Supporting Information

Space-resolved tissue analysis by solid phase microextraction coupled to high resolution mass spectrometry via desorption electrospray ionization

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1. Chemicals and materials

MS-grade solvents: methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), and water were purchased from Fisher Scientific. MS-grade formic acid, polyacrylonitrile (PAN), and N,N-dimethylformamide (DMF) were purchased form Millipore Sigma (Oakville, ON, Canada), as were the components that were needed to prepare the 1 M phosphate buffered saline solution (PBS; pH 7.4): sodium chloride, potassium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate. The standards of the pharmaceuticals and drugs of abuse used in this study and their deuterated isotopologues, which were used as internal standards (IS), were purchased from Cerilliant Corporation (Round Rock, TX, USA). The target analytes and their most relevant properties are listed in table T1 in the Supporting Information. Working solutions were prepared by mixing the analytes in ACN/MeOH 1:1. The stock and working solutions were stored at -80 °C. SPME fibers were manufactured in house using a dip-coating procedure reported elsewhere.[1] A supporting nitinol wire (SE508 alloy, 198 µm diameter, Confluent Medical, Fremont, CA, USA) was coated with hydrophilic-lipophilic balance (HLB) polymer particles to create a biocompatible extraction phase with a total thickness of 27.5 ± 2.5 µm and a length of 4 mm or 15 mm. The monodisperse Oasis HLB particles (5 µm diameter) were kindly provided by Waters Corporation. The agar used to prepare gel models used in this study was purchased from BioShop (Burlington, ON, Canada).

2. Target analytes

Table S-1: Summary of drugs and pharmaceuticals targeted in this study, including their corresponding internal standards. Table shows hydrophilicity, expressed as logP, as well as dissociation constants, and monitored Tof-MRM transitions.

Compound	logP*	pKa**	Precursor ion [m/z]	Fragment ion [m/z]	Collision energy [eV]
Benzoylecgonine	1.71	9.54	290	168.1025	18
Citalopram	3.58	9.78	325	109.0448	27
Clenbuterol	2.94	9.63	277	203.0159	15
Cocaine	1.97	8.85	304	182.1189	18
Cocaethylene	2.53	8.77	318	196.1372	20
Fluoxetine	4.09	9.8	310	148.1126	8
Propranolol	3.03	9.67	260	116.1084	17
Benzoylecgonine-D3	n/r		293	171.1126	18
Citalopram-D6	n/r		331	109.0448	25
Clenbuterol-D9	n/r		286	204.0223	17
Cocaine-D3	n/r		307	185.1394	19
Cocaethylene-D3	n/r		321	85.0839	27
Fluoxetine-D6	n/r		316	154.1513	8
Propranolol-D7	n/r		267	116.1062	17

*values from ALOPPS 2.1; ** basic pKa; values from DrugBank (version 5.1.1); n/r- not relevant

3. Sampling procedure

SPME probes were preconditioned in MeOH/H_2O 1:1 for 1 h prior to extraction. Following preconditioning, internal standards were per-loaded onto the probes by extracting the deuterated compounds from 600 µL of water spiked at 250 ng/mL. For convenience, all probes used in the experiments were pre-loaded overnight (≈ 15 h), dried, and subsequently stored in the freezer at -80°C until use. The samples were spiked with the appropriate amounts of analytes, while ensuring that the total amount of organic solvent never exceeded 1% (v/v) of the matrix's total volume. SPME probes were inserted directly into the sample and extraction was carried out for 8 min in static mode. The probes were quickly withdrawn, wiped with a lint-free tissue to remove any loosely attached matrix, and rinsed by vortexing in ultrapure water for 3 s. The probes were then analysed as quickly as possible via DESI-MS to avoid any analyte diffusion within the SPME coating. Although freezing is hypothesized to slow down the diffusion process across the coating, evaluation of the effect of storage stability was outside of the scope of this study and will be investigated in the future.

The agar gel was prepared by dissolving agar in PBS buffer (2%, w/v) at elevated temperature. After cooling the gel to ≈50°C it was spiked with analytes, vortexed, sonicated to remove any air bubbles, and left to set at room temperature.

Following preliminary evaluation of the carry-over of analytes between the gel layers induced by the physical force of the fiber insertion (data not shown) it was concluded that transfer of analytes does not occur at high speed of fiber introduction. Therefore, during all experiments the SPME fibers were inserted into and withdrawn from the model samples by one fast, confident movement.

4. SPME-DESI-MS interface and SPME probe holder assembly

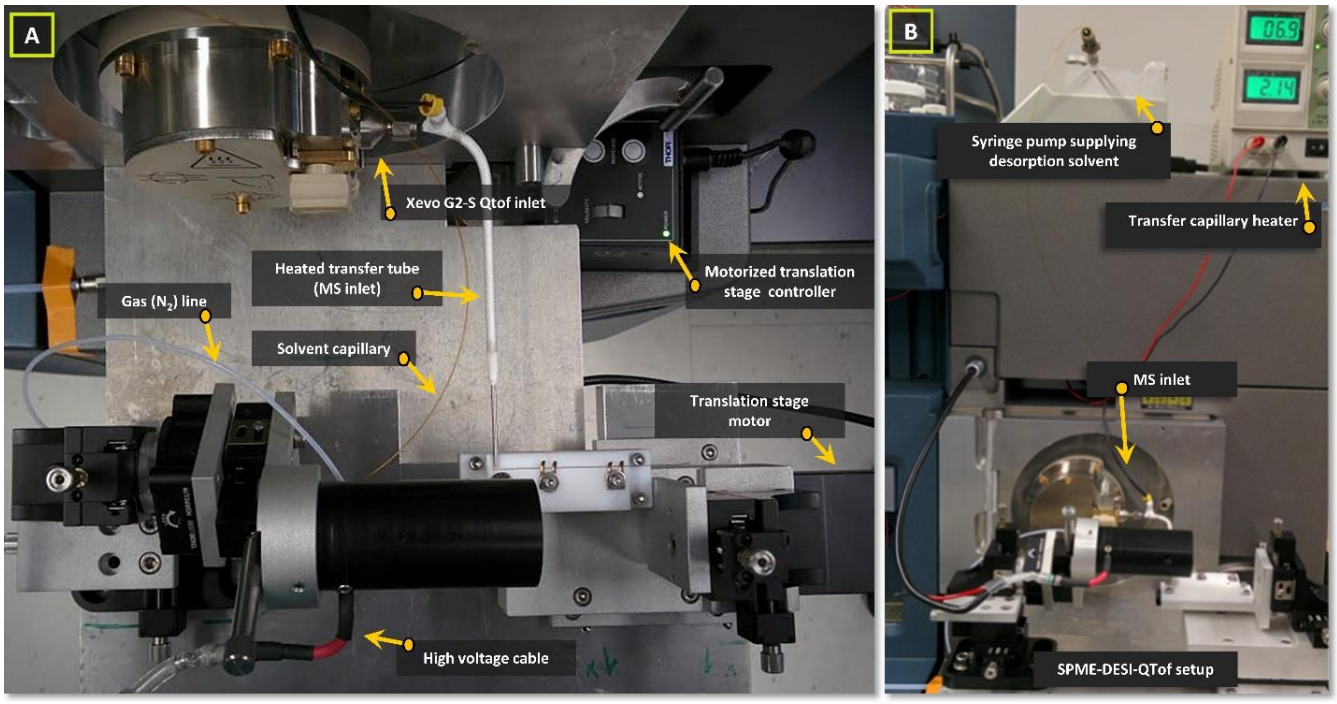

Figure S-1. In-house-built SPME-DESI interface designed for the Xevo G2-S mass spectrometer. A) view of the SPME-DESI source from above with all main elements labelled; B) view of the SPME-DESI source mounted on the MS showing the solvent supply system and external transfer tube heater.

The steps undertaken to improve the signal's stability and intensity, and to maximize the spatial resolution included optimization of the desorption solvent flow rate, positioning and distance between the sprayer tip and the SPME probe, and the distance between the SPME probe and the MS inlet. The parameters were tested with changes in the LeuEnk signal being observed and acquired in real time.

Table S-2. Experimental conditions used for SPME-DESI-MS/MS.

Xevo G2-S QTof MS	Spray voltage	3 kV
	Cone voltage	40 V
	Source offset	80 V
	Heated capillary temperature	250 ˚C
	Source block temperature	100 ˚C
	Acquisition mode	Sensitivity; MS/MS (Tof MRM)
	Scan time	250 ms
	Mass range	70-400 m/z
	Mass resolution	22000
	Acquisition time	5 min
	LockMass acquisition	LeuEnk (fragment m/z 120.0813); scan time 300 ms; interval 5 s; 3 scans to average
	Mass calibration	0.5 mM sodium formate in MS/MS mode between 70-400 m/z (weekly); real-time correction with LockMass (LeuEnk included in desorption solvent)
DESI source	Nebulizing gas pressure	100 psi
	Solvent flow rate	3 µL min-1
	Spray solvent	methanol/water (95:5, v/v) + 0.1% FA + 5 ppm LeuEnk
	Tip-to-surface height	2 mm
	Distance inlet-sample	2 mm
	Angle tip-sample	45 °
	Angle inlet	10 °
	Distance tip-inlet	4.5 mm
	Fiber scanning speed	50 µm/s
SPME fibers	Extracting phase	Hydrophilic-lipophilic balance (HLB)
	Coating length	4 mm or 15 mm
	Coating thickness	27.5 µm ± 2.5 µm
	Max. probe diameter	255 µm ± 5 µm

The holder assembly guaranteed that the probe would remain stable and secure during desorption and analysis. This was achieved by encapsulating the non-coated portion of the SPME probe inside of a two-piece polytetrafluoroethylene (PTFE) block that was held together with two screws. To provide further stability, the probe was inserted into a tight-fitting sleeve, which fits into a groove that had been carved inside of the PTFE block. Both of these measures (along with the stopper located at the front end of the holder assembly) successfully restricted the probe's freedom of movement during the analysis (especially against vibrations produced by gas blow). Moreover, the holder assembly was equipped with a lower-level segment located below the level on which the coated probe was placed in order to support the heated transfer capillary. Seating the transfer capillary on this flat, smooth surface during the movement of the whole holder assembly proved to be an effective solution to issues associated with baseline signal instability. This combination of solutions ensured that fiber placement was reproducible, and, as there was only one possible and correct way to place the probe in front of the MS interface, it also ensured minimal sensitivity to inter-operator variability. The SPME probes were fixed in position by simply inserting their non-coated side (back-loading) into the tight-fitting sleeve. The holder assembly then positioned the coating orthogonally in front of the MS transfer capillary.

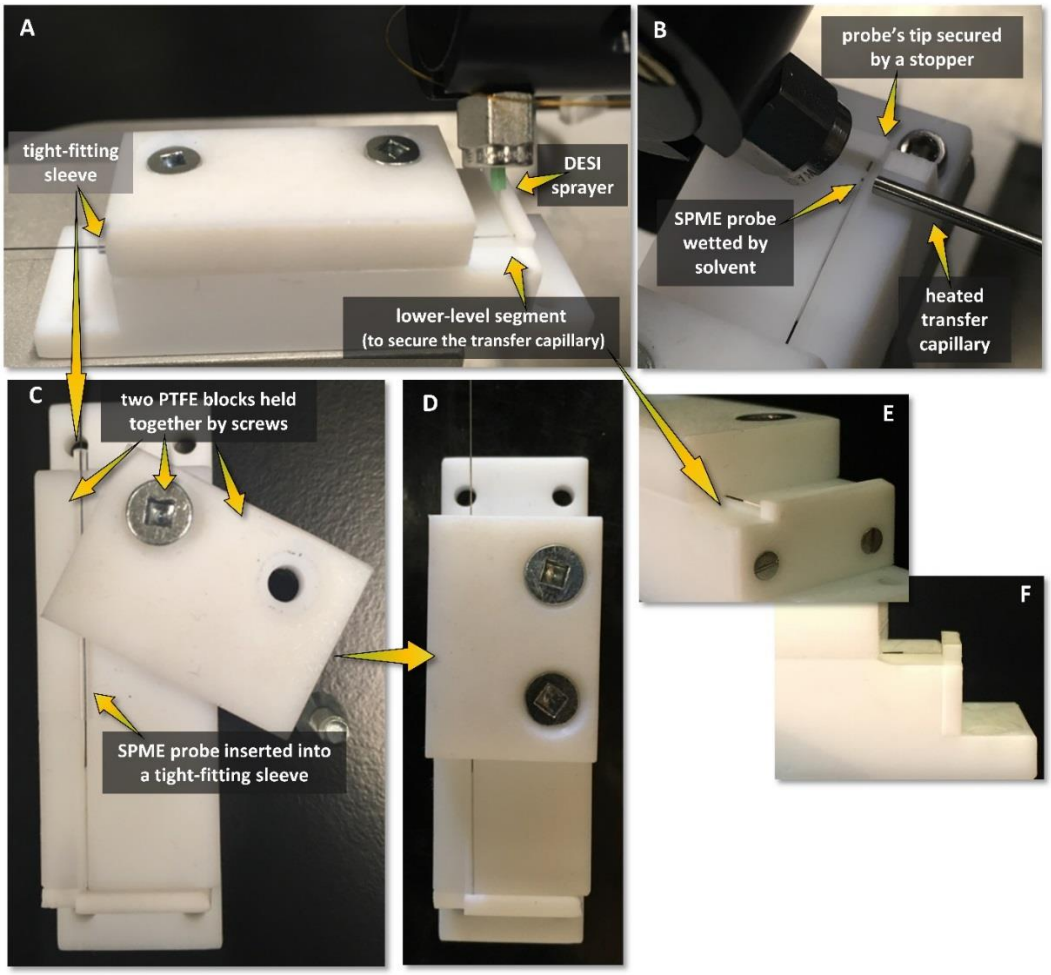

Figure S-2. SPME probe holder assembly. A) holder capable of accommodating 4 mm coated probes; B) 15 mm coated probe being analyzed. This image shows the alignment of the spray plume with the SPME probe and the heated transfer capillary; C) partially disassembled holder assembly ready for probe replacement; D) SPME probe secured in position inside of the holder assembly; E) and F) detailed view of the lower-level segment, which was carved into a PTFE block in order to seat the extended MS inlet (heated transfer tube) in an appropriate position for ion transfer.

5. SPME probes manufacturing and selection of the most appropriate coating thickness

The SPME probes used in this study were manufactured utilizing a dip-coating technique reported elsewhere.[2] The 5 µm Oasis HLB particle suspension was prepared using a ratio of 10% particles in PAN-DMF binder (7% PAN in DMF, *w/v*). In accordance with the fundamentals of dip-coating, the thickness of the deposited layer is proportional (among several other parameters) to the speed at which the supporting material was withdrawn from the suspension.[3] In this study, the ratio of particles to the binder and the suspension's viscosity (which also influence the coating thickness) were kept constant. Thus, the coating's thickness was varied by simply changing the withdrawal speed and the number of deposited layers.

The use of thin and uniform SPME coatings has been critical to achieving fast compound extraction, as well as rapid and efficient desorption/ionization by DESI. The latter is particularly important for enhancing sensitivity, which may be inherently diminished as a result of SPME-DESI coupling (for example, in some cases less than one percent of analyte amount is extracted, several percent of that fraction is then desorbed per unit of time, and no more than a small fraction of the desorbed analyte is then effectively

moved into the gas phase, ionized, and introduced into the MS^4).

In order to select a coating thickness that would ensure fast and efficient desorption upon contact with the solvent deposited by DESI sprayer and good signal reproducibility, three sets of probes were compared based on the extraction of a model compound: fluoxetine. The IS (fluoxetine-D6) was preloaded onto the SPME probes by performing extractions from 1 mL of water spiked at 500 ng/mL for 30 min in static mode. The extraction of fluoxetine was carried out for 8 min in static mode from PBS spiked at 1 µg/mL. As described in main text, an extraction time of 8 min was selected to ensure that sufficient amounts of analytes were extracted, while keeping the diffusion path length below 1 mm.[5] After a quick rinse with H_2O, the probes were analysed via DESI-MS. The compared sets of probes differed in terms of number of layers of HLB coating, withdrawal speed from the particle suspension during the coating preparation, and total coating thickness. The results of the aforementioned tests are presented in Figure S-3.

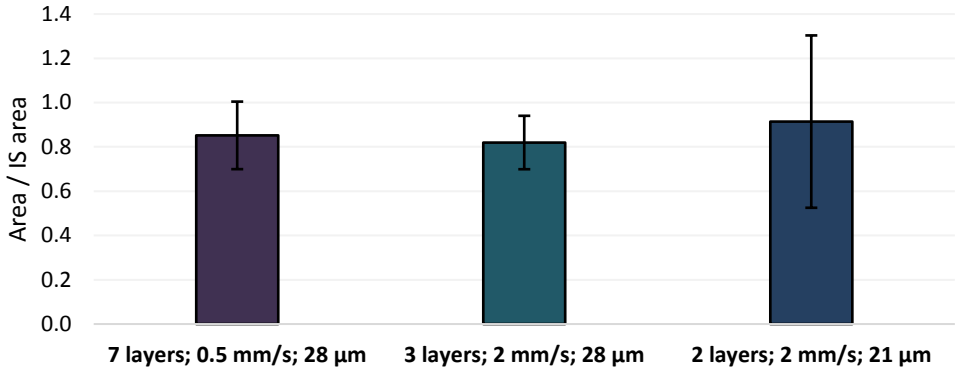

Figure S-3. Comparison of response and response reproducibility corresponding to the extraction of fluoxetine from PBS using three sets of SPME probes with different coating thicknesses and manufacturing methods (n=5).

As Figure S-2 shows, the most reproducible measurements were obtained by the probes that had been manufactured using 3 layers of coating applied at a withdrawal speed of 2 mm/s for a final thickness of 28 µm. Although it is desirable to use as few layers as possible because it decreases manufacturing time and labour, the probes featuring only 2 layers of coating yielded results that were less reproducible.

6. Signal stabilization and correction by preloading IS on the SPME fiber

During the preliminary experiments, the SPME fibers were preloaded with IS and scanned in both directions (starting from the tip of the fiber first and then going back) in order to confirm that the high amounts of pre-loaded internal standard enabled strong signals to be recorded from a single fiber multiple times (**Fig. S3**). This incomplete desorption of substantial amounts of analyte extracted onto the fiber is an effect of very short interaction times between the desorption spray plume and the SPME coating, additionally occurring on a spot size as small as several hundreds of micrometres. The ion chronograms acquired in both directions were symmetrical, which suggests that the source of signal instability lies in the local imperfections of the fiber's surface (**Fig. S4**).

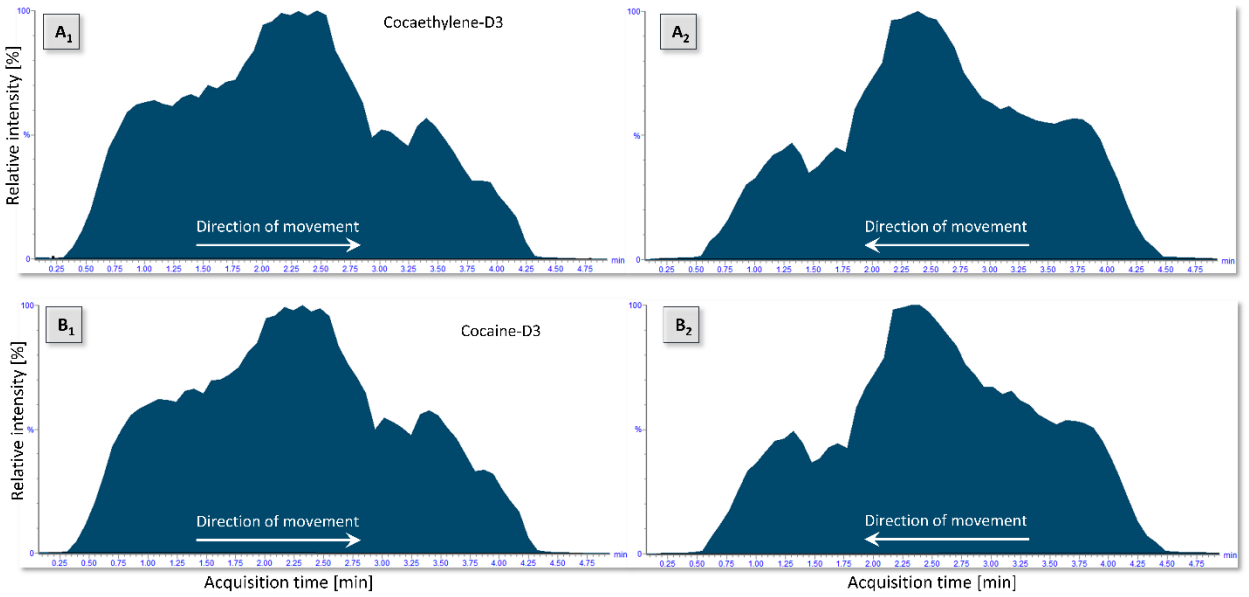

Figure S-4. Ion chronograms obtained by scanning SPME fibers preloaded with IS in both directions. The high amounts of preloaded deuterated analyte analogues enabled multiple strong signals to be recorded, unveiling a symmetrical signal-fluctuation profile (the entire fiber was uniformly preloaded with compounds; therefore, in absence of signal instability sources, the profiles were expected to be stable). An additional factor that could potentially contribute to this observation is microscale bending of the probe mounted on the holder.

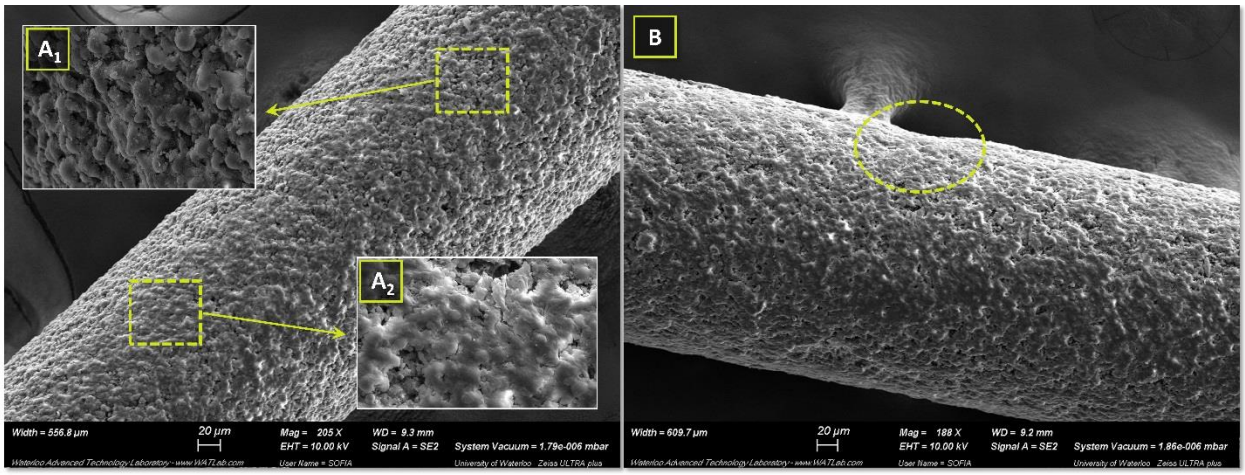

Figure S-5. Field emission scanning electron microscope images of the HLB-coated SPME fibers used in this study, emphasizing areas with surface imperfections as the source of signal fluctuations (acquired on an FE-SEM Zeiss UltraPlus instrument; Carl Zeiss Meditec AG, Jena, Germany). The samples were coated with a gold layer prior to FE-SEM analysis, and images were captured with the secondary electron detector at 10 kV. A1 and A2 represent areas on the coating surface with more exposed particles or biocompatible binder, respectively. B shows an area with coating indentation. Since these effects impact the different sides of the SPME probes differently, uniform IS pre-loading ensures signal correction independently on fiber rotation.

Table S-3: Improvement in measurement reproducibility achieved by preloading IS onto the fiber, investigated via fluoxetine extraction from agar gel (IS was preloaded for 30 min from 1 mL of water spiked with Fluoxetine-D6 at 500 ng/mL; 8 min extraction from 1 mL of 2% agar gel spiked with fluoxetine at 1 µg/mL).

Compound	Ion chronogram signal area [a.u.]				%RSD
	Rep. 1	Rep. 1	Rep. 3	Average	
Fluoxetine	4538	1335	5844	3906	59
Fluoxetine-D6 (IS)	1115	364	1306	928	54
Fluoxetine/IS	4.1	3.7	4.4	4.4	10

7. Diffusion-driven spatial resolution of SPME measurements

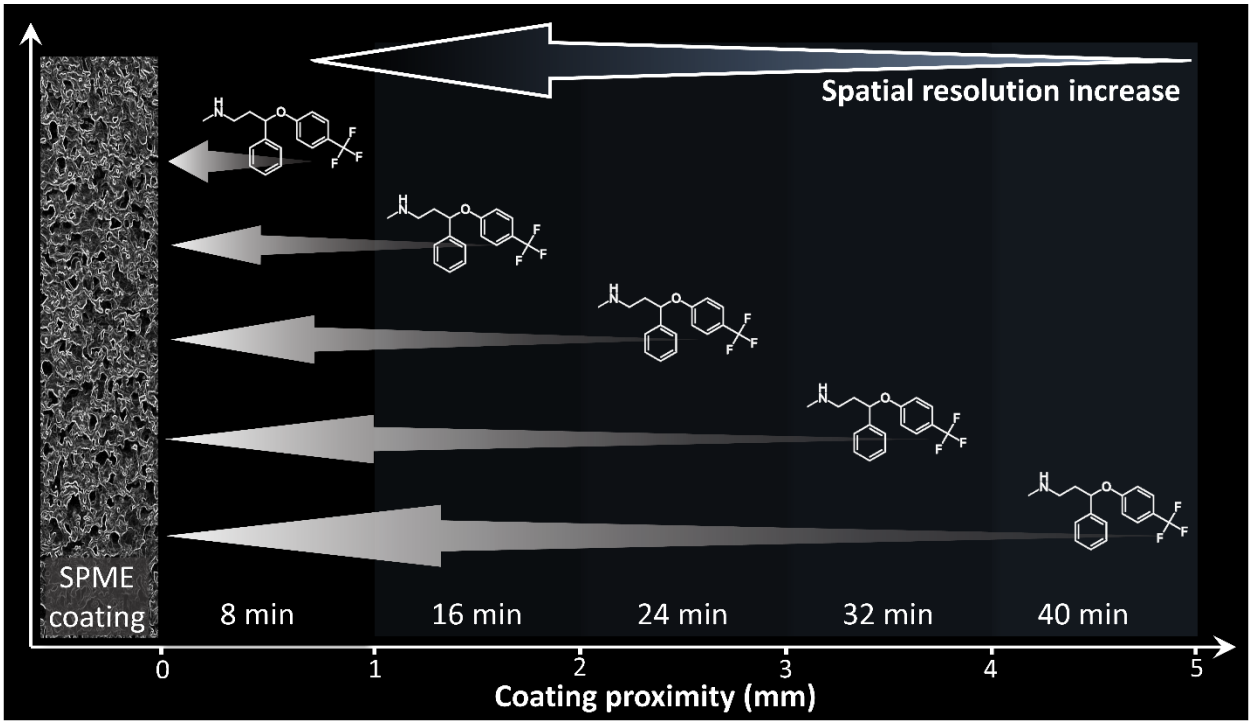

Figure S-6. The relationship between the spatial resolution of SPME measurements and extraction time in gel matrix. The negative trend, wherein spatial resolution decreases as exposure time increases, is driven by the migration of analyte molecules from the bulk matrix towards the SPME coating as the local pool of analyte nearest to a coating becomes depleted due to extraction.

As outlined in the section *Spatial resolution: The art of compromise* higher spatial resolution of the SPME measurements are expected in the tissue as compared to the gel matrix, for compounds characterized by high binding in particular. The effective diffusion coefficient D_{eff} given by equation S1 describes the analyte adsorption or desorption in the coating,[6] which can be extended analogously to the analyte's behaviour in the tissue in the presence of binding matrix.

$$D_{eff} = \frac{\mathrm{D}}{1+\mathrm{k}} \quad \text{(S1)}$$

$$k = \frac{K}{V_s/V_f} \quad \text{(S1.1)}$$

Where: D is absolute diffusion coefficient, K is partition coefficient of analyte between intercellular fluid and the investigated tissue, where V_s is volume of the binding matrix and V_f is volume of the intracellular fluid.

The analytes` adsorption onto the binding matrix present in the tissue limits or eliminates their diffusion, analogously as in the case of their adsorption onto the SPME coating during storage or DESI-MS analysis. The binding properties of the analyte in a tissue with unknown concentration of the binding matrix have been described by numerical modeling and experimental approach elsewhere.[7] The discussion above indicates that the spatial resolution will be different for different compounds and will depend on their affinity to the matrix and the SPME coating, defined by K.

8. Quantitation of drugs and pharmaceuticals in agar gel layers by SPME-DESI-MS

Table S-4. The order of layers in each configuration of the gel stack used in the experiments depicted in Figure 2 of the main text and Figure S-7.

Layer	**Config. 1**	**Config. 2**	**Config. 3**
Bottom	⦸	⦸	1000 ng/mL
Middle	500 ng/mL	1000 ng/mL	⦸
Top	1000 ng/mL	500 ng/mL	500 ng/mL

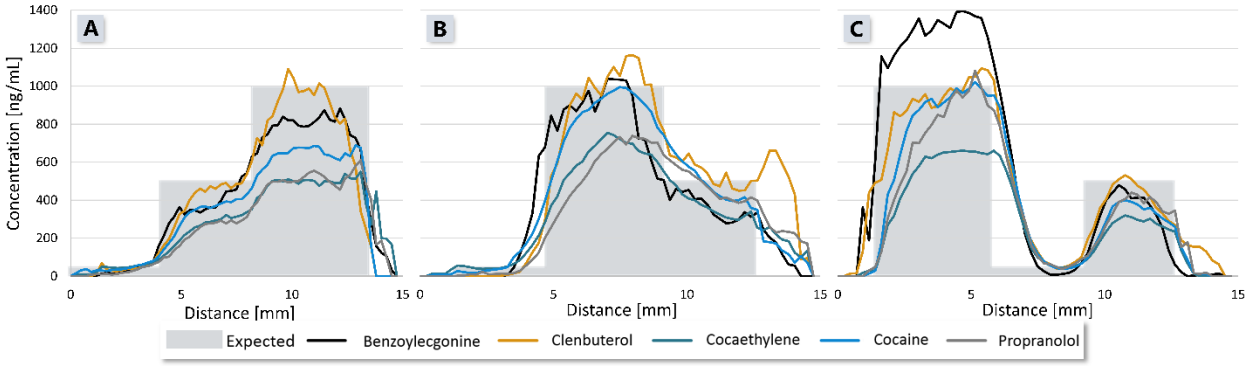

Figure S-7. Gel layer profiles acquired via SPME-DESI-MS for all tested compounds. Each profile represents the average of 3 collected fibers for every gel stack configuration. A), B), and C) correspond to gel stack configurations included in Table S-4: Config. 1, Config. 2, and Config. 3, respectively.

Limits of quantitation (LOQ) were defined as the lowest concentration of an analyte producing a signal-to-noise ratio ≥ 5, with a relative standard deviation (RSD) of 4 replicate measurements below 20%, and an accuracy within 20% of the relative error.[8]

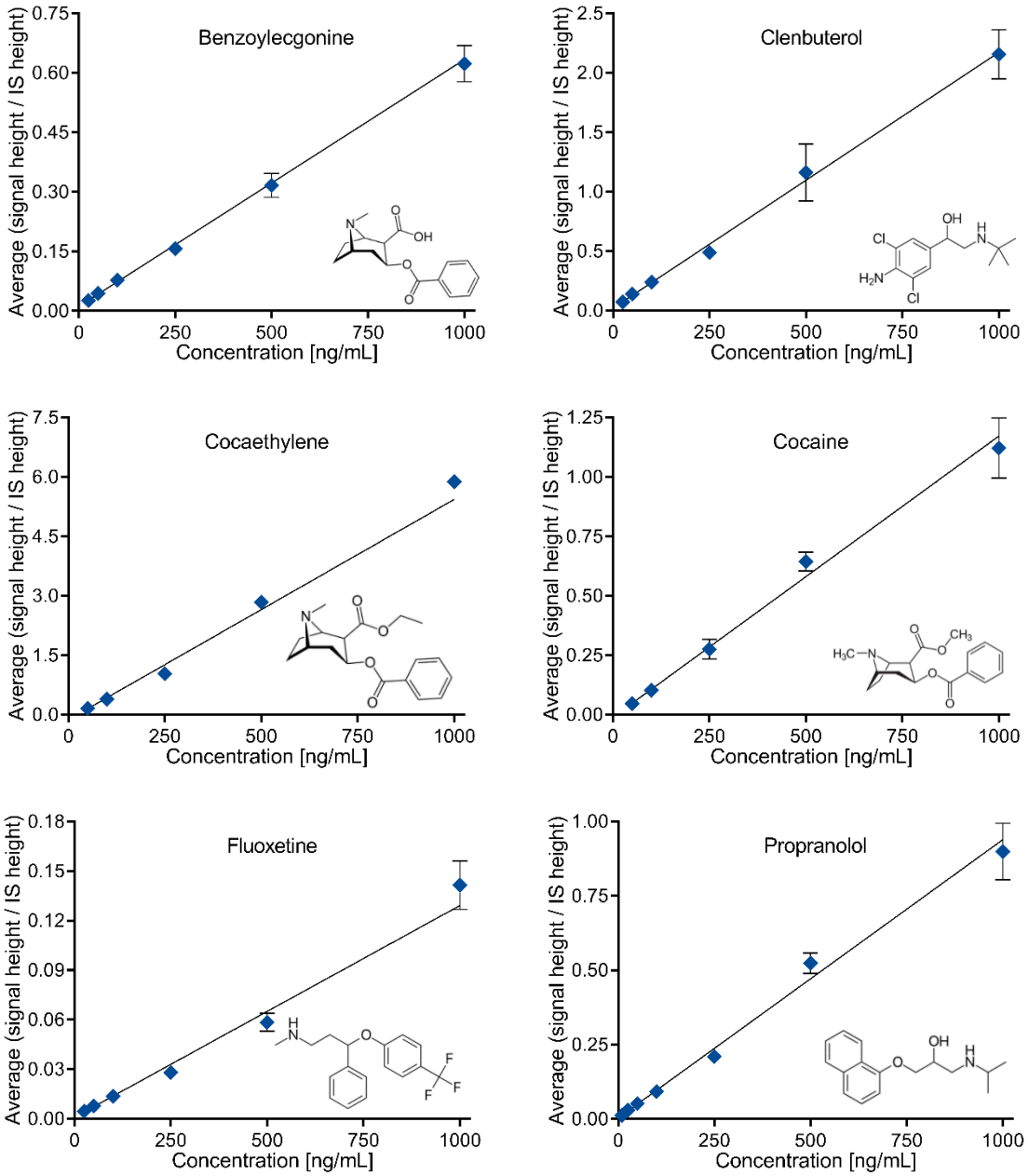

Figure S-8. Calibration curves for the quantitation of target analytes in agar gel matrix via SPME-DESI-MS with 4 mm long fibers. The slope, intercept, and R^2 for each calibration function can be found in Table S-4.

9. Quantitation of drugs and pharmaceuticals in surrogate brain matrix

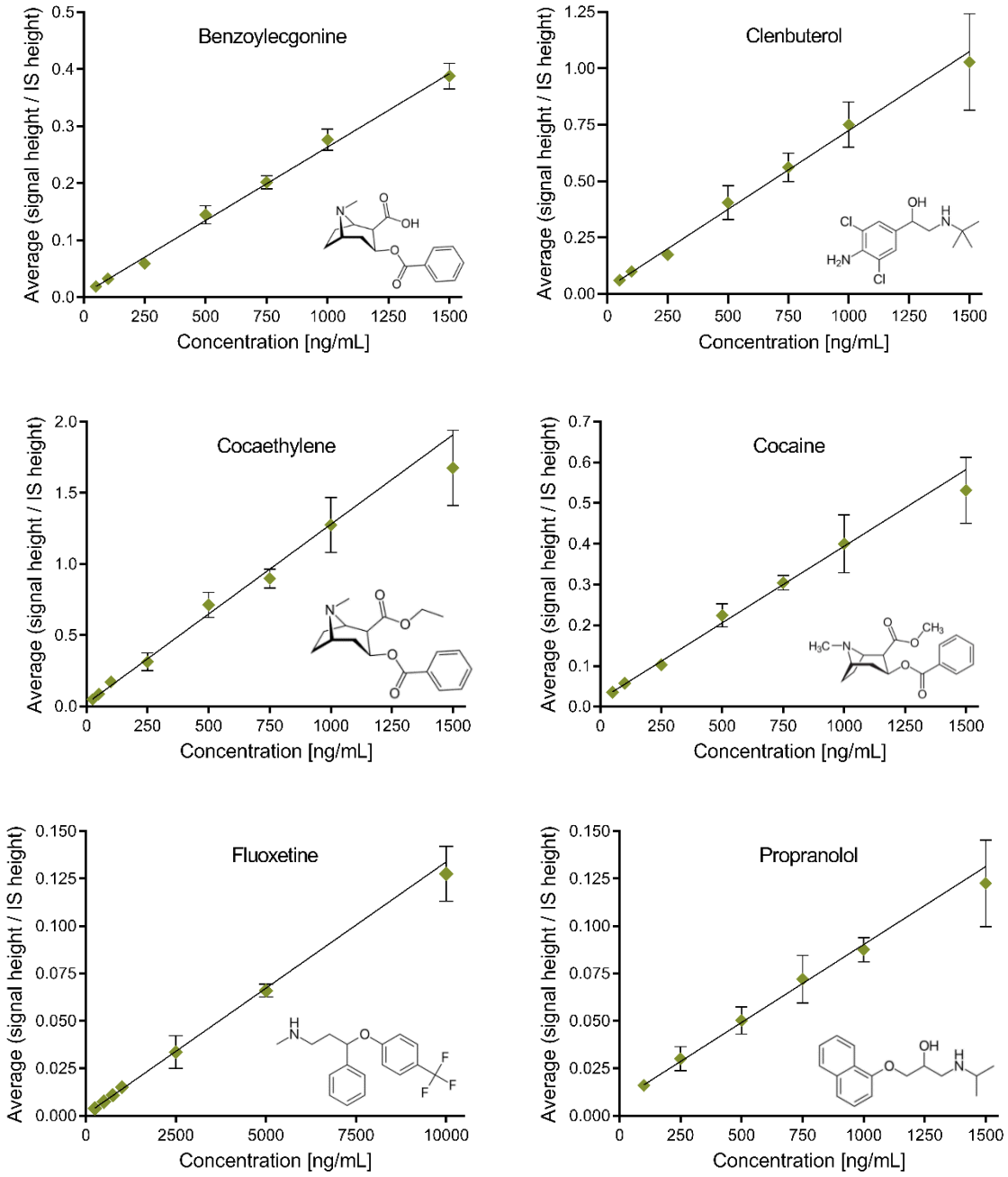

Figure S-9. Calibration curves for the quantitation of target analytes in brain surrogate matrix via SPME-DESI-MS with 4 mm long fibers. The slope, intercept, and R^2 for each calibration function can be found in Table S-4.

Table S-5: Figures of merit for the quantitation of drugs and pharmaceuticals in gel and surrogate brain matrices via SPME-DESI-MS.

Analyte	Gel matrix					Surrogate brain matrix				
	slope	intercept	R^2	weighing	LOQ [ng/mL]	slope	intercept	R^2	weighing	LOQ [ng/mL]
benzoylecgonine	0.0006	0.0113	0.979	$1/x^2$	25	0.0003	0.0069	0.984	$1/x^2$	50
clenbuterol	0.0022	0.02010	0.965	$1/x^2$	25	0.0007	0.0187	0.967	$1/x^2$	50
cocaine	0.0012	-0.0111	0.981	1/x	50	0.0004	0.0142	0.973	$1/x^2$	50
cocaethylene	0.0056	-0.1282	0.997	$1/x^2$	50	0.0013	0.0224	0.979	$1/x^2$	25
propranolol	0.0009	0.0033	0.980	$1/x^2$	10	0.0001	0.0077	0.977	$1/x^2$	100
fluoxetine	0.0001	0.0009	0.975	1/x	25	0.0001	0.0007	0.950	$1/x^2$	250

10. Quantitation of space-weighted average of drugs and pharmaceuticals in agar gel layers via SPME-nanoESI-MS

The in-house-built SPME-nanoESI-MS interface, which has been described elsewhere[9,10] was modified to fit the front end of the QTof instrument used in this study. The nanospray emitters (GlassTip coated: 1.0 mm OD, 0.78 mm ID, 4 µm tip ID) were obtained from New Objective Inc. (Woburn, MA, USA). The optimal ionization voltage was determined by testing voltages ranging between 800 and 2100 V (step size = 100 V) while spraying the desorption solution spiked with analytes. Ultimately, 1200 V was found to provide the highest and most stable signal for most of the target analytes. With the exception of the concentration and time of IS preloading used in the extractions for nanoESI-MS analysis (30 min extraction from 600 µL of water spiked with IS mixture at 5 ppb), all extractions from the gel layers were carried out identically for both nanoESI-MS and DESI-MS analysis. Immediately following each extraction, the probes were wiped, rinsed with water, and placed in glass capillaries (1 mm ID) filled with 10 µL of desorption solvent containing 500 ppb LeuEnk. Desorption was then carried out for 20 min with agitation at 1500 rpm, and the extracts were subsequently transferred to nanoESI emitters using a micro-syringe and analyzed.

Table S-6: Xevo G2-S QTof acquisition parameters used for nanoESI-MS analysis.

Parameter	Value
Spray voltage	1.2 kV
Cone voltage	40 V
Source offset	80 V
Heated capillary temperature	250 ˚C
Source block temperature	100 ˚C
Acquisition mode	Sensitivity; MS/MS (Tof MRM)
Scan time	200 ms
Mass range	70-400 m/z
Mass resolution	22000
Acquisition time	1 min
LockMass acquisition	LeuEnk (fragment m/z 120.0813); scan time 200 ms; interval 5 s; 3 scans to average

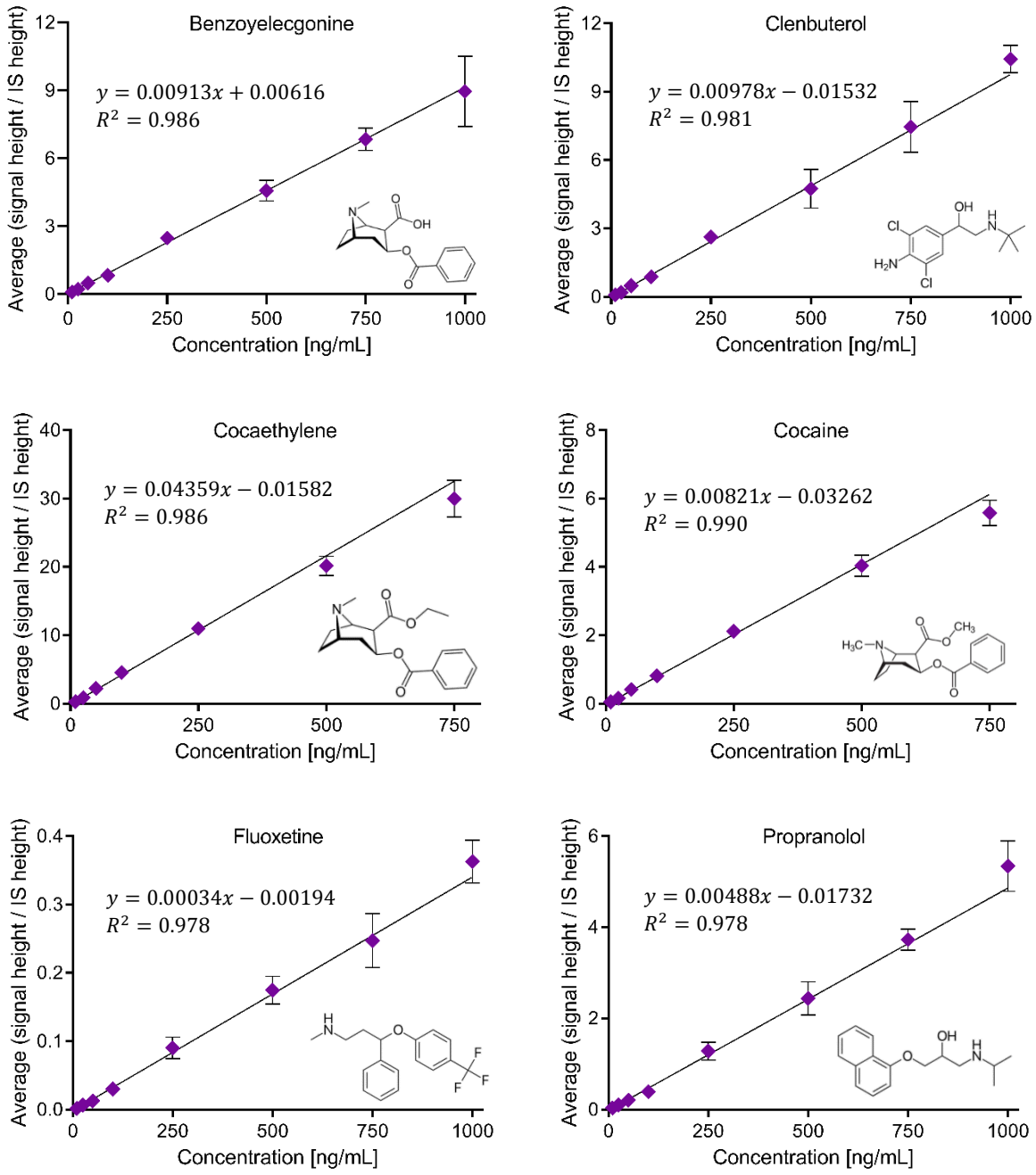

Figure S-10. Calibration curves for the quantitation of target analytes in agar gel matrix via SPME-nanoESI-MS with 4 mm long fibers.

11. Spatial distribution of fluoxetine in rat brain

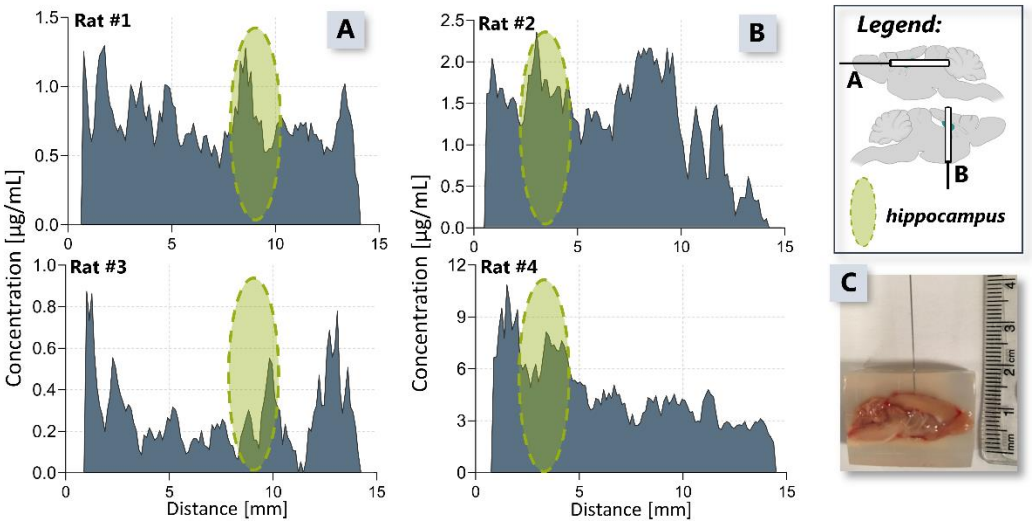

Figure S-11. Space-resolved quantitative profiles of fluoxetine in brains of 4 rats measured *ex vivo* (after *in vivo* 10 mg/kg drug administration). A) profiles acquired from long fibers inserted along the sagittal plane; B) profiles acquired from fibers inserted along the coronal plane; C) experimental setup with rat brain half embedded in agar gel block and the probe piercing through the hippocampus. The areas of the SPME fibers that were extracting from hippocampus were marked with green circles.

Numerous studies have found evidence to suggest that fluoxetine positively influences hippocampal synaptic plasticity and long-term potentiation,[11] enhances neurogenesis,[12] has neuroprotective effects,[13] including anti-inflammatory properties,[14] and prevents oxidative stress.[13] As shown in **Figure S-11**, the inter-animal variability causes identical doses of an administered drug to result in different concentrations in the hippocampus of each animal. Despite our attempts to aid the targeting of the hippocampus by embedding the brain samples in gel blocks, manual fiber insertion *ex vivo* remains prone to poor positioning precision (positioning was based on the stereotaxic coordinates for rat brains[15]). We postulated that brain's inherent heterogeneity and the area-specificity of matrix effects, may cause problems with proper interpretation of spatial profiles of the drug in brain without proper IS correction. Cerebrospinal fluid and white and grey brain matter can significantly differ in their lipid[16] and metabolite compositions, cation concentrations,[17] and metabolism,[18] thus creating different local environments that interact with the analytes and the extraction phase. Moreover, the diffusion coefficients of solutes can vary depending on the sampled brain area or compartment,[19] the brain's pathophysiology, and the subject's age,[20] thus affecting the spatial resolution of measurements.

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