Received: 17 May 2019 | Revised: 22 November 2019 | Accepted: 8 December 2019

DOI: 10.1002/jms.4488

APPLICATION NOTE

Journal of MASS SPECTROMETRY WILEY

Immersion by rotation-based application of the matrix for fast and reproducible sample preparations and robust results in mass spectrometry imaging

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Abstract

Automated matrix deposition for matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) is crucial for producing reproducible analyte ion signals. Here we report an innovative method employing an automated immersion apparatus, which enables a robust matrix deposition within 5 minutes and with scalable throughput by using MAPS matrix and non-polar solvents. MSI results received from mouse heart and rat brain tissues were qualitatively similar to those from nozzle sprayed samples with respect to peak number and quality of the ion images. Overall, the immersion-method enables a fast and careful matrix deposition and has the future potential for implementation in clinical tissue diagnostics.

KEYWORDS

immersion/dipping, MALDI-TOF MSI, mass spectrometry imaging, matrix application/deposition, tissue preparation

1 | INTRODUCTION

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) is a molecular analytical technology for analyzing the spatial distribution of molecules directly in a tissue section.[1-4] Tissue slices are typically coated with a MALDI matrix in order to extract analytes of interest from the tissue. The function of the matrix is considered to be required for both absorbing the laser energy and for facilitating desorption/ionization of the analyte molecules.[5] The resulting dataset can be viewed as an ion-intensity map displaying the relative abundance of each ion on a color-intensity scale at each coordinate location (pixel) across the tissue.

In the last decades different matrix application methods have been successfully applied varying from microspotting[6] and sublimation[7,8] to spray coating.[9] The reproducibility of the matrix deposition is of great importance to ensure that several samples can be analyzed with minimum technical variability. Various automated systems have been developed and marketed including SunCollect,[10] HTX TM Sprayer (nozzle spraying technology),[11] Bruker Image Prep (vibrational vaporization),[12] Shimadzu CHIP-1000 (inkjet printing technology),[13] and Labcyte Portrait (acoustic droplet technology).[14] The advantage of automated systems over manual applications is the accurate control of application variables such as coating cycles and drying times. In order to realize the implementation of MSI as standard application in translational medicine, the sample preparation must be robust, easy to be integrated into the clinical workflow routines and the throughput should be scalable. The usage of these automated systems is still demanding in terms of qualified personnel and high maintenance.

The choice of the matrix plays a key role in MALDI imaging experiments. The most conventional MALDI matrices for both positive-ion (+) and negative-ion (–) MSI of proteins, lipids, and other low-molecular weight (MW) endogenous and exogenous compounds have been

α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxybenzoic acid (DHB).[15] Limitations of these most widely used matrices are, eg, their relatively low ionization efficiencies in the (–) mode, and high background signals in the low mass-range that interfere with the detection of low-MW compounds and peptides.[16]

Over the last few years, new organic compound matrices for MALDI-MS were developed, for example 1,8-bis (dimethyl-amino) naphthalene (DMAN) for the study of acidic analytes from dry droplet samples. However, DMAN is recognized as an unstable matrix in vacuum because of its relatively high sublimation rate and is therefore not suitable for long-term MS imaging experiments.[17,18] Another novel matrix is the maleic anhydride proton sponge (MAPS), which is suitable for the visualization of small analytes (less than *m/z* 250) such as lactate, 2-hydroxyglutarate, chloride anions,[19] and protein fragments.

The chemical characteristics of MAPS offer the opportunity for an innovative matrix deposition method. MAPS matrix can be dissolved in solvents with low polarity as chloroform. Since the previously mentioned polar analytes of interest (less than *m/z* 250) do not dissolve in this solvent, the tissue sections can be immersed in the matrix solution. The immersion of tissue sections mounted on glass slides results in a thin and uniform layer of matrix on the samples.

In order to standardize the process of matrix application by immersion, an automated technique would be beneficial. For this reason, we developed an innovative matrix deposition apparatus with an automatic rotation mechanism. This immersion device allows a feasible and low maintenance procedure of matrix deposition in about 5 minutes.

2 | METHODS

2.1 | Tissue sectioning

Mouse heart and rat brain were snap-frozen in liquid nitrogen and stored at −80°C until sectioning. The specimens were sectioned sequentially to a thickness of 10 μm at −20°C using a cryo-microtome and transferred onto conductive slides (indium tin oxide [ITO]–coated glass slides), after which they were dried in a vacuum desiccator. Each experiment was performed in three replicates, for which consecutive tissue sections were prepared.

2.2 | MAPS synthesis

The matrix maleic anhydride proton sponge (MAPS) was synthesized according to previously reported procedures,[19,20] and solutions for immersion were prepared at a concentration of 5 mg/mL by dissolving in chloroform, dichloromethane, or toluene.

2.3 | Matrix deposition for MALDI-MSI

An immersion apparatus was constructed to allow a reproducible and uniform coating of the slides (MSI Diagnostics GmbH, Bielefeld, Germany; Figure 1). By using the developed program code, it is possible to set the following parameters: rotation number, speed, stop time, and angle of the carrier during a stop. The settings used here have been adapted optimally to the test setup. The slide was deposed with matrix by six automated inverse rotations at a speed of 10 seconds per rotation through the container comprising 30 mL MAPS-solvent solution. After each rotation, there was a stop of the carrier in the horizontal until the solvent had evaporated. The drying times were adjusted to the solvent used: dichloromethane, 10 seconds; chloroform, 15 seconds; and toluene, 45 seconds. For the matrix deposition using the nozzle spraying method, the TM Sprayer (HTX Technologies, North Carolina, USA) was used with MAPS dissolved in toluene. Spray parameters were flow rate, 100 μL/min; pressure, 10 psi; track spacing, 3 mm; velocity, 600 mm/min; and nozzle temperature, 30°C. After, matrix application slides were placed in a desiccator until MALDI-MSI analysis.

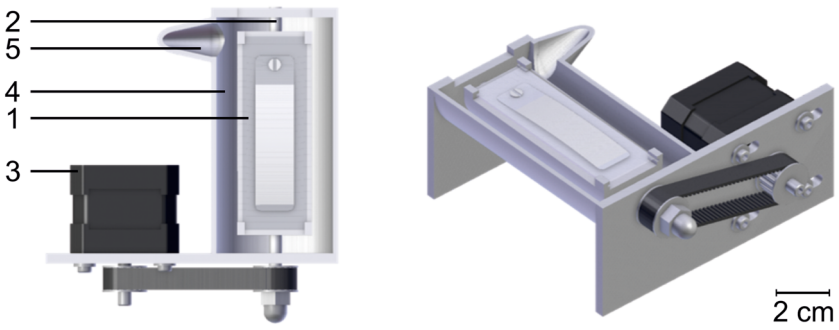

FIGURE 1 The immersion apparatus in stainless steel has a turning device consisting of a carrier with an integrated rail for fixing the object slide (1). The carrier is located on a shaft (2) rotated by a motor (3) through a container (4) with an outlet (5) comprising matrix. At the press of a button, the immersion process starts with a consistent rotation depositing matrix on the target mounted tissue. Until the solvent is completely evaporated, an automatic rotation in the opposite direction follows with a total number of rotations of six. The method parameters can be set via a program code. This technology allows a matrix deposition of maleic anhydride proton sponge (MAPS) matrix in about 5 minutes regarding the used solvent. A customer-friendly immersion device is available from MSI Diagnostics GmbH (Bielefeld, Germany). Scale bar, 2 cm [Colour figure can be viewed at wileyonlinelibrary.com]

3 | MALDI-TOF MSI

The Bruker Daltonics ultrafleXtreme MALDI time-of-flight (TOF) mass spectrometer, which is equipped with smartbeam laser technology (Bruker Daltonik, Bremen, Germany) was used. MALDI-TOF mass spectra were obtained in negative-ion reflector mode in the *m/z* range between 10 and 1900 by averaging 250 laser shots with a frequency of 1000 Hz. The laser size "small" was used with a raster step of 20 µm. Global attenuator offset was set to 72% and laser intensity was adjusted to the tissue by using a laser intensity of 70% for all mouse heart sections and 90% for all rat brain sections. The reflector detector voltage was 3147 kV. External mass calibration was performed using red phosphorus.[21] For image creation, controlling, and data analysis, flexImaging ver. 4.1 (Bruker, Germany), flexControl ver. 3.4 (Bruker, Germany), and CoralDraw X5 were used. Obtained mass spectra were compared by using mMass ver. 5.5.0. All experiments conducted in this study were processed three times.

4 | RESULTS

The comparison of MALDI-TOF mass spectra from MSI measurements of rat brain or mouse heart tissue processed by either immersion or spray-coating with MAPS matrix, which has been dissolved in toluene, shows no qualitative differences between the two sample preparations (Figure 2). Mass spectra display no apparent differences in peak number within the scanned regions (Figure 2).

In order to show that rotational immersion is suitable as a matrix deposition method and additionally comparable with common spray coating, exemplary selected mass channels were analyzed, and no significant differences were found regarding intensity and lateral resolution. The MALDI-MSI images of the ion at *m/z* value 35 are displayed in Figure 3, which is tentatively identified as chloride because it was observed with a chloride-specific isotopic abundancy (Supporting information, Figure S1). There is no obvious difference in lateral resolution and quality between images of rat brain tissue immersed or nozzle sprayed with MAPS dissolved in toluene (Figure 3A,B). In addition, immersion with MAPS dissolved in three different solvents (dichloromethane, chloroform, and toluene) was tested. The ion intensities of *m/z* 35 showed higher scales of the same tissue immersed with MAPS dissolved in both chloroform or dichloromethane (Figure 3C,D) compared with immersion or nozzle spraying using toluene as solvent (Figure 3A,B). Furthermore, the same experimental setup was used for the analysis of the mouse heart tissue, and MALDI-TOF images of channel 35.0 Da ± 0.1 Da are shown (Figure 3E-H). Here, a higher MALDI-TOF intensity was detected after immersion of the mouse heart tissue using MAPS dissolved in toluene in comparison with the MALDI-TOF intensity after nozzle spraying (Figure 3E,F). After immersion with solvents toluene or dichloromethane, MALDI-TOF intensities of mouse heart tissue of channel 35.0 Da ± 0.1 Da are higher (Figure 3F,H) than intensities after immersion with chloroform as solvent (Figure 3G). Analysis of the matrix solutions alone as controls showed no analyte signals (Figure 3).

Furthermore, MALDI-TOF images of two other mass channels (89.0 Da ± 0.1 Da and 690.4 Da ± 0.1 Da) are shown exemplary for the automated immersion method by using rat brain tissue (Figure 4). There is no obvious difference in the quality of the MALDI-TOF MSI results between sprayed and immersed samples (Figure 4A,B,E,F). The MALDI-TOF MSI intensities are higher in the rat brain tissue after immersion with MAPS dissolved in dichloromethane or chloroform (Figure 4C,D,G,H) than

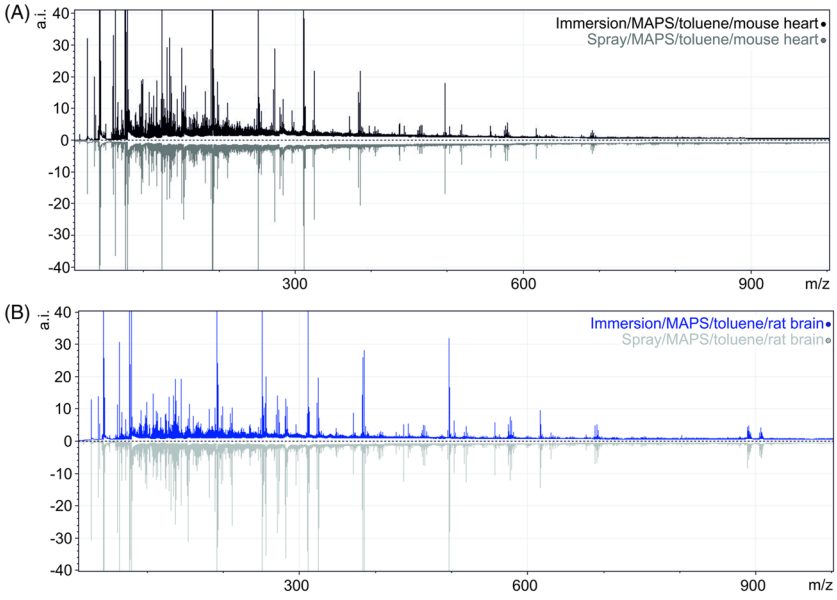

FIGURE 2 Comparative representation of the peak number of the TIC-normalized matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of mouse heart (A) and rat brain tissue (B) sprayed or immersed with MAPS matrix (5 mg/mL) dissolved in toluene. The mass-to-charge ratio (*m/z*) to absolute intensity (a.i.) is shown [Colour figure can be viewed at wileyonlinelibrary.com]

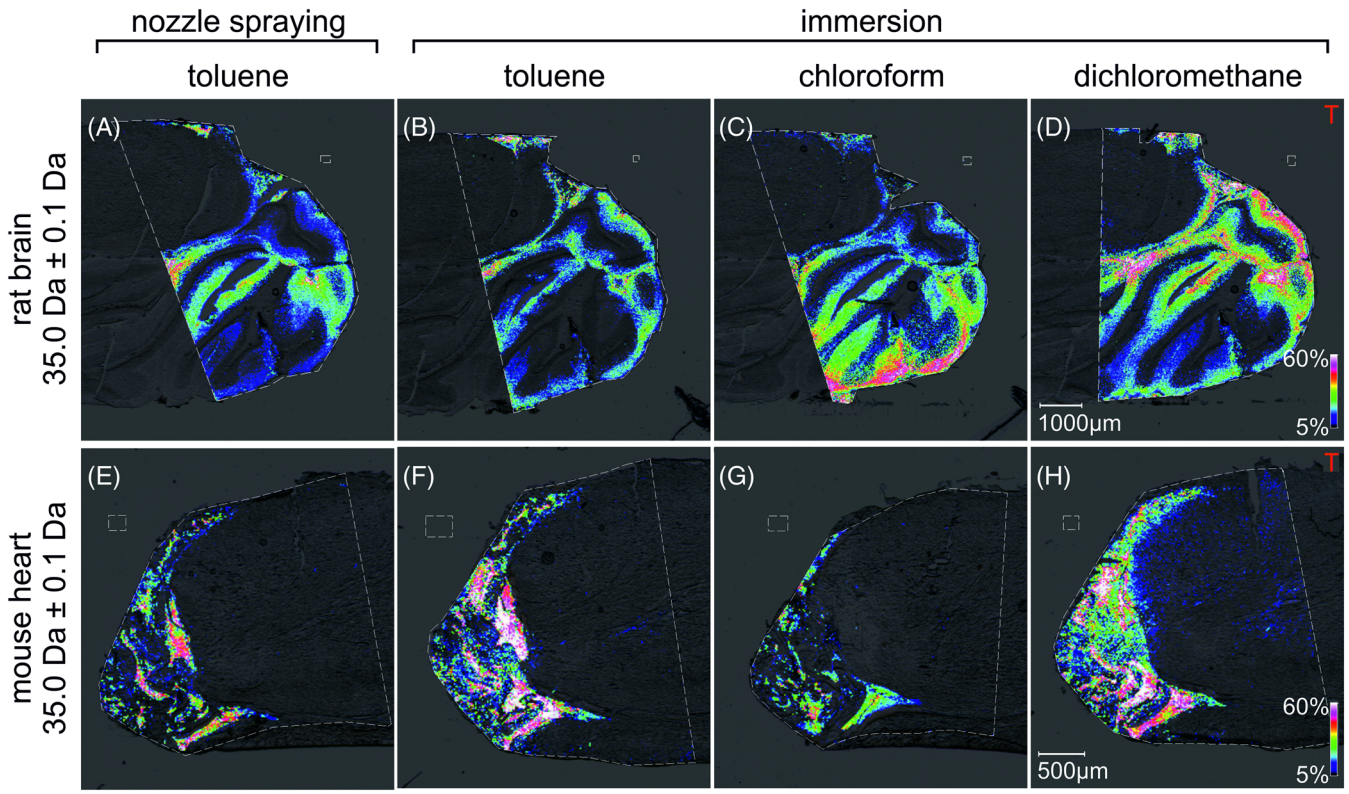

FIGURE 3 Mass spectrometry imaging (MSI) comparison between immersion and nozzle spraying the maleic anhydride proton sponge (MAPS) matrix (5 mg/ml) by using different solvents and fresh section cuts (10 µm) from tissues. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) signal distributions are exemplarily shown for the mass channel 35.0 Da ± 0.1 Da of rat brain tissue nozzle sprayed with MAPS dissolved in toluene (A) or immersed with MAPS dissolved in toluene (B) or in chloroform (C) or in dichloromethane (D). MALDI-TOF intensities for the mass channel 35.0 Da ± 0.1 Da of mouse heart tissue nozzle sprayed with MAPS dissolved in toluene (E) or immersed with MAPS dissolved in toluene (F) or in chloroform (G) or in dichloromethane (H) are shown. Ion images are TIC-normalized. Scale bars, 500 and 1000 µm

immersed or sprayed using the solvent toluene (Figure 4A,B,E,F). Control analysis of the matrix solutions showed no analyte signals (Figure 4).

5 | DISCUSSION

Fast and feasible sample preparation for MALDI-MSI analysis is becoming increasingly important for routine analysis. The use of classical matrices for the MALDI-MSI detection of low molecular weight compounds leads to strong background signals in the low mass range of the spectrum due to the ionization of the matrix, related clustering processes, and because of susceptibility to in-source fragmentation of small molecules.[22] The MAPS matrix is suitable for the MALDI-MSI analysis of small molecules lower than 250 Da.[19] In the study of Giampà et al, it is shown by scanning electron microscope (SEM) images that MAPS matrix produces a thin film when applied on tissues, rather than a crystalline structure as, eg, in case of DHB.[19] Apparently, the lack of visible crystals does not hinder the adequate ionization of metabolites. In the contrary, because of this fact, there are no limitations regarding the matrix crystal size or crystal distributions, which enables novel high-resolution MALDI-based imaging methods.

The immersion method in combination with MAPS matrix is suitable as a preparation method for MALDI-TOF MSI. Here, three potential analytes were selected for the proof of concept. Chloride is an example of a particularly low molecular weight analyte (*m/z* 35). In addition, chloride is a major inorganic anion in the brain and has already been selected for characterization of tumor tissue with MAPS as matrix.[19] Since the automated immersion method offers future potential as a supportive preparation for clinical diagnostics, lactate was selected as the second exemplary molecule, because of its role as marker for physiological conditions within tissues as, eg, for hypoxia, traumatic brain injury,[23] and for the phenomenon known as the Warburg effect.[24] Components with higher mass than *m/z* 250 can also be detected as it is exemplary shown in the ion map of *m/z* 690.4.

Rat brain and its fine structured gyri is exemplary shown in order to prove the lack of delocalization of molecules within the tissue. Heart tissue was selected for presentation due to its largely uniform structure with distinct histological areas as the blood vessels and lymph nodes in the cranial part of the heart. However,

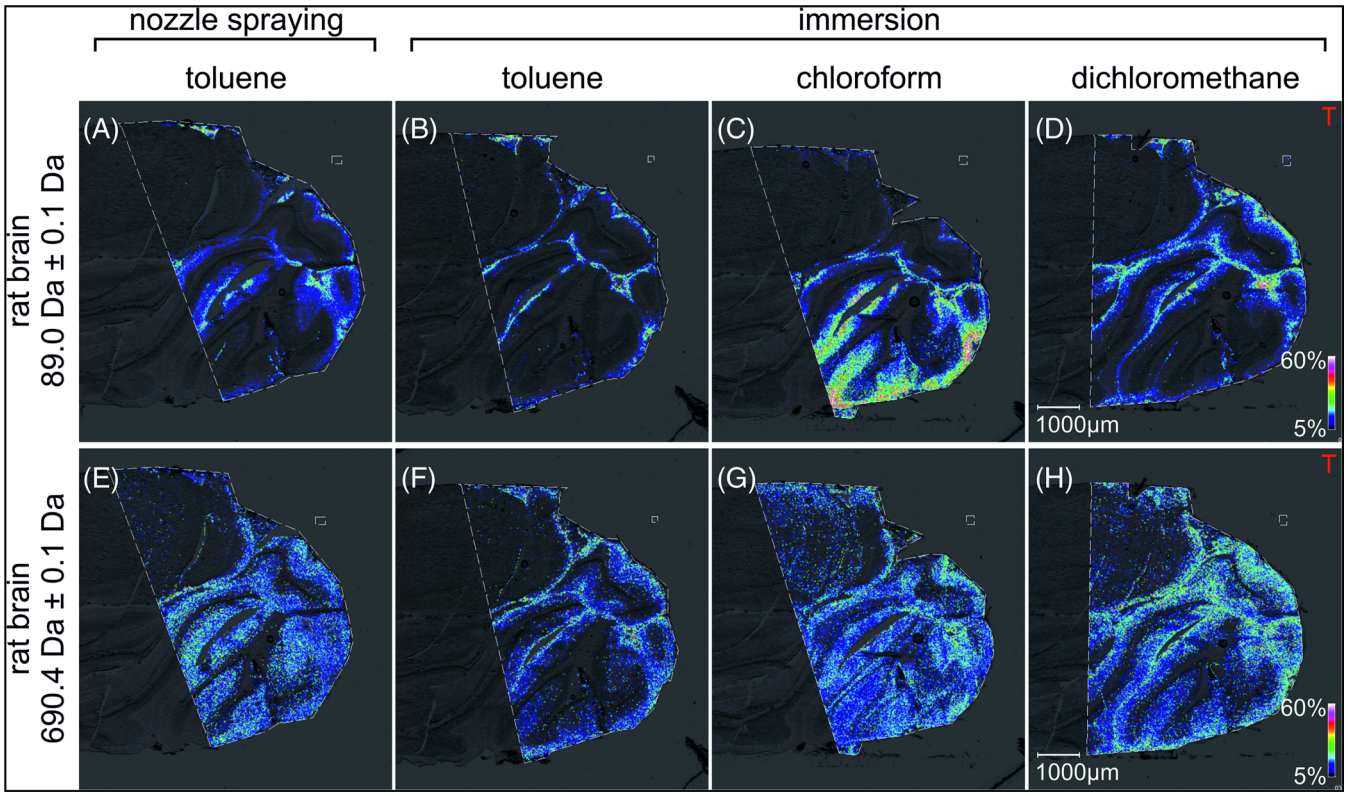

FIGURE 4 Mass spectrometry imaging (MSI) comparison between immersion and nozzle spraying the maleic anhydride proton sponge (MAPS) matrix (5 mg/mL) by using different solvents and fresh section cuts (10 µm) from rat brain tissue showing two further exemplary mass channels. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) signal distributions and intensities of the mass channel 89.0 Da ± 0.1 Da of tissue nozzle sprayed with MAPS dissolved in toluene (A) or immersed with MAPS dissolved in toluene (B) or in chloroform (C) or in dichloromethane (D). MALDI-TOF signal distributions and intensities of the mass channel 690.4 Da ± 0.1 Da of tissue nozzle sprayed with MAPS dissolved in toluene (E) or immersed with MAPS dissolved in toluene (F) or in chloroform (G) or in dichloromethane (H). Ion images are TIC normalized. Scale bar, 1000 µm [Colour figure can be viewed at wileyonlinelibrary.com]

other tissues such as liver or kidney were also tested as suitable for the immersion method (data not shown).

The ion intensities measured here differ between rat brain and mouse heart tissue, using the same matrix but various solvents (dichloromethane, chloroform, or toluene). The underlying composition and physiology of the tissues differ and, accordingly, also the interaction between the matrix and the ionized compounds. Our results indicate that the type of the solvent used for the matrix solution has a crucial role not only on the ionization processes[25] but also on the matrix-tissue surface morphology.[26] This observation can be explained by a solvent-dependent rearrangement of the matrix molecules during the solvent evaporation[27,28] and then a different interaction with the analytes.[29] In general, there are evidences that the morphology of co-crystal analyte-matrix influence the ionization efficiency precluding a quantitative analysis in MALDI-MS.[30,31] For this reason, in order to influence the morphology of the matrix layer, several techniques were developed such as fast evaporation,[32] seeded layer,[33] sandwich,[34] electrospray deposition,[35] and sublimation.[8]

In principle, it is conceivable that also other matrices than MAPS can be used for immersion with the apparatus. Several factors need to be considered, as the physicochemical properties of the analyte of interest, the complexity of the biomolecular interactions in a tissue, matrix solubility, and the solvent evaporation/matrix crystallization process.

Areas of application of the MALDI-MSI are mainly pathology,[36,37] medical,[38] pharmaceutical,[39,40] and fundamental research.[41] In addition, MALDI-MSI is gaining interest for the detection of metabolites in frozen tissue sections in a diagnostic setting.[42] Fast MALDI-MSI instruments as the Bruker rapifleX MALDI Tissuetyper[43] have the potential to offer a supporting tool for diagnostics and decision-making during surgeries. For this reason, a robust matrix is required, which efficiently detects target molecules, and a rapid and feasible tissue preparation is needed for routine examinations.

In conclusion, the immersion apparatus in combination with the MAPS matrix represents a robust and manageable matrix deposition method that facilitates the sample preparation in MALDI-MSI workflow, exhibits no obvious qualitative difference compared with MALDI-MSI results from sprayed specimens, and provides a future potential as supportive preparation tool for clinical diagnostics.

ACKNOWLEDGMENT

We thank all members of the Department for Proteome and Metabolome Research for fruitful discussions. The concept idea was supported with an "EXIST-Gründerstipendium" by the Federal Ministry of Economics and Energy. The grant was also cofinanced by the European Social Fund.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Schäfermann J, Kliewer G, Lösch J, Bednarz H, Giampà M, Niehaus K. Immersion by rotation-based application of the matrix for fast and reproducible sample preparations and robust results in mass spectrometry imaging. *J Mass Spectrom*. 2020;55:e4488. https://doi.org/10.1002/jms.4488