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A one-step matrix application method for MALDI mass spectrometry imaging of bacterial colony biofilms

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Abstract

MALDI imaging of biofilms cultured on agar plates is challenging because of problems related to matrix deposition onto agar. We describe a one-step, spray-based application of a 2,5-dihydroxybenzoic acid solution for direct MALDI imaging of hydrated *Bacillus subtilis* biofilms on agar. Using both an optimized airbrush and a home-built automatic sprayer, region-specific distributions of signaling metabolites and cannibalistic factors were visualized from *B. subtilis* cells cultivated on biofilm-promoting medium. The approach provides a homogeneous, relatively dry coating on hydrated samples, improving spot to spot signal repeatability compared to sieved matrix application, and is easily adapted for imaging a range of agar-based biofilms.

Keywords

Airbrush; Automatic sprayer; Biofilms; MALDI MSI; Matrix application

Introduction

Mass spectrometry imaging (MSI) is a label-free molecular imaging technique with broad applications in clinical and pharmaceutical research, and food science.[1–5] With its unique capacity for untargeted, spatio-chemical characterization with a high degree of specificity, MSI is increasingly employed in microbiology.[6,7] Several ionization modalities are suitable for MSI of microbial communities, including matrix-assisted laser desorption/ionization (MALDI),[8,9] secondary ionization mass spectrometry (SIMS),[10–12] and ambient ionization techniques such as desorption electrospray ionization (DESI).[6,13] Because of its commercial availability and performance specifications, and broad coverage of proteins, peptides, and a variety of metabolites, MALDI is the most commonly used ionization mode for MSI.[14]

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Supporting Information

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Microbial cells and their associated extracellular material generate a complex chemical environment. Microbes are traditionally grown on agar-based media; however, the application of MALDI matrix directly onto a wet agar biofilm is problematic because hydrated agar facilitates lateral diffusion, causing delocalization of endogenous analytes. A wet agar medium also tends to absorb MALDI matrices, limiting sample extraction and coverage of colonies.

Directly sieving matrix powders onto wet, agar-based cultures is an innovative, quick and simple sample preparation method for MALDI MSI,[15] and has worked well.[8,9] Alternative methods include indirect MSI via imprinting or blotting analytes onto an appropriate surface, such as a silicon wafer or filter membrane.[16,17] Imprinting circumvents many of the difficulties associated with analyzing agar cultures with DESI imaging.[16,18] The agar film can also be desiccated with reduced pressure or heat; a dried sample can then be treated with matrix solution for MALDI imaging.[19,20] While these approaches allow MS-based imaging of microbial colonies/biofilms grown on agar media, each has limitations. The sieving method results in an uneven coating and matrix aggregation that may produce artifacts in the ion images obtained.[19] In the imprinting technique, selective analyte transfer may bias the resulting images and the overall process may lead to chemical redistribution. The drying processes require careful attention to prevent sample deformation, degradation of labile compounds, and delocalization of analytes. Perhaps because of these issues, many studies have performed MSI of bacteria cultured on non-agar-based surfaces.[10,21]

The goal of this work was to create an improved, easy to use approach for direct, one-step sample preparation to enable MSI of native distributions of metabolites across microbial colonies/biofilms grown on thin-layered agar. Two spray-based matrix application techniques were optimized for application of MALDI matrix onto hydrated colony biofilms grown on biofilm-inducing MSgg media (minimal salts glycerol glutamate medium).[22] With a concentrated matrix solution and optimized gas flow rates, a relatively dry coating was achieved without prior dehydration of the agar cultures. The one-step sample preparation facilitated direct imaging, circumventing issues related to heat-induced degradation or biased extraction of metabolites caused by imprinting the heterogeneous sample surface. A number of lipopeptides, including surfactins and plipastatins, as well as two peptides, sporulation killing factor (SKF) and sporulation delaying protein (SDP), were detected and localized using the two spray-coating approaches. Furthermore, signal stability, mass accuracy, and resolution were improved due to the homogenous matrix coating on the agar sample surface. Airbrush-based matrix application is simple to perform and allows for wide-spread utilization. A robotic sprayer, also developed for this work, provides a more robust and repeatable method for matrix application on agar samples. These cost-effective, spray-based matrix application methods are rapid and straightforward approaches for *in situ* exploration of microbial colony biofilms with MALDI MSI.

Materials and methods

Reagents and chemicals

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Formation of *Bacillus subtilis* biofilm on thin-layered agar

B. subtilis wild-type isolate NCIB3610 was grown in biofilm-inducing MSgg media (5 mM potassium phosphate (pH 7), 100 mM MOPS (pH 7), 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 50 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg mL^{-1} tryptophan, 50 μg mL^{-1} phenylalanine). For biofilm growth, an overnight cell culture (0.5 μL) grown in Luria broth media at 37 °C and 250 rpm was spotted onto MSgg media supplemented with 1.5% agar and incubated at 37 °C for 24 h. To facilitate MSI analysis, thin layers of solid media were produced by dispensing 5 mL of agar-containing MSgg media into 10 cm diameter petri dishes to form agar, approximately 1.7 mm thick. Petri dishes were sealed with Parafilm M laboratory film (Bemis, Neenah, WI) to prevent drying during biofilm cultivation.

Sample preparation

Biofilms were harvested by cutting 0.8 cm^2 sections from the agar and transferring them to a stainless steel target plate. For matrix crystal size measurements, an inoculated agar section was placed onto an indium tin oxide (ITO) coated glass slide for matrix coating and optical images were taken with an inverted microscope (Axiovert 25, Carl Zeiss, Zena, Germany). An artist's airbrush with a 0.2 mm nozzle (Paasche Airbrush Company, Chicago, IL) and a home-built automatic sprayer were used for spraying the MALDI matrix. Matrix sieving was performed using a 53-μm sieve (Hogentogler & Co., Columbia, MD) according to the reported protocol,[15] with minor modifications. Two MALDI matrices, 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA), were evaluated before selecting DHB for the spray-based methodology. A total of 30 biofilm samples were used during method optimization and the results confirmed using three independent biofilms via our optimized protocols.

For airbrush application, several parameters were optimized to ensure even deposition of matrix on top of the biofilm. The N_2 pressure was set at 40 psi and the agar samples were spray coated with 250 mg/mL DHB dissolved in pure ethanol at a distance of 30–35 cm. After spraying 2 mL of the DHB solution with the airbrush, the sample was allowed to dry for 1 min to avoid sample over-wetting, which could lead to analyte delocalization. A total of approximately 36 mL of DHB solution was applied over 20 min per target plate to an approximate amount of 4.0 mg/cm^2. A saturated solution of CHCA in pure ethanol was also tested; the agar samples were airbrush spray coated with approximately 50 mL of the CHCA solution using the same parameters described above for the DHB solution.

To facilitate higher-throughput, more reproducible coatings than are typically possible with an artistic airbrush, an automated matrix application system was designed and built. The nebulizer construction is similar to prototype DESI sources [23] and applies 35 mL/hr of 200

mg/mL of DHB dissolved in ethanol-water (9:1, v/v) with a 100 psi nitrogen sheath gas. Samples are affixed to a disk and rotated at ~7 Hz with a DC electric motor. The matrix nebulizer moves along the radius of the disk via a linear actuator to cover the samples. As the nebulizer approaches the center of the disk, its velocity is increased proportional to $1/r$ to maintain uniform coating over the sample area. For deposition onto the wet agar samples, the nebulizer was held 11 cm above the sample and oscillated over the plate 45 times. Afterwards, the sample was dried on the spinning plate for 2 min before applying another 45 rounds of matrix. The application and drying intervals were repeated for a total of 180 nebulizer passes; up to 12 samples (e.g., size of square, 9 mm × 9 mm) on 4 standard ITO glass slides (75 mm × 25 mm) were coated with 4.4 mg/cm^2 of DHB in 28 min. Dried biofilm samples were stored in a vacuum desiccator until MALDI imaging analysis.

MALDI MSI

All measurements were performed using an ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA) with a frequency tripled Nd:YAG solid state laser (λ=355 nm). The laser was set to the "Ultra" footprint setting at an ~100 μm diameter. Mass spectrometer calibration was performed using a Peptide Calibration Standard Kit II (Bruker Daltonics). Data acquisition was performed in positive reflectron mode with pulsed ion extraction, a 200-μm laser step size, and a mass range of 500–4800 Da. Biofilms were analyzed with 1000 laser shots fired at 1000 Hz. Spectra were baseline-corrected and analyzed in flexAnalysis 3 (Bruker Daltonics). MALDI imaging was performed using flexImaging 4 (Bruker Daltonics).

Results and discussion

The components of microbial culture media are often tailored to enable specific characteristics, such as swarming or synthesis of particular biochemical targets. Accordingly, MSI approaches typically require optimization for each application, including the screening of appropriate matrices. Our initial work focused on the selection of suitable chemical matrices for MALDI imaging of *B. subtilis* biofilms grown in MSgg agar media. Two commonly used matrices, DHB and CHCA, were sprayed to saturate agar media using an airbrush for subsequent MALDI-TOF analyses. As shown in Figure 1, the two matrices resulted in comparable signal intensity for lipopeptides, but two peptides, SKF and SDP, were detected exclusively with DHB. Therefore, DHB was utilized for subsequent development of the spray-based matrix application due to its wider analyte coverage.

Direct application of matrix onto wet biofilms is challenging because of the absorbent, semi-solid properties of agar, as well as the distinct surface topologies between agar and biofilms. Two-step matrix application procedures for MSI of agar samples have been developed in which the sample is dried in vacuo at room temperature or in an oven at no more than 40 °C prior to matrix deposition with a commercial sprayer such as an ImagePrep system[19,24] or HTX TM-Sprayer.[25] However, enzymatic degradation of endogenous metabolites may occur during dehydration, particularly with prolonged heat treatments. Therefore, to preserve native metabolite profiles and improve sample throughput, we evaluated one-step matrix application methods. Airbrush application was initially investigated because it is

commonly used for MSI of tissue. The simplicity of the airbrush compared to other commercial sprayers makes the technology easy to adapt. A sufficiently thin layer of agar aids in creating the required surface conductivity and minimizing surface charging.[15] In our initial experiments we applied a direct spray of 50 mg/mL of DHB in ethanol-water (7:3, v/v) onto the sample. Although effective, this approach was time-consuming and occasionally produced uneven crystallization due to its high water content. To shorten the deposition time, solvent composition and matrix concentration were optimized for the airbrush. Instead of the ethanol-water mixture, pure ethanol was used to minimize the incubation time and 100 mg/mL, 200 mg/mL, 250 mg/mL, and saturated DHB solutions were tested. The 250 mg/mL DHB solution was ultimately selected, as this composition provided the shortest application time without clogging the emitter. In most cases, after application of the matrix solution, the agar sample was dehydrated due to solvent evaporation and the N_2 gas flow. However, an hour of vacuum drying at room temperature is recommended to further minimize the risk of sample loss in the high vacuum of the source chamber of the mass spectrometer.

A drawback of manual spray application is that the outcome depends on the specific individual applying the matrix. Although the airbrush generates favorable coatings, the results are limited by a lack of reproducibility, especially between users (Figure S1). Thus, an automatic matrix deposition system was assessed, based on the aforementioned airbrush method, with the addition of a laboratory-built robotic motion control. Three major parameters—N_2 gas pressure, solvent flow rate, and the distance of spray emitter to the sample stage—were optimized to achieve even matrix coatings on the agar samples in a short period of time.[19,25] The concentration and composition of the solvent were modified slightly in order to prevent clogging of the sprayer head. Specifically, the concentration of the DHB solution was reduced and the water content was gradually increased until clogging was no longer observed. The optimized solution for automatic matrix application consisted of 200 mg/mL DHB in ethanol-water (9:1, v/v). Next, the solvent flow rate, gas pressure, and tip-to-stage distance were optimized to produce a relatively drier spray and small crystal sizes (Figure 2). Decreasing the tip-to-stage distance limits evaporation as the nebulized droplets travel to the surface, generating larger droplets and therefore larger crystal sizes. The solvent flow rate was optimized within the range of 30–60 mL/hr to prevent clogging and minimize matrix application time. At the optimized distance (11 cm) and flow rate (35 mL/hr), the gas pressure was set to 100 psi, which resulted in a dry spray and homogeneous sample coverage, as well as accelerated dehydration of the wet agar sample. Adjusting the number of sweeps proportionally affects the matrix density to further tune the MS signal. The automatic sprayer system provides excellent reproducibility and consistency in crystal size and coverage. Compared to sieving (Figure 2a), the homogenous coating obtained using either of the two spray-based methods yielded more reproducible mass accuracy and peak shape (Figure 2b, c). During MSI, the improved reproducibility resulted in less pixel-to-pixel variation, which is typically problematic with linear (non-orthogonal acceleration) MALDI-TOF instruments. The average crystal size for DHB is limited to 50–100 μm with the spraying methods.

An additional concern with spray-based matrix application is the possibility of analyte delocalization across the wet surface, even with the relatively dry spray applied in this work.

To estimate the magnitude of delocalization, the increase in the spot diameter of a colorant directly deposited onto agar media was measured with MALDI MSI (adapted from previous work[19]). Applying 1 μL of a methanolic extract of permanent marker (containing crystal violet from a black Sharpie®) onto hydrated agar produced spots of approximately 3.0 mm and 3.4 mm diameter in two technical replicates (Figure 3). Manual airbrush and automatic matrix deposition were performed using the optimized methods, and the increased diameters were measured from ion images of crystal violet (*m/z* 372) to assess analyte spreading from the original spot. Slight broadening of the spot boundary was observed with both spraying methods; for the airbrush application, the spot diameter increased from 3.0 mm to 3.1 mm, and for the automatic sprayer, from 3.4 mm to 3.6 mm. The measured delocalization is within the raster size for these images, suggesting that analyte redistribution should not affect image quality at the current image resolution. These increases may reflect differences in the coating process or diffusion of crystal violet when soaked into the agar; only a few pixels were detected outside of the core region. These results indicate that little analyte delocalization occurs for both spraying methods.

Finally, as shown in Figure 4, both spray-based methods provided comparable ion images and the agar samples are evenly covered by the matrix layer. Surfactins, plipastatins, as well as the two peptides SKF and SDP, were detected with well-resolved, region-specific spatial distributions, which is consistent with our recent work.[26]

Conclusions

The simple one-step matrix-application protocol for MALDI MSI of biofilms cultured with agar media described here eliminates the need for a separate dehydration step. Delocalization of analytes was limited by employing an optimized set of spraying parameters related to the solvent, matrix concentration, and gas pressure. The simplicity and affordable cost of the airbrush makes the method practical and readily adaptable by other users. For a more robust and repeatable matrix deposition, a custom-built robotic sprayer was used to apply matrix onto 12 samples simultaneously. Both techniques yielded clear and well-resolved ion images of metabolites located in biofilm samples, and offer potential for capturing spatially-resolved molecular snapshots of labile metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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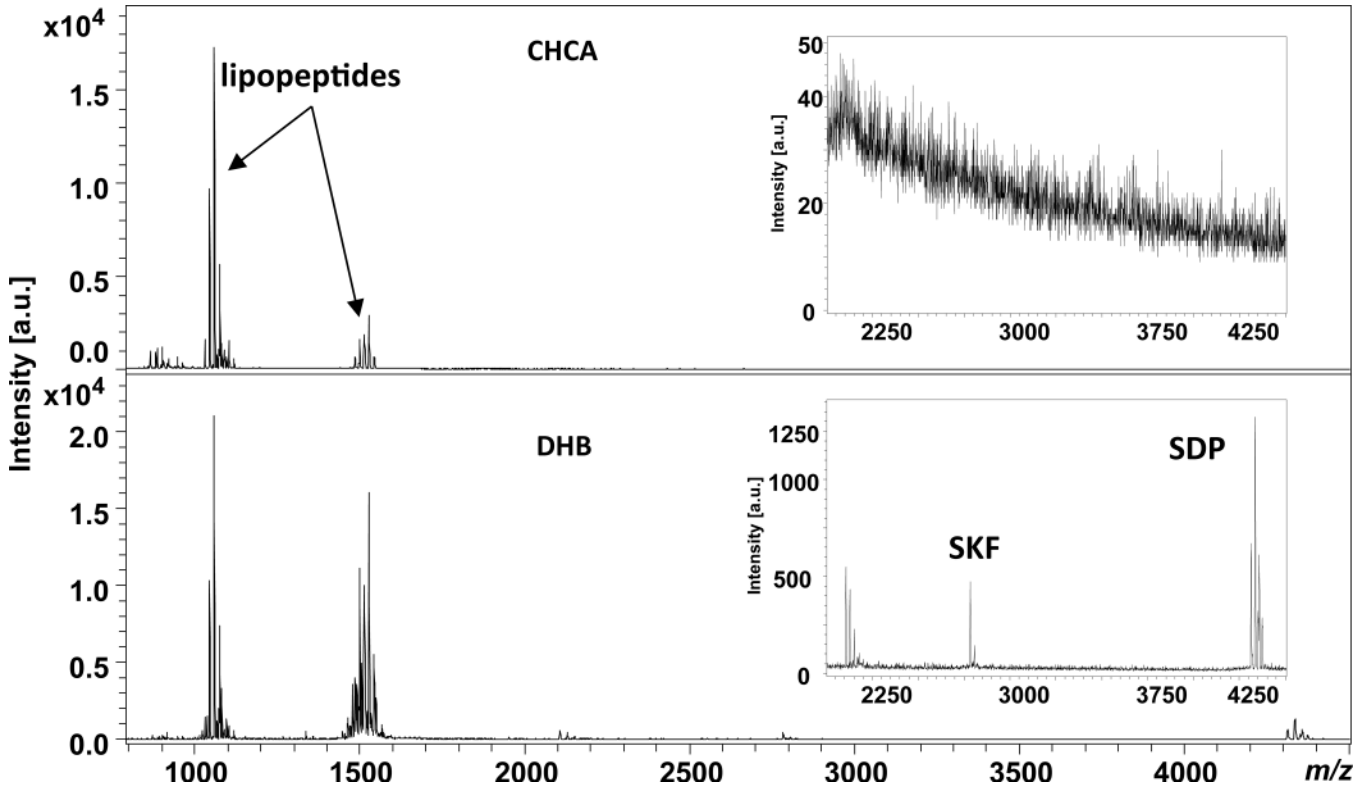

Figure 1.

Representative MALDI-TOF mass spectra obtained from the center region of *B. subtilis* biofilms coated with airbrush spray: a) saturated CHCA solution and b) DHB solution (250 mg/mL). The insets for each mass spectrum are a close-up of the mass range between 2000–4500.

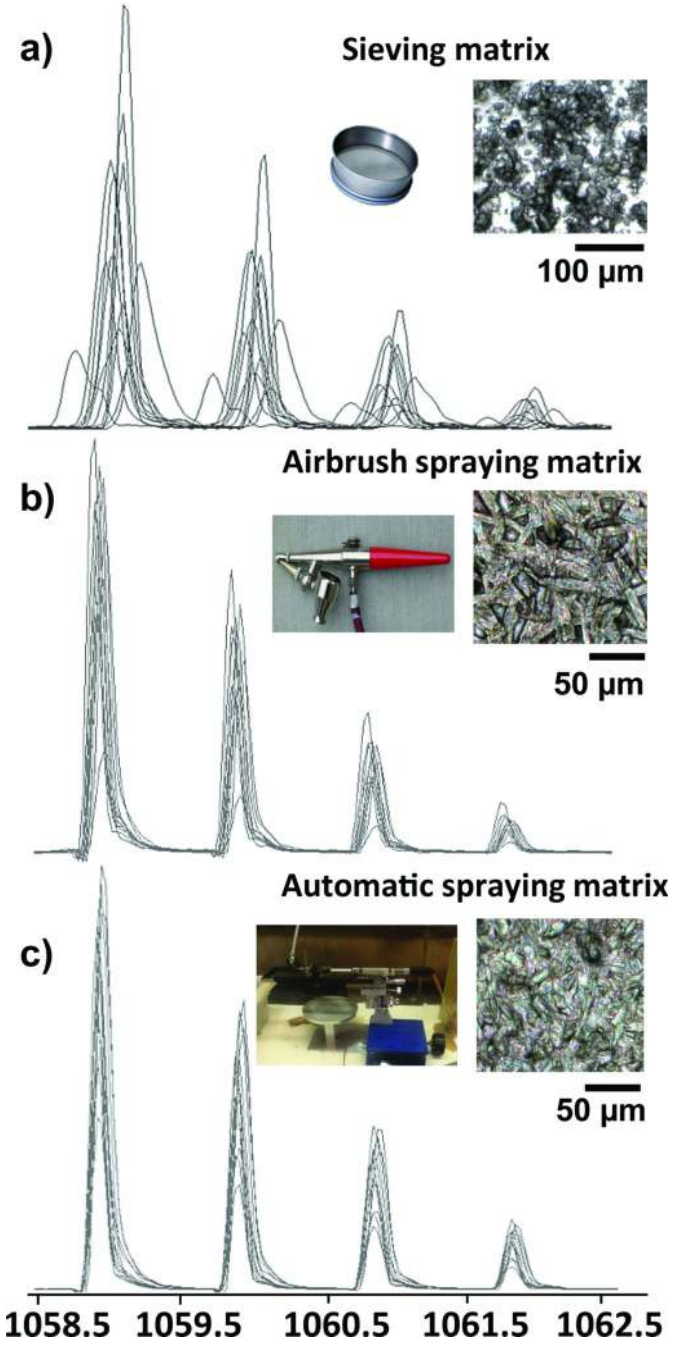

Figure 2.

Overlay of 12 individual pixel mass spectra of *m/z* 1058 (surfactin-C_{15}, $[M+Na]^+$) using three different approaches for matrix application and the corresponding optical images of DHB crystals from agar samples; a) sieving of DHB powder, and b) airbrush spraying and, and c) automatic spraying of DHB solution onto hydrated agar. The overlaid spectra are individual pixel spectra from the same biofilm image.

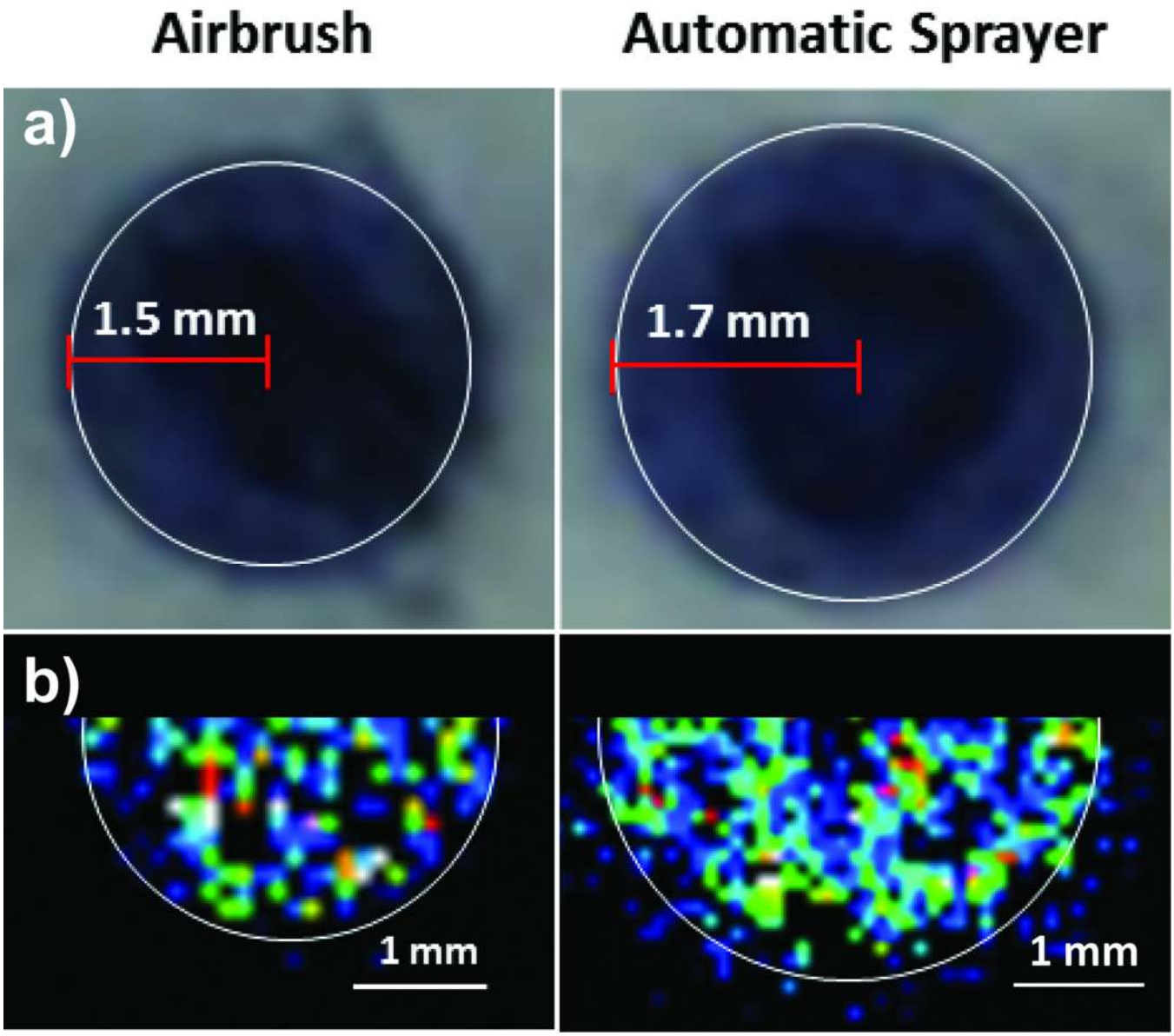

Figure 3.

Measurement of analyte delocalization after matrix coating. a) Optical images of agar samples after spotting 1 μL of methanolic extract on hydrated agars. b) Ion images were created with crystal violet (*m/z* 372, $[M\text{-}Cl]^+$). Step size: 125 μm.

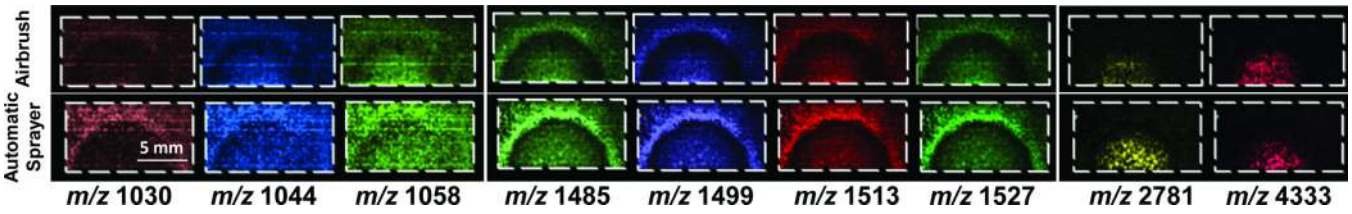

Figure 4.

Selected ion images of wild type *B. subtilis* biofilms using spray-based matrix application. From left to right: *m/z* 1030 (surfactin-C13, $[M+Na^+]$; *m/z* 1044 (surfactin-C14, $[M+Na]^+$); *m/z* 1058 (surfactin-C15, $[M+Na]^+$); *m/z* 1485 (plipastatin-C16-Ala, $[M+Na]^+$); *m/z* 1499 (plipastatin-C17-Ala, $[M+Na]^+$); *m/z* 1513 (plipastatin-C16-Val, $[M+Na]^+$); *m/z* 1527 (plipastatin-C17-Val, $[M+Na]^+$); *m/z* 2781 (SKF, $[M+H]^+$); and *m/z* 4333 (SDP, $[M+Na]^+$) The ion intensity is reflected by the intensity of the colors. Each column of ions is displayed using the same intensity scale, optimized per each metabolite.