Supporting Information

Cross-Validated Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging Quantitation Protocol for a Pharmaceutical Drug and Its Drug-Target Effects in the Brain Using Time-of-Flight and Fourier Transform Ion Cyclotron Resonance Analyzers

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Supporting Information Content

Note S1. Optimization of method for applying calibration standards, internal standard and matrix for MALDI-MSI analysis of citalopram in mouse brain tissue sections.

To optimize the method for applying the calibration standards, quality control (QC) samples, internal standard (IS), and MALDI matrix for MALDI-MSI analysis, we tested different methodologies (as illustrated in **Figure S2**) and compared the results with those from LC-MS/MS analysis. We used a chemical inkjet printer to apply the calibration standards and QC samples onto tissue for MALDI-MSI analysis. A volumetric measurement protocol was developed for the chemical inkjet printer. In this optimization study, we report protocols for preparing samples for citalopram analysis by MALDI-MSI as well as LC-MS/MS. These protocols were subsequently used in a complete validation of our MALDI-MSI method, using the developed LC-MS/MS method as the reference method.

Methods & Results

The chemicals and reagents were as described in the Experimental Section.

Preparation of Stock solutions and Liquid Handling. The preparation of stock and working stock solutions is described in the Experimental Section. However, 50 % b.w. methanol solution was used because of its defined properties. It was prepared by weighing both water and methanol with high precision and had the following physical properties at 20 °C: density (ρ) = 0.9156 g/mL, [1] viscosity (η) = 1.761 cP [1] and surface tension (γ) = 33.37 mN/m. [2] For comparison, the corresponding physical properties of water are ρ = 0.9982 g/mL, [1] η = 1.002 cP [1] and γ = 72.75 mN/m, [3] whereas those of 100 % methanol are ρ = 0.7917 g/mL, [1] η = 0.586 cP [1] and γ = 22.60 mN/m. [4] When adding the 50 % b.w. methanol gravimetrically at 20 °C, it was easy to determine its volume with high accuracy. The 50 % b.w. methanol solution was handled well by both the chemical inkjet printer and manual pipetting.

Animal Preparation. Male mice (C57BL/6J) of age 7-8 weeks and average weight 23.1 ±1.6 g (±SD, n = 5) were used in the optimization study. The animals were housed in air-conditioned rooms providing 12 h dark/12 h light cycles at 20 °C with 53 % relative humidity and food ad libitum. The mice were administered S-citalopram oxalate (30 mg/kg, n = 3) or saline solution (n = 2) via intraperitoneal (i.p.) injection and euthanized by decapitation 30 min after injection. Following euthanasia, brain tissues were rapidly dissected out, frozen in dry ice-cooled isopentane and stored at -80 °C until further use. The study was conducted in accordance with European Communities Council Directives 86/609/EEC and 2010/63/EU on the ethical use of animals and were approved by the local ethical committee at the Karolinska Institute (approval nos. N350/08 and N40/13).

Tissue Preparation. The tissue cutting and cutting scheme as well as IS application are described in the Experimental Section, **Figure 1**, and **Figure S2**. The tissue sections collected for MALDI-MSI were thaw-mounted onto indium-tin-oxide (ITO) glass slides (Bruker Daltonics, Bremen, Germany) in methods **A** and **B** (**Figure S2**). In methods **C**, **D** and **F**, the IS (citalopram-d_6) was uniformly applied before thaw-mounting the tissue sections on the ITO-glass slides. For methods **E** and **F**, calibration standards and quality control (QC) samples were also applied before thaw-mounting tissue sections. The ITO glass slides were stored at -80 °C until further analysis.

MALDI-MSI Sample Preparation Protocol Optimization. Standards for generating a calibration curve for citalopram were applied either manually by a pipette or automatically using a Chip-1000 chemical inkjet printer (Shimadzu Corp., Tokyo, Japan) on two control coronal brain tissue sections. QC samples were deposited on a third coronal brain control tissue section. Citalopram was then quantified in three coronal brain tissue sections from animals that had been administered the drug *in vivo* using an ultrafleXtreme MALDI TOF/TOF mass spectrometer (Bruker Daltonics). Mass spectra were acquired using flexControl 3.4 and flexImaging 4.1 software supplied by

Bruker Daltonics, and msIQuant software, [5] developed in-house was used for data processing, normalization, and quantitation. Regions-of-interest (ROIs) were manually defined using both the optical images and MS imaging data. [6]

Each series of quantitative measurements started and ended with measurement of standards to generate a calibration curve. In addition, QC samples were measured after each citalopram-dosed tissue section to assess the accuracy and precision of the known concentrations in the biological matrix. A deuterated analogue of citalopram (citalopram-d_6) was used as IS to normalize the citalopram signal in each pixel. Citalopram-d_6 **(Figure S1C)** was selected as IS as no interfering matrix or endogenous compound peaks were observed in the *m/z* 331.2 ±0.1 range **(Figure S4)**. Six methods **(Figure S2)** were tested for applying calibration standards and QC samples onto **(A-D)** or under **(E, F)** the tissue. The IS was applied as follows: **(A)** mixed with the matrix, **(B, E)** onto the tissue, **(C, F)** under the tissue, or **(D)** under the tissue with wet application (50 % acetonitrile, 0.2 % trifluoroacetic acid, in water) on top of the tissue. Results obtained with the MALDI-MSI protocol following these applications **(Figure S2)** were compared with those obtained by LC-MS/MS. The results obtained with methods **(B)**, **(B, manual)** and **(D)** did not differ significantly from the LC-MS/MS results **(Figure S3)**. Signal-to-noise (S/N) ratios of an ROI (striatum or thalamus) were calculated (by dividing mean signal intensities by their standard deviations [3]) and annotated **(Figure S4)**. The results showed that methods **(A)**, **(B)**, **(B, manual)** and **(E)** provided S/N ratios ≥ 3, with method **(B)** showing the highest ratios.

Hence, method **(B)** was subsequently used for applying the calibration standards, QC samples, IS, and MALDI matrix for MALDI-MSI analyses. Applying calibration standards with the chemical inkjet printer or by manual pipetting gave similar results. Since standards can be applied in smaller volumes by a chemical inkjet printer than by manual pipetting (the applied droplet diameter is smaller and can be applied with higher precision on small tissue structures), we decided to use this application method.

LC-MS/MS Sample Preparation Protocol Optimization. The sample preparation protocol for LC-MS/MS is described in the Experimental Section and is a combination of three previously published protocols. [7-9] The preparation protocols for calibration standards, QC samples, and brain tissue sections of mice administered citalopram *in vivo* are also described in **Note S3**. In the LC-MS/MS analysis of citalopram, citalopram-d_4 was used as the IS. The four deuterium atoms of this analog are localized on citalopram's aromatic ring and remained on the product ions formed in the MS/MS analysis **(Figure S1B)**. In addition, the product ions from the analyte and IS did not interfere with each other in the MS/MS analysis.

Volumetric Calibration of Pipettes. Three specific pipettes with specified pipette tips were used during the experiments: Pipetman P20, P200 and P1000 adjustable pipettes, with capacities up to 20, 200 and 1000 µL, respectively (Gilson Inc., Middleton, WI, USA). The pipette tips used were Gilson Diamond D200 and D1000, 200 and 1000 µL tips. The pipettes were calibrated for each liquid used (50 % b.w. methanol, 100 % methanol, 2 % methanol + 1 % acetic acid, and pure water).

Procedure for Gravimetric Measurement of Microcentrifuge Tubes. To measure condensed water vapor remaining on microcentrifuge tubes containing cut brain tissue, stored on dry ice (-78 °C), 12 dry, closed, empty microcentrifuge tubes were weighed on a Sartorius 1712 analytical scale (Sartorius AG, Göttingen, Germany). Each measurement was repeated three times and the mean weight and standard deviation were recorded. To simulate the sublimated water vapor, the tubes were put onto dry ice in a Styrofoam box for 1 hour. Each tube was taken out from the box, dried and cleaned with soft tissue paper and quickly measured on the analytical scale (Sartorius 1712, Sartorius AG, Göttingen, Germany).

Each tube was weighed and re-weighed three times following this procedure, and the mean calculated mass difference, assumed to equal the weight of residual moisture (mean ±SD, n = 12) 0.75 ±0.18 mg, was subtracted from every tissue weight measurement. Nine coronal brain-tissue sections were collected in each microcentrifuge tube, and the average weight of the tissue was (mean ±SD, n = 39) 6.32 ±0.49 mg.

Protocol for Volume Calibration of the Chemical Inkjet Printer. A requirement for accurate MALDI-MSI quantitation was deposition of expected volumes by the chemical inkjet printer. Gravimetric measurement of the volume was impossible because a 50 nL droplet of 50 % b.w. methanol has a large surface area to volume ratio and evaporates quickly in an ambient atmosphere at room temperature. Thus, we developed a nano-volume measurement protocol that was insensitive to liquid evaporation.

The chemical inkjet printer was set to deliver a volume of 50 nL (of 50 % b.w. methanol). Two dilution series of citalopram were prepared (for printer and manual addition) and added to a 96-well microtiter plate. Calibrated manual pipetting was used to obtain reference volumes for the printer volume determination. A fixed amount of IS (0.875 pmol citalopram-d_4) was added to each portion of citalopram solution.

The samples were then transferred to microcentrifuge tubes, dried in a Concentrator 5301 centrifugal evaporator (Eppendorf AG, Hamburg, Germany), reconstituted in 20 µL of 10 % acetonitrile and 0.25 % acetic acid, and vortex-mixed at maximum power for 10 s. The addition of 20 µL 0.25 % acetic acid was repeated twice.

Next, 2 µL of the sample was injected during each nano-LC run. The ratio of analyte to IS signal intensities was determined by LC-MS/MS. From the resulting standard curves, generated using the chemical inkjet printer and manual pipetting (used as a reference), the volume delivered by the printer was calculated (see detailed protocol in **Note S2**). If the set volume of the chemical inkjet printer was correct, the slope of the generated standard curve had the same slope as the manually pipetted reference standard curve. The slopes obtained from the standard curves generated from the manually pipetted reference standards and printer-deposited standards were 1.239 (R^2 = 0.999) and 1.143 (R^2 = 0.964), respectively. Since the set volume was 50.0 nL, the resulting calibrated volume was 46.1 nL according to equation (1).

$$\frac{Slope\ (cal.)}{Slope\ (ref.)} \times V_{set} = \frac{1.143}{1.239} \times 50.0 = 46.1\ \text{nL} \qquad (1)$$

Hence, this volume was used for all calculations pertaining to the validation and quantitation measurements.

Note S2. Determination of spotter volume by LC-MS/MS measurement.

Preparation before sample preparation

- Per sample, annotate two Eppendorf tubes.
- 96-well microtiter plate for Shimadzu CHIP-1000 spotter.
- 20 µL manual pipette.

Liquids to prepare

- 20 µM CIT solution in 50 % b.w. MeOH (analyte) – [20 pmol/µL].
- 100 nM CIT solution in 50 % b.w. MeOH (analyte) – [100 fmol/µL].
- 50 nM CIT-d_4 solution in 50 % b.w. MeOH (internal standard) – [50 fmol/µL].
- 50 % b.w. MeOH.
- 10 % ACN, 0.25 % HAc.
- 0.25 % HAc.

Volume preparation

1.	Dilute portions of the 20 µM CIT solution to 10 and 5 µM CIT. From these, prepare a 15 µM CIT solution. From the 10 and 5 µM solutions, prepare a 7.5 µM CIT solution. This dilution series is for the CHIP spotter.
2.	Dilute portions of the 100 nM CIT solution to 50 and 25 nM CIT. From the 100 and 50 nM solutions, prepare a 75 nM CIT solution. From the 50 and 25 nM solutions, prepare a 37.5 nM CIT solution. This dilution series is for the manual spotting.
3.	Mount the 96-well microtiter plate in the CHIP-1000 spotter.
4.	Add (spot) 50 nL portions of the 5, 7.5, 10, 15 and 20 µM CIT solutions (0.375, 0.5, 0.75 and 1 pmol) in separate wells using the CHIP spotter, with at least 3 wells per concentration.
5.	Manually add 20 µL of the dilution series for manual spotting in wells, with at least 3 replicates per concentration.
6.	Manually add 20 µL of the internal standard (50 nM CIT-d_4) to each spotted well (both manually and CHIP spotted).
7.	Add 200 µL 50 % b.w. MeOH to each well and put the microtiter plate on a shaker for 10 min.
8.	Transfer the liquid to an Eppendorf tube.
9.	Repeat steps 7 and 8 twice more so that the total liquid volume is about 600 µL.
10.	Dry the liquid in a speedvac (for 30 - 75 min) at ambient temperature.
11.	Reconstitute the sample by adding 20 µL of 10 % ACN, 0.25 % HAc.
12.	Vortex-mix the solution at maximum power and centrifuge the tube.
13.	Add another 20 µL of 0.25 % HAc.
14.	Vortex-mix at maximum power and centrifuge the tube again.
15.	Inject and analyze 2 µL sample on the EASY-nLC coupled to LTQ mass spectrometer. Citalo_20min_2ul, Cita_MRM_3frag_20min.meth

Abbreviations

ACN – acetonitrile
b.w. – by weight
CIT – citalopram
CIT-d_4 – citalopram-d_4
HAc – acetic acid
MeOH – methanol

Note S3. LC-MS/MS sample preparation of standards, QC samples, and dosed tissue for citalopram quantitation.

Preparation before standard sample preparation

- Per sample, annotate two Eppendorf tubes with sample number plus A and B. Annotate a 10 kDa MWCO filter plus filter holder with sample number and C.
- Weigh Eppendorf tube (A) on an analytical scale and place at -20 °C.
- Styrofoam box with dry ice.
- Styrofoam box with ice.

Liquids to prepare

Working stock solutions:

- Standard curve: 1.6, 3.1, 12.5, 25, 50, 75, 100, 150 and 200 µM CIT together with 100 µM CIT-d_4 in 100 % MeOH.
- LLOQ, LQC, MQC and HQC: 0.8, 2.3, 75 and 150 µM CIT together with 100 µM CIT-d_4 in 100 % MeOH.
- Extraction of brain tissues from animals administered CIT *in vivo*: 100 µM CIT-d_4 in 100 % MeOH.

Other solutions and chemicals:

- 1 % HAc with 2 % working stock solution.
- NaCl (solid).
- 100 % ACN.
- 10 % ACN, 0.25 % HAc.
- 0.25 % HAc.

Sample prep

1.	Section control/vehicle brain tissue on cryomicrotome, -20 °C, up to a mass of 8 mg (equal to 5 - 10 sagittal or coronal mouse brain sections, 14 µm thick)**.**
2.	Transfer tissue to -20 °C cooled tube **(A)**. Check that tissue is at the bottom of the tube. Close the lid and put the tube on dry ice.
3.	Weigh tube **(A)** with the tissue.
4.	Add ice-cooled 1 % HAc with 2 % working stock solution [25 µL/mg tissue] and 10 mg NaCl/mg† tissue to tube **(A).**
5.	Add ice cooled ACN [50 µL/mg tissue] to tube **(A).**
6.	Sonicate until tissue is homogenized. This step is very important and needs enough time to be sure to eliminate all the brain residues.
7.	Centrifuge tube **(A)** @ rcf 14,000 for 15 - 30 min at 4 °C.
8.	Transfer the organic phase from tube **(A)** to a new Eppendorf tube **(B).**
9.	Add 200 µL ACN a second time to tube **(A).**
10.	Vortex-mix solution in tube **(A)** at maximum power for 1 min.
11.	Centrifuge tube **(A)** in a table centrifuge for 2 min.
12.	Transfer organic phase from tube **(A)** to tube **(B).**
13.	Add 200 µL ACN a third time to Eppendorf tube **(A).**
14.	Vortex-mix solution in tube **(A)** at maximum power for 1 min.
15.	Centrifuge tube **(A)** in a table centrifuge for 2 min.
16.	Transfer organic phase in tube **(A)** to tube **(B).**
17.	Dry the organic liquid in tube **(B)** containing CIT in a speedvac for 30 - 75 min at ambient temperature.
18.	Reconstitute the sample by adding 10 % ACN, 0.25 % HAc [25 µL/mg tissue] in tube **(B).**
19.	Vortex-mix the solution in tube **(B)** at maximum power for 1 min.
20.	Transfer the whole volume from tube **(B)** to a 10 kDa MWCO filter with filter holder **(C).**
21.	Add 0.25 % HAc [25 µL/mg tissue] to tube **(B).**
22.	Vortex-mix solution in tube **(B)** at maximum power for 1 min.
23.	Transfer the whole volume from tube **(B)** to 10 kDa MWCO filter with filter holder **(C).**
24.	Filter the liquid @ rcf 14,000 for 60 min at 4 °C.
25.	Transfer 10 µL of the filtrate to insert for LC-MS/MS analysis.
26.	Freeze the sample in -20 °C freezer for later analysis.
27.	Inject 1 µL sample on the EASY-nLC -LTQ. Citalo_20min_1ul, Cita_MRM_3frag_20min.meth

Abbreviations

ACN – acetonitrile
CIT – citalopram
CIT-d_4 – citalopram-d_4
HQC – high concentration QC sample
kDa – kilo Dalton
HAc – acetic acid
LLOQ – lower level of quantitation
LQC – low concentration QC sample
MeOH – methanol
MWCO – molecular weight cut-off
MQC – medium concentration QC sample
NaCl – sodium chloride
rcf – relative centrifugation force

† The amount of NaCl is in excess and must be added quickly. Measure weight of NaCl on a spatula, e.g., if a spatula scoop provides 25 mg NaCl and mass of tissue is 5 mg, add two scoops of NaCl with the spatula.

Note S4. LC and MS/MS settings.

LC Settings

Instrument Meth: Citalo_20min_1ul
Citalo_20min_2ul

Buffer A: water, 0.1 % formic acid (FA)
Buffer B: 100 % ACN, 0.1 % FA
Sample pump buffer: Same as buffer A

Precolumn equilibration
Volume: 12.00 µL
Max. pressure: 200 bar
Liquid: Buffer A

Analytical column equilibration
Volume: 3.00 µL
Max. pressure: 200 bar
Liquid: Buffer A

Sample loading (*total volume to transfer the sample from sample loop to precolumn*)
Volume: 20.00 µL
Max. pressure: 250 bar

Sample pickup (*to the sample loop*)
Volume: 1.00 or 2.00 µL
Flow: 20.00 µL/min

Sample loading (*total volume to transfer the sample from sample loop to precolumn*)
Volume: 20.00 µL
Max. pressure: 250 bar

Gradient

Time	Duration	Flow [nL/min]	%B
00:00	N/A	250	2
20:00	20:00	250	55
22:00	02:00	250	100
32:00	10:00	250	100

Autosampler wash (custom wash)

Order	Source	Volume [µL]	Cycles
1	2	250	2
2	1	250	55
3	3	250	100

Source 1: 50 % isopropanol (IPA)
Source 2: 80 % ACN
Source 3: 0.25 % HAc

MS/MS Settings

Instrument Meth:
Cita_MRM_3frag_20min.meth
LTQ Instrument Method

MS Run Time (min): 20.00
Sequence override of method parameters: not enabled
Divert Valve: not used during run
Contact Closure: not used during run
Syringe Pump: not used during run
MS Detector Settings
Real-time modifications to method: disabled
Stepped collision energy: not enabled

Additional Microscans:

MS2	0	0
MS3	0	0
MS4	0	0
MS5	0	0
MS6	0	0
MS7	0	0
MS8	0	0
MS9	0	0
MS10	0	0

Segment 1 Information

Duration (min): 20.00
Number of Scan Events: 2
Tune Method: TuneFile
Scan Event Details
1: ITMS + c norm · (325.17)->oS
(260.6-263.6 278.6-281.6 305.6-308.6)
MS/MS: AT CID CE 35.0 % Q 0.250
Time 30.000 IsoW 2.0
CV = 0.0V

2: ITMS + c norm · (329.20)->oS
(264.6-267.6 282.5-285.5 309.5-312.5)
MS/MS: AT CID CE 35.0 % Q 0.250
Time 30.000 IsoW 2.0
CV = 0.0V
Custom Data Dependent Settings: not enabled

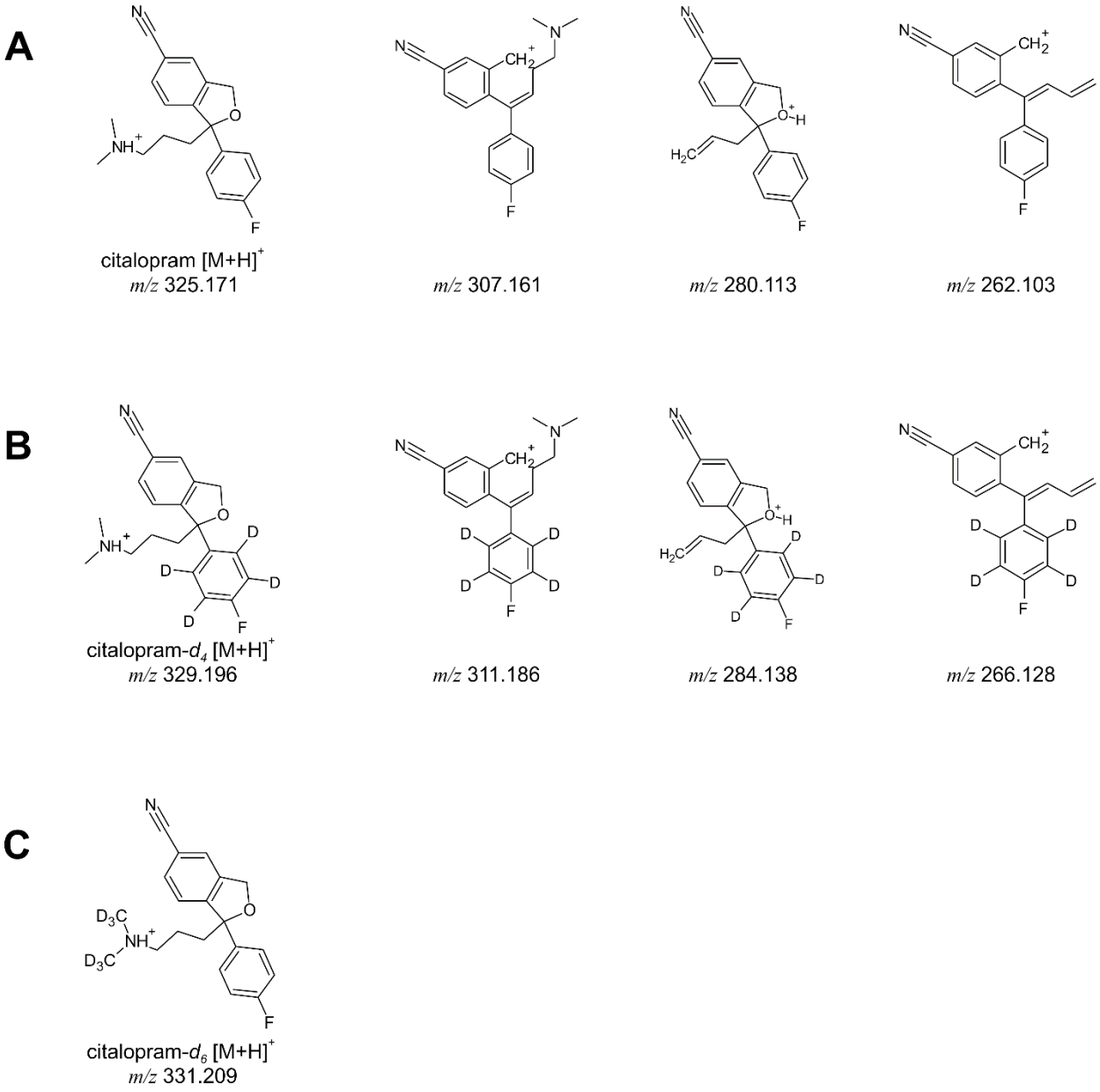

Figure S1. Chemical structures of citalopram and internal standards (citalopram-d_4 and citalopram-d_6) used for method validation.

(A) In the LC-MS/MS study, the molecular ion (*m/z* 325.171) was set as the citalopram precursor ion, the three main product ions (*m/z* 307.161, 280.113 and 262.103) generated by collision-induced dissociation were monitored, and the chemical structure of each product ion was confirmed. [10] **(B)** Three main product ions (*m/z* 311.186, 284.138 and 266.128) were also produced from the main precursor ion of citalopram-d_4 (*m/z* 329.196). **(C)** The internal standard citalopram-d_6 (*m/z* 331.209) was used for MALDI-MSI analyses.

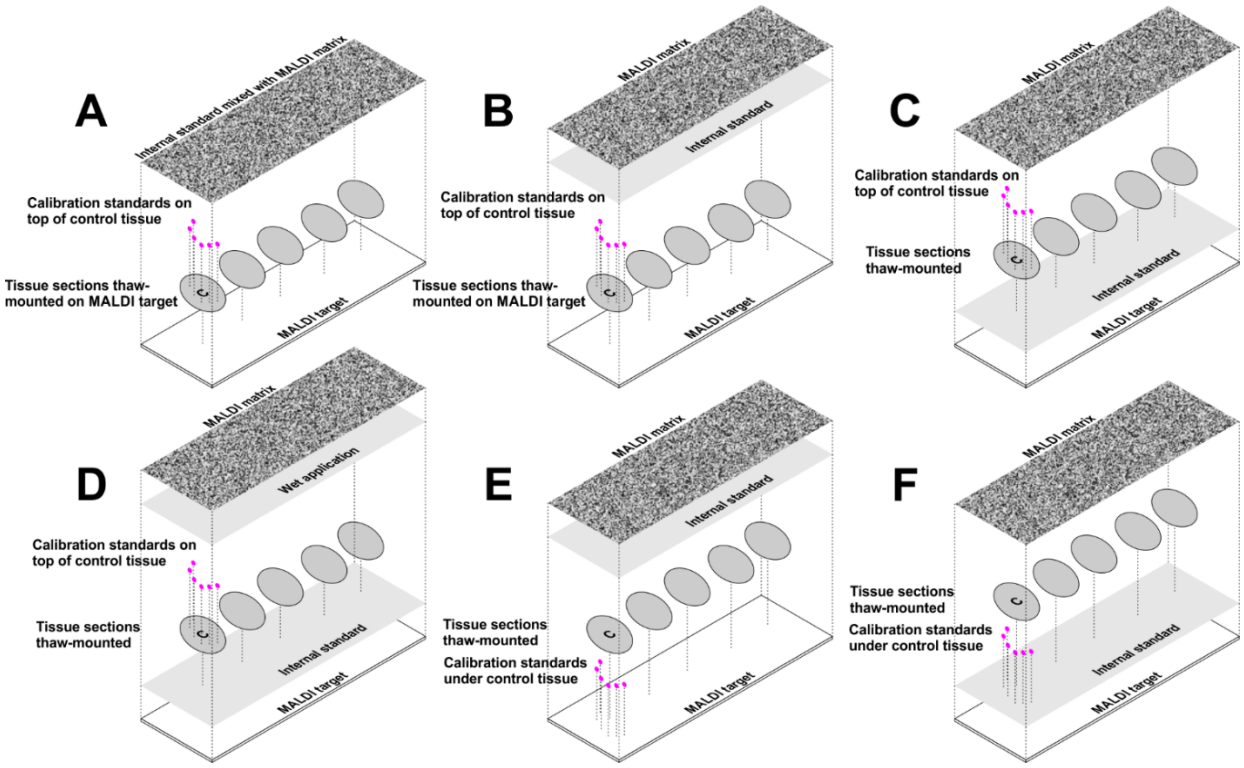

Figure S2. Investigation of optimal protocols for applying calibration and internal standards.

(A) Calibration standards (for generating a standard curve) applied on top of the tissue and internal standard (IS) mixed with the MALDI matrix. **(B)** Calibration standards and IS applied on top of the tissue before matrix application. **(C)** Calibration standards applied on top of the tissue and IS under the tissue (sprayed on the glass slide before applying the tissue) prior to matrix application. **(D)** Calibration standards applied on top of the tissue and IS under the tissue with wet application of matrix on the tissue. **(E)** Calibration standards applied under the tissue (applied onto the glass slide before the tissue) and IS applied on top of the tissue before matrix application. **(F)** Calibration standards and IS applied under the tissue and matrix applied on top of the tissue.

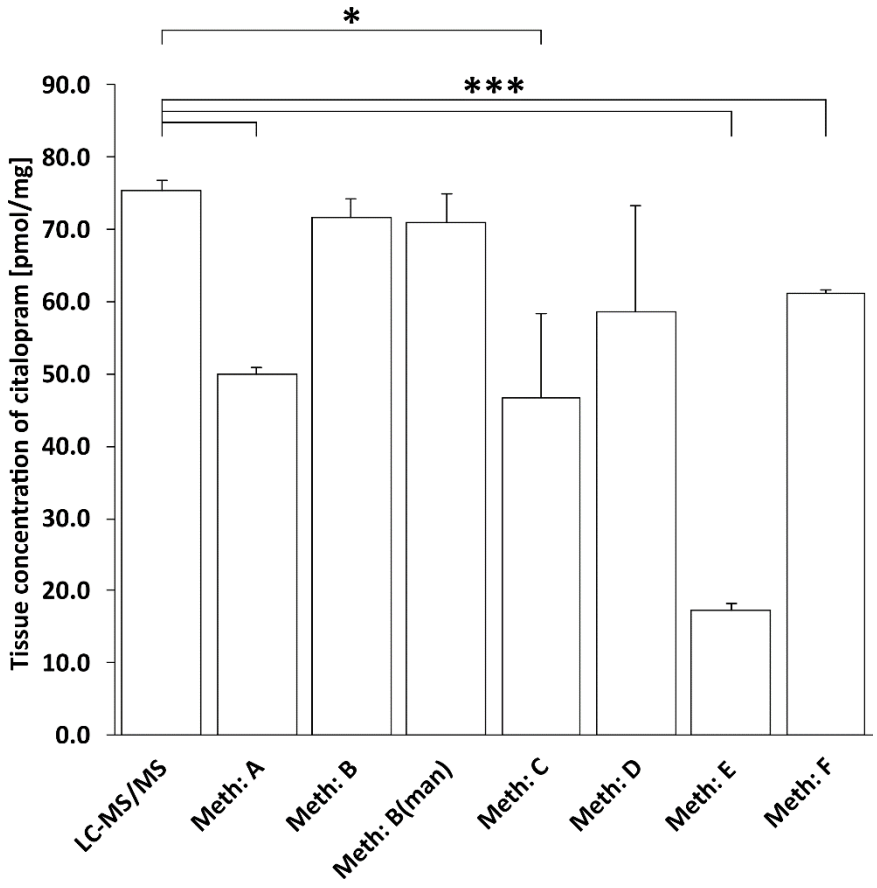

Figure S3. Citalopram concentrations determined by MALDI-MSI using the indicated application protocols and reference LC-MS/MS method.

Bar chart showing average tissue concentrations of citalopram obtained from coronal tissue sections (distance from bregma 0.20 to -1.4 mm), [11] by MALDI-MSI following different preparation protocols **(A-F)** described in **Figure S2** and LC-MS/MS. The quantitation results (pmol/mg mean ±SD, $n = 3$) were as follows: LC-MS/MS 75.4 ±1.1, method **(A)** 50.0 ±0.9 ($p < 0.001$), method **(B)** 71.6 ±2.6 ($p = 0.11$), method **(B**, manual) 71.0 ±3.9 ($p = 0.18$), method **(C)** 46.8 ±11.5 ($p < 0.05$), method **(D)** 58.5 ±14.7 ($p = 0.18$), method **(E)** 17.4 ±0.9 ($p < 0.001$), and method **(F)** 61.2 ±0.4 ($p < 0.001$). The asterisks indicate significant differences compared to results obtained with the LC-MS/MS reference method according to two-tailed *t*-tests with unequal variances: *$p < 0.05$, ***$p < 0.001$.

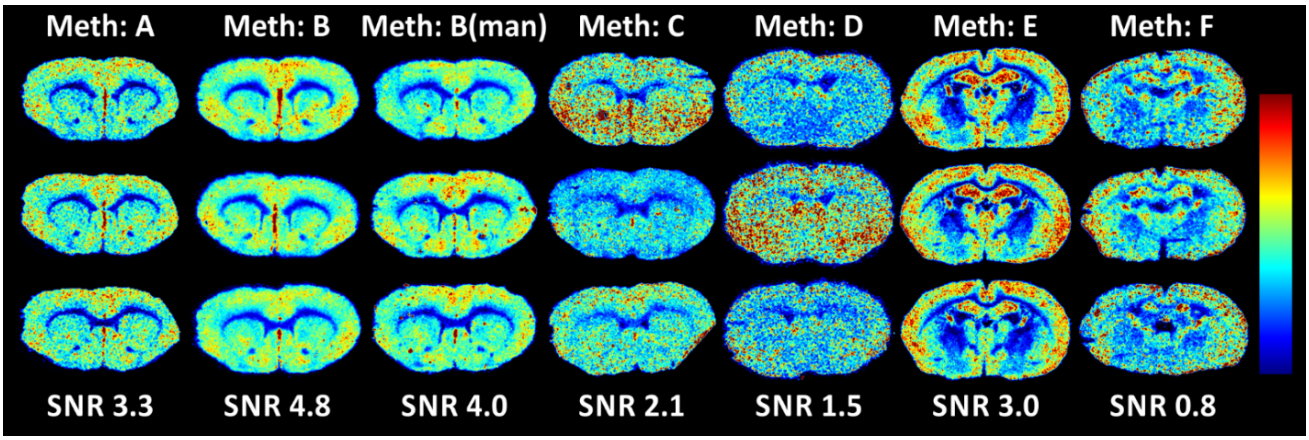

Figure S4. MALDI-MS images of citalopram distributions in brain sections following the indicated methods for applying the internal standard.

Ion distribution images of citalopram $[M+H]^+$ (*m/z* 325.2) normalized with respect to the internal standard, citalopram-d_6 $[M+H]^+$ (*m/z* 331.2) obtained from coronal tissue sections (distance from bregma, 0.20 to -1.4 mm). [11] IS was applied by methods **(A-F)** described in **Figure S2**. All images are scaled to the same maximum intensity. Signal-to-noise ratios (SNR) were calculated by dividing mean values of intensities of IS normalized citalopram signals in subregions of either the striatum or thalamus by their standard deviations of the noise [12-15].

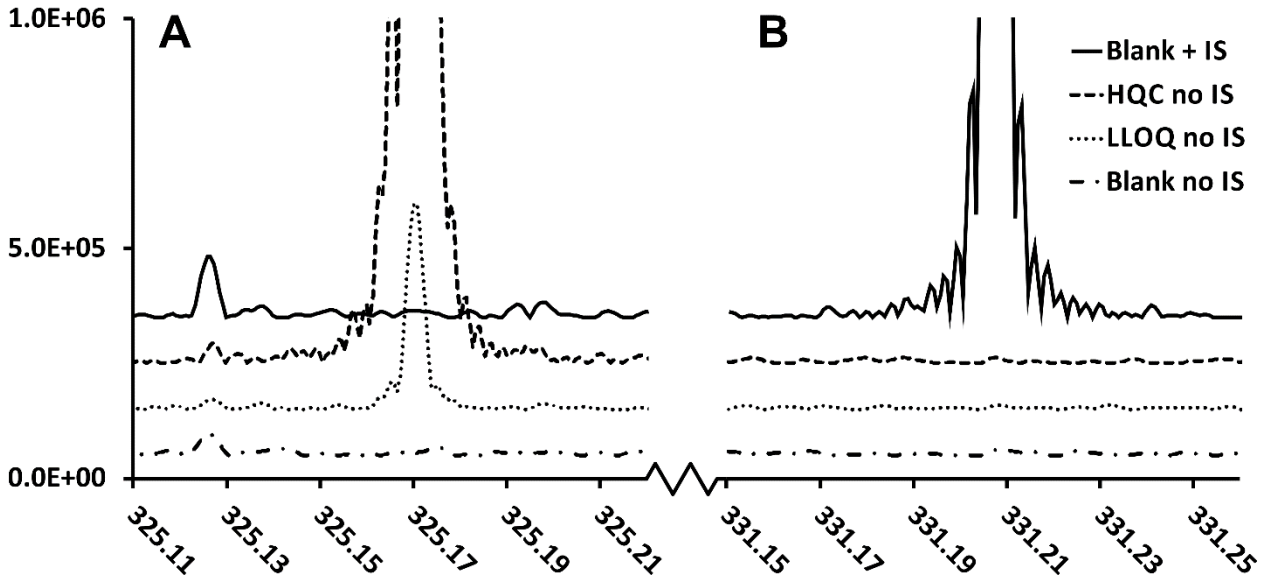

Figure S5. Investigation of citalopram and IS (citalopram-d_6) MSI peak interference.

Peak interference between citalopram $[M+H]^+$ (*m/z* 325.1711) and citalopram-d_6 $[M+H]^+$ (*m/z* 331.2087) illustrated by comparison of four mass spectra from FTICR-MSI analysis. **(A, B)** Mass spectra acquired from ROIs containing blank and IS (solid line); the highest quality control (HQC) concentration and no IS (dashed line); and LLOQ with no IS (dotted line); and blank and no IS (dash-dotted line). The four spectra are baseline separated by 1×10^5 au to avoid overlapping. The intensity axis is truncated at 1×10^6 au to visualize baseline levels. **(A)** There were no interfering peaks from IS or endogenous compounds at the mono-isotopic mass for citalopram. The citalopram peak intensity (HQC) was 7.4×10^6 au. The intensity from the citalopram peak (LLOQ) was 4.5×10^5 au, and the intensity from the blank with no IS background was 1.2×10^4 au (the selectivity, i.e., the LLOQ and blank sample ratio was estimated to 38). **(B)** Citalopram-d_6 was selected as the IS since there was no interference with its peak from citalopram or endogenous compounds. The IS peak intensity was 6.3×10^6 au.

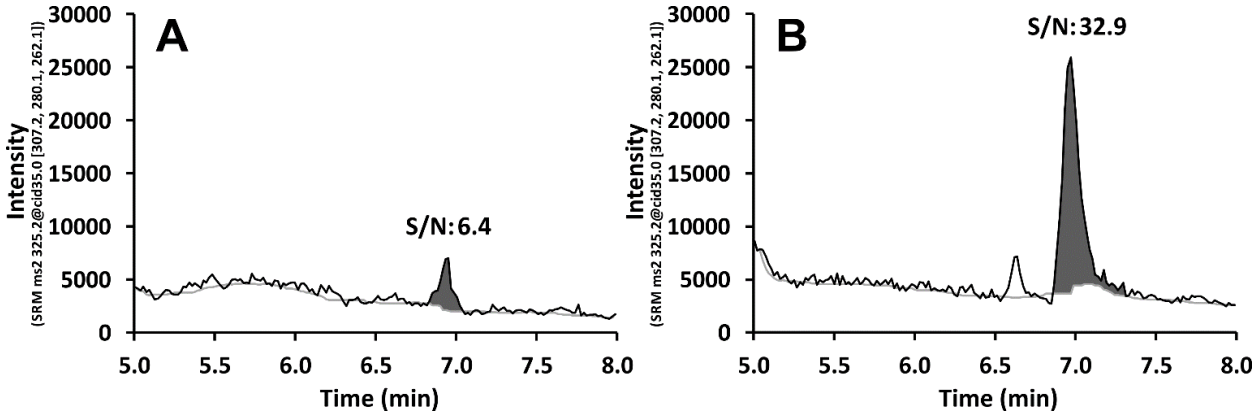

Figure S6. Ion chromatogram from LC-MS/MS analysis showing results of the selectivity test (blank sample *vs*. citalopram at the lowest level of quantitation, LLOQ).

Citalopram (*m/z* 325.17) was eluted at 6.96 min and the ion intensity is displayed as the total intensity of the product ions, *m/z* 307.2, 280.1 and 262.1. **(A)** Typical ion chromatogram of a blank sample. The peak at 6.95 min is due to some carry over effect. **(B)** The signal at LLOQ was more than five times stronger than the signal from the blank sample.

Table S1. Accuracy, precision, and coefficient of determination of citalopram measurements obtained by MALDI-MSI and LC-MS/MS analysis as part of quality control (QC) validation.

			MALDI-TOF-MSI			MALDI-FTICR-MSI			LC-MS/MS
			Slide Q1	Slide Q2	Slide Q3	Slide Q4	Slide Q5	Slide Q6	
Accuracy (*n* = 3)									
LLOQ	(≤ ±20 %)	[%]	6.7	10.9	(23.9)	-3.7	15.5	5.9	-4.8
LQC	(≤ ±15 %)	[%]	-7.5	3.7	0.8	-10.1	3.5	1.1	10.5
MQC	(≤ ±15 %)	[%]	(-17.9)	2.7	-0.3	-1.0	-2.0	-3.9	-1.4
HQC	(≤ ±15 %)	[%]	-1.1	(-16.3)	12.0	-2.9	5.9	-5.2	-0.1
Precision (*n* = 3)									
LLOQ	(≤ 20 %)	[%]	3.2	14.3	6.8	9.0	6.7	17.7	11.8
LQC	(≤ 15 %)	[%]	10.1	2.9	2.7	7.1	0.7	1.1	6.8
MQC	(≤ 15 %)	[%]	12.6	(18.3)	6.5	3.4	0.3	5.6	2.2
HQC	(≤ 15 %)	[%]	12.5	13.4	9.2	2.9	0.5	2.9	1.1
Coefficient of determination									
R^2			0.982	0.986	0.966	0.998	0.986	0.995	0.999

QC measurements were obtained for each analyzed slide, including the coefficient of determination of the standard curve. QC values outside the limits for a validated method (shown in parentheses) were only obtained from MALDI-TOF-MSI analyses.

Table S2. Cross-validation of MALDI-TOF-MSI, MALDI-FTICR-MSI, and LC-MS/MS quantitation.

	Animal D1	Animal D2	Animal D3	Animal D4	Animal D5	Animal D6
MALDI-TOF-MSI						
Slide Q1 [pmol/mg]	47.1	44.6	50.9	44.4	48.8	38.8
Slide Q2 [pmol/mg]	63.0	58.1	58.2	58.9	58.6	47.6
Slide Q3 [pmol/mg]	52.3	49.8	50.6	51.1	51.4	40.7
Average	54.1	50.8	53.2	51.5	52.9	42.4
St. dev.	8.1	6.8	4.3	7.3	5.1	4.6
MALDI-FTICR-MSI						
Slide Q4 [pmol/mg]	66.2	63.6	60.9	65.9	63.3	47.1
Slide Q5 [pmol/mg]	57.4	56.3	57.8	56.3	47.0	45.5
Slide Q6 [pmol/mg]	58.6	53.6	59.0	56.7	57.9	42.5
Average	60.7	57.8	59.2	59.6	56.1	45.0
St. dev.	4.8	5.2	1.6	5.4	8.3	2.3
LC-MS/MS						
	53.0	60.3	58.9	54.5	55.6	45.9
Block 1 triplicate [pmol/mg]	54.9	60.0	59.2	55.0	56.2	46.8
	59.9	59.0	57.6	53.9	58.2	43.9
	59.7	60.0	58.7	54.2	55.2	46.4
Block 2 triplicate [pmol/mg]	52.2	59.3	58.8	53.0	55.8	46.3
	56.0	58.3	56.3	54.2	56.5	44.7
Average	55.9	59.5	58.3	54.1	56.3	45.7
St. dev.	3.3	0.8	1.1	0.7	1.1	1.1
***t*-test [p-value]**						
MALDI-TOF-MSI vs LC-MS/MS	0.739	0.156	0.177	0.593	0.372	0.346
MALDI-FTICR-MSI vs LC-MS/MS	0.218	0.635	0.409	0.221	0.971	0.695

Citalopram was quantified in coronal brain tissue sections (distance from bregma, 1.6 to -1.4 mm) [11] from six animals. Each animal was administered an intraperitoneal (i.p.) injection of 30 mg/kg S-citalopram. Amounts of the drug in sections from each animal ($n = 6$) on three glass slides were quantified and compared with amounts obtained using LC-MS/MS by two-tailed *t*-tests with unequal variances. No significant differences were found between results from the MALDI-MS methods and the reference LC-MS/MS method.

Table S3. Cross-validation of MALDI-TOF-MSI, MALDI-FTICR-MSI, and LC-MS/MS analyses of biological variance.

	Animal D1	Animal D2	Animal D3	Animal D4	Animal D5	Animal D6
MALDI-TOF-MSI [pmol/mg]	54.1	50.8	53.2	51.5	52.9	42.4
Average [pmol/mg]	51.1					
St. dev.	4.4					
MALDI-FTICR-MSI [pmol/mg]	60.7	57.8	59.2	59.6	56.1	45.0
Average [pmol/mg]	56.9					
St. dev.	6.0					
LC-MS/MS [pmol/mg]	55.9	59.5	58.3	54.1	56.3	45.7
Average [pmol/mg]	55.0					
St. dev.	4.9					
***t*-test [p-value]**						
MALDI-TOF-MSI vs LC-MS/MS	0.153					
MALDI-FTICR-MSI vs LC-MS/MS	0.648					

Amounts of citalopram in coronal brain tissue sections from six animals on three glass slides were quantified by MALDI-TOF-MSI and MALDI-FTICR-MSI, then compared to amounts obtained using LC-MS/MS. Mean intensity values were compared by two-tailed *t*-tests with unequal variances. No significant differences were found between results from the MALDI-MSI methods and the reference LC-MS/MS method.

Table S4. Results of tests of citalopram's long-term stability in brain tissue sections.

	Animal D1	Animal D2	Animal D3	Animal D4	Animal D5	Animal D6
MALDI-TOF-MSI						
Analyses dated February 2017						
Slide T-Q1 [pmol/mg]	54.6	53.5	49.3	40.7	48.6	35.0
Slide T-Q2 [pmol/mg]	54.3	49.4	51.3	50.5	47.1	41.9
Slide T-Q2 [pmol/mg]	57.5	59.1	51.9	55.4	52.3	34.5
Average	55.5	54.0	50.8	48.9	49.3	37.1
St. dev.	1.8	4.9	1.4	7.5	2.7	4.1
MALDI-TOF-MSI						
Analyses dated March 2018						
Slide Q1 [pmol/mg]	47.1	44.6	50.9	44.4	48.8	38.8
Slide Q2 [pmol/mg]	63.0	58.1	58.2	58.9	58.6	47.6
Slide Q2 [pmol/mg]	52.3	49.8	50.6	51.1	51.4	40.7
Average	54.1	50.8	53.2	51.5	52.9	42.4
St. dev.	8.1	6.8	4.3	7.3	5.1	4.6
***t*-test [p-value]**						
Feb-2017 vs Mar-2018	0.802	0.550	0.447	0.686	0.360	0.217

Citalopram in coronal brain tissue sections from six dosed mice was quantified by MALDI-TOF-MSI twice at a 13-month interval (February 2017 and March 2018). A two-tailed *t*-test with unequal variance detected no significant differences between measured concentrations at the two time points.

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