Supporting Information for

Maleic Anhydride Proton Sponge as a Novel MALDI Matrix for the Visualization of Small Molecules (< 250 m/z) in Brain Tumors by Routine MALDI ToF Imaging Mass Spectrometry

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Experimental Section

Synthesis of 4-Maleicanhydridoproton Sponge (MAPS)

1,8-Bis(dimethylamino)naphthalene (5,43 g, 25 mmol) in 45 mL of tetrahydrofuran (THF) was added to bromomaleic anhydride (2,30g, 13 mmol) in 11 mL of THF and stirred. Thereupon, the reaction mixture immediately turned deep red. After the mixture was stirred for 1 hour and 15 min, the solvent was removed under reduced pressure, and the residue was re-dissolved in 60 mL of THF and filtered to remove PSHBr. The solvent was removed under reduced pressure, yielding a purple hygroscopic solid (3,7 g, 66%) with the following properties: mp 115-124 ^{o}C (mp 116-127 (Swor et al., 2010)). Found: C, 69.84; N, 9.02. Calc. for C18H18N2O3: C, 69.66; N, 9.03%

MALDI measurements of acidic compounds

Stock solution of citric acid was made in respective HPLC-grade ethanol at 100 µM. MAPS and DMNA were made up at the concentration as analytes in HPLC-grade acetonitrile. One microliter of the analyte was premixed with 1 microliter of the matrix and the resulting mixture was spot-ted on the target plate Anchor Chip (Bruker Daltonik, Bremen, Germany) and analysed by MALDI-TOF-MS. Mass spectrometric analyses were performed in the reflectron negative mode accelerating potential on a time-of-flight mass spectrometer (Bruker UltraflExtreme TOF-TOF; Bruker Daltonik, Bremen, Germany. Ion source 1 voltage -20,01 kV, ion source 2 voltage -17,81 kV, reflector 1 voltage -21,25 kV, reflector 2 voltage -10,78 kV and lens voltage -8,70 kV) which was equipped with a Smartbeam laser (Nd:YAG 355 nm) capable of operating at a repetition rate of 1000 Hz with optimized delayed extraction time and laser beam size was set to small. Laser energy was optimized for signal-to-noise in each preparation.

MALDI Imaging experiments

Tissue samples were obtained from five patients suffering from brain tumors (i.e. diffuse astrocytoma, anaplastic astrocytoma, or glioblastoma) undergoing therapeutic tumor resection at the Clinic for Neurosurgery, University Hospital Essen. After removal the tumor samples were immediately dissected and surplus tissue not needed for diagnostic purposes was frozen in liquid nitrogen and stored at −80°C until further processing. The study was approved by the local ethics committee. Informed consent was received from all tissue donors.

Cryo-sections of human brain tumors were mounted onto an indium tin oxide coated conductive glass slide (ITO; Bruker Daltonics, Bremen, Germany) and immediately dried in a vacuum desiccator for 24 h. Samples were scanned (Plustec optiLab 850, Ahrensburg, Germany) with an image resolution of 2,400 dpi and covered with a maleic anhydride proton sponge (MAPS) matrix (5 mg/ml in 90% (v/v) acetonitrile/chloroform) using the ImagePrep™ device (Bruker Daltonics, Bremen, Germany). Mass spectrometric analyses were performed in the reflectron negative mode accelerating potential on a time-of-flight mass spectrometer (Bruker ultrafleXtreme™; Bruker Daltonics, Bremen, Germany) with the following parameters: ion source 1 voltage -25.00 kV, ion source 2 voltage -22.10 kV, reflector 1 voltage -26.50 kV, reflector 2 voltage -13.50 kV and lens voltage -8.30 kV. The mass spectrometer was equipped with a Smartbeam laser (Nd:YAG 355 nm) capable of operating at a repetition rate of 1000 Hz with optimized delayed extraction time and laser beam size was set to large. Laser energy was optimized for signal-to-noise in each preparation. Mass spectral data sets were acquired over a whole section using flexImaging™ software (Bruker Daltonics, Bremen, Germany) in the mass range of m/z 20 to 1,000, with a raster step size of 70 µm, 400 laser shots per spectrum and with a sample rate of 4 GHz. After data acquisition, molecular images were reconstituted using the flexImaging™ software. Each data set consists of approximately 20,000 individual sampling locations, or pixels. Data were normalized using the flexImaging™ software. For display purposes, data were interpolated and pixel intensities were rescaled in flexImaging™ to utilize the entire dynamic range. For device calibration, 2 µl of 5.0 mM ascorbic acid, citric acid and potassium chloride were mixed with 2 µl of MAPS solution and 1

µl was spotted both onto a ground steel MALDI target (Bruker Daltonics, Bremen, Germany) and onto ITO slides outside brain sections.

FTICR MS measurement

FTICR MS was performed on a solariX XR 12T Fourier transform mass spectrometer (FTMS) (Bruker Daltonics) equipped with a dual ESI/MALDI ion source. External linear calibration was carried out using $NaCF_3COOH$ clusters in negative electrospray mode. MALDI spectra were acquired in negative ion mode with a 2 MW acquisition size followed by a single zero filling and a sin apodization (mass range 75 <m/z< 1500). A single scan was recorded using the ions generated by 400 laser shots for each spectrum. The Laser was running at 1 KHz and the ions were accumulated externally (hexapole) before being transferred into the ICR cell. For MALDI-MS measurements the prepared slides were mounted into a Slide Adapter (Bruker Daltonics) and loaded into the dual source. Data acquisition was carried out using FTMSControl 2.1 (Bruker Daltonics), sum formula generation was accomplished using DataAnalysis 4.4 (Bruker Daltonics).

SEM Microscopy

The cryo-sections of tumorous tissue were mounted on a conventional microscopy object slide and matrices were applied with a common protocol by ImagePrep™ (Bruker). The slides were subsequently covered with 30nm layer of gold in a BAL-TEC sputter coater (SCD 005). The prepared samples were analysed in a S-450 scanning electron microscope (Hitachi) at 15kV.

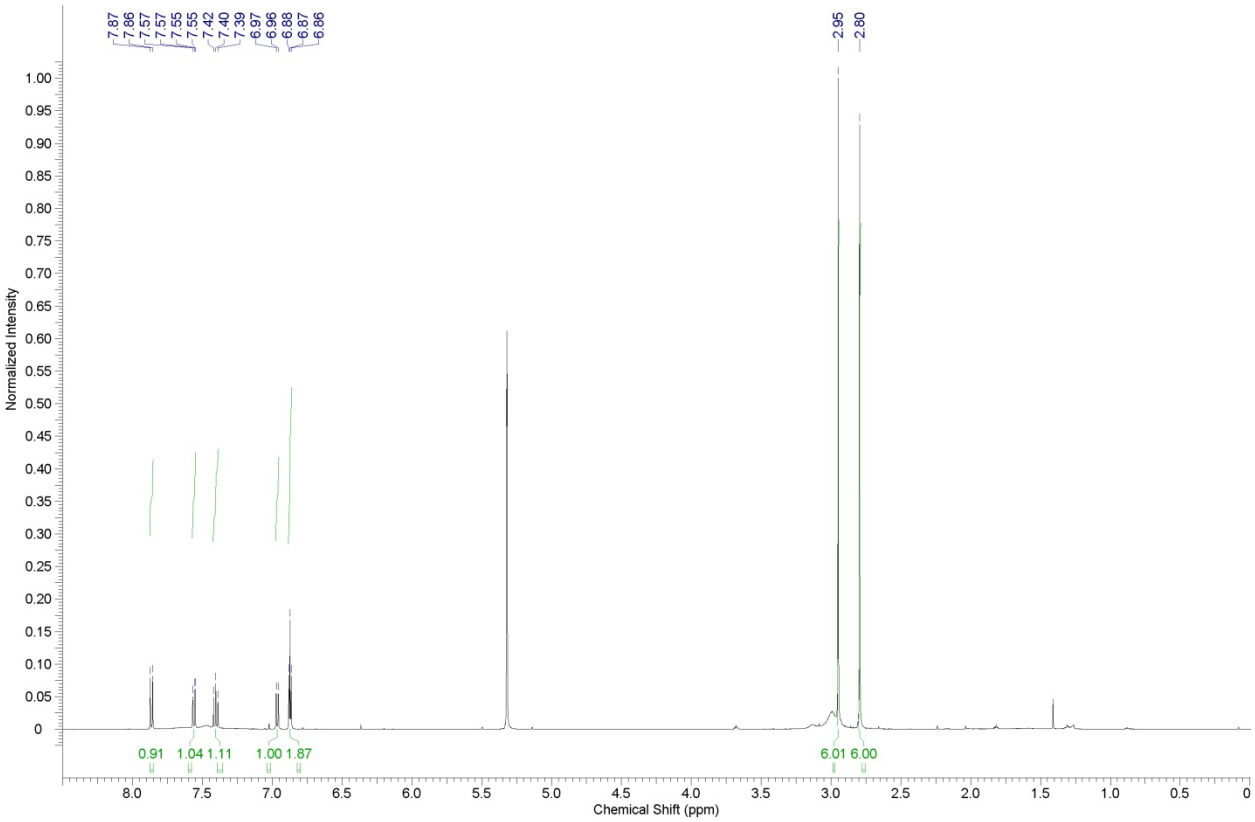


Figure S1: ^{1}H NMR (500 MHz, DICHLOROMETHANE-d_2) δ ppm 2.80 (s, 2CH_3), 2.95 (s, 2 CH_3), 6.87 (d, *J*=8.48 Hz, H2), 6.87 (s, H14), 6.96 (d, *J*=7.54 Hz, H7), 7.40 (t, *J*=8.01 Hz, H6), 7.56 (d, *J*=8.48, 0.63 Hz, 1 H5), 7.86 (d, *J*=8.48 Hz, H3).

NMR Spectra were recorded on an Avance III 500MHz Bruker® NMR spectrometer. Chemical shift values are reported in ppm using $CDCl_3$ as an internal reference.

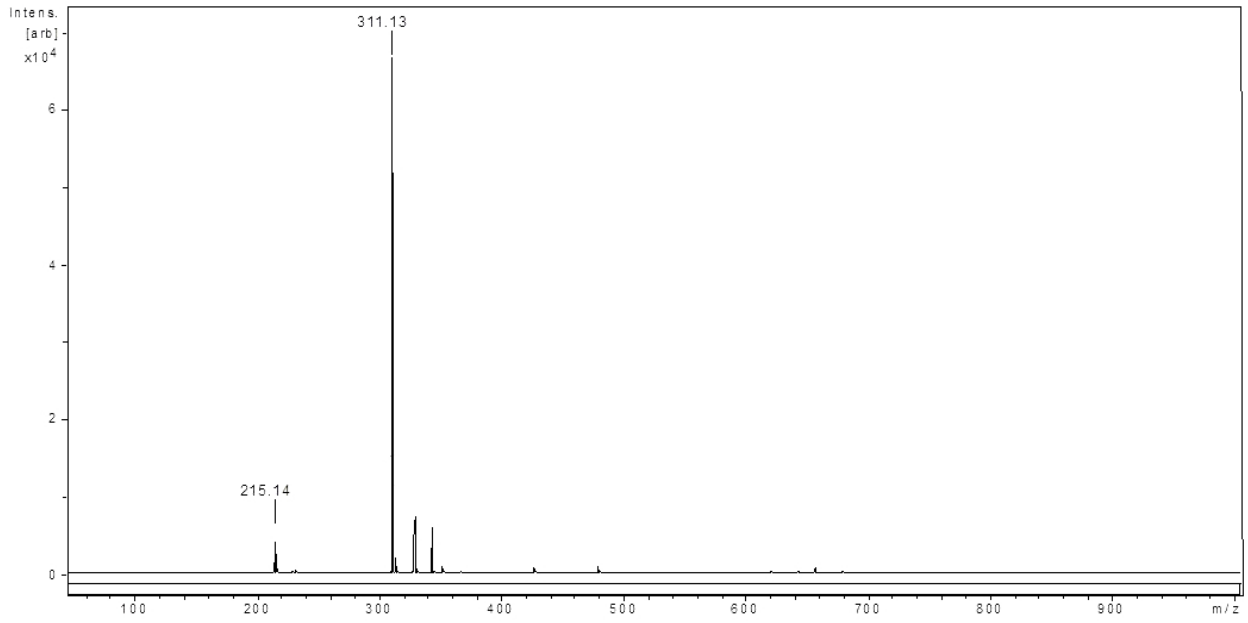
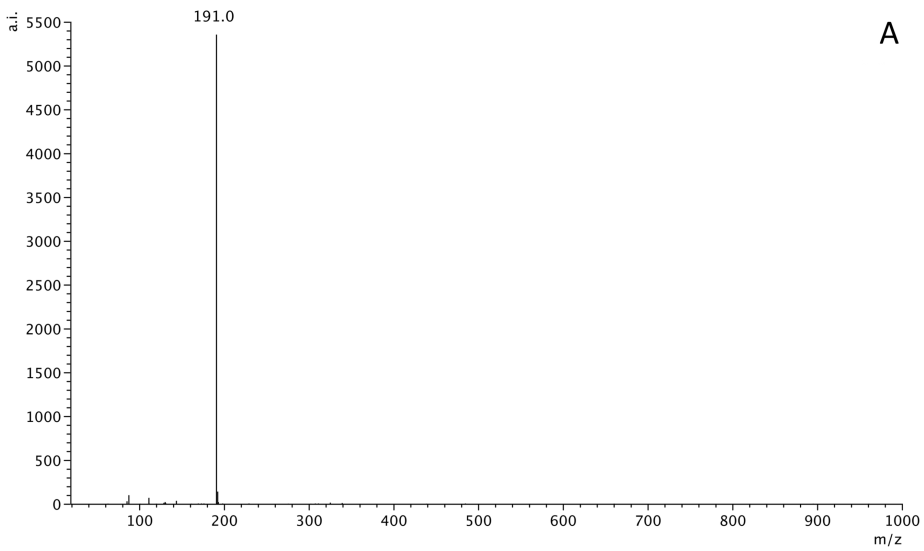


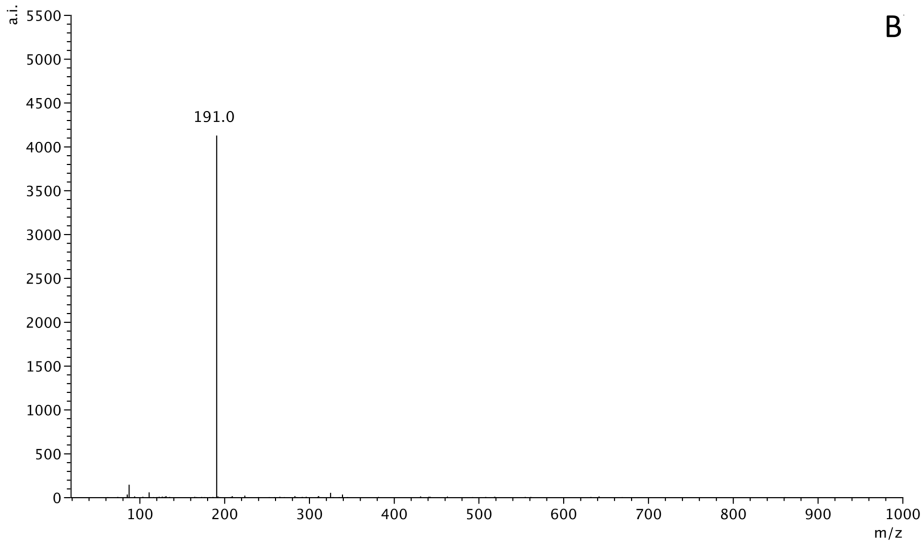
Figure S2: ESI MS spectrum of MAPS in CH_3CN (10 µM) and formic acid in positive mode. The m/z signal of 311 is $[MAPS+H]^+$ and m/z 215 is $[DMNA+H]^+$ in the impurity intensity range.

ESI MS experiments were carried out using a micrOTOF-Q Bruker® mass spectrometer coupled with ESI source in positive mode. The solution was directly injected in the ESI source.

Table S1: Parameters for ESI MS (microTOF control, Bruker) in full scan MS mode

Mode	**Scan Mode**	MS	**Ion Polarity**	Positive
	Mass Range	50-1000 m/z	**Rolling Average**	off
	Spectra Acquisition	Save Spectra	**Absolute Threshold**	10
	Include Profile Spectra	Always	**Peak Summation Width**	5 pts
	Focus	Inactive	**Acquisition Rate**	1.0 Hz
Source	**Endplate Offset**	-500 V	**Dry Gas**	1.8 L / min
	Capillary	-5000 V	**Dry Temp**	180 °C
	Nebulizer	11.6 psi		
Transfer	**Funnel 1 RF**	180.0 Vpp	**ISCID Energy**	0.0 eV
	Funnel 2 RF	200.0 Vpp	**Hexapole RF**	100.0 Vpp
Quadrupole	**Ion Energy**	5.0 eV	**Low Mass**	100.00 m/z
Collision Cell	**Collision Energy**	10.0 eV	**Collision RF**	150.0 Vpp
	Transfer Time	70.0 µs	**Pre Puls Storage**	7.0 µs


A
a.i.
191.0
m/z


B
a.i.
191.0
m/z

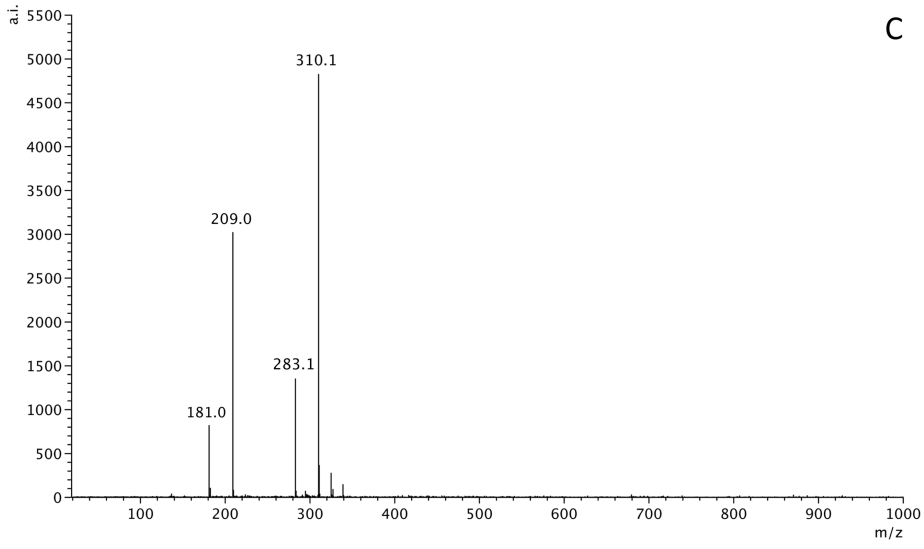

C
a.i.
310.1
209.0
283.1
181.0
m/z

Figure S3: MALDI-TOF spectra of citric acid using a) DMNA and b) MAPS as matrix by dried droplet method. In figure c, MALDI spectrum of only MAPS was registered as negative control. In presence of analyte complete ion matrix suppression and detection of citric acid as deprotonate form were observed. This experiment shows the high basicity of MAPS and DMNA as well. In the mass spectrum c, the ion at m/z value 310 is the radical anion form of MAPS and ions at values m/z 283, 209 and 181 were not identified.

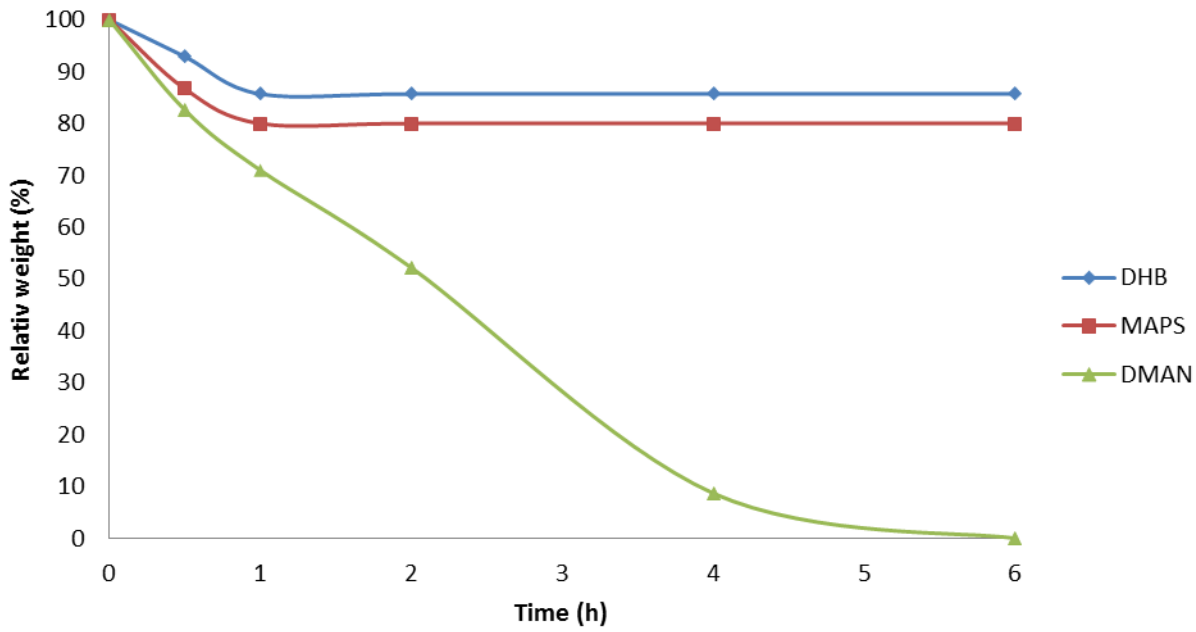


Figure S4: Vacuum stability curves of DHB, MAPS and DMAN. Each slide was weighed prior to and after matrix deposition. The slides were placed under vacuum for max. 6 hours. The respective weights of slide were measured at different time points to obtain the curves of vacuum stability for the three tested matrices. DHB and MAPS have similar robust vacuum stability properties; instead DMNA evaporates considerably and gradually up to complete evaporation after six hours under vacuum.

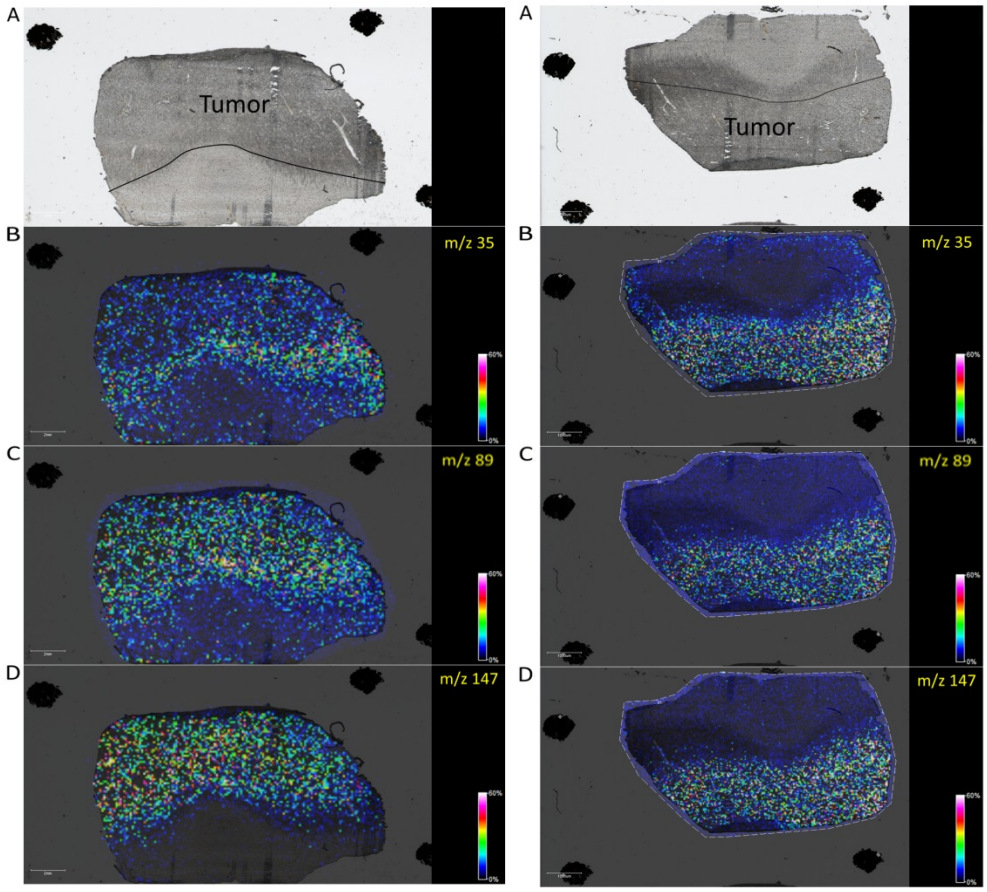


Figure S5: Other examples of MALDI imaging of glioma on two consecutive tissue sections derived from the same patient, where onto optical picture A), chloride B), lactate C) and 2-hydroxyglutarate D were visualized in the tumor area.

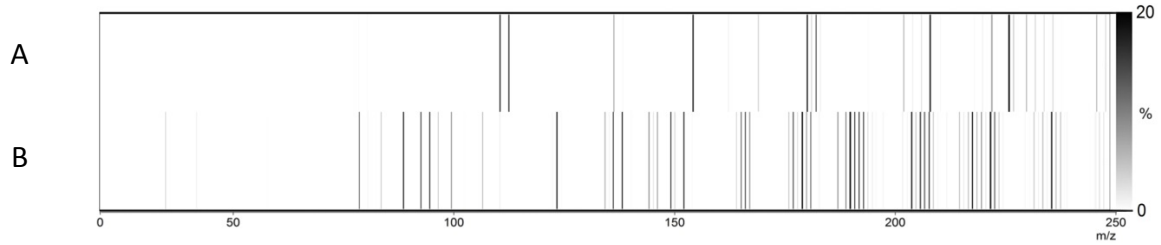


Figure S6: Two-dimensional (2D) density plots in of a) MAPS and b) glioma regions. The peaks derived from the MAPS matrix do not contribute to the visible density plot of the signals from the coated tissue.

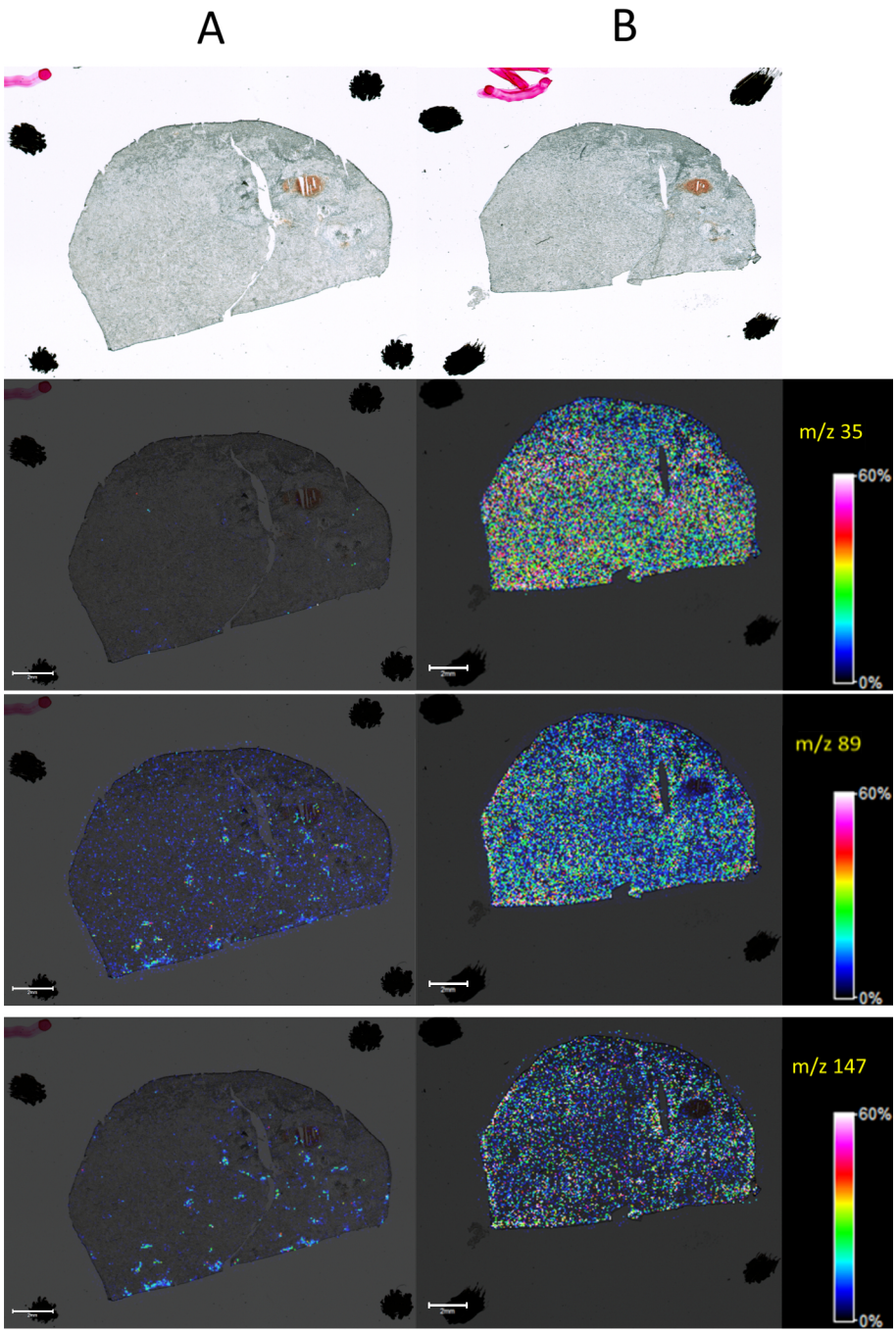


Figure S7: Comparison of MS-imaging results in brain tumor tissue sections processed using a classical matrix DHB (column A) and novel matrix MAPS (column B). By using DHB as matrix substance the localization of metabolites is undefined. A clear ionic map in tissues by using MAPS was observed. The selected m/z channels present chloride (m/z 35), lactate (m/z 89) and 2-hydroxyglutarate (m/z 147).

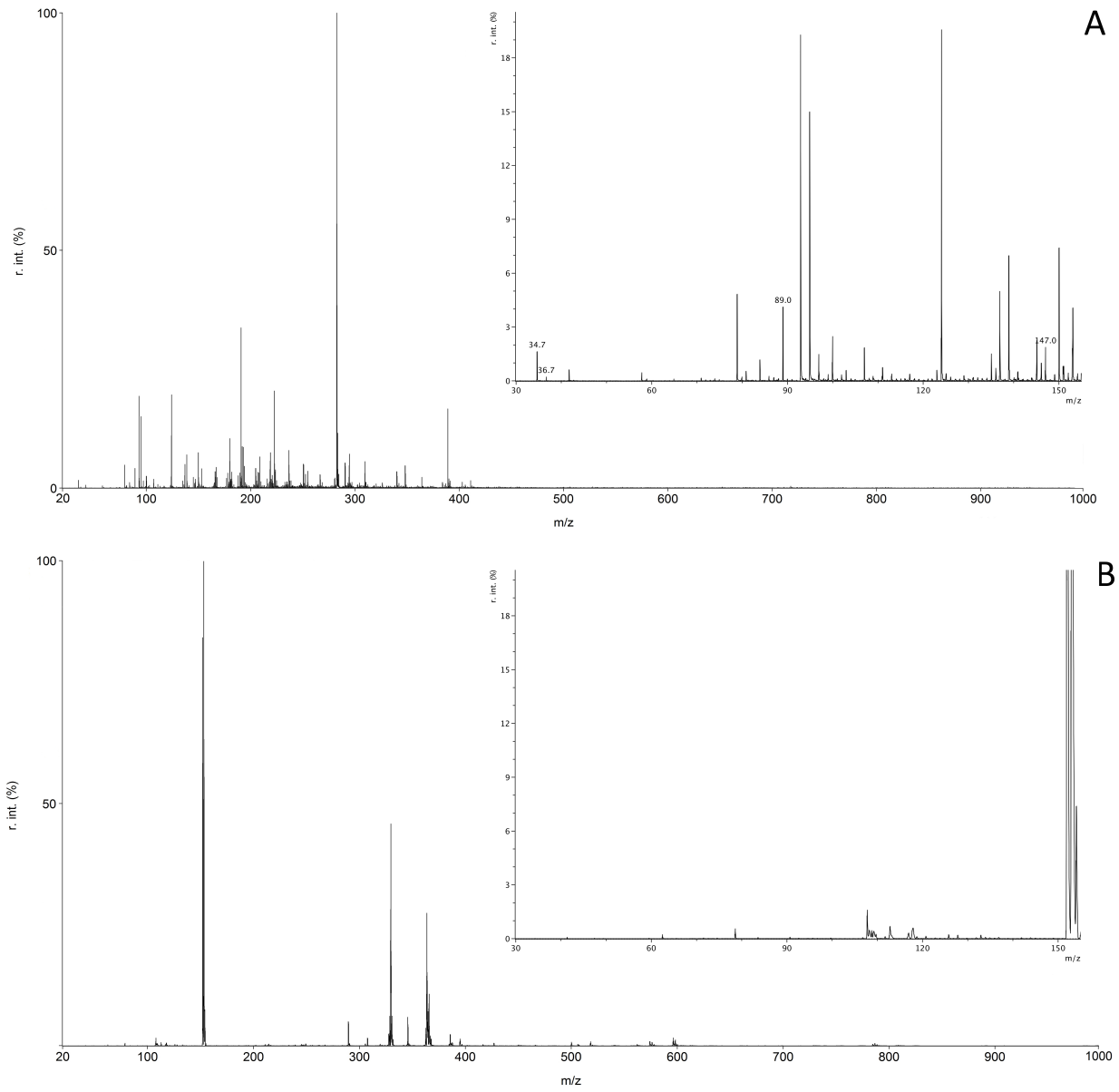


Figure S8: Overall average spectrum and respective enlargment of diffuse glioma tissue MSI of figure S5 using A) MAPS B) DHB in reflectron negative mode.

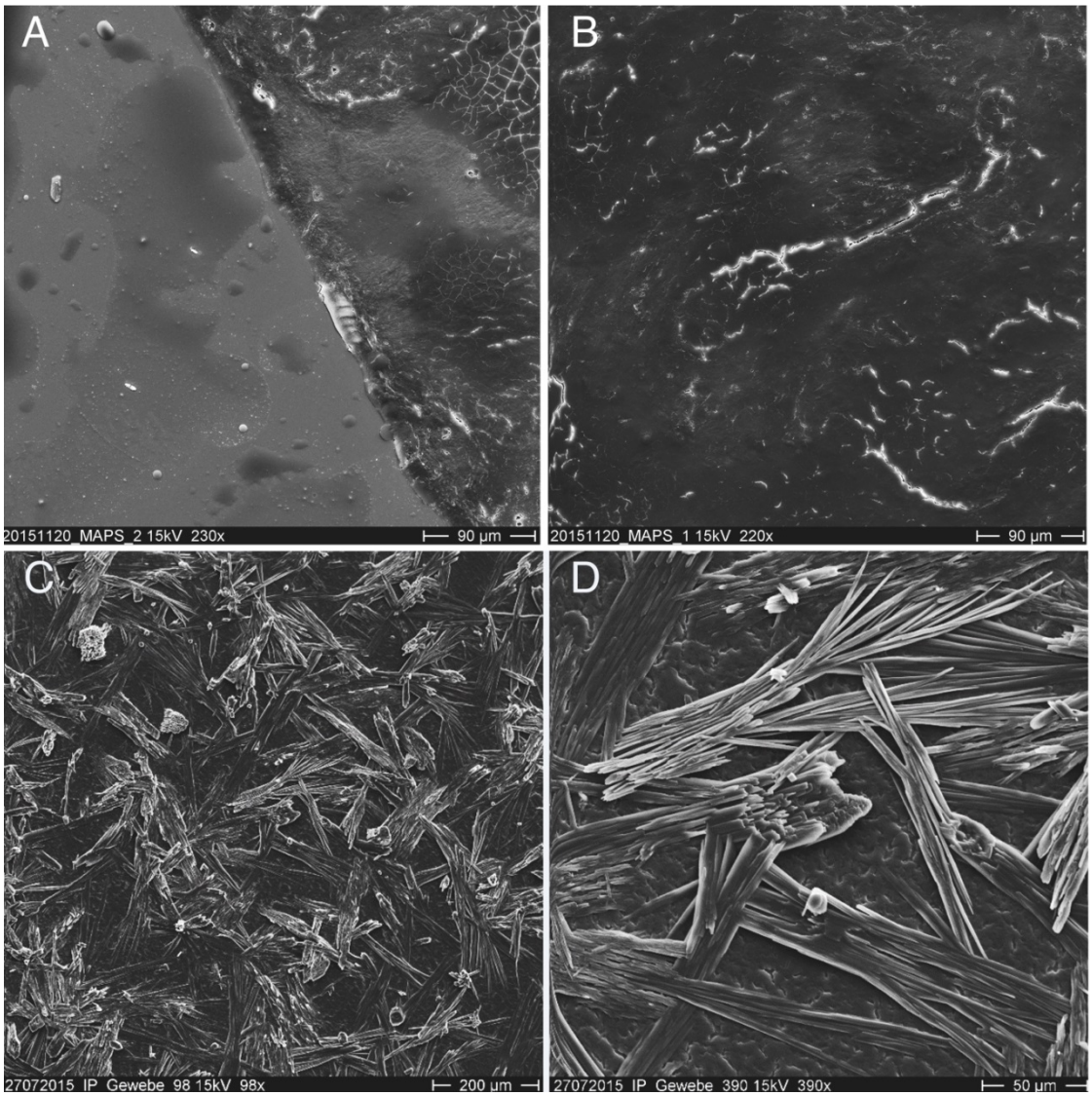


Figure S9: SEM microscopy of a series of matrix depositions on slides with human glioma tissue sections. A: The interface between the glass slide (left) and the tissue section (right), coated with the MAPS matrix. B: Surface of the tissue sample coated with MAPS; the matrix produces a homogenous layer rather than crystals, due to acetonitrile/chloroform containing solvent. The lack of visible crystals apparently does not hinder the adequate ionization of metabolites. C: In comparison to the uniform MAPS layer, DHB coating produces a mesh of crystals on the tissue. D: DHB coating produces matrix-free spaces between the crystals of approx. 50µm in size, which may contribute to limitations in imaging resolution.