Supporting Information

Understanding the pharmacokinetics of synthetic cathinones: evaluation of the blood-brain barrier permeability of 13 related compounds in rats.

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UHPLC-MS/MS description and optimization

Chromatographic separation was performed in reverse phase, using a Cortecs UPLC™ T3 2.1 x 100 mm, 1.6 µm analytical column (Waters Corp, Wexford, Ireland), maintained at 40 ºC. Mobile phases were water (solvent A) and acetonitrile (solvent B), both with 0.1% formic acid, at a flow rate of 0.4 mL/min and changing as follows: 0 min 10% B, 0.5 min 10% B, 5.5 min 40% B, 5.6 min 99% B, 8.0 min 99% B, 8.1 min 10% B (total run time 10 min). Injection volume was 20 µL.

ESI was operated in positive ionization mode using a capillary voltage of 1.0 kV. Nitrogen was used as desolvation and cone gas, at 1200 L/h and 250 L/h, respectively. Source temperature was 150 ºC, and desolvation temperature 650 ºC. Cone voltage and collision energies were optimized for each compound. Argon (99.995 %, Praxair) was used as collision gas. 3 Selected reaction monitoring (SRM) transitions were acquired per compound. Dwell times were automatically selected in order to acquire 12 points/peak, being at least 0.08 s/transition.

UHPLC-MS/MS data were acquired and processed using MassLynx 4.1 software (Waters Corp, Manchester, UK) and TargetLynx application (Waters Corp, Manchester, UK).

MS/MS optimization was performed by direct infusion into the MS system of individual solutions of the cathinones at 1 µg/L. An ESI source was selected due to the presence of an easily-protonatable nitrogen in all the synthetic cathinones. The capillary voltage was optimized using pentedrone, MDPV and naphyrone (the latter not being included in the *in vivo* study) as model compounds. Cone voltage and precursor ion selection was performed using individual solutions, testing different cone voltages from 10 to 50 V. As expected, the precursor ion selected was, in all the cases, the protonated molecule ($[M+H]^+$). Once the precursor ion as selected, different collision energies (from

5 to 50 eV, in steps of 5 eV) were tested in order to evaluate the fragmentation of the compounds and thus, select the most specific and sensitive product ions. Up to 3 SRM transitions (*Q* quantification transition; q_1 and q_2 first and second confirmation transitions, respectively) were selected for each cathinone in order to increase the confidence of compound identification based on the calculation of two ion ratios (Q/q_1 and Q/q_2). The optimized MS/MS conditions for the 14 synthetic cathinones (the 13 used for treating the animals plus naphyrone) are shown in **Table S1**.

Chromatographic separation was accurately optimized in order to separate two pairs of isomeric cathinones (*N*,*N*-dimethylpentylone vs *N*-ethyl-pentylone, and *N*-ethyl-hexedrone vs *N*-ethyl-4-methylpentedrone) that present interferences in their SRM transitions. In order to enhance chromatographic resolution, a Cortecs UPLC™ T3 2.1 x 100 mm, 1.6 µm analytical column was selected. Initially, chromatographic performance was assessed by comparing peak shape and sensitivity using H_2O:methanol and H_2O:acetonitrile with a generic elution gradient (0.3 mL/min, 0% organic solvent at 0 min linearly increased to 99% at 10 min). Peak shape and sensitivity was higher for all the compounds using acetonitrile. After that, acidity of the solvents was tested using formic acid. In this case, a concentration of 0.1% of formic acid produced the highest sensitivity and also the narrowest peaks. Finally, the addition of NH_4Ac was also assessed, but no improvements were observed and thus, the use of this modifier was discarded.

Based on the retention times observed for the cathinones, different elution gradients were evaluated. The best chromatographic separation for the two pairs of isomeric cathinones was achieved using the gradient described in the Instrumentation section: maintained from 0 to 0.5 min at 10% of acetonitrile, and linearly increased until 40% at 5.5 min. Finally, flow rate was slightly optimized in order to obtain narrower peaks and

enhance the separation of the isomeric cathinones. A flow rate of 0.4 mL/min was selected as the optimal flow, as it provided narrower peaks and did not produce the co-elution of these isomers. This chromatographic method allowed the chromatographic separation of these isomers at 5% of baseline, as can be observed in **Figure S1**.

Method validation

The analytical methodology was validated in terms of specificity, linearity, matrix effect, accuracy, precision, lower limits of quantification (LLOQs) and limits of detection (LODs).

Specificity was assessed by the analysis of blank telencephalon samples. No chromatographic peaks at the expected retention time for all the compounds were observed for the selected SRM transitions.

Linearity was evaluated by analyzing matrix-matched calibration curves at 10 concentration levels, from 1 to 1000 ng/L. A linear model was accepted if, upon back-calculation, >75% of the standards from the true concentrations was within the 15% of the nominal value [27,28].

By comparing the absolute areas of peaks and slopes of the standard lines between solvent and matrix-matched calibration, the absence or presence of "absolute" *matrix effect* was assessed. A value of 100% indicates that no absolute matrix effect was observed. A value of >100% indicates an ionization enhancement and a value of <100% an ionization suppression, following the procedure available in the literature [29,30].

Accuracy of the analytical procedure was evaluated by means of recovery experiments using blank brain samples spiked at 1 and 10 ng/g, in quintuplicate for each spiked level. Recoveries between 85 and 115% (80-120% at LLOQ) were considered satisfactory.

Precision was evaluated at 2 concentration levels (1 and 10 ng/g) as the repeatability in terms of relative standard deviations (RSD), considering RSDs lower than 15% (20% at LLOQ) as satisfactory.

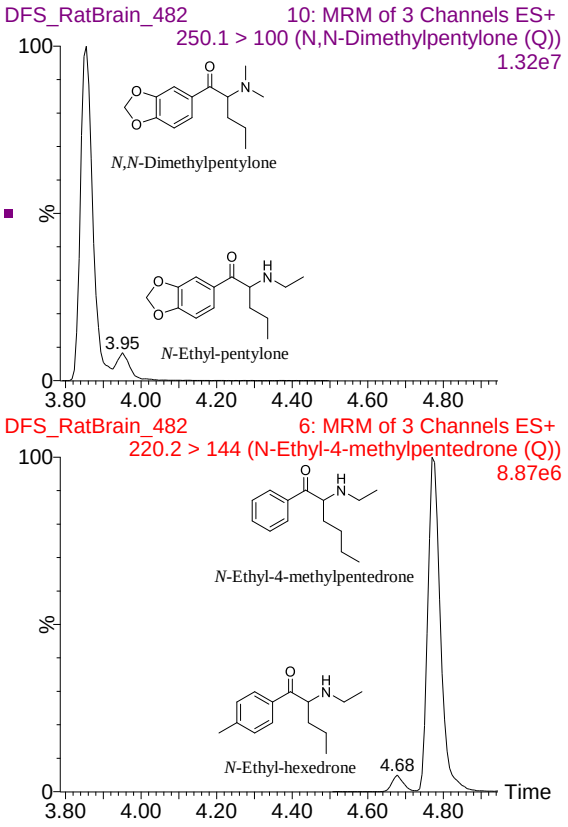
LLOQs were established as the lowest level validated with acceptable accuracy and precision (%bias and %RSD within 20%), while LODs were established for a signal-to-noise ratio (*S/N*) of 3 from the chromatographic peak of the sample spiked at the LLOQ.

For the fortification of a blank brain sample, a mix of the 14 synthetic cathinones was prepared in acetone, in order to promote the miscibility of the compounds in the crushed telencephalon tissue. After 60 min equilibration, fortified samples were extracted using the described procedure.

Table S2 shows the results obtained during the validation of the method, including recoveries and RSD at the two levels studied, LODs, LLOQs and correlation coefficients. For all the compounds, correlation coefficients higher than 0.99 were obtained in the range of 1 to 1000 ng/L using matrix-matched calibration.

Table S3 shows the matrix-effect for *N*-ethyl-pentylone, including the information on the signal suppression at each concentration level for both solvent and matrix-matched calibration lines, and also for their slopes. Additionally, **Table S4** shows a summary of the matrix effect study for all the cathinones validated. It is important to highlight that the matrix effects were overall limited (between 91% and 106%, except for buphedrone (121.6%) and pentedrone (125.1%)), with CV's below 15%. Recoveries ranged from 82 to 113% for the 1 ng/g level (LLOQ), and from 85 to 108% for the 10 ng/g level (**Table S2**). Importantly, in both cases, RSDs were lower than 10% (**Table S2**), illustrating the high precision of the developed methodology. **Figure S2** shows the SRM transitions for the 14 validated compounds at the LLOQ level.

The LLOQ for all the synthetic cathinones was set at 1 ng/g, while the LODs, calculated theoretically based on the S/N ratio obtained for the LLOQ level, were between 0.2 and 23 pg/g (**Table S2**), further indicating the high sensitivity of synthetic cathinones analyzed by ESI-MS/MS.



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129 **Figure S1.** Chromatographic separation of isomeric cathinones at 5% of baseline. **Top**
130 SRM for the quantification trace of *N*,*N*-dimethylpentylone (3.8 min). A chromatographic
131 peak can be observed at 3.95 min, corresponding to *N*-ethyl-pentylone. **Bottom** SRM for
132 the quantification trace of *N*-ethyl-4-methylpentedrone (4.78 min). A chromatographic
133 peak can be observed at 4.68 min, corresponding to *N*-ethyl-hexedrone.

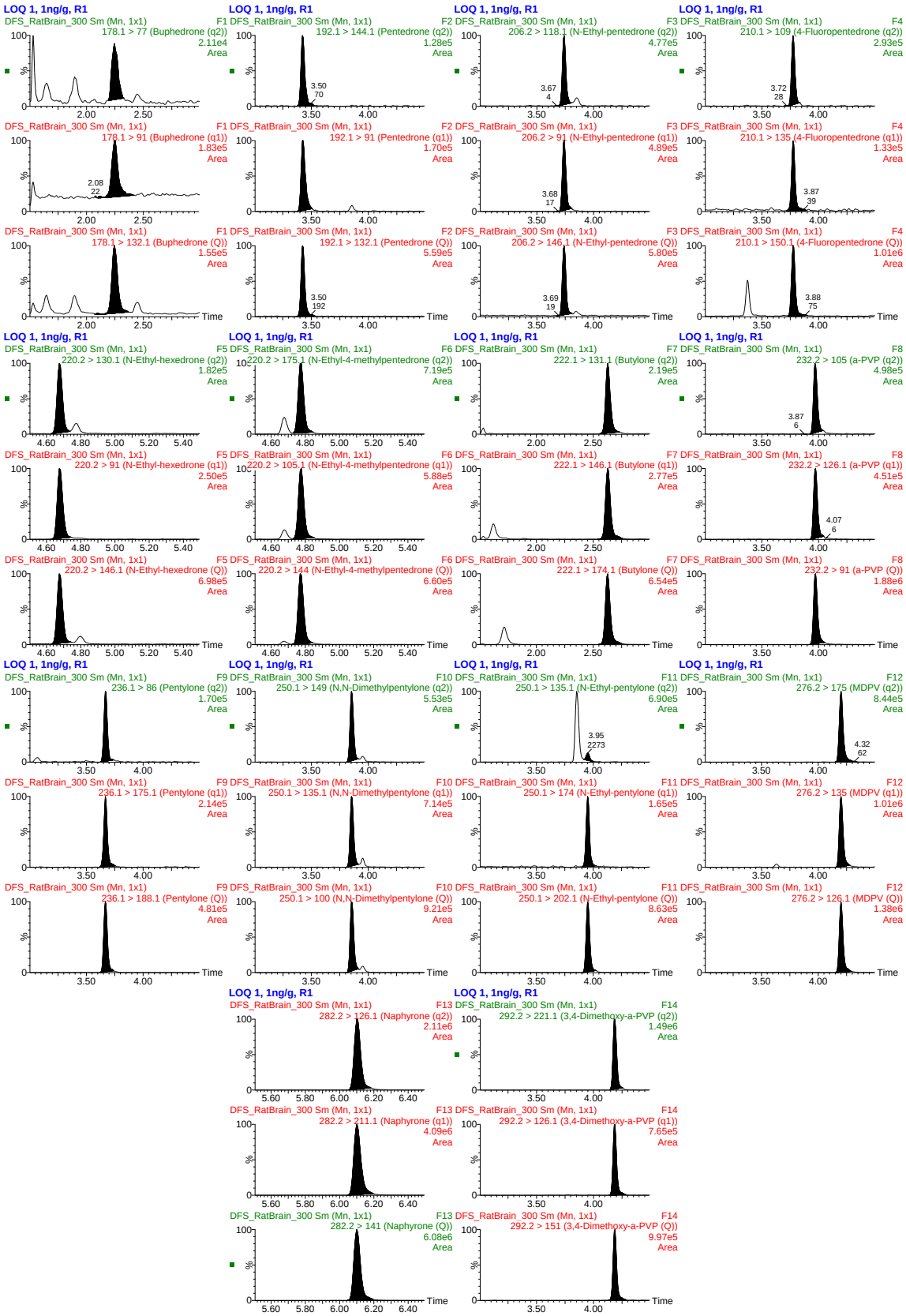

Figure S2. LC-MS/MS (SRM) chromatograms obtained for the 14 synthetic cathinones at the LLOQ level (1 ng/g).

Table S1. Selected SRM transitions for the validated compounds. Retention time (RT), cone voltage (CV) and collision energy (CE) are included.

Compound	Elemental Composition	RT (min)	Precursor ion	CV (V)	*Q* transition		q_1 transition		q_2 transition	
					Product ion	CE (eV)	Product ion (q_1/*Q*)	CE (eV)	Product ion (q_2/*Q*)	CE (eV)
Buphedrone	$C_{11}H_{15}NO$	2.3	178.1	30	132.1	15	91.0 (0.98)	20	77.0 (0.12)	30
Butylone	$C_{12}H_{15}NO_3$	2.6	222.1	20	174.1	20	146.1 (0.42)	20	131.1 (0.32)	30
Pentedrone	$C_{12}H_{17}NO$	3.4	192.1	20	132.1	15	91.0 (0.36)	15	144.1 (0.22)	25
Pentylone	$C_{13}H_{17}NO_3$	3.7	236.1	30	188.1	20	175.1 (0.42)	25	86.0 (0.30)	20
N-Ethyl-pentedrone	$C_{13}H_{19}NO$	3.7	206.2	30	146.1	15	91.0 (0.96)	15	118.1 (0.83)	20
4-Fluoropentedrone	$C_{13}H_{16}FNO$	3.8	210.1	20	150.1	15	109.0 (0.11)	15	135.0 (0.26)	25
N,*N*-Dimethylpentylone	$C_{14}H_{19}NO_3$	3.9	250.1	30	100.0	20	135.1 (0.72)	20	149.0 (0.56)	20
α-PVP	$C_{15}H_{21}NO$	4.0	232.2	30	91.0	20	126.1 (0.23)	20	105.0 (0.27)	25
N-Ethyl-pentylone	$C_{14}H_{19}NO_3$	4.0	250.1	20	202.1	15	174.0 (0.17)	30	135.1 (0.11)	20
MDPV	$C_{16}H_{21}NO_3$	4.2	276.2	20	126.1	25	135.0 (0.73)	25	175.0 (0.61)	20
3,4-dimethoxy-α-PVP	$C_{17}H_{25}NO_3$	4.2	292.2	20	151.0	30	126.1 (0.73)	30	221.1 (1.55)	15
N-Ethyl-hexedrone	$C_{14}H_{21}NO$	4.7	220.2	20	146.1	15	91.0 (0.35)	15	130.1 (0.25)	25
N-Ethyl-4-methylpentedrone	$C_{14}H_{21}NO$	4.8	220.2	20	144.0	30	105.1 (0.88)	20	175.1 (1.12)	10
Naphyrone	$C_{19}H_{23}NO$	6.1	282.2	20	141.0	30	211.1 (0.76)	15	126.1 (0.34)	30

Table S2. UHPLC-MS/MS method validation results for the selected synthetic cathinones in telencephalon tissue samples (n=5).

Compound	Recovery (RSD) (%)		LOD (pg/g)	LOQ (ng/g)	Correlation coefficient (r)
	1 ng/g	10 ng/g			
Buphedrone	103 (6)	85 (8)	22.7	1	0.99985
Butylone	101(8)	89 (9)	1.3	1	0.99995
Pentedrone	97 (9)	96 (8)	8.2	1	0.99983
Pentylone	105 (8)	89 (8)	4.2	1	0.99982
N-Ethyl-pentedrone	98 (9)	98 (7)	14.4	1	0.99960
4-Fluoropentedrone	112 (7)	86 (8)	8.6	1	0.99897
N,N-Dimethylpentylone	100 (8)	92 (8)	2.5	1	0.99993
α-PVP	107 (8)	100 (8)	1.9	1	0.99995
N-Ethyl-pentylone	104 (8)	95 (8)	4.0	1	0.99998
MDPV	111 (7)	96 (8)	1.9	1	0.99990
3,4-dimethoxy-α-PVP	82 (6)	96 (9)	3.5	1	0.99716
N-Ethyl-hexedrone	106 (8)	102 (8)	4.0	1	0.99983
N-Ethyl-4-methylpentedrone	113 (7)	94 (8)	1.8	1	0.99958
Naphyrone	111 (8)	108 (9)	0.2	1	0.99965

Table S3. Matrix effect observed for *N*-ethyl-pentylone at each concentration level, and, for the slope, for solvent and matrix-matched calibration curves.

St. Conc. (ppt)	Peak area		Matrix effect (%)[b]
	Solvent	Matrix-matched	
1	1289.6	1260.6	97.7
2.5	2165.6	2114.4	97.6
5	4110.0	3975.6	96.7
10	6999.1	6843.5	97.8
25	16881.3	16633.7	98.5
50	33606.2	33342.0	99.2
100	66933.6	66551.9	99.4
250	170041.9	162359.1	95.5
500	334794.2	326551.1	97.5
1000	638222.2	621185.5	97.3
Mean			97.7
S.D. (±)			1.2
C.V. (%)			1.2
Slope[a]	642.61	625.22	97.3
R^2	0.9994	0.9994	

S.D. = standard deviation.

C.V. = coefficient of variation.

[a] Calculated from the equation y=mx + b; each standard line was constructed using ten different concentrations.

[b] Matrix effect calculated as ME (%) = M/S x 100, where M is matrix-matched area and S solvent area. A value of >100% indicates ionization enhancement, and a value of <100% signal suppression.

Table S4 (1/2). Matrix effect observed for the validated cathinones.

Compound	Parameter[a]	Solvent	Matrix-matched	Matrix effect (%)[a]
Buphedrone	Mean			121.6
	S.D. (±)			33.3
	C.V. (%)			27.4
	Slope	248.71	235.45	94.7
Pentedrone	Mean			125.1
	S.D. (±)			43.8
	C.V. (%)			35.0
	Slope	593.02	572.63	96.6
N-ethyl-pentedrone	Mean			92.8
	S.D. (±)			12.8
	C.V. (%)			13.8
	Slope	523.24	508.69	97.2
4-fluoropentedrone	Mean			93.1
	S.D. (±)			8.9
	C.V. (%)			9.6
	Slope	1036.04	997.32	96.3
N-ethyl-hexedrone	Mean			92.7
	S.D. (±)			3.0
	C.V. (%)			3.2
	Slope	540.81	519.20	96.0
N-ethyl-4-methylpentedrone	Mean			101.0
	S.D. (±)			15.8
	C.V. (%)			15.7
	Slope	837.42	791.15	94.5
Butylone	Mean			95.1
	S.D. (±)			2.9
	C.V. (%)			3.1
	Slope	867.81	848.64	97.8

S.D. = standard deviation.
C.V. = coefficient of variation.
[a] Calculated as in **Table S3.**

156 **Table S5 (2/2).** Matrix effect observed for the validated cathinones.

Compound	Parameter[a]	Solvent	Matrix-matched	Matrix effect (%)[a]
α-PVP	Mean			92.0
	S.D. (±)			4.4
	C.V. (%)			4.8
	Slope	1978.08	1890.81	95.6
Pentylone	Mean			106.0
	S.D. (±)			9.9
	C.V. (%)			9.4
	Slope	480.33	475.77	99.1
N,*N*-dimethylpentylone	Mean			91.5
	S.D. (±)			7.3
	C.V. (%)			8.0
	Slope	1049.18	1048.83	100.0
N-ethyl-pentylone	Mean			97.7
	S.D. (±)			1.2
	C.V. (%)			1.2
	Slope	642.61	625.22	97.3
MDPV	Mean			97.7
	S.D. (±)			3.6
	C.V. (%)			3.7
	Slope	1252.12	1229.22	98.2
3,4-dimethoxy-α-PVP	Mean			92.8
	S.D. (±)			5.3
	C.V. (%)			5.7
	Slope	1010.13	978.44	96.9
Naphyrone	Mean			91.5
	S.D. (±)			3.4
	C.V. (%)			3.7
	Slope	6671.27	6290.21	94.3

157 S.D. = standard deviation.
158 C.V. = coefficient of variation.
159 [a] Calculated as in **Table S3.**
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