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Characterization of the recently detected cathinone *N*-cyclohexyl butylone: From structure elucidation to *in silico* supported pharmacological/ toxicological considerations

María Mata-Pesquera [a], David Fabregat-Safont [a,b], Cristina Gil [c], Mireia Ventura [c], Fabian P. Steinmetz [d], María Ibáñez [a,*]

[a] *Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, 12071 Castelló de la Plana, Spain*
[b] *Applied Metabolomics Research Group, IMIM-Hospital del Mar Medical Research Institute, 88 Doctor Aiguader, 08003 Barcelona, Spain*
[c] *Energy Control (Asociación Bienestar y Desarrollo), c/ Independencia 384, 08041 Barcelona, Spain*
[d] *Delphic HSE (Europe) B.V., The Base B, Evert van de Beekstraat 104, 1118CN Schiphol, Netherlands*

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ABSTRACT

One of the most widely consumed new psychoactive substances (NPS) families in Europe are synthetic cathinones. Cathinone structure can be easily modified resulting in new derivatives that rapidly reach drug markets. In this work, the recently detected synthetic cathinone *N*-cyclohexyl butylone has been characterized by gas chromatography coupled to mass spectrometry (GC–MS), liquid chromatography coupled to high-resolution mass spectrometry (HRMS), nuclear magnetic resonance (NMR) and Fourier-transformed infrared (FTIR) spectroscopy, using research chemicals samples collected by the drug analysis service Energy Control from anonymous users. Compound identification was performed by the combination of HRMS and NMR data. The elemental composition and putative moieties of the compound were determined based on the accurate-mass ions observed by HRMS. Then, different NMR experiments, including bidimensional, allowed the establishment of the chemical structure and confirmation of compound identity. Furthermore, FTIR spectrum was also acquired in order to provide a complete analytical characterization of the novel cathinone. Finally, pharmacological/toxicological characterization was attempted using *in silico* methods. Based on these predictions, *N*-cyclohexyl butylone probably has similar effects to stimulants like MDMA.

1. Introduction

New psychoactive substances (NPS) are designer drugs that try to produce similar effects to classical ones (stimulation, hallucination, or sedation). As these novel compounds are not detected by the drug tests typically used and most of them are not covered by on-going legislation, they have emerged in the drug market as "legal" alternatives to controlled drugs. Due to their widespread consumption all over the world, together with the fact that there are few data regarding toxicity and potency, NPS use has become an important public health problem in the last years[1]. In fact, the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) was monitoring approximately 880 NPS at the end of 2021; 52 of these were detected for the first time in Europe during that year [2].

The NPS can be classified in different families according to their chemical structure, being synthetic cannabinoids the most consumed in Europe nowadays, followed by synthetic cathinones[2]. Synthetic cathinones are based on the cathinone structure, a natural alkaloid presents in the *Catha edulis* plant[3]. These compounds act as stimulants, increasing dopamine and serotonin levels and thus, producing similar effects to cocaine[4] (euphoria, constant state of alert, etc.). However, they also produce adverse effects like tachycardia, anxiety, or aggressive behaviours, having been reported in the last years numerous intoxication cases[5–7].

Synthetic cathinones are usually found as crystal or powder (65% of the seizures in 2020 in Europe were cathinone powders)[2]. Therefore, they are usually administrated orally or snorted, although they are commercialised as "not for human consumption" to avoid the legislation

* Corresponding author.
E-mail address: ibanezm@uji.es (M. Ibáñez).

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[8]. Until 2020, the large-scale production of these substances took place in China but, due to the legal controls introduced in recent years in this country, most of the synthetic cathinones are nowadays produced in India[2].

Chemically, cathinones are formed by a benzyl group, a keto group on the β-carbon and an amine termination[9,10]. This structure allows to obtain a wide variety of synthetic cathinones by modifying *N*-functionalization, α-carbon alkyl chain length, and aromatic ring substitution[11] (see Fig. 1**A**). These small modifications result in the appearance of new derivatives with similar structures in the drug market each year[12,13], making very difficult the continuous identification and regulation of these substances. Therefore, their detection and characterization become a challenge for analytical chemists and toxicologists[3,12], as different but complementary advanced analytical techniques are required.

For monitoring synthetic cathinones in legal highs samples, various analytical approaches have been reported[13,14]. One of the most powerful techniques is ultra-high performance liquid chromatography combined to high-resolution mass spectrometry (UHPLC-HRMS), as it allows the detection of all the ionisable compounds of a certain sample and their tentative identification without available reference standard. Furthermore, it allows the structural elucidation of unknown substances by using the elemental composition of the (de)protonated compound and its accurate-mass fragments. Nevertheless, HRMS data are not enough for performing an unequivocal identification without analytical standard, being necessary an orthogonal technique such as nuclear magnetic resonance (NMR). Also, in certain cases, HRMS data cannot provide enough evidence for proposing the chemical structure of an unknown compound, being mandatory the use of bidimensional NMR experiments.

In the present study, the new synthetic cathinone *N*-cyclohexyl butylone (see Fig. 1**B**) recently reported by the first time by the Center for Forensic Science Research & Education (CFSRE)[15] was fully characterized in samples collected from anonymous users by the drug analysis service Energy Control. The characterization was performed by gas chromatography coupled to mass spectrometry (GC–MS), UHPLC-HRMS and different NMR experiments, including bidimensional. In addition, accurate-mass fragmentation data and Fourier-transformed infrared (FTIR) spectrum were also assessed in order to obtain as much information as possible for this novel cathinone.

2. Materials and methods

2.1. Reagents and chemicals

For UHPLC-HRMS analysis, an Ultramatic Plus GR from Wasserlab (Navarra, Spain) was used to obtain ultrapure water by purifying demineralized water. LC-MS grade methanol (MeOH) and acetonitrile (ACN), acetone, formic acid (HCOOH), sodium hydroxide (NaOH) and deuterium oxide (D_2O) were purchased from Scharlau (Scharlab, Barcelona, Spain). Leucine enkephalin acetate salt hydrated (>95%) was purchased from Merck (Darmstadt, Germany).

2.2. Collection of samples

Drug samples were received by the Balearic Islands headquarter of Energy Control, a harm reduction project within the Spanish NGO "Asociación Bienestar y Desarrollo"[16]. Samples were submitted by anonymous Spanish users to Energy Control's drop-in service for their analysis. The format of these samples was the following: two samples were yellow pills with the logo of Snapchat (also containing caffeine), one brown crystal sample was containing also MDMA and finally two were in white crystal form. All of them were acquired as MDMA.

2.3. Sample treatment

For GC–MS, approximately 10 mg of the sample were dissolved in 10 mL of methanol.

For UHPLC-HRMS analysis, 10–20 mg of sample were extracted with 1 mL of acetone and vortexed for 1 min. Extracts were centrifuged and supernatant was 1000-fold diluted with ultrapure water.

For NMR analysis, approximately 20 mg of sample were dissolved in 0.75 mL of D_2O.

For FTIR, sample was directly analyzed in its solid form using an attenuated total reflection (ATR) accessory.

2.4. Instrumentation

For the GC–MS analysis, an Agilent 7890B Gas Chromatograph coupled to a 5977A quadrupole mass spectrometer detector (Agilent; Santa Clara, CA, USA) was used. The mass spectrometer was in electronic ionization mode at 70 eV. MS system worked in SCAN acquisition mode, acquiring from *m/z* 40 to 550 Da.

UHPLC-HRMS analyses were performed using an ACQUITY UPLC system (Waters, Mildford, MA, USA) coupled to a Xevo G2 QTOF mass spectrometer (Waters, Manchester, UK) equipped with a hybrid quadrupole time-of-flight mass analyzer. Data were acquired from *m/z* 50 to 1000 in data-independent acquisition mode, in order to obtain information about the protonated molecule and adducts (if present) and fragment ions in a single run.

For NMR experiments, a Bruker Ascend 400 MHz spectrometer equipped with a SampleCase autosampler (Bruker, Etlingen, Germany) was used, performing different NMR experiments (^{1}H NMR, ^{13}C NMR, correlated spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC)).

For FTIR measurements, a Jasco FT/IR spectrometer with the accessory ATR Pro One (Jasco Inc., Easton, MD, USA) was used, acquiring data between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1}.

Further details about GC–MS, UHPLC-HRMS, NMR and FTIR, including an accurate description of the instrumental methods and

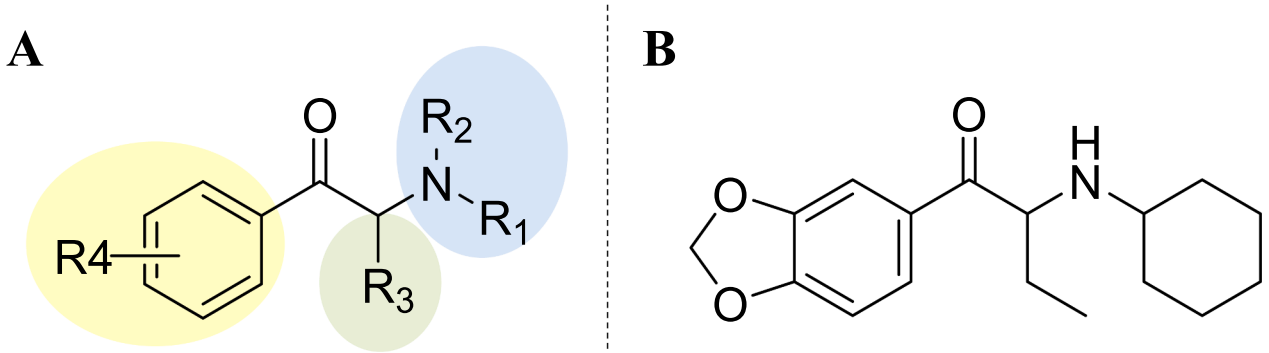

Fig. 1. General chemical structure of synthetic cathinones (A) and *N*-cyclohexyl butylone chemical structure (B).

conditions used, can be found in previous studies[13,17,18].

2.5. Computational tools for pharmacological/toxicological predictions

Similar compounds to *N*-cyclohexyl butylone were identified with ChemSpider[19]. These compounds were investigated for relevant substructures and pharmacological activities. Additionally, two typical targets for psychedelic amphetamines, the serotonin reuptake transporter (SERT) and a subtype of the serotonin receptor (5-HTR$_{2A}$) were docked using DockThor[20] and protein structures (6VRH and 6WHA) from the RCSB's Protein Data Bank[21]. Metabolic predictions were conducted with QSAR Toolbox 4.2[22] applying the *in vivo* rat metabolism simulator. Furthermore, *N*-cyclohexyl butylone was screened for structural alerts using Toxtree v3.1.0[23].

3. Results and discussion

3.1. Compound identification and analytical characterization

A chromatographic peak was observed at 8.465 min in the total ion current (TIC) chromatogram when injecting the sample extract into the GC–MS (Fig. S1**A**). The EI spectra (Fig. S1**B**) showed a base peak at *m/z* 140.1 and minor peaks at *m/z* 58.1, 41.1, 121.0, 83.1 and 232.1, from highest to lowest intensity. This spectrum was compared to the Searchable Mass Spectral Library NIST/EPA/NIH Mass Spectral Library, Data Version: NIST 14; Searchable Mass Spectral Library Version 3.9 [24], Searchable Mass Spectral Library Cayman Spectral Library (CSL) [25], European project RESPONSE database[26] and Energy Control's internal mass spectral library; but no match was found. Therefore, HRMS was used for tentative compound identification.

When injecting the compound in the UHPLC-HRMS system, a single

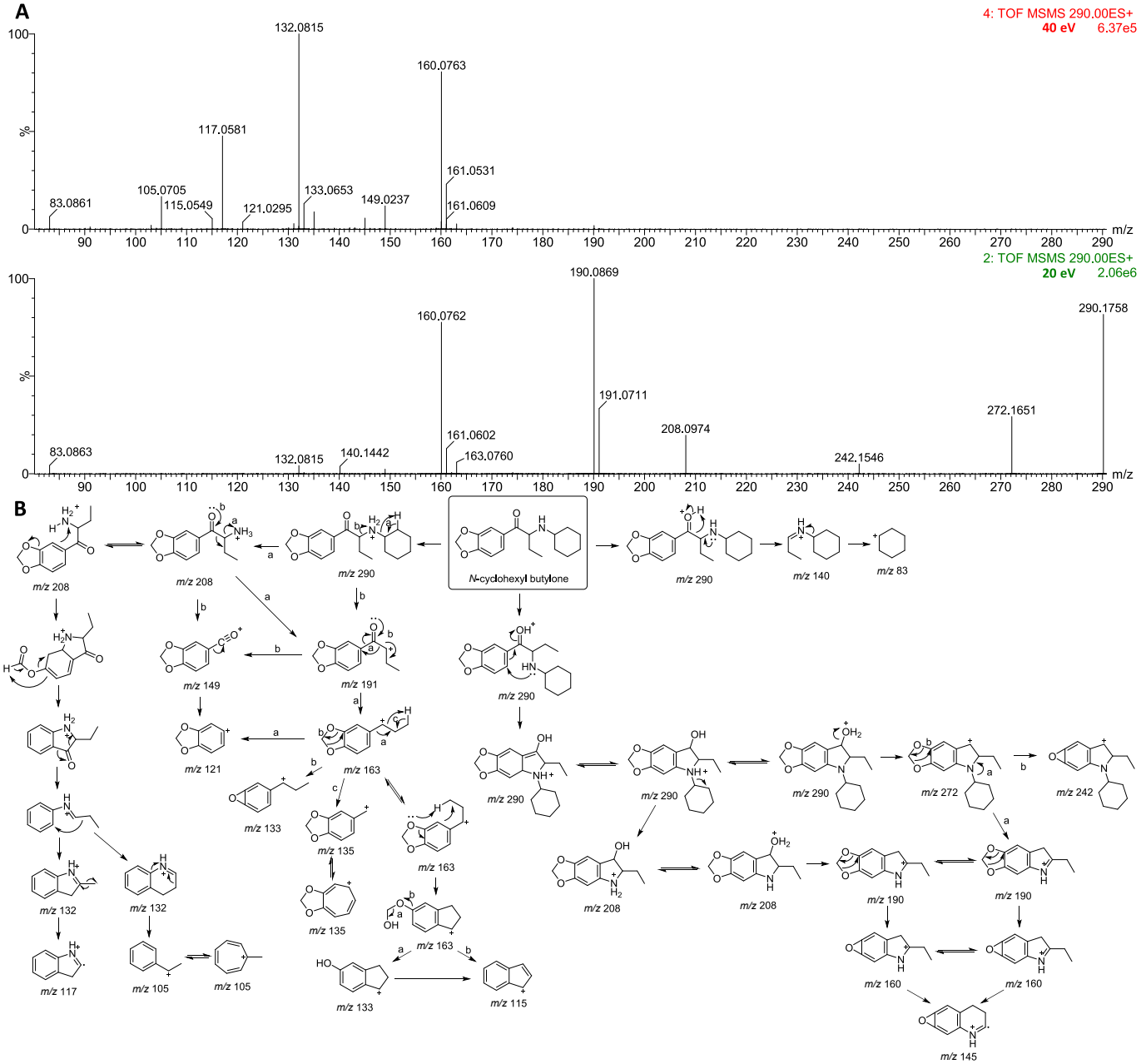

Fig. 2. HRMS data for *N*-cyclohexyl butylone. MS/MS spectra at 20 and 40 eV (A) and proposed fragmentation pathway (B).

chromatographic peak at 5.74 min was observed in the TIC of LE, corresponding to *m/z* 290.1758. So, the elemental composition of the $[M + H]^+$ was determined to be $C_{17}H_{24}NO_3^+$ (0.2 mDa). Considering that NPS follow similar fragmentation pathways as reported for synthetic cathinones[27], synthetic cannabinoids[28] and synthetic opioids[29], the elemental composition for the observed accurate-mass fragments (HE spectrum) suggested that the unknown compound was a synthetic cathinone. Briefly, aromatic ring substitution was established as 3′,4′-methylenedioxy based on the presence of fragment ion at *m/z* 149 after the disconnection of carbonyl and α-carbon. Amine functionalization was determined by a neutral loss of 82 Da together with a fragment ion at *m/z* 83 (disconnection of amine functionalization). Alkyl chain length was proposed knowing the amine moiety and the presence of fragment ions at *m/z* 140 (disconnection of carbonyl and α-carbon) and *m/z* 191 (loss of amino group, once established aromatic ring substitution)[27].

Some fragments observed in HE had a 1 Da difference between them, and a mass defect and relative intensity that could be produced by ^{13}C isotope pattern. In order to correctly assess compound fragmentation, MS/MS experiments were performed at different collision energies (10–50 eV) using a 1 Da mass isolation window to avoid ions coming from the compound isotope pattern. Fig. 2**A** shows the MS/MS spectra at 20 and 40 eV, while Table 1 summarizes the accurate-mass, elemental composition, and mass error of each product ion (PI).

PI 1 (*m/z* 272.1651, $C_{17}H_{22}NO_2^+$, 0 mDa) corresponds to the loss of water commonly detected in synthetic cathinones with secondary amines[30,31]. The loss of water followed by a formaldehyde loss from the methylenedioxy group, leads to PI 2 (*m/z* 242.1546, $C_{16}H_{20}NO^+$, 0.1 mDa)[8]. PI 3 (*m/z* 208.0974, $C_{11}H_{14}NO_3^+$, 0 mDa) would derive from the protonated molecule after a cyclohexyl loss, as previously supposed based on HE fragmentation spectrum. In addition, cyclohexylium ion was also observed, corresponding to PI 19 (*m/z* 83.0861, $C_6H_{11}^+$, 0 mDa). Regarding PI 3, it can then undergo a loss of water, leading to the abundant PI 5 (*m/z* 190.0869, $C_{11}H_{12}NO_2^+$, 0.1 mDa). The loss of formaldehyde from PI 5 originates PI 8 (*m/z* 160.0762, $C_{10}H_{10}NO^+$, 0 mDa), in the same way as reported by Ibáñez et al for MDPV[32], and the posterior loss of a methyl group produces PI 10 (*m/z* 145.0533, $C_9H_7NO^{+\cdot}$, 0.5 mDa). From PI 3, and after the disconnection of carbonyl and α-carbon, the PI 9 (*m/z* 149.0237, $C_8H_5O_3^+$, 0.2 mDa) is obtained, indicating thus the functionalization of the aromatic ring. On the other hand, PI 14 (*m/z* 132.0815, $C_9H_{10}N^+$, 0.2 mDa) originates from PI 3 (*m/z* 208), after a molecular rearrangement for cyclization and charge stabilization. PI 16 (*m/z* 117.0581, $C_8H_7N^{+\cdot}$, 0.3 mDa) and 18 (*m/z* 105.0705, $C_8H_9^+$, 0.1 mDa) are obtained after the loss of a methyl group and cyanide acid from PI 14 (*m/z* 132), respectively. Finally, the disconnection of α-carbon and amine group generated PI 11 (*m/z* 140.1442, $C_9H_{18}N^+$, 0.3 mDa) and the posterior loss of propylamine, the PI 19 (83.0861, $C_6H_{11}^+$, 0 mDa). The whole proposed fragmentation pathway of this cathinone can be checked in Fig. 2**B**.

Table 1
Accurate mass, elemental composition and mass error of the protonated molecule and product ions.

Ion	Accurate mass	Elemental composition	Mass error (mDa)	Mass error (ppm)
$[M + H]^+$	290.1758	$C_{17}H_{24}NO_3^+$	0.2	0.7
PI 1	272.1651	$C_{17}H_{22}NO_2^+$	0.0	0.0
PI 2	242.1546	$C_{16}H_{20}NO^+$	0.1	0.4
PI 3	208.0974	$C_{11}H_{14}NO_3^+$	0.0	0.0
PI 4	191.0711	$C_{11}H_{11}O_3^+$	0.3	1.6
PI 5	190.0869	$C_{11}H_{12}NO_2^+$	0.1	0.5
PI 6	163.0760	$C_{10}H_{11}O_2^+$	0.1	0.6
PI 7	161.0602	$C_{10}H_9O_2^+$	−0.1	−0.6
PI 8	160.0762	$C_{10}H_{10}NO^+$	0.0	0.0
PI 9	149.0237	$C_8H_5O_3^+$	0.2	−1.3
PI 10	145.0533	$C_9H_7NO^{+\cdot}$	0.5	3.4
PI 11	140.1442	$C_9H_{18}N^+$	0.3	2.1
PI 12	135.0446	$C_8H_7O_2^+$	0.0	0.0
PI 13	133.0653	$C_9H_9O^+$	0.0	0.0
PI 14	132.0815	$C_9H_{10}N^+$	0.2	1.5
PI 15	121.0295	$C_7H_5O_2^+$	0.5	4.1
PI 16	117.0581	$C_8H_7N^{+\cdot}$	0.3	2.6
PI 17	115.0549	$C_9H_7^+$	0.1	0.9
PI 18	105.0705	$C_8H_9^+$	0.1	1.0
PI 19	83.0861	$C_6H_{11}^+$	0.0	0.0

However, the unequivocal confirmation of the structure could not be made only based on the accurate-mass fragmentation, as potential isomers such as the position of the methylenedioxy group and the length of α-carbon alkyl chain (can be both ethyl and dimethyl) would fit also with the observed fragmentation. Therefore, bidimensional NMR experiments were required in order to confirm the proposed structure**.** Fig. 3 shows the signal assignment observed in COSY (**A**) and HSQC (**B**) for *N*-cyclohexyl butylone. These assignments were based on chemical shifts (*δ*, ppm) of ^1H and ^{13}C, on the multiplicity patterns of proton resonances depicted by the J couplings (Hz), and on correlation between ^1H–^1H (COSY) and ^1H–^{13}C (HSQC). In addition, single ^1H **(Fig. S2)** and ^{13}C NMR **(Fig. S3)** experiments were also performed in order to obtain information regarding proton integration and additional carbon atoms out of range of the HSQC experiment (such as carbonyl), respectively. According to the ^1H spectrum **(Fig. S2),** the triplet at $\delta = 0.85$ ppm, which integration was 3, indicated the presence of a methyl group in the structure. As expected for this moiety, the carbon atom of this methyl was at $\delta = 7.99$ ppm in the ^{13}C spectrum, as stablished by the HSQC spectrum (Fig. 3**B**). Based on the couplings observed in the COSY **(**Fig. 3**A),** this methyl group is near to a secondary carbon corresponding to the multiplet at δ around 2.04 ppm in ^1H spectrum, which is overlapped with the cyclohexyl signals (δ between 1 and 2.2 ppm) (Fig. 3**A**). This information, together with the couplings observed in bidimensional experiments, indicated that the α-carbon chain must be an ethyl group. Similarly, the position of the 3′,4′-methylenedioxy group was confirmed based on the signals observed in COSY (Fig. 3**A**).

After establishing the structure of this compound, all the remaining NMR signals observed in the bidimensional experiments (COSY and HSQC) were assigned to the proposed structure (see Fig. 3). This new cathinone is a trisubstituted aromatic compound characterized by one doublet at 7.02 ppm (H-5), one singlet at 7.48 ppm (H-3) and one doublet at 7.71 ppm (H-6), as can be seen in **Fig. S2**. The singlet at 6.11 ppm (H-1) allows the identification of the methylenedioxy group attached to the aromatic ring, while the triplet at 5.12 ppm corresponds to the methine proton (H-9) located between the carbonyl group and the nitrogen atom. The multiplets of the methylene protons H-13, H-14, H-15, H-16 and H-17 of the cyclohexyl group appeared as separated signals between 1.25 and 2.04 ppm, partially overlapped with the methylene protons H-10 of the alkyl side chain. The COSY experiment confirmed that the singlet at 0.85 ppm, which integrates 3 protons, corresponds to the terminal methyl group of the alkyl side chain (H-11). Finally, the multiplet at 3.07 ppm which integration was two corresponds to the H-12.

Regarding the ^{13}C NMR spectrum (**Fig. S3**), the signals from the 17 carbon atoms were assigned based on the HSQC spectrum (Fig. 3**B**). The methylenedioxy group (C-1) produced a signal at around 102.75 ppm, while the signals observed between 107.98 and 153.82 ppm correspond to the phenyl group (C-2, C-3, C-4, C-5, C-6 and C-7). The carbonyl carbon (C-8) was found at 194.83 ppm, the methine carbon (C-9) at 59.94 ppm and the cyclohexyl carbon bonded to the nitrogen (C-12) appeared at 57.51 ppm. Finally, the carbon of the methyl group (C-11) appeared at 7.99 ppm, whereas the corresponding to the methylene group of the alkyl chain (C-10) and the cyclohexyl (C-13, C-14, C-15, C-16) appeared between 24.03 and 29.59 ppm.

In addition, the complete characterization of the compound was provided by FTIR analysis. This information is highly useful as FTIR has been highly used in the last years for the rapid and in-situ identification of NPS in seizures[33,34]. Fig. 4 shows the FTIR spectrum of *N*-cyclohexyl butylone, where the most characteristic signals can be identified.

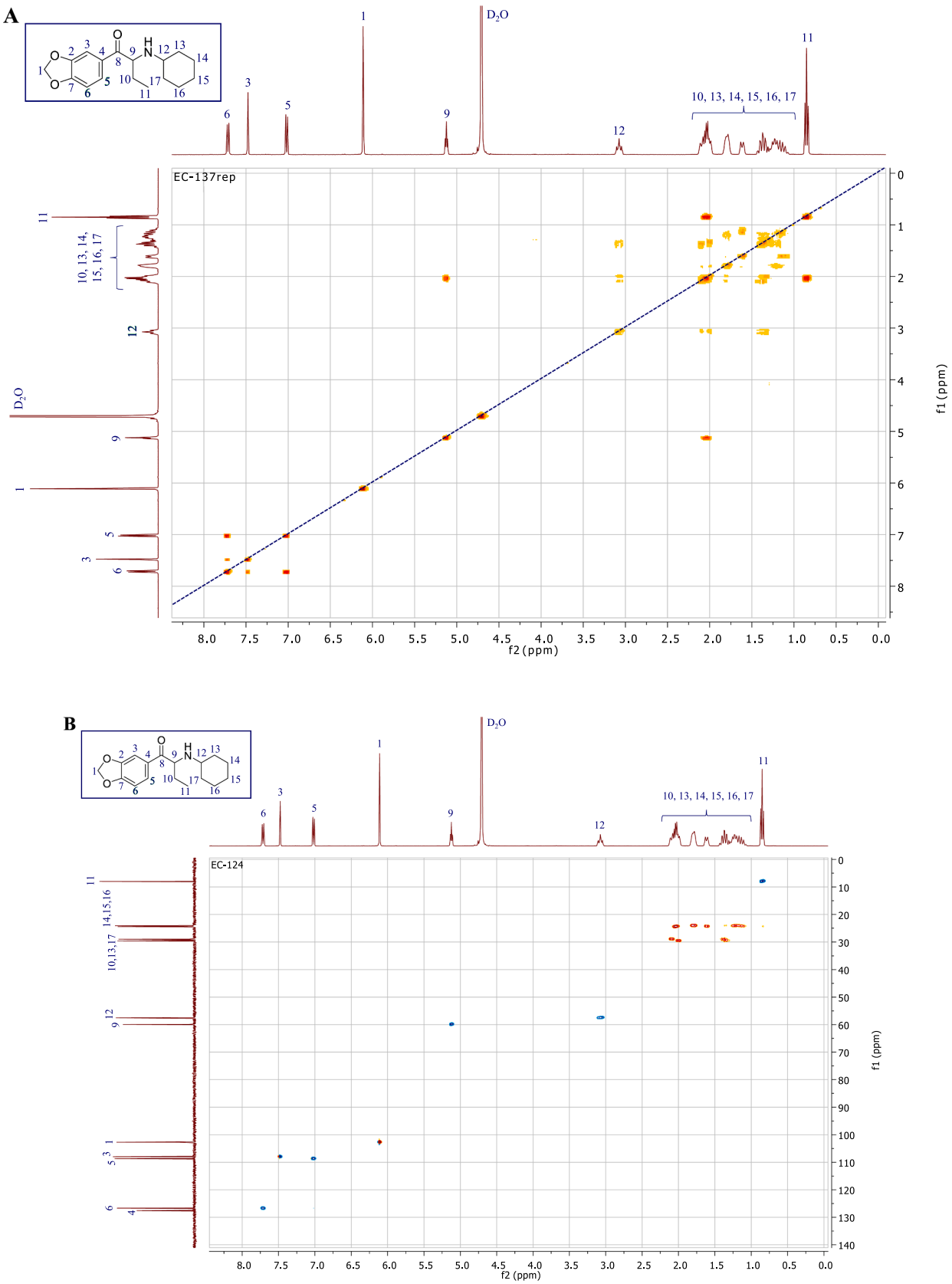

Fig. 3. Bidimensional NMR experiments, with signal assignation, performed for the identification of the *N*-cyclohexyl butylone. (A) COSY spectrum. (B) HSCQ spectrum.

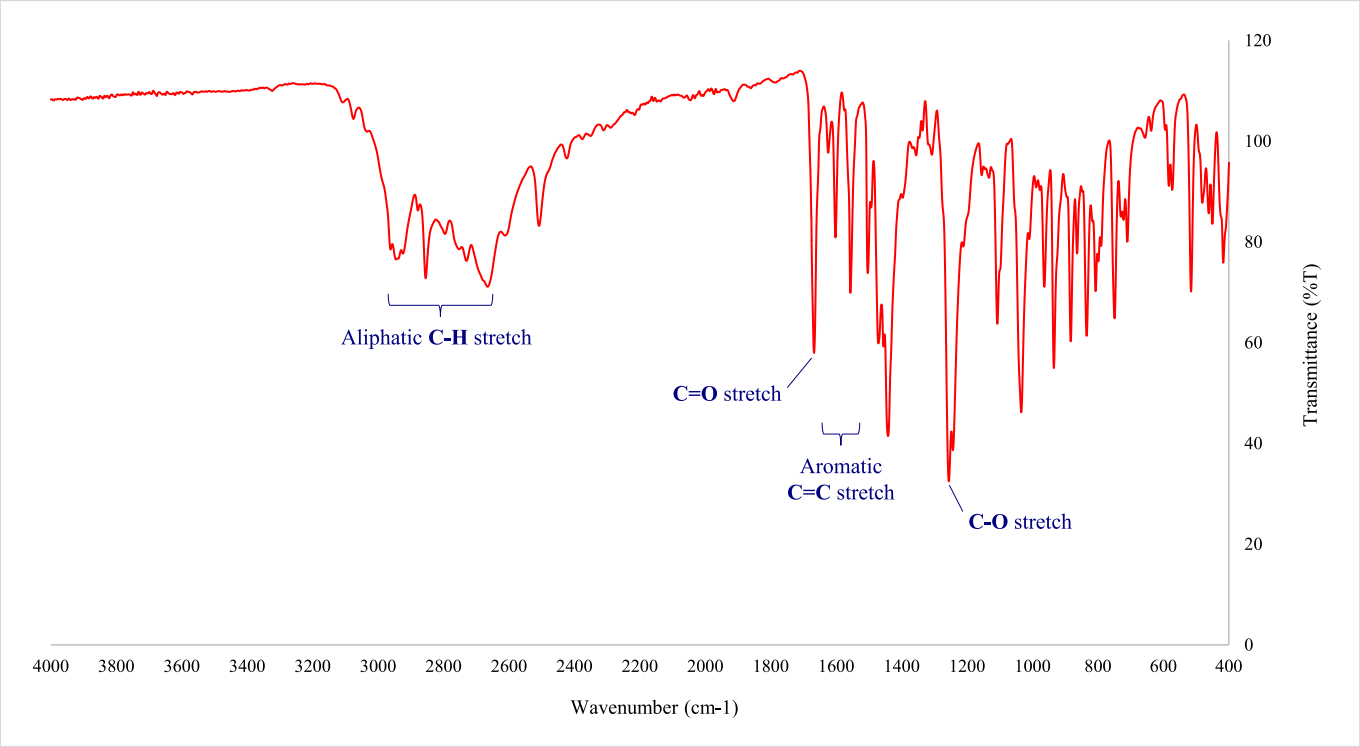

Fig. 4. ATR-FTIR spectrum of the *N*-cyclohexyl butylone.

The spectrum contains a set of bands between 2700 and 3000 cm^{-1} that corresponds to C-H stretch of the cyclohexyl ring. It is also worthy to note the characteristic band of the carbonyl group at 1667 cm^{-1}, and the bands between 1556 and 1603 cm^{-1} indicating the C = C stretch of the aromatic ring. Additionally, an intense band can be observed at 1255 cm^{-1} that can be assigned to the C-O stretch of the methylenedioxy group.

3.2. *In silico predictions and limitations*

Several similar compounds were identified via ChemSpider[19] with 1-(1,3-benzodioxol-5-yl)-2-(1-pyrrolidinyl)-1-butanone (MDPBP) covering most substructures while still having a high Tanimoto similarity (≥95%). When considering MDPBP an appropriate read-across compound, then it can be assumed that the effects, compared to ecstasy (MDMA), are moderately greater on the dopamine uptake transporter (DAT) but less on SERT. Furthermore, MDPBP, while being a potent inhibitor for those targets, is not associated with active serotonin and dopamine efflux[35]. MDPBP is chemically closely related to the more popular 3,4-methylenedioxypyrovalerone (MDPV).

Trace amine-associated receptor 1 (TAAR1), a trace amine-associated receptor present in the human central nervous system, is also considered a relevant target for some effects of amphetamine-type stimulants, particularly amphetamine, methamphetamine[36]. However, there is limited data on chemically more closely related compounds affecting TAAR1, and there is also lack of *in silico* data/tools to address this potential target.

Another consideration are psychedelic effects. The *N*-cyclohexyl group of *N*-cyclohexyl butylone may remind one of the highly potent stimulants of the NBOMe family, such as 2-(2H-1,3-Benzodioxol-5-yl)-N-[(2-methoxyphenyl)methyl]ethan-1-amine (MDPEA-NBOMe). However, this additional ring structure of *N*-cyclohexyl butylone is neither aromatic nor does it contain any oxygen in *para*-position, which seem to be perquisites for 5-HTR_{2A}-related activity cliffs[37]. Overall, the docking results for the targets for SERT and 5-HTR_{2A} do not suggest any significant steric hindrance (see **Fig. S4 and S5**). However, these results do not predict potency. The metabolites predicted via OECD QSAR Toolbox are summarized in **Fig. S6**. Most interesting are the hydroxy-adducts at the *N*-cyclohexyl. If the hydroxy metabolites are produced in significant quantities, then 5-HTR_{2A} agonism could be a clinically relevant mode of action (cf. classic psychedelics).

When assessing the risk of NPS, it is important to consider additional toxicity-driving substructures. Toxicity beyond a baseline is then often referred to as "excess toxicity". However, in this case, no structural alerts suggest excess toxicity, such as skin sensitization or mutagenicity. That means that toxicity is most likely driven by the same modes of action as MDMA and related cathinones, e.g. MDPBP. Nevertheless, structural alerts are limited to already identified substructures of concern. Therefore, it cannot be fully excluded that *N*-cyclohexyl butylone triggers adverse outcome pathway, e.g. endocrine targets.

3.3. *Effects reported by users*

Regarding effects, user 1 reported a mild effect similar to MDMA after taking two and a half pills. However, the effects only lasted for 1.5 h and the user noted a lack of typical pleasant effects. User 2 took three and a half pills with similar effects to MDMA but lacking euphoria and empathogenic feelings. User 3 took 250 mg of crystal form and did not report any effect. This fact may be due to this user took a lower dose, compared to the two previous cases.

4. Conclusions

This work presents the detection and characterization of the novel synthetic cathinone *N*-cyclohexyl butylone by combining GC–MS, UHPLC-HRMS, NMR and FTIR spectroscopy, in order to provide full analytical data that will help forensic and toxicological laboratories for the identification of this compound.

In addition, pharmacological/toxicological characterization was attempted based on *in silico* predictions and literature review, suggesting that *N*-cyclohexyl butylone is likely to have similar effects to other empathogens/stimulants, such as MDMA, MDPV, MDPBP etc.

Furthermore, it is unclear if *N*-cyclohexyl butylone or its metabolites may trigger psychedelic effects via 5-HTR_{2A} agonism. Considering the potential DAT > SERT selectivity[38], *N*-cyclohexyl butylone might have a moderately higher abuse liability than MDMA. This and the plethora of pharmacological/toxicological uncertainties associated with *N*-cyclohexyl butylone should remind policymakers of the need for regulation.

CRediT authorship contribution statement

María Mata-Pesquera: Investigation, Data curation, Writing – original draft, Visualization. **David Fabregat-Safont:** Methodology, Data curation, Writing – review & editing, Visualization. **Cristina Gil:** Conceptualization, Methodology, Resources, Writing – original draft. **Mireia Ventura:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision. **Fabian P. Steinmetz:** Formal analysis, Investigation, Writing – original draft. **María Ibáñez:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.108577.

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