Review

Bioanalytical methods for the determination of synthetic cannabinoids and metabolites in biological specimens

Abstract

Recently the use of synthetic cannabinoids (SCs) has increased around the world. As a result, the importance for accurate analysis of SCs in human biological matrices is evident and continues to be especially challenging due to their chemical structures being constantly modified. Many methods have been published recently for the analysis of SCs in human biological samples. This review provides an overview of the analytical methods used for the analysis of SCs and their metabolites in biological specimens with a special focus on chromatographic analysis and sample preparation. Liquid chromatography assay is the most commonly used for confirmation purposes of SCs and their metabolites in biological matrices. In blood and oral fluid, analysis of SCs must be very sensitive. In urine, SCs have extensive metabolism pathways; therefore the main target compounds are their hydroxyl and carboxyl metabolites, which is important to recognise when establishing clinical and forensic toxicology analytical methods.

Synthetic Cannabinoids, GC-MS, LC-MS/MS, analysis, review

Contents

Abbreviations

AAIs	Aminoalkylindoles
AAPCC	American Association of Poison Control Centres
AB-001	Adamantan-1-yl(1-pentyl-1H-indol-3-yl)methanone
AB-CHMINACA	N-[(1S)-1-(Aminocarbonyl)-2-methylpropyl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide
AB-FUBINACA	N-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide
AB-PINACA	N-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide
ADB-CHMINACA	(*N*-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-(cyclohexylmethyl)-1H- indazole-3-carboxamide)
ADB-FUBINACA	N-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide
ADBICA	N-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-pentyl-1H-indole-3-carboxamine
ADB-PINACA	N-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-pentyl-1Hindazole-3-carboxamide
AKB48	N-(1-Adamantyl)-1-pentyl-1Hindazole-3-carboxamide
AM-1220	[1-[(1-Methylpiperidin-2-yl)methyl]-1H-indol-3-yl]-(naphthalen-1-yl)methanone
AM-1248	Adamantan-1-yl[1-[(1-methyl-2-piperidinyl)methyl]-1H-indol-3-yl]methanone
AM-1241	(2-Iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1Hindol-3-yl]methanone
AM-2201	[1-(5-Fluoropentyl)-1H-indol-3-yl]-1-naphthalenylmethanone
AM-2233	(2-Iodophenyl)[1-[(1-methyl-2-piperidinyl)methyl]-1H-indol-3-yl]-methanone
AM-679	(2-Iodophenyl)(1-pentyl-1H-indol-3-yl)methanone
AM-694	1-[(5-Fluoropentyl)-1H-indol-3-yl]-(2-iodophenyl)methanone
APCI	Atmospheric pressure chemical ionisation
APICA	N-(1-Adamantyl)-1-pentyl-1Hindole-3-carboxamide
CI	Chemical ionization
CP47, 497	(2-[(1R,3S)-3-Hydroxycyclohexyl]-5-(2-methyloctan-2-yl)phenol)
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction's
ESI	Electrospray ionization
EWDTS	European Workplace Drug Testing Society
5F-AKB48	N-(1-Adamantyl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide
5F-PB-22	1-(5-Fluoropentyl)-8-quinolinyl ester-1H-indole-3-carboxylic acid
FID	Flame ionization detector
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
HEIA	Homogeneous enzyme immunoassay
HU-210	3-(1,10-Dimethylheptyl)- 6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6Hdibenzo[b,d]pyran-9-methanol
JWH-015	1-Naphthalenyl(2-methyl-1-propyl-1H-indol-3-yl)methanone
JWH-018	1-Naphthalenyl(1-pentyl-1H-indol-3-yl)methanone
JWH-019	1-Naphthalenyl(1-hexyl-1H-indol-3-yl)methanone
JWH-020	1-heptyl-1H-indol-3-yl-1-naphthalenyl-methanone
JWH-073	1-Naphthalenyl(1-butyl-1H-indol-3-yl)methanone
JWH-081	4-methoxynaphthalen- 1-yl- (1-pentylindol- 3-yl)methanone
JWH-122	4-methylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone
JWH-200	1-Naphthalenyl[1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl]methanone
JWH-203	2-(2-Chlorophenyl)-1-(1-pentyl-1Hindol-3-yl)ethanone
JWH-210	4-ethylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone
JWH-250	2-(2-Methoxyphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone
JWH-251	2-(2-Methylphenyl)-1-(1-pentyl-1Hindol-3-yl)ethanone
JWH-307	[5-(2-Fluorophenyl)-1-pentyl-1Hpyrrol-3-yl](naphthalene-1-yl)methanone
JWH-387	(4-bromo-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone
JWH-398	1-pentyl-3-(4-chloro-1-naphthoyl)indole
JWH-412	(4-fluoro-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone
LC	Liquid chromatography
LC/MS	Liquid chromatography/mass spectrometry
LC–MS/MS	Liquid chromatography/tandem mass spectrometry
LC–HR/MS	Liquid chromatography–high resolution mass spectrometry
LLE	Liquid–liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MAM-2201	[1-(5-Fluoropentyl)-1H-indol-3-yl](4-methyl-1-naphthalenyl)methanone
MRM	Multiple reaction monitoring

MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
NPD	Nitrogen phosphorus detector
NPS	New psychoactive substances
PB-22	1-Pentyl-1*H*-indole-3-carboxylic acid 8-quinolinyl ester
QUCHIC	Quinolin-8-yl 1-(cyclohexylmethyl)-1H-indole-3-carboxylate
RCS-4	(4-Methoxyphenyl)(1-pentyl-1Hindol-3-yl)methanone
RCS-8	1-(2-cyclohexylethyl)-3-(2-methoxyphenylacetyl)indole
SALLE	Salting-out liquid–liquid extraction
SAMHSA	Substance Abuse and Mental Health Services Administration
SCs	Synthetic cannabinoids
SIM	Selected ion monitoring
SLE	Supported liquid extraction
SoHT	Society of Hair Testing
SPE	Solid-phase extraction
THC	Tetrahydrocannabinol
TOF/MS	Time-of-flight/ mass spectrometry
UR-144	(1-Pentyl-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone
WADA	World Anti-Doping Agency
WIN48, 098	(4-methoxyphenyl)-[2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]methanone
WIN55 212-2	*R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1 naphthalenyl)methanone mesylate
XLR-11	[1-(5-Fluoropentyl)-1H-indol-3-yl](2,2,3,3- tetramethylcyclopropyl)methanone

1. Introduction

From 2004, illicit drugs manufacturers began to increasingly produce herbal mixtures under a variety of brand names, e.g. “Spice” and “K2” [1]. It has been sold in European countries such as Switzerland, Austria and Germany since at least 2006 [2]. To produce these, chemical additives are sprayed onto dried plant materials to achieve psychoactive effects, and these products started being marketed throughout the world as safe and legal alternatives to cannabis [3]. Mixtures of these drugs commonly have labels such as ‘incense’, ‘not for human consumption’ or ‘for aromatherapy only’ to try to avoid regulatory oversight [4]. They also often come without declaration of ingredients and some may still come with ingredients list, however it does not contain the true contents because SCs are omitted from them [5]. They have been easy to buy for several years over the internet, or in gas stations or “head shops" [6].

It was not really until 2008 that these new products became popular, coinciding with the time that media in Germany claimed that they were a ‘legal’ alternative to marijuana but could not be detected by common drug-screening tests [7]. By the end of 2008, forensic researchers had detected several synthetic cannabinoids (SCs) in these herbal smoking mixtures [8]. The most commonly detected compounds in the first generation of “Spice” products were CP-47,497 and the JWH- series [9], named after John William Huffman, a professor at Clemson University who first synthesised them in the laboratory during 1990s while studying human endocannabinoid receptor systems and their potential as therapeutic agents for scientific research [10]. Since then, the number and type of Spice products and new SCs have increased continuously.

SCs are the largest drugs group monitored by the European Monitoring Centre for Drugs and Drug Addiction’s (EMCDDA) early drug warning system [11]. In 2013, 21,495 seizures of SCs were reported across Europe which represents 40 percent of the total number of seizures of new psychoactive substances (NPS) [11]. During 2014, a total of 101 NPS were notified for the first time through the organisation’s early warning system (EWS), and 30 of these compounds were SCs [11]. According to the American Association of Poison Control Centres (AAPCC), the number of calls related to SCs exposure sharp increased from 2096 calls in 2010 to 6968 in 2011, and decreased to 5230 in 2012, and dropped to 2668 in 2013; the trend of the exposures of SCs was increased again with 3,677 calls in 2014 [12]. Many SCs are now designated Schedule 1 Drugs under the US Controlled Substance Act and similar legislation has been put in place in many countries in a bid to stem the flow of them [13]. The World Anti-Doping Agency (WADA) has classified designer cannabimimetics as prohibited substances in sports [14].

SCs may cause serious toxicity and impairment of health, and as such, the development of analytical methods for the detection of these compounds in different biological matrices is very important for clinical and forensic purposes. However, the detection of SCs and metabolites in biological specimens using common drug-screening tests is difficult because SCs are structurally different from delta-9 tetrahydrocannabinol (THC), the primary psychoactive compound of cannabis [15, 16]. The analytical method development of SCs in biological specimens is challenging for many reasons; lack of available reference materials, the low concentrations of SCs in the body due to high potency, and the similarities of the structures of various SCs that may cause interference. Recently, several reviews on the toxicity, pharmacology and detection methods of SCs have been published [17-19]. This review will focus on the chromatographic analysis and sample preparation techniques of SCs and their metabolites in biological specimens.

2. Pharmacological and toxicological aspects of synthetic cannabinoids

After the completion of THC synthesis in 1964 by Raphael Mechoulam [20] and the identified of cannabinoid receptors CB_1 [21] and CB_2 in 1980s [22], the interest in synthesising cannabinoids has increased to the yield of more than 100 substances. SCs contain a great variety of structurally dissimilar compounds and this makes the classification of SCs is quite complex.

The classification of SCs, based on the chemical structures was published by Thakur *et al*. [23] and Howlett *et al*. [24] and includes classical cannabinoids (e.g. HU-210, AM-906, nabilone), nonclassical cannabinoids (e.g. CP47,497), eicosanoids (e.g. Methanandamide), aminoalkylindoles (AAIs) and others (e.g. JWH-307, AKB48) (Figure 1). AAIs can be categorised into sub-groups includes naphthoylindoles (e.g. JWH-018, JWH-073, JWH-200, JWH-122, JWH-210, JWH-398, AM-2201), phenylacetylindoles (e.g. JWH-250, JWH-203, JWH-251, RCS-8), benzoylindoles (e.g. AM-694, RCS-4), naphthylmethylindoles (e.g. JWH-184), naphthylmethylindenes, adamantoylindoles (e.g. AM-1284), cyclohexylphenols, and tetramethylcyclopropylindoles.

These compounds have structural features that allow binding to CB_1 or CB_2 or to both of them in the brain [25] which results in the creation of cannabis-like effects (e.g. impaired sense of time, paranoia, sedation, hallucinations, anxiety and tachycardia) [26, 27]. In *In vitro* studies, it was found that some of SCs bind to cannabinoid receptors (mostly CB_1) more avidly than THC as measured by the affinity constant K_i and the effect of this is to produce more severe intoxication [27, 28].

Some users reported and/or showed additional unique effects from SCs such as agitation [27], hypokalaemia, hyperglycemia, seizures and emesis [29, 30], acute kidney injury [31], stroke [32], myocardial ischemia secondary [33] and myocardial infarction [34]. SCs have also been reported in

association with deaths in several publications. Behonick *et al*. [35] reported four deaths following use of 5F-PB-22. One death related to use of AM-2201 has been reported by Patton *et al*. [36]. Recently, ADB-CHMINACA (*N*-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide) has been reported in association with two deaths; one has been reported by the Hungarian National Focal Point [37] and the other one has been reported by Hasegawa *et al.* [38] that occurred in Japan.

The usual mode of administration for the SCs is smoking via a conventional cannabis pipe, in cigarette papers or via a water pipe [3]. Teske *et al.* [39] reported the maximum concentration of SCs in serum was in the range of 10 ng/ml after smoking approximately 50 µg/kg of JWH-018, and then decreases rapidly to less than 10 % of original concentration within 3 hours. SCs are easily metabolised to hydroxyl or carboxyl derivatives of the *N*-alkyl side chain or aromatic ring [40, 41]. Saito *et al.* [42] reported the accumulation of some SCs such as MAM-2201 in the adipose tissue due to their high lipophilicity.

Due to extensive metabolism, urine analysis requires identification of their probable metabolites. The metabolism studies of SCs are based on *in vivo* experiments [40, 43-53] or on *in vitro* experiments with human or rat liver microsomes [24, 41, 43, 44, 53-63]. Due to the speed of both the emergence and spread of SCs on the world market, the pharmacokinetics properties of some SCs have not been satisfactorily investigated.

3. Sample preparation

Sample analysis workflow should include a process of sample collection, transportation, preparation, analysis and report generation. Forensic laboratories should follow procedures and recommendations for the selection of the appropriate specimens and on how to be collected and tested that managed by guidelines, such as those of the Substance Abuse and Mental Health Services Administration (SAMHSA), European Workplace Drug Testing Society (EWDTS) or Royal College of Pathologists (UK) in order to minimize the risk of errors. During transporting and storage, it should be considered that loss of analyte from samples could adversely affect the quality of test results. For the analysis of SC and metabolites in serum, urine and oral fluid, samples should be refrigerated or frozen to minimise degradation. For example, PB-22 and 5-F-PB-22 was reported to degrade significantly due to the instability of the ester bond in the structures [54].

Sample preparation is an essential stage of the analytical process to convert the biological specimen into a form that is suitable for analytical investigation. The extraction step is the main part of the procedure of sample preparation, and presents two major advantages for the analysis process [64].

Firstly, it removes interfering matrix compounds (such as proteins, salts and phospholipids) which reduces background noise. Secondly, it concentrates the target drugs, increasing sensitivity and achieving lower limits of detection. In liquid chromatography-tandem mass spectrometry (LC-MS/MS), suitable sample preparation is important to reduce matrix effect, which is a change in the ionisation process of the drug due to endogenous matrix components. Matrix effects may alter the MS response of the target drug resulting in ion enhancement (gain in signal) or suppression (loss of signal) leading to effect on the precision, accuracy and robustness of the method.

Simple dilution, protein precipitation, liquid-liquid extraction (LLE), solid phase extraction (SPE), supported liquid extraction (SLE) and salting-out LLE (SALLE) have been used for the extraction of SCs.

3.1 Protein precipitation

Protein precipitation has been found to be more useful when analysing protein rich matrices, such as whole blood, serum and plasma. Some authors have used protein precipitation for the extraction of SCs [36, 65]. An organic solvent is added to the specimen to reduce the solubility of the solute and precipitate the protein, and then it can be removed from the specimen by centrifugation or filtration. Protein precipitation is a rapid and simple extraction technique; however, it does not remove many of the matrix interferences.

3.2 Liquid-liquid extraction

LLE is applicable with most types of matrices. In SCs extraction, LLE is usually used because of their high hydrophobicity. It has been used for the extraction of SCs in the following matrices: blood [35, 66-72], serum [39, 73-76], plasma [77], urine [40, 41, 47, 49, 51, 59, 72, 78-83], oral fluid [84, 85] and hair [86-90]. It involves adding an immiscible organic solvent such as *tert*-butyl methyl ether [41, 82], chloroform [50] and diethyl ether [40, 52, 79] to the sample, and mixing, removing the organic solvent. The target compounds transfer from the sample layer to the organic layer. A sufficient amount of solvent should be used to capture all of the drugs from the original sample. The two phases are agitated by shaking the mixture, and then the organic phase is allowed to separate.

LLE offers better clean up than protein precipitation and it also removes protein. In addition, it can be optimised for different compound classes. However, it requires an evaporation step to remove excess of solvents prior to analysis and can be difficult to automate.

3.3 Solid phase extraction

In more recent years, SPE has become more popular as an extraction technique in analysing illicit drugs. Extraction of SCs using SPE has been used in blood and serum [91], urine [92-96] and oral fluid [45, 97, 98]. Different types of SPE columns can be used depending on the cost, availability and the nature of the analytes of interest. The sample matrix is allowed to pass via the sorbent to waste, with the target analytes being retained. A series of washing steps are essential to remove matrix interferences, and then target analytes are eluted back off and collected in a clean vial.

SPE is the only extraction type that will remove the endogenous compounds that cause technical problems such as co-elution which may cause matrix effects. Ionisation of the compound occurs at the interface of the LC-MS. The using of suitable sample preparation (e.g. SPE) and deuterated internal standards can reduce matrix effects. SPE also concentrates and cleans up the samples. However, Grigoryev *et al.* [99] reported nearly identical amounts of matrix elements for LLE and SPE in the extracts of SCs in urine samples. Rigdon *et al*. [100] reported SPE give better recovery of carboxylated metabolites of JWH-018 and JWH-073 in urine samples than LLE. The disadvantages of SPE are the cost and time of method development and extraction.

4. Analytical methods

Immunoassays (e.g. enzyme linked immunosorbent assays 'ELISA') and separation methods such as gas chromatography mass spectrometry (GC-MS), LS-MS/MS have been used for studying SCs and metabolites in biological samples.

4.1 Immunoassay technique

Immunoassay is a rapid technique used as an initial screening test to eliminate negative samples. Whilst quick, it has limitations, such as lower specificity. Moreover, cross-reactivity and false positives can occur, and as such, toxicologists recommend that all positive immunoassay findings must be retested and confirmed by chromatographic analysis. Immunoassay tests have been used for the screening of SCs in oral fluid [101] and their metabolites in urine samples [82, 93, 102-104].

Different commercial kits have been available for detecting SCs and metabolites, including those made by Randox Toxicology, Neogen Corporation, Cayman Chemical, Immunalysis Corporation and National Medical Services [103]. The main limitation of immunoassay is the inability to cross-react with newer analogues of SC as they become more widely used. It is difficult for the manufactures to keep up within the rapidly changing analogues in use. Rodrigues *et al*. [101] evaluated an ELISA targeting JWH-200 in oral fluid. They reported that in oral fluid ELISA targeting JWH-200 had limited cross-reactivity to JWH-018, JWH-073, JWH-015, JWH- 022, AM2201, AM2232 and AM1220.

4.2 Gas chromatography

GC-MS is a commonly used separation technique for identification and quantitation of drugs of abuse in biological samples in most laboratories. However, GC-MS has some disadvantages such as high retention and limitation on the masses of the analytes. In addition, compounds which are thermally labile might decompose in the GC injection port. Many compounds with a polar functional group such as an amino or hydroxyl groups can also cause a polar interaction between the compound and the column stationary phase that leading to poor detection of the analyte. To overcome these problems, derivatisation step must be added onto the extraction method for these compounds. Derivatisation can change the functional groups and chemical properties of the molecule, making them less reactive with the stationary phase and more stable. Derivatisation is also used to increase sensitivity and specificity, improve peak shape, reduces tailing of polar compounds and rise volatilisation and ionization to promote chromatography. However, this adding step consumes more time and reagent which can be toxic. Moreover, it may introduce another source of possible error.

In SCs analysis, it seems essential to include a derivatisation step prior to GC analysis to increase detectability and stability of compounds and help to improve analytical efficiency [105]. SCs are organic compounds containing active and polar functional groups, and are allowed to react with different types of derivatisation reagents. The following derivatisations have been used for SCs: trimethylsilylation [9, 46, 80, 99], methylation [80], acetylation [46, 99] or trifluoroacetylation [46, 99]. Acetylation is preferred to routine analyses despite the slightly lower sensitivity because trifluoroacetylation can cause low thermal stability for JWH-018, JWH-073, JWH-250 and their metabolites [99]. MS detector is mostly carried out for SCs detection. Other types in use are nitrogen phosphorus detector (NPD) [4] which is suitable for most types of aminoalkylindoles, and flame ionization detector (FID) [106, 107].

Electron impact (EI) and chemical ionization (CI) are the most common ionization techniques in GC-MS. Most of EI fragments molecules at 70 electron volts (eV), producing several fragmentation of molecules and more than those acquired by LC–MS that can provide useful structural information.

Mass spectra under EI conditions differ significantly from one SC to another due to the great variety of their chemical structures. The fragmentations of SCs can take place on both sides of the carbonyl group. The mass-spectral fragmentation pattern of SCs also involves formations of different immonium ions at m/z = 127 and 155 (naphthyl and naphthoyl moieties) or 144 (indole moiety).

Indeed, SCs are not ideal compounds for GC-MS analysis because they are neutral to weakly acidic compounds and have high molecular weight. Some SCs are very difficult to analyse using GC-MS. For example, ester analogs (e.g. PB-22) decompose (or participates in the ester-exchange reactions) in the injection port. Another example is that cyclopropyl (e.g. UR-144) undergoes a thermal degradation (isomerization) mainly in GC column [108, 109]. This is indicated by shape of chromatographic peaks and variation of GC conditions. Therefore, appropriate strategies for the identification of the thermal degradation product of SCs (e.g. UR or XLR series) should be used during method development stage. Suitable derivatisation agent should be added to the extracted drug prior to analysis. The injection port temperature can be reduced to prevent injection port degradation. Generally, the sensitivity of GC-MS is low, which makes the determination of SCs in biological samples particularly blood more challenging.

4.3 Liquid chromatography

LC is the most popular technique for the analyses of different types of human matrices containing SCs and their metabolites. In SCs analysis, as with most LC-MS methods, methanol or acetonitrile are commonly used for the organic solvent of the mobile phase. Acetic acid, formic acid or their salts are mainly chosen as buffer additives to enhance the ionisation leading to increase the sensitivity. C18 columns are mainly performed for the separation of SCs and their metabolites during LC.

The combination of LC with mass spectrometry (MS) can yield a powerful analytical tool for identification purposes. It is clear that tandem mass spectrometry is the preferred instrument for the detection of SCs because they offer more information about chemical structures than acquired by single mass spectrometry.

Electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) are usually used for ionisation. The fragmentation of the parent drug and their metabolites is similar for ESI+collision and EI modes. However, the m/z of the fragments is different.

It was found that most of SCs are affected by the matrix compounds found in the sample [69, 79], particularly when ESI is used [110]. It has been recommended that the most effective way to minimise ME is to use of isotope-labeled internal standards [111]. However, this is not always possible due to the rapid proliferation of SCs on the world market and absence of certified reference material. Another solution to minimise the ME is to choose suitable sample preparation procedure. Due to the different ionisation processes, APCI seems to be less susceptible to matrix effects than ESI [112]. However, it was found that the responses of SCs are lower using APCI than with ESI [73].

ESI and APCI interfaces operate in positive and negative polarity. In SCs' analysis, positive polarity is commonly performed. The negative ionisation mode has been used for HU-210, CP 47,497 and its homologue due to the absence of nitrogen in the chemical structures of these compounds in contrast to almost all other SCs [82].

MS detection can be performed in full scan, product ion scan mode, selected ion monitoring (SIM) and multiple reaction monitoring (MRM). Full scan mode reduces sensitivity however, and this may cause false negative result for analytes in low concentrations. Fragment ions are observed using product ion mode method when the protonated molecular ion ($[M+H]^+$) is utilized as the precursor ion. Two product ions of acceptable abundance should be determined to ensure that the correct compound and achieve good sensitivity. SIM and MRM modes elevate sensitivity however, it is important to have information about the compounds and fragmentation patterns.

4.4 Analytical challenges and solutions

The speed of the emergence of SCs has resulted in several analytical challenges for clinical and forensic laboratories. Reference material for use in positive identifications is not readily available. In blood and oral fluid, parent compounds are found at extremely low concentrations therefore methods for analysis of SCs in these matrices must be very sensitive. To the best of our knowledge, libraries of reference mass spectra were not commercially available to assist the identifications, especially for the most recent drugs. Similar or overlapped mass spectra of SCs are sometimes obtained by GC-MS due to their structural similarities and isomeric forms. The misidentification of these drugs might increase when using only the information from the mass spectra. The identification of new compounds in a biological material without knowledge about their structure is very difficult, even if analysing using LS-MS/MS.

To overcome these emerging challenges, different strategies should be applied. Forensic communities must monitor the world drug market to ensure that clinical and forensic laboratories are keeping up with the illegal drug suppliers. When new compounds appear in the drug-user marketplace, toxicologists need to respond quickly and identify suitable biomarkers via *in vitro* studies to detect intake. Toxicologists need also to address the synthesis of reference standard for these new compounds.

Analytical methods for the identification of SCs should be continuously updated, improved and revalidated to cover the new drug. Because SCs contain a variety of functional groups, the extraction of SCs requires a general procedure. Very limited data on cut-offs, detection windows or expected concentrations of SCs have been published. Therefore, LODs should be determined as low as

analytically possible. Nowadays, laboratories have gained more experience with these drugs. Therefore, toxicologists should collect larger databases of searchable mass spectral library of many SCs and their metabolites and biomarkers that can indicate SC use.

To overcome the misidentification of various SCs, which have similar chemical structures that can produce very similar fragmentation patterns or to identify new compounds in a biological material, High Resolution (HR) MS or TOFMS can be used to estimate the structure and screen of parent drugs and their metabolites of SCs in specimens. The development of non-targeted methods using HRMS technique facilitates the detection of known or unknown compounds at the time of analysis. It can distinguish between different substances with the similar nominal molecular mass. Unlike MS analysers, new HRMS technology enables the identification of analytes based on the exact molecular formula because it offers mass assignment with an accuracy of 0.001 atomic mass units (amu) [113]. This new technology allows for retrospective data inquiry after library updates. The main challenge of non-targeted LC-HRMS screening methods is to achieve sufficient selectivity to identify low concentrations of SC [19]. Several non-targeted methods using HRMS technique for screening of drugs of abuse including SCs in biological matrices were published [89, 94, 114, 115]. These methods are enabling forensic laboratories to obtain accurate data of MS and MS/MS without pre-selecting substances.

5. Synthetic cannabinoids detection in biological specimens

The identification and determination of SCs and their metabolites can be performed in the analysis of various body fluids, including blood, urine, hair and oral fluid. Seized products, such as herbal blends and powders, can also be tested for the presence of SCs. The choice of the kind of sample matrix is a critical component of forensic toxicology investigation. It depends on many factors such as the availability of specimens and the purpose of analysis. Figure 2 illustrates the number of publications for each biological matrix since 2010. A summary of the published methods for determination of SCs and their metabolites in blood/serum/plasma, urine, oral fluid and hair are summarised in Tables 1, 2, 3 and 4 respectively.

5.1 Blood, serum and plasma

Blood can offer unique advantages over other matrices. It can be used for the determination of recent drug use as in the case for identifying drivers under the influence of illicit drugs [70, 116]. It also can be used for the determination of the concentrations and ratios of parent drug and metabolite that could yield useful information relating to acute or chronic drug use. However, blood sampling requires the presence of medical staff for the collection and there being a risk of infection.

Hermanns-Clausen *et al.* [27] studied the clinical and laboratory findings for 29 patients seeking emergency treatment after use of SCs between September 2008 and February 2011. They found in poisoning cases, the concentrations of CP-47,497-C8, JWH-018, JWH-073, JWH-250, JWH-122, JWH-081, JWH-015, JWH-210 and AM 694 in serum were between 0.1 and 190 ng/mL.

In fatal cases, the concentrations of SCs in blood were 0.1-199, 0.1-68.3, 12, 1.1-1.5, 12.4 and 6.05-10.6 ng/mL for JWH-018 [67], JWH-073 [67], AM-2201 [36], 5F-PB-22 [35], MAM-2201 [42], and ADB-CHMINACA [38], respectively. However, the lethal doses for many SCs are still unknown. The concentrations of SCs in blood are usually very low however, and as such, analytical methods for detection need to be very sensitive. Another difficulty in determining SCs in blood specimens is the window for detecting acute intake is short (hours–days) [39]. In the case of long-term use of SCs, the windows for detection can be longer due to the accumulation of its presence in fatty tissues [117].

A qualitative method for the detection of 20 SCs in blood using LC-TOF/MS and automated SPE was developed by Guale *et al.* [91]. Covering a wider scope, a qualitative method for the detection of 46 SC and 8 labelled analogs in serum utilizing LC-MS/MS and heated electrospray ionization (ESI) to enhance the sensitivity was developed by Huppertz *et al*. [76]. LODs ranged from 0.1 ng/ml to 0.5 ng/ml.

Kneisel *et al*. [75] developed a quantified method for the determination of 30 SCs in serum using LC-MS/MS following an alkaline LLE with n-hexane/ethyl acetate (90:10) mixture. Recoveries were in the range of between 94% and 111%. LOQs and LODs were 0.1 - 2.0 and 0.01 – 2.0 ng/mL respectively. 833 authentic serum specimens from forensic cases were analysed between August 2011 and January 2012. 227 (27.2 %) were positive for at least one of the following SCs: AM-2201, JWH-018, JWH-073, JWH-307, JWH-210, JWH-203, JWH-200, JWH-122, JWH-081, JWH-019 and RCS-4. JWH-210 was the most prevalent of SCs in positive samples (80 %), then JWH-122 (63 %) and AM-2201 (29%).

An LC-MS/MS method after LLE for the quantitation of JWH-018, JWH-073, JWH 200, JWH-250, JWH-081, JWH-015, WIN 55,212-2 and methanandamide and the detection of JWH-020 and JWH-019 in serum was developed by Dresen *et al*. [73]. All analytes were stable in serum under storage conditions of −20 °C. However, degradation of JWH-018 and JWH-081 and the respective internal standard JWH-018-d_{11} in serum samples stored in the autosampler at 4 °C by more than 15 percent after 5 hours. They also observed a degradation of JWH-073, JWH-081 and methanandamide in serum samples kept at room temperature in polypropylene and glass tubes by more than 15 percent after 72 hours. 101 authentic serum samples from 80 subject provided by police authorities and

therapy and forensic psychiatric centres were analysed. 57 samples (56.4%) of the samples were found positive for at least one SC. Methanandamide, WIN 55,212-2, JWH-200, JWH-020 and JWH-019 were not detected in any samples.

5.2 Urine

Urine is the most widely used matrix for toxicological analysis in providing evidence of drug use and assessing drug exposure. It is the preferred specimen for many laboratories because the collection of urine samples is easy and non-invasive and the concentrations of analytes are often higher when compared with sample of blood or oral fluid. Moreover, the detection time in urine is longer than blood (days–weeks). The prevalence of positive results in urine specimen for some compounds is higher than those observed in blood because the detection windows are longer for urine samples. However, there are some disadvantages with using urine as a sample, and these include the possibility of adulteration or substitution, and it is not suitable for identifying drivers suspected of being under the influence of drugs as only gives evidence of past use.

The parent compounds of SCs may not be detectable in urine; therefore it seems essential to focus on metabolites [41]. The metabolisms of some of SCs are still under investigation which also makes analysis from urine samples more complicated. Hydrolysis with acids [46, 80, 99] or enzymes [40, 50, 51, 59, 118] is required in the procedure of urine sample preparation for SCs because it was found that the percentage of conjugated form excreted of the hydroxylated metabolites of JWH-018, JWH-073 and JWH-250 is very high [46, 51, 119]. JWH-122 and its respective fluorinated analogue, MAM-2201 produced common metabolites, *N*-4-hydroxylated, *N*-5-hydroxylated and carboxylated JWH-122 metabolites. The key factor to prove JWH-122 or MAM-2201 abuse is the relative concentrations of JWH-122 *N*-4-OH M and JWH-122 *N*-5-OH M [120]. *N*-4-hydroxylated JWH-122 metabolite is the predominant in JWH-122 metabolism, whereas *N*-5-hydroxylated JWH-122 metabolite was the primary metabolite of MAM-2201 [120].

The first publication to identify the JWH-018 urinary markers after the smoking of illicit herbal mixtures was by Sobolevsky *et al.* in 2010 [40]. Urine samples were collected from three intoxicated people taken into police custody. The parent compound JWH-018 was not detected whereas the phase-1 metabolites were identified after de-conjugation. The Authors suggested a low content of the metabolites phase-1 in urine.

Another confirmation LC-MS/MS method was developed to detect 9 hydroxypentyl, carboxylated and hydroxyindole metabolites from 8 parent SCs [79]. Samples were treated by enzymatic

hydrolysis and LLE. The method was applied to urine samples obtained from a volunteer who smoked a mixture blend.

An LC-MS/MS method using SPE and enzymatic hydrolysis for simultaneous quantification of 37 SCs metabolites such as *N*-hydroxypentyl and carboxy metabolites in urine was developed by Jang *et al*. [121]. All SCs metabolites were stable in urine stored at 4 °C and −20 °C for 14 days, and after three freeze–thaw cycles. LODs were between 0.1 and 1 ng/ml, and extraction recoveries were 65-99%. Wohlfarth *et al*. [122] developed an LC-MS/MS method using a library search for qualitative detection of 9 SC and 20 metabolites in human urine. Protein precipitation and enzymatic hydrolysis have been used for sample preparation. LODs ranged from 0.5 to 10 ng/ml. Recoveries were in the range of between 53 and 95% and matrix effects were between 95 and 122%.

An LC-MS/MS method using SLE+ extraction for the simultaneous quantification of 20 SCs and 21 metabolites, and semi-quantification of 12 alkyl hydroxy metabolites in urine was developed by Scheidweiler *et al.* [123]. SLE is analogous to traditional LLE. SLE+ which is a product of SLE containing plates and/or columns provides inherently cleaner extracts than other extraction techniques and high analyte recoveries. The method was conducted in two MRM injections; one in negative for HU-210 ionized and CP 47,497 compounds and one in positive ESI Mode for all other analytes. LODs for the evaluated drugs ranged from 0.1 to 1 ng/ml. Matrix effects and extraction efficiencies were - 73 to 52% and 44 to 110%, respectively.

Kronstrand *et al*. [114] compared the performance of an immunoassay screening for SCs with a new developed non-targeted qualitative LC-HR/MS confirmation method in urine. Enzymatic hydrolysis and SALLE have been used for sample preparation for LC-HR/MS method. They observed no cross-reactivity with UR-144 metabolites but there was cross-reactivity with JWH-122 and MAM-2201 metabolites for the immunoassay. They found the immunoassay performed well for SCs present in the urine samples tested. Due to the rapidly changing of SCs which may cause problems for immunoassays and for confirmation methods, the using of time-of-flight mass spectrometry is necessary for forensic laboratories as the new SC can be quickly included to the method and identified.

5.3 Oral fluid

Illegal drugs are readily detectible in oral fluid specimens. Oral fluid can be used as an alternative to blood for identifying drivers that are suspected of driving under the influence, and in place of urine samples when testing for drugs usage in the work place. It is easy and rapid to obtain and is less prone to adulteration [84]. In addition, oral fluid containing parent SCs make the sample a good

choice for testing in cases where metabolite references may be lacking, or if the target metabolites are still unknown [101]. However, there are some disadvantages; the sample volume must be sufficient for the analysis, and this amount could be difficult to obtain from some SCs abuser. Losses of the sample's volume can also occur without adequate devices for collecting the sample.

Different collection devices have been used for oral fluid collection including DCD 5000 Dräger [84], Intercept [85] and Quantisal™ [97, 101, 124]. Kneisel et al. [84] studied the detection of 30 SCs using LLE and LC-MS/MS in oral fluid. The Dräger DCD 5000 device was used for oral fluid sample collection. The linearity and accuracy were acceptable for 28 of the 30 SCs covered by the method. LOQs were in the range from 0.15 to 3 ng/ml and LODs ranged from 0.015 to 0.9 ng/ml. 264 authentic oral fluid samples were tested. 31 (12%) samples were found positive for at least one of the following SCs: JWH-018, JWH-250, JWH-203, JWH-122, JWH-081, JWH-019, JWH-307, JWH-210, AM-694, AM-2201, MAM-2201, and RCS-4. JWH-210 was identified in 31 samples, whereas JWH-122, JWH-081 and JWH-018 were identified in 17, 8 and 7 samples respectively.

Coulter *et al*. [97] developed a quantitative method for the detection of 6 SCs in oral fluid using SPE following LC-MS/MS with MRM in ESI+ and ESI- mode, and achieved 0.5 ng/ml LOQ. They observed a degradation for JWH-250 in oral fluid specimens kept at room temperature for one week by 25 percent of the initial concentration, while with refrigerated samples, the loss was only 10 percent. They also observed some degradation for JWH-073 and JWH-018 when stored at room temperature for one week, while when refrigerated, samples were stable. HU-210, CP 47,497 and its C8 homologue showed losses of 9 to 14 percent at both storage conditions over one week.

5.4 Hair

Hair was first described as an alternative specimen for drugs testing in the field of toxicology in 1979, when used to document long-term drug exposure. Hair testing provides long-term histories via segmental analysis, because hair offers a larger detection window when compared with all the other types of specimens (up to months) [125]. Another advantage of hair as a sample is low potential for donor manipulation. However, hair testing has its disadvantages too. Environmental contamination especially through smoking of drug, for example, can have an effect on hair analysis, leading to false-positive result [126]. According to the Society of Hair Testing (SoHT), preparation of a hair sample should include a washing step in the procedure prior to analysis to remove the superficial or surface drug [127]. This allows for demonstrating that the drug being recovered by the extraction procedure is an internally consumed drug.

Hutter *et al*. [128] developed a comprehensive quantitative method using LC-MS/MS for the determination of 22 SCs in hair. The hair specimen was washed with water, petroleum ether and acetone, and then dried, and cut into pieces. Finally, it was extracted with using LLE prior to analysis. LOQ was 0.5 pg/mg for all analytes, except JWH-398, which was 5 pg/mg.

Another quantitative method using LLE for the detection of 23 SCs in hair was developed by Salomone *et al*. [88]. The hair specimen was washed with dichloromethane, and extracted with n-hexan/ethyl acetate (90/10). An LC-MS/MS and SRM in ESI+ mode were used for analysis. LOQ were in a range of 0.7 and 4.3 pg/mg except for HU-210, at 80 pg/mg. In 344 authentic hair specimens obtained in 2011 from individuals with drug abuse histories and drivers with suspended licences, 4.36% (N=15) of the specimens were positive for at least one SC. The most prevalent compounds in positive samples was JWH-073 (N=11) at concentrations ranging from 1.6 to 50.5 pg/mg.

An LC-HR/MS screening method for the detection of 50 NPS including 8 SCs that had been reported by the National Early Warning System in Italy between 2009 and 2011 in hair was developed by Gottardo *et al*. [89]. For sample preparation, hair underwent NaOH digestion overnight and LLE prior to analysis. LOD was 10 pg/mg. The method was applied to 435 authentic hair specimens from individuals with suspended driving licences in year 2010. 8 were found positive for: JWH-018, JWH-081, JWH-073, JWH-122, JWH-250, with concentrations rate 0.010 - 1.28 ng/mg.

6. Conclusion

Due to the recurring threat of SCs to public health and their rapidly increasing usage worldwide, it is necessary to develop reliable analytical methods for their detection in different biological matrices. SCs are constantly being modified and rapidly becoming widely available; therefore laboratories should update their scope for detecting the most prevalent compounds at specific time.

LLE and SPE are the most widely used sample clean up procedures for the detection of SCs and their metabolites in biological specimens. The separation techniques GC-MS, LC–MS/MS and LC-QTOF/MS are mainly used to detect and quantify SCs present in different biological specimens as well as in “Spice” or “K2” products. LC–MS/MS is more convenient for the detection of SC and metabolites because it provides better sensitivity. Matrix effects must be evaluated during method validation.

Selection of the appropriate specimen is an important step in forensic toxicology. In blood and oral fluid samples, parent compounds are found at extremely low concentrations therefore methods for analysis of SCs in these matrices must be very sensitive. Urine is the most commonly collected sample for monitoring recent exposure. However, the analysis of drugs in urine is complex because it requires knowledge and understanding of the drug’s metabolites. Hydrolysis with acids or enzymes

should be performed in urine sample to enhance the sensitivity of detection. Blood and urine are the first choice of sample for SCs testing. Hair is often used as alternative sample in documenting drug exposure. Due to the stability issue of SCs, it is recommended to keep samples frozen whenever possible and sample extracts should be analysed shortly after extraction. We recommend that it is necessary to use HRMS to look for unknowns or keep updating your targeted method to include relevant analytes.

Acknowledgement

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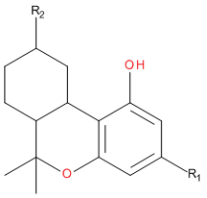
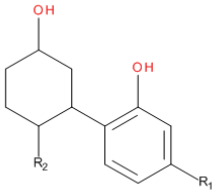
Conflict of interest

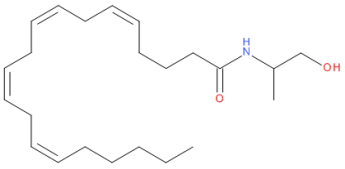
There are no financial or other relations that could lead to a conflict of interest.



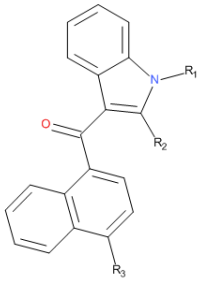
1. Classical cannabinoids



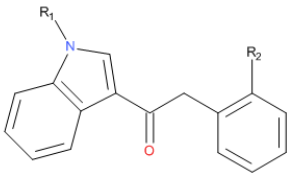
2. Nonclassical cannabinoids



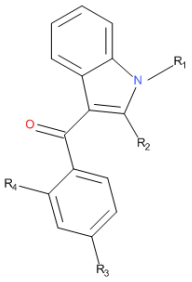
3. Eicosanoids



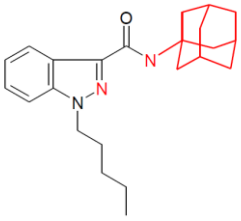
4 (1). Aminoalkylindoles - Naphthoylindoles



4 (2). Aminoalkylindoles - Phenylacetylindoles



4 (3). Aminoalkylindoles - Benzoylindoles



5. Others (e.g. AKB48)

Figure 1: Structures classes of SCs.

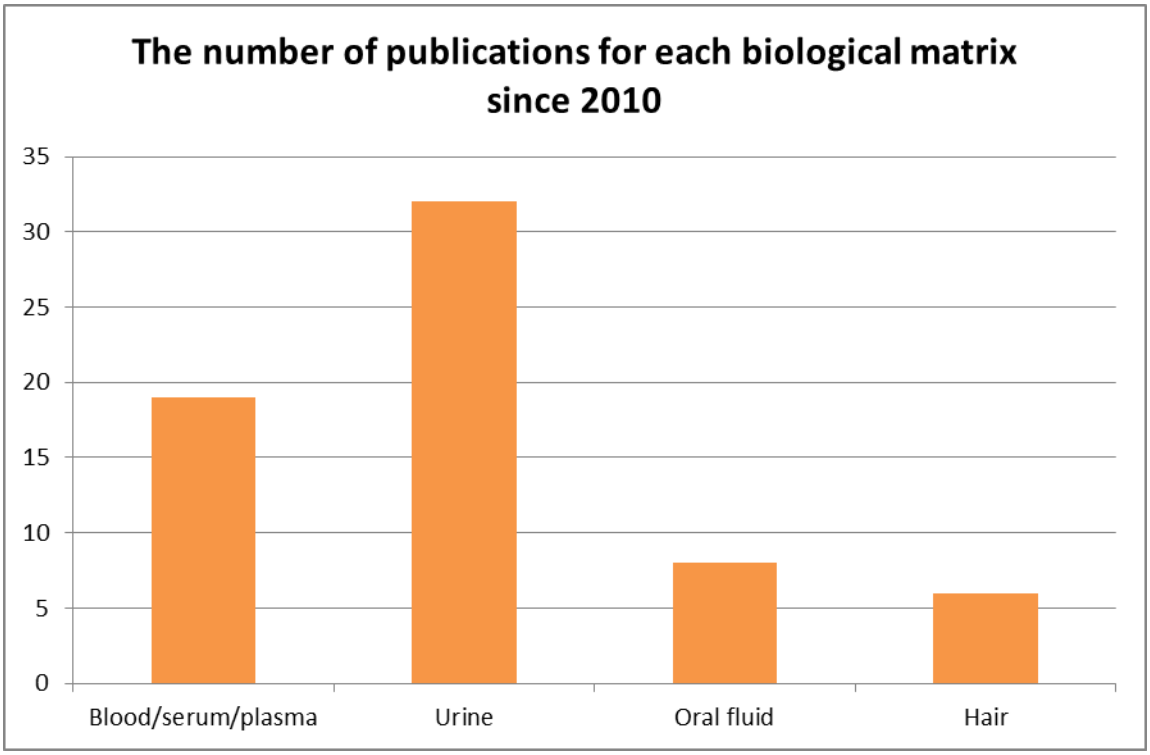


Figure 2: Methods in different biological specimens for SC detection published between 2010 and 2015.

Table 1: Bioanalytical procedures for determination of SCs and metabolites in blood/serum/plasma samples.

SCs	Matrix	Sample preparation	Detection mode	LOD (ng/mL) ; LOQ (ng/mL)	References
(25 SCs): JWH-018, JWH-073, JWH-081, JWH-203, JWH-210, JWH-250, JWH-251, JWH-007, JWH-015, JWH-019, JWH-030, JWH-302, JWH-398, CP47, 497, CP47, 497 C8-homolog, HU-210, AM-694, AM-1241, RCS-4, RCS-4 2- and 3-methoxy homolog, RCS-8, RCS-4-C-4-homolog, WIN48 098, WIN55 212-2 mesylate	Whole blood	LLE	LC-MS/MS-ESI+/-	LOQ = 0.5 and 5	[66]
(29 SCs): JWH-018, JWH-019, JWH-020, JWH-073, JWH-122, JWH-147, JWH-200, JWH-210, JWH-250, JWH-251, JWH-398, JWH-007, JWH-203, JWH-015, JWH-073-methyl, JWH-081, JWH-098, AM-1220, AM-1241, AM-2201, AM-2233, MAM-2201, AB-001, AM-694, RCS-4, RCS-4 ortho, RCS-8, UR-144, WIN55 212-2	Whole blood	LLE	LC-MS/MS-ESI+	Not specified	[68]
(2 SCs): JWH-018, JWH-073	Whole blood	LLE	LC-MS/MS-ESI+	0.01 ; 0.05	[67]
(4 SCs): JWH-018, JWH-073, JWH-019, JWH-250	Whole blood	LLE	LC-MS/MS-ESI+	0.006 ; 0.016	[69]
(20 SCs and 7 metabolites): AM-2201, AM-2201-*N*-4-OH-pentyl, JWH-018, JWH-210, JWH-203, JWH-250, JWH-251, JWH-081, JWH-122, JWH-122-*N*-5-OHpentyl, JWH-018-*N*-4- and 5-OH-pentyl, JWH-147, JWH-200,JWH-200-4-OHindole, JWH-201, JWH-302, JWH-398, JWH-412, MAM-2201, RCS-4, RCS-4 ortho isomer, RCS-4-*N*-5- OH-pentyl, RCS-4-*N*-COOH, RCS-8, WIN48 098, WIN55 212	Whole blood	Protein precipitation	LC-MS/MS-ESI+	Not specified	[36]
(18 SCs): 5F-PB-22, JWH-018, JWH-073, JWH-019, JWH-122, JWH-210, JWH-250, JWH-015, JWH-081, AM-2201, Cl-2201, MAM-2201, UR-144, XLR-11, BB-22, PB-22, AB-PINACA, ADB-PINACA,	Whole blood	LLE	LC-MS/MS	Not specified	[35]
(13 SCs): JWH-018, JWH-073, JWH-081, JWH-019, JWH-122, JWH-200, JWH-210, JWH-250, JWH-175, AM-2201, AM-694, RCS-4, RCS-8	Whole blood	LLE	LC-MS/MS-ESI+	Not specified	[70]
(18 SCs): JWH-018, JWH-073, JWH-081, JWH-250, JWH-251, JWH-122, JWH-200, JWH-210, JWH-015, JWH-019, JWH-020, CP47, 497, HU-210, AM-694, AM-2201, RCS-4, RCS-4-C4, RCS-8, WIN55,212-2	Whole blood	LLE	LC-MS/MS-ESI+	Not specified	[71]
UR-144 and pyrolysis product [1- (1-pentyl-1H-indol-3-yl)-3-methyl-2-(propan-2yl)but-3-en-1-one]	Whole Blood	LLE	LC-MS/MS-ESI+	0.15 ; 0.5	[72]
(10 SCs): JWH-018, JWH-073, JWH-081, JWH-250, JWH-015, JWH-200, JWH-019, JWH-020, WIN55 212-2, methanandamide	Serum	LLE	LC-MS/MS-ESI	0.1 ; 0.1 - 0.6	[73]
(15 SCs): JWH-018, JWH-073, JWH-081, JWH-019, JWH-122, JWH-200, JWH-210, JWH-250, JWH-251, JWH-203, JWH-307, JWH-015, AM-1220, AM-2201, AM-694	Serum	LLE	LC-MS/MS-ESI+/-	0.02 - 0.4 ; 0.05 - 0.5	[74]
JWH-018	Serum	LLE	LC-MS/MS-ESI+	0.07 ; 0.21	[39]

(2 SCs): PB-22 and UR-144	Serum	Not specified	LC-TOF-MS	Not specified	[129]
(30 SCs): JWH-018, JWH-018 adamantyl derivative, JWH-073, JWH-081, JWH-122, JWH-122 5-fluoropentyl derivative, JWH-200, JWH-210, JWH-250, JWH-251, JWH-015, JWH-019, JWH-020, JWH-203, JWH-307, JWH-387, JWH-398, JWH-007, AM-694, AM-1220, AM-2201, AM-2233, MAM-2201, methanandamide, CRA-13, RCS-4, RCS-4 ortho isomer, RCS-8, WIN 48 098, WIN 55 212-2	Serum	LLE	LC-MS/MS-ESI+	0.01 - 2.0 ; 0.1 - 2.0	[75]
(46 SCs): JWH-018, JWH-073, JWH-081, JWH-122, JWH-182, JWH-200, JWH-007, JWH-015, JWH-019, JWH-210, JWH-250, JWH-251, JWH-020, JWH-022, JWH-203, JWH-307, JWH-370, JWH-387, JWH-398, JWH-412, AM-694, AM-1220, AM-1220 azepane isomer, AM-2201, APICA, AM-1248, AM-2232, AM-2233, MAM-2201, Methanandamide, AB-001, Cannabipiperidie- thanone, CRA-13, RCS-4, RCS-4-C4, RCS-4 ortho isomer, RCS-8, STS-135, UR- 144, UR-144 isomer, XLR-11, XLR-11 isomer, WIN48 098, WIN55 212-2, AKB48, 5F-AKB-48	Serum	LLE	LC-TOF/MS-ESI+	LOD = 0.1 - 0.5	[76]
(5 SCs): JWH-122, PB-22, AM-2233, BB-22, 5F-PB-22	Plasma	LLE	LC-HR-MS	Not specified	[77]
(20 SCs): JWH-018, JWH-073, JWH-081, JWH-122, JWH-200, JWH-007, JWH-015, JWH-016, JWH-018-6-MeO, JWH-098, JWH-210, JWH-250, JWH-022, JWH-203, AM-694, AM-2201, AM-1241, RCS-4, RCS-8, WIN 48 098	Blood, serum	SPE	LC-TOF/MS-ESI+	Not specified	[91]

Table 2: Bioanalytical procedures for determination of SCs and metabolites in urine samples.

SCs	Sample preparation	Detection mode	LOD (ng/mL) ; LOQ (ng/mL)	References
(5 metabolites of JWH-018): JWH-018 *N*-5-OH-pentyl, 5-OHindole, *N*-COOH, *N*-dealkylated-5-OH-indole and 2-OH-naphthoyl	Hydrolysis, LLE	LC-MS/MS-ESI+	0.1 ; 0.5 (Only for JWH-018-*N*-OH-pentyl and *N*-COOH)	[49]
Metabolites of JWH-018 and AM-2201	Hydrolysis, LLE	LC-HR/MS	Not specified	[78]
(3 metabolites of JWH-018): *N*-5-OH-pentyl, *N*-COOH, 6-OH-indole	Hydrolysis, LLE	LC-MS/MS-ESI+	0.01 - 0.1 ; 1	[50]
(10 metabolites of 7 SCs): JWH-018 *N*-5-OH-pentyl, -COOH, JWH-073 *N*-4-OH-butyl, -COOH, JWH-250 5-OH-indole, *N*-COOH, JWH-122 *N*-5-OH-pentyl, JWH-200 5-OH-indole, JWH-019 5-OH-indole, RCS-4 *N*-5- OH-pentyl	Hydrolysis, LLE	LC-MS/MS-ESI+	LOQ = 0.1	[79]
JWH-018 and its metabolites	Hydrolysis, LLE	LC-MS/MS heated-ESI	Not specified	[40]
(JWH-018 and 7 metabolites): JWH-018 *N*-5-OH-pentyl, *N*-COOH, 2-, 4-,5-,6- and -7-OH-indole **(JWH-073 and 6 metabolites)**: JWH-073 *N*-4-OHbutyl, *N*-COOH, 4-, 5-, 6- and -7-OH-indole	LLE/SPE	LC-MS/MS-ESI+	LOD = 1	[92]
Metabolites of UR-144	Hydrolysis	GC-MS, LC-MS/MS	Not specified	[109]
CP47, 497	Dilution	LC-MS/MS-ESI-	10 ; 20	[130]
(JWH-018 and 6 metabolites): JWH-018 *N*-5-OH-pentyl, –COOH and 4-, 5-, 6-, 7-OH-indole **(JWH-073 and 6 metabolites)**: JWH-073 *N*-4-OH-butyl, -COOH and 4-,5-,6-,7-OH-indole	Hydrolysis, LLE	LC-MS/MS-ESI+	< 2 ; 1.8- 10.8	[51]
(3 metabolites of JWH-018): JWH-018 *N*-4- and 5-OH-pentyl and COOH **(3 metabolites of JWH-073):** JWH-073 *N*-3- and 4-OH-butyl and COOH.	Hydrolysis, SPE	LC-MS/MS-ESI+	0.1 ; 0.1	[119]
JWH-018 and 19 metabolites (mainly hydroxylated and *N*-dealkylatedmetabolites)	Hydrolysis, LLE	LC-MS/MS-ESI+	LOD = 0.1	[59]
AB-001 and 7 metabolites	Hydrolysis, LLE	GC-MS	Not specified	[80]
(4 metabolites of JWH-018): JWH-018 *N*-COOH, 6-OH-indole, *N*-4- and 5 OH-pentyl **(4 metabolites of JWH-073):** JWH-073 *N*-COOH, 6-OH-indole, *N*-3- and 4-OH-butyl	Dilution, Hydrolysis, SPE	ELISA, LC-MS/MSESI+	0.025 – 0.1 ; 2.5	[93]
(UR-144 and 5 metabolites): despentyl-OH-UR-144, di- OH-UR-144, despentyl-UR-144, dehydrated OH-UR-144, OH-UR-144 **(AM2201 and 7 metabolites):** JWH-018 *N*-(5-OH-pentyl), *N*-(5-OH-pentyl) dihydrodiol-JWH-018, di-OH-AM-2201, dihydrodiol-AM-2201, OH-AM-2201,despentyl-AM-2201, JWH-018 *N*-COOH	Hydrolysis, LLE	LC-MS/MS-ESI+	Not specified	[47]
RCS-4 and its metabolites	Hydrolysis, LLE,	GC-MS	Not specified	[81]
(8 metabolites of 5 SCs): JWH-018 *N*-4- and 5-OH-pentyl, JWH-073 *N*-3- and 4-OH-butyl, JWH-250 *N*-4-OHpentyl, JWH-019 *N*-5- and 6-OH-hexyl, AM-2201 *N*-4-OH-pentyl	Hydrolysis, LLE	ELISA, LC-MS/MS-ESI+	Not specified	[82]
5-F-AKB48 and its metabolites	Hydrolysis, LLE	LC-HRAM-MS	Not specified	[131]

(37 metabolites of 17 SCs): JWH-018 *N*-COOH, -6-OH-indole, *N*-4- and 5 OH-pentyl, JWH-073 *N*-COOH, 6-OH-indole, *N*-3- and 4-OH-butyl, JWH-250 *N*-COOH, *N*-4- and 5 OH-pentyl, JWH-122 *N*-4- and 5 OH-pentyl, JWH-019 *N*-6 OH-pentyl, JWH-210 *N*-4- and 5 OH-pentyl, JWH-081 *N*-5 OH-pentyl , JWH-398 *N*-4- and 5 OH-pentyl, JWH-203 *N*-COOH, *N*-4- and 5 OH-pentyl, AM-2201-6-OH-indole, *N*-4 OH-pentyl, MAM-2201 *N*-COOH, *N*-4 OH-pentyl, UR-144 *N*-COOH, *N*-4- and 5 OH-pentyl, XLR-11 *N*-4 OH-pentyl, AB-PINACA *N*-COOH, *N*-4 OH-pentyl, 5F-AB-PINACA *N*-4 OH-pentyl, AKB48 *N*-COOH, *N*-4- and 5 OH-pentyl, 5F-AKB48 *N*-4 OH-pentyl	Hydrolysis, SPE	LC-MS/MS-ESI+	LOD = 0.1 - 1	[121]
Major metabolites of JWH-018, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, RCS-4	Hydrolysis, LLE	LC-MS/MS-ESI+, LC-TOF/MS-ESI+	Not specified	[41]
(2 metabolites of JWH-018): JWH-018 *N*-4-OH-pentyl, *N*-COOH, **(2 metabolites of JWH-073):** JWH-073 *N*-3-OH-butyl, *N*-COOH	Hydrolysis, LLE	LC-MS/MS-ESI+	LOQ = 4	[83]
(4 metabolites of JWH-018): JWH-018 *N*-4- and 5- OH-pentyl, *N*-COOH, methyl ester **(2 metabolites of JWH-073):** JWH-073 *N*-3-OH-butyl, *N*-COOH JWH-072 *N*-COOH	Hydrolysis	LC-MS/MS-ESI+	LOQ = 4	[132]
(47 metabolites of 21 SCs): JWH-018 *N*-OH-pentyl, *N*-COOH, 5- and 6-OH-indole, JWH-073 *N*-OH-butyl, 5- and 6-OH-indole, *N*COOH, JWH-122 *N*-OHpentyl, *N*-COOH, JWH-250 5-OH-indole, *N*-OH-pentyl, *N*-COOH, JWH-210 *N*-COOH, *N*-OH-pentyl, JWH-200 5- and 6-OH-indole, JWH-398 *N*-OH-pentyl, *N*-COOH, JWH-019 5-OH-indole, -OH-hexyl, JWH-081 *N* OH-pentyl, AM-2201 6-OH-indole, *N*-OH-pentyl, MAM-2201 *N*-OH-pentyl, RCS-4 *N*-OH-pentyl, *N*-COOH, M9, UR-144 *N*-OH-pentyl, *N*-COOH, degradant-COOH, XLR-11 *N*-OH-pentyl, 6-OH-indole, ADB-PINACA *N*-OH-pentyl, AKB48 *N*-OH-pentyl, *N*-COOH, 5F-AKB48 *N*-OH-pentyl, 5F-AB-PINACA *N*-OH-pentyl, AB-PINACA *N*-OH-pentyl, -COOH, PB-22 *N*-OH-pentyl, *N*-COOH, *N*-OH-pentyl-3-COOH-indole, *N*-COOH-3-COOHindole, 3-COOH-indole, 5F-PB-22 3-COOH indole	Dilution, Hydrolysis, SLE+	LC-TOF/MS-ESI+	LOD = 0.25 - 20	[115]
(35 SCs and 19 metabolites): JWH-018, JWH-018 *N*-4- and 5-OH-pentyl, *N*-COOH, -4-, 5-, 6- and 7-OH-indole, 6-methoxy-indole, -1-methyl-hexyl, JWH-073, JWH-073 *N*-3- and 4-OH-butyl, -4-, 5-, 6-, and 7-OH-indole, *N*-COOH, 2- and 3-methyl homology, JWH-122, JWH-122 *N*-5-OH-pentyl, JWH-200, JWH-200 4-OH-indole, JWH-250, JWH-251, JWH-081, JWH-147, JWH-007, JWH-015, JWH-019, JWH-201, JWH-210, JWH-203, JWH-302, JWH-398, JWH-412, CP47, 497,CP47, 497-C8, CP55,940, HU-210, AM-694, AM-1220, AM-1220 azepane isomer, AM-2201, MAM-2201, RCS-4, RCS-4 ortho isomer, *N*-5-OH-pentyl, *N*-COOH, RCS-8, AB-0101, WIN48 098, WIN55 212	Hydrolysis, SPE	LC-TOF-MS-ESI+	Not specified	[94]
22 SC and 37 metabolites	No sample preparation	Biochip array techn-ology immunoassay	Not specified	[133]
(9 SC and 20 metabolites): JWH-018, JWH-018 *N*-5-OH-pentyl, 5- and 6-OH-indole, *N*-COOH, JWH-073, JWH-073 *N*-4-OH-butyl, *N*-COOH, 5- and 6-OH-indole, JWH-250 *N*-4- and 5-OH-pentyl, 5-OH-indole, JWH-200 5- and 6-OH-indole, JWH-081, JWH-081 *N*-5-OH-pentyl, JWH-210, JWH-210 *N*-4- and 5-OH-pentyl, *N*-COOH, JWH-122, JWH-122 *N*-5-OH-pentyl, AM-2201, AM-2201 6-OH-indole, *N*-4-OH-pentyl, MAM2201, RCS-4, RCS-4 *N*-5-OH-pentyl, *N*-COOH	Protein precipitation, Hydrolysis	LC-MS/MS-ESI+	LOD = 0.5 - 10	[122]
JWH-018 *N*-COOH (calibrator)	No sample	ELISA	Not specified	[103]

	preparation			
(5 SC and 6 metabolites): JWH-018, JWH-018 *N*-4- and 5-OHpentyl, 4- and 5-OH-indole, JWH-073, JWH-073 *N*-3- and 4-OH-butyl, JWH-250, HU-210, AM-2201	Hydrolysis, SPE	LC-MS/MS-ESI+	0.01 - 0.5 ; 0.05 - 5	[95]
(20 SC and 33 metabolites): JWH-018, JWH-018 *N*-OH-pentyl, *N*-COOH, 5- and 6-OH-indole, JWH-073, JWH-073 *N*- OH-butyl, *N*-COOH, 5- and 6-OH-indole, JWH-019, JWH-019 5-OH-indole, -*N*-OH-hexyl, JWH-250 *N*-OH-pentyl, *N*-COOH, -5-OH-indole, JWH-081, JWH-081 *N*-OH-pentyl, JWH-210, JWH-210 *N*-OH-pentyl, *N*-COOH, 5-OH-indole, JWH-200 5- and 6-OH-indole, JWH-122, JWH-122 *N*-OH-pentyl, JWH-398, JWH-398 *N*-OH-pentyl, *N*-COOH, JWH-203, CP47, 497-C7, CP47, 497-C7-OH, CP47, 497-C8, CP47, 497-C8-OH dimethyloctyl, HU-210, AM-694, AM-2201, AM-2201 6-OH-indole, *N*-OH-pentyl, MAM-2201, MAM-2201 *N*-OH-pentyl, *N*-COOH, UR-144 *N*-OH-pentyl, *N*-COOH, RCS-4, RCS-4 *N*-OH-pentyl, M9, M10, *N*-COOH,	Dilution, Hydrolysis, SLE	LC-MS/MS-ESI+/-	0.05 - 1 ; 0.1 - 1	[123]
(38 metabolites of 12 SCs): JWH-018 *N*-4- and 5-OH-pentyl, *N*-COOH, 5-, 6- and 7-OH-indole, JWH-073 *N*-3- and 4-OH-butyl, *N*-COOH, 5-, 6- and 7-OH-indole, JWH-250 *N*-4- and 5-OH-pentyl, *N*-COOH, JWH-019 *N*-5- and 6-OH-hexyl, JWH-210 *N*-4- and 5-OH-pentyl, *N*-COOH, JWH-122 *N*-4-OH-pentyl, *N*-5-OH-pentyl, JWH-081 *N*-5-OH-pentyl, *N*-COOH, JWH-398 *N*-4- and 5-OH-pentyl, *N*-COOH, AM-2201 *N*-4-OHpentyl, 6- and 7-OH-indole, MAM-2201 *N*-4-OHpentyl, *N*-COOH, UR-144 4- and 5-OH-pentyl, *N*-COOH, RCS-4 4- and 5-OH-pentyl, *N*-COOH	SALLE	HEIA, LC-TOF/MS-ESI+	Not specified	[114]
(8 metabolites of 3 SCs): JWH-018 *N*-4 and 5-OH-pentyl, *N*-COOH, *N*-5-OH-pentyl β-Gluc, 6-OH-indole, JWH-073 *N*-COOH, AM-2201 *N*-4-OH-pentyl, 6-OH-indole	Hydrolysis, SPE	LC-MS/MS-ESI+	0.1 ; 2.5	[96]
(14 SC and 15 metabolites): JWH-018 *N*-4-OH-pentyl, *N*-COOH, JWH-073 *N*-3-OH-butyl, -*N*-COOH, JWH-250 *N*-4-OH-pentyl, JWH-122, JWH-122 *N*-5-OH-pentyl, JWH-081, JWH-081 *N*-5-OH-pentyl, JWH-120, JWH-210 *N*-4-OH-pentyl, JWH-019, JWH-019 *N*-5-OH-hexyl, JWH-200 6-OH-indole, HU-210, AM-694 *N*-COOH, AM-1248, AM-2201, AM-2201 *N*-4-OHpentyl, UR-144, UR-144 *N*-COOH, XLR-11, XLR-11 6-OH-indole, XLR-12, RCS-4, RCS-4 *N*-5-OH-pentyl, RCS-8, AB-FUBINACA, PB-22	Hydrolysis, Dilution	LC-MS/MS-ESI+	LOD/LOQ = 1 - 5	[134]
(UR-144 and 7 metabolites): UR-144 *N*-COOH, di-OH-UR-144, UR-144 *N*-5-OH-pentyl- β-Gluc, despentyl-UR-144, despentyl-OH-UR-144, dehydrated-OH-UR-144, OH-UR-144	Hydrolysis, LLE	LC-TOF-MS-ESI+	0.15 ; 0.5	[72]

Table 3: Bioanalytical procedures for determination of SCs and metabolites in oral fluid samples.

SCs	Sample preparation	Detection mode	LOD (ng/mL); LOQ (ng/mL)	References
(10 SCs): JWH-018, JWH-073, JWH-250, JWH-122, JWH-019, JWH-200, HU-210, CP47,497, nabilone, AM-694	Dilution	LC-MS/MS-ESI+	LOD = 1 - 20	[135]
(10 SCs): JWH-018, JWH-073, JWH-250, JWH-200, JWH-081, HU-210, CP47,497, CP47,497 (C8), AM-2201, RCS-4	Dilution	ELISA, LC-MS/MS-ESI+	0.1 ; 0.25	[101]
(28 SCs): JWH-018, JWH-073, JWH-250, JWH-200, JWH-122, JWH-019, JWH-210, JWH-007, JWH-015, JWH-387, JWH-398, JWH-251, JWH-307, JWH-081, JWH-203, JWH-020, JWH-412, AM-694, AM-1220, AM-2233, AM-2201, MAM-2201, methanandamide, RCS-4, RCS-8, RCS-4 ortho isomer, WIN48, 098, WIN55 212-2	Protein precipitation	LC-MS/MS-ESI+	0.015 - 0.9 ; 0.15 - 3	[65]
(30 SCs): JWH-018, JWH-018 adamantyl derivative, JWH-073, JWH-250, JWH-081, JWH-122, JWH-007, JWH-015, JWH-387, JWH-398, JWH-251, JWH-307, JWH-210, JWH-200, JWH-203, JWH-019, JWH-020, AM-2201, AM-2233, AM-694, AM-1220, CRA-13, AB-001, MAM-2201, methanandamide, RCS-8, RCS-4, RCS-4 ortho isomer, WIN48 098, WIN55 212-2	LLE	LC-MS/MS-ESI+	0.015 - 0.9 ; 0.15 - 30	[84]
(18 SCs): JWH-018, JWH-073, JWH-250, JWH-122, JWH-019, JWH-200, JWH-015, JWH-020, JWH-210, JWH-081, JWH-251, HU-210, AM-2201, AM-694, RCS- 4, RCS-4-C4, RCS-8, WIN55 212-2	LLE	LC-MS/MS-ESI+	LOD = 0.05 - 1.2	[85]
(UR-144 and 2 metabolites): UR-144 4-OH-pentyl, pyrolysis product of UR-144 **(XLR-11 and 2 metabolites):** XLR-11 4-OHpentyl and pyrolysis product of XLR-11	SPE	LC-MS/MS-ESI+	0.35 - 1.93 ; 5	[45]
(6 SCs): JWH-018, JWH-073, JWH-250, HU-210, CP47,497, CP47,497 (C8)	SPE	LC-MS/MS-ESI+/-	LOQ = 0.5	[97]
(7 SCs): JWH-018, JWH-073, JWH-250, JWH-200, HU-211, CP47,497, CP47,497 (C8)	SPE	LC-MS/MS-ESI+/-	0.025 - 1.0 ; 0.1 - 2.5	[98]

Table 4: Bioanalytical procedures for determination of SCs and metabolites in hair samples.

SCs	Sample preparation	Detection mode	LOD (pg/mg); LOQ (pg/mg)	References
(18 SCs): JWH-018, JWH-073, JWH-018-*N*-NCOOH, JWH-250, JWH-200, JWH-122, JWH-210, JWH-081, JWH-015, JWH-020, JWH-019, JWH-203, JWH-007, HU-210, CP47,497, AM-2201, AM-694,WIN55 212-2	Washing, Digestion	LC-MS/MS-ESI+	LOQ = 500	[136]
(JWH-018 and 3 metabolites): JWH-018 *N*-4- and 5-OH-pentyl, *N*-NCOOH **(JWH-073 and 3 metabolites)**: JWH-073 *N*-3- and -4-OHbutyl, *N*-COOH	Washing, LLE	LC-MS/MS-ESI+	LOD = 0.05	[86]
(5 SC and 11 metabolites): JWH-018, JWH-073, JWH-122, AM2201, MAM2201, JWH-018 *N*-4- and 5-OH-pentyl, *N*-NCOOH, JWH-073 *N*-3- and -4-OHbutyl, *N*-COOH, JWH-122 *N*-5-OH-pentyl, *N*-COOH, AM-2201 *N*-4-OH-pentyl, 6-OH-indole, MAM-2201 *N*-4-OHpentyl	Washing, LLE	LC-MS/MS-ESI+	0.05 ; 0.1	[87]
(23 SCs): JWH-018, JWH-073, JWH-250, JWH-122, JWH-019, JWH-200, JWH-015, JWH-020, JWH-210, JWH-081, JWH-251, JWH-307, JWH-007, JWH-398, JWH-203, HU-210, AM-694, AM-2201, AM-1220, RCS- 4, RCS-8, WIN55 212-2, WIN48 098	Washing, Digestion, LLE	LC-MS/MS-ESI+	0.2 – 1.3 ; 0.7 – 4.3 except (HU-210 = 80 LOQ)	[88]
(8 SCs): JWH-018, JWH-073, JWH-250, JWH-200, JWH-122, JWH-210, JWH-081, AM-694	Base hydrolysis, LLE	LC-TOF-MS-ESI+	LOD = 10	[89]
(5 SCs): JWH-018, JWH-073, JWH-250, JWH-200, HU-210	Washing, digestion, LLE	LC-MS/MS-ESI+	0.02 – 0.18 ; 0.07 - 18	[90]

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