Characterisation of hallucinogenic phenethylamines using high-resolution mass spectrometry for non-targeted screening purposes

Daniel Pasin[1], Adam Cawley[2], Sergei Bidny[3], Shanlin Fu[1*]

[1] Centre for Forensic Science, University of Technology Sydney, Broadway NSW 2007, Australia

[2] Australian Racing Forensic Laboratory, Racing NSW, Sydney NSW 2000, Australia

[3] Forensic Toxicology Laboratory, NSW Forensic and Analytical Science Service, Lidcombe, NSW 2141, Australia

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*Author to whom correspondence should be addressed:

Shanlin Fu

University of Technology Sydney

15 Broadway, Sydney NSW 2007, Australia

Email : Shanlin.Fu@uts.edu.au

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Abstract

Hallucinogenic phenethylamines such as 2,5-dimethoxyphenethylamines (2C-X) and their *N*-(2-methoxybenzyl) derivatives (25X-NBOMe) has seen an increase in novel analogues in recent years. These rapidly changing analogues make it difficult for laboratories to rely on traditional targeted screening methods to detect unknown new psychoactive substances (NPS). In this study, twelve 2C-X, six 2,5-dimethoxyamphetamines (DOX) and fourteen 25X-NBOMe derivatives, including two deuterated derivatives (2C-B-d_6 and 25I-NBOMe-d_9), were analysed using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS). Collision-induced dissociation (CID) experiments were performed using collision energies set at 10, 20 and 40 eV. For 2C-X and DOX derivatives, common losses were observed including neutral and radical losses such as NH_3 (17.0265 Da), $\bullet CH_6N$ (32.0500 Da), C_2H_7N (45.0578 Da) and C_2H_9N (47.0735 Da). 2C-X derivatives displayed common product ions at *m/z* 164.0837 ($[C_{10}H_{12}O_2]^{+\bullet}$), 149.0603 ($[C_9H_9O_2]^+$) and 134.0732 ($[C_9H_{10}O]^{+\bullet}$) while DOX derivatives had common product ions at *m/z* 178.0994 ($[C_{11}H_{14}O_2]^{+\bullet}$), 163.0754 ($[C_{10}H_{11}O_2]^+$), 147.0804 ($[C_{10}H_{11}O]^+$) and 135.0810 ($[C_9H_{11}O]^+$). 25X-NBOMe had characteristic product ions at *m/z* 121.0654 ($[C_8H_9O]^+$) and 91.0548 ($[C_7H_7]^+$) with minor common losses corresponding to 2-methylanisole ($C_8H_{10}O$, 122.0732 Da), 2-methoxybenzylamine ($C_8H_{11}NO$, 137.0847 Da) and $\bullet C_9H_{14}NO$ (152.1074 Da). Novel analogues of the selected classes can be detected by applying neutral loss filters (NLFs) and extracting the common product ions.

Introduction

Hallucinogenic phenethylamines have recently become popular due to their potent serotonergic activity, giving users a sense of euphoria and intense hallucinogenic episodes.[1-3] Their popularity is also attributed to literature such as the notable book published by Alexander Shulgin and Ann Shulgin, PiHKAL (***P**henethylamines **i** **H**ave **K**nown **A**nd **L**oved*) which outlines the synthesis of almost 200 ring-substituted phenethylamine derivatives and anecdotal information on the effects at certain doses from self-administration.[4] The most prevalent are the 2,5-dimethoxyphenethylamines, colloquially known as "2C's" or "2C-X", where "2C" refers to the 2 carbons atoms between the benzene ring and amine group and "X" refers to a letter or number corresponding to a possible substituent, e.g. 'B' (bromo), 'C' (chloro), and 'I' (iodo). Typically, these compounds are modified at the *para*-position by the addition of halogens, alkyl and thioalkyl groups.[5] In addition to the 2C's, PiHKAL also outlines the synthesis of 2,5-dimethoxyamphetamines or "DOX" derivatives which are structurally similar to the 2C's and only differ by the addition of an α-methyl group.[4,6,7]

Recently, more concerning derivatives of the ring-substituted phenethylamine class are the *N*-(2-methoxybenzyl) or 25X-NBOMe derivatives of the 2C-X compounds.[8] These compounds were first synthesized by Glennon *et al.*[9] and extensively studied by Ralf Heim [10] and Martin Hansen [11] as selective serotonin 2A (5-HT_{2A}) agonists. Generally, 25X-NBOMe derivatives were synthesised through reductive alkylation of selected 2C-X derivatives with 2-methoxybenzaldehyde.[8,10] The potency of these compounds has been demonstrated in literature, detailing the severe hallucinogenic episodes experienced by users of 25X-NBOMe derivatives, some of which have led to deaths as a result of their own actions.[12-15] Additionally, these compounds have also been investigated as 5-HT_{2A} and 5-HT_{2C} receptor agonist radioligands for positron emission tomography (PET).[16,17]

In the 2016 European Drug Report by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 98 NPS were reported for the first time to the European Union (EU) Early Warning System (EWS) in 2015 with 9 new phenethylamines (9%) reported. The number of phenethylamine analogues was lower than previous years with 14 new analogues reported out of 74 NPS in 2012 (19%) and 14 out of 81 in 2013 (17%). While phenethylamines made up only 9% of the new analogues reported in 2015, synthetic cathinones were the most prevalent with 36 new analogues (37%) and synthetic cannabinoids having 24 analogues reported (24%).[18] PiHKAL, Heim's and Hansen's theses are freely available online, allowing clandestine laboratory operators to obtain detailed synthetic procedures which can allow them to modify these molecules at their discretion. This proliferation of NPS can potentially make it difficult for forensic laboratories to detect novel compounds using high throughput traditional targeted screening methods that typically rely on the availability of certified reference materials (CRMs) and online databases. It is, therefore, crucial that class-based detection strategies are developed, in particular the determination of common product ions and losses through collision-induced dissociation (CID) studies using high-resolution mass spectrometry (HRMS) for identification of product ion formulae.

There have been several comprehensive CID studies on synthetic cathinones[19-21] using HRMS. There has been some literature published on single and multi-analyte characterization of hallucinogenic phenethylamines[22-28] and synthetic cannabinoids[29-31] using this technique, however, there have been no publications on the comprehensive characterization of these classes with explicitly stated rules or guidelines for the detection of novel analogues in a non-targeted screening approach. The aim of this study was to fill this current gap in research and to investigate the CID pathways of 2C-X, DOX and 25X-NBOMe derivatives using high-

resolution mass spectrometry (HRMS) in order to develop a non-targeted strategy for the detection of new emerging analogues.

Experimental

Chemicals and Reagents

Hydrochloride salts of 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 2,5-dimethoxy-3,4-dimethylphenethylamine (2C-G), 2,5-dimethoxy-4-methylthiophenethylamine (2C-T), 2-(3,4-dimethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25G-NBOMe), 2-(4-methylthio-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25T-NBOMe), 2,5-dimethoxyamphetamine (DOH/2,5-DMA), 4-bromo-2,5-dimethoxyamphetamine (DOB), 2,5-dimethoxy-4-methylamphetamine (DOM) and 2,5-dimethoxy-4-methylthioamphetamine (DOT) were purchased as powders from the National Measurement Institute (NMI, North Ryde, NSW, Australia). Hydrochloride salts of 4-chloro-2,5-dimethoxyphenethylamine (2C-C), 2,5-dimethoxy-4-methylphenethylamine (2C-D), 4-ethyl-2,5-dimethoxyphenethylamine (2C-E), 2,5-dimethoxy-4-propylphenethylamine (2C-P), 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25C-NBOMe), 2-(4-methyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25D-NBOMe), 2-(4-ethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25E-NBOMe), 2-(2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25H-NBOMe), 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe), 2-(4-nitro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25N-NBOMe), 2-(4-propyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25P-NBOMe), 2-(4-ethylthio-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25T2-NBOMe), 2-(4-isoproprylthio-2,5-

dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25T4-NBOMe), 2-(4-propylthio-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25T7-NBOMe), 4-ethyl-2,5-dimethoxyamphetamine (DOET) and 4-iodo-2,5-dimethoxyamphetamine (DOI) were purchased as powders from PM Separations (Capalaba, QLD, Australia). Deuterated standards, 2C-B-d_6 and 25I-NBOMe-d_9, were purchased as 1 mg/mL methanolic solutions from PM Separations. LC-MS grade acetonitrile was purchased from Chem-Supply Pty Ltd (Gillman, SA, Australia) and LC-MS grade formic acid and AR grade ammonium formate was purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Ultrapure grade water (18.2 MΩ cm^{-1}) was obtained from a Sartorius arium® pro ultra-pure water system (Goettingen, Germany).

Sample preparation

Standards that were obtained as 1 mg/mL methanolic solutions were transferred into 2-mL amber sample vials. Purchased powders were weighed into 1 mg portions and dissolved in 1 mL of methanol to achieve a concentration of 1 mg/mL. Working solutions were prepared by further dilution to 1 mg/L in 2-mL sample vials and capped. All samples were stored in a refrigerator (4^{o}C) until analysis.

Instrumental Analysis

Chromatographic separation was achieved using an Agilent Technologies 1290 Infinity series ultra-performance liquid chromatograph (UPLC), consisting of an Inifinity II high speed pump (G4220A), thermostat (FC/ALS G1330B), column compartment (G1316C, 25^{o}C) and autosampler compartment (G4226A, 8^{o}C) coupled to an Agilent Technologies 6510 quadrupole time-of-flight mass spectrometer (QTOF-MS) fitted with a dual electrospray ionization (ESI) source. Instrument control and data acquisition was performed using Agilent Technologies MassHunter LC-MS Data Acquisition Software (Version B.05.01). A sample

volume of 1 µL was injected onto an Agilent Technologies Poroshell 120 C18 column (2.1 x 75 mm, 2.7 µm particle size) using a gradient elution with a flow rate of 0.4 mL/min with a total run time of 17 minutes. Mobile phase A consisted of 20 mM ammonium formate and mobile phase B consisted of acetonitrile containing 0.1% (% v/v) formic acid. Initial mobile phase composition was 87% A which was held for 0.5 min and then decreased linearly to 50% A over 9.5 min. It was then decreased to 5% A over 0.75 min, held for 1.5 min and then returned to the initial conditions over 0.25 min with a final hold time of 2.5 min and post-run equilibration time of 2 min.

The QTOF-MS was operated in positive electrospray ionisation (ESI+) mode using Extended Dynamic Range (2 GHz) with capillary and fragmentor voltages set to 3500 V and 180 V, respectively. An Auto-MS/MS (data-dependent) acquisition mode was used over a mass range of *m/z* 50-1000 for both MS and MS/MS experiments with scan rates of 1 and 3 spectra/s, respectively. A maximum of 3 precursors from the MS scan were selected for CID per cycle with a cycle time of 2.1 s and an abundance threshold of 200 counts. CID experiments were performed at collision energies (CE) of 10, 20 and 40 eV in separate analyses with nitrogen as the collision gas.

Data Analysis

All data acquired was processed using Agilent Technologies MassHunter Qualitative Analysis Software (Version B.06.00). The Find by Auto-MS/MS function was used to generate MS and MS/MS spectra for all precursors selected for CID. Molecular formulae for product ion mass-to-charge (*m/z*) values were generated using MassHunter's molecular formula generator (MFG) algorithms with elements of interest set to $C_{(7\rightarrow25)}$, $N_{(0\rightarrow2)}$, $O_{(0\rightarrow5)}$, $H_{(7\rightarrow30)}$, $S_{(0\rightarrow1)}$, $Cl_{(0\rightarrow1)}$, $Br_{(0\rightarrow1)}$, $I_{(0\rightarrow1)}$ and $D_{(0\rightarrow9)}$.

Results and Discussion

In this study, twelve 2C-X, six DOX and fourteen 25X-NBOMe analogues were analysed by UPLC-QTOF-MS in order to evaluate their CID pathways and assess the applicability of the generated product ions for non-targeted detection of novel hallucinogenic phenethylamine analogues. The panel of selected analytes also contained isotopically labeled 2C-B-d_6 and 25I-NBOMe-d_9 which were used to aid in the elucidation of product ions. Structures, retention times and precursor ion data for all analytes are listed in Supporting Information, Table S1.

Chromatographic analysis of selected hallunicogenic phenethylamines

All selected analytes eluted within the first 10 min of the chromatographic analysis with the least substituted 2C-X derivative (2C-H) eluting first at 1.98 min and the propyl 25X-NBOMe derivative (25P-NBOMe) eluting last at 9.40 min. All 2C-X and DOX derivatives eluted within the first 6 min of analysis with the latter typically having longer retention times than the respective 2C-X derivative. For example, 2C-B eluted at 3.82 min while its α-methyl derivative, DOB, eluted later at 4.28 min due to the additional methyl group resulting in greater retention on the reversed phase Poroshell EC120 C18 column. This was also observed for 2C-E/DOET, 2C-H/DOH, 2C-I/DOI, 2C-D/DOM and 2C-T/DOT. The panel of selected analytes had several isobaric pairs that were successfully resolved in the 15 minute chromatographic analysis. For example, isobaric analytes 2C-E (t_r = 4.56 min), 2C-G (t_r = 4.17 min) and DOM (t_r = 3.86 min) were all baseline separated. Interestingly, it can be observed that the addition of alkyl groups to the *para*-position on the 2,5-dimethoxyphenylamine moiety have a greater retaining effect compared to the substitution at the α-carbon. For example, isobaric derivatives 2C-D (*para*-methyl) and DOH (α-methyl) have retention times of 3.25 and 2.44 min, respectively. The 25X-NBOMe compounds

generally eluted towards the end of the chromatographic run due to their larger structure with the least substituted derivative (25H-NBOMe) eluting first at 5.97 min and the propyl derivative (25P-NBOMe) eluting last at 9.40 min as mentioned previously.

CID of hallucinogenic phenethylamines

The main advantage of using HRMS is that losses and product ions which have the same nominal mass can be differentiated to provide greater confidence in elucidating product ions structures. For example, losses of -NH_3 and -OH would give a nominal mass change of 17 Da but an accurate mass change of 17.0265 and 17.0027 Da, respectively, which can be differentiated by an instrument that has sufficient mass resolution.

When determining the CID pathways and elucidating the structures of product ions using ESI, it is important to consider the basic rules for CID which have been established in literature.[32-34] In addition, the 'even-electron' rule stipulates that even electron (EE) ions will typically fragment to EE ions via neutral losses (NLs) under electron ionization (EI) in gas chromatography – mass spectrometry (GC-MS). It has also been observed that odd electron (OE) ions such as radical cations ($M^{+\bullet}$)[35] can form other OE ions through NLs but OE ions typically do not form EE ions due to radical losses other than in a limited number of examples.[36] The use of HRMS can assist in determining whether a product ion is an EE or OE ion by using information such as the chemical formula and double bond equivalents. The MFG algorithm in the MassHunter software can designate a product ion as EE or OE.

Evaluation of product ion spectra at each of the selected collision energies showed that a CE of 20 eV provided a wide range of product ions for all analytes. In some cases, the precursor ion ($[M+H]^+$) could still be observed whilst also having lower mass product ions such as the tropylium cation ($[C_7H_7]^+$, *m/z* 91.0542) observable. Therefore, all results presented herein will be based on those produced at a collision energy of 20 eV.

General CID of 2C-X and DOX compounds

The product ion data for all 2C-X and DOX analytes are listed in Table S2 and the representative product ion spectra are illustrated in Figure S1. The CID pathways for 2C-X and DOX analytes are illustrated in Figure 1. For all 2C-X and DOX analytes, the first product ion was generated by simple inductive cleavage of ammonia (NH_3, 17.0265 Da) with charge-migration fragmentation (CMF) to the α-carbon to produce the EE ion **1** ([M-NH_3+H]$^+$ or [$C_{10}H_{12}O_2R^1R^2R^3$]$^+$). Where R^1 corresponds to the "primary" or *para*-substituent (alkyl, thioalkyl, halogen), R^2 is the "secondary" or *meta*-substituent and R^3 is the α-carbon substituent (R^3 = H and R^3 = CH_3 for 2C-X and DOX derivatives, respectively). For DOX analytes, there is also β-cleavage of the phenethylamine chain with a NL of C_2H_7N (45.0578 Da) to give product ion **2** ([M-C_2H_7N+H]$^+$ or ([$C_9H_9O_2R^1R^2$]$^+$). A base peak for many of the selected analytes was the loss of NH_3 followed by radical cleavage of the ether methyl group (•CH_3, 15.0235 Da) to produce the distonic OE product ion **3** ([M-CH_6N+H]$^{+\bullet}$ or [$C_9H_9O_2R^1R^2R^3$]$^{+\bullet}$; total loss of 32.0500 Da from the precursor ion). Evaluation of the product ion spectra for 2C-B-d_6 (which has deuterium atoms incorporated into both methoxy groups) confirmed radical cleavage of the aromatic methoxy groups located at the 2- or 5-position. The precursor ion exhibited a loss of 35.0689 Da as opposed to 32.0500 Da corresponding to the loss of a deuterated methyl radical (•CD_3, 18.0424) and NH_3. The formation of an OE ion from an EE ion is a violation of the previously mentioned heuristic 'even-electron' rule, however, a study by Thurman *et al.*[36] demonstrated that OE ions can be formed from EE ions under ESI by losses of stable radicals such as •CH_3 and •OCH_3 from aromatic methoxy groups. Additionally, it is postulated by Levsen *et al.*[37] that aromatic OE ions may be more stable than their EE counterparts. In this case, the location of the radical (oxygen adjacent to the aromatic ring) may stabilize the OE ion due to resonance of the radical around the aromatic ring. Subsequent radical cleavage of •CH_3 from the alternate

methoxy group following the losses of •CH_3 and NH_3 was observed to yield product ion **4** ($[M\text{-}C_2H_9N+H]^+$ or $[C_8H_6O_2R^1R^2R^3]^+$), corresponding to a loss of 47.0735 Da. This was confirmed by the product ion spectra for 2C-B-d_6 which showed a loss of 53.1114 Da from the precursor ion corresponding to NH_3 and two •CD_3 radicals. A study by Karni *et al.*[35] stated that subsequent radical losses can occur if a state of aromaticity can be achieved. In this case, the formation of **4** would initially result in an EE diradical cation ($M^{+\bullet\bullet}$) but it is postulated that radical migration would occur in order to generate an aromatic EE cation. The order of radical cleavage could not be investigated in this study and would need to be confirmed by isotopically labeled analytes of which only one methoxy group contained deuterium atoms.

Product ions **1**-**4** resulted from NLs producing different *m/z* values for selected analytes excluding isobaric compounds, however, there are some product ions which are common to the majority of analytes. There is an OE product ion (**5**) observed at *m/z* 164.0837 ($[C_{10}H_{12}O_2]^{+\bullet}$) and *m/z* 178.0994 ($[C_{11}H_{14}O_2]^{+\bullet}$) for 2C-X and DOX analytes, respectively, corresponding to the loss of NH_3 and radical cleavage of the *para*-substituent (•R^1). The loss of •R^1 was also confirmed by the absence of the $[M+2+H]^+$ isotope for the bromo and chloro derivatives 2C-B/DOB and 2C-C, respectively. It was also observed that the *meta*-substituent (R^2) is retained with 2C-G (R^2 = CH_3) having a product ion at *m/z* 178.1009 ($[C_{11}H_{14}O_2]^{+\bullet}$, +8.4 ppm) rather than *m/z* 164.0837. A subsequent loss of •CH_3 following the losses of NH_3 and •R^1 produced the diradical EE product ion at *m/z* 149.0603 ($[C_9H_9O_2]^+$) for 2C-X derivatives and *m/z* 163.0754 ($[C_{10}H_{11}O_2]^+$) for DOX derivatives and 2C-G. It is likely the resulting diradical cations underwent radical migration to form cations **6a** and **6b** corresponding to cleavages at the 2- and 5-methoxy groups, respectively.

For 2C-X analytes only, a product ion corresponding the to the NL of formaldehyde (CH_2O) following the loss of NH_3 and •R^1 to yield the OE ion **7** at *m/z* 134.0732 ($[C_9H_{10}O]^{+\bullet}$) and *m/z*

148.0883 ($[C_{10}H_{12}O]^{+\bullet}$) for 2C-G. These proposed losses were supported by the product ion spectra of 2C-B-d_6 which had a product ion at *m/z* 138.0980 corresponding to $[C_9H_6D_4O]^+$, suggesting that there is rearrangement of a deuterium atom from the methoxy group to the aromatic ring. Based on the proposed CID pathways presented for product ions **1-4**, it could have been hypothesized that from product ion **5**, stepwise cleavage of $\bullet CH_3$ radicals would occur, however, that would have yielded an OE product ion at *m/z* 134.0368 ($[C_8H_{10}O_2]^{+\bullet}$). It is likely that this ion was not formed as it would have involved three radical cleavages which may not be considered energetically favourable.

Similar to **7** for 2C-X analytes, there is an EE product ion observable at *m/z* 147.0804 ($[C_{10}H_{11}O]^+$) for DOX analytes which is proposed to be formed by the radical cleavages of $\bullet R^1$ and $\bullet OCH_3$ from the 5-position to yield two adjacent radicals which is proposed to form the benzyne cation **8**. Another product ion exclusive to the DOX class is the EE product ion **9** ($[C_9H_{11}O]^+$, *m/z* 135.0810) which is likely formed by the NL of CH_2O and radical cleavages of $\bullet R^1$ and $\bullet CH_3$ followed by radical rearrangement.

The isobaric thioalkyl derivatives, 2C-T-4 and 2C-T-7 ($[M+H]^+$ = *m/z* 256.1371), did not have product ions corresponding to the loss of C_2H_9N present. However, both had an EE product ion at *m/z* 197.0631 ($[C_{10}H_{13}O_2S]^+$) corresponding to the loss of C_3H_9N which was proposed to be from the NL of NH_3 followed by the loss of 1-propene or 2-propene from 2C-T-4 and 2C-T-7, respectively. These product ions subsequently underwent radical cleavage of the methoxy group to yield the OE ion $[C_9H_{10}O_2S]^{+\bullet}$ (*m/z* 182.0401). The methylthio derivative, 2C-T, did not have a product ion at *m/z* 197.0631 since this ion is formed by the loss of the alkene and is not possible by a methyl derivative. Extrapolation to the ethylthio derivative (2C-T-2), which was not included in this study would suggest that it may form *m/z* 197.0631 by the loss of ethylene.

There have been studies on 2C-X compounds using GC-MS published by Theobald *et al*.[38-40], however, due to the different ionization techniques and the requirement for derivatization for GC-MS, product ion spectra can differ significantly and cannot be translated to LC-MS necessitating re-characterization. Zuba *et al*.[26] analysed 2C-E and 2C-G by LC-QTOF-MS and also observed product ions **1**,**3** and **4** at a collision energy of 15 eV. Similar to present study, they postulated that these product ions formed from the loss of NH_3, $\bullet CH_6N$ and CH_9N However, they did not suggest the location where the methyl radicals were lost (product ions **3** and **4)**. A study conducted by Boumrah et al.[22] identified that 2C-B was a metabolic product of 25B-NBOMe when analysed by HRMS. They postulated that product ion *m/z* 217.9551 was formed by the β-cleavage of the amine followed by cleavage of a methyl group from one of the aromatic methoxy groups which contrasts to what was determined in this study. In addition, the CID pathway for *m/z* 227.9786 was not determined in this study.

A limitation of this study is that only six DOX derivatives were analysed so it is recommended that in future studies the panel would be extended to include newly available DOX CRMs to provide a more representative overview of the CID pathways for this class. In addition, there have been other ring-substituted phenethylamine derivatives that have been encountered such as the dihydrofuran derivative, 2-(4-bromo-2,3,6,7-tetrahydrofuro[2,3-f][1]benzofuran-8-yl)ethanamine (2C-B-Fly), and difuranyl derivative 1-(8-bromobenzo[1,2-*b*; 4,5-*b*']difuran-4-yl)-2-aminopropane (Bromo-DragonFLY) which could have the bromine atom replaced with other functional groups.[7,41,42]

Collision induced dissociation (CID) of NBOMe derivatives

The product ion data for selected 25X-NBOMe analytes are listed in Table S3 and their CID pathways are illustrated in Figure 2. For all 25X-NBOMe analytes, there is a base peak corresponding to the CMF of the 2,5-dimethoxyphenethylamine moiety to give the EE 2-methoxybenzyl cation **10** ($[C_8H_9O]^+$, *m/z* 121.0654) followed by the NL of CH_2O to yield the EE tropylium cation **11** ($[C_7H_7]^+$, *m/z* 91.0548). In addition to these major product ions, there is to a small extent charge retention fragmentation (CRF) through α-cleavage of the 2-methylanisole moiety ($C_8H_{10}O$, 122.0732 Da) to yield the EE product ion **12** ($[C_{10}H_{12}NO_2R^1R^2]^+$) . There is also α-cleavage of the 2-methoxybenzylamine moiety ($C_8H_{11}NO$, 137.0847 Da) to produce the EE product ion **13** ($[C_{10}H_{11}O_2R^1R^2]^+$) which then undergoes radical cleavage of $\bullet CH_3$ ($C_9H_{14}NO$, 152.1074 Da) to yield the OE product ion **14** $[C_9H_9O_2R^1R^2]^{+\bullet}$. The CID pathways for 25X-NBOMe compounds are relatively characteristic and do not fragment as extensively as their 2C-X counterparts, following mostly EE to EE transitions. Figure S2 illustrates the characteristic product ion spectra for selected 25X-NBOMe derivatives. Zuba *et al.*[43] analysed 25D-NBOMe, 25E-NBOMe and 25G-NBOMe by LC-QTOF-MS and observed product ions **10**-**14** for all three analytes. Poklis *et al.*[44] utilized Direct Analysis in Real Time AccuTOF™ mass spectrometry (DART-MS) for the analysis of 25X-NBOMe derivatives on blotter paper and observed product ions **10**, **11** and **13**. In addition, they also observed the loss of iodine to produce *m/z* 302.1776 for 25I-NBOMe which was not observed in the present study.

The major product ions for the selected 25X-NBOMe analytes are the 2-methoxybenzyl cation (*m/z* 121.0654) and tropylium cation (*m/z* 91.0548) which are indicative of the *N*-substituted moiety. Therefore, it may be reasoned that reductive alkylation of a 2C-X derivative with a different benzaldehyde would produce a different product ions corresponding to the benzylic cation of the substituted benzaldehyde. There have been novel derivatives reported in literature which were produced by the reductive alkylation of 2C-X derivatives with 2 -fluorobenzaldehyde and 1,3-benzodioxole-4-carbaldehyde to produce the *N*-substituted derivatives *N*-(2-fluorobenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine (25I-NBF) and *N*-(2,3-methylenedioxybenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine (25I-NBMD), respectively.[44,45] Sekula et al.[45] analysed 25I-NBMD by LC-QTOF-MS and found that it had a base peak corresponding to the 1,3-benzodioxole-4-methyl cation ($[C_8H_7O_2]^+$, *m/z* 135.0447) but did not observe the tropylium cation when the precursor ion was subjected to a CE of 15 eV. Therefore, if a characteristic "25X-NBOMe-like" product ion spectra is observed which has a product ion at *m/z* 91.0548 and has a major product ion which is not *m/z* 121.0654 it may indicate the presence of a novel analogue which is not a *N*-(2-methoxybenzyl) derivative, however, this would need to be validated by characterization of different *N*-substituted derivatives.

Non-targeted screening approach

The recent proliferation of NPS and the lack of CRMs available for novel analogues typically results in these compounds going undetected in conventional targeted screening techniques where retention time, precursor and product ion data are collected from CRMs or databases. Therefore, a holistic or unbiased approach is required to successfully detect and tentatively identify novel compounds so these samples of interest can be flagged and archived until suitable CRMs become available for confirmation. Non-targeted, or untargeted screening, typically refers to the method of data acquisition: data-independent acquisition (DIA)

involves CID of all precursor ions whereas data-dependent acquisition (DDA) involves the CID of a limited number of precursor ions which are above user-defined thresholds. Most often this data is then compared to either in-house databases populated with retention times and mass data obtained from previous analyses of CRMs or comprehensive online databases for compound detection and identification.[46,47] However, in the case of the work presented here, a non-targeted screening approach refers to the interrogation of DIA/DDA mass spectrometry data to detect novel analogues using class-based mass spectrometric characteristics such as common NLs and product ions which is not CRM or database-driven. This strategy is applied to MS/MS or MS^2 data and, therefore, its efficacy relies on the use of an appropriate data acquisition method. Both DIA and DDA can be utilized for non-targeted data acquisition purposes, however, the use of DDA, such as the technique which was used in this study (Auto MS/MS), will limit the amount of precursor ions selected for CID and, therefore, reduce the likelihood that analytes of interest will be detected, particularly if there are more abundant precursor ions from matrix components within the same scan. It can be reasoned that the number of precursors selected for CID per cycle/scan may be increased, however, the scan rate (spectra/s) is limited by the instruments data processing capabilities. DIA provides better coverage of precursor ion CID and, therefore, improves the likelihood that a novel analogue will be subjected to CID. Many instrumentation vendors have DIA options available such as All Ions MS/MS (Agilent Technologies), MS^E (Waters Corporation) and broadband CID (bbCID, Bruker) that acquire data by alternating between low and high energy channels. Additionally, a DIA method called sequential window acquisition of all theoretical mass spectra (SWATH™, AB Sciex), acquires data by sequentially allowing increments of a selected mass range through the quadrupole and into the collision cell. The major advantage of DIA is that comprehensive full scan data is acquired that can be

retrospectively interrogated for new analytes of interest without the need for re-extraction and re-analysis

Detection of novel hallucinogenic phenethylamine derivatives can be achieved by the application of basic data processing techniques to acquired MS/MS data such as extracted ion chromatograms (EIC or XIC) and neutral loss filtering (NLF). The generation of NLFs and EICs was done manually in this case but MassHunter offers the ability to automate user-defined data processing steps which can be applied to multiple data files for rapid screening. NLF is typically a data processing technique which is offered in metabolomics software such as Metabolynx (Waters Corp). Due to its usefulness in undertaking non-targeted screening of unknown compounds, NLF may find its integration into more MS data processing software in the future. Table 1 summarises what potential core structures may be present based on the detection of particular NLs and product ions. The presence of chromatographic peaks after NLF application of 17.0265, 32.0500 and 47.0735 Da NLFs may indicate if any 2C-X or DOX derivatives are present but will not necessarily differentiate between the two classes. However, the presence of a chromatographic peak with the NLF application of 45.0578 Da may indicate that the derivative is likely a DOX derivative. Figure 3 illustrates an example for the application of the precursor neutral loss chromatogram (pNLC) function in MassHunter using the isobaric analytes, 2C-D and DOH. If NLF is unavailable, detection of novel analogues can be achieved by generating EICs of the common product ions such as *m/z* 164.0837, 149.0603 and 134.0732 for 2C-X derivatives and *m/z* 178.0994, 163.0754, 147.0804 and 135.0810 for DOX derivatives. However, if *m/z* 178.0994 and 163.0754 are present but *m/z* 147.0804 and 135.0810 (which are exclusive to the DOX class) are absent, it may indicate a 2C-X derivative where $R^2 = CH_3$. Additionally, presence of peaks in the 17.0265 and 32.0500 Da NLF and the EICs for *m/z* 197.0631 and 182.0401 may indicate the

presence of a thio derivative. Figure 4 illustrates an example of the application EICs for common product ions using 2C-I and DOI.

Similarly, detection of 25X-NBOMe derivatives can be achieved by the application of NLFs for 122.0732, 137.0847 and 152.1074 Da, however, the absence of chromatographic peaks for some of these NLFs may not necessarily indicate a false negative as some of the product ions formed by the NLs have low relative abundances and will depend on the sensitivity of the NLF. Detection of 25X-NBOMe derivatives would be better achieved by generating EICs for *m/z* 91.0548 and 121.0654 and checking whether the product ion spectra contains only those ions since the tropylium cation and 2-methoxybenzyl cation are not exclusive to the 25X-NBOMe hallucinogenic phenethylamines.

Conclusion

Hallucinogenic phenethylamines were successfully characterized by LC-QTOF-MS using CID at different CEs. The 2C-X and DOX derivatives had common losses of NH_3, CH_6N and C_2H_9N and common product ions at *m/z* 164.0837, 149.0603 and 134.0732 for 2C-X derivatives and *m/z* 178.0994, 163.0754, 147.0804 and 135.0810 for DOX derivatives. The 25X-NBOMe derivatives had characteristic product ion spectra with abundant ions at *m/z* 121.0654 and 91.0548, together with minor NLs corresponding to 2-methylanisole and 2-methoxybenzylamine and $C_9H_{14}NO$. Screening for these common NLs and product ions can be used in a non-targeted screening approach to detect and tentatively identify novel analogues.

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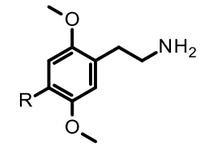
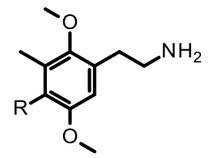
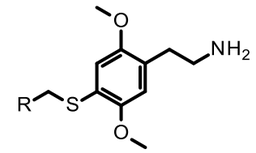
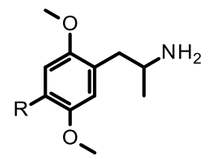
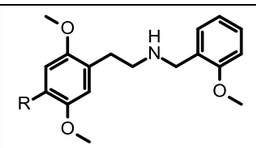
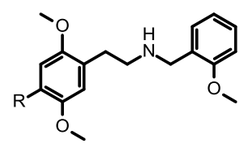
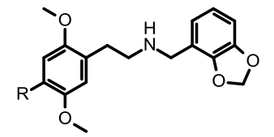
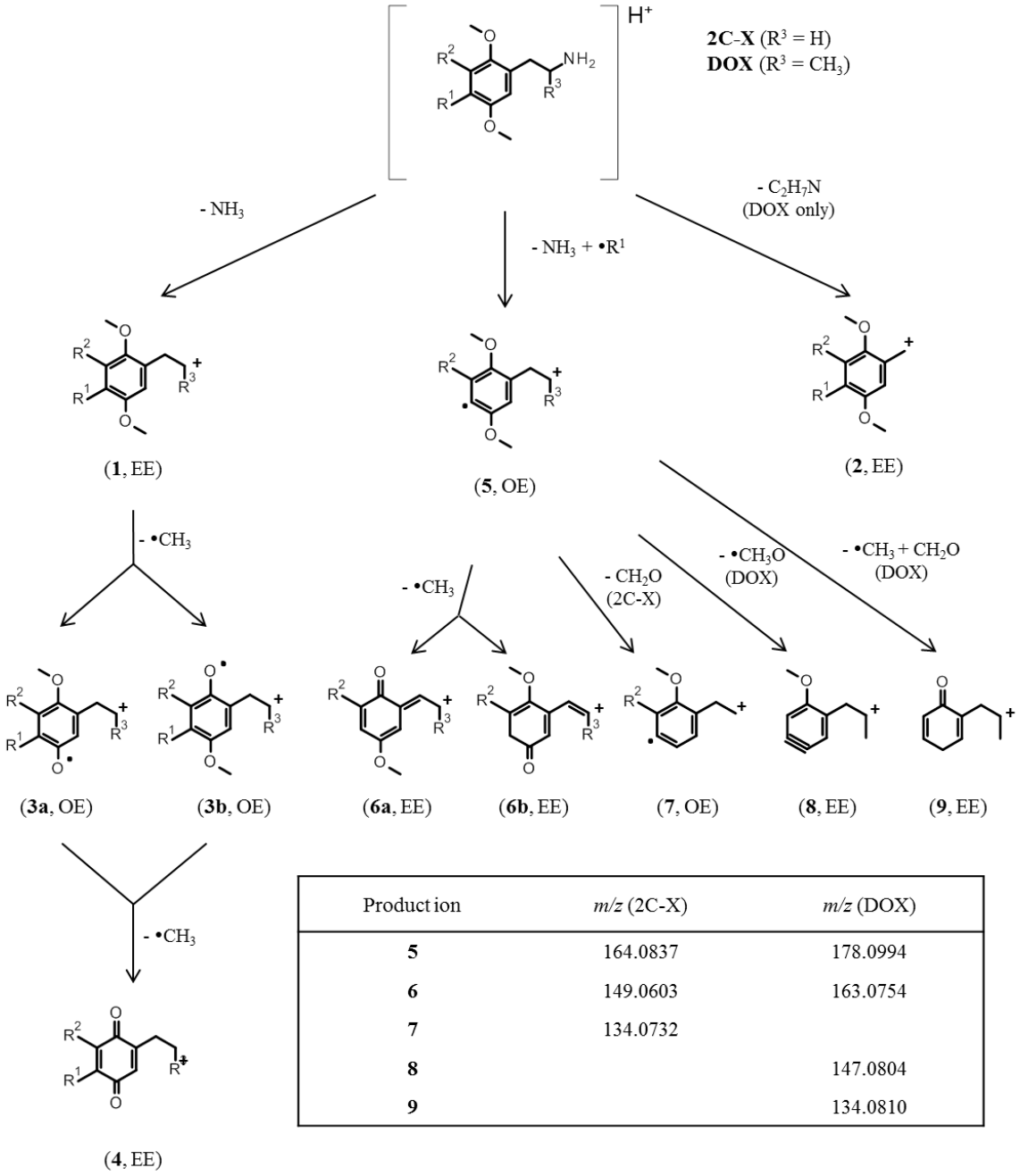
Table 1. Potential core structures of hallucinogenic phenethylamines associated with the presence of neutral losses (Da) and product ions (*m/z*)

Common losses (Da)				Product ions (*m/z*)										Potential Core Structure
17.0265	32.0500	45.0578	47.0735	134.0732	135.0810	147.0804	148.0883	149.0603	163.0754	164.0837	178.0994	197.0631	182.0401	
Y	Y		Y	Y				Y		Y				
Y	Y		Y				Y		Y		Y			
Y	Y			Y				Y		Y		Y	Y	
Y	Y	Y	Y		Y	Y			Y		Y			

Common losses (Da)					Product ions (*m/z*)			Potential Core Structure
122.0732	137.0847	152.1074	136.0524 [a]	151.0633 [a]	91.0548	121.0654	135.0441 [a]	
					Y	Y		
Y		Y		Y	Y	Y		

	Y	Y		Y	

[a]Identified in the study by Sekuła *et al.*[45]



Product ion	*m/z* (2C-X)	*m/z* (DOX)
5	164.0837	178.0994
6	149.0603	163.0754
7	134.0732	
8		147.0804
9		134.0810

Figure 1. CID pathways for 2C-X and DOX derivatives at 20 eV

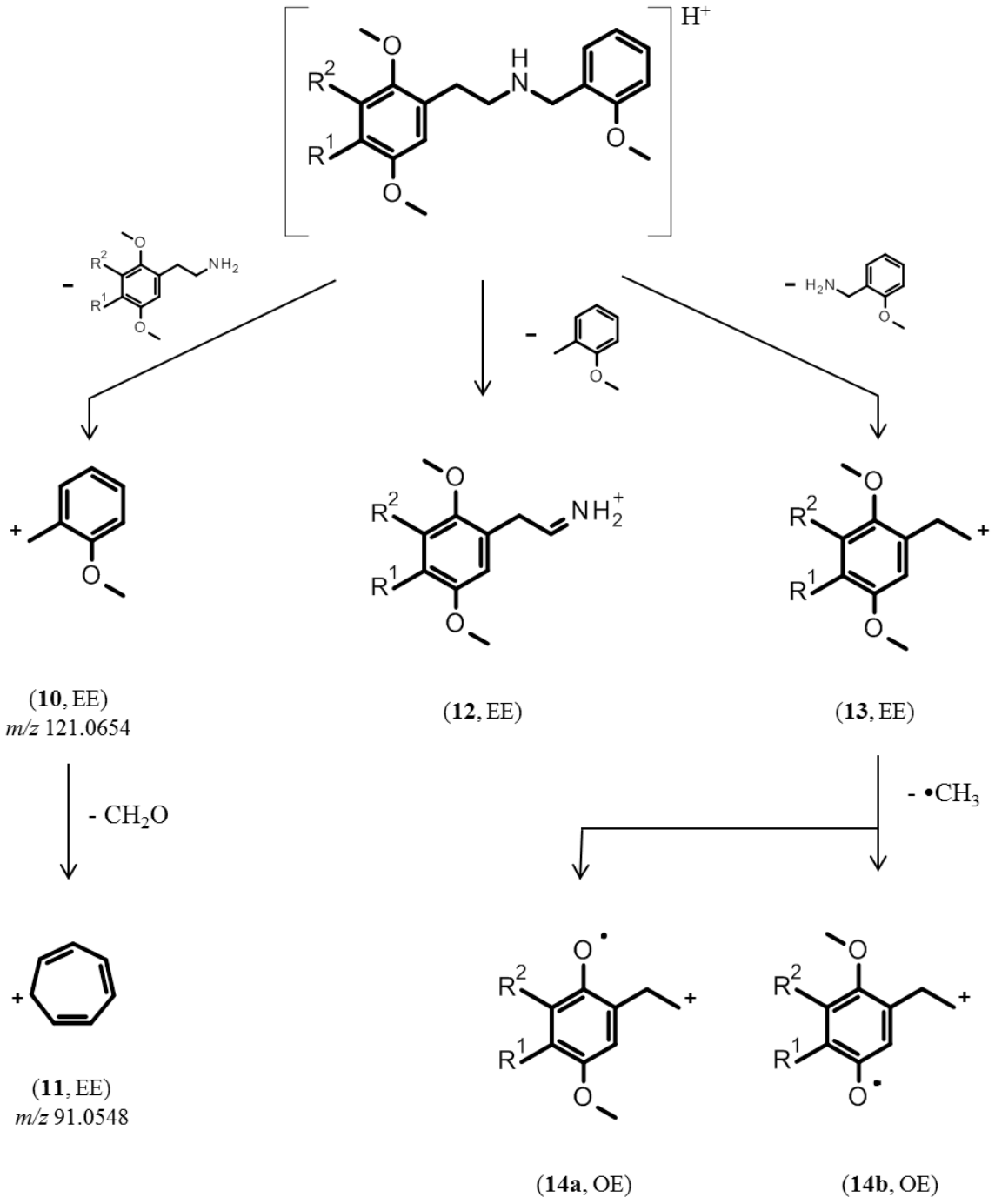


Figure 2. CID pathways for 25X-NBOMe derivatives at 20 eV

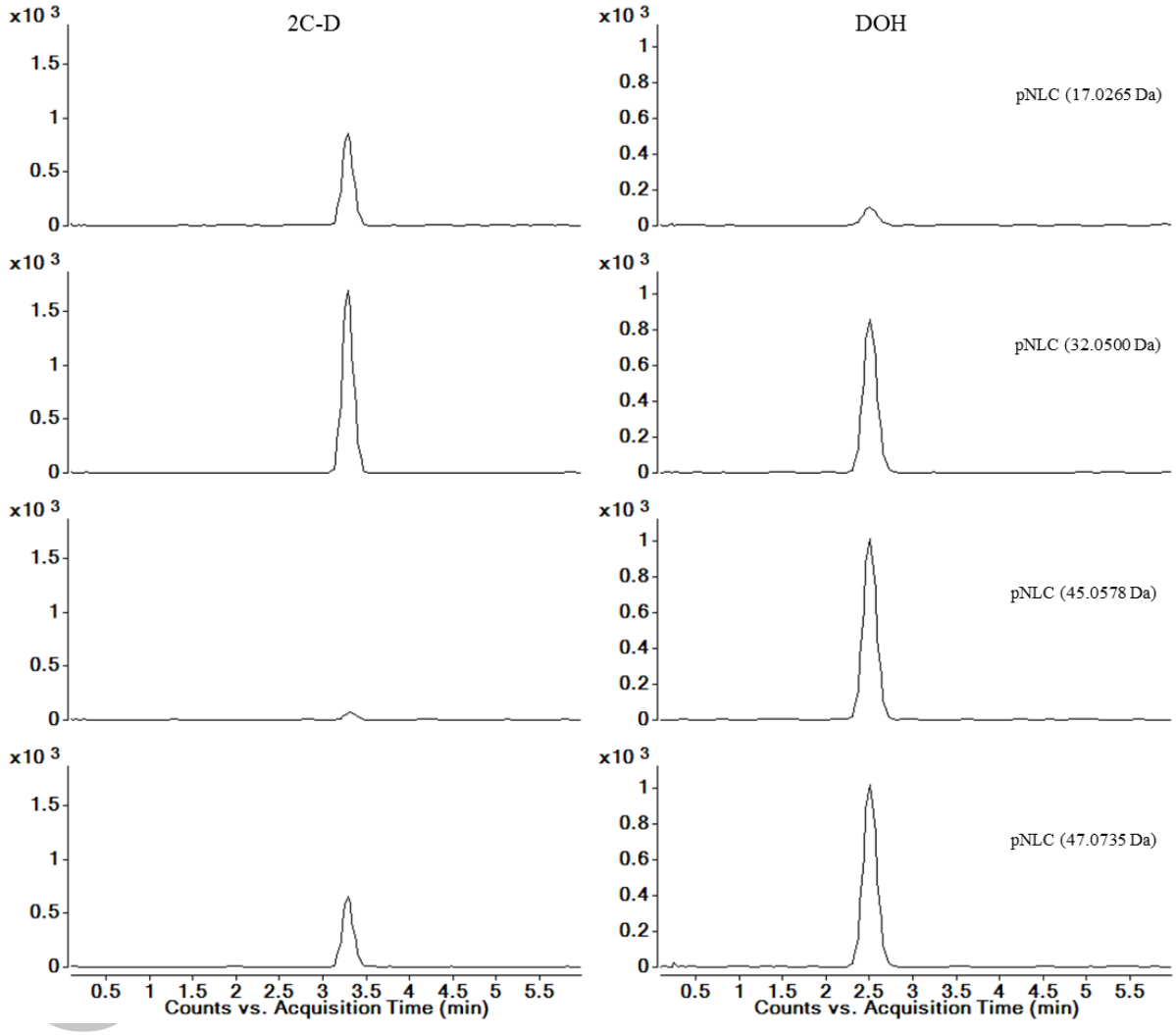


Figure 3. pNLCs of 17.0265, 32.0500, 45.0576 and 47.0735 Da for 2C-D and DOH at 20 eV

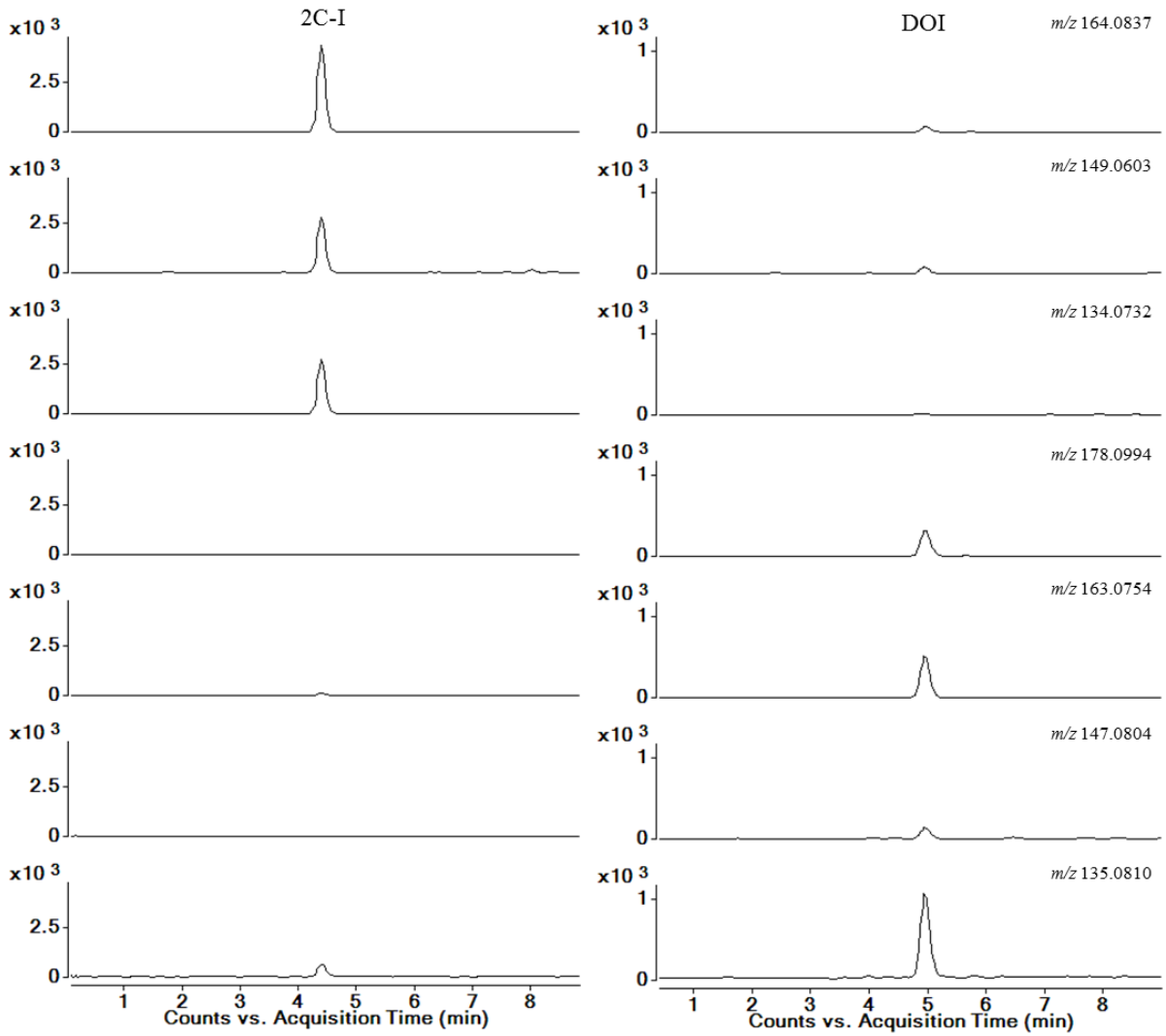


Figure 4. EICs for 2C-I and DOI at m/z 164.0837, 149.0603, 134.0732, 178.0994, 163.0804, 147.0804 and 135.0810 at 20 eV