Meanwhile, Parson et al. added deuterated chloroquine to the extraction solvent for LESA-MS/MS analysis then studied the intensity variations of the internal standard depending on tissue type.⁵⁵ Their study shows that only 8%, on average, of the internal standard intensity is observed after extraction of endogenous molecules present in the tissue section compared to the signal prior to extraction. Signal suppression observed in LESA-MS/MS⁵⁵ experiments is similar to signal suppression in DESI MSI and nano-DESI MSI (discussed earlier herein). It follows that although various MS/MS scan modes may be used to increase the signal-to-noise ratio of the analyte of interest—hence, the dynamic range of ambient liquid extraction experiments—MS/MS analysis does not eliminate matrix effects. A similar study has been performed using LESA MS of lung tissue.¹²⁶ Although lung tissue is heterogeneous, the study did not detect significant ion suppression effects between different locations of the lung, possibly due to the large area being sampled by LESA at each location.¹²⁶

Efficient compensation for matrix effects requires that the selected internal standard has similar ionization efficiency as the analyte of interest. It is assumed that an internal standard having the same ionization efficiency as the analyte will experience equivalent matrix effects during ionization. Meanwhile, a mixture of internal standards representing different classes of molecules may be used to compensate for matrix effects in ionization of the corresponding classes of endogenous analytes during MSI.

3.2 Quantification

Quantitative mass spectrometry imaging (Q-MSI) is an emerging field that enables accurate measurements of concentration gradients of molecules in complex samples.^{127,128} Q-MSI experiments commonly are performed by spotting a standard onto the sample, such as a thin tissue section, at different concentrations.¹²⁸ Then, an external calibration curve is generated based on the observed signal intensity and the known concentration of the spotted standard. To ensure similar response of the analyte and the standard, the standard should have analogous ionization efficiency as the analyte of interest. As such, isotopically labeled standards are commonly used in Q-MSI experiments. Muramoto et al. deposited cocaine standard at different concentrations on paper to generate an external calibration curve for DESI MSI experiments.¹²⁴ Based on the external calibration curve, they performed Q-MSI of cocaine in fingerprints on paper (shown in Figure 2). The intensity scale in the figure represents the absolute abundance of cocaine in pg/pixel determined in these experiments.¹²⁴ Spotting a standard on paper also has been used to generate calibration curves for the quantification of components in biodiesel using EASI.^{129,130} For quantification of administered clozapine, Vismeh et al. investigated deposition of a standard onto rat brain tissue.¹²³ First, DESI MSI experiments were performed to determine clozapine localization in the tissue. Next, the internal standard loxapine, a close analogue of clozapine, was spotted onto the exact location of the drug in the brain tissue using a micropipette. Finally, the clozapine concentration at each location was calculated based on the known loxapine concentration, the ratio of ion intensities of the analyte and the standard, and the known sampling spot size. The simultaneous detection of both the drug and the standard in each

spectrum helped reduce matrix effects and generate calibration curves with high linearity and low scan-to-scan variability.¹²³ The dynamic range of quantitative DESI has been determined to be, at least, three orders of magnitude, demonstrating the suitability of DESI for Q-MSI of tissue sections.¹²³

Deposition of a standard onto the sample surface for quantification is relatively time consuming and may introduce uncertainty in Q-MSI experiments. For example, it is difficult to rule out uneven distribution of the standard on the tissue and diffusion of endogenous molecules in the region where the standard is deposited, which may be detrimental to MSI experiments. Furthermore, if the standard and analyte are not perfectly co-localized, they may experience different matrix effects during ionization. To overcome these barriers and compensate for unevenly distributed standard, Lebeau et al. acquired spectra at several locations of the standard spot using DESI.¹³¹ They then used the average signal from the different locations to generate an external standard curve. This approach provided a calibration curve with high linearity and good correlation coefficient values, enabling the quantification of EDTA in concrete.¹³¹ Similarly, Mandal et al. investigated the applicability of sheath flow PESI and LESA for quantification of agrochemicals in plants by analyzing a concentration range of standards spotted onto Teflon tape.¹³² Their study concluded that the spot-to-spot signal intensity of the standard varies more using sheath flow PESI than LESA. This result has been attributed to larger variations in the amount of extracted material in sheath flow PESI compared to LESA, in which the extraction volume is better controlled.¹³² Thus, the sample's complex chemical matrix and uncertainties introduced by depositing a standard onto the sample surface are not the only obstacles for Q-MSI. The suitability of an MSI technique for quantification also depends on its reproducibility and robustness in extracting analyte molecules from the sample.

The ability to add multiple standards to the extraction solvent makes ambient liquid extraction techniques particularly suitable for Q-MSI experiments. In addition, the experimental approach of adding standards into the extraction solvent substantially simplifies Q-MSI experiments. Indeed, because the standard does not have to be spotted onto the sample, no added complexity is introduced to the sample preparation step. As previously discussed, the presence of internal standards in the extraction solvent may be used to compensate for matrix effects that originate from variations in the chemical composition of the complex molecular mixture present during the ionization step. When the internal standard has similar ionization efficiency as the analyte of interest, its signal intensity may be used for quantification. Specifically, in Q-MSI experiments, where the standard is continuously supplied with the extraction solvent, quantification is performed by normalizing the signal of the analyte of interest to that of the standard. Several studies (described herein) have employed this approach for Q-MSI and quantification combined with localized analyses.

Deng et al. used a surface-coated PESI probe to quantify perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) in *Daphnia magna*.¹¹⁰ They constructed calibration curves by spiking the compounds, in a range of concentrations, into the organism's body fluid. The compounds were extracted from the body fluid by the probe, and ¹³C₄-PFOS and ¹³C₄-PFOA were added to the spray solvent to enable quantification

using internal standard calibration curves. Despite the complex chemical matrix, linearity and correlation coefficient values were found to be satisfactory with good repeatability and low relative standard deviations, indicating the suitability of solid-phase microextraction for quantification.¹¹⁰ Gong et al. have also used PESI to analyze compounds in plant cells.¹³³ Quantitative analysis of sucrose in *Allium cepa* cells was performed using arabinose as an internal standard added to the extraction solvent. Quantification was based on an external standard curve of sucrose, and the results were in good agreement with literature values.¹³³

The use of structural analogues as quantification standards must be evaluated carefully because of differences in matrix effects experienced by different molecules. Therefore, an isotopically labelled standard is preferred for Q-MSI experiments. To quantify the drug chloroquine and its metabolites in rat organs post-dose using LESA-MS/MS, Parson et al. used a deuterated standard, chloroquine-d4, in the extraction solvent. After a 24-hour time-course study was conducted,⁵⁵ the samples were analyzed and the relative amounts of chloroquine in tissues were calculated by normalizing the signal of the drug, or its metabolite, to the signal of the internal standard, chloroquine-d4. Quantitative data then revealed that drug metabolism was most prominent during the first six hours post-dose.⁵⁵

Using nano-DESI, Lanekoff et al. reported Q-MSI of nicotine in rat brain tissue sections 15 min post-dose.¹³⁴ In the experiments, the isotopically labeled nicotine internal standard was added to the extraction solvent and continuously supplied throughout the analysis. The amount of nicotine, in moles, in each pixel in the image was calculated using Eq. 1 from the intensity ratio of nicotine (I_{nicotine}) to deuterated nicotine (I_{std}), accounting for the concentration of deuterated nicotine in the extraction solvent (C_{std}), the flowrate ($\text{Flowrate}_{\text{std}}$), and the ion accumulation time (IT) for each scan in the Orbitrap:¹³⁴

$$\text{amount nicotine} \left(\frac{\text{moles}}{\text{pixel}} \right) = C_{\text{std}} \times \frac{I_{\text{nicotine}}}{I_{\text{std}}} \times \text{Flowrate}_{\text{std}} \times \text{IT} \quad (1)$$

The amount of nicotine extracted from the tissue during nano-DESI MSI was found to be ~0.34 fmol per pixel, where the pixel size was approximately $27 \mu\text{m} \times 150 \mu\text{m}$. The ~9% extraction efficiency was estimated by comparing the total amount of nicotine observed in Q-MSI experiments with the amount of nicotine extracted from tissue homogenates. This study has established an approach for Q-MSI of selected drugs or metabolites using nano-DESI MSI that can be readily extended to other ambient liquid extraction techniques.¹³⁴

A related approach also has been developed for shotgun-like quantification of endogenous PC species using nano-DESI MSI.¹³⁵ In a typical shotgun quantification experiment, the sample of interest is homogenized, and the molecular class of interest is extracted. The crude extract is spiked with internal standards and analyzed by ESI mass spectrometry or MS/MS through direct infusion in a high-throughput manner.¹³⁶ Quantification of lipids within the same class is achieved by comparing the signal observed for the identified species with that of the corresponding standard.¹³⁶ Using nano-DESI, Lanekoff et al. have extended this approach to Q-MSI of endogenous PC species in rat brain tissue by adding two PC standards to the extraction solvent.¹³⁵ The two internal standards bracket the range of acyl chain

lengths in endogenous PC species, which accounts for the effect of the chain length on the ionization efficiency of individual PC species using carbon factors (CF) given by Eq. 2:¹³⁵

$$CF_{PC} = CF_{std} + \gamma \times \Delta N_C \quad (2)$$

In Eq. 2, the carbon factor for the standard (CF_{std}) is set to 1, γ is a factor that accounts for the effect of chain length on the ionization efficiency, and N_C is the difference between the number of carbons in the fatty acid chains of the endogenous PC and number of carbon atoms in the tail groups of the standard.¹³⁵ Eq. 3, obtained by incorporating CF into Eq. 1, can calculate the amount of each PC species in each pixel in the image:¹³⁵

$$\text{amount PC} \left(\frac{\text{moles}}{\text{pixel}} \right) = C_{std} \times \frac{I_{PC}}{I_{std}} \times \text{Flowrate}_{std} \times IT \times \frac{CF_{PC}}{CF_{std}} \quad (3)$$

This shotgun-like Q-MSI approach has enabled simultaneous imaging and quantification of 22 endogenous PC species in seven different brain regions without sample preparation. Figure 3a depicts quantitative ion images of four selected PC species with the intensity scale bars in fmoles/pixel. Q-MSI affords direct comparison of the absolute abundances of endogenous molecules in different regions of interest in the ion images. For example, Figure 3b highlights four regions of interest, and Figure 3c shows the average amounts of the four PC in each region of the brain. These results demonstrate that PC 34:1 is the most abundant species in all four regions, whereas PC 36:1 is the second most abundant species in the white matter. Interestingly, PC 36:1 is twice as abundant in the neocortical layers V-VI as in the neocortical layers I-IV. Comparison of Q-MSI data obtained for 22 endogenous PC species indicates that acyl chain unsaturation has a pronounced effect on the localization of PC species in brain regions. Specifically, PC with four double bonds are most abundant in the hippocampus and least abundant in the thalamus. Saturated PC are abundant in the neocortical layers I-IV, while PC with one and six double bonds are most abundant in the thalamus.¹³⁵ This study demonstrates the unique power of Q-MSI for studying regional variations of endogenous lipids important to understanding the role of lipids in health and disease.¹³⁵ By adding an appropriate mixture of standards to the extraction solvent, shotgun-like quantification of lipids can be readily combined with metabolite quantification.

Almeida et al. have presented quantification of several lipid classes using PLESA MS/MS experiments, where lipids were extracted from different locations on a rat brain tissue section.⁸⁷ In these experiments, 16 lipid standards, one for each lipid class, were incorporated into the extraction solvent. The presence of lipids standards during extraction and ionization enabled quantification of 170 different lipid species from 11 lipid classes at each location.⁸⁷ The concentrations of the endogenous lipids were calculated using the known concentration of the internal standard of the lipid class and the intensity ratio of the endogenous lipid over the standard.¹³⁷ These experiments have demonstrated that the most abundant lipid class in all brain regions was PC, followed by phosphatidylethanolamine (PE). Furthermore, the authors found that phosphatidylinositol (PI) and phosphatidylserine (PS) are enhanced in the thalamus.⁸⁷

The Q-MSI approaches discussed in this section generally apply to the entire class of ambient liquid extraction techniques. Accurate quantification may be obtained using either isotopically labeled standards or standards that are structurally similar to the analyte of interest. The addition of internal standards into the extraction solvent is not limited to one class of molecules. Moreover, shotgun-like lipid quantification may be combined with targeted metabolite quantification in the same Q-MSI or spatial profiling experiment, providing for broad coverage of analytes.

4. REACTIVE ANALYSIS

Another advantage of ambient liquid extraction techniques is the ability to perform *in situ* chemical derivatization of the extracted analyte molecules by doping the extraction solvent with an appropriate reagent.^{21,138} *In situ* derivatization enables sensitive analysis of molecules that are not readily ionized using soft ionization techniques.^{139,140} For example, reactive DESI experiments with Girard T (GT) reagent¹⁴⁰ and betaine aldehyde (BA)¹⁴¹ (shown in Scheme 1) have been used for detecting and imaging cholesterol in tissue sections. These experiments take advantage of a dramatic increase in reaction rates in microdroplets compared to the bulk solution.¹⁴² For instance, an at least two orders of magnitude increase in product yields was obtained using reactive DESI of cortisone with GT as reagent in comparison with product yields observed in the bulk cortisone/GT solution.¹⁴² Rapid chemical reactions in small droplets result in enhanced product yields of online chemical derivatization in ambient liquid extraction experiments. We also note that quantitative conversion of analyte molecules into products enhances the sensitivity of reactive analysis.

Both GT and BA form covalent bonds with analyte molecules. However, covalent bond formation is not required for reactive analysis. For example, dicationic compounds that form strong electrostatic bonds with negatively charged lipids have been successfully used for enhancing lipid coverage in positive mode DESI MSI experiments.¹⁴³ As shown in Scheme 1, a dicationic compound, DC9, has been used for enhancing signals of fatty acids and PE species in DESI MSI of rat brain and zebra fish tissues.¹⁴⁴ Figure 4 shows mass spectra obtained using positive (panel a), negative (panel b), and reactive (panel c) DESI MSI analysis of rat brain tissue. In the positive mode, the spectrum is dominated by PC species, while the negative mode spectrum shows the presence of fatty acids (FA) at lower m/z and PE species at higher m/z . Reactive DESI (Figure 4c) contains all three lipid classes (e.g., PC, PE, and FA), thereby illustrating improved coverage of endogenous compounds in the imaging mode.¹⁴⁴ Interestingly, different images were obtained for abundant PE species in negative mode compared to positive mode reactive DESI MSI experiments, indicating differences in the matrix effects experienced by PE species in these experiments.

Reactive nano-DESI experiments have been used for selective derivatization of carbonyl compounds in complex organic aerosol (OA) mixtures collected on substrates.⁶⁸ Furthermore, it has been demonstrated that reactive nano-DESI enables quantification of individual constituents in a complex OA mixture.⁶⁸ In these experiments, the analysis was performed by adding varying amounts of the charged GT reagent to the solvent and performing multiple experiments on the same sample using localized liquid extraction. The

signals of the products increased with increasing GT concentration and reached saturation above a ~10- μ M concentration of the reagent. Based on the observed signals of individual products in the saturation region and the estimated ionization efficiency of the reaction products, concentrations of more than 100 carbonyl compounds in a complex OA mixture were quantified using only ~11 pg of material. This approach enables quantitative analysis of selected classes of compounds in complex mixtures, for which internal standards do not exist, and it possibly can be extended to imaging applications.

Using a transparent LMJ-SSP probe operated in a plug injection mode, ElNaggar and Van Berkel were able to visualize a redox reaction between 2,6-dichlorophenol indophenol (DCIP) and ascorbic acid.⁵¹ In this reaction, reduction of the oxidized form of DCIP that has blue color generates a colorless product. This color change was observed both when a dried spot of DCIP was collected into the probe and subsequently mixed with ascorbic acid introduced through the probe, as well as when DCIP was delivered through the probe to a dried spot of ascorbic acid. These experiments demonstrate efficient transfer of analyte molecules from the sample into the LMJ-SSP probe, mixing within a small volume inside the probe, and rapid reactivity of DCIP and ascorbic acid in the small volume of the plug. Furthermore, reactive analysis can be readily extended to other types of reagents.^{19,21} Scheme 1 shows examples of several reactions that may be used in reactive MSI experiments. These experiments may be used for either improved coverage or selective detection of specific classes of compounds, which is particularly advantageous for targeted analysis and may help improve the dynamic range of low-abundance species that are difficult to detect in conventional MSI experiments.

5. TANDEM MASS SPECTROMETRY IMAGING

MS/MS is commonly used for the structural characterization of molecules observed in MSI experiments. In the absence of any separation prior to detection, typical of MSI experiments, many isomeric and isobaric peaks often are present at the same nominal m/z . High-resolution mass spectrometry combined with MSI experiments may be used for separation of isobaric peaks. However, some isobaric species present in complex mixtures remain difficult to separate, even using modern high-resolution mass spectrometry instrumentation. In addition, isomeric peaks with the same exact molecular formula cannot be resolved using mass spectrometry. Both isobaric and isomeric peaks may have different spatial localization in the sample, which complicates the interpretation of individual images obtained in MSI mode. As such, MS/MS imaging is ideally suited for simultaneous spatial localization and identification of molecules extracted from substrates. However, most MS/MS imaging experiments are performed using a targeted list of compounds, usually limited to less than 10 species using single- and multiple-reaction monitoring (SRM and MRM).^{145,146} For example, DESI MS/MS has been used to distinguish between two isobaric red pigments, rhodamine 6G and rhodamine B at m/z 443, based on their respective fragment ions at m/z 415 and 399.¹⁴⁷ More recently, DESI SRM was used for imaging drug penetration into skin,¹⁴⁸ and AFADESI MRM experiments provided information on the spatial localization of an antitumor drug.⁴⁰ Meanwhile, LESA MS/MS experiments performed in MRM mode have been used for imaging drugs in tissue sections with a spatial resolution of ~1 mm.⁹⁷ Key findings from these studies will be discussed in the next sections.

When coupled to a high-speed mass spectrometer, nano-DESI provides an opportunity for simultaneous acquisition of a large number of MS/MS imaging data sets.¹⁴⁹ In fact, the number of m/z windows selected in these experiments is limited only by the acquisition speed of a mass spectrometer and the desired spatial resolution.¹⁴⁹ For example, in a study by Lanekoff et al.,¹⁴⁹ a targeted list of 92 m/z values was subjected to sequential isolation and fragmentation using higher-energy collision-induced dissociation (HCD) while the sample was scanned under the nano-DESI probe at a rate of 10 $\mu\text{m/s}$. Because of the presence of multiple features in each m/z window, this experiment generated MS/MS images for more than 300 individual molecules with a spatial resolution of $\sim 150\text{ }\mu\text{m}$ for each isolated m/z window.¹⁴⁹ The results demonstrated the enhanced dynamic range of MS/MS imaging, which enabled spatial localization of the minor metabolite, ornithine, not observed in the MSI mode. In Figure 5, simultaneous imaging of isobaric species difficult to separate using high-resolution mass spectrometry is depicted for two phospholipids, plasmenyl PE (P-16:0/22:6) at m/z 770.5095 and $[\text{M}+\text{K}]^+$ ion of PC 32:1 at m/z 770.5095, which would require a mass resolving power of 3,850,000 in MSI mode. Moreover, MS/MS imaging can enable separation of isomeric lipids, PC 18:0/18:2 and PC 18:1/18:1, based on the observed losses of fatty acid tail groups.

6. LOCALIZED ANALYSIS AND IMAGING APPLICATIONS

6.1 Tissue Imaging

Liquid extraction techniques have been used extensively for molecular imaging of tissue sections. In these experiments, thin tissue sections produced by cryo-sectioning are thaw mounted onto glass slides. Then, MSI is employed to map distributions of administered drugs and/or endogenous compounds, such as metabolites, lipids, and proteins, directly from the surface of the tissue section without additional sample pretreatment.

6.1.1 Drug Localization—Drug localization studies are essential to drug discovery and development. MSI provides information about the localization of administered drugs, drug metabolites, and endogenous species in tissues in a single imaging experiment. DESI MSI has been used to study the distribution of administered clozapine in rat brain¹²³ and in whole body section of a neonate mouse.¹⁵⁰ In their work, Lui et al. imaged both administered clozapine and its N-oxide metabolite in mouse tissues and found them co-localized in the brain, spinal cord, liver, heart, and lungs.¹⁵⁰ Because of its blood-brain barrier similarities with mammals, the locust has been evaluated as a future model species for drug development.¹⁵¹ Using DESI MSI, terfenadine and its metabolite terfenadine acid were mapped in the whole body of a locust.¹⁵¹ Interestingly, the ion images of the drug and its metabolite did not correlate. The metabolite was only detected in unexcreted feces, suggesting an immediate delocalization of the metabolite upon formation.¹⁵¹ Due to the significant anatomical differences between mammals and insects, these results may not be directly transferrable to mammals. Nevertheless, they provide insights on biochemical processes associated with terfenadine administration.

In their experiments, D'Alvise et al. investigated the distribution of lidocaine and its metabolite 3-OH lidocaine in pig ear skin.¹⁵² Biopsies of pig ear, post application of

Xylocain ointment, were frozen, cryo-sectioned, and imaged with DESI MS/MS. The study successfully observed follicular transport of lidocaine and its metabolism in adipose tissue of pig ear skin.¹⁵² In a related study, Eberlin et al. investigated the penetration of several analgesics into human skin using DESI MSI.¹⁵³ Post application, the skin was frozen and cross-sectioned to examine the penetration depth. Lidocaine, aconitine, and batrachotoxin were found to penetrate the deep dermis layer, while saxitoxin did not exhibit any penetration of the skin, probably because of its charged hydrophilic nature. Notably, a derivate of saxitoxin, with a long-chain glycerol ether group, showed transdermal penetration but only in a dimethyl sulfoxide vehicle. Studies such as these are important in drug delivery development, specifically for local anesthetics.¹⁵³

In addition, AFADESI in MRM mode was employed to map the distribution of an anticancer drug, CAT, and its metabolite in whole body rat tissue sections.⁴⁰ At 20 minutes post administration, the drug was localized to the brain, adrenal gland, and kidney. At two hours post dosing, the drug began to metabolize, showing metabolites localized to the stomach and intestine.⁴⁰ A related study used AFADESI MSI to examine the effect of drug administration on the endogenous metabolites in whole body rat tissue sections.¹⁵⁴ Metabolite abundances in a control tissue section and another from a rat administered the anti-insomnia drug candidate, NHBAB, were examined using AFADESI MSI.¹⁵⁴ Principal component analysis (PCA) of the mass spectra identified six endogenous metabolites, which were altered upon drug administration. Spatiotemporal post-dosing MS/MS imaging of the endogenous metabolites and NHBAB found significant time-dependent metabolic alterations that may aid in understanding drug action mechanisms and pathways.¹⁵⁴ This study demonstrates the strength of MSI for biomedical studies because no pre-labeling of molecules is required.

In their work, Schadt et al. used isotopically labeled figopitant to correlate drug distributions in whole body rat sections with autoradiography and LESA MS post drug administration.⁸⁶ While autoradiography enabled quantitative high-resolution imaging of the labeled figopitant, it could not distinguish between the drug and drug metabolites as both contained the isotopic label. LESA MS proved to be superior for the identification of drug and drug metabolites.⁸⁶ In another study, Prideaux et al. performed imaging of levofloxacin in rat lung tissue infected with tuberculosis using Flowprobe MSI.¹⁵⁵ The tissue was imaged by extracting material from positions separated by 630 μm over the entire tissue section. Figures 6A and 6B show interpolated raw images, while Figures 6C and 6D show the unprocessed ion images at 2 and 24 hours post drug administration, respectively. Figures 6E and 8F depict the optical images of the tissue sections used for MSI. The study concluded that despite the modest spatial resolution obtained using Flowprobe MSI, the analysis was rapid and sensitive with no sample preparation post cryo-sectioning needed, making the technique a good choice for high-throughput imaging of tissue sections.¹⁵⁵

One drawback to using MSI for drug discovery and development studies is that it requires each of the subject animals to be sacrificed, which is both costly and laborious, especially when conducting time-course studies.⁹⁷ To increase throughput and potentially reduce costs, Swales et al. simultaneously administered four drugs to a rat model.⁹⁷ MSI was performed on rat brain and kidney tissue sections post drug administration at two time points. Figure 6G shows LESA MS/MS images of four drug compounds in rat kidney at 2 and 6 hours post

dosing. LESA images were acquired with a spatial resolution of 1 mm by extracting material from approximately 125 locations over the tissue section. The study concluded that the strategy of dosing multiple drugs simultaneously successfully enabled both pharmacokinetic and distribution data using fewer resources.⁹⁷

6.1.2. Endogenous Compounds—MSI of endogenous compounds provides valuable information about biochemical processes and metabolic pathways occurring in tissues. Ambient liquid extraction techniques enable image acquisition of endogenous compounds, such as lipids,^{156–158} metabolites,^{157,159} and proteins in tissue sections.¹⁶⁰ Rodent brain tissue sections are often used in MSI studies to examine the distribution of endogenous molecules and administered drugs in animal models. Chemically heterogeneous brain tissues have been reproducibly mapped using different liquid-extraction-based techniques. The distribution of major lipids in mouse and rat brain tissue sections is well established, making rodent brain tissue sections ideal samples for developing novel liquid extraction techniques and other technical advancements in MSI.^{22,30,36,41,60,73,104,105,107,125} Lipid distributions in brain tissue sections using both DESI MSI and EASI MSI have been reported in two related studies.^{22,30} Both studies concluded that although the acquired ion images showed extreme similarities between the two techniques, higher ion intensity and dynamic range can be achieved using DESI.^{22,30} Additional increases in sensitivity in the presence of air flow in AFADESI experiments have enabled rapid whole-body imaging and sensitive metabolite imaging in tissue sections.^{40,161} Sensitive lipid imaging with a signal-to-noise ratio of up to 100 in each pixel and a spatial resolution of ~12 μm has been reported using nano-DESI MSI.⁶¹ Lipid imaging with a spatial resolution down to ~8 μm has been reported using single-probe MSI.⁷³ Improved lipid coverage has been achieved using reactive DESI MSI.¹⁴⁴ These studies provide benchmark information for applications of ambient liquid extraction MSI in biological and clinical research.

MSI and spatial profiling using ambient liquid extraction ionization have been engaged in numerous studies relevant to clinical research.^{6,162} For example, significant efforts have been dedicated to obtaining cancer-specific DESI mass spectrometry and MSI data that can differentiate between necrotic and healthy tissue. DESI MSI has shown it affords acceptable repeatability and reproducibility for clinical studies and diagnostics.¹⁶³ Specifically, repeatability of $22\pm 7\%$ and reproducibility of $18\pm 8\%$ in lipid imaging has been reported by comparing DESI MSI of human oesophageal cancer tissue samples performed on two different instruments by two distinct operators.¹⁶³ The ability to perform rapid and reproducible ambient analysis of molecules in thin tissue sections has established DESI MSI as a tool for studying both cancer biology and clinical diagnostics.

DESI MSI of mouse cancer models has been used for understanding cancer cell biochemistry. For instance, Eberlin et al. examined the effect of the v-myc avian myelocytomatosis viral oncogene homolog (MYC) on the lipid and metabolite profile in mouse cancer models.^{164–166} MYC overexpression is strongly correlated with the aggressiveness of human cancers. The study of a mouse model of hepatocellular carcinoma demonstrated substantial enhancement of glycerophosphoglycerols in MYC-induced lymphoma tissues compared to tissue samples from the control mice.¹⁶⁵ Furthermore, a higher abundance of monounsaturated fatty acyl chains in glycerophospholipids and

cardiolipins was observed in cancer tissue compared to healthy tissue.¹⁶⁵ The similarity of lipid profiles obtained for the mouse model and MYC-associated human lymphoma samples suggests that the transgenic mouse model recapitulates key molecular signatures of human cancer. In addition, glutamine—but not glucose—was enhanced in the MYC-induced model of renal cell carcinoma (RCC),¹⁶⁶ suggesting that RCC cells use glutamine as an energy source. It also has been suggested that inhibition of glutamine metabolism may be used for treatment of MYC-induced RCC.¹⁶⁶ t-SPEI MSI of mouse pancreatic cancer tissue sections also have revealed several unique markers of cancerous regions of the tissue, including polyamines, several metabolites, and small proteins.¹⁶⁷

Ambient liquid extraction MSI also has been utilized for studying molecular signatures of human cancer. For example, Eberlin et al. used DESI MSI of 36 human glioma samples to examine molecular signatures of human brain tumors.¹⁶⁸ Figures 7A–7F show ion images of selected lipid species in a heterogeneous glioblastoma section, and Figure 7I shows the hematoxylin and eosin (H&E)-stained tissue with two marked regions, blue and red, with the border magnified. Tumor cell density in the blue region is nearly 100%, while it is only about 30% the red region that mainly consists of gray matter infiltrated by glioma cells. As suggested by the ion images in Figures 7A–7F, lipid profiles in the two regions are distinctly different, indicating the complexity of these systems and the need for molecular classifications to distinguish between healthy and necrotic tissue.¹⁶⁸ In other work, Li et al. examined 52 lung cancer tissue samples using AFADESI.¹⁶¹ Several lung cancer biomarkers, including choline, PC species, proline, and carnitine, have been identified and validated in that study. Nano-DESI MSI also has been used to examine human RCC samples.⁶¹ Cholesterol and cholesteryl esters known to be enhanced in RCC tissues have been observed as dominant species in the nano-DESI MSI experiments.⁶¹ DESI MSI of patient biopsies have indicated the disruption of normal lipid metabolism as the main marker of the altered microenvironment in necrotic tissue.^{161,168–171} In a related study, DESI MSI of surgical biopsies has been used for diagnostics of human breast cancer¹⁷¹ and canine non-Hodgkin's lymphoma.¹⁷²

Because the cellular chemistry in cancer may be altered before the tissue morphology changes, DESI MSI has been used to address the difficulties faced by surgical teams in finding margins between the tumor and healthy surrounding tissue.¹⁶² Several studies have found that tumor margins can be quickly identified using molecular markers observed in DESI MSI experiments.^{162,173–178} These molecular markers typically are identified using PCA of mass spectra collected on healthy and cancerous tissues. For example, Calligaris et al. used DESI MSI for breast cancer margin analysis.¹⁷³ They found that several fatty acids were more abundant in the tumor than in healthy tissue. Molecular tumor margins determined using DESI MSI were consistent with the results on H&E staining.¹⁷³ In the absence of known markers, contrast agent MSI may be used to examine tumor margins and heterogeneity.¹⁷⁸ For example, Tata et al. used a standard magnetic resonance imaging (MRI) contrast agent, gadoteridol, to examine the heterogeneity of human breast cancer tumors grown in mice.¹⁷⁸ In that study, DESI MSI data were complemented with *in vivo* MRI, H&E staining, and inductively coupled plasma (ICP) MSI of gadolinium in tissues. Complementary information obtained from the multimodal characterization of tissue samples has enabled confident mapping of vasculature and margins in different tumor

subregions. These studies establish DESI MSI as a valuable tool for surgical decision-making processes.^{162,173–178}

Lipid and metabolite imaging also has been engaged to examine early stages of embryo development in mice. In these studies, nano-DESI MSI has helped generate three dimensional and MS/MS ion images of lipids and metabolites in mouse embryo implantation sites on Day 6 of pregnancy.^{63,149} The spatial distribution of free fatty acids and lipids in uterine tissue were found to depend highly on the composition of the fatty acyl tail group moiety. Specifically, both acyl chains containing an 18:2 moiety and acetylcholine had enhanced abundance in the primary decidual zone of the uteri, suggesting the importance of specific molecules in decidual cell signaling.⁶³ Nano-DESI MSI demonstrated differences in localization of choline, phosphocholine, and acetylcholine, which has been attributed to the different roles these compounds play in the decidualization process. The results suggest that acetylcholine and FA 18:2 play important roles in decidual cell signaling, while choline and phosphocholine are involved in PC synthesis.

Although protein imaging using ambient ionization MSI¹⁶⁰ is less established than imaging of lipids and metabolites, several examples have been reported in the literature. For example, Sarsby et al. profiled proteins correlating with nonalcoholic liver disease in liver tissue using LESA MS.¹⁷⁹ After cryo-sectioning, the tissue sections were washed to remove lipids and metabolites for an improved protein signal. Healthy and diseased tissues were analyzed by top-down proteomic LESA MS. Liver fatty acid binding protein (FABP1) and its variants were successfully identified in the tissue sections by MS/MS. In particular, the FABP1 variant FABP_{TA}, which is implicated in nonalcoholic liver disease, was reliably identified. The results indicate that LESA MS is well suited for profiling and detection of protein biomarkers of nonalcoholic liver disease in human liver biopsies.¹⁷⁹ Top-down LESA MS also has been used for direct analysis of hemoglobin variants in dried blood spots,¹⁸⁰ and the reproducible identification of hemoglobin substructures established LESA MS as a promising technique for diagnosing hemoglobinopathies from dried blood spots.¹⁸⁰

In another experiment, Hsu et al. used nano-DESI MS to detect hemoglobin and other endogenous molecules, including peptides, proteins, lipids, sugars, and metabolites, in developing mouse spinal cord, from embryonic until adult stages.¹⁸¹ They were able to follow the transition of fetal hemoglobin subunits to adult isoforms. Specific locations on the tissue were identified using a microscope followed by site specific mapping of endogenous molecules with nano-DESI MS. Spatiotemporal mapping of ion distributions then revealed molecular gradients of two β -thymosin isoforms in the spinal cord during embryonic development, suggesting their role in spinal cord development.¹⁸¹ Nano-DESI MSI also has been used to study protein alteration in MYC-induced lymphomas.¹⁸² Ubiquitin and thymosin missing C-terminal amino acid residues have been identified in lymphomas while full-length proteins have been observed in healthy tissues.¹⁸² Identification of truncated proteins, difficult to detect using traditional immune-based methods, and their spatial mapping using nano-DESI MSI provides important information on biochemical processes in cancer cells.

6.2. Imaging of Plant Tissues

MSI also has been used for spatial localization of plant metabolites.^{9,183,184} The presence of plant cuticle, a protective lipid polymer layer impregnated with waxes on the outer surfaces of leaves and petals, makes ambient MSI of plant tissues particularly challenging.^{159,184} To enable molecule extraction from under this protective layer, direct analysis of plant tissues requires energetic bombardment.^{184–187} Li et al. examined the effect of solvent composition on the efficiency of direct DESI MSI of plant tissues.¹⁸⁵ A ternary chloroform-acetonitrile-water (1:1:0.04) solution has shown the best performance for direct plant tissue imaging.¹⁸⁵ Using this solvent composition, the authors detected a number of metabolites in plant tissues. Very long chain fatty acids (VLCFAs) localized on the leaves and flower cuticles have been mapped by DESI MSI. The ability to detect VLCFAs not observed in plant imprints demonstrates the advantage of direct plant tissues analysis by DESI MSI.¹⁸⁵ Alternatively, prior to analysis, plant tissues may be cryo-sectioned¹⁸⁷ or imprinted^{184,188,189} onto a substrate, such as a TLC plate,^{190–193} tape,¹⁹⁴ or porous Teflon.¹⁸⁷ The latter approach has been extensively used for DESI MSI of lipids and metabolites in plant tissues. For example, Hemalatha and Pradeep have reported DESI MSI of leaves and petals imprinted onto TLC plates.¹⁹² They identified several alkaloids and metabolites in plant tissues using MS/MS and database searching. Tata et al. have used DESI MSI of plant tissue imprints to study plant-pathogen interactions¹⁸⁹ and observed a decrease in the level of glycoalkaloids that became more pronounced as the disease progressed. DESI MSI also has been used to map pesticides in plant tissues¹⁹⁵ and has shown that imidacloprid accumulated in the plant tissue surface while methiocarb distributed homogeneously in the tissue. Similarly, pesticides in plants have been mapped using LESA MS.¹³² In that study, a mixture of pesticides was supplied during plant watering. After one week, pesticides were detected only in lower parts of stem and leaves, indicating that the upper portion of the plant was protected against foreign material not used for growth.

6.3 Localized Analysis and Imaging of Microbial Communities

Ambient liquid extraction techniques provide a unique opportunity for direct spatial profiling of molecules in living microorganisms, such as bacteria, fungi, yeast, and algae.^{7,8} The gentle surface microextraction of nano-DESI has enabled characterization and identification of molecular signatures of microbial interactions by analyzing living colonies directly from agar plates.^{65–67,196–198} Watrous et al. used nano-DESI to spatially characterize and identify molecular signatures in two bacterial strains, *Streptomyces coelicolor* A3 and *Bacillus subtilis* PY79, co-cultured on an agar plate.⁶⁵ Molecules were profiled at two locations, facing away from and toward the other colony. A molecular network of metabolites released during bacterial interaction was established using nano-DESI MS/MS data.⁶⁵ Molecular networks are obtained by clustering structurally related compounds based on the observed fragments in MS/MS. In this approach, both common ionic fragments and neutral losses help determine similarity scores for individual compounds. Identification of previously unknown compounds then could be performed based on their classification. For example, a new surfactin analog was identified for *Bacillus Subtilis* based on the molecular network obtained for this system.⁶⁵ In addition, nano-DESI MS of *Pseudomonas* sp. strain SH-C52 combined with molecular networking enabled detection of a predicted—but never experimentally observed—lipopeptide, thanamycin.⁶⁵

Spatial profiling of living microbial colonies coupled with MS/MS experiments facilitates understanding of molecular interactions in and between microbial communities.^{7,8} Lanekoff et al. examined diffusion of metabolites from living *Synechococcus* sp. PCC 7002 colonies using spatially resolved nano-DESI MSI analysis of the colonies and the surrounding agar in a time-course experiment.⁶⁶ In this study, several novel monogalactosyldiacylglycerol and digalactosyldiacylglycerol species have been identified in the colonies. Chemical gradients of metabolites on the surrounding agar, acquired using a one-dimensional scan, identified two metabolites, glycosylglycerol and sucrose, released by the *Synechococcus* sp. PCC 7002 colony onto agar. Nano-DESI experiments further indicated that metabolite diffusion correlates with colony age.⁶⁶ Watrous et al. have shown nano-DESI MSI of metabolites in two different living bacterial colonies, *Shewanella oneidensis* MR-1 and *Bacillus subtilis* 3610, and in one colony where the two strains were mixed.⁶⁷ By establishing the molecular network of detected metabolites, the study mapped several metabolites associated with metal oxide reduction by *S. oneidensis* MR-1, including putrebactin and riboflavin specific to the *S. oneidensis* MR-1 strain.⁶⁷ Molecular networks of endogenous species detected by nano-DESI MS/MS afford insights into metabolic pathways of both individual and co-cultured living microbial communities.^{196,197} Nano-DESI MS also has enabled identification and characterization of molecules in gut bacteria of microbiota mouse models¹⁹⁹ and pathogenic fungi living on the skin of bats with white-nose syndrome.²⁰⁰ Additionally, Flowprobe MS has helped map endogenous microbial metabolites of more than 30 different microorganisms from around the world, showcasing the technique's ability for high-throughput characterization of molecules in microbial colonies.⁵⁷ Using TS mass spectrometry directly from a swab, Jarmusch et al. also achieved rapid analysis of bacteria in patients' throats.²⁰¹

In other work, Kai et al. used LESA MS to profile endogenous antibiotic compounds produced by bacterial colonies living on agar.²⁰² Microbial signaling and production of antibiotics were explored by co-culturing bacterial strains and monitoring production of antibiotic-like compounds. By optimizing the extraction solvent, several antibiotic compounds, such as multithiomycins and sulfomycins, could be readily detected in the colonies.²⁰² Similarly, using LESA MS, Menezes et al. investigated signaling and secondary metabolite release between competing fungal strains *in vivo*.²⁰³ Two fungal strains, *Schizophyllum commune* and *Hypholoma fasciculare*, were co-cultured on an agar plate followed by molecular profiling of the fungal colonies and their interaction area. Molecular signatures in different locations were compared using PCA, revealing significant differences between three locations. Specifically, indigo, indirubin, and isatin were identified in the interaction area between the two fungi colonies.²⁰³ LESA MS also has been employed for top-down proteomic analysis of living *E. coli* colonies using a "contact" LESA approach.⁸⁴

The high gas and droplet velocity in DESI and EASI typically requires a hard surface to obtain a consistent spray over the sample.^{67,204–206} Angiolini et al. investigated sample preparation protocols for direct analysis of microbial colonies using these spray-based techniques.²⁰⁴ Specifically, they compared imprinting bacterial colonies onto a polytetrafluoroethylene (PTFE) membrane with agar dehydration. The study found that imprinting generates a non-consistent spatial distribution of molecules and loss of molecular information, while dehydrated agar enables well-resolved ion images with high signal intensity using both DESI MSI and EASI MSI.²⁰⁴ However, imprinting has been used

successfully to explore metabolite production in plants as a response to phytopathogen invasion.^{192,194} Using DESI MSI of samples imprinted onto a tape, Tata et al. investigated potato sprouts infected with pathogenic fungi.¹⁹⁴ As the infection progressed, glycoalkaloids were found to decrease in abundance, suggesting that the fungi *Pythium ultimum* can tolerate these glycotoxins.¹⁹⁴ Similarly, Hemalatha et al. imprinted basil leaves onto TLC plates to investigate metabolites originating from the fungal infection of *Fusarium*.¹⁹¹ Several metabolites associated with fungal infection may be distinguished by comparing ion signals from diseased plants with those from healthy plants. That study found that fusaric acid was the main characteristic toxic metabolite.¹⁹¹

In search of natural products targeting virulence in methicillin-resistant *S. aureus* (MRSA), metabolites produced by the guttate-forming fungi *Penicillium restrictum* (Figure 8A and 8B) were investigated using DESI MSI.²⁰⁵ Fungal colonies were imprinted onto PTFE membranes, and ion images of nine different quorum-sensing inhibitors, polyhydroxyanthraquinones, were found mainly localized to the guttate. To investigate the spatial depth distribution of polyhydroxyanthraquinones within the fungal colony, the culture was frozen and cryo-cross-sectioned prior to DESI MSI. Figure 8C shows the optical image of the colony cross-section and ion images of eight investigated polyhydroxyanthraquinones. Interestingly, distribution of the most potent quorum-sensing inhibitors was concentrated to the fungal surface, while the less-potent inhibitors were distributed throughout the colony. These results suggest that polyhydroxyanthraquinones are produced by the fungal mycelia and distributed to the colony's surface to facilitate interactions with the surrounding environment.²⁰⁵

6.4 Single-cell Analysis

Ambient liquid extraction techniques have been used for studying molecular heterogeneity within and between single cells. Fukano et al. used live single-cell mass spectrometry to study single-cell heterogeneity of tafluprost metabolism in primary human hepatocytes.²⁰⁷ In that study, one picoliter of cellular content was collected on a fine tip, which was backfilled with 1 μ l of extraction solvent and positioned in front of a mass spectrometer. Five tafluprost metabolites were detected, but the drug-to-metabolite ratio differed significantly between individual single cells, reflecting heterogeneity in cell metabolism.²⁰⁷ Date et al. used video-mass spectrometry, similar to live single-cell mass spectrometry, to monitor subcellular metabolism of the drug tamoxifen in single human hepatocellular carcinoma (HepG2) cells.²⁰⁸ The drug was discovered inside cell vacuoles, but the drug metabolites were detected only in the cytoplasm, suggesting differences in subcellular metabolism.²⁰⁸ The endogenous contents of plant cells also have been investigated using live single-cell mass spectrometry.²⁰⁹ In that study, molecules from inside a single cell of plant tissue were collected from a leaf, stem, and flower petal, respectively. Using PCA, molecules of different origin were distinguished. The study found that blumenol B, not previously reported in *Pelargonium zonale*, was specific to the flower petal cells.²⁰⁹

In a related study, Tsuyama et al. used PCA to compare endogenous compound extracted from single cells from seven different cell lines using live single-cell MS.²¹⁰ The PCA score plot revealed unique peaks for single cells originating from each cell line, indicating the

metabolic heterogeneity between cell lines. The live single-cell mass spectrometry setup also has also been engaged to study both internal and external metabolites in individual single cells trapped in a microdroplet array.²¹¹ Molecules, such as dopamine and epinephrine, were successfully identified from single rat pheochromocytoma (PC12) cells, suggesting the technique's applicability to single-cell secretomics.²¹¹ A similar approach, called capillary microsampling, has been used for metabolic profiling of single *Arabidopsis thaliana* plant cells from different regions of a leaf.²¹² In that study, cell content was harvested into a fine tip, backfilled with 1- μ l electrospray solution. A platinum wire was inserted into the tip before being placed in front of a mass spectrometer. Distinctly different mass spectra were observed from three different cell types of the leaf, and ion mobility spectrometry enabled separation of isobaric ions.²¹² An oil-filled, pressurized fine quartz capillary tip, or the pressure probe, was employed to collect molecular content from single cells in a tulip bulb.²¹³ Once the cell solution was collected, the tip was positioned at the front of the mass spectrometer, and the cell solution was directly ionized by atmospheric pressure ionization (API). The cellular metabolome was rapidly analyzed, and amino acids, sugars, and fatty acids were detected from a single cell.²¹³ Gong et al. conducted single-cell analysis using PESI.¹³³ They inserted a sharp needle probe inside a single *Allium cepa* plant cell for 30 s to accumulate endogenous molecules on its surface. When withdrawn, the probe was placed in front of the mass spectrometer for analysis, detecting PC and PE species and metabolites, such as flavones. Methylene blue was added to the cells, and subcellular analysis concluded that the intensity of methylene blue in the nucleus was 20 times higher than in the cytoplasm, establishing PESI mass spectrometry as a technique for subcellular analysis.¹³³

Pan et al. also demonstrated single-cell analysis using single-probe mass spectrometry of cervical cancer (HeLa) cells.⁷² Endogenous lipids, such as PC, sphingomyelin, and diacylglycerol, and metabolites, such as AMP, ADP, and ATP, were detected from individual cells. HeLa cells then were treated with anticancer drugs. Both the administered drug and its metabolite could be detected in single cells, indicating the viability of single-probe mass spectrometry for single-cell analysis.⁷² Recent advances in single-cell analysis by mass spectrometry provide great opportunities for future studies of metabolic pathways in single cells. In this context, ambient liquid extraction techniques will facilitate understanding of how cellular function and behavior are influenced by the surrounding molecular environment.

Ellis et al. used LESA MS for ambient profiling of living single cells in microarrays produced by inkjet printing.⁹⁰ LESA MS generated stable prolonged signals from individual inkjet printed cells, which enabled signal averaging and MS/MS characterization of lipids extracted from the cells. Different cells types were readily separated using PCA indicating substantial differences in lipid profiles. The authors proposed that LESA MS combined with inkjet printing may be used for high-throughput cell profiling.

6.5 Spatially Resolved Analysis of Environmental Samples

Ambient liquid extraction techniques have been extensively used in analyzing aerosol samples collected on substrates.²¹⁴ In these experiments, aerosol samples generated under controlled laboratory conditions or collected in field studies are analyzed directly from either

aluminum foil or Teflon substrates using ambient liquid extraction techniques. Spatially resolved ambient liquid extraction, where analyte molecules are extracted into a very small volume of the extraction solvent, is particularly advantageous for detecting analyte molecules in small/sparse samples,^{69,215} analysis of time-resolved aerosol samples collected using drum impactors,²¹⁶ and reactive analysis from multiple locations on the sample (described earlier).⁶⁸ It also has been demonstrated that DESI and nano-DESI preserve chemically labile compounds in organic aerosol samples susceptible to hydrolysis in solution.^{62,217} Using spatially resolved LESA MSI, Fuller et al. examined variations in the chemical composition of atmospheric organic particles collected in California's San Joaquin Valley with meteorological conditions.²¹⁶ They identified several nitrogen-containing compounds originating from a local emission source and a series of compounds attributed to biomass burning. Meanwhile, Boone et al. used nano-DESI for localized extraction of very small atmospheric aerosol samples collected onboard a research aircraft over a forested region of Alabama.⁶⁹ The dominant class of compounds observed in these samples was identified as organosulfates produced in atmospheric oxidation of isoprene and monoterpenes. Using nano-DESI coupled to a high-resolution mass spectrometer, O'Brien et al. identified organonitrates, organosulfates, and nitroxy organosulfates in negative mode and low-volatility products of diesel fuel and isoprene oxidation in positive mode mass spectra of small aerosol samples collected in Bakersfield and Los Angeles, California.^{79,215}

CONCLUSIONS

Ambient liquid extraction ionization has been established as powerful tool for spatial profiling and imaging of complex biological and environmental samples. In this review, we have summarized key developments and applications of ambient liquid extraction techniques reported in the past four years. Specifically, we have discussed approaches for quantification, reactive analysis, and MS/MS imaging along with distinct applications of these techniques in drug development, imaging of endogenous compounds in tissues and microbial communities, single-cell studies, and environmental research. In the past four years, there has been substantial growth in the number of new ambient liquid extraction techniques and their applications, indicating increasing interest among the scientific community in the application of novel tools for solving challenging problems in biology, clinical studies, and environmental research. Robust approaches for quantitative chemical analysis using ambient liquid extraction and the ability to couple these techniques to any mass spectrometer provide unique advantages for a range of applications. In particular, a diversity of probes that have been developed in the past decade, selective ionization and quantification using reactive analysis, and straightforward quantification approaches make liquid extraction approaches especially advantageous for studies in biology, environmental sciences, and forensics.

In this review, we also have discussed the importance of matrix effects in MSI experiments and described approaches that account for ion suppression during ionization by doping the extraction solvent with appropriate standards. These approaches can enable accurate determination of concentration gradients of various classes of molecules in complex samples. Similar approaches using ambient liquid extraction ionization techniques also can be employed for quantitative imaging and spatial profiling. Q-MSI is an emerging area of

research where liquid extraction techniques will play an important role. The ability to tailor the extraction solvent's composition is particularly advantageous for Q-MSI experiments, where both individual molecules and selected classes of extracted compounds may be quantified. Reactive analysis provides yet another powerful tool to facilitate ionization and structural characterization of species that can be difficult to ionize using traditional soft-ionization approaches. Reactive analysis also may enable quantification of selected classes of analytes, for which internal standards cannot be readily obtained. We anticipate continuing developments of liquid extraction techniques to improve the spatial resolution and enable accurate determination of the extraction efficiency for absolute quantification in Q-MSI. Such developments will continue to establish ambient liquid extraction ionization as a viable and distinct tool for addressing complex problems in a variety of research applications.

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Ingela Lanekoff received her M.Sc. degree in chemical engineering from Mälardalen University, Eskilstuna, Sweden in 2001 and her Ph.D. degree in analytical chemistry from the University of Gothenburg, Sweden in 2011. She was a postdoctoral fellow at Pacific Northwest National Laboratory (PNNL, 2011–2014) and currently is an assistant professor at Uppsala University in Sweden. Her research focuses on development of ambient surface sampling coupled to mass spectrometry and mass spectrometry imaging with applications in the field of biomedicine.

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Figure 1.

A) Ion suppression effect in performing DESI on 1.5 ng of clozapine from the surface of different animal tissue sections. Average ion intensities are normalized to Omni-Slide. Error bars represent standard deviation from the mean for four replicates. Reproduced from Vismeh, R.; Waldon, D. J.; Teffera, Y.; Zhao, Z., Localization and Quantification of Drugs in Animal Tissues by Use of Desorption Electrospray Ionization Mass Spectrometry Imaging. *Anal. Chem.* 2012, 84, 5439–5445 (ref. 123). Copyright 2012 American Chemical Society. (B) Optical image of the brain tissue section with marked areas: pink = ischemic region and blue = white matter regions. (C) Ion images of sodium and potassium adducts, $[M + Na]^+$

and $[M + K]^+$, of standard PC 43:6 normalized to the TIC. (D) Ion images of sodium and potassium adducts, $[M + Na]^+$ and $[M + K]^+$, of standard PC 43:6 normalized to the standard PC 25:0. Lateral scale bar is 2 mm. Intensity scale bar ranges from 0 (black) to 100% (light yellow) signal intensity of an individual peak. Panels B-C are reproduced from Lanekoff, I.; Stevens, S. L.; Stenzel-Poore, M. P.; Laskin, J., Matrix Effects in Biological Mass Spectrometry Imaging: Identification and Compensation. *Analyst* 2014, 139, 3528–3532 (ref. 125), with permission of The Royal Society of Chemistry (Copyright, 2014).

Figure 2.

DESI MSI of cocaine traces in fingerprint with the intensity color coded in pg/pixel. Image size is 14×9 mm. Reproduced with permission from Muramoto, S.; Forbes, T. P.; van Asten, A. C.; Gillen, G., Test Sample for the Spatially Resolved Quantification of Illicit Drugs on Fingerprints Using Imaging Mass Spectrometry. *Anal. Chem.* 2015, 87, 5444–5450 (ref. 124). Copyright 2015 American Chemical Society.

Figure 3.

(A) Four quantitative nano-DESI images of endogenous PC. From left to right: PC 32:0, PC 34:1, PC 36:4, and PC 36:1 are displayed as $[M+K]^+$ signals normalized to $[PC\ 43:6+K]^+$. The values in the intensity scale bar show fmol/pixel and vary between ion images. Scale bar shows 2 mm. (B) Regions of interest are color coded as: yellow, neocortical layers I–IV (I–IV); green, neocortical layers V–VI (V–VI); red, white matter (WM); and blue, hippocampus (Hipp). (C) Graphs showing the average amount of the corresponding PC in each region of the brain in fmoles per pixel (color coded as described in B). Error bars show standard deviations of individual pixels in the respective region. Number of pixels within each region: I–IV, $n = 258$; V–VI, $n = 356$; WM, $n = 249$; and Hipp, $n = 477$. Reproduced from Lanekoff, I.; Thomas, M.; Laskin, J., Shotgun Approach for Quantitative Imaging of Phospholipids Using Nanospray Desorption Electrospray Ionization Mass Spectrometry. *Anal. Chem.* 2014, 86, 1872–1880 (ref.135). Copyright 2014 American Chemical Society.

Figure 4.

Representative DESI mass spectra of lipids in rat brain. (a) PC present in rat brain in positive ion mode control. (b) Lipids in rat brain in negative ion mode. (c) Reactive DESI mass spectrum of enhanced intensity DC9-lipid ion pairs in rat brain in positive ion mode.

Reproduced from Lostun, D.; Perez, C. J.; Licence, P.; Barrett, D. A.; Ifa, D. R., Reactive Desi-Ms Imaging of Biological Tissues with Dicationic Ion-Pairing Compounds. *Anal. Chem.* 2015, 87, 3286–3293 (ref.144). Copyright 2015 American Chemical Society.

Figure 5.

a) Average MS/MS spectrum of m/z 770 $\pm$ 1. b) 2D images of selected fragment ions of the $[M+Na]^+$ ion of the PE(P-16:0/22:6): A= m/z 727.48 (NL 43.04), B= m/z 629.49 (NL 141.03), and C= m/z 146.98. 2D images of selected fragment ions of the $[M+K]^+$ ion of the PC(14:0/18:1) lipid: D= m/z 711.43 (NL 59.07), E= m/z 549.48 (NL 221.02), and F= m/z 162.95. Light blue lines show a trace of the optical image. Lateral scale bar 1 mm. Intensity scale bar ranges from 0 (black) to 100% (light yellow) signal intensity of an individual peak. Reproduced from Lanekoff, I.; Burnum-Johnson, K.; Thomas, M.; Short, J.; Carson, J. P.; Cha, J.; Dey, S. K.; Yang, P.; Conaway, M. C. P.; Laskin, J., High-Speed Tandem Mass

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Figure 6.

Examples A–F show flow probe imaging of levofloxacin in rat lung tissue. A) Display interpolation of the raw image C) acquired from tissue E). B) Display interpolation of the raw image D) acquired from tissue F). Scale bar shows 5 mm. Repinted from *Int. J. Mass Spectrom.* Vol 377, 2015, Prideaux, B.; ElNaggar, M. S.; Zimmerman, M.; Wiseman, J. M.; Li, X.; Dartois, V., *Mass Spectrometry Imaging of Levofloxacin Distribution in Tb-Infected Pulmonary Lesions by MALDI-MSI and Continuous Liquid Microjunction Surface Sampling*, pp. 699–708 (ref. 155). Copyright 2015, with permission from Elsevier. G) LESA imaging of four simultaneously administered drugs in rat kidney at two different time points. Scale bar is 3 mm. Reproduced from Swales, J. G.; Tucker, J. W.; Strittmatter, N.; Nilsson, A.; Cobice, D.; Clench, M. R.; Mackay, C. L.; Andren, P. E.; Takats, Z.; Webborn, P. J. H.; Goodwin, R. J. A., *Mass Spectrometry Imaging of Cassette-Dosed Drugs for Higher Throughput Pharmacokinetic and Biodistribution Analysis*. *Anal. Chem.* 2014, 86, 8473–8480 (ref. 97). Copyright 2014 American Chemical Society.

Figure 7.

Heterogeneity of a glioblastoma acquired by DESI MSI. Examples A–F show ion images of specific lipids. Image I shows the two different regions, blue and red, within the sample.

Reprinted by permission from the American Association for Cancer Research: Eberlin, L. S.; Norton, I.; Dill, A. L.; Golby, A. J.; Ligon, K. L.; Santagata, S.; Cooks, R. G.; Agar, N. Y. R., Classifying Human Brain Tumors by Lipid Imaging with Mass Spectrometry. *Cancer Res.* 2012, 72, 645–654. DOI: 10.1158/0008-5472.can-11-2465 (ref. 168).

Figure 8.

Examples A) and B) are images of the guttate-forming fungi *Penicillium restrictum*. Example C) shows the optical image of a cross-sectioned fungal community and DESI ion images of eight compounds shown to be quorum-sensing inhibitors. Reproduced from Figueroa, M.; Jarmusch, A. K.; Raja, H. A.; El-Elmat, T.; Kavanaugh, J. S.; Horswill, A. R.; Cooks, R. G.; Cech, N. B.; Oberlies, N. H., Polyhydroxyanthraquinones as Quorum Sensing Inhibitors from the Guttates of *Penicillium Restrictum* and Their Analysis by Desorption Electrospray Ionization Mass Spectrometry. *J. Nat. Prod.* 2014, 77, 1351–1358 (ref. 205) Copyright 2014 American Chemical Society.

Scheme 1.

Reagents commonly used in reactive analysis and examples of reactions used in reactive DESI experiments to improve sensitivity in detecting cholesterol, anabolic steroids, cis-diols, phosphonate esters, and cyclic acetals. Reproduced with modifications from Ifa, D. R.; Wu, C. P.; Ouyang, Z.; Cooks, R. G., Desorption Electrospray Ionization and Other Ambient Ionization Methods: Current Progress and Preview. *Analyst* **2010**, *135*, 669–681 (ref. 19), with permission of The Royal Society of Chemistry (Copyright, 2010).

Table 1

Experimental approaches for liquid extraction from solid and liquid targets

Technique	Schematic Drawing	Characteristics	References
<i>Spray-Based Techniques</i>			
Desorption electrospray ionization (DESI)		<ul style="list-style-type: none"> A fast stream of primary droplets generates secondary droplets containing analyte molecules Commercially available, broadly used Spatial resolution 150–250 μm (typical), 35 μm (best reported) 	19,23
Easy ambient sonic spray ionization (EASI)		<ul style="list-style-type: none"> Similar to DESI but the sensitivity is typically lower The absence of high voltage may be advantageous for sensitive samples 	28,29
Air-flow-assisted DESI (AFADESI)		<ul style="list-style-type: none"> Similar to DESI, improved sensitivity 	40
<i>Direct Liquid Extraction Techniques</i>			
Liquid microjunction surface sampling probe (LMJ/SSP), Flow probe		<ul style="list-style-type: none"> Analyte extraction into a continuous liquid flow Electrospray ionization Commercially available (Flowprobe) Spatial resolution ~500–1000 μm Probe position relative to the sample is important 	47,50,56,155

Technique	Schematic Drawing	Characteristics	References
Nanospray Desorption Electrospray Ionization (nano-DESI)		<ul style="list-style-type: none"> Analyte extraction into a continuous liquid flow Nanospray ionization Spatial resolution 100–150 μm (typical), 12 μm (best reported) Independent optimization of the capillary positions is advantageous for improved spatial resolution Probe position relative to the sample is important 	59–61
Swan Probe		<ul style="list-style-type: none"> Analyte extraction into a continuous liquid flow Nanospray ionization Spatial resolution is determined by the aperture size, typically $\sim 200 \mu\text{m}$ Primary and secondary capillaries incorporated into a single device Probe position relative to the sample is important 	70
Single-probe		<ul style="list-style-type: none"> Analyte extraction into a continuous liquid flow Nanospray ionization Primary and secondary capillaries incorporated into a single device Spatial resolution 8.5 μm (best reported) Probe position relative to the sample is important 	72,73

Technique	Schematic Drawing	Characteristics	References
Laser ablation liquid capture surface analysis (LA/LCSA)		<ul style="list-style-type: none"> Capture of material desorbed by LA into a static droplet or continuous liquid flow Spatial resolution 50 μm (best reported) Electrospray ionization 	76,77,80
Liquid extraction surface analysis probe (LESA) and pressurized LESA (PLESA)		<ul style="list-style-type: none"> Analyte extraction into a static solvent droplet Nanospray ionization Commercially available Spatial resolution 110 μm (best reported) 	83,85
Electrostatic spray ionization, polarization-induced ESI		<ul style="list-style-type: none"> Analyte extraction into a continuously replenished solvent droplet Nanospray ionization directly from the droplet Spatial resolution 1 mm (typical), 350 μm (best reported) 	100–102
Scanning Probe Electrospray Ionization (SPESI)		<ul style="list-style-type: none"> Analyte extraction into a continuously replenished solvent droplet Nanospray ionization from the droplet formed on the other side of the probe capillary Spatial resolution 35 μm (best reported) 	103,104
<i>Substrate Spray Techniques</i>			

Technique	Schematic Drawing	Characteristics	References
Probe electrospray ionization (PESI), touch spray ionization (TS)		<ul style="list-style-type: none"> • Direct transfer of analyte molecules to a sharp solid needle or analysis from sharp objects naturally present in the sample • Nanospray ionization directly from the needle or ESI-assisted ionization • Enhanced sensitivity and selectivity achieved by applying different coatings to the probe • Spatial resolution 60 μm (best reported) • Depth profiling with 20 μm resolution 	106,132,133
Scanning Probe		<ul style="list-style-type: none"> • Direct sampling from liquid surfaces and buried liquid interfaces using a short tapered capillary • Nanospray ionization directly from the capillary 	121
Substrate spray		<ul style="list-style-type: none"> • Extraction of analytes directly from plants into a capillary • Nanospray ionization directly from the capillary 	119,120