

Spatial Localization of Vitamin D Metabolites in Mouse Kidney by Mass Spectrometry Imaging

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1. Supplementary Methods

1.1 Off-tissue analysis of derivatized vitamin D metabolites

Deuterated labelled 25-(OH)-D₃ standard (100 µg/ml in acetonitrile; d₆-25(OH)-D₃) was independently mixed in an Eppendorf tube with PTAD (1 mg/ml), DMEQ (0.5 mg/ml) and Amplifex (~1 mg/ml) at a 1:2 (v/v) ratio of vitamin D to derivatisation reagent. Each reaction was left for 2 h at room temperature (RT) under dark conditions as recommended in the literature. The reaction solution was then mixed with a 1:1 (v/v) ratio of CHCA matrix (5 mg/ml, 60:40 (v/v) ACN:H₂O + 0.1% FA (v/v)) and spotted (0.5 µL) onto a MALDI target plate using the dried droplet method and MALDI-FTICR-MS analysis was performed as described in **section 2.8**. DESI-qTOF-MS analysis was also carried out on the 3 reaction mixtures on off-tissue spots, with the same reaction solutions as mentioned above. The reaction solutions were then spotted on to Presolia Micro-24 glass slides (Presolia, Indianapolis, IN, USA) slides and analysed by with DESI-MS (spotting mode) using the line scan function as described in **Supplementary section 1.4**.

1.2 On-tissue spotting analysis of Vitamin D-Amplifex and DMEQ-TAD derivatives and Amplifex reaction conditions optimization

From -80 °C, control tissue sections (blank) were dried in a vacuum desiccator (20 min). Using same solutions as previously described in **Supplementary section 1.1** A 2:1 (v/v) mixture of Amplifex: d₆-25-(OH)-D₃ or DMEQ-TAD: d₆-25-(OH)-D₃ was prepared and 0.5 µL of the reaction was manually spotted onto the tissue and allowed to dry under nitrogen gas. Once dried, CHCA matrix was applied as described in **section 2.6** and MALDI-FTICR-MS analysis was performed as described in **section 2.8**. DESI-qTOF-MS analysis was also performed on the tissue spots, with the same reaction solutions as mentioned above. The reaction solutions were then spotted on to Presolia Micro-24 glass slides (Presolia, Indianapolis, IN, USA) slides and analysed by with DESI-MS (imaging mode) using the line scan function as described in **Supplementary section 1.5**.

1.3 Optimization of Amplifex reaction conditions

Upon assessing that Amplifex was the most suitable candidate for further optimization using MALDI-MSI, reaction optimisation experiments (time and temperature) were carried out using same solutions as described in **Supplementary section 1.2** with the addition of 25-(OH)₂-D₃ (100 µg/ml, in acetonitrile) acting as target and d₆-25-(OH)-D₃ as internal standard (ISTD). Conditions tested were at 0-3 h at both RT and 40 °C for off-tissue and on-tissue assessment and samples were prepared and analysed as previously described in **Supplementary section 1.1** (off-tissue) and **Supplementary section 1.2** (on-tissue) with the exception that analysis was carried out using Applied Biosystems / MDS Sciex 4800 MALDI TOF/TOF mass

spectrometer (Foster City, California, USA) equipped with a Nd:YAG laser operating at 355 nm (200 Hz repetition rate). The mass range was acquired from 400-800 Da, focusing on the appropriate base peak ion in positive reflector mode. 200 Laser shots per spot in random walk mode were accumulated for ITSD and target compound signal intensity calculations using data explorer software. Instrument was calibrated using CAL MIX 5 as per manufacture guidelines.

1.4 Confirmatory LC-MS/MS of 1,25-dihydroxyvitamin D₃ in mouse kidney homogenate

Mouse kidney (~100 mg) was homogenized by adding 700 μ L of hexane/acetone mixture (1:1, v/v) and using ultra sonication for 1 min, amplitude 80%, and interval 0.5-0.9 s (UP50H, MMTG, US) in a 1.2 mL Eppendorf, and add 20 μ L of internal standard (1ng/ml of d₃-1,25-(OH)₂-D₃). Sample is kept in an ice bath during homogenization. 300 μ L of distilled water is added to the mixture, vortexed for 30 s, ultrasonicated for 10 min and centrifuge for 10 min at 15,000 RPM. The supernatant (600 μ L) was dried down by evaporation using nitrogen gas flow at RT. The sample was reconstituted in 50:50 Mobile phase (50:50 (v/v) water:methanol containing 2 mM lithium acetate; 200 μ L) in an HPLC insert, in an amber HPLC vial. Stock solutions of 1 mg/ml of 1,25-(OH)₂-D₃ and d₃-1,25-(OH)₂-D₃ were dissolved in methanol. Working standards of 0, 2, 20, 100, 200 and 1000 pg/g were created by serial dilution. The standards and samples were separated on a Luna-extended C18 (50 x 2.1mm, 3.0 μ m) at 40 °C on an ACQUITY ultra high-pressure liquid chromatography (UHPLC; Waters, Manchester, UK). using mobile phases (A) water + 2 mM lithium acetate and (B) methanol + 2 mM lithium acetate. See gradient profile in **Table S1**. Samples were kept at 5 °C and injection volume at 20 μ L. Mass spectrometry analysis was carried out in positive mode electrospray ionization on a ABSciex 6500 QTrap (ABSciex, US). Ionization and fragmentation conditions were set as follows: CAD (Arb): 6, CUR (Arb):40, GSI (Arb): 35; GSII (Arb) 40; IS:(V) 5500; TEMP (°C): 500. See MRM transitions in **Table S2**.

1.5 DESI-qTOF-MS analysis: tissue spotting and MSI

Ambient mass spectrometry imaging experiments were performed on a commercial DESI 2D source (OmniSpray 2D Source, Prosolia Inc, Indianapolis, IN, USA) mounted on a Waters Xevo G2-XS mass spectrometer (Waters Corporation, Manchester, UK). The instrument was operated in positive ion mode, the spray voltage was 4 kV, the nitrogen gas pressure was 5 bar and the capillary temperature was 150 °C. The spray solvent was MeOH: H₂O (98:2, v/v), which was directed onto the sample at an angle of 80 °C at a flow rate 2 μ L/min. Images were acquired at a spatial resolution of 100 x 100 μ m and a scan rate of 100 μ m/sec resulting in a scan time of 0.986 s. Images were generated using the HDImaging 1.4 software (Waters Corporation) and normalized with the TIC (total ion current).

2 Supplementary Figures

Figure S1: VitD metabolites chemical derivatization reaction schemes of a) Amplifex and b) 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) c) 4-(2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxaliny)ethyl)-1,2,4-triazoline-3,5-dione (DMEQ-TAD) with a deuterated vitamin D metabolite and internal standard (d_6 -25(OH)-D₃)

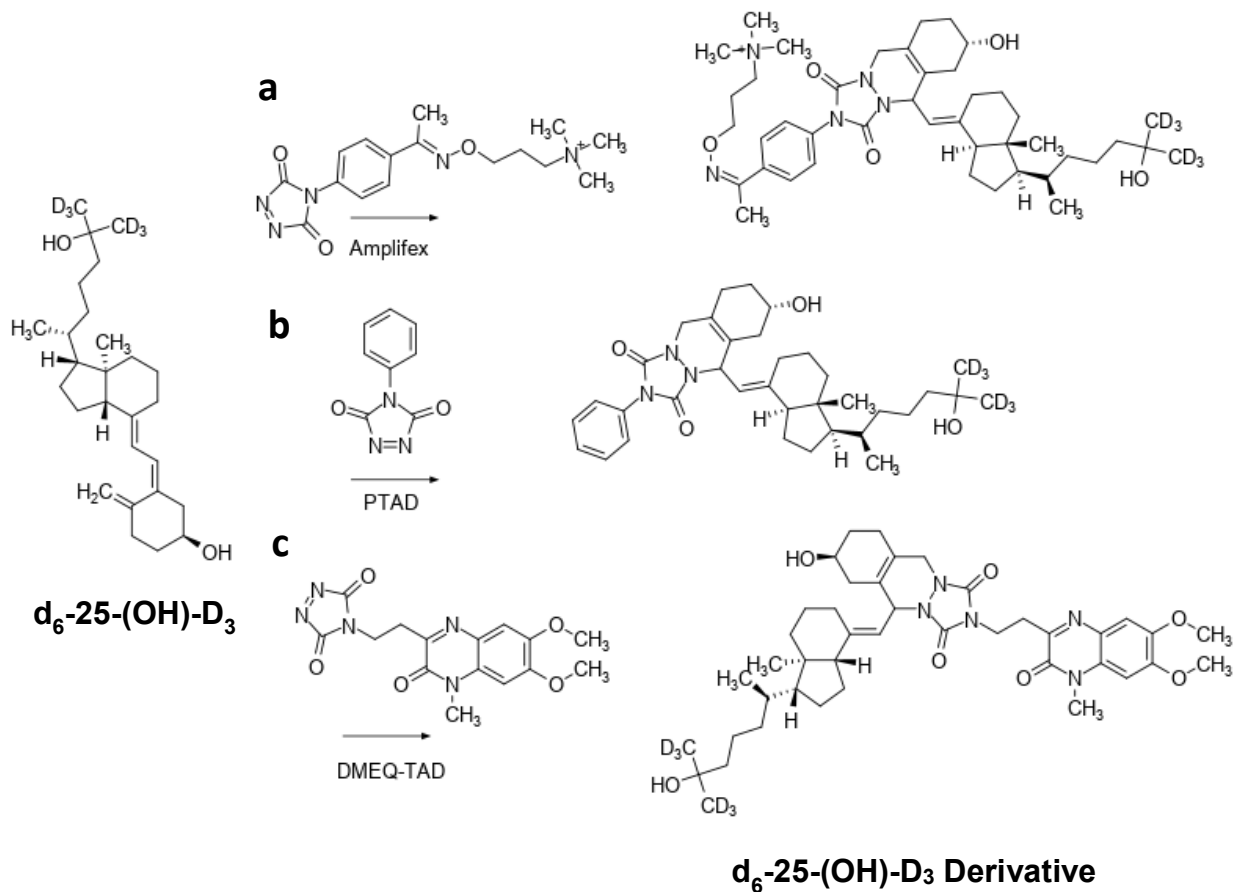


Figure S2: Off-tissue spotting experiments using Amplifex as derivatization reagent. a) Theoretical monoisotopic mass of d_6 -25-(OH)- D_3 -Amplifex derivative at m/z 738.54351; **b)** Representative MALDI-FTICR-MS spectrum of VitD-Amplifex derivative at m/z 738.5437 with a mass accuracy of 0.27 ppm; **c)** Representative DESI-qTOF-MS spectrum of VitD-Amplifex derivative using DESI-qTOF-MS at m/z 738.5447 with a mass accuracy of 1.62 ppm.

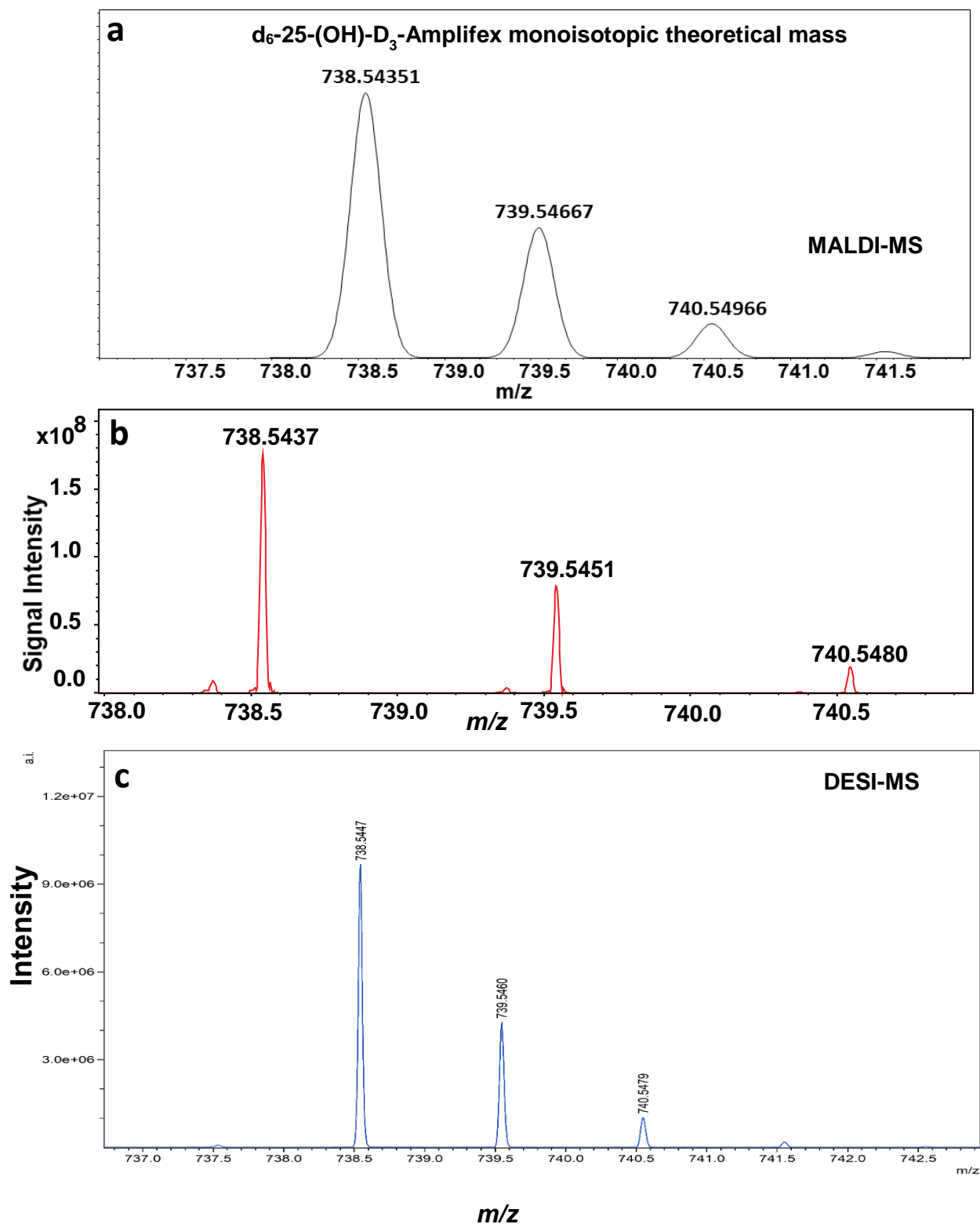


Figure S3: Off-tissue spotting experiments using DMEQ-TAD as derivatization reagent.

a) Theoretical monoisotopic mass of d_6 -25-(OH)- D_3 -DMEQ-TAD derivative at m/z 752.48639;

b) Representative MALDI-FTICR-MS spectrum of VitD-DMEQ-TAD derivative at m/z 752.4863 with a mass accuracy of 0.12 ppm;

c) Representative DESI-qTOF-MS spectrum of VitD-DMEQ-TAD derivative using DESI-qTOF-MS at m/z 752.4843 with a mass accuracy of 2.78 ppm.

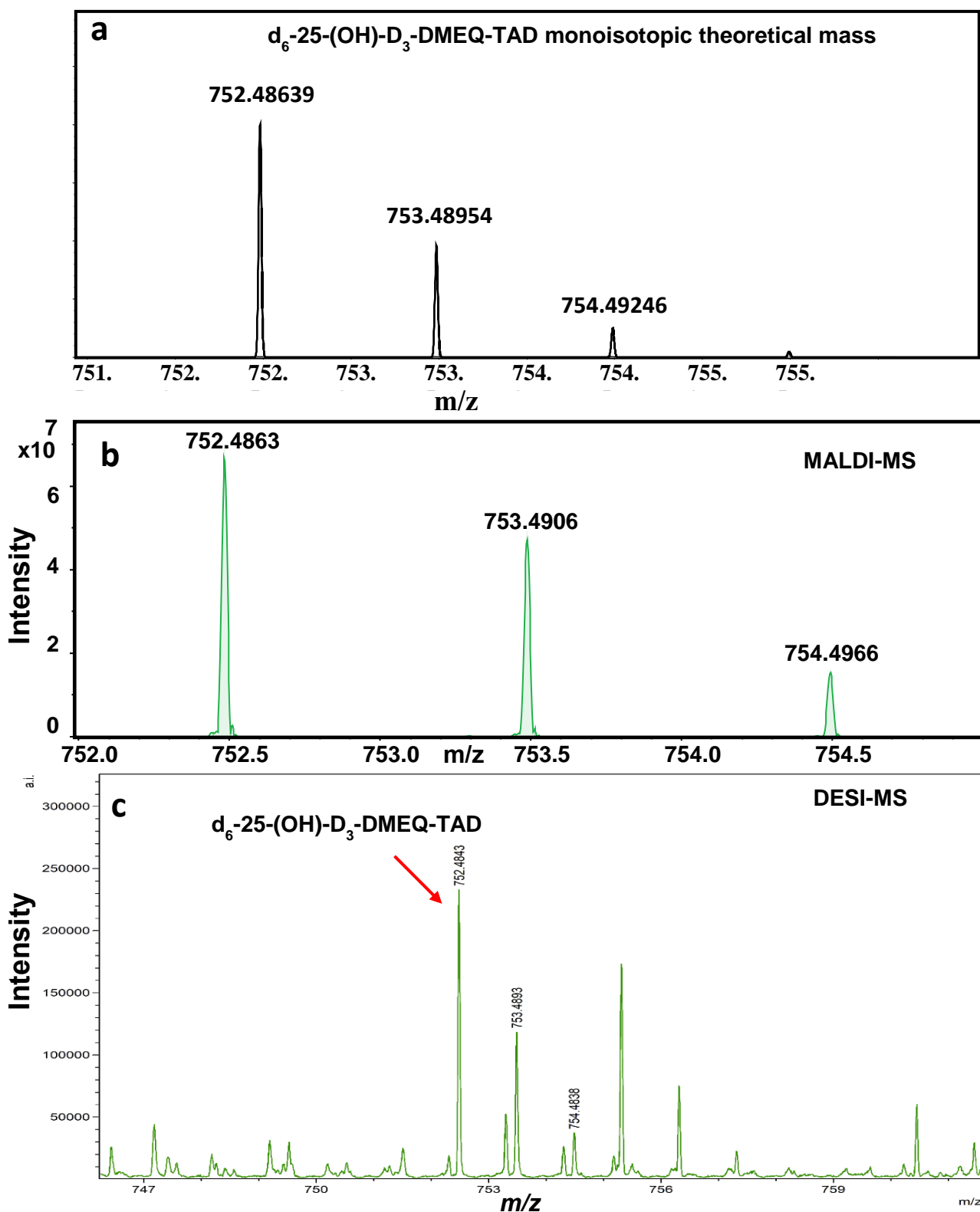


Figure S4: On-tissue ionization assessment using MALDI-FTICR-MSI of Amplifex and DMEQ-TAD derivatives of d_6 -25-(OH)-D₃ (ISTD) spots on control tissue section. **a)** Optical image of control tissue spotted with ISTD-Amplifex derivative; **b)** Molecular distribution map of ISTD-Amplifex derivative (m/z 738.5444 \pm 0.005); **c)** Optical image of control tissue spotted with ISTD- DMEQ-TAD derivative; **d)** Molecular distribution map of ISTD-DMEQ-TAD derivative (m/z 752.4848 \pm 0.005); **e)** Representative mass spectrum for ISTD-Amplifex derivative with mass accuracy of 1.21 ppm against theoretical monoisotopic mass (shown inset); **f)** Representative mass spectrum for ISTD-DMEQ-TAD derivative with a mass accuracy of 2.11 ppm against theoretical monoisotopic mass (shown inset). Data was normalised to TIC. Spatial resolution was analysed at 100 μ m, with a scale bars shown. Signal intensity is depicted by colour on the scale shown. Spectra were post-calibrated to CHCA cluster matrix ion at m/z 417.04834.

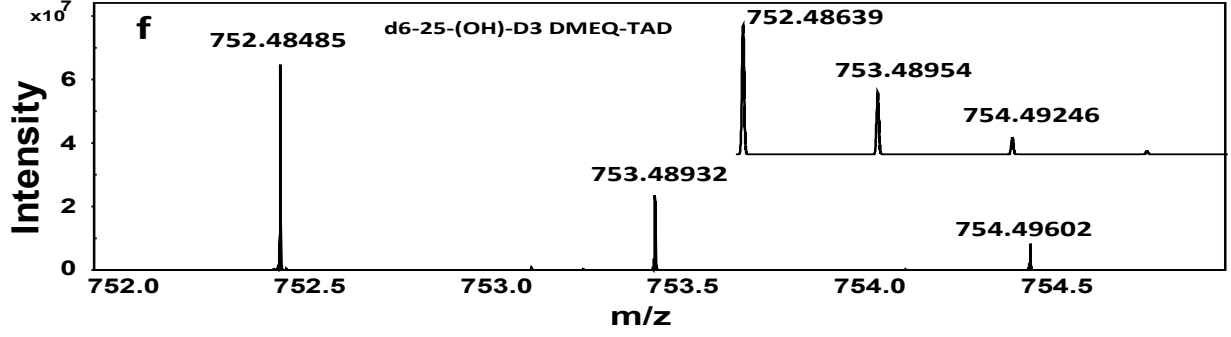
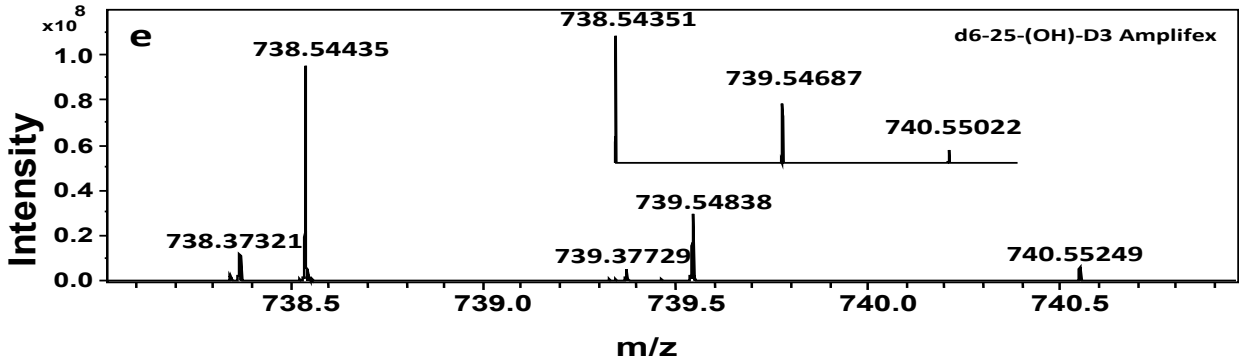
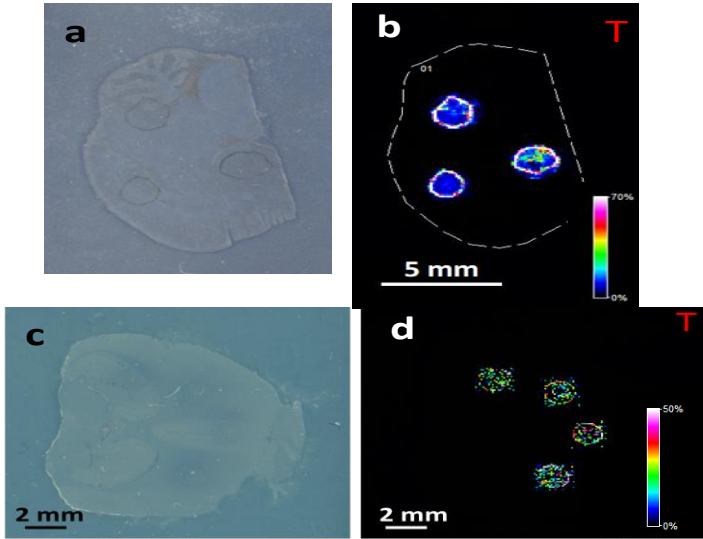


Figure S5: On-tissue ionization assessment using DESI-qTOF-MSI of Amplifex and DMEQ-TAD derivatives of d_6 -25-(OH)-D₃ (ISTD) on a control tissue section. a) Molecular distribution map of ISTD-Amplifex derivative (m/z 738.543 \pm 0.005 – green signal). b) Molecular distribution map of ISTD-DMEQ-TAD derivative (m/z 752.4730 \pm 0.005). c) Representative mass spectrum for ISTD-Amplifex derivative with a mass accuracy of 0.8 ppm. d) Representative mass spectrum for ISTD-DMEQ-TAD derivative with a mass accuracy of 1.7 ppm. Data is normalised by TIC. Scale bar is shown, and signal intensity is depicted by the colour scale shown.

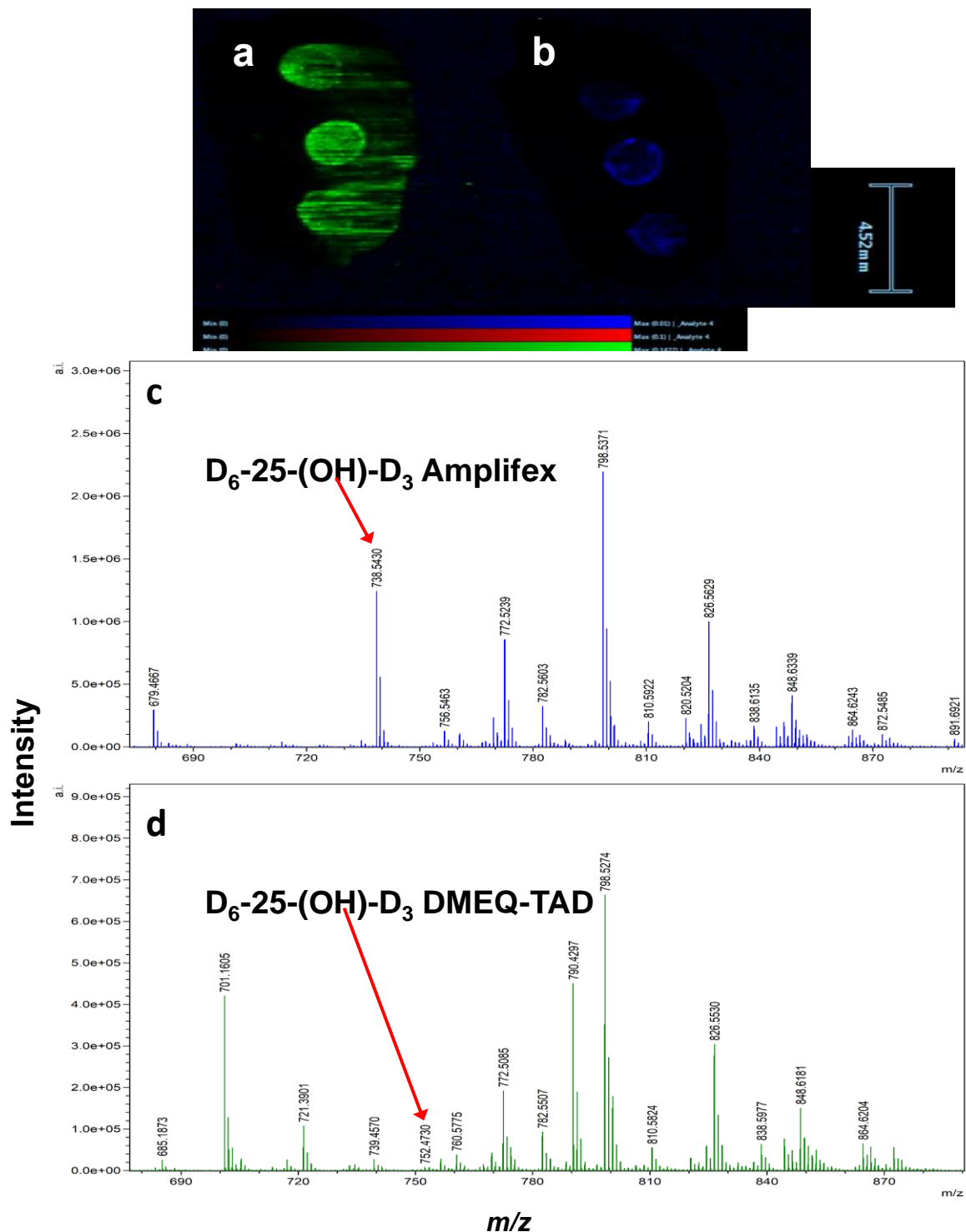
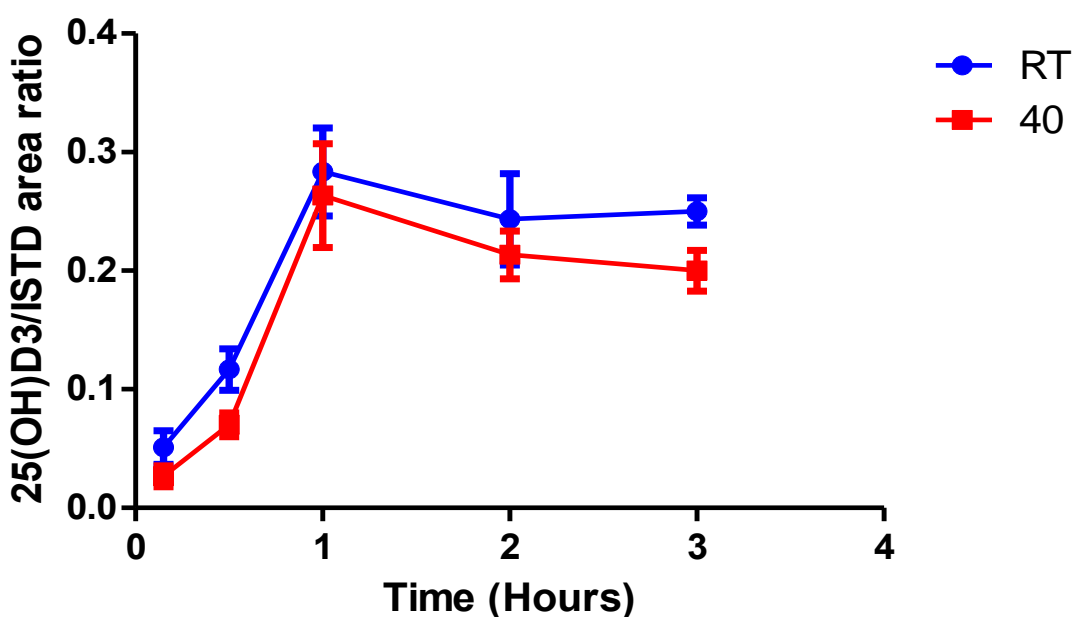


Figure S6: VitD-Amplifex Reaction conditions optimization: Temperature and time were screened (a) off and (b) on-tissue using 25-(OH)-D₃ as target VitD metabolite and d₆-25-(OH)-D₃ as ISTD for optimal Amplifex reaction conditions in MALDI-MS. Samples were analysed as described in **Supplementary section 1.3** Signal detection of vitamin D metabolite 25-(OH)-D₃ was normalised using an ISTD (d₆-25-(OH)-D₃). Optimal chemical derivatization condition achieved at 1 h at RT for both off-tissue and on-tissue derivatization. Samples were n=3 for each time point, with mean values ± SEM displayed.

a Off-Tissue reaction conditions optimization



b On-Tissue reaction conditions optimization

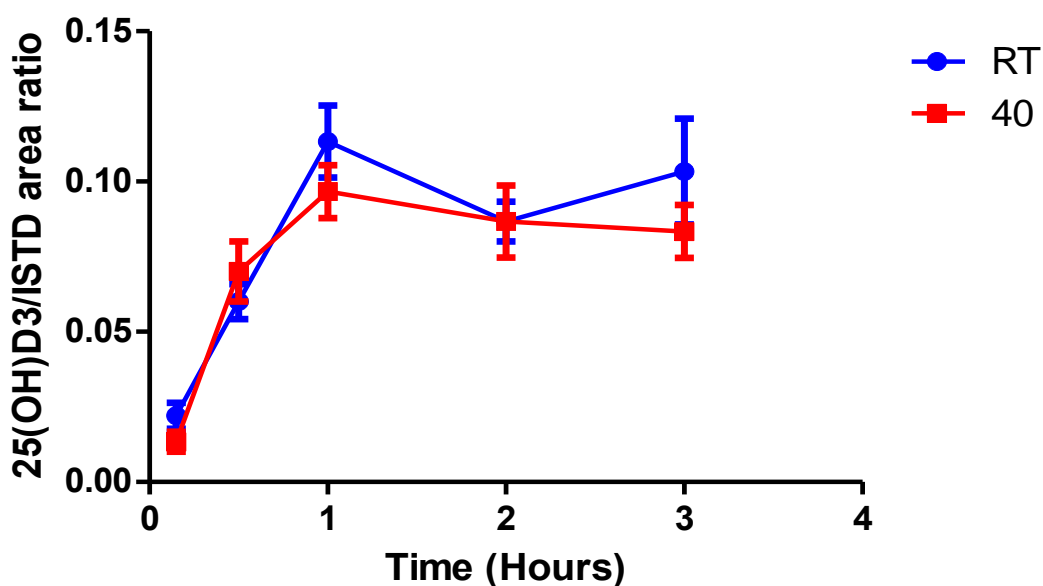


Figure S7 : Optimization of OTCD of Amplifex on mouse kidney sections using the ImagePrep and airbrush. Molecular distribution map of 1-25-(OH)₂-D3-Amplifex derivative **a)** Using ImagePrep at m/z 748.500 \pm 0.005; **b)** Using artistic airbrush at m/z 748.4974 \pm 0.005. **c)** Theoretical monoisotopic mass of 1-25-(OH)₂-D3-Amplifex derivative at m/z 748.5007; **d)** Superimposition of representative single pixel (highlighted in white) mass spectra of 1-25-(OH)₂-D3-Amplifex derivative acquired by ImagePrep (**red**) and airbrush (**blue**) OTCD methods. Mass accuracy was 3.62 ppm for both applications. Data was normalised to TIC. Spatial resolution was analysed at 100 μ m, with the scale bar indicating 5 mm. Signal intensity is depicted by colour on the scale shown. Spectra were post-calibrated to CHCA cluster matrix ion at m/z 417.04834.

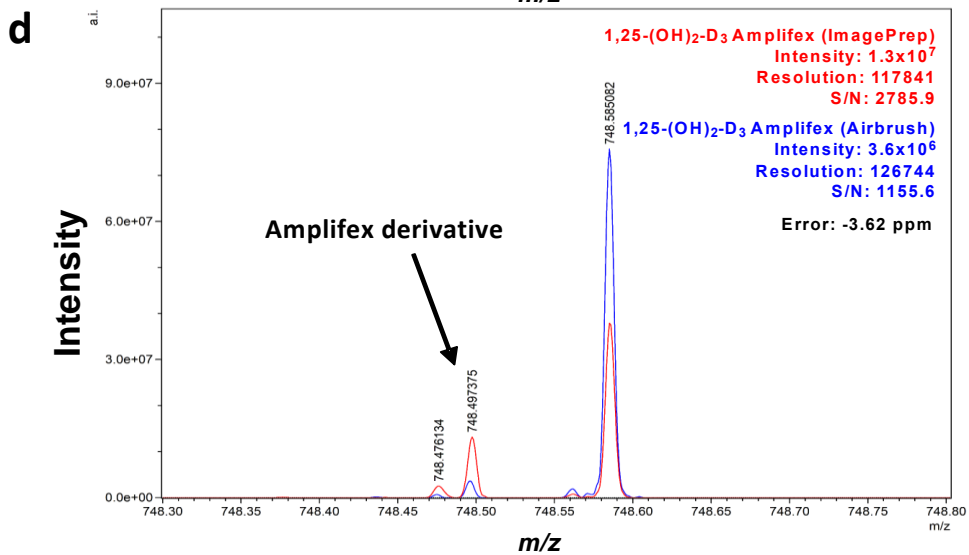
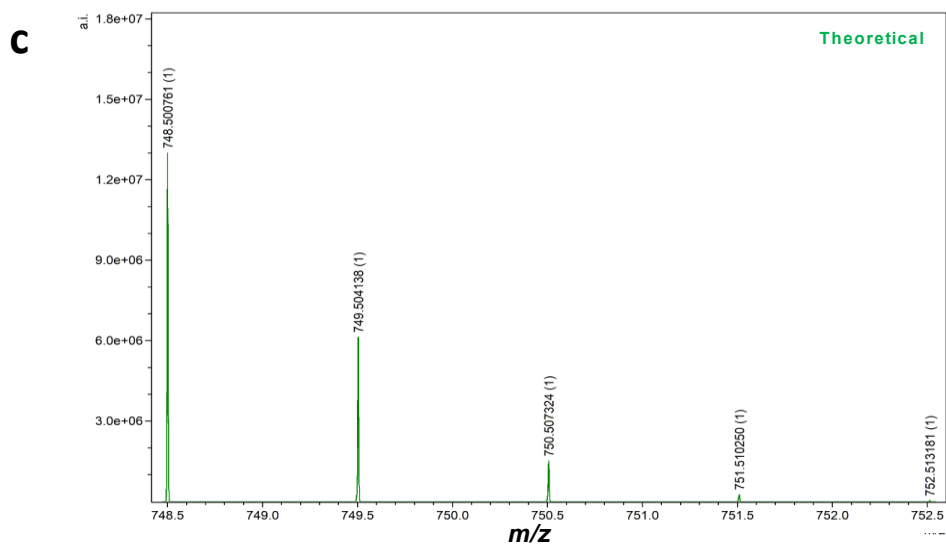
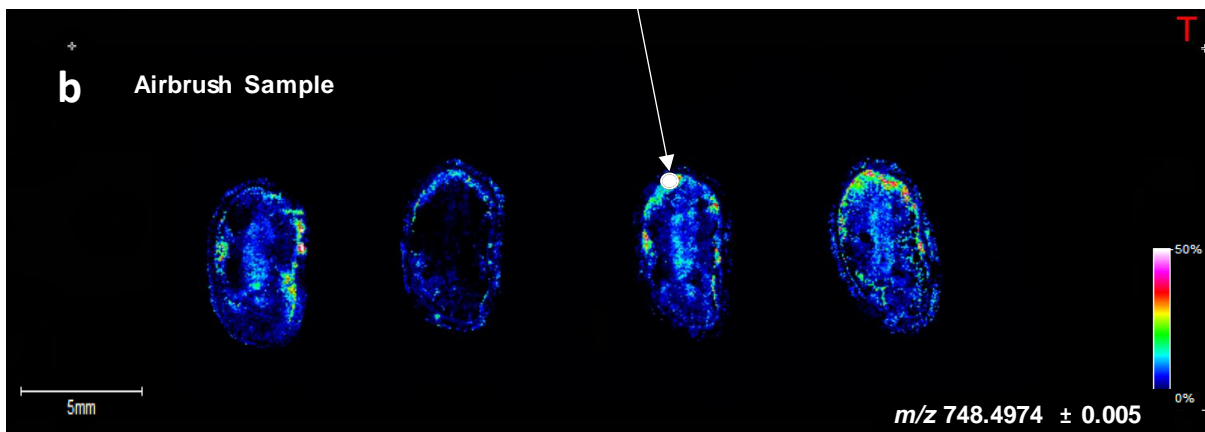
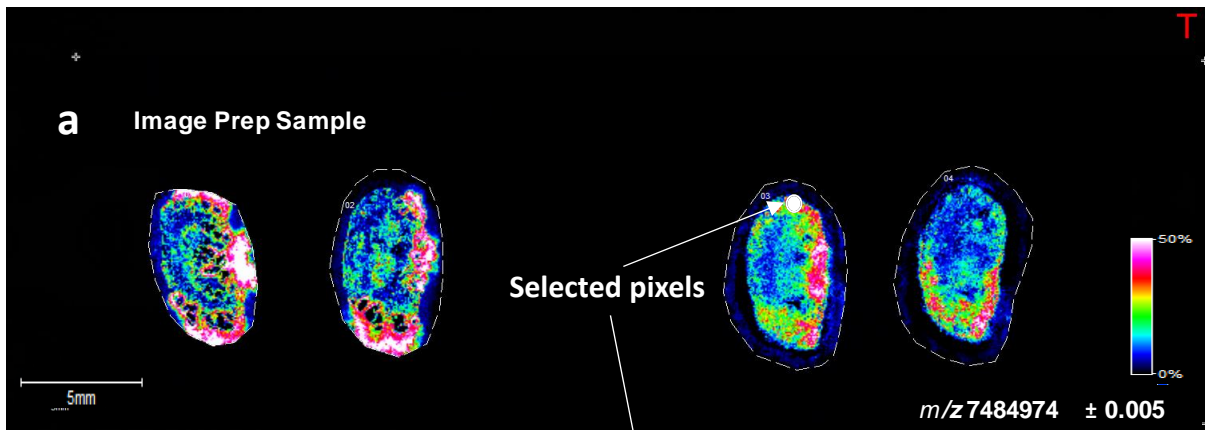
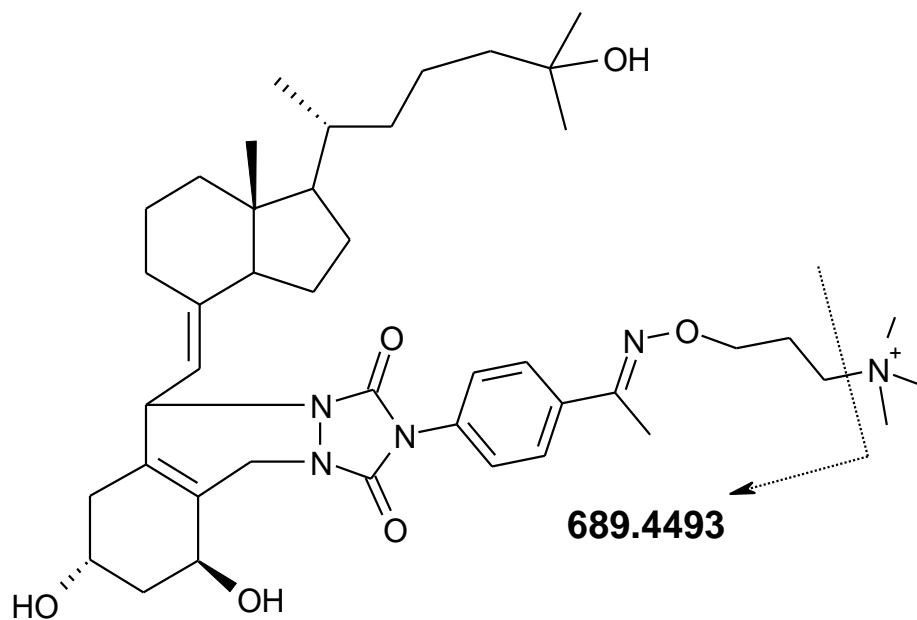
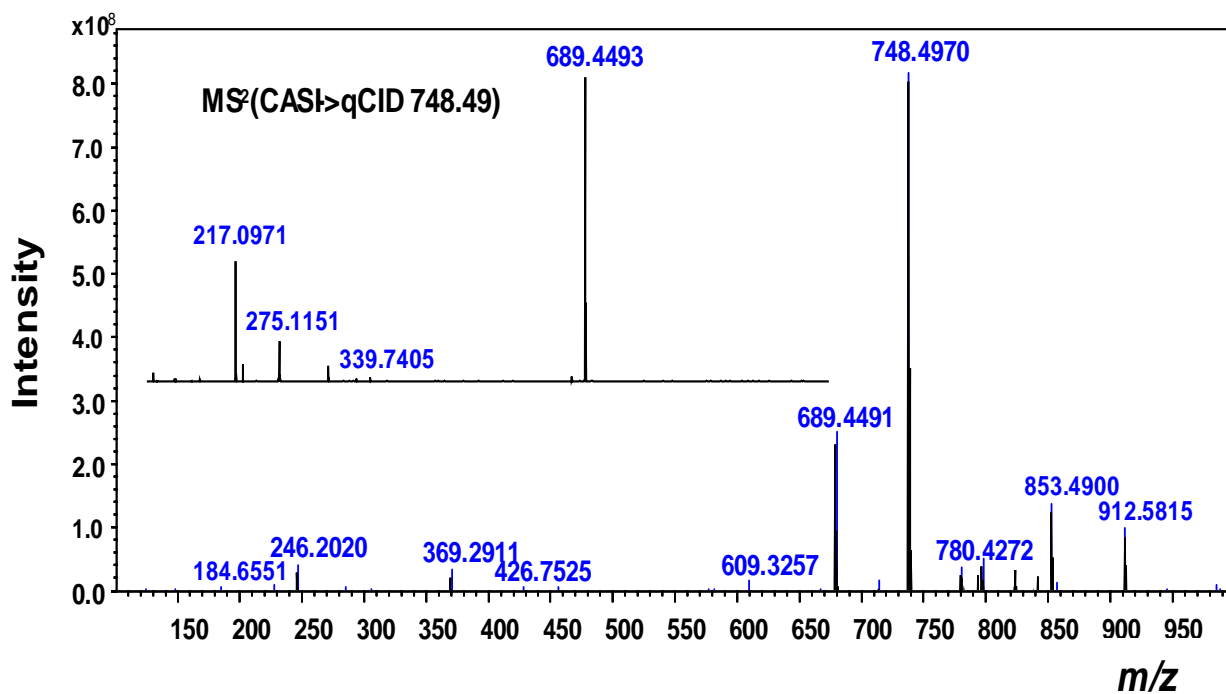


Figure S8: Confirmative collision induced dissociation (CID) experiments of endogenous 1,25-(OH)₂-D₃-Amplifex derivative on a kidney tissue section. CID experiments were performed on region of interest (ROI) within the tissue section. CID on M⁺ at *m/z* 748.49 shown a main fragment at *m/z* 689.4493, corresponding to a loss of the trimethylamine moiety (59 Da).



3. Supplementary Tables

Table S1: LC gradient

Time (min)	Flow (ml/min)	B (%)
0.1	0.35	50
3.0	0.35	50
10.0	0.35	100
10.1	0.35	50
12.1	0.35	50

Table S2: Multiple reaction monitoring conditions

Compound	Parent (m/z)	Quantifier (m/z)	Qualifier (m/z)	CE (V)	DP (V)	CXP (V)	EP (V)
1,25 (OH) ₂ D ₃	423.1	369.0	161.2	23/49	96	10/12	10
ISTD	426.1	372.1	N/A	23	96	10	10

Table S3: Off-tissue reagent screening results

Derivatization Reagent	Off-tissue Protonated mass ion intensity/ signal/noise (S/N) of d ₆ -25-(OH)-D ₃ by MALDI-FTICR-MS	Off-tissue Protonated mass ion intensity/ signal/noise (S/N) of d ₆ -25-(OH)-D ₃ by DESI-qTOF
PTAD	Non-detectable	Non-detectable
DMEQ-TAD	$7.2 \times 10^7 / 2.2e^5$	$2.5 \times 10^5 / 1342$
Amplifex	$1.8 \times 10^8 / 1.1e^8$	$1.1 \times 10^7 / 1.7e^6$

Table S4: On-tissue ionization suppression assessment

Derivatization Reagent	On-tissue Protonated mass ion intensity signal/noise (S/N) of d ₆ -25-(OH)-D ₃ by MALDI-FTICR-MS	On-tissue Protonated mass ion intensity signal/noise (S/N) of d ₆ -25-(OH)-D ₃ by DESI-qTOF
DMEQ-TAD	$6.3 \times 10^7 / 1.2e^4$	$0.8 \times 10^4 / 340$
Amplifex	$1.1 \times 10^8 / 1.0e^6$	$1.2 \times 10^6 / 4427$