

Metabolism of JWH-015, JWH-098, JWH-251, and JWH-307 in silico and in vitro: a pilot study for the detection of unknown synthetic cannabinoids metabolites

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Abstract This pilot study was performed to study the main metabolic reactions of four synthetic cannabinoids: JWH-015, JWH-098, JWH-251, and JWH-307 in order to setup a screening method for the detection of main metabolites in biological fluids. In silico prediction of main metabolic reactions was performed using MetaSite™ software. To evaluate the agreement between software prediction and experimental reactions, we performed in vitro experiments on the same JWHs using rat liver slices. The obtained samples were analyzed by liquid chromatography-quadrupole time-of-flight and the identification of metabolites was executed using Mass-MetaSite™ software that automatically assigned the metabolite structures to the peaks detected based on their accurate masses and fragmentation. A comparison between the experimental findings and the in silico metabolism prediction using MetaSite™ software showed a good accordance between experimental and in silico data. Thus, the use of in silico metabolism prediction might represent a useful tool for the forensic and clinical toxicologist to identify possible main biomarkers for synthetic cannabinoids in biological fluids, especially urine, following their administration.

Keywords Synthetic cannabinoids · In silico metabolism prediction · In vitro metabolism · Forensic toxicology

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Introduction

In the last two decades, a large number of new drugs with psychoactive properties from several drug classes have appeared on the illicit drug market. The “challenge of new psychoactive substances” is in fact a worldwide problem, and UNODC recently published a report on this issue [1]. New psychoactive substances (NPS) are synthesized by slight modifications of the known psychoactive “parent” compound, to obtain similar—or even stronger—psychoactive effects and to circumvent the laws, being not included in the lists of controlled substances yet. Among them, synthetic cannabinoids, known also as “herbal highs” and often sold as “air fresheners”, flooded the market of illicit drugs, and are mainly sold over the internet or in so-called “smart shops”, but also at street level. The first synthetic cannabinoids began to appear on the market in 2004, and nowadays the majority of them are considered illicit substances in many countries, as at national level, many of them are included in the lists of controlled substances.

Beside the identification of seized drugs, the challenge for forensic and clinical toxicologists is their identification in biological fluids, mainly urine, following the administration of such substances. Only limited data are available about the metabolism of the huge variety of synthetic cannabinoids. In fact, their administration is not possible to volunteers and, moreover, real urine specimens from abusers are seldom disposable. Studies performed both in vivo and in vitro on a limited number of these compounds showed that they are extensively metabolized, being excreted as mono-, di-, and tri-hydroxylated, carboxylated, *N*-dealkylated metabolites, and subsequently also conjugated with glucuronic acid. No unmetabolized parent drug is generally detected in urine samples from human studies [2–14]. The knowledge of the metabolism of all these compounds is therefore critical to search

for synthetic cannabinoid derivatives in urine. In vitro studies have been performed in order to elucidate the main metabolic pathways of some of these cannabinoids, demonstrating the suitability of this approach to be used as an alternative to human studies [12–16]. In metabolism studies, the elucidation of the metabolites' chemical structure is usually labor-intensive and time consuming, due to the necessity of processing and interpreting huge amounts of data obtained with MS techniques. To overcome this step, software for supporting and improving drug metabolism studies were developed; they can be classified in two categories:

- (a) Software for in silico metabolism prediction
- (b) Software for fast and reliable experimental data handling.

The first category aims at predicting the most probable sites of metabolism for a chemical compound, and thus to rank the metabolite formation likelihood. Examples of software comprised in this class are MetaSite™ (Molecular Discovery Ltd, UK) and Meteor™ (Lhasa Limited, UK). In particular, MetaSite™ software, used in this study, predicts the structure of the metabolites formed from phase I reactions without any need for experimental data: the software considers the enzyme–substrate recognition and the chemical transformations induced by the CYP enzymes on the most reactive sites, then it attributes the more probable sites of metabolism (SoM), and therefore predicts the main metabolites that can be formed in various human tissues (liver, skin, brain, and lungs). Hence, the metabolites predicted are listed with a likelihood ranking. An extensive description of the method and several applications are reported elsewhere [17–19].

The second category aims at generating structural assignment of metabolites from spectral data [19–21], and these software are usually directly provided together with the MS instruments, like Metabolynx™ (Waters Corporation, MA, USA), MetabolitePilot™ (ABSCIEX, MA, USA) MetWorks™ (Thermo Scientific, MA, USA), or Mass Hunter Metabolite IDTM (Agilent Technologies, CA, USA). A recent application of in silico metabolism prediction followed by in vitro studies on four designer drugs (2-desoxypipradol, 3,4-dimethylmethcathinone, alpha-pyrrolidinvalerophenone, and methiopropamine) was reported by Tyrkkö et al., who used Meteor™ software for prediction and identification in samples from in vitro experiments and real urine samples [20]. The study demonstrated the applicability of this approach for the identification of metabolites of new drugs, although with some limitations, mainly due to the high number of metabolites predicted by the software, and by slight differences of metabolites encountered in real human urine samples respect to in vitro experiments. Recently, Bonn et al. [21] presented an application of the Mass-MetaSite™ computational technique to automatically process the experimental data (mass spectra

obtained using UHPLC-HRMS and MS/MS acquisition), in order to significantly speed up the structure elucidation process in the study of metabolic transformations related to cytochrome-mediated reactions. The main advantage of Mass-MetaSite™ is that it is platform independent, being able to process raw data from the most used HRMS instruments and to handle different acquisition modes. Mass-MetaSite™ is an extension of MetaSite™, which combines the information from MS/MS spectral data with SoM predictions to automatically assess the structures of formed metabolites. Mass-MetaSite™ looks for the best matches between the experimental fragmentation pattern for each chromatographic peak and the in silico-predicted fragmentation pattern for the parent compound and its metabolites. Moreover, it can handle phases I and II metabolites up to the third generation of metabolites. Mass-MetaSite™ can be used to interpret data from in vitro or in vivo experiments [22].

The aim of the present study was to investigate the main metabolites formed from four synthetic cannabinoids (JWH-015, JWH-098, JWH-251, and JWH-307), chosen among others as they have different chemical structures, to provide information on possible biomarkers that could be useful for their rapid urinary screening. Only the most abundant metabolites were therefore investigated, using in silico metabolic predictions by means of MetaSite™ and in vitro metabolism experiments. The studies were performed on four synthetic cannabinoids in rat liver slices to identify the main phases I and II metabolites formed. The MS/MS spectral data obtained using a liquid chromatography-quadrupole time-of-flight (LC-QTOF) instrument were analyzed by Mass-MetaSite™. Moreover, scan data were manually revised, also considering the metabolism reactions reported on available literature data not found by the automatic search performed by the software. Thus, a comparison between the experimental findings and a totally in silico metabolism prediction using MetaSite™ is reported to evaluate if the in silico approach can be applied to predict the main metabolites formed for a JWH compound, also in absence of experimental data or reference standards. Based on the results obtained by LC-QTOF analyses, an LC-MS/MS method in multiple reaction monitoring (MRM) mode was setup for the screening of the main urinary JWHs metabolites detected.

Materials and methods

Chemicals and reagents

JWH-015, JWH-098, JWH-251, and JWH-307 were obtained from LGC Standards (Milan, Italy). Standard solutions in methanol at a concentration of 1 µg/mL were prepared as reference standards to optimize the analytical conditions. Standard compounds were stored according to supplier

recommendations. Dimethyl sulfoxide (DMSO), water, acetonitrile, formic acid, NADPH, methionine, insulin, gentamicin, hydrocortisone 21-hemisuccinate, and methanol were purchased from Sigma-Aldrich (Milan, Italy); ammonium formate was from Agilent Technologies (Santa Clara, CA, USA). RPMI medium was purchased from CAMBREX (Milan, Italy). All other chemicals and solvents were of the highest grade available and obtained from common commercial sources.

LC-QTOF method

The LC-QTOF system was an Agilent 6540 UHD Accurate-Mass QTOF with a dual Jet Stream electrospray ionization source, equipped with an Agilent 1290 Infinity LC system (Agilent Technologies). The LC consisted in a binary pump with integrated vacuum degasser, high-performance well-plate autosampler, and thermostated column compartment modules. The column was a 100×4.6 mm Widespore C4, 3.6 μm (Phenomenex, Bologna, Italy). Column temperature was set at 40 °C, and injection volume was 1 μL. Mobile phase A (water containing 0.1 % formic acid) and mobile phase B (acetonitrile with 0.1 % of formic acid) were utilized with a linear gradient from 5 % B to 95 % B within 6 min, held for 4 min, column re-equilibration was performed with linear gradient to 5 % A in 1.0 min, held for 3.0 min. The flow rate was set to 350 μL/min and the eluate was introduced into the mass spectrometer by means of a Dual Jet Stream electrospray ionization source (ESI) in positive mode. Source parameters were the following: capillary voltage was set to 4,000 V, the ion source temperature was set at 300 °C; nitrogen was used as nebulizing and collision gas at 4 L/min and 35 psi, respectively. Fragmentor voltage was set to 90 V and nozzle voltage at 0 V, sheath gas flow was set to 9 mL/min, and sheath gas temperature at 320 °C.

The LC-QTOF was governed by Agilent MassHunter software (B.05.00); acquisition of spectrometric data was obtained in AutoMSMS mode, performing the acquisition simultaneously in MS full scan and in MS/MS scan at different fragmentation energies (20, 30, and 40 V); exact protonated masses ($[M+H]^+$) of precursor ions to be fragmented were isolated according to a preferred list ions generated by Mass-MetaSite™ program (version 2.2.0, Molecular Discovery Ltd.) including all potential metabolites predicted up to the third generation.

LC-triple quadrupole MS/MS method

The LC-triple quadrupole system was an Agilent 6460 triple quadrupole mass spectrometer with the Jet Stream electrospray ionization source and Agilent 1290 Infinity LC system (Agilent Technologies). The column was a superficially porous Kinetex C18 column (2.6 μm, 100×2.1 mm from Phenomenex, Bologna, Italy). Column temperature was set to

40 °C, and injection volume was 10 μL. Mobile phase A (5 mM ammonium formate containing 0.05 % formic acid) and mobile phase B (methanol/acetonitrile 1:1 with 0.1 % of formic acid) were utilized at a flow rate was of 350 μL/min. Mobile phase gradient was as follows: 50 % A for 1 min, linear gradient to 100 % B in 8 min, held for 1.0 min, column re-equilibration was performed with linear gradient to 50 % A in 1.0 min, held for 3.0 min. The eluate was introduced into the mass spectrometer by means a Dual Jet Stream ESI in positive mode with the following parameters: capillary voltage was set to 4,000 eV, the ion source was heated up to 350 °C and nitrogen was used as nebulizing and collision gas at 12 L/min and 40 psi, respectively; EM voltage was set to +1,000 V and nozzle voltage at 2,000 V. The detector operated in MRM mode. Transitions, collision energies, and retention times are reported in Table 3.

Modeling synthetic cannabinoids metabolism

The MetaSite™ software (version 4.1.1, Molecular Discovery Ltd.) was used for metabolism prediction. The 2D structures of the four compounds were imported in MetaSite™ to predict their phase I metabolism in liver. The algorithm was set to consider both the reactivity of the positions of the molecule and its interaction with the enzyme. Accordingly, the software assigned a likelihood ranking to the metabolites predicted. Only metabolites with a molecular mass higher than 100 and with a likelihood ranking >20 were considered.

In vitro experiments: metabolite profiling in rat liver slices

All experiments were performed in strict compliance with the recommendation 2010/63/EU for the care and use of laboratory animals. Rat livers slices (250 μm thick, 15 mg for each experiment) from male Wistar albino rats were incubated at 37 °C in 12-well culture plates containing 750 μl of RPMI 1640 supplemented with 5 % fetal calf serum, 0.5 mM L-methionine, 1 μM insulin, 50 μg/ml gentamicin, and 0.1 mM hydrocortisone 21-hemisuccinate [23]. The plates were continuously shaken horizontally at 100 rpm. Following an initial 30-min pre-incubation aimed at equilibrating the slices, the medium was replaced with a fresh vehicle containing 50 μM of each of tested compounds dissolved in DMSO and incubated up to 4 h. At the end of the incubation, the slices and incubation media were collected and homogenized. Seven hundred-fifty microliters of acetonitrile was added to the samples to stop enzymatic reaction and precipitate all proteic material. The samples were then centrifuged at 10,600×g for 10 min. Samples were conserved until use at -20 °C. Blank was prepared incubating rat liver slices in absence of the investigated compounds. A chemical control sample without the liver slices was prepared to study the eventual spontaneous formation of metabolites. The standard solutions in mobile phase A were also analyzed in

order to check if they were free of impurities. The samples were analyzed using LC-QTOF and the metabolites detection was performed using the Mass-MetaSite™ software (version 2.2.0, Molecular Discovery Ltd.), followed by a manual revision of mass spectrometric data.

Results

In silico phase I metabolites prediction for synthetic cannabinoids

MetaSite™ software predicted the main SoM for JWH-015, JWH-098, JWH-251, and JWH-307 and their main metabolic reactions. In its current version, MetaSite™ reports only the phase I metabolite structures. Thus, the SoMs and main metabolites with the relative rank of likelihood are reported in Table 1.

For JWH-015, the most probable site of metabolism was the carbon in position 1 of the side chain, followed by various sites of the methyl-indole moiety and of the naphthalene. Main metabolites predicted were therefore those derived by the cleavage of the N-C bond leading to *N*-dealkylation, oxidation, hydroxylation, and carboxylation on the position 1 of the alkyl chain (all of them with an equal likelihood of

100 %), and hydroxylation on various sites of the methyl-indole and of the naphthalene moieties.

For JWH-098, the most probable site of metabolism was the methoxy group located on naphthalene moiety, followed by the ω -1 position of aliphatic chain and then by the C1 of the side chain. Hence, the predicted metabolic reactions were *O*-demethylation (ranking, 92 %), aliphatic hydroxylation in position ω -1 (72 % of likelihood), *N*-dealkylation, oxidation, dehydrogenation, and reduction on the C1 with an equal 37 % ranking.

The most probable site of metabolism of JWH-251 was the ω -1 position of aliphatic chain, followed by the C1, by the methyl on the benzyl group, and by the aromatic ring of the indole moiety, all of them with the same ranking. The most likely metabolites were therefore those formed by alkyl and benzyl hydroxylation and carboxylation at the methyl of the substituted benzyl group.

Finally, for JWH-307, MetaSite™ predicted the ω -1 position of aliphatic chain as the most probable site of metabolism, followed by the C1 position, by the *para* position of the fluorobenzene ring and by different positions of the naphthalene. The main metabolites predicted were therefore those hydroxylated on the ω -1 position (100 %), and hydroxylated, dehydrogenated, oxidated on the C1 position (ranking, 49 %), and hydroxylated on fluorobenzene ring (45 %) or on naphthalene (20 %).

Table 1 Most probable sites of metabolism (darkest circles correspond to the most probable SoMs) and metabolites predicted

| SoM | Most probable metabolites predicted (n=7) and respective likelihood ranking | | | | | | |
|--------|---|------|------|------|-----|-----|-----|
| JWH015 | 100% | 100% | 100% | 100% | 36% | 36% | 36% |
| JWH098 | 92% | 72% | 37% | 37% | 37% | 37% | 21% |
| JWH251 | 100% | 77% | 77% | 77% | 54% | 54% | 54% |
| JWH307 | 100% | 49% | 49% | 49% | 49% | 45% | 20% |

Table 2 Main metabolites identified in rat liver slices by HRMS: exact mass, elemental composition, accurate mass and mass error, predicted or not by MetaSite (only phase one reactions), characteristic fragments and retention times RT

| Compound | Exact [M + H] ⁺ | Elemental composition | Accurate [M + H] ⁺ | Δppm | Predicted | Characteristic fragments | RT (min) |
|---------------------------|----------------------------|--|-------------------------------|------|----------------------|--|------------------|
| JWH015 | 328.1696 | C ₂₃ H ₂₁ NO | 328.1712 | 0.05 | | 155.0492; 127.0541; 200.1069 | 7.39 |
| JWH015 <i>N</i> -desalkyl | 286.1226 | C ₂₀ H ₁₅ NO | 286.1233 | 0.02 | Yes | 155.0489; 127.0540; 158.0599 | 6.65 |
| JWH015-OH (three peaks) | 344.1645 | C ₂₃ H ₂₁ NO ₂ | 344.1641 | 0.01 | Yes | 155.0494; 127.0537; 216.1016 | 6.69, 7.04, 6.55 |
| JWH015 -OH glucuronated | 520.1966 | C ₂₉ H ₂₉ NO ₈ | 520.1971 | 0.01 | n.a. | 155.0493; 127.0537; 216.1019; 344.1648 | 5.82 |
| JWH098 | 386.2115 | C ₂₆ H ₂₇ NO ₂ | 386.2130 | 0.04 | – | 185.0600; 228.1385; 157.0650 | 7.85 |
| JWH098 <i>O</i> -demethyl | 372.1958 | C ₂₅ H ₂₅ NO ₂ | 372.1963 | 0.01 | Yes | 171.0444; 228.1385; 143.0499 | 7.30 |
| JWH098 <i>N</i> -desalkyl | 316.1332 | C ₂₁ H ₁₈ NO ₂ | 316.1344 | 0.04 | Yes | 185.0590; 158.0595; 130.0663; 157.0650 | 6.72 |
| JWH098 -OH (naphthalene) | 402.2064 | C ₂₆ H ₂₇ NO ₃ | 402.2070 | 0.01 | No | 228.1390; 173.0603; 201.0549 | 7.64 |
| JWH098 -OH (alkylindole) | 402.2064 | C ₂₆ H ₂₇ NO ₃ | 402.2063 | 0.01 | Yes | 185.0610; 85.0282; 157.0672; 244.1338 | 6.81, 7.0, 7.24 |
| JWH098 ketone formation | 400.1907 | C ₂₆ H ₂₅ NO ₃ | 400.1907 | 0 | Yes | 185.0594; 85.0648; 157.0628 | 7.03 |
| JWH098 demethyl, -OH | 388.1907 | C ₂₅ H ₂₅ NO ₃ | 388.1906 | 0 | No | 171.0446; 143.0497; 244.1338 | 6.32 |
| JWH098 -OH glucuronated | 578.2385 | C ₃₂ H ₃₅ NO ₉ | 578.2383 | 0 | n.a. | 402.2051; 185.0599; 228.1366 | 5.10 |
| JWH251 | 320.2009 | C ₂₂ H ₂₅ NO | 320.2021 | 0.04 | – | 214.1232; 144.0447; 105.0700 | 8.92 |
| JWH251 -OH alkylindol | 336.1958 | C ₂₂ H ₂₅ NO ₂ | 336.1961 | 0.01 | Yes | 186.1278; 230.1167; 105.0700 | 6.63, 6.79 |
| JWH251 -OH benzylic group | 336.1958 | C ₂₂ H ₂₅ NO ₂ | 336.1961 | 0.01 | Yes | 121.0638; 214.1227; 103.0535; 290.1900 | 7.06 |
| JWH251 -OH glucuronated | 512.2279 | C ₂₈ H ₃₃ NO ₈ | 512.2291 | 0.02 | n.a. | 318.1860; 290.1905; 214.1227 | 6.28 |
| JWH251 <i>N</i> -desalkyl | 250.1226 | C ₁₇ H ₁₅ NO | 250.1225 | 0 | Yes | 144.0449; 105.0704; 91.0546 | 6.51 |
| JWH251-ketone formation | 334.1802 | C ₂₂ H ₂₃ NO ₂ | 334.1808 | 0.02 | Not in that position | 105.0704; 228.1025; 85.0653 | 6.84 |
| JWH251 -COOH | 350.1751 | C ₂₂ H ₂₃ NO ₃ | 350.1770 | 0.05 | Not in that position | 245.1046; 105.0704; 304.1696 | 6.55 |
| JWH251 -COOH glucuronated | 526.2072 | C ₂₈ H ₃₁ NO ₉ | 526.2072 | 0 | n.a. | 332.1653; 304.1720 | 5.30 |
| JWH307 | 386.4807 | C ₂₆ H ₂₄ FNO | 386.4934 | 0.32 | | 155.0493; 127.0541; 258.1283 | 7.95 |
| JWH307 -OH | 402.1864 | C ₂₆ H ₂₄ FNO ₂ | 402.1871 | 0.02 | Yes | 155.0493; 127.0533; 274.1240 | 6.95 7.37 |
| JWH307-ketone formation | 400.1707 | C ₂₆ H ₂₂ FNO ₂ | 400.1711 | 0.01 | Yes | 155.0490; 127.0540 | 7.12, 7.20 |
| JWH307-insaturation | 384.1758 | C ₂₆ H ₂₂ FNO | 384.1765 | 0.02 | Yes | 155.0486; 127.0545 | 7.70 |
| JWH307-diOH | 418.1813 | C ₂₆ H ₂₄ FNO ₃ | 418.1817 | 0.01 | No | 155.0499; 127.0542; 171.0437 | 6.56 |

Metabolism study in rat liver slices

In order to evaluate if phase I *in silico* prediction of liver metabolic reactions on the investigated JWHs agreed with experimental results, *in vitro* experiments on rat liver slices were performed.

The samples from *in vitro* metabolism studies of JWH-015, JWH-098, JWH-251, and JWH-307 were analyzed by LC-QTOF; the raw data were then processed by MassMetaSite™ software, which assigned the most probable chemical structures to the peaks detected based on its metabolism predictions, on accurate masses of parent ions obtained by HRMS, and on characteristic fragments obtained after fragmentation. A manual revision of scan data was also performed in order to search for other expected metabolic reactions not found by the automatic search, such as

di- and tri-hydroxylation and *N*-dealkylation plus hydroxylation. Main metabolites identified in rat liver slices by HRMS and their respective accurate masses and characteristic fragments are reported in Table 2.

JWH-015

Figure 1 depicts the HR MS/MS spectra of two main metabolites of JWH-015 obtained in rat liver slices, with postulated structures and fragments. JWH-015 was mainly metabolized by *N*-dealkylation ($\Delta M = -42.0469$ Da respect to JWH-015, accurate mass, 286.1233; see Fig. 1a). We detected three peaks with a protonated molecular mass $\Delta M = +15.9949$ Da (accurate mass, 344.1648), corresponding to hydroxylation on different sites of the molecule. These peaks are attributable to hydroxylation on different sites of alkyl-indol moiety as

demonstrated by the presence of the intact naphthyl group (m/z 155.0497) and of the hydroxy-2methyl, propylindole carbonyl moiety (m/z 216.1025) as shown in Fig. 1b. The fragmentation pattern, analog for all these metabolites, did not allow to determine the exact site of hydroxylation. Hydroxylated metabolites were also present as glucuronic acid conjugated. We did not detect any carboxylated metabolite, neither in the free or glucuronated form, nor di or tri-hydroxy compounds, even after a manual revision of full-scan data files. These metabolites found by Zhang et al. [16] are probably formed in lower amounts, not detectable with our method designed for screening of the more abundant metabolites. It is interesting to notice that the MetaSite™ software predicted also a metabolite with an insaturation on the aliphatic chain that was found in that study, but not in our in vitro experiments. Main metabolites predicted in silico and detected after in vitro experiments are anyway in accordance with the results described by Zhang.

JWH-098

Figure 2 shows the HRMS/MS spectra of the main metabolites of JWH-098 with postulated structures and fragments. The main metabolite of JWH-098 was the *O*-demethylated compound ($\Delta M = -14.0156$ Da than JWH-098; accurate mass, 372.1963), shown in Fig. 2a. Its structure was confirmed by the presence of the fragment with accurate mass 171.0442, corresponding to the hydroxy-naphthyl carbonyl moiety, of the fragment at m/z 228.1385, corresponding to the intact pentylindole carbonyl moiety, including the lateral chain, and m/z 143.0489, corresponding to the hydroxy-naphthyl group. Other metabolic reactions included *N*-dealkylation (Fig. 2b; $\Delta M = -70.0783$; accurate mass, 316.1344), hydroxylation on three different sites of the lateral chain or of the indole, and on the naphthyl moiety ($\Delta M = +15.9949$ Da than JWH-098; accurate mass, 402.2063). Hydroxylation on the alkyl chain or on the indole was demonstrated by the fragments with m/z 185.0610 and 157.0640, corresponding to the methoxy naphthyl and naphthyl moieties, respectively, and with m/z 244.1323, corresponding to the hydroxylated other side of the molecule, including the carbonyl group (Fig. 2c). No other characteristic fragments could explain the exact site of hydroxylation in the peaks detected. Another site of hydroxylation could be the naphthyl moiety: although not predicted by the software, we detected another peak with accurate mass 402.2063 corresponding to a hydroxylated metabolite, showing in this case the fragment with accurate mass m/z 201.0549, corresponding to the OH methoxynaphthyl group (see Table 2). Hydroxylation occurred also on the demethylated metabolite ($\Delta M = +2$; accurate mass, m/z 388.1906), probably on the aliphatic chain or on the indole, as supposed by the

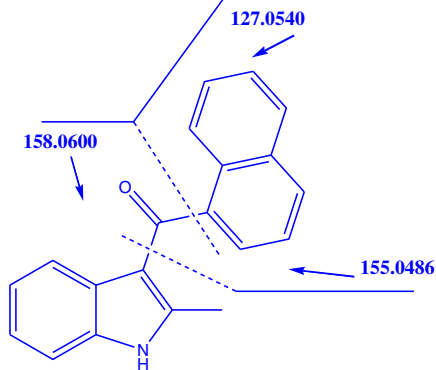
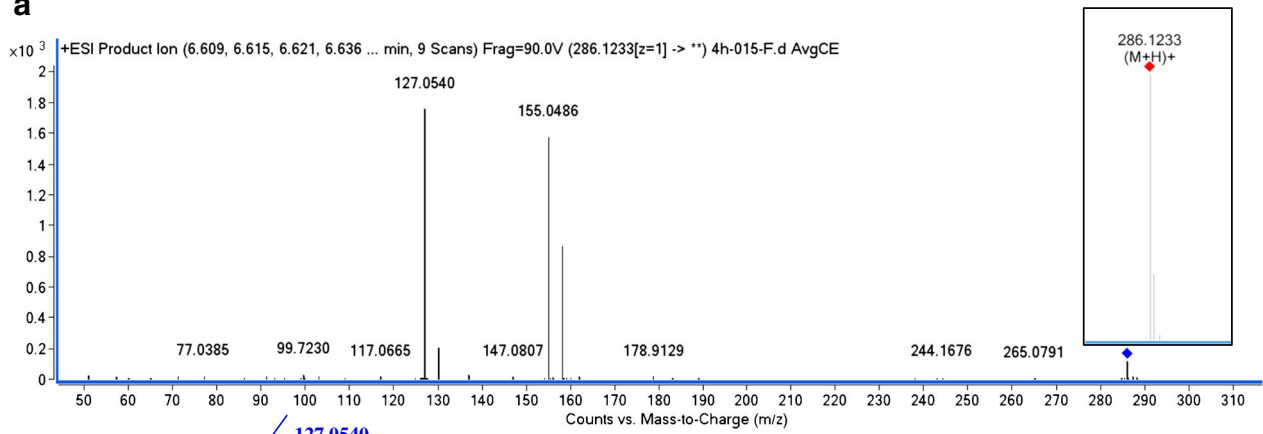
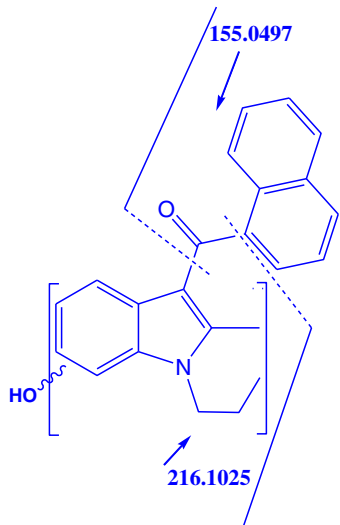
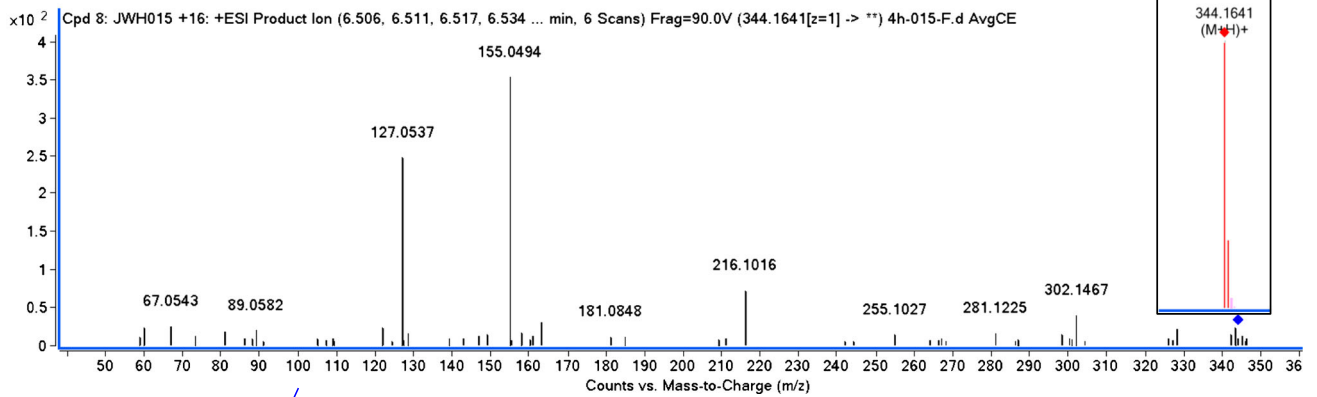
presence of fragment m/z 244.1338 (Table 2). We also detected a metabolite formed by carbonylation of the side chain ($\Delta M = +14$; accurate mass, 400.1907), shown in Fig. 2d. Hydroxyl metabolites were detected also as glucuroconjugates. Their main fragments are reported in Table 2.

JWH-251

The HRMS/MS spectra of JWH-251 main metabolites and their postulated structures/fragments are reported in Fig. 3. JWH-251 underwent hydroxylation on various sites of the molecule ($\Delta M = +15.9949$; accurate mass, 336.1963), giving at least four hydroxyl metabolites. Hydroxylation probably took place on the side chain as demonstrated by the presence of the intact methylphenyl and carbonylindole moieties (m/z 105.0700 and m/z 144.0446, respectively) in two metabolites and on the indole moiety as demonstrated by the presence in the molecules of the intact dimethylphenyl group and of the hydroxy-*N*-alkylindole (m/z 230.1167) shown in Fig. 3a. Hydroxylation took place also on the substituted phenyl group suggested by the presence of the fragment with m/z 121.0653, corresponding to the hydroxylation on that part of the molecule, and the intact other part (m/z 214.1232; see Table 2).

JWH-251 also gave a *N*-dealkylated metabolite ($\Delta M = -70.0783$), with accurate mass 250.1225, corresponding to the loss of the alkyl chain, shown in Fig. 3b. One carbonylated metabolite was detected ($\Delta M = +13.9792$; accurate mass, 334.1808), shown in Fig. 3c; carbonylation took probably place on the side chain as demonstrated by the presence of the intact 1,3 dimethylphenyl group (m/z 105.0704) and *N*-carbonylpentyl-3-indole moiety with accurate mass 228.1025 and of carbonylated aliphatic chain (m/z 85.0653). As can be seen in Table 2, other metabolic reactions observed were carboxylation of the side chain ($\Delta M = +29.9742$), MH^+ 350.1770, and dihydroxylation ($\Delta M = +31.9898$; accurate mass, 352.1907). Hydroxylated and carboxylated metabolites were subsequently glucuronated (accurate masses, 512.2291 and 526.2072, respectively). These results are in agreement with those recently reported by Kavanagh et al. on human studies [8]. Main metabolite detected in that study was hydroxylated on the pentyl chain, and was chosen as a marker of intake of JWH-251; other metabolites detected in lower amount were monohydroxylated and carboxylated, dihydroxylated, dealkylated and hydroxylated, carbonylated, and hydroxylated.

Fig. 1 Main JWH-015 metabolites detected in LC/QTOF-HRMS: MS/MS spectra, accurate MH^+ (in the right corner) and postulated fragmentation. **a** *N*-desalkyl JWH-015; **b** alkyindole-OH-JWH-015

a**b**

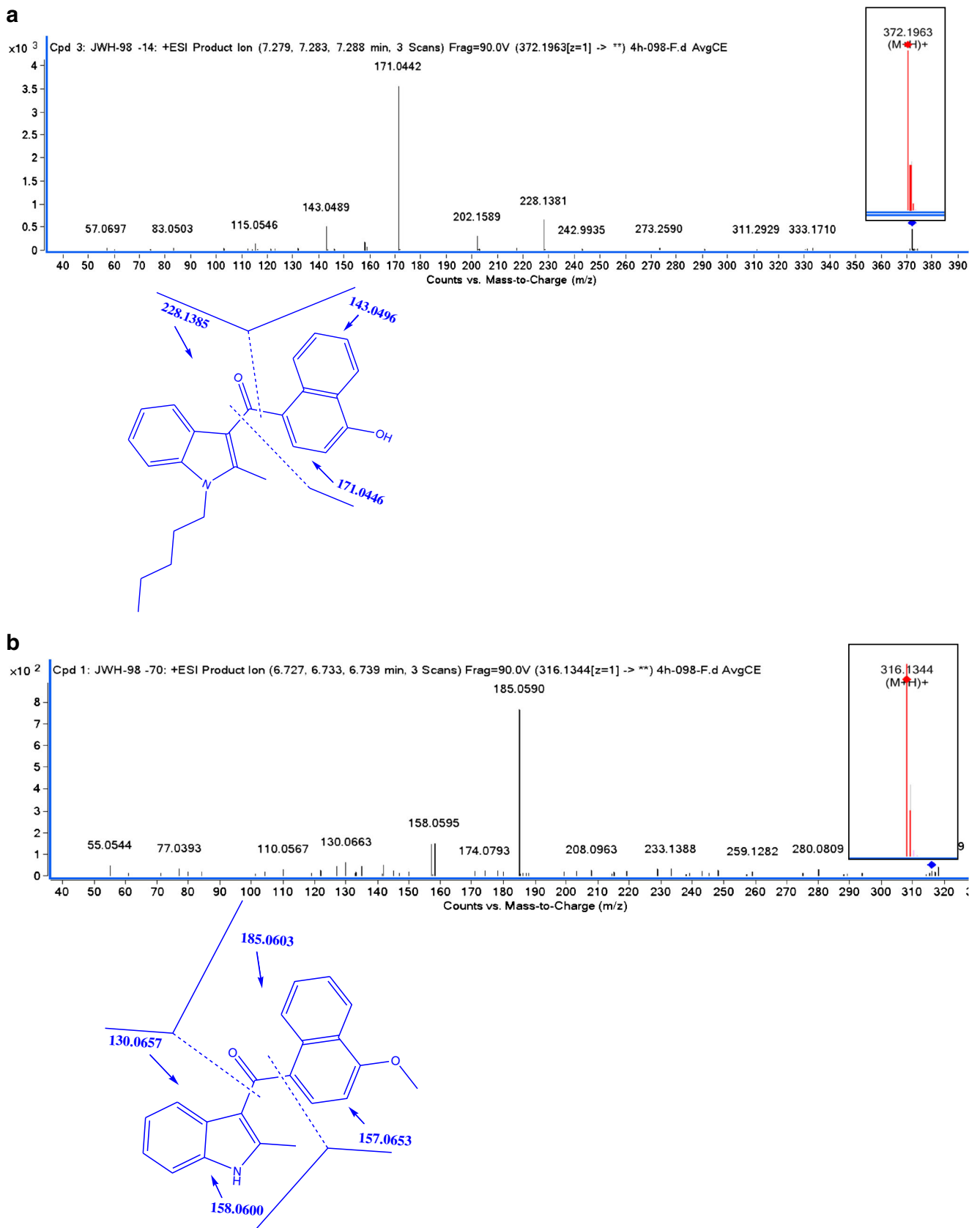


Fig. 2 Main JWH-98 metabolites detected in LC/QTOF-HRMS: MS/MS spectra, accurate MH^+ (in the right corner) and postulated fragmentation. **a** *O*-demethyl-JWH-98, **b** *N*-desalkyl JWH-98, **c** alkylindole-OH JWH-98, **d** alkylindole-ketone JWH-98

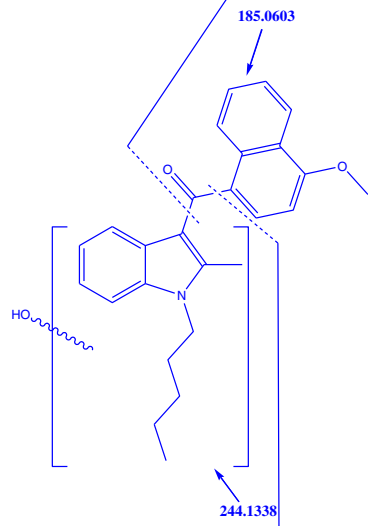
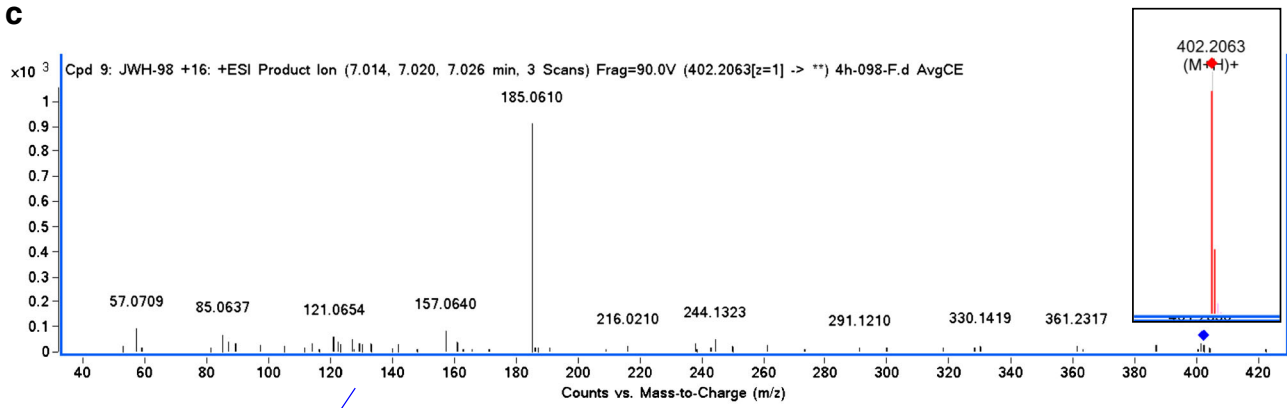
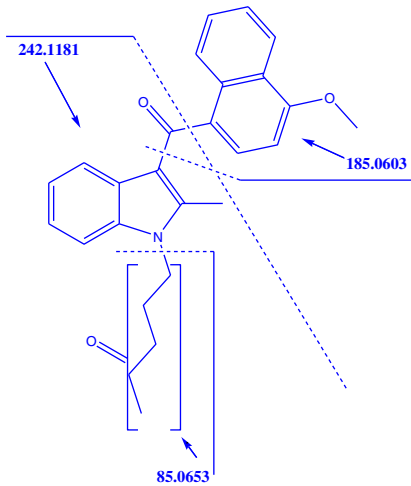
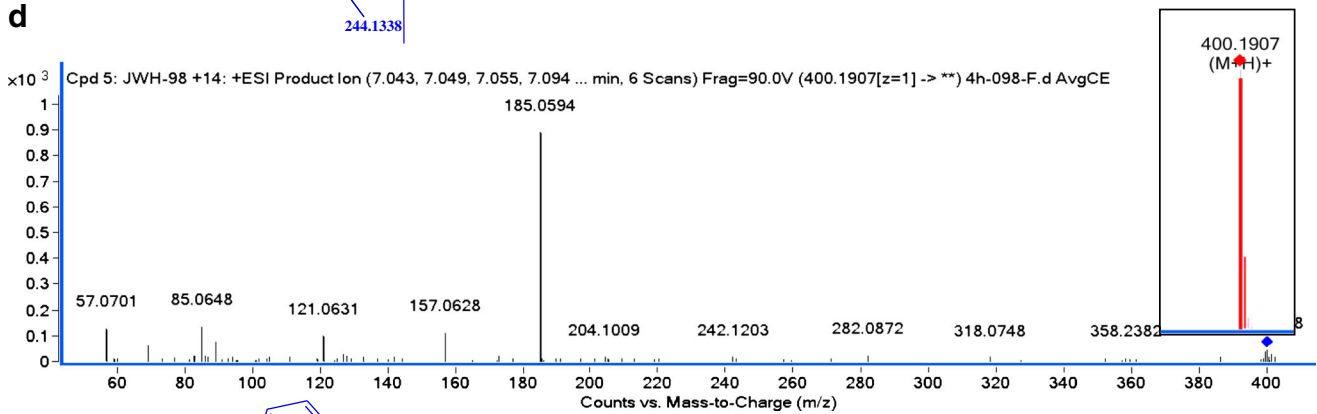
c**d**

Fig. 2 (continued)

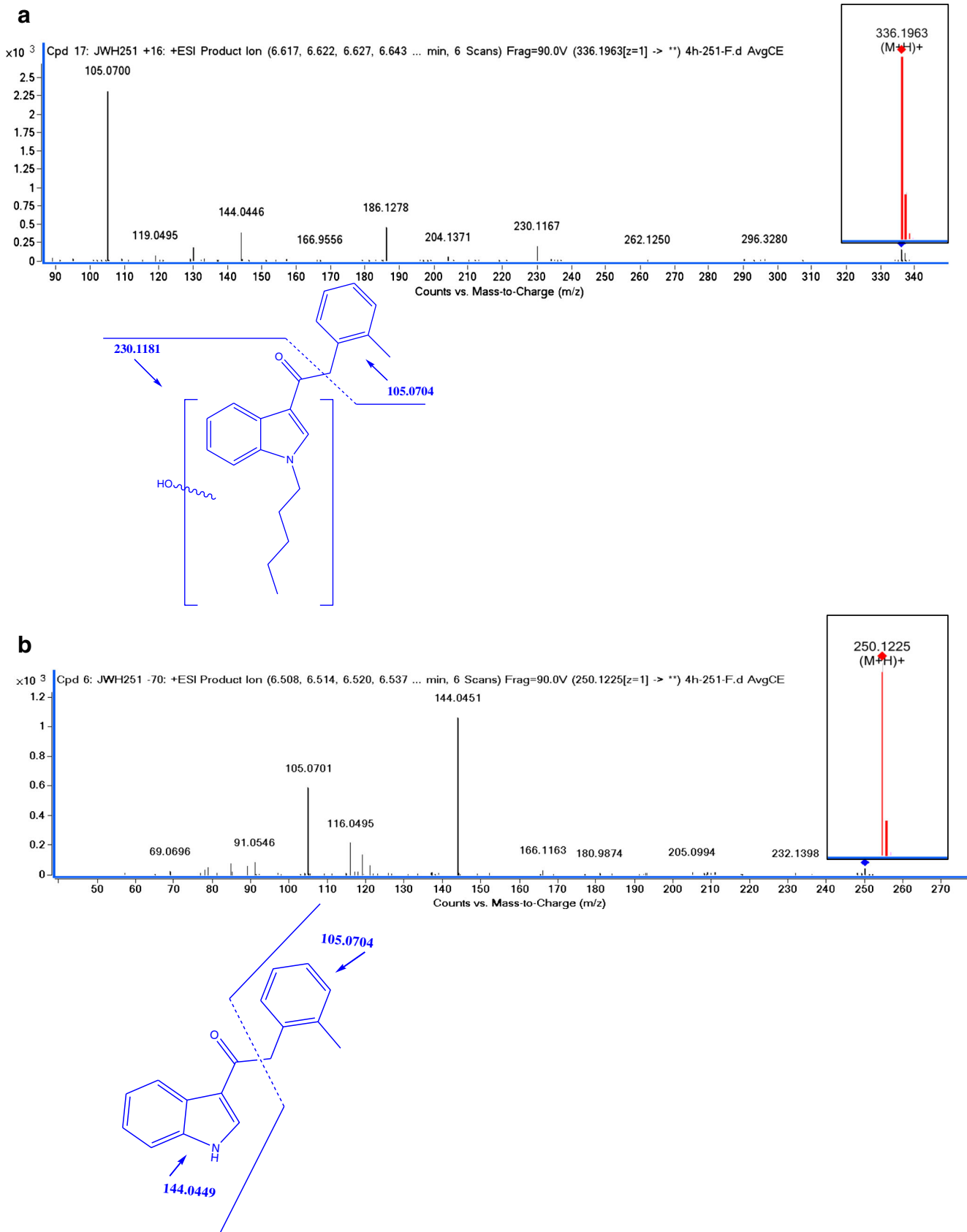


Fig. 3 Main JWH-251 metabolites detected in LC/QTOF-HRMS: MS/MS spectra, accurate MH^+ (in the right corner) and postulated fragmentation. **a** JWH-251-alkylindole-OH, **b** *N*-desalkyl JWH-251, **c** alkylindole-ketone JWH-251

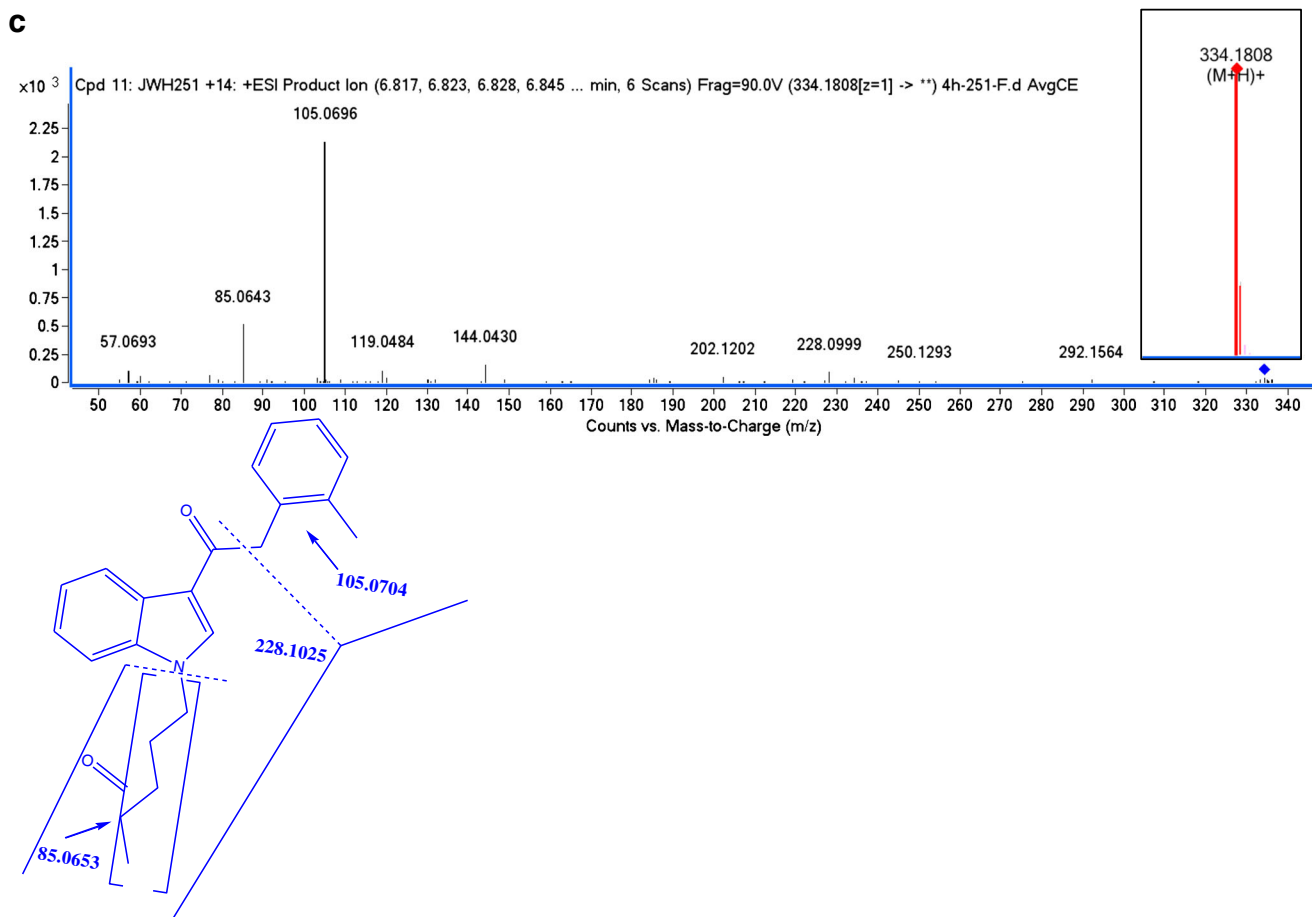


Fig. 3 (continued)

JWH-307

Figure 4 shows HRMS/MS spectra of JWH-307 main metabolites and their postulated structures and fragments. JWH-307 underwent aliphatic carbonylation ($\Delta M = +13.9792$ Da than JWH-307; accurate mass, 400.1711), shown in Fig. 4a, and hydroxylation ($\Delta M = +15.9949$ Da, 402.1871) in Fig. 4b, both in two different sites of the side chain, giving two peaks for each metabolite and hydroxylation likely on the fluorobenzene ring. As reported in Table 2, it was also formed a di-hydroxylated metabolite ($\Delta M = +31.9898$ Da, 418.1817), and a metabolite with an insaturation on the side chain ($\Delta M = -2.0157$; accurate mass, 384.1765). All the metabolites detected showed the presence of the intact carbonyl-naphtalene group, m/z 155.0497, demonstrating that all the metabolic reactions took place on the alkyl chain, on the pyrrole, and/or on the fluorobenzyl moieties. No *N*-dealkylated metabolite was detected for this molecule.

LC-MS/MS qualitative screening method

We set up an LC-MS/MS method using a triple quadrupole in MRM mode for the screening of the studied cannabinoids and

respective metabolites based on the $[M + H]^+$ of the main metabolites identified and on the characteristic fragments obtained in QTOF-HRMS. This kind of instrument is in fact more likely to be available in laboratories performing forensic and clinical toxicological analyses. Chromatographic conditions were optimized for the detection of synthetic cannabinoids and metabolites. The ion transitions and respective collision energies were chosen according to the best conditions obtained in the experiments performed by LC-QTOF, being the configuration of both source and collision cells the same for these two instruments; transitions, collision energies, and retention times (RT) of main identified metabolites and parent compounds, included in the screening method are scheduled in Table 3. Figure 5 shows extracted ionic chromatograms of the MRM analyses of the main metabolites of JWH-015, JWH-098, JWH-251, and JWH-307 obtained from *in vitro* experiments.

Discussion

In this pilot study, we studied the main metabolic behavior of four JWH synthetic cannabinoids, comparing *in silico*

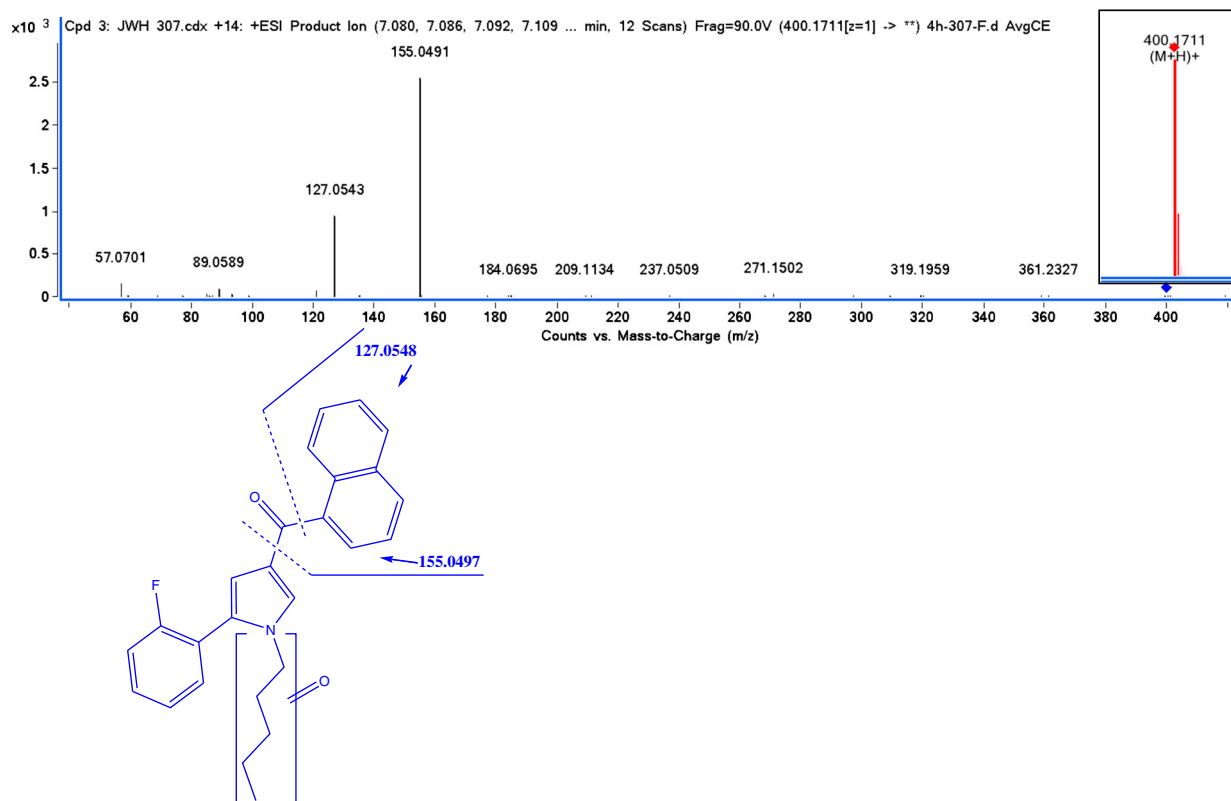
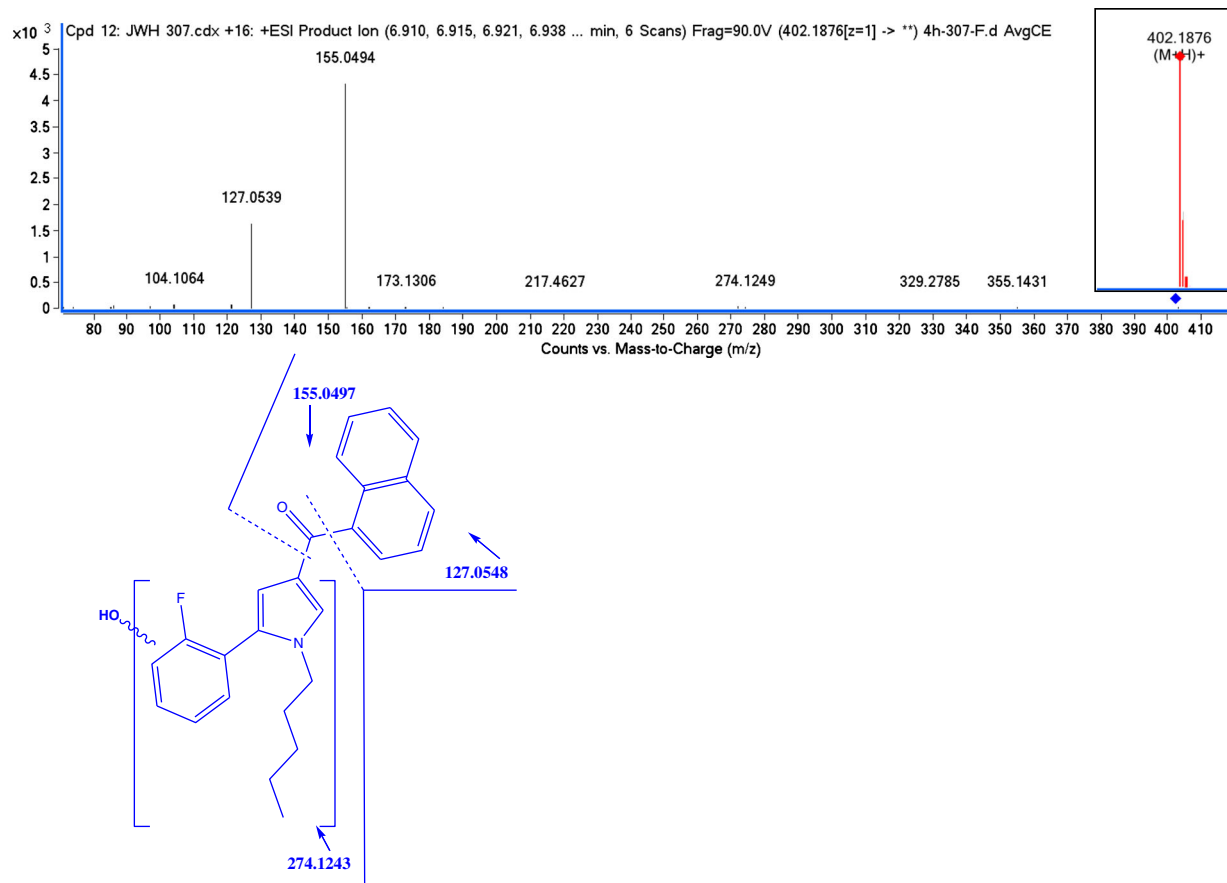
a**b**

Fig. 4 Main JWH-307 metabolites detected in LC/QTOF-HRMS: MS/MS spectra, accurate MH^+ (in the right corner), and postulated fragmentation. **a** Hydroxy-JWH-307, **b** keto-JWH-307

prediction and in vitro formation of their main metabolites, in order to evaluate the reliability of in silico prediction on JWHs metabolism and to develop analytical methods for the detection in biological fluids, mainly urine, of possible biomarkers of synthetic cannabinoids abuse. The in silico prediction gave preliminary information on the most probable metabolites expected to be formed in human liver for each new compound investigated.

On the other hand, the use of rat liver slices allowed the evaluation of the main metabolites formed in vitro, although with the inherent limitation of in vitro studies [24], also considering that the murine metabolism could be slightly different than human one, as demonstrated by De Brabanter and coworkers and by Grigoryev and coworkers [5, 13].

The Mass-MetaSite™ software was used to analyze the raw LC-QTOF data files from the analysis of in vitro samples, resulting as a valid tool to significantly speed up the assignation of the metabolite structures from LC-HR-MS/MS data, particularly when new molecules with an unknown metabolic pathway are investigated. None of the described metabolites

were found in blanks samples and samples incubated without rat liver.

The comparison between experimental findings and the in silico prediction obtained using MetaSite™ showed that the predicted metabolic reactions were in the majority of cases confirmed by in vitro results. In Table 2, it is reported when a metabolite detected experimentally matched with the software prediction. The predicted metabolites ranked as the most probable were always detected in the samples from in vitro studies. Occasionally, false predictions also occurred, but that was due to the prediction of the same most-probable SoM of the various possible metabolic reactions that not always occurred (e.g., oxidation, dealkylation, hydroxylation, and insaturation, predicted with the same likelihood ranking), while only one of the possible metabolites was produced experimentally in detectable amount.

Based on our studies, some general considerations could be drawn. *N*-dealkylation is very likely to occur for methylindole and indole cannabinoids; otherwise from the others, we did not detect any *N*-desalkyl metabolite for JWH-307.

Hydroxylation occurred for all the cannabinoids studied, and, depending on the structure of the molecule, it could take place on the aliphatic chain (ω -1 and C1 are the favorite positions, depending on the chain length), and/or on the indole, and/or on naphthalene or substituted aromatic ring.

Table 3 Transitions, collision energies, and retention times of JW015, JWH019, JWH251, JWH307, and main metabolites in the triple quadrupole MS/MS method in MRM mode

| Analyte | Transitions | CE | RT (min) |
|---|--------------------------|----|------------------|
| JWH015 | 328 → 200, 155, 127 | 30 | 6.9 |
| JWH015-OH (indole) | 344 → 216, 155, 127 | 40 | 3.6; 3.8; 4; 5.6 |
| JWH015 <i>N</i> -desalkyl | 286 → 155, 127, 158 | 30 | 3.7 |
| JWH098 | 386 → 228, 185, 157 | 20 | 8.4 |
| JWH098 <i>O</i> -demethyl | 372 → 171, 143, 228 | 30 | 7.5 |
| JWH098 <i>N</i> -desalkyl | 316 → 185, 158, 130 | 30 | 4.3 |
| JWH098 -OH (naphthyl) | 402 → 228, 201, 57, 173 | 40 | 7.2 |
| JWH098 -OH (aliphatic chain, methyl indole) | 402 → 57, 244, 185 | 40 | 5.2; 6; 6.6; 6.8 |
| JWH098 CO | 400 → 185, 242, 157 | 40 | 5.6 |
| JWH098 demethyl, OH | 388 → 244, 171, 143, 115 | 40 | 3; 3.6 |
| JWH251 | 320 → 214, 143, 105 | 20 | 4.72 |
| JWH251 -OH (aliphatic chain) | 336 → 318, 262, 186 | 30 | 3.8; 5.6 |
| JWH251 -OH (indole) | 336 → 230, 186 | 30 | 4.4; 5.9 |
| JWH251 -OH (methylphenyl group) | 336 → 318, 290, 214, 121 | 30 | 3.9; 5.7; 6.1 |
| JWH251 <i>N</i> -desalkyl | 250 → 144, 116, 105 | 40 | 2.9 |
| JWH251 -CO (aliphatic chain) | 334 → 105, 85, 234 | 40 | 4.2 |
| JWH251 -COOH (aliphatic chain) | 350 → 304, 245, 105, 144 | 20 | 3.6 |
| JWH307 | 386 → 258, 155, 127 | 10 | 8.4 |
| JWH307 -OH (alkyl-pyrrole, <i>F</i> -benzene) | 402 → 155, 127 | 30 | 5.4, 6.1 |
| JWH307 -OH (naphthyl group) | 402 → 304, 171, 143 | 30 | 7.2 |
| JWH307-diOH | 418 → 155, 127 | 40 | 3.9, 4.2 |
| JWH307-CO | 400 → 155, 127, 271 | 30 | 5.7, 6.4 |
| JWH307-dehydro | 385 → 327, 155, 127 | 40 | 8.5 |

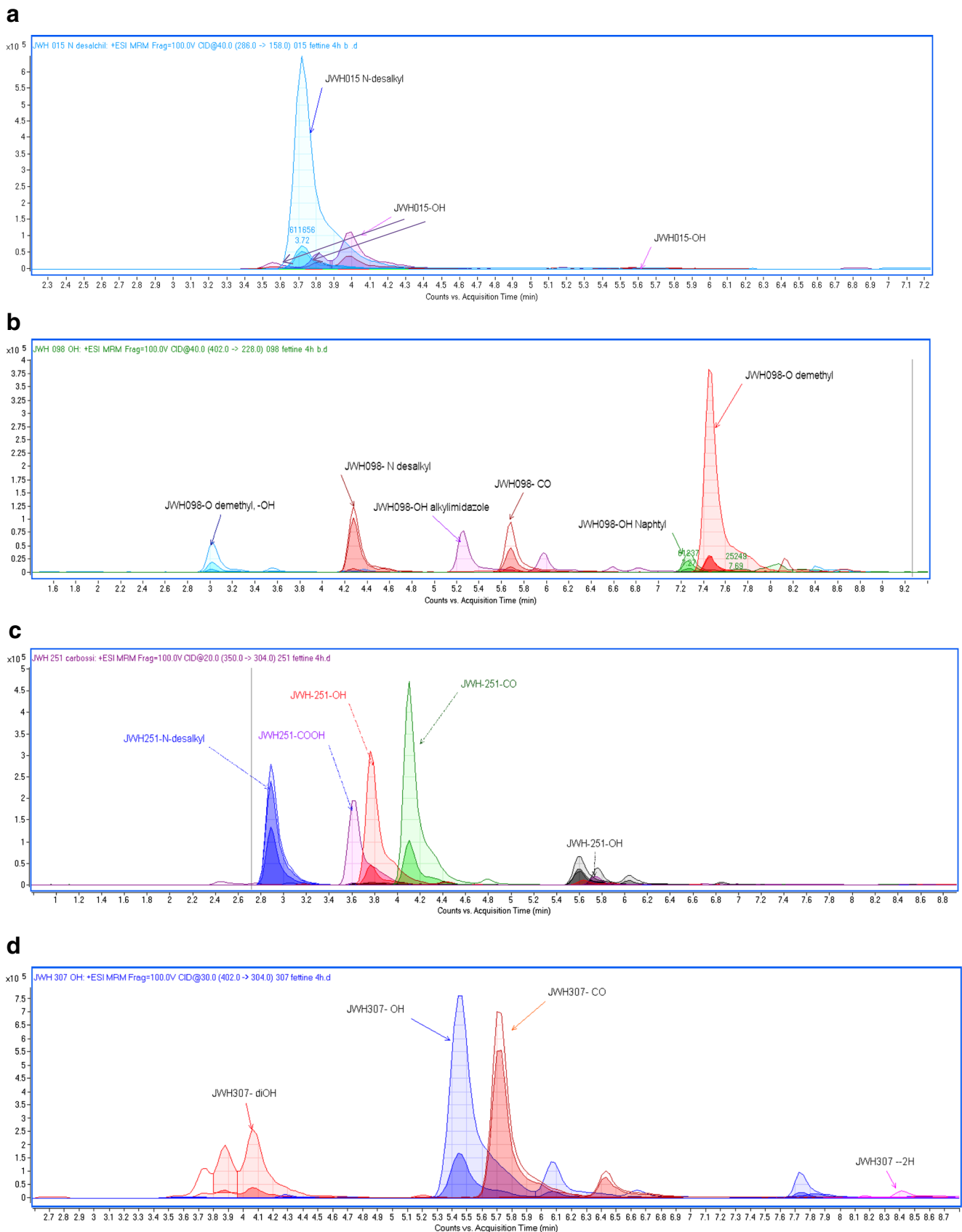


Fig. 5 LC-MS/MS in MRM mode analysis of samples from *in vitro* metabolism studies. Extracted ionic chromatograms of the characteristic transitions of each metabolite. **a** JWH-015, **b** JWH-098, **c** JWH-251, and **d** JWH-307

Hydroxyl groups could be further metabolized to carboxylic acids. Other metabolic reactions include oxidation and insaturation of the side chain, and hydrolysis of ethers, as for JWH-098. Hydroxylated and carboxylated metabolites can undergo glucuronic acid conjugation. A prior hydrolysis of the samples or including glucuronic acid conjugates in the routine analytical methods is therefore advisable.

JWH-015 metabolism was previously studied *in vitro* by Zhang et al. [16], using rat liver microsomes; in that paper they detected a higher number of metabolites. This is probably due on one side to the different conditions of the microsomal incubation compared to rat liver slices, where we used a lower amount of substrate, therefore probably the amount of these further minor metabolites were too low to be detected with the analytical method used in our study, that is set to inject only small amounts of sample to safeguard the efficiency of the equipment and therefore is aimed only at the detection of more abundant metabolites. Main metabolites, useful for the diagnosis of JWH-015 intake, were anyway predicted and detected in our experiments in accordance of what reported by Zhang.

Main JWH-251 metabolites obtained, both *in silico* and *in vitro*, confirmed the results described by Tyrkko, confirming the reliability of MetaSite™ software in metabolites predictions, also compared to Meteor™ prediction software, and by Kavanagh [7]. To our knowledge, no data are available till now on the metabolism of JWH-098 and JWH-307. The theoretic prediction and the *in vitro* experiments performed allow a qualitative determination of their main metabolites in urine samples.

The experiments performed both on molecules with a known metabolism and on new molecules demonstrate the usefulness of the theoretic metabolism prediction, which can be a starting point for developing methods to detect the most likely metabolites. The results obtained suggest that MetaSite™ could be a useful tool to predict the main phase I metabolic pattern of synthetic cannabinoids, especially when the human data will be very limited in terms of availability. This approach could hence be used for setting up qualitative screening methods for synthetic cannabinoids analysis in urine, by the detection of the most probable metabolites predicted. The aim of the study was in fact the identification of main metabolites to be used as target compounds for synthetic cannabinoids consumption diagnosis in biological specimens, mainly urine. It must anyway be taken into account that experiments were performed only on murine liver models, and human metabolism could be different, both in kind and in relative abundances of produced metabolites. Further experiments are planned in order to evaluate human metabolism.

Finally, we developed a qualitative LC-MS/MS screening method in MRM mode for the studied compounds that could in principle be extended to the detection of the metabolites of other synthetic cannabinoids. This could be done based on the prediction of the metabolism of each compound performed by

MetaSite™. The analytical method could be developed on the basis of the molecular weight of main predicted metabolites and on their respective characteristic fragments, which can be deduced by their chemical structure. Moreover, the fragmentation behavior of this class of molecules appears to be similar in all the synthetic cannabinoids studied till now [2–16]. The typical ion fragments obtained for all cannabinoids and metabolites are related to the naphthoyl or benzoyl groups, depending on the base structure, with eventual substitutions, and to the indole and carbonyl-indole moieties, with and without retention of the alkyl chain. This can be a good preliminary starting point for the development of screening methods for other synthetic cannabinoids and their possible main metabolites. Further experiments are in progress in order to increase the number of synthetic cannabinoids and respective metabolites to be screened, with the aim to routinely apply it on authentic urine samples.

Conclusions

In conclusion, this pilot study has demonstrated the possibility to theoretically predict and practically detect the main metabolites of four synthetic cannabinoids with different structures. The results obtained confirm the value of the MetaSite™ *in silico* approach, that can give very useful information on the most probable metabolites to be screened in all those cases where the exact metabolism of a compound is unknown, and, furthermore, help in the interpretation of results. The approach proposed in this study appears very promising, allowing the identification of main metabolites of JWH-015, JWH-098, JWH-251, and JWH-307, which are extensively metabolized as demonstrated by the *in vitro* experiments performed.

The MS fragments obtained are typical of this class of substances, as also reported in other studies involving synthetic cannabinoids. This could in principle allow the development of screening methods based on most probable fragments of main metabolites, predicted *in silico*, in those cases where analytical standards are not available.

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