Supporting Information for:

A Localized *In-Situ* Hydrogel-Mediated Protein Digestion and Extraction For Imaging Mass Spectrometry

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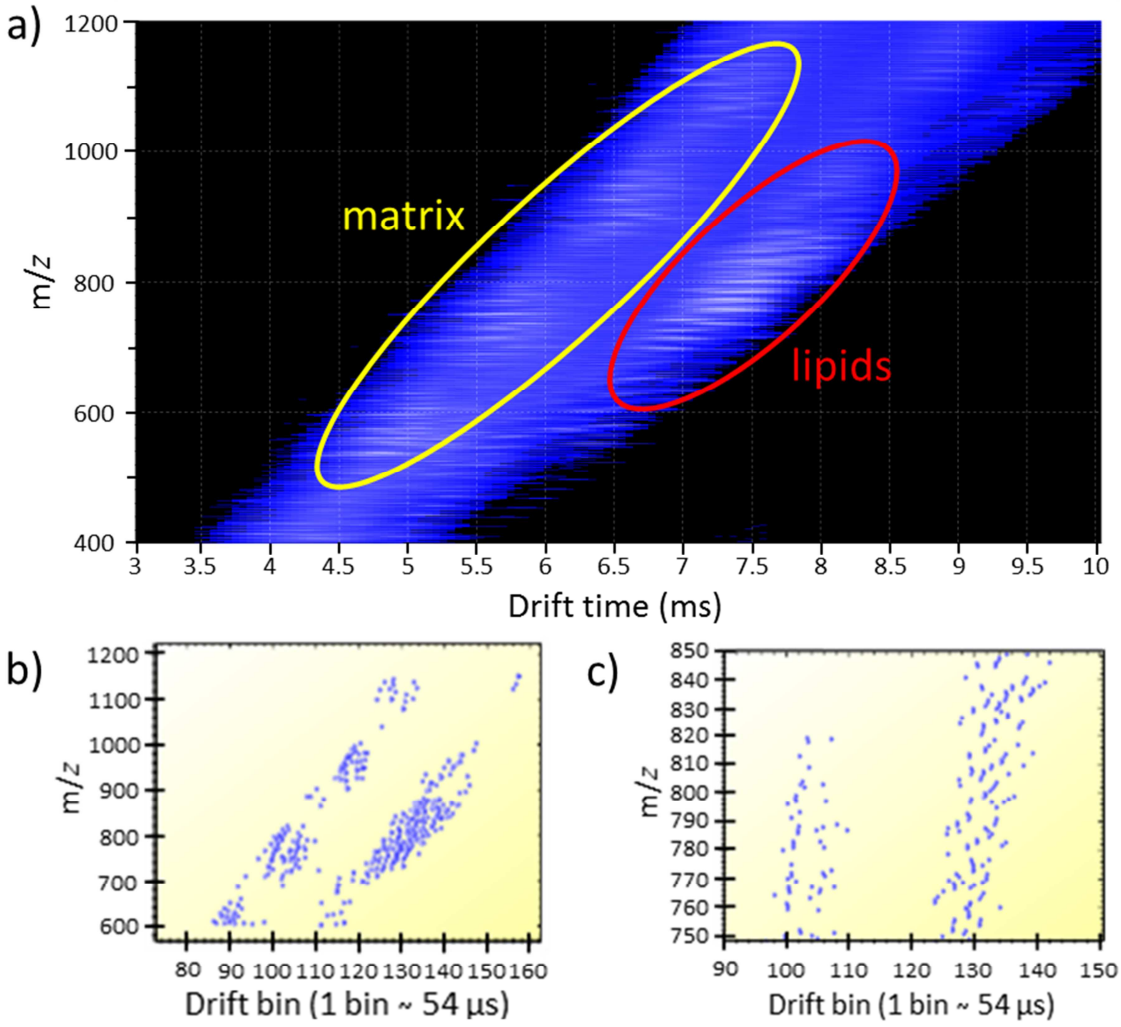

Supporting information Figure S1: a) Two-dimensional ion mobility-MS distribution plot outlining the matrix and lipid species acquired during the image acquisition shown in Figure 3 of the main manuscript (scaled to top 75 % of total ion signal). Additional peak selected distribution plots generated in the Waters HD Imaging software are shown for the b) top 250 most intense peaks in the m/z 600-1200 range and c) zoomed in region in the m/z 750-850 range.

Supporting Information Appendix on MS/MS Identification of Lipids

Protonated PC 36:1 was identified from the MS/MS base peak at m/*z* 184.08 relating to the characteristic protonated phosphatidylcholine head group fragment ion and two other ions at m/*z* 605.61 and 729.60 belonging to the neutral losses of phosphatidylcholine (-183 Da) and choline (-59 Da), respectively (Figure 3b).[1] Potassiated PC 34:1 had fragment ions associated with the potassiated cyclic 1,2-phosphodiester ion (m/*z* 162.96), the neutral loss phosphatidylcholine and choline ions (m/*z* 615.56 and 739.47, respectively) and the protonated phosphatidylcholine head group ion (m/*z* 184.08) which is sometimes seen in positive mode CID analysis of metal adducts of *in-situ* lipids (Figure 3c).[2] Sodiated PC 36:4 had fragment ions relating to the sodiated cyclic 1,2-phosphodiester ion (m/*z* 146.99), the neutral loss phosphatidylcholine and choline ions (m/*z* 621.52 and 745.54, respectively) and the protonated phosphatidylcholine head group ion (m/*z* 184.08) (Figure 3d).

For two of the identified lipid species, the MS/MS spectra revealed additional adducts not expected to be seen given their charge carrier adduct. The protonated species PC 36:1 had a fragment ion at m/*z* 162.96, which is usually present only in the presence of a potassiated precursor PC lipid since this ion is commonly observed as the potassiated cyclic 1,2-phosphodiester ion.[3] Investigating further into the ion mobility dimension of the IMS experiment, it was revealed that a matrix species was also present at m/*z* 788.94 and was likely selected in the quadrupole when it was set to m/*z* 788.6. The ion image of both species clearly shows the spatial separation of the two ions, with the lipid species only present in the mylinated regions of the rat brain and the matrix species found only off tissue and in a small tear within the tissue (Supporting information Figure S2 a). Additional confirmation was given when the matrix ion had an arrival time (t_D = 5.8 ms) consistent with the observed DHB matrix-associated trend

line completely separate from the lipid trend line and the arrival time of PC 36:1 (t_D =7.2 ms, Supporting information Figure S2 b).

Likewise, a small amount of the sodiated cyclic 1,2-phosphodiester ion was observed in the MS/MS spectrum of PC 34:1 (potassiated adduct lipid). The ion mobility dimension revealed a potential sodiated lipid species at m/z 798.72 which was probably selected with the precursor ion when the quadrupole when set to m/z 798.5. Adding to the complexity was the co-localization of both ions (Supporting information Figure S3 a and b). Although it is clear in the mass spectrum that there are two species (Supporting information Figure S3 c) and their relative abundance is different, they are likely structurally similar since they have the same arrival time (t_D = 7.1 ms) for the ion mobility settings used in this study (Supporting information Figure S3 d). From the mass spectrum signal levels, the unidentified species had a 5 % relative intensity to PC 34:1 during the sum of all spectra acquired in the imaging experiment. The MS/MS intensity levels of other diagnostic sodiated ions were not observed which could have aided in the identification of the suspected lipid species at m/z 798.72.

REFERENCES

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2. Zemski Berry, K. A.; Hankin, J. A.; Barkley, R. M.; Spraggins, J. M.; Caprioli, R. M.; Murphy, R. C. *Chem. Rev.* **2011**, *111*, 6491-6512.

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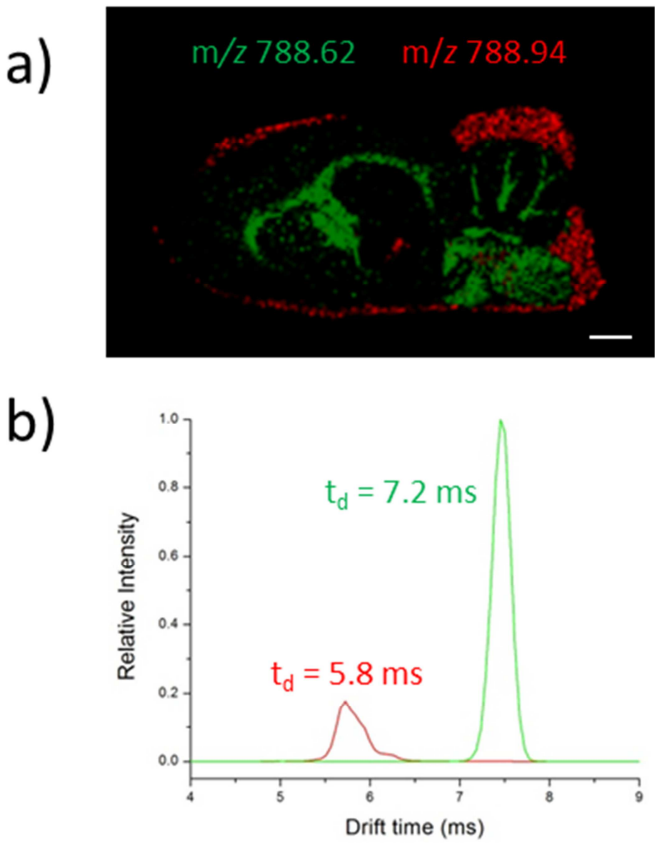
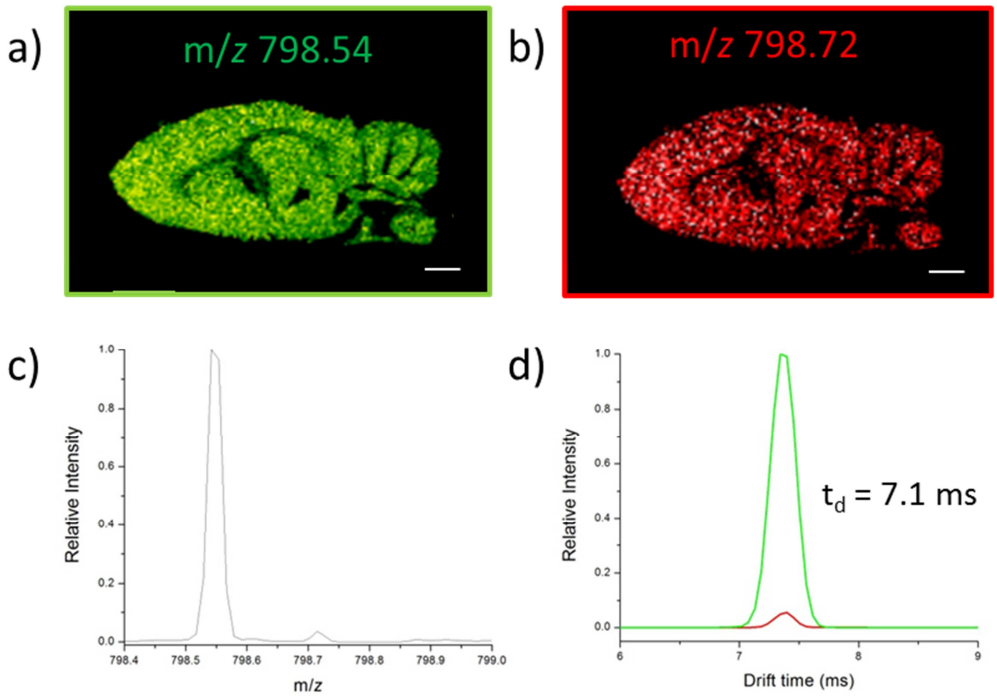
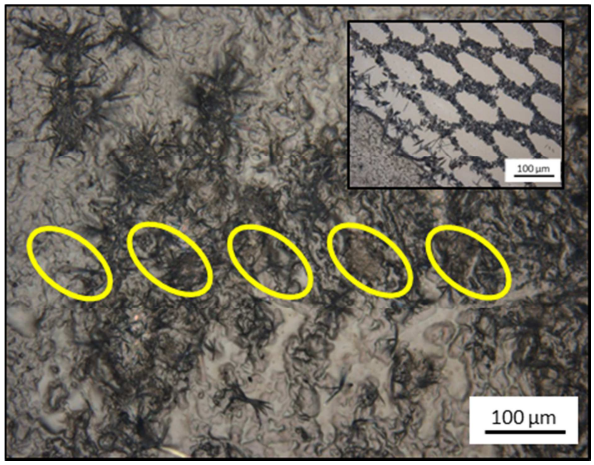


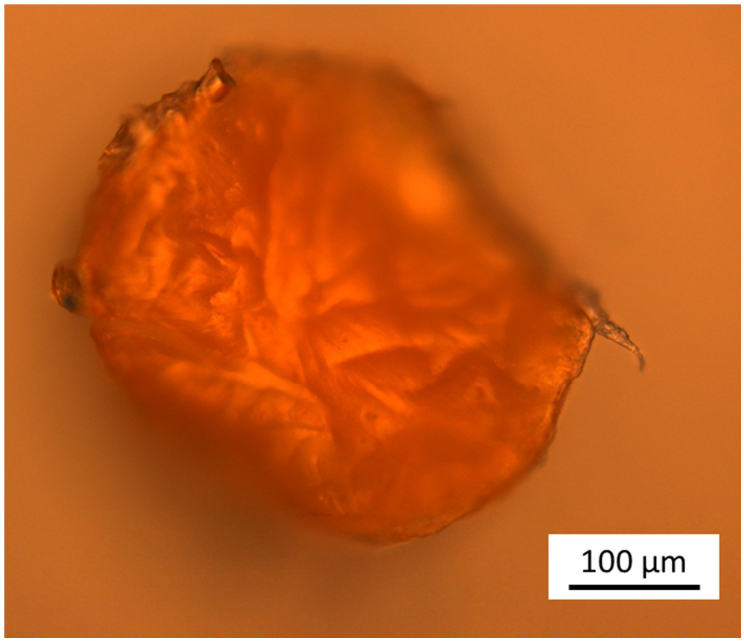
Supporting information Figure S2: a) Image overlay of two ions obtained from ion mobility analysis at nominal m/z 788 and b) arrival time distributions of both ions that were both transmitted in the precursor ion selection for the MS/MS identification of PC 36:1 (m/z 788.62, green). The red species at m/z 788.94 was only imaged outside the tissue in regions where only the matrix was present, and its arrival time at t_d = 5.8 ms falls in line with all other matrix-related species in the faster moving ions in the ion mobility dimension. Scale bar is 2 mm.



Supporting information Figure S3: Ion images from ion mobility experiments of a) PC 34:1 (green) and b) an un-identified lipid species (red) that co-localizes. Both ions were transmitted in the precursor ion selection for PC 34:1 and led to the additional fragment sodium adducts observed in the MS/MS spectrum in Figure 3. Via mass accuracy measurements, it is clear in the mass spectrum (c) that two species are present, but these species are structurally similar since they have identical arrival times in the ion mobility dimension (d). Scale bars are 2 mm.



Supporting information Figure S4: After the ion mobility MALDI IMS experiments, an optical microscope image of the rat brain cerebellum was collected to show the laser sampling regions along one imaged row. The yellow ovals indicate the laser ablation regions. The inset shows a region outside the tissue where only the matrix was present on the glass microscope slide. It is evident that no significant damage was done to the tissue from laser ablation during the image acquisition and prior to the on-tissue hydrogel incubation process and staining.



Supporting information Figure S5: Sub-millimeter alginate hydrogel made manually with a gel-loader pipette tip spotting onto laser printed chromatography paper.