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**A simultaneous quantitative determination of both natural and synthetic
cannabinoids in bio-matrix by ultra-high pressure liquid chromatography
tandem mass spectrometry**

by

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Magister Scientiae

in

Pharmacology

in the

Faculty of Health Sciences

at the

University of Pretoria

Supervisor: Professor AD Cromarty

Declaration of originality

I, Heiko Detmar Schillack, hereby declare that all the work done for this dissertation, which is submitted for fulfilment of the requirements for the degree MSc in Pharmacology is my own original work and has not been submitted for any academic award to any other institution of higher learning. This includes the calculations, preparation of standards and samples as well as the calibration and optimization of all the UPLC-MS/MS methods reported.

HD Schillack

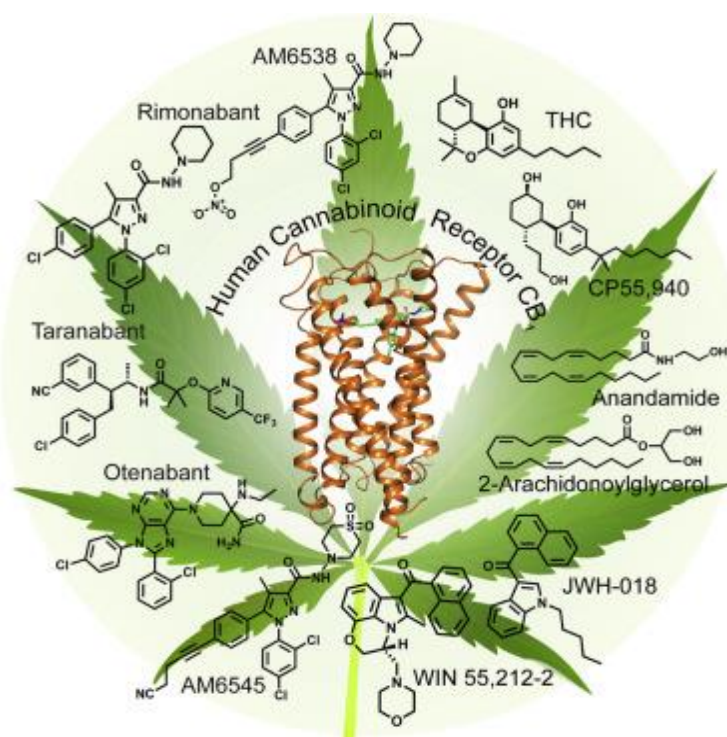


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Abbreviations

11-Hydroxy-THC	11-Hydroxy tetrahydrocannabinol
11-Hydroxy-THC D3	Tri-deuterium labelled 11-Hydroxy tetrahydrocannabinol
11-Nor- Δ^9 -THC-COOH	11-Nor-carboxy- Δ^9 -THC metabolite
11-Nor- Δ^9 -THC-COOH D9	Nono-deuterium labelled 11-Nor- Δ^9 -carboxy-tetrahydrocannabinol
Δ^9 -THC	Δ^9 -tetrahydrocannabinol
Δ^9 -THC D3	Tri-deuterium labelled Δ^9 -tetrahydrocannabinol
Δ^9 -THC-COOH –glucuronide	11-Nor-carboxy- Δ^9 -THC-glucuronide
CBN	Cannabinol
CBN-D3	Tri-deuterium labelled cannabinol
CBD	Cannabidiol
CBD-D3	Tri-deuterium labelled Cannabidiol D3
DDH2O	Double deionized water /reverse osmosis
ELISA	Enzyme-linked immune sorbent assay
HLB SPE	Hydrophilic-Lipophilic-Balanced solid phase extraction cartridge
HU-210	HU 210 Spice cannabis
JWH-018	JWH-018 Spice cannabinoid
JWH-018-4-OH-pentyl D5	Penta-deuterium labelled JWH-018-4-hydroxypentyl
JWH-073	JWH-073 Spice cannabis
JWH-073-4-OH butyl metabolite	JWH-073-4-hydroxybutyl metabolite
JWH-073-3-OH-butyl D5	Penta-deuterium labelled JWH-073-3-Hydroxybutyl metabolite
JWH-210-4-OH-pentyl metabolite	JWH-210-4-Hydroxypentyl metabolite
JWH-210-5-OH-pentyl metabolite	JWH-210-5-Hydroxypentyl metabolite
UPLC-MS/MS	Ultra high pressure liquid chromatography with tandem mass detector

Ethical aspects

Samples from patients that were submitted for cannabinoid analysis by ELISA based methods were anonymously retested using the Ultra high pressure liquid chromatography with tandem mass detector (UPLC-MS/MS) method developed during this study to prove applicability to real life samples. All data from patients testing positive for the synthetic cannabinoids, JWH-073, JWH-018 and HU-210 or their metabolites were kept confidential.

No positive result was issued unless the overseeing pathologist specifically requested the information.

Since the safety profile of synthetic cannabinoid compounds is largely unknown, the ability to perform consented human studies to determine cannabinoid and synthetic cannabinoid concentrations in both urine and plasma or their physiological effects presents an ethical challenge. Identifying individuals who abuse these drugs can be difficult. (1)

1 ABSTRACT

Numerous publications outlining the quantitative determination and semi-quantitative screening for both parent and metabolites of the natural plant derived cannabinoids have been reviewed due to the widespread use and abuse of cannabinoid containing substances. Synthetic cannabinoids have become fairly easily accessible as recreational drugs during recent times and are continually being structurally changed to increase the hallucinogenic effects and to avoid being specifically listed as illegal compounds.

The most common sample type to be used for identification and quantification of cannabinoids in humans is urine, which is currently tested using immunology-based methods. These methods require specific antibodies for each possible new synthetic cannabinoid in addition to the many different possible metabolites that form after oral or inhaled administration. These antibody-based assays are in general expensive, show cross-reactivity and are prone to high analyst-based variations. Advanced analytical techniques such as ultra-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) can be a more cost effective method and can be applied routinely for qualitative and quantitative analysis of the ever-changing combinations of cannabinoid compounds being abused.

The need in the laboratory for testing for each of the known plant derived cannabinoids as well as for several synthetic cannabinoids has in recent times increased. Herbal products sold legally in some retail shops, tobacco shops, bottle stores and via the internet, have been shown to contain both natural and synthetic cannabinoid compounds. This study developed and optimised a rapid ultra-performance liquid chromatographic tandem mass spectrometry method to quantitatively determine low concentrations of the natural cannabinoids and three of the most commonly used synthetic cannabinoid compounds, namely JWH-018; JWH-073 and HU-210 and their respective metabolites in both human plasma and urine. Chromatographic separation was achieved on an Acuity UPLC Cortex C18 Phenyl 100 X 2.1 mm 1.6 µm column. Urine samples were initially enzymatically hydrolysed to release the glucuronide and sulphate groups that are commonly conjugated to the metabolites, using standard enzymatic hydrolysis procedures, then analysed directly following dilution with 1% formic acid in methanol. Plasma samples were initially extracted using solid phase extraction prior to analysis. Both urine and plasma analysis used stable-isotope labelled analogues of the natural and synthetic cannabinoid analytes as internal standards. Analyses of the samples were performed by tandem mass spectrometry in positive electrospray ionization mode with selected reaction monitoring.

The total analytical run time was 14 minutes. The linear dynamic range was between 1-100 µg/L with a lower limit of detection in urine for JWH-073-4-hydroxy-butyl of 1.31 µg/L; 11-hydroxy THC was 8.66 µg/L; 11-Nor-Delta-Carboxy THC was 2.66 µg/L; JWH-210-4-hydroxy-pentyl was 1.18 µg/L; JWH-210-5-hydroxy-pentyl was 2.53 µg/L respectively. The elimination half-life of the cannabinoid metabolites in plasma is very short due to the effective renal excretion of these metabolites, and therefore only the precursor cannabinoids could be identified in plasma using the developed methods. Detection limits for the natural cannabinoids in plasma were: Cannabinol was 6.78 µg/L; Cannabidiol was 5.56 µg/L; Delta 9 THC was 2.40 µg/L; while for the synthetic cannabinoids: JWH-073 was 0.15 µg/L; JWH-018 was 8.71 µg/L and HU-210 was 1.03 µg/L.

Intra-run imprecision at concentration levels of 10 and 50 µg/L for JWH-073-4-hydroxy butyl was 1.32 µg/L and 1.57 µg/L respectively; 11-Hydroxy-THC was 3.02 µg/L and 4.02 µg/L respectively; 11-Nor 9 carboxy THC was 2.17 µg/L and 4.55 µg/L respectively; JWH-210-4-hydroxy-pentyl was 2.21 µg/L and 1.93 µg/L respectively; HU-210-5-hydroxy-pentyl was 2.0 µg/L and 2.65 µg/L respectively. The intra-run imprecisions for the non-metabolised compounds at the same concentration levels were slightly higher with: Cannabinol at 2.43 µg/L and 8.72 µg/L respectively; Cannabidiol at 1.54 µg/L and 3.79 µg/L respectively; Delta 9 THC at 1.96 µg/L and 3.03 µg/L respectively; JWH-073 at 2.25 µg/L and 3.57 µg/L; JWH-018 at 2.64 µg/L and 3.83 µg/L respectively and HU-210 at 5.49 µg/L and 5.64 µg/L respectively

In conclusion the method performance compares to methods reported in literature where different classes of cannabinoids have been analysed. In addition, improvement in turn-around time and reduction in analysis cost could be implemented. The newly developed method, used in an analytical laboratory will aid to confirm whether the patients have indeed been administered natural or synthetic cannabinoids. This can help a referring General Practitioner to make quicker decisions to minimize long-term effects of the cannabinoids. In practice, analytical laboratory performance is dependent on implementation of Good Laboratory Practise (GLP) that includes following well documented standard operating procedures of validated analytical methods. With pharmacokinetic studies a validated protocol must be followed for a study to be accepted.

2 INTRODUCTION

Cannabis contains a mixture of natural plant alkaloids (phyto-cannabinoids) and is also known as marijuana or THC. Three constituents, delta-9-tetrahydrocannabinol, (Δ^9 -THC), cannabidiol (CBD) and cannabinol (CBN) are the most abundant cannabinoid constituents in cannabis. The two most common cannabis plants (*Cannabis sativa*) and (*Cannabis indica*) grow wild and are commonly illegally harvested or commercially cultivated.

Cannabis sativa contains the highest amount of the active component, Δ^9 -THC, but can vary in percentage of this active cannabinoid depending on the area and climate where it is cultivated. The *Cannabis sativa* contains more Δ^9 -THC and less CBD whereas *C. indica* has less Δ^9 -THC relative to the CBD. The physiological effects of the different strains of cannabis in humans is very important to the user. For instance, *C. sativa* has a sedative effect (2) whereas cannabis strains with relatively higher CBD cause more anxiety. If the ratio of Δ^9 -THC to CBD is 2:5 the effects are likely to be more relaxing and produce less anxiety (3). The debate to legalize Δ^9 -THC has long been under medical and legal discussion. Hundreds of articles suggest that cannabis may have a positive medical effect that can be beneficial to patients with cancer, chronic pain (analgesic), insomnia and may be prescribed as an appetite stimulant (4-8). In the Netherlands cannabis can be used legally at certain street cafés. In South Africa there have been some heated debates to legalize the use of cannabis. Although prescription cannabis products are available for patients (Dronabinol; Marinol) it is still illegal to consume, trade or commercially grow cannabis. Several questions still remain; is the effect experienced by the patient psychological or is there a true positive clinical response to cannabinoid treatment and which of the metabolites are responsible for this positive effect and at what concentration. Genetically modified cannabis, herbal cannabis or sinsemilla are known to be highly potent cannabis products from selected female plants. These plants are specially hybridised, tended and kept seedless by preventing pollination to induce a high resin content. Very often portable cannabis grinders are used to break herbal cannabis and sinsemilla into a suitably fine texture for smoking.



Fig 1: Example of finely ground cannabis for smoking or to be pressed into tablets. Open access image from <http://www.ilovegrowingmarijuana.com/what-is-marijuana-kief/> (accessed 25/02/2018)

When cannabis is ingested for medical purposes, it is in tablet or capsule form and administered orally resulting in an initial lag-time in the therapeutic and hallucinogenic effects, similar to those experienced when taking illegal cannabis products orally. The effect of orally dosed cannabis is that it lasts for a longer period. Smoking cannabis gives a quick effect due to the rapid uptake from the large lung surface area. The main difference is that the herbal powders or dried leaves are often adulterated and contain a synthetic cannabinoid or contaminants such as fungal mycotoxins and even pesticides. This has the potential to cause lung disease among immune compromised individuals, and possibly respiratory problems. Given that cannabis is becoming a commercial crop, it is likely that plant growth stimulants and other substances will be added to grow and maximize yield and quality of the cannabis plant.

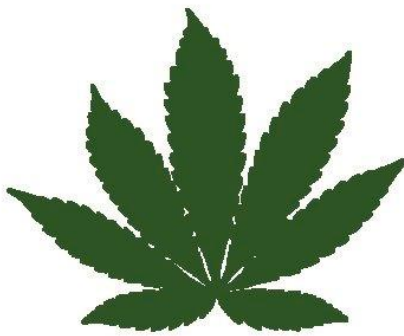
The cannabis genus consists of a number of species, *C. indica* and *C. sativa*, with several different hybrids due to crossing these two species. One example of a hybrid is *Cannabis ruderalis* (9) that is “considered as its own species”. The idea of cultivating hybrid species was to cultivate different hybrids that would increase the relative yield and ratio of psychoactive cannabinoids by growing different plants near to each other to stimulate cross pollination and this interaction with each other giving rise to smaller plant hybrids (9). *Ruderalis* produces very little Δ^9 -THC, which is less appealing for recreational users. *C. ruderalis* strains have been found to possess a chemical profile similar to wild Hemp.

Types Of Cannabis



SATIVA

Cannabis Sativa Sativa is characterized by leaflets that are more narrow, branches that are farther apart, and coloration that tends more toward spring green. Sativa Sativa plants tend to be taller and produce fewer flowers.



INDICA

Cannabis Sativa Indica is characterized by broad leaflets that offer overlap, branches that are closer together, and coloration that tends more toward deep olive green. Sativa Indica plants tend to be shorter and bushier, producing fuller, denser flower buds.



RUDERALIS

Cannabis Ruderalis is characterized by varied leaflets in the mature leaves, a shorter stature and generally small size. This subspecies is used to create S. Sativa or S. Indica hybrids with select desired traits.

Fig 2: A list of the characteristic features of the different cannabis plants commonly used for recreational purposes.

C. sativa: Uplifting and energising, thought focusing, generally feel “awesome” best suited for day use.

C. indica: Relaxing and calming body buzz or “couch lock” best suited for night use.

C. ruderalis: A group of hybrids that vary in their Δ^9 -THC, CBD and other cannabinoids content, and often referred to based on the major cannabinoid compound ratio.

Image with permission from <http://www.whtcla.com/types-of-cannabis/> accessed 24/02/2018)

The Δ^9 -THC content of *C. sativa* and *C. indica* is highest in the flowering tops, declining in concentration in the newest leaves, still lower in older leaves than the stems. Dried plant material or simple extracts are smoked alone or mixed with tobacco and are normally inhaled deep into the lungs and held for at least 15 to 30 seconds, giving smokers the ability to easily control their toxicity

levels. Because effective levels of the active cannabinoids are rapidly achieved in the brain due to the highly lipophilic nature of these compounds, it is easy to control the intake (10). The second most common means of intoxication is orally administered products but Δ^9 -THC is absorbed much more rapidly when smoking the Δ^9 -THC product.

The metabolism of the most active cannabinoid constituent, Δ^9 -THC in humans follows the following sequence: Phase I metabolism where hydroxylation of Δ^9 -THC at the C-11 position takes place rapidly followed by a second oxidation resulting in further conversion to the yet more water soluble carboxylic acid derivative. The metabolic pathway for Δ^9 -THC is illustrated in Figure 3.

In its natural state, the cannabis plant contains more than 400 chemical compounds, which includes a wide range of other cannabinoids that have no psychological effects. Depending on cultivation techniques, time of harvesting and species of plant and methods for processing the plant material, the Δ^9 -THC content in commercial Δ^9 -THC containing products range from 0.4-20%. Δ^9 -THC in general has several pharmacological actions that have been identified and are illustrated in Figure 4.

Many of the metabolites summarized in Figure 4 are excreted in the form of phase I and phase II metabolic conjugates in the urine. For example, the metabolite, 11-hydroxy- Δ^9 -THC, which is the first primary metabolite, induces psychomimetic activity when Δ^9 -THC is smoked and this metabolite plus products resulting from further metabolism are excreted in the urine.

Analytical techniques for the measurements of the natural cannabinoids and their metabolites in urine, biological fluids and tissues have improved over the last decade due to increased sensitivity, very high selectivity, freedom of interferences and reduction in background noise through the use of very selective detection systems especially UPLC-MS/MS techniques. This has led to a better understanding of the underlying principles involved in distribution and metabolism of these highly lipophilic and potent psychoactive substances.

Metabolism Peaks of THC after Oral Ingestion

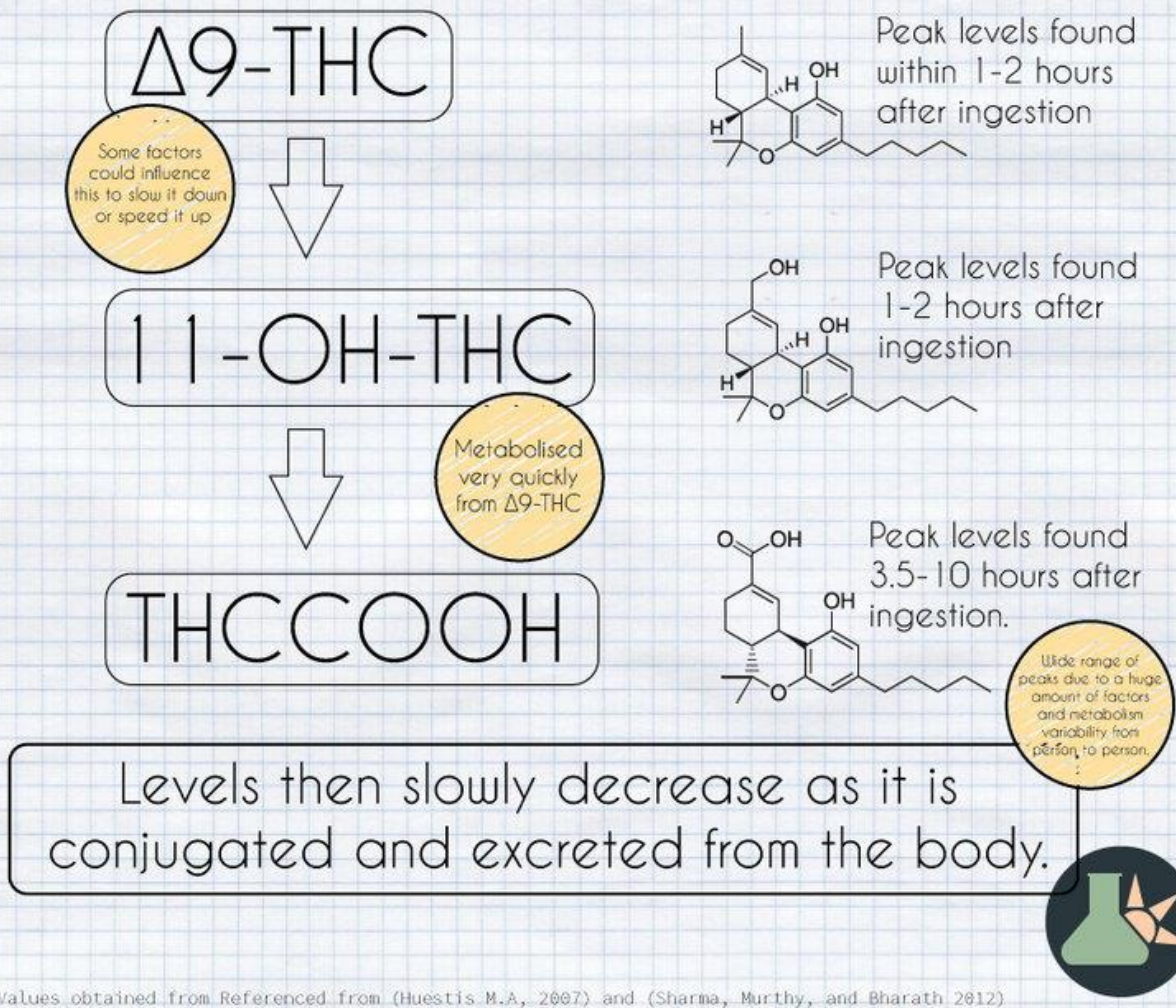


Fig 3: A diagram illustrating the major metabolic pathway of orally ingested Δ^9 -THC. Diagram from <https://thesunlightexperiment.com/blog/2016/4/5/how-is-thc-metabolised> (accessed on 24/02/2018) based on pharmacokinetic data reported in (11),(12).

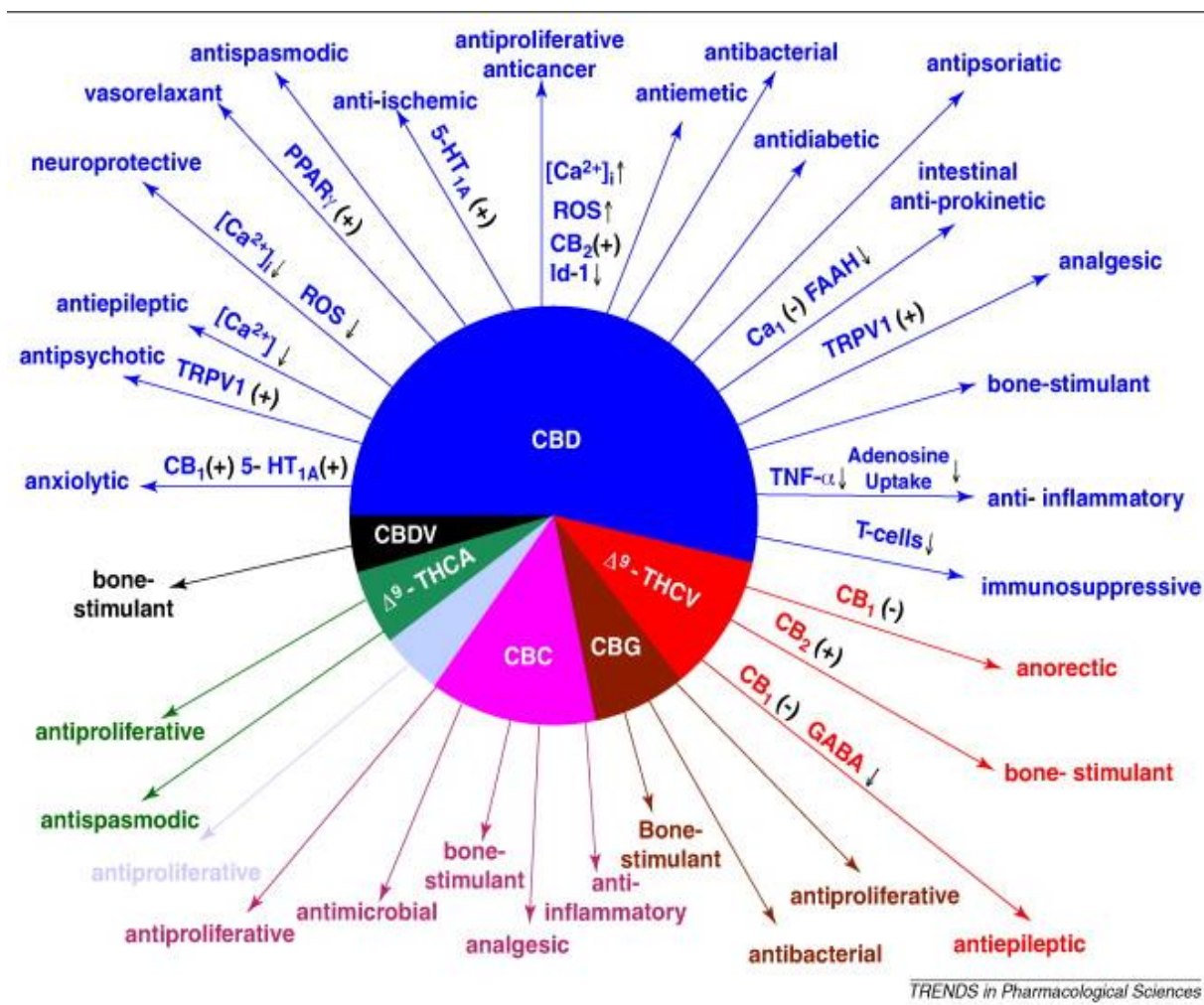


Fig 4: Pharmacological actions of the many non-psychotropic cannabinoids with an indication of the proposed mechanisms of action. Izzo, AA. Trends in Pharmacological Sciences, 30, 10, 515-527. With permission.

Abbreviations: CBN, cannabitol; CBD, cannabidiol; Δ^9 -THCV, Δ^9 -tetrahydrocannabivarin; CBC, cannabichromene; CBG, cannabigerol; Δ^9 -THCA, Δ^9 -tetrahydrocannabinolic acid; CBDA, cannabidiolic acid; TRPV1, transient receptor potential vanilloid type 1; PPAR γ , peroxisome proliferator-activated receptor γ ; ROS, reactive oxygen species; 5-HT $_{1A}$, 5-hydroxy tryptamine receptor subtype 1A; FAAH, fatty acid amidohydrolase. (+), direct or indirect activation; \uparrow , increase; \downarrow , decrease (13). Used with permission.

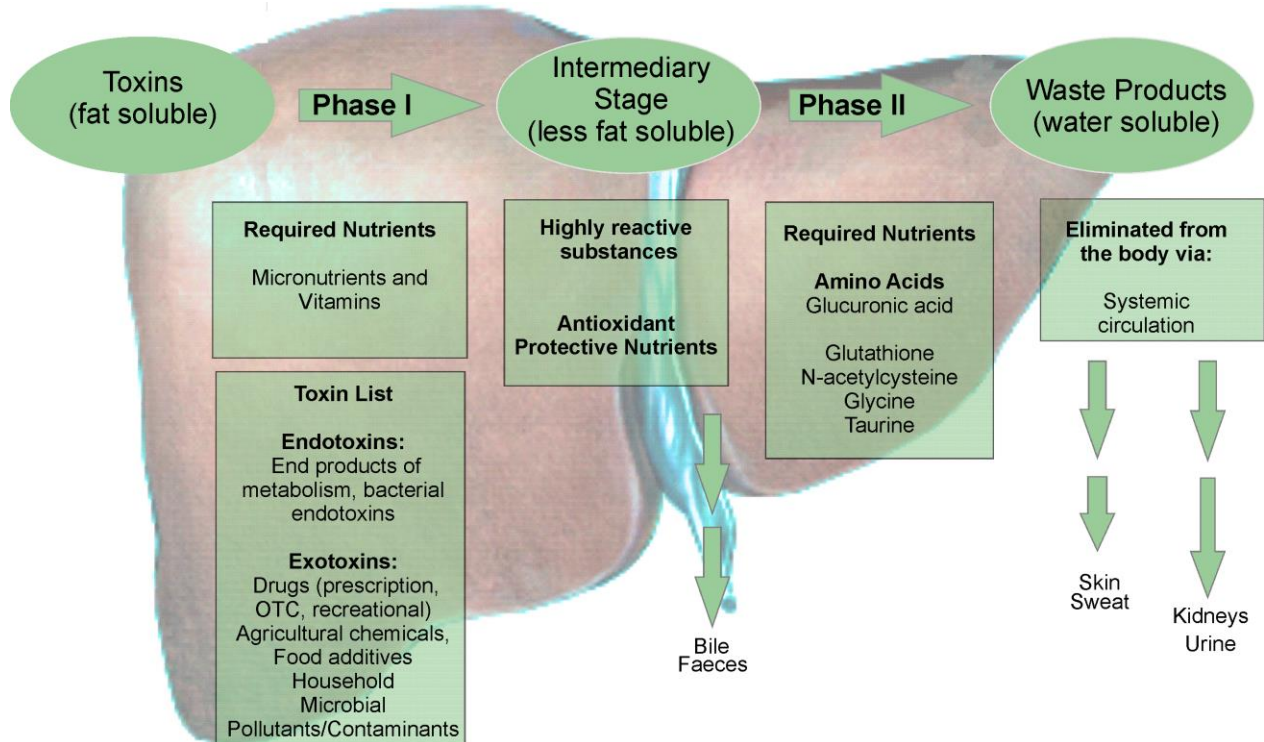


Fig 5: Metabolisms and excretion of endogenous and exogenous compounds by the liver. Distribution of THC into fatty organs is rapid, easily crossing the blood brain barrier (BBB) and placenta. Phase I metabolism in the liver by CYP450 enzymes which is a normal detoxification step leads to other active metabolites which can be followed by Phase II metabolism to form inactive water soluble glucuronides.

Testing of urine samples has been the preferred biological matrix due to the accumulation effect of the more polar metabolites over time while the bladder fills as opposed to the single time point concentration seen in a plasma sample. The most common analytical technique for detecting marijuana constituents is by measurement of the primary Δ^9 -THC metabolite (11-nor- Δ^9 -THC carboxylic acid) in urine by immuno-assay based enzyme-linked immune sorbent assay (ELISA) screening. However, this method has disadvantages, which affects the reliability, specificity and sensitivity of this technique. For instance, the commonly used ELISA to test for Δ^9 -THC uses polyclonal antibodies that are sensitive, but not highly selective. These antibody based techniques target different chemical moieties of the aminoalkylindole cannabinoids (14). Preliminary data from the manufacturer shows good reactivity with metabolites of eleven of the synthetic cannabinoids but are prone to be less specific if the samples have been compromised or spiked with certain chemicals that destroy the binding efficiency of the specific antibodies. There is also currently limited data available on the ability of the assay to detect metabolites of the synthetic cannabinoids (15) or whether any of the synthetic cannabinoid metabolites can mask the active binding sites of the antibodies.

A chronic user of marijuana requires ever increasing doses of the cannabinoids to achieve a similar response, whereas recreational users consume small quantities of Δ^9 -THC to experience a state of euphoria, alteration of time and space perception and a more vivid perception of their surroundings. Chronic high dose smokers of marijuana experience symptoms of hallucinations, anxiety and very often lose touch with reality, experiencing illusions and exhibiting bizarre behaviour. Both recreational and chronic users commonly report these effects (16).

2.1 PHARMACEUTICAL APPLICATIONS UNDER INVESTIGATION:

In the recent past there have been a number of studies carried out to confirm the efficacy of cannabinoids for various clinical indications. Many of the studies have been flawed, yet there is sufficient evidence to confirm that there are several indications for which cannabinoids do show efficacy with limited adverse side effects being reported. The most studied indications with respect to clinical outcomes has been related to suppression of nausea and vomiting, the suppression of pain, attenuation of inflammation and effects on the neural cells. These effects are mediated through the agonistic activity of the cannabinoids for the two well characterised G-protein coupled cannabinoid receptors (CB), with two different receptors showing distinct tissue specific distribution. The CB1 receptor is dominant in the central and peripheral neural tissue while CB2 appears to be predominantly immune-cell associated. Other non-CB receptors that have been confirmed to be activated by cannabinoids are the transient receptor potential vanilloid type 1 (TRPV1), the CB2-like receptors that mediate anti-nociception and the "abnormal cannabidiol" receptors that mediate vaso-relaxation.

Endogenous cannabinoids are involved in suppression of nausea and vomiting, anti-convulsive, analgesia, cognition, memory, locomotor activity, appetite stimulation, and in controlling inflammatory and immune responses. The endogenous cannabinoids include the naturally occurring lipid mediators; anandamide, 2-arachidonoylglycerol and palmitoyl ethanolamide.

2.1.1 Antiemetic effect

THC and synthetic analogues have been found to be effective in alleviating nausea and vomiting, especially following chemotherapy. Side-effects of THC itself can still be a problem, but evidence of its efficiency for nausea treatment led to the Food and Drug Administration of the USA (FDA) registering cannabinoid capsules for this indication, available as a prescription drug under the trade name Marinol (17).

2.1.2 Anticonvulsant effect

The discovery that cannabidiol (a major component of marijuana with no hallucinogenic effects with practically no Δ^9 -THC like activity) exhibited anticonvulsant activity in animals, generated considerable attention (18). However, this activity could not be demonstrated with sufficient efficacy in humans.

2.1.3 Analgesia

Cannabinoids were consistently shown to improve mood, sleep and coping ability. This is not the case for pain management. Cannabinoids are not first-line analgesics, although they may be useful for some patients for coping in their illness (19). Cannabinoids elicited a number of side effects in the effective dose range during controlled clinical trials evaluating cannabis as an analgesic. All tested active substances were cannabinoids. Orally administered Δ^9 -THC or a synthetic analogue of THC at 5-20 mg and 1 mg respectively together with intramuscular levonantradol at 1.5-3 mg were about as effective as orally administered codeine 50-120 mg. Compared to the high concentration of codeine the Δ^9 -THC with injected levonantradol were just as effective for pain relief in much lower concentrations. In comparison orally administered benzopyranoperidine at 2-4 mg was less effective than codeine and no better than placebo. Psychotropic adverse effects were however commonly reported for the cannabinoid treatment (20). Cannabinoids can be as effective as codeine in treating cancer or post-operative pain, but the adverse effects are unacceptable to many patients (21)

Cannabinoids appear to be no more effective than codeine in controlling pain and exhibit depressant effects on the central nervous system that limit their use. The reasoning leading to the use of cannabinoid for pain management in clinical practice is therefore tenuous. Before cannabinoids can be considered for routinely treating spasticity and neuropathic pain, further well designed and valid randomized controlled studies are needed (20).

The first direct evidence for a cannabinoid receptor was made possible by the synthesis and radiolabelling of the potent bicyclic cannabinoid CP-55,940 (22) which led to the identification of CB1 and CB2 receptors in rat brain membranes. The CB2 receptor is found predominantly in the spleen and in haematopoietic cells and stem cells. CB2 has only 44% overall gene sequence identity with the CB1 receptor (23). The existence of this receptor provided the molecular basis for the immunosuppressive actions of marijuana.

The CB1 receptor mediates inhibition of adenylate cyclase, inhibition of N- and P/Q-type calcium channels, stimulation of potassium channels (24), and activation of the mitogen-activated protein

kinase pathway (25). The CB2 receptor, like the CB1 receptor, mediates inhibition of adenylate cyclase and activation of mitogen-activated protein kinase.

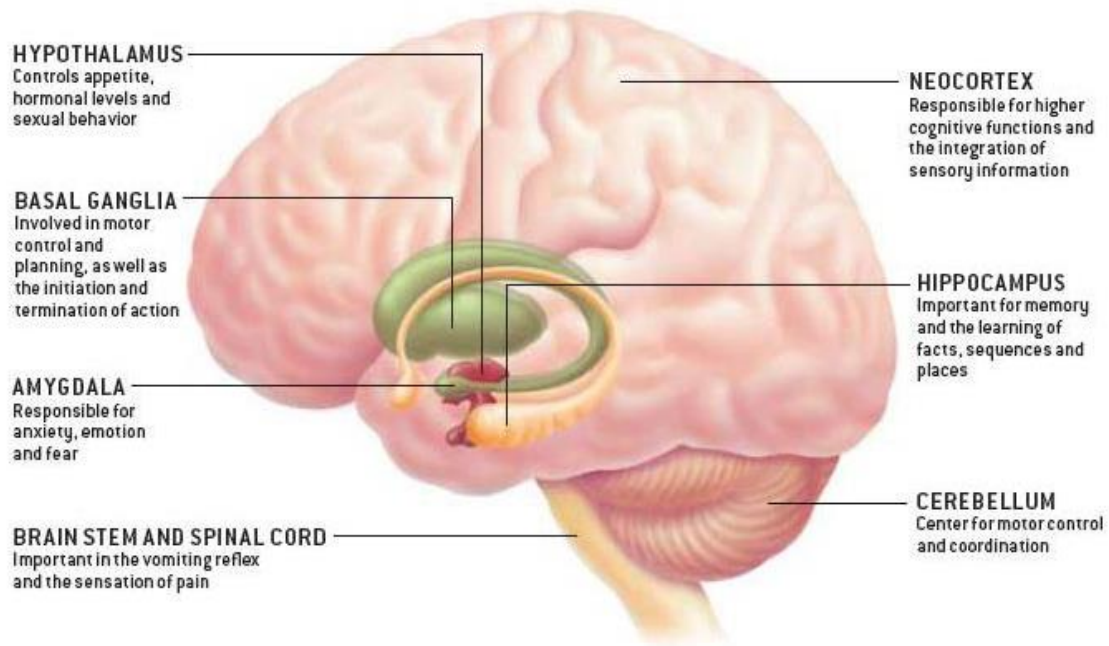


Fig 6: Illustration of where synthetic cannabinoids act as potent cannabinoid receptor agonists for CB1 that are generally located in the brain. (With permission Chen, A. Scientific American 2009)

Anandamide is released from neurons after depolarization through a mechanism that requires a calcium-dependent neuronal membrane phospholipid precursor cleavage (26). The released anandamide results in a rapid increase in the plasma concentration and is hydrolysed by the fatty-acid amidohydrolase (FAAH) enzyme. The psychoactive cannabinoids increase the activity of dopaminergic neurons in the ventral tegmental area affecting the mesolimbic pathway (also known as the reward pathway). Since these dopaminergic circuits are known to play an important role in mediating and reinforcing of the rewarding effects of the most drugs of abuse, the enhanced dopaminergic drive elicited by the cannabinoids is thought to underlie the abuse properties of marijuana (Fig 7). Interestingly, cannabidiol does not activate either CB1 or CB2, but instead activates GPR55 and the 5-HT₆ serotonin receptor. As a result, cannabidiol lacks the psychotropic effects of Δ^9 -THC and, in fact, has some distinct, antagonistic effects for Δ^9 -THC. (27)

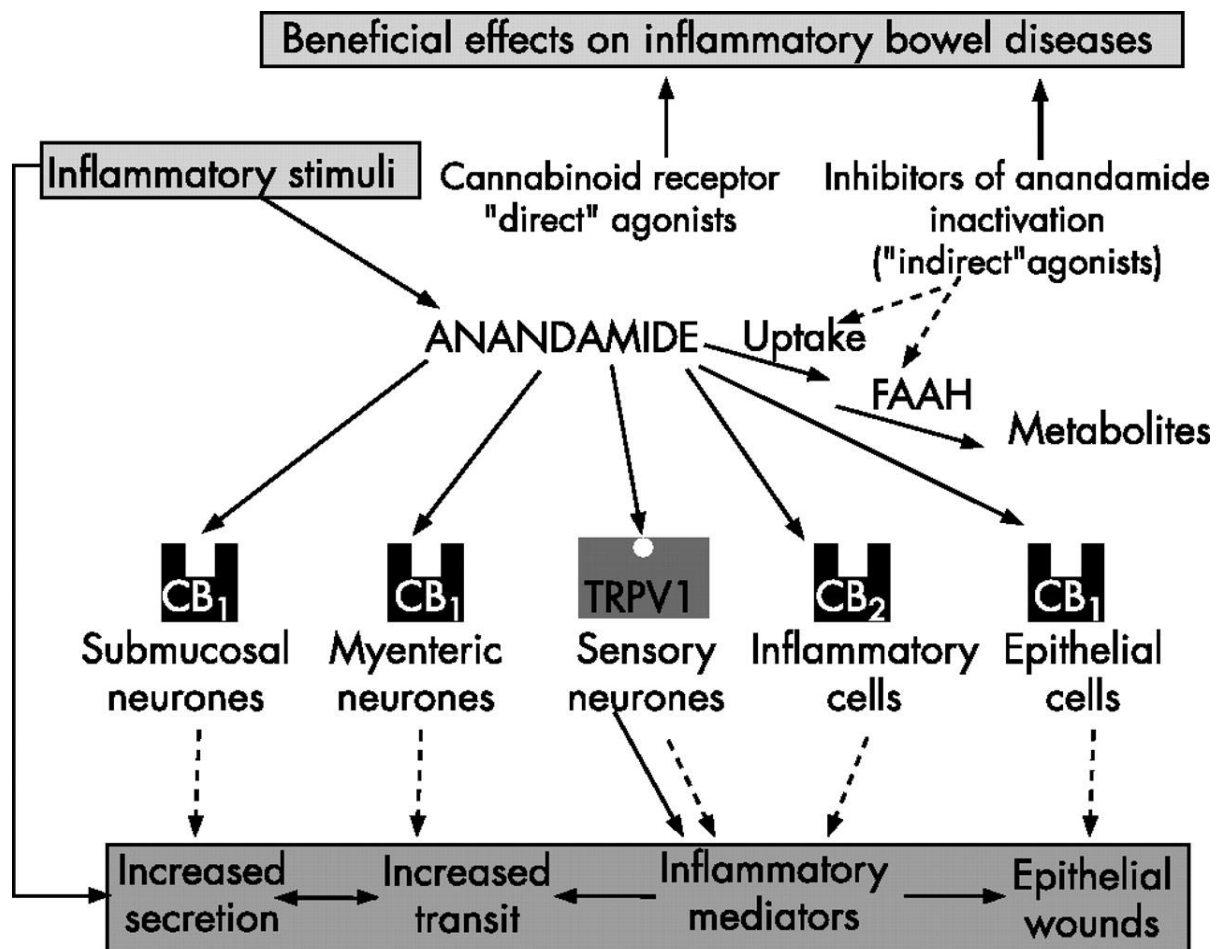


Fig 7: Anandamide, an endogenous ligand for cannabinoid receptor 1 (CB1), is released from neurons on depolarization but is rapidly inactivated. Anandamide inactivation is not completely understood, but appears to occur by transport into cells or by enzymatic hydrolysis. Adapted from (26)

Cannabinoids share a final common neuronal action with other drugs of abuse such as morphine, ethanol and nicotine by facilitation of the mesolimbic pathway (28).

Binding in the different regions of the neural system explains cannabinoid dependant alteration of motor function and the effect on short-term memory in humans. This observation is consistent with the intermediate levels of CB1 receptors in the pyramidal cell layers of the hippocampus as well as layers 1 and 6 of the brain cortex. The effect of cannabinoids on memory and cognition are consistent with receptor localization in the hippocampus and the cortex. The hippocampus is involved in memory as well as coding of sensory information. The CB1 cannabinoid receptors are generally found in regions of the brain showing enhanced abundance of dopamine associated neurons (29)

The results of numerous studies suggest that many neuro- transmitters and -modulators have a role to play in the neuropharmacology of cannabinoids (26). These neurotransmitters include substances like acetylcholine, histamine, GABA, dopamine, norepinephrine, opioids, neuroactive peptides and prostaglandins. The basis of some of these cannabinoid interactions were studied to determine the interaction of cannabinoids and other drugs that can bind to different receptor types or even drugs that alter the storage, synthesis, release or metabolism of neural modulators and transmitters. Cannabinoids have the ability to enhance the synthesis of norepinephrine, dopamine and 5-hydroxytryptamine (30). Cannabinoids also stimulated the release of dopamine from corpus stratum and from the nucleus accumbens and the medial prefrontal cortex. GABA turnover in the presence of cannabinoids is enhanced (31).

Interpretation of the action of cannabinoids on the neurotransmitter systems is normally very difficult but experimental evidence shows that cannabinoids can both inhibit or stimulate neuronal uptake of neurotransmitters (32).

2.2 RECREATIONAL USE /DRUG ABUSE

An on-going national research and reporting program in which secondary school children, college students and young adults have been surveyed on a yearly basis consistently reveals that alcohol is the most frequently used substance and that marijuana is the most frequently used illicit substance (33). High school children especially were monitored closely since 1975. Prevalence of use was measured in terms of lifetime, annual, 30 days as well as daily use. The prevalence of marijuana use peaked during the late 1970's where more than 60% of high school seniors reported that they had smoked marijuana at least once in their lifetime (34). More importantly, 10% of those students smoked marijuana on a daily basis. Lifetime use for 8th and 10th grade learners were 16.7% and 30.4%, respectively in 1994. Marijuana was also reported to be widely used by young adults, although the use tends to decline with age. The most common form of marijuana use in the United States is in the form of cigarettes. It is also added into cookies or brownies and ingested. In this oral administration mode, the effects after ingestion do not start immediately and peak brain levels are only reached approximately 2 hours after ingestion after which the effects last longer.

THC is mostly used for recreational purposes, relaxation and euphoria effects, to suppress inhibitions, to give a sense of well-being and disorientation, altered time and space perception, lack of concentration, learning problems, alterations in thought formation and expression, drowsiness, sedation, mood changes such as panic reactions and paranoia, a more vivid sense of

taste, sight, smell, and hearing. In general, higher doses intensify these effects and may cause fluctuating emotions, flights of fragmentary thoughts with disturbed associations, a dulling of attention despite an illusion of heightened insight, image distortion and psychosis (16).

The most frequent effects include vasodilatation, increased heart rate, reddening of the eyes, dry throat and mouth and increased appetite (munchies).

Negative effects like fatigue, paranoia, memory loss, depersonalization, mood alterations, urinary retention, constipation, decreased motor coordination, lethargy, slurred speech and dizziness have commonly been reported. Impaired health including lung damage, behavioural changes, and reproductive, cardiovascular and immunological effects have been associated with regular marijuana use. Regular and chronic marijuana smokers may have many of the same respiratory problems that tobacco smokers have (daily cough and phlegm, symptoms of chronic bronchitis) as the amount of tar inhaled and the level of carbon monoxide absorbed by marijuana smokers is 3 to 5 times greater than among tobacco smokers. Smoking marijuana while simultaneously administering cocaine has the potential to cause severe increases in heart rate and blood pressure (16). It has also been established that sinus tachycardia results from the administration of THC or by smoking marijuana. After acute inhalation of marijuana, bronchodilation in asthmatic as well as normal patients has been observed (35).

Recreational users of cannabinoid compounds who use cannabis based products, which can impact on the work being performed or in the case of athletes in enhanced performance are often monitored through urine or blood tests requested by employers or authorised monitors. These tests should be validated tests that limit the possibility of false positive detection of cannabinoids in urine or plasma and must account for the possibility of several potential interfering substances. Many of the available methods are not sensitive to the synthetic cannabinoids or not validated to be interference free, which is a major concern as the use of cannabinoids in sport and certain working environments is illegal.

Anti-inflammatory drugs have been reported to interfere with cannabinoid assays causing false-positive results for cannabinoids in some enzyme-linked immuno assay (ELISA) and other assay systems, although conflicting results have been reported among studies (36). In a study that tested 510 urine samples from patients who had received ibuprofen, naproxen or fenoprofen at therapeutic dosing regimens (single-dose and long-term chronic ingestion), two false-positive

results for cannabinoids were found, 1 during the short-term ingestion of ibuprofen (1200 mg for 1 day) and the other after long-term use of naproxen (37, 38)

The relatively high popularity of marijuana and the variable cannabinoid content ratios of the different forms of illicit cannabis products makes it difficult to determine the origin of the product used. This is further complicated by the growing use of synthetic cannabinoids and products that contain both natural and synthetic cannabinoids for which many existing methods are not able to identify.

2.3 CANNABIS-OIL

Cannabis has been investigated for decades for medicinal constituents, and this has made it a controversial subject with active debates regarding the benefits or contra-indications for the use of different cannabinoid components present in cannabis plants. The USA Drug Enforcement Administration Controlled Substances Act still (2017) classifies cannabinoids as Schedule I, which means that these compounds are only available as investigational drugs, despite some states legalising the use of cannabis. In addition to the social aspects of the use of cannabinoids and the abuse potential, the issue of approving cannabis as a therapeutic agent is further questionable due to the complexity of the mixture of chemicals found in different plant parts and the extraction techniques used.

The major active component is delta-9-tetrahydrocannabinol (Δ^9 -THC) which is highly enriched in several available products (39). Cannabis essential oil, is commonly used in the perfume industry and is one of the more unusual essential oils because of the plant from which it is derived. Cannabis essential oils are commonly isolated by steam distillation (24) and this method produces different cannabis essential oils that contain a relatively low Δ^9 -THC concentration of <0.5%. There are many different compounds found in these essential oils, including high concentrations of monoterpenes, of which myrcene, limonene, α -pinene, α -terpinolene are the dominant compounds of this class, and sesquiterpenes with trans-caryophyllene and caryophyllene oxide being the dominant sesquiterpene compounds. The concentration of the alkaloids is relatively low due to the steam distillation process. Two of the hemp essential oil components, myrcene and α -pinene, are natural flavonoids that have an insect repellent effect (26).

In contrast to the steam distillation of plant material, there are methods used for the selective extraction of the active alkaloids falling into the cannabinoid class and which are mostly used for illegal recreational purposes and often involves chemical processing to enhance the Δ^9 -THC

concentration. These methods generally involve extraction using lipophilic solvents or even supercritical fluid extraction using carbon dioxide. The plant age at harvest time, the pollination status, the weather conditions "just-before-harvest" and the plant parts extracted all have an influence on the cannabinoid content of the essential oil. Solvent extraction consistently produces extracts with Δ^9 -THC concentrations between 12 - 18%, making these extracts useful for medical applications. By using specific chemical processing and selective re-extraction these concentrations can be increased to greater than 60%. This is based on the theory that sniffer dogs are more likely to smell the volatile sesquiterpene, caryophyllene oxide instead of Δ^9 -THC (25).

Recently there has been strong lobbying for the legal use of cannabinoids that are being proposed for use as therapeutic agents (40) with more consistent data than that provided before 1990. The structures of the active chemical compounds derived from cannabis are now known, the mechanisms of action on the nervous system have mostly been elucidated and the discovery of an endogenous cannabinoid signalling system add to the understanding of the different effective treatments which are now essentially scientifically validated although some safety aspects remain controversial.

2.4 SYNTHETIC CANNABINOIDS

Following the discovery of the cannabinoid receptors, compounds like compounds JWH-018, JWH-073 and HU-210, that mimic the natural receptor ligands and which elicit potent CB1 agonist activity similar to Δ^9 -THC (41) were designed and synthesised. These synthetic cannabinoids are highly potent compounds with higher binding affinity for CB1 receptors than Δ^9 -THC that specifically trigger the CB1 effects at concentrations that can be as much as 100 times lower than Δ^9 -THC. These drugs are referred to as synthetic cannabinoids and have found their way into the illicit drug trade and has been used to spike several herbal products to attain psychological effects similar to those obtained by smoking Δ^9 -THC. There are two classes of compounds typical of the synthetic cannabinoids. These are the aminoalkylindole naphthalenes and the tetrahydrocannabinol analogues.

Because Δ^9 -THC is one of the most popular drugs of abuse worldwide, the primary focus of manufacturing these synthetic cannabinoid drugs is to enhance the supply and to provide alternative drugs that are easy to come by, not regulated and that cannot be easily detected and has the same or greater effect than Δ^9 -THC (42). The idea by cannabis (and other drug) users, to evade detection and legal prosecution has made many online available products such as "drug-

free urine products” popular as the legislation and justice systems are slow to respond to the release of new designer type drugs onto the market. Redesigned and synthesized drugs have the same or even greater effects than the natural plant-derived cannabinoids and some of these products have been sold openly in liquor stores as “K2”, “Spice Gold” and “Spice Diamond” (42)

The synthetic compound JWH-018 is the most investigated synthetic cannabinoid and is both a CB1 and CB2 agonist with a higher affinity for CB1 than Δ^9 -THC but HU-210 is reported to be approximately 100 times more potent than Δ^9 -THC (43).

The synthetic cannabinoid HU-210 also displays a range of biochemical, pharmacological, and neural effects, most of which have been established to be dependent on potent agonist activity at CB1 cannabinoid receptors and involve the main neurotransmitter systems (44). Results obtained in various studies suggest several potential clinical applications of this potent CB1/2 agonist (e.g. as an antipyretic, anti-inflammatory, analgesic, antiemetic and antipsychotic agent) as well as its usefulness in research aimed to develop a better understanding of the association of the endogenous cannabinoid system in a number of pathophysiological conditions. HU-210 has shown another significant concept in pharmacology and shows a confirmed reverse agonist effect at the receptor (45). Reverse agonist effects are best explained by the model where receptors exist in a balance between activated and inactive states, with a portion of the receptors being in the active state at any given time. In the absence of an agonist, there will be a low level of signalling mediated by the receptor (46). Increasing concentrations of agonists will trigger greater numbers of receptors to the active state, and show a cumulative signalling effect. However, an inverse agonist binds to the receptor and triggers an atypical response that is effectively the opposite to the expected agonist induced response.

To date, there are several methods published detailing the identification of synthetic cannabinoids in blood and urine samples following Spice administration with more than 20 cannabinoid related compounds being detected following Spice administration using gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS/MS) (47, 48). The challenge is to develop a sensitive and robust LC-MS/MS method capable of detecting and quantitating the synthetic cannabinoids in the presence of the less active natural plant-based cannabinoids in biological matrices. Because of the world-wide prevalence of only a few synthetic cannabinoid products, namely JWH-018, JWH-073 and HU-210, an analytical method to determine

these very potent synthetic drugs in plasma and urine by UPLC-MS/MS will be of benefit to the general practitioner.

Planned pharmacokinetic profiling of these synthetic cannabinoids is difficult to investigate, partly because of the ethical considerations due to the unknown safety and toxic effects, the potential for abuse, the extremely high potency of these compounds, the scheduling of these compounds, the lack of understanding of the long-term effects.

Pharmacokinetic assessment of the synthetic cannabinoids using either plasma or urine samples will be challenging due to the low concentrations that would be administered and the large number of possible metabolites that can be formed at both the phase I and phase II metabolic phases. No studies in humans with these compounds has been performed in South Africa as of 2017.

Drugs that inhibit CYP450 activity will increase the plasma concentrations of any medications requiring the inhibited enzyme for metabolic conversion to less active and more polar compounds. For example, any reduction of CYP2C9 activity can result in adverse drug effects such as increased clotting time despite stable warfarin treatment.

Figure 9 illustrates the effect of metabolism of both the synthetic cannabinoid JWH-018 and Δ^9 -THC showing which metabolites can still bind to and elicit a normal response and which metabolites show no response.

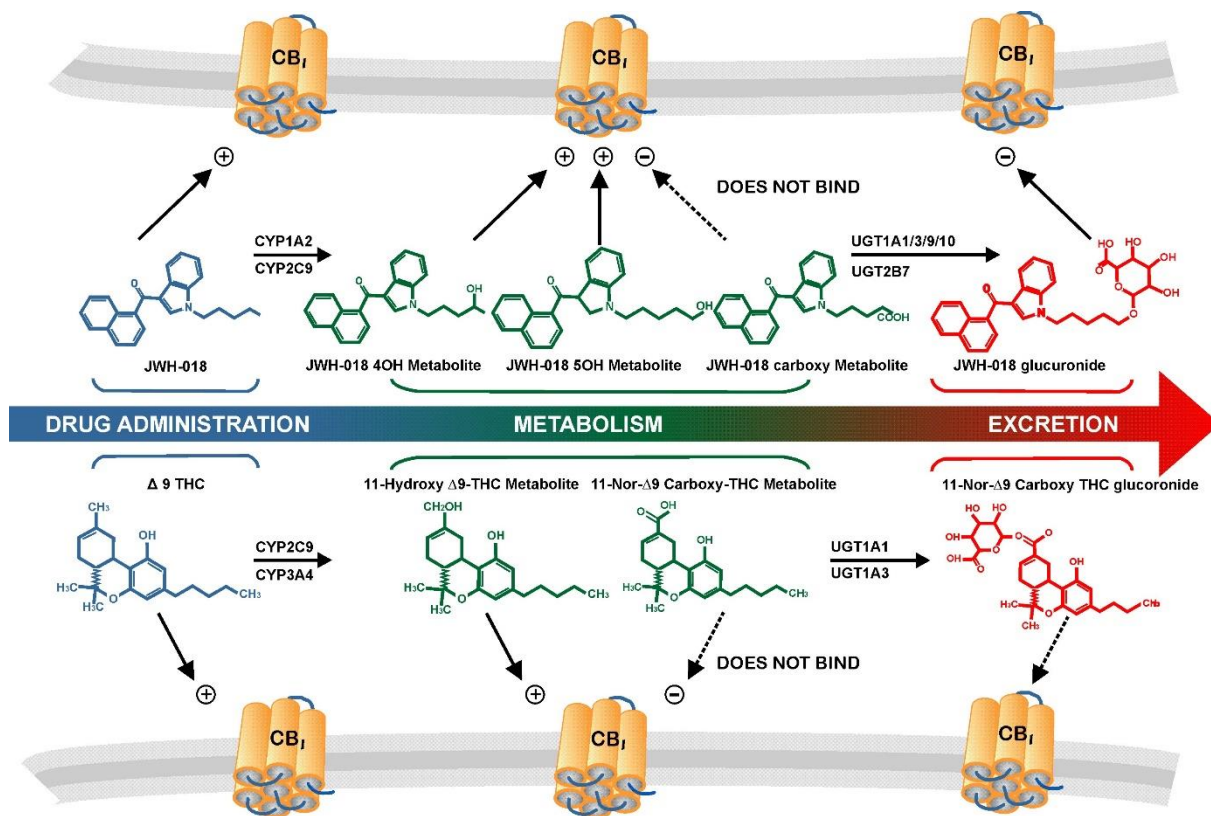


Fig 9: A schematic representation that summarizes what is known about JWH-018 (upper) and Δ⁹-tetrahydrocannabinol (THC) (lower) metabolism, excretion, and potential downstream interactions with cannabinoid type-1 (CB₁) receptors (“+” indicates agonist, “-” indicates antagonist). CYP2C9 metabolism of JWH-018 produces several products that retain affinity and intrinsic activity at CB₁ receptors. CYP2C9 metabolism of Δ⁹-THC results in the production of a single active metabolite.

The following three synthetic cannabinoids are the most commonly recreationally used compounds despite being illegal in most countries. Note the difference between the JWH-018 and JWH-073 with respect to the N-alkyl chain length being a pentyl or butyl chain respectively. Their structures are shown in Fig10 below (49).

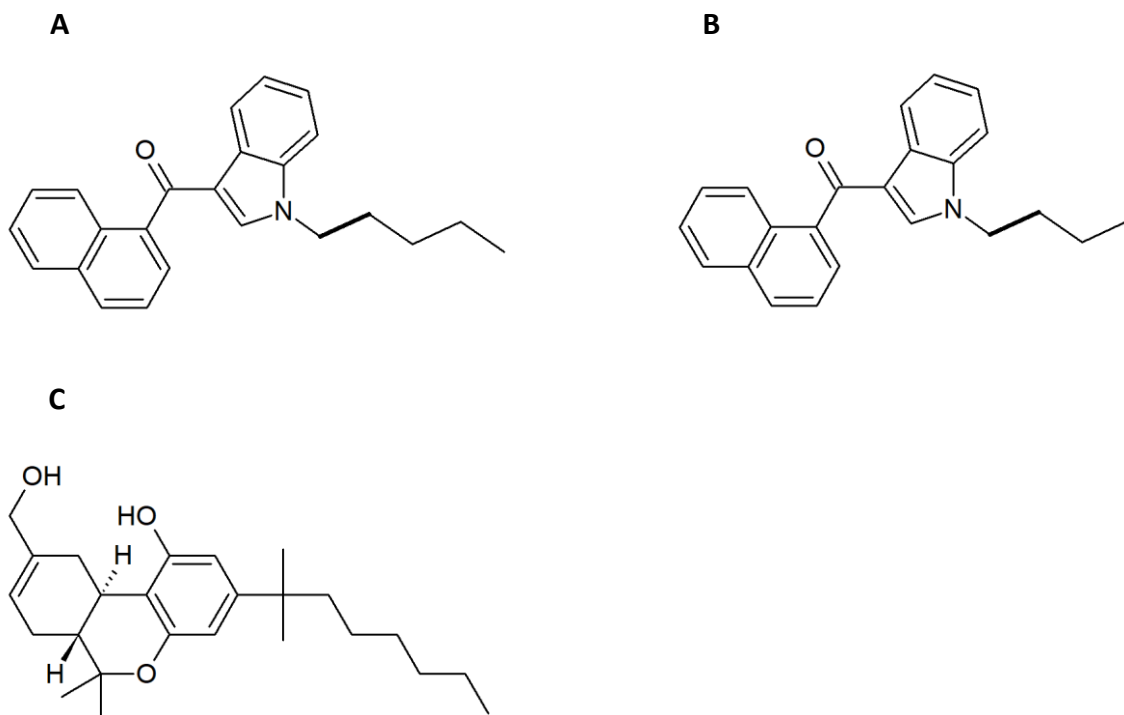


Fig 10: The synthetic cannabinoids most commonly supplied in various forms and used recreationally. The two aminoalkylindole naphthalenes A.) JWH-018; B.) JWH-073 and the delta 8 cannabinoid analogue C.) HU-210 that was incubated with human liver microsomes yielded 24 identifiable metabolites when the reaction mixture was analyzed using LC-MS/MS. (50)

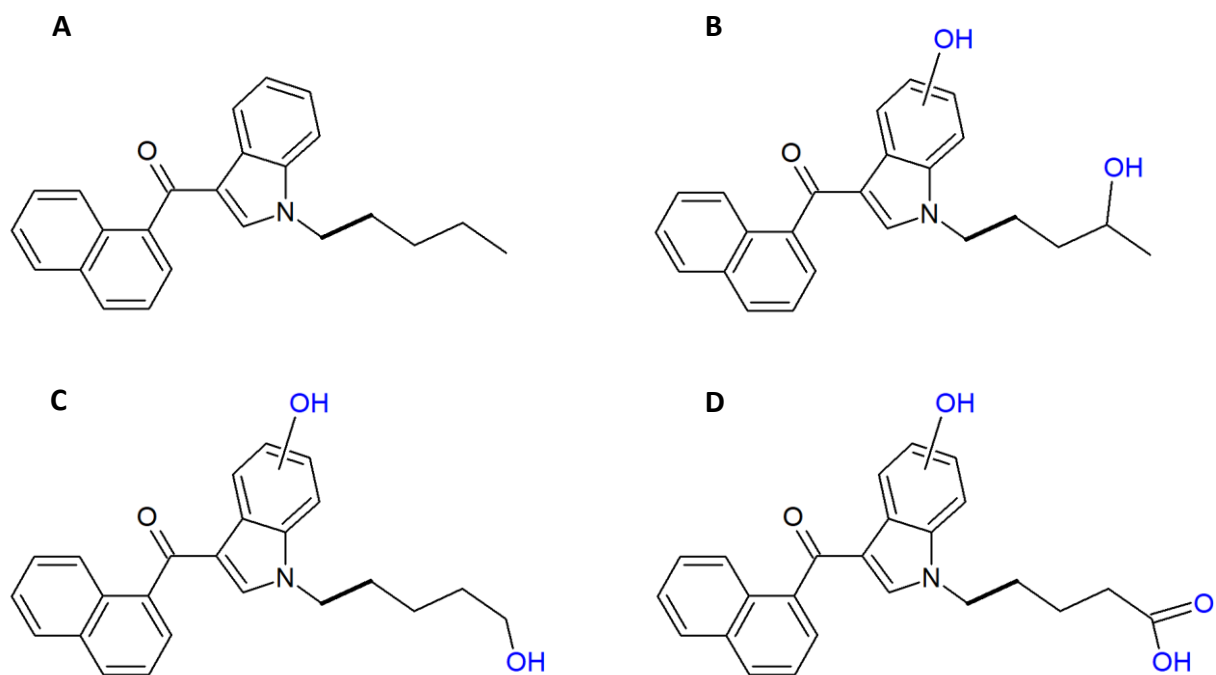


Fig 11: A.) Sites of chemical modifications of the major metabolites detected in human urine resulting from hydroxylation or oxidative products from JWH-018. The hydroxylation of the N-pentyl chain can take place at the C4 or C5 position and may be further oxidised to a 5 carboxy metabolite. In addition to these alkyl oxidations, hydroxylation of the phenolic ring of the indole can take place on any of positions 4, 5, 6 or 7 in addition and simultaneously to the alkyl chain hydroxylation. Structures were drawn with ChemSketch 2017

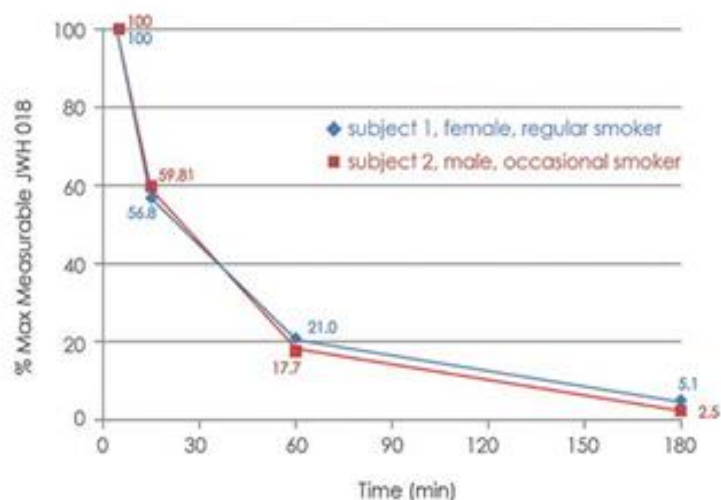


Fig 12: The typical pharmacokinetic profile of the elimination of JWH-018 from human subjects following inhalation showing a first order elimination.

Vaporization of cannabinoids from synthetic cannabinoid impregnated candles are a popular alternative instead of the traditional smoking of the products and is one of the methods exploited as a means of selling the synthetic cannabinoids. Vaporising is the process where the synthetic cannabinoids are vaporised by heating in a candle. Levels of each ingredient in the composition, including the cannabinoid can be controlled. Since vaporisation does not involve combustion of the harmful chemical, the user can to a certain extent control the amount of cannabinoid inhaled.

The active ingredient is generally free of harmful high levels of tar and various toxins associated with smoking the herbal mixture spiked with the synthetic cannabinoid (51)

Vaporisation also avoids sudden overdosing, as can occur during in rapid deep inhalation during smoking and avoids many of the respiratory disadvantages associated with smoking. Vaporisation occurs at approximately 180-190°C which significantly reduces the polyaromatic hydrocarbon content of the vapour.

The vaporisation method may additionally involve both cannabinoid and acetylcholine receptor inhibitors (51).

2.5 PHARMACOLOGIC EFFECTS

One challenge to general practitioners is the lack of clinical and pharmacological information available for the synthetic cannabinoids. Pharmacokinetic information requires sensitive and accurate detection and classification of the synthetic cannabinoids and their metabolites in samples collected from human subjects under controlled conditions and is of high importance (1). Data of metabolic pathways of the synthetic cannabinoids is very limited. Some reports show that the Phase I metabolites are excreted as hydroxylated derivatives of the parent drug compounds. The presence of glucuronic acid conjugates (49) implies effective Phase II metabolism. Oxidative metabolism and hydroxylation (Phase I) often serves to prepare compounds for Phase II reactions where conjugation of many active xenobiotic compounds to glucuronic acid inhibits the biological activity and increases the rate of excretion (52).

Both hydroxyl- and carboxy- metabolites of JWH-018 and JWH-073 have been identified and confirmed as glucuronide conjugates in urine samples from users of these synthetic cannabinoids (53).

2.6 USE OF LC-MS/MS

For LC-MS/MS analysis, the initial separation takes place on a liquid chromatography system where the analytes are separated from each other based on differential solubility of the analyte between the stationary and the mobile phases. The mobile phases have the strict limitation that any additives used for their buffering or ionisation-promoting characteristics must be volatile if detection is to be by mass spectrometry. The chromatographic separation will also only be successful if the analytes are soluble in the mobile phase. The advantage of LC-MS/MS method is that no intensive derivatization is needed to make the analyte volatile. This makes the LC-MS/MS methods more universally applicable and can often avoid the requirement for any hydrolysis or derivatization. Sample preparation remains important and especially so for conjugated biological compounds. The use of a standard operating procedure (SOP) for urine analysis in most laboratories includes the hydrolysis of urine specimens with β -glucuronidase/arylsulfatase to release the polar and highly charged conjugated compounds such as glucuronic acid and sulphate groups. The analyte, whether a parent drug or metabolite is then extracted using liquid-liquid or solid-phase extraction. Solid phase extraction reduces the number of sample preparation steps, concentrates the analytes several fold and reduces the matrix effects resulting from ionic and inorganic compounds. This then minimises interferences and gives a cleaner final matrix. Testing of complex plasma samples is also possible by using a modified solid phase extraction protocol followed by LC-MS/MS analysis that enhances the detection of the precursor synthetic cannabinoids. LC-MS/MS performed in positive ionisation mode adds a single proton and a positive charge to the molecule to create a pseudo-ion with a mass one unit higher than the precursor molecule that is then selected during the mass analysis. This ionisation process is illustrated in Figure 13 below. The charged pseudo-ion, $[M+H]^+$ then enters the mass spectrometer where the initial precursor molecule is transferred into the first mass spectrometer by applying a suitable cone voltage. Different compounds require different optimal cone voltages, therefore it is of importance that each analyte be optimized separately. Fragmentation of selected precursors takes place in the collision cell (Q2) to obtain several product ions that are characteristic of the analytes and that allows the increase in sensitivity and reduction of the background noise. The initial conditions used are mentioned in UPLC-MS/MS method.

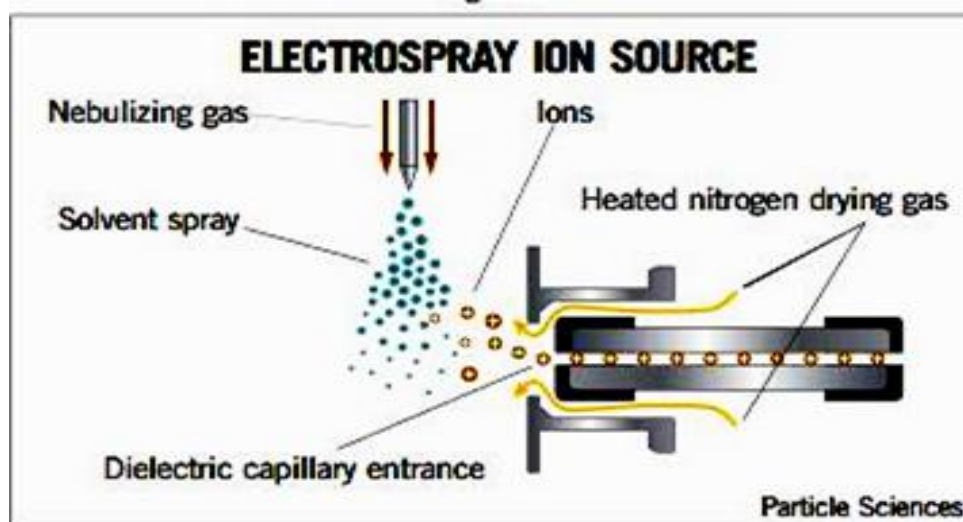


Fig 12: Schematic image for the ionization source for an ES+ of an LC-MS/MS

The anthropomorphic terms for ions involved in fragmentation reactions, for example, “daughter ion”, have fallen into disuse after strong sentiments against the use of the term were voiced two decades ago. The term “product ion” is recommended in place of “daughter ion” and “precursor ion” in place of “parent ion” or “mother ion”. The use of n^{th} -generation product ion is recommended in place of granddaughter ion and similar terms.

2.7 GC-MS VERSUS LC-MS/MS METHODS FOR CANNABINOID ANALYSIS

Extraction of the natural and synthetic cannabinoids and their metabolites from urine can be accomplished through hydrolysis of the glucuronate and sulphate conjugates of these drugs and their respective drug metabolites. When analysing urine samples for drug metabolites a hydrolysis step at 37°C for 8 hours at pH 5-7 (54) using β -Glucuronidase (from *Helix pomatia*, *Haliotis rufescens* or *Escherichia coli*) is generally included before extraction to provide the best drug and drug metabolite recovery. After enzymatic hydrolysis, the pH of individual samples is adjusted by addition of 1.0 M phosphate buffer (pH 4) and vortex mixed before addition of hexane:ethyl acetate (5:1) for lipophilic drug extraction. Samples are clarified after extraction by centrifuging at 400 g for 5 minutes (55). The organic layer is removed into clean tubes and evaporated under a stream of nitrogen. Derivatization for GC-MS is performed by adding TMCS and BSTFA in a closed vessel and placing all the tubes into a dry heating block at 90°C for 20 minutes. The vials were removed to cool then excess derivatization reagent was evaporated under a stream of nitrogen, 200 μL ethyl acetate was added and transferred to GC-MS vials. An injection volume of 1.0 μL was used on the GC-MS.

LC-MS/MS methods are particularly applicable for this type of analysis as either the conjugated glucuronide of both the JWH-018 and JWH-073 metabolites can be detected without enzyme hydrolysis or derivatization as required for GC-MS methods. The advantage of not requiring hydrolysis can simplify the sample preparation thereby reducing the variability and can increase the sensitivity and selectivity of the methods leading to more significant clinical research. or injected directly into an LC-MS/MS system after protein precipitation. Vortex mixed and clarified at 400 g for 5 minutes. The injection volume used is 1 μ L.

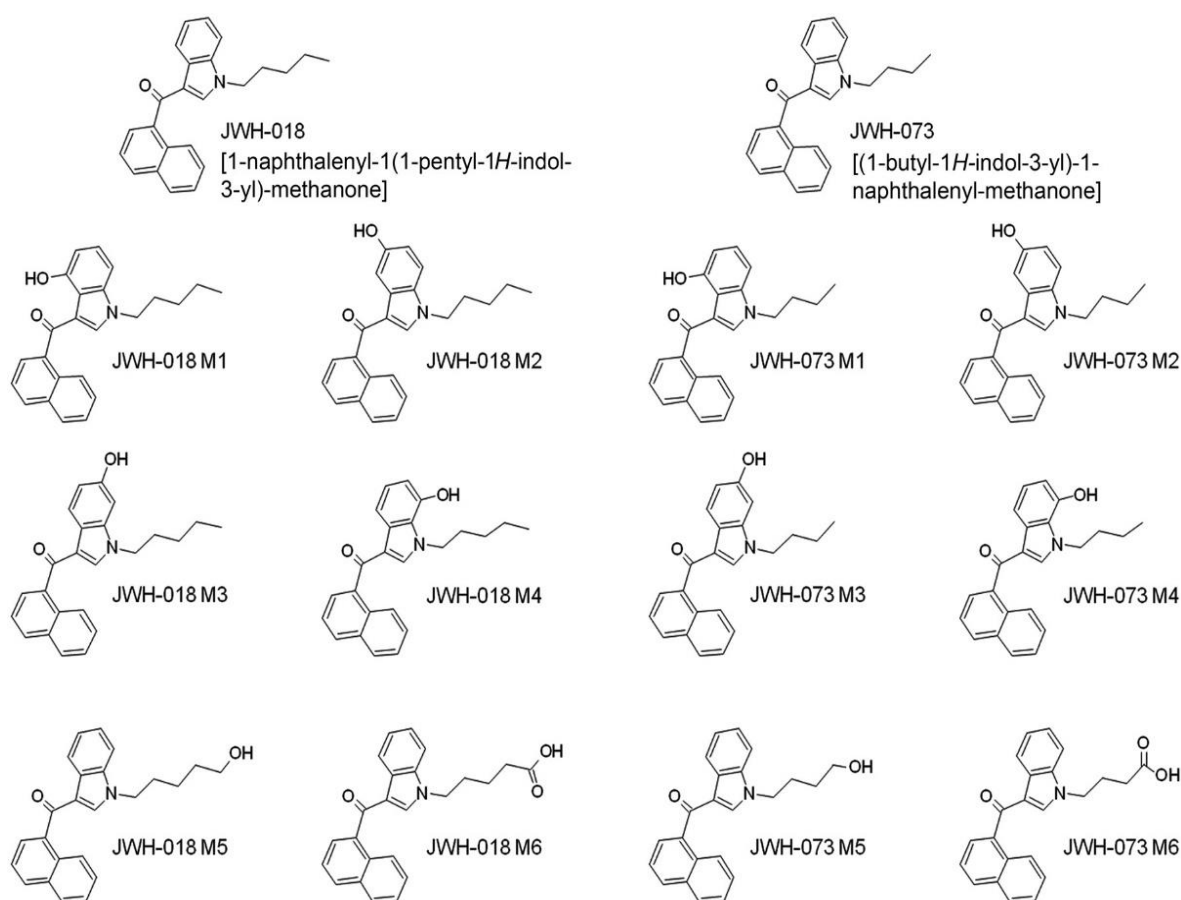


Fig 13: Structures of JWH-018 and JWH-073 synthetic cannabinoids and the common metabolites for both molecules. Major metabolites of synthetic cannabinoids are mono-hydroxylated on the indole ring (M1, M2, and M3), or on the alkyl chain (M5 and M7), or terminally mono-carboxylated on the alkyl chain (M6).

Compounds JWH-018 and JWH-073 differ only in the length of the alkyl side chain attached to the nitrogen of the indole ring. All of these potential hydroxylation sites can be glucuronidated. Several hydroxylated “Phase I” metabolites are shown in the Figure 14 below (53).

The fragmentation of both JWH-018 and JWH-073 provide major fragments of mass 155.0 and 127.0 and are fragments cleaving the naphthyl group from the indole ring (56).

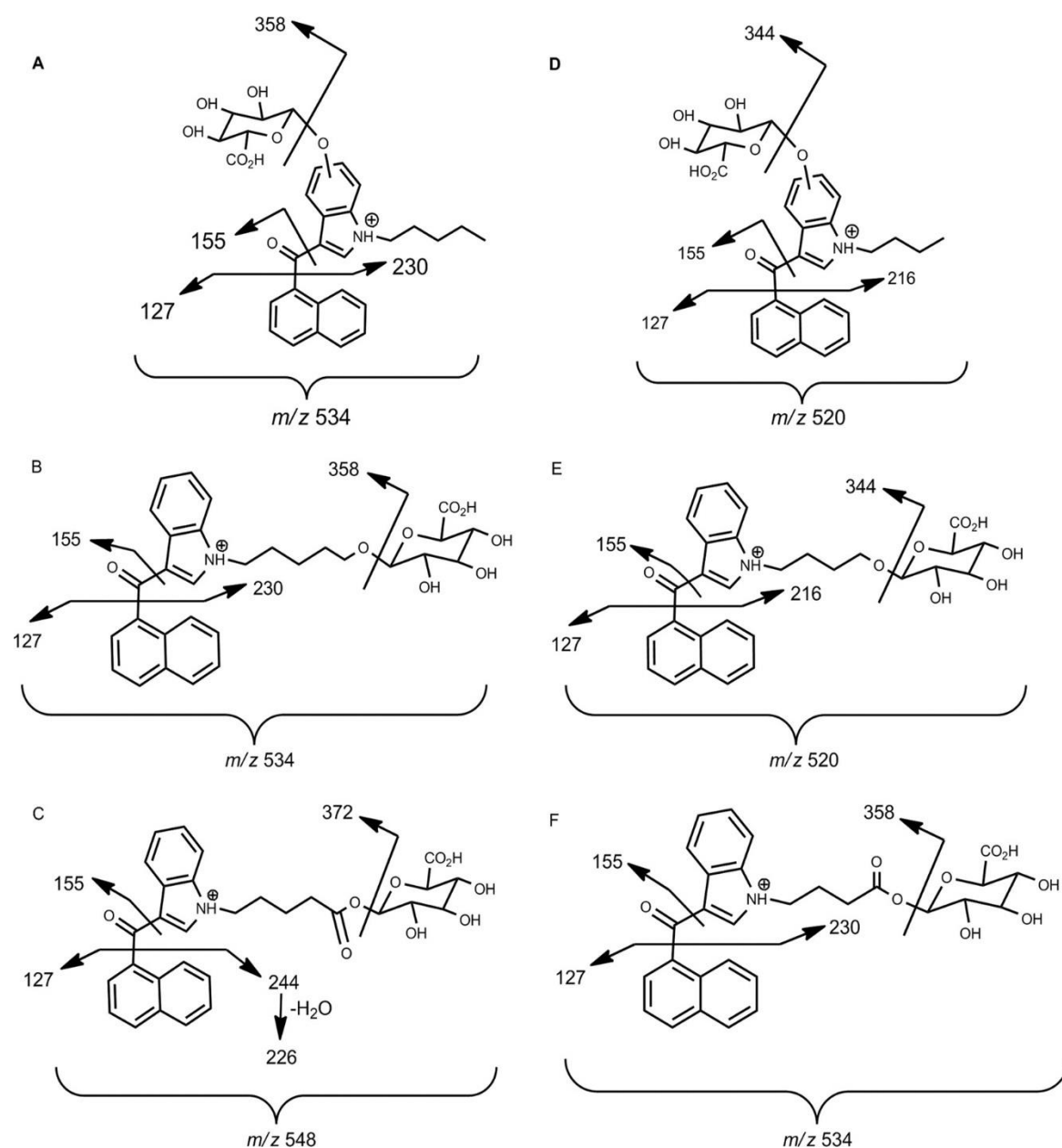


Fig 14: MS/MS fragmentation pathways for the glucuronidated hydroxy-metabolites of JWH-018 and JWH-073. Shown are JWH-018 M2-M4 (A), JWH-018 M5 (B), JWH-018 M6 (C), JWH-073 M2-M4 (D), JWH-073 M5 (E), and JWH-073 M6 (F) (56).

2.8 THE RESEARCH PROBLEM

At present there are no validated methods available for determining the presence of both the natural and the synthetic cannabinoids in the same biological sample using a single UPLC-MS/MS method. The ever-changing designer drugs that mimic the cannabinoids or that are designed to specifically target the CB1 cannabinoid receptors are becoming a bigger problem for both clinical and drug abuse monitoring. The most used method for cannabinoid analysis at present in daily use in most pathology and forensic laboratories is the ELISA based method that relies on specific antibodies that target the natural cannabinoids but not yet available for the synthetic cannabinoids.(57)

One way to address the problem of assessing the presence of drugs in biological matrices with a rapidly changing availability of selected drugs and to monitor abuse of these drugs is to devise a generic type of assay that will have the selectivity and sensitivity to be able to detect and quantitate numerous different cannabinoid compounds. Urine is the most common biological matrix assayed at present due to the concentration effect over time, but contains mostly more polar and conjugated cannabinoid metabolites. Analysis of blood samples, where the parent cannabinoid compounds can still be detected at low concentrations also needs to be addressed. The two methods that could match the requirements for this type of generic assay are mass spectrometry based and differ only in the separation method used prior to the introduction into the mass spectrometer. These methods are GC-MS and the LC-MS/MS methods, each having advantages and disadvantages which can influence the final choice of method. As many metabolites are polar and not naturally volatile, an LC-MS/MS method has several advantages. At present no LC-MS/MS method has been validated for the simultaneous quantitation of both natural and synthetic cannabinoids and their respective major metabolites. This study set out to provide such a method using stable isotope labelled internal standards where available and to assess the sample preparation and stability of the different analytes.

It is often the case in emergency rooms to inform the GP (General Practitioner) of the type of cannabinoids the patient taken. The patient can progress from sick to critical ill if he indigested HU-210. For these reasons, these compounds are also of forensic significance and the modern forensic toxicology laboratory must be able to detect and quantitate synthetic cannabinoids in the typical biological matrices, including whole blood.

3 AIMS

The primary aim of this study was to develop and validate a sensitive, accurate UPLC-MS/MS method to quantify the common plant cannabinoids and the three most common synthetic cannabinoids, JWH-018, JWH-073 and HU-210 and their respective metabolites in a single limited volume plasma or urine sample.

To confirm that natural and synthetic cannabinoid metabolites can be detected in urine using a non-derivatizing sample preparation methodology followed by an LC-MS/MS separation and detection method.

To assess the effectiveness of the developed method for medical diagnostics and assess whether sample turn-around times can be improved.

4 MATERIALS AND METHODS

4.1 MATERIALS

All organic solvents used were of ultra-high purity with the methanol and acetonitrile (Romil, Microsep, Jhb, RSA). Mass spectrometer grade formic acid was from Sigma-Aldrich (St Louis, Mo, USA). Ultra-pure water was obtained from an in-house Purelab reverse osmosis and deionisation water system which provided water with conductivity of $>18 \Omega\text{Ohms}$.

The following certified reference standards were purchased from Cerilliant, a Sigma Aldrich company.

- Δ^9 -Tetrahydrocannabinol (**Δ^9 -THC**)
- (+)-11-Nor- Δ^9 -carboxylic acid glucuronide (**THC-COOH Glu**)
- Synthetic cannabinoid (**JWH-018**)
- Synthetic cannabinoid (**HU-210**)
- Synthetic Cannabinoid (**JWH-073**)

Certified Reference Material for natural and synthetic cannabinoid metabolites

- JWH-210-4-Hydroxypentyl metabolite (**JWH-210–4-hydroxy pentyl**)
- JWH-210-5-Hydroxypentyl metabolite (**JWH-210–5-hydroxy pentyl**)
- JWH-073-4-Hydroxybutyl metabolite (**JWH-073–4-hydroxy butyl**)
- Cannabidiol (**CBD**)
- Cannabinol (**CBN**)

The following stable isotope labelled internal standards were purchased Cayman Chemical (Ann Arbor, MI, USA),

- (\pm)-cis-11-Nor-9-carboxy- Δ^9 -THC glucuronide-D3
- JWH-073 3-Hydroxybutyl metabolite-D5
- Cannabidiol-D3
- JWH-018-4-hydroxypentyl metabolite-D5
- Δ^9 -THC-D3

All the standards were made up in methanol and stored at -20°C .

Drug free blank urine was purchased from UTAK Laboratories (Valencia, CA, USA) and stored between 2 - 8°C. The urine contained sodium azide 0.01% as preservative.

Drug free canine plasma was purchased from Onderstepoort Veterinarian Clinic, Pretoria.

A Cortecs C18 phenyl column and the Oasis solid phase extraction cartridges (SPE) HLB 30 µm 60 mg/3cc were purchased from Waters (Milford, Ma, USA).

4.2 INSTRUMENTATION

A StepWave™ Acquity Xevo TQ-S triple quadrupole tandem mass spectrometer from Waters (Milford, Ma, USA) was used for this study. The mass spectrometer has an electrospray ionization (ESI) source capable of both ES+ and ES- ionisation. The UPLC system coupled to the mass spectrometer was an Acquity binary solvent manager and sample manager module. The sample manager temperature was set at 8°C and the analytical column compartment was kept at a constant 35°C. Keeping the column and sample manager at constant temperature prevents thermal effects if there is any change in the ambient temperature. The sample manager used two wash steps for cleaning the sample probe, a strong 80% methanol in water and a weak organic wash at 20% methanol in water between samples. Masslynx 4.1 software installed on the control module was used to control the instruments as well as for data collection for the qualification and quantification of the data.

4.3 UPLC-MS/MS CONDITIONS

Gradient elution was performed on an Acquity UPLC Cortecs C18, 100 x 2.1 mm 1.6 µm column (Waters, Milford, MA, USA). The following elution buffers were used for separation:

Mobile phase A: 0.1% formic acid in double deionised water

Mobile phase B: 0.1% formic acid in 100% acetonitrile

Both mobile phases were filtered through 25 mm diameter 0.2 µm polyvinylidene difluoride (PVDF) non-reactive thermoplastic fluoropolymer membrane filters.

The gradient used is set out in Table 1.

Table 1: Gradient elution program used for the separation of the cannabinoids and their metabolites

	Time (min)	Flow mL/min	% A	%B	Curve
1	0.0	0.250	80	20	1
2	2.0	0.250	80	20	1
3	3.0	0.250	40	60	6
4	8.0	0.250	0	100	1
5	10.0	0.250	0	100	1
6	10.5	0.250	80	20	1
7	14.0	0.250	80	20	1

The mass spectrometer settings for optimal ionisation to achieve maximum sensitivity were as follows:

- Capillary 3.25 kV (ES+)
- Source offset 60 V
- Source temperature 150°C
- Desolvation temperature 350°C
- Cone gas flow 150 L/Hr
- Desolvation gas flow 600 L/Hr
- Collision gas flow 0.17 ml/Min
- Nebulizer gas flow 7.0 (bar).

Cone voltage and the collision voltage were varied for different analytes. The ion transitions are set out in the Table 2 below.

Table 2: The different analytes with the retention times on the Cortecs C18 column and the precursor and product ions used for each. The specific cone and collision voltages for each analyte are also provided.

Component	RT (min)	Precursor Ion (m/z)	Product Ion (m/z)	Dwell time (sec)	Cone voltage (V)	Collision voltage (V)
JWH-073-4-OH butyl	8.37	344.1	155.1/127.1	0.007	39	37
JWH-073-3-OH butyl D5	8.58	349.1	150.1/167.3	0.007	39	30
JWH-018-4-OH butyl D5	8.69	347.2	160.1	0.007	32	24
JWH-018-4-OH pentyl D5	8.69	363	155.0	0.007	32	24
JWH-210-4-OH pentyl	9.43	386	183.3/155.0	0.007	34	28
JWH-210-5-OH pentyl	9.50	386.2	155.0/183.3	0.007	34	28
11-Hydroxy-THC	9.87	331.1	313.1	0.007	29	14
11-Hydroxy-THC D3	9.87	334.4	201.0	0.007	29	30
11-Nor-carboxy- Δ^9 -THC D9	9.90	354.5	308.6	0.007	30	27
11-Nor-carboxy- Δ^9 -THC	9.94	345.2	193.1/299.5	0.007	27	23
JWH-073	10.53	328.0	155	0.007	34	22
Cannabidiol	10.76	315.0	280.7	0.007	50	17
Cannabidiol D3	10.76	318.2	196.1	0.007	45	28
HU 210	10.97	387.3	201.0/243.0	0.007	45	30
Cannabinol D3	11.32	314.2	193.1	0.007	45	28
Cannabinol	11.33	311.2	195.4	0.007	50	17
JWH-018	11.67	342.2	127.1/155.1	0.007	32	45
Δ^9 -THC	11.87	315.3	193.3	0.007	30	35
Δ^9 -THC D3	11.87	318.3	196.2	0.007	30	35
11-Nor-carboxy- Δ^9 Delta Gluconide		521.1	327.1	0.007	18	24

4.4 PREPARATION OF STANDARDS

The certified reference standards were accurately diluted to 1000 $\mu\text{g/L}$ in blank urine purchased from UTAK Laboratories (Valencia, CA, USA), marked as stock solutions and stored in sealed amber glass vials at -20°C . The following set of calibration standards were prepared in analyte free blank urine: 1.0, 2.5, 5.0, 10.0, 30.0, 50.0, 70.0 and 100.0 $\mu\text{g/L}$ to prepare a matrix matched calibration curve set. Stable isotope labelled internal standards for each component, excluding HU-210, were prepared in methanol at a final concentration of 1000 $\mu\text{g/L}$. This was spiked into the calibration

standards at a fixed concentration of 20 µg/L. In addition, a set of spiked quality control standards were prepared in drug free blank urine with concentrations of 10, 50 and 100 µg/L for each analyte. These controls were stored at -20°C and the set of all three QC standards were run with every calibration curve generated and with every sample batch analysed. The standards were analysed in triplicate on a single day to generate a set of calibration curves for precision assessment and included a commercial external urine QC control for the free natural (±)-cis-11-Nor-9-carboxy- Δ^9 -THC the unconjugated metabolite of Δ^9 -THC. This external QC was purchased from Bio-Rad (Bio-Rad, USA) as the Liquichek™ Urine Toxicology Control Level 3 with a concentration of 18.5 µg/L of the (±)-cis-11-Nor-9-carboxy- Δ^9 -THC. All patient samples positive for natural cannabinoids would initially have the (+)-11-nor-carboxy- Δ^9 -THC glucuronide present in the urine which is converted to the free metabolite during enzyme hydrolysis using β -glucuronidase/arylsulfatase as described in the protocol below.

4.5 SAMPLE PREPARATION (URINE)

For validation of the developed method the urine extract was enzymatically hydrolysed with β -glucuronidase/arylsulfatase in a water bath at 37°C for 8 hours.

Two hundred microliters (200 µL) of the spiked urine standards, QC controls and patient samples were aliquoted into individual 1.5 mL Eppendorf tubes, then 50.0 µL of the internal standard mix solution accurately added followed by 750 µL of 0.1% formic acid in 100% methanol. Each vial was vortex mixed for at least 1 min then centrifuged at 3500 g for 5 min. An aliquot was removed and filtered through a 0.22 µm syringe filter. The filtrate was transferred into 1.5 mL amber autosampler vials, capped and placed into the sample manager at 8°C. An injection volume of 2.5 µL was used for the analysis.

4.6 SAMPLE PREPARATION (PLASMA)

The solid phase extraction methodology was optimised to ensure that the highly lipophilic cannabinoids were effectively recovered from the plasma using HLB mixed mode extraction cartridges. Despite the vendor claiming that the cartridges do not require preconditioning it was found during initial extraction and recovery tests that the use of methanol to pre-wet and remove the trapped air had a positive effect on the extraction efficiency of the cartridges. Proper preconditioning with acidic aqueous phase also improved the extraction of the lipophilic compounds. The sample flow rate during the extraction process was assessed and it was confirmed that the

best extraction was achieved at slow flow under gravity and not using vacuum to draw the sample through the cartridges.

Washing with both acidic aqueous phase followed by washes of 10% incremental methanol concentrations showed that minimal analyte elution occurred at less than 40% methanol in the wash solution, however at 50% significant analyte elution could be detected.

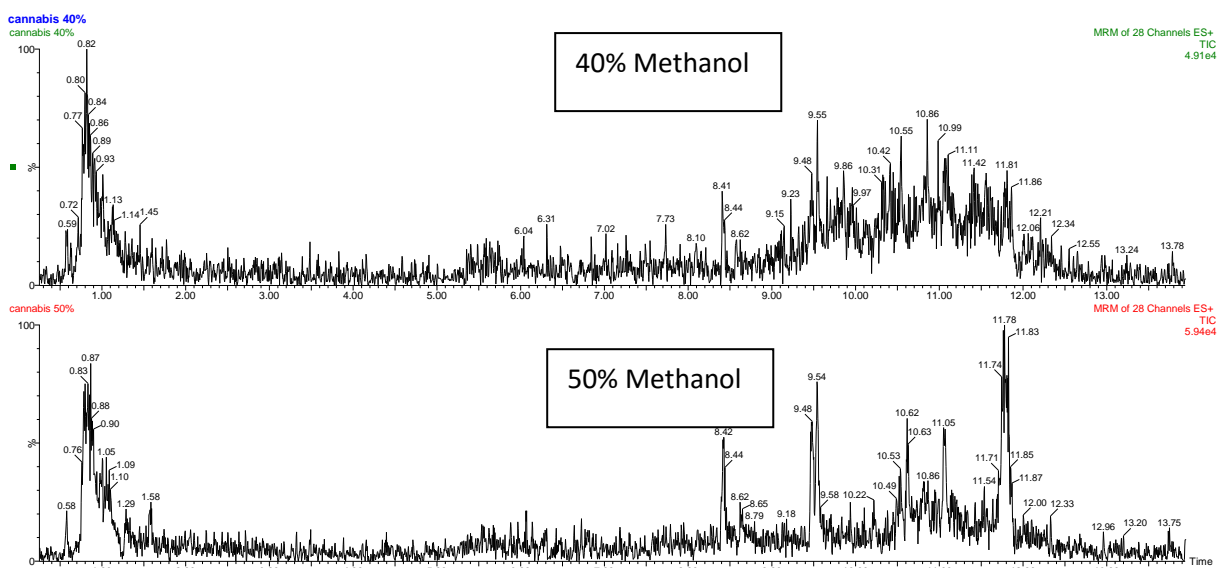


Fig 15: Analysis of the SPE eluent by UPLC-MS/MS to determinate the methanol percentage at which the cannabinoid analytes elute from the Oasis HLB mixed mode SPE cartridges. The elution volume used was 1.0 mL

Optimum conditions for HLB mixed mode SPE extraction of plasma

- Pre-conditioning: 1.0 mL methanol followed by 1mL water – must not run dry.
- Add 1 mL 4% phosphoric acid in DDH₂O and allow to run through before adding 200 μ L undiluted plasma sample.
- Run through under gravity.
- Wash step: 1mL of 40% methanol in DDH₂O
- Dry under vacuum for 5 minutes
- Elute with 400 μ L of 100% methanol
- Dry under nitrogen and reconstitute in 200 μ L Mobile Phase A

The same concentration range of analytes used for urine analysis was made in drug free plasma by spiking purchased blank canine plasma (Onderstepoort, Pretoria) with the analyte standard stock solutions. This concentration range was prepared in triplicate. The prepared standards were extracted using HLB SPE cartridges as described below. In-house prepared controls of 10.0, 50.0 and 100.0 µg/L were used to verify accuracy and for inter-batch verification.

Extraction of the standards, QC controls and positive plasma cannabinoid plasma samples were prepared in the following way: Oasis HLB (Hydrophilic-Lipophilic-Balanced Copolymer) cartridges were conditioned with 1.0 mL methanol followed by 1.0 mL deionized water. One millilitre of 4% phosphoric acid was passed through the SPE followed by 200 µL of plasma accurately spiked with 50 µL internal standards stock solution and run through under gravity. Centrifuged at 8000 *g*. Add wash step using 1.0 mL of 40% methanol was then run through the cartridge under gravity and allowed to run dry. Elution of the analytes was performed twice using 200 µL of 100% methanol each. The eluent from both elution steps was collected into a test tube and evaporated to dryness under nitrogen. The residue was dissolved in 200 µL mobile phase A and aliquot to autosampler vials. Injection volume of was 2.5 µL was used for the analysis.

4.7 STATISTICS

All raw data was analysed statistically. This included all the data needed for validation of the methods and the raw data prior to and after integration. The data was analysed using the built in statistical functions of MassLynx 4.1 that determines: accuracy, precision and performs the linear regression calculation for the best fit formulas for the calibration curves as well as the correlation coefficient and coefficient of determination.

Validation of the method was according to the current validation process performed in Esoteric Science Laboratory of AMPATH Laboratories in Centurion Pretoria which is an ISO 15189 certified laboratory.

Results obtained from the mass spectrometer were analysed by the MassLynx 4.1 software package with all the appropriate add-ons.

The validation process follows the guidelines as set out by FDA (58) and the ICH (59) for analytical methods for the analysis of drugs and for forensic samples. For method validation three triplicate eight-point calibration curves of each component were analysed on the same day and on three separate days to determine the following:

Validation Parameters Studied:

1. Linear dynamic range
2. Linearity
3. Calibration uncertainties
4. Limit of detection (LOD)
5. Lower limit of quantification (LLOQ)
6. Accuracy (No CRM or Consensus CRM)
7. With-in run precision and repeatability (imprecision)

Validation Criteria

1. Working range of the standards: 0-100 µg/L
2. Number of standards: n = 8 plus a blank
3. Blank and double blank must be included
4. Linearity must be proven
 - Correlation coefficient (r) should be close to 1
 - Decision rules:
 - If $F_{\text{calc}} < F_{\text{crit}}$ – accept H_0 (not linear)
 - If $F_{\text{calc}} > F_{\text{crit}}$ – accept H_1 (linear)
 - If $t_{\text{calc}} < t_{\text{crit}}$ – accept H_0 (not linear)
 - If $t_{\text{calc}} > t_{\text{crit}}$ – accept H_1 (linear)
5. Calibration uncertainties must be determined and evaluated.
 - S_b and $S_a < S_{y/x}$ = good precision
 - $S_b < S_a$ = working range wide enough
 - S_a/S_b = positive high value indicate enough standards close to the blank (lowest standard)
6. LOD has to be determined
7. LLOQ has to be determined
8. Calibration sensitivity $b \neq 0$
9. Accuracy
 - %DV < 20% (Reference total allowable error data base)
 - Decision rules:
 - If $t_{\text{calc}} < t_{\text{crit}}$ – accept H_0 (no difference between target and experimental mean)
 - If $t_{\text{calc}} > t_{\text{crit}}$ – accept H_1 (difference between target and experimental mean)
10. With-in run precision and repeatability (imprecision): < 20%

4.8 ANALYTICAL DATA AND METHOD VALIDATION

After initial tuning of the mass spectrometer parameters these were again optimised with respect to the tuning parameters at the elution conditions of the different standards from the chromatographic system with changes introduced for the gradient, different solvents and different

stationary phases which were re-optimized to give the best separation in as short a time as possible while retaining a relatively rapid turnaround time from injection to injection.

These assays were tweaked while using mixed analyte standards and were aimed at achieving the best resolution between closely eluting critical pairs. It was found that using high solvent percentages in the initial starting conditions resulted in poor analyte resolution despite the advantage of faster elution times. With the optimised conditions that were regarded as a good compromise there were still two analytes that partially co-eluted and these were 11-hydroxy- Δ^9 -THC and 11-Nor-9-carboxy- Δ^9 -THC-COOH together with the deuterated internal standard of the later analyte, 11 -Nor-9-carboxy- Δ^9 -THC COOH-D9.

The chromatographic peaks were well resolved and generally gave sharp peaks with minimal tailing although the natural cannabinoids did appear to show some degree of tailing that was not evident for the synthetic cannabinoids.

The first eluting peaks only eluted after seven minutes, which was after approximately half the run time. What was of interest is that the synthetic cannabinoids appeared to show greater sensitivity, probably due to more efficient ionization but that they mostly eluted before the natural cannabinoids. Figure 15 shows a typical chromatogram of the cannabinoid standards and the most prevalent metabolites for which standards were available. Note the co-elution of the analytes at about 10.1 minutes and the general higher sensitivity for the synthetic cannabinoids. All analytes were present at a concentration of 10 $\mu\text{g/L}$ and were spiked into a purchased drug free blank urine sample. The lower expanded chromatogram shows the area of co-eluting analytes where the deuterated standard shows some separation from the non-labelled analogue.

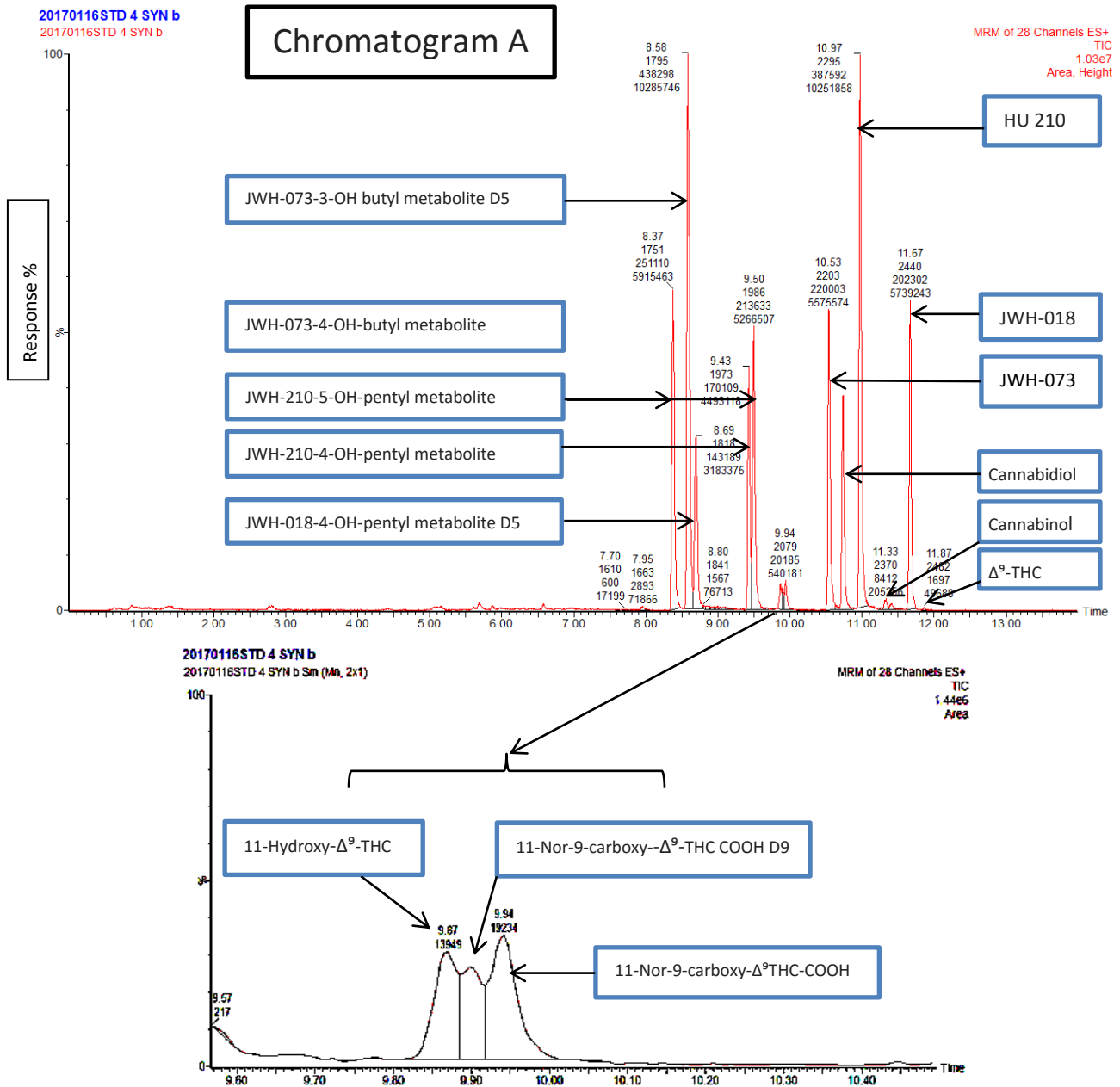


Fig 15: Total Ion Chromatogram (TIC) of a mixed natural and synthetic cannabinoid standard in urine using a Cortecs UPLC C18 2.1 x 100 mm 1.6 μm column at a concentration of 10 μg/L each. Chromatograph A: Represent the TIC all the cannabinoids in Table 2 in the UPLC-MS/MS. The enlarged portion of the chromatogram shows the co-elution of 11-hydroxy-Δ⁹-THC, 11-Nor-9-carboxy-Δ⁹-THC COOH and 11-Nor-9-carboxy-Δ⁹-THC COOH-D9 between 9.8 and 10 minutes.

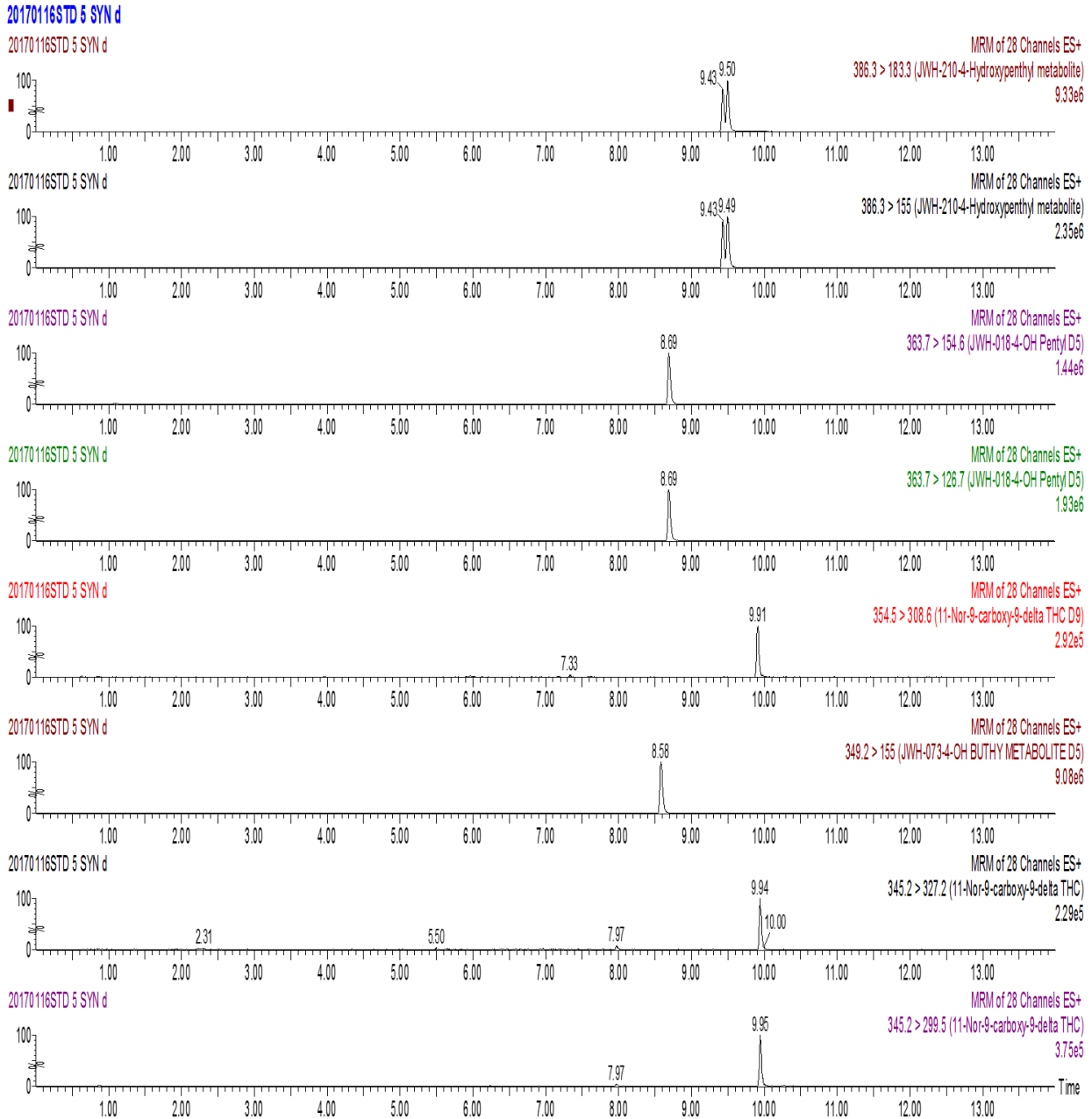


Fig 16: Extracted Ion Chromatograms (XIC): individual SRM transitions of synthetic cannabinoids including THC-COOH metabolites all at a concentration of 10 µg/L in urine.

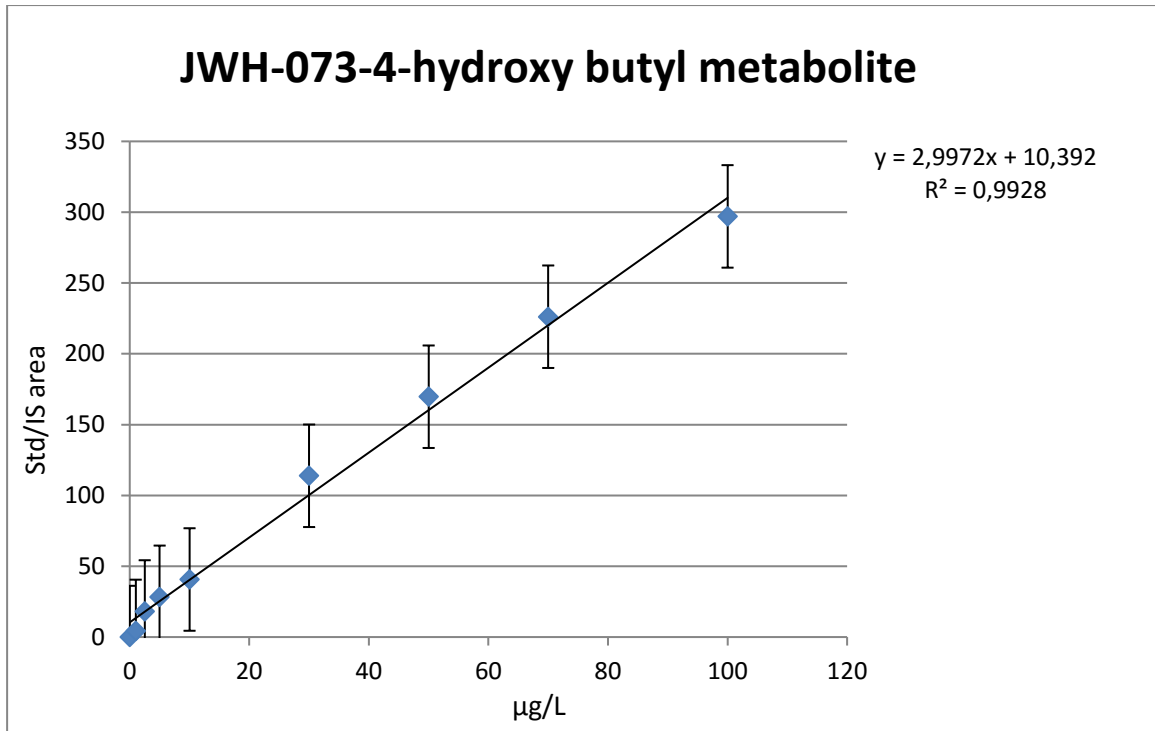


Fig 17: Calibration curve for JWH-073-4-hydroxy butyl metabolite in the working range 0 – 100 µg/L.
n = 3

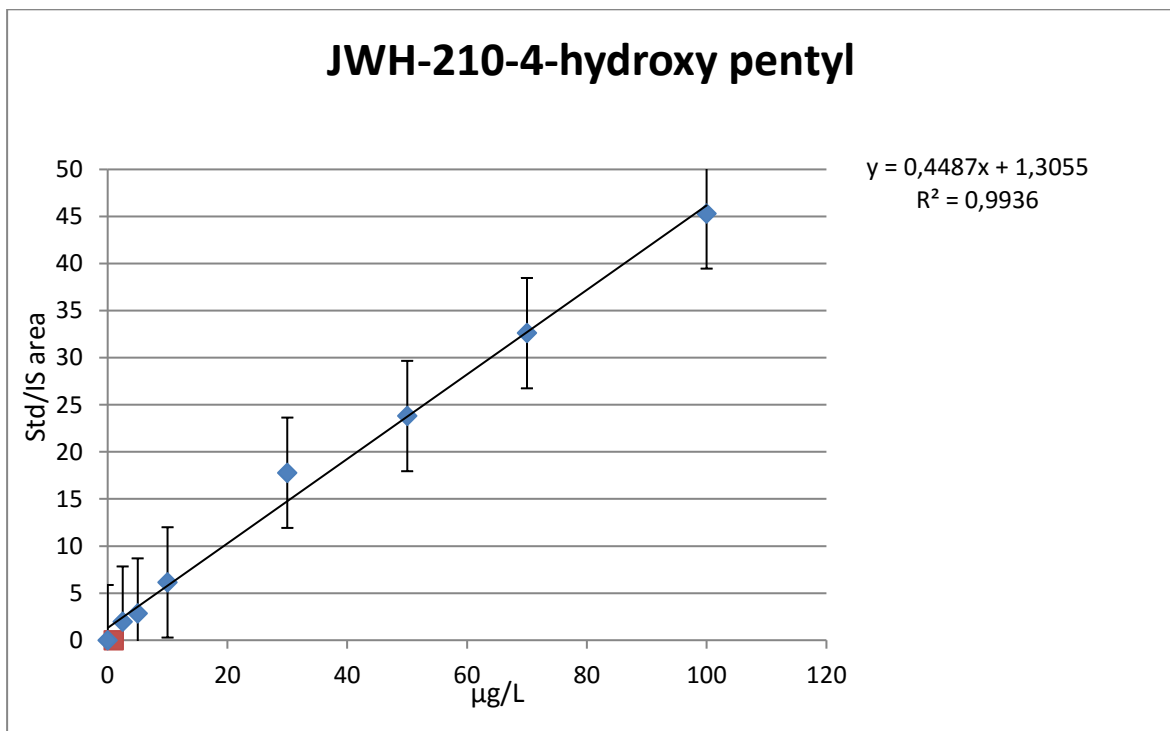


Fig 18: Calibration curve for the JWH-210-4-OH pentyl metabolite working range 0 – 100 µg/L.
n = 3

Table 3: Summary of the regression of the metabolites of the synthetic cannabinoids in urine.

Component	Slope b	Intercept a	r ²	Calibration Uncertainty(Sy /x)	Slope Uncertainty Sb	Intercept Uncertainty Sa	Sa/Sb	LOD	LOQ
JWH-073-4-Hydroxy butyl metabolite	0.0366	0.0390	0.998	0.0345	0.00049	0.0160	32.5	1.3	4.4
11-Hydroxy-THC	0.019	0.0400	0.979	0.109	0.0011	0.054	48.0	8.7	29.0
11-Nor-9-carboxy-9-delta THC	0.060	-0.0210	0.997	0.1082	0.0011	0.053	48.0	2.7	8.9
JWH-210-4-Hydroxy pentyl metabolite	0.006	0.0005	0.999	0.0056	0.00008	0.0026	32.5	1.2	3.9
JWH-210-5-Hydroxy pentyl metabolite	0.011	0.0098	0.998	0.0211	0.00021	0.0093	45.3	2.6	8.4

The values of Sb and Sa are smaller than Sy/x indicating good precision. If Sb < Sa indicates range of calibration is wide enough and Sa/Sb with high values indicate that standards are close to blank.

Table 4: The values for the intercept (a) and slope (b) and the 95% confidence level of each for the major metabolites in urine.

	JWH-073-4-hydroxy butyl metabolite		11-Hydroxy- Δ^9 -THC		11-Nor-9-carboxy- Δ^9 -THC COOH	
Intercept (a)	0.039±0.039	0.0004<a<0.079	0.040±0.133	-0.093<a<0.173	-0.021±0.131	-0.152<a<0.109
Slope (b)	0.036±0.001	0.035<b<0.038	0.019±0.003	0.016<b<0.022	0.060±0.0027	0.058<b<0.063
95% CL	JWH-210-5-hydroxy pentyl metabolite		JWH-210-4-hydroxy pentyl metabolite			
Intercept (a)	0.009±0.025	-0.012<a<0.032	-0.0005±0.0067	-0.0069<a<0.0058		
Slope (b)	0.011±0.0005	0.011<b<0.012	0.006±0.0002	0.006<b<0.007		

Table 5: Significant linear if: F-calc > F-crit. If T-calc > T-crit correlation significant linear

Component	F calc	F crit	T-cal	T- crit
JWH-073-4-Hydroxy butyl metabolite	5477.74	5.99	67.56	2.447
11-Hydroxy-THC	276.38		15.18	
11-Nor-9-carboxy-9-delta THC	2937.58		49.48	
JWH-210-4-Hydroxy pentyl metabolite	6811.66		57.34	
JWH-210-5-Hydroxy pentyl metabolite	2881.81		49.70	

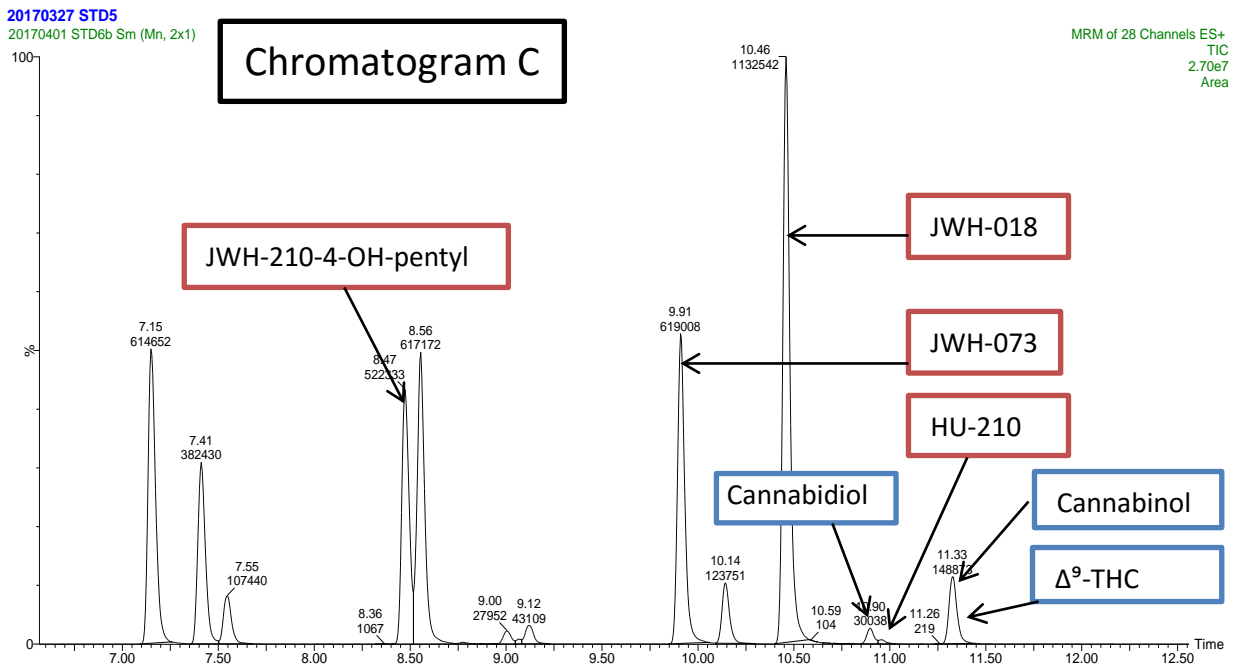


Fig 19: TIC of the separation of cannabinoids on a Cortecs UPLC C18 2.1 x 100 mm 1.6 µm column with all the cannabinoid standards at a concentration of 10 µg/L in plasma. Synthetic cannabinoids are labelled in brown boxes.

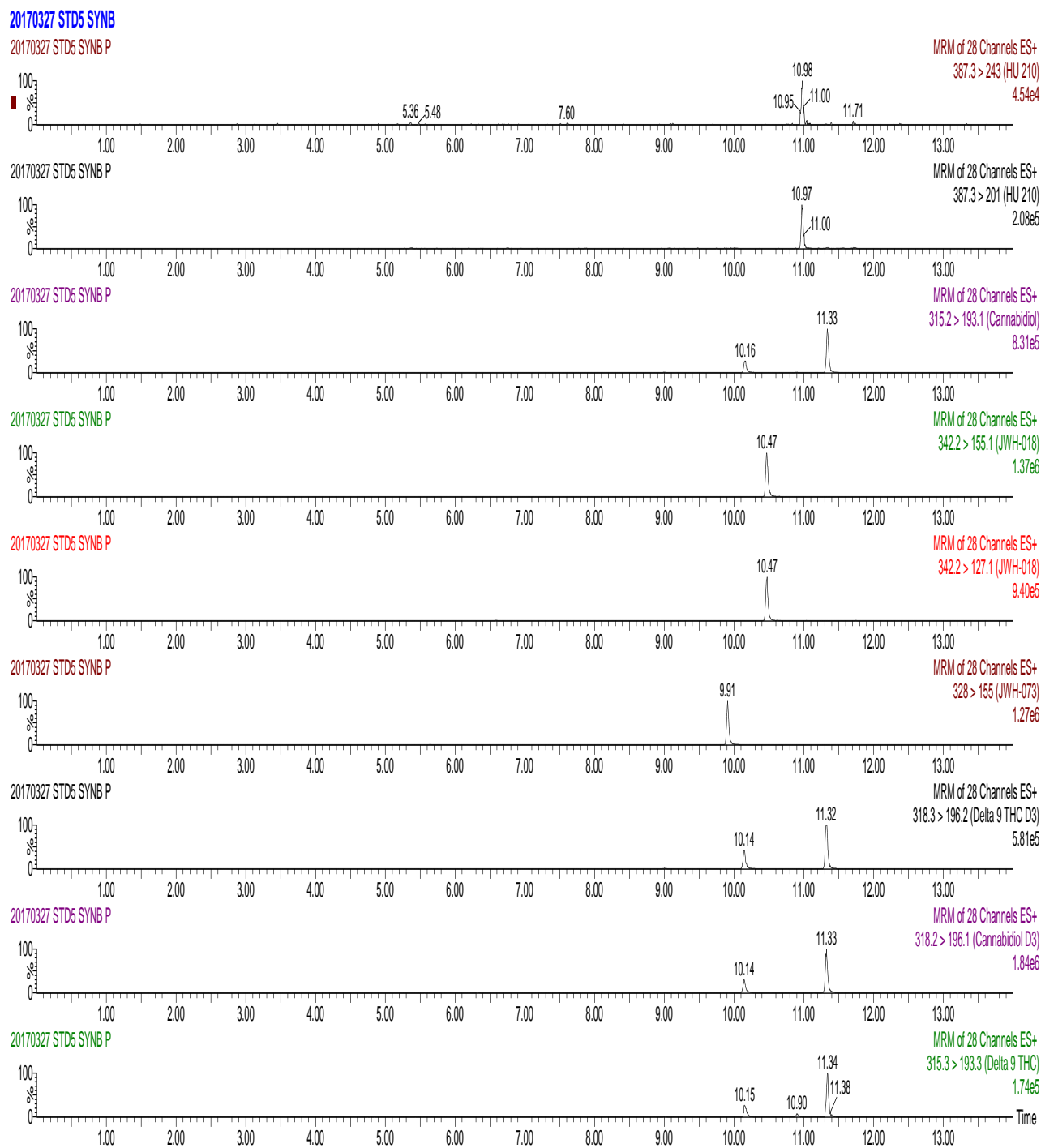


Fig 20: Extracted Ion Chromatograms (XIC): individual SRM transitions of synthetic cannabinoids including THC-COOH metabolites all at a concentration of 10 µg/L in plasma.

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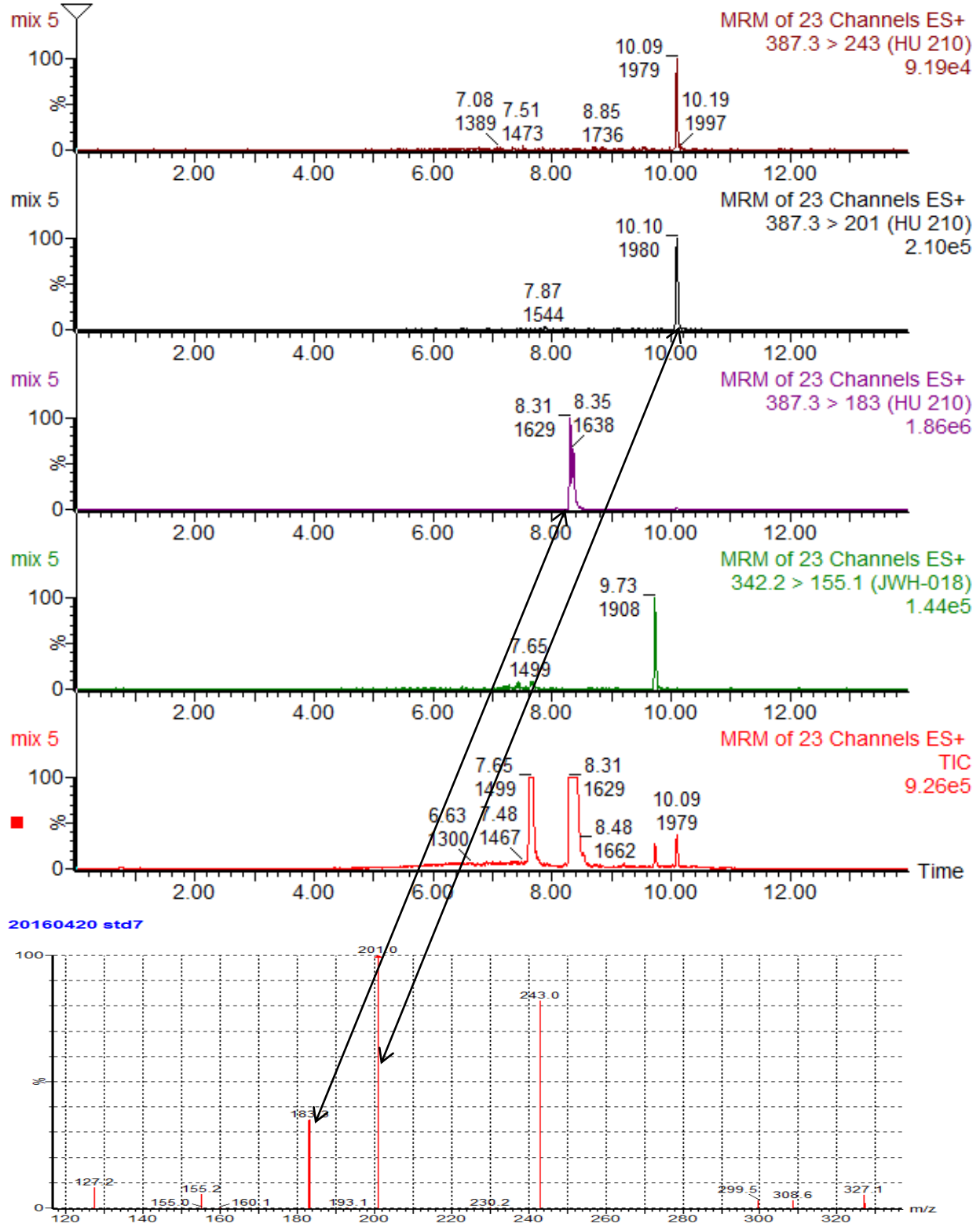


Fig 21: Chromatogram E: Production of HU-210 metabolites. Mass 387.3 > 201/243 with the same RT 10.1 and mass 387.3 > 183 with retention time of 8.31 min.

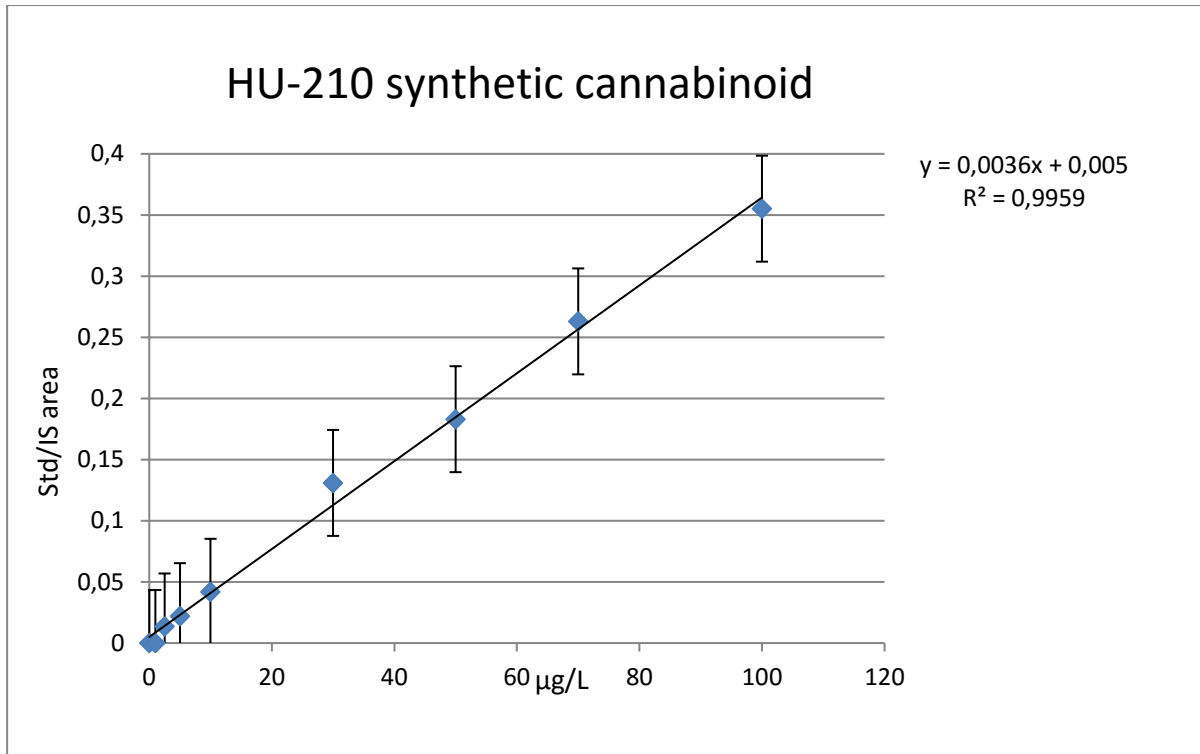


Fig 22: Calibration curve: HU-210 synthetic cannabinoid with concentration range of 0 – 100 µg/L.
n = 3

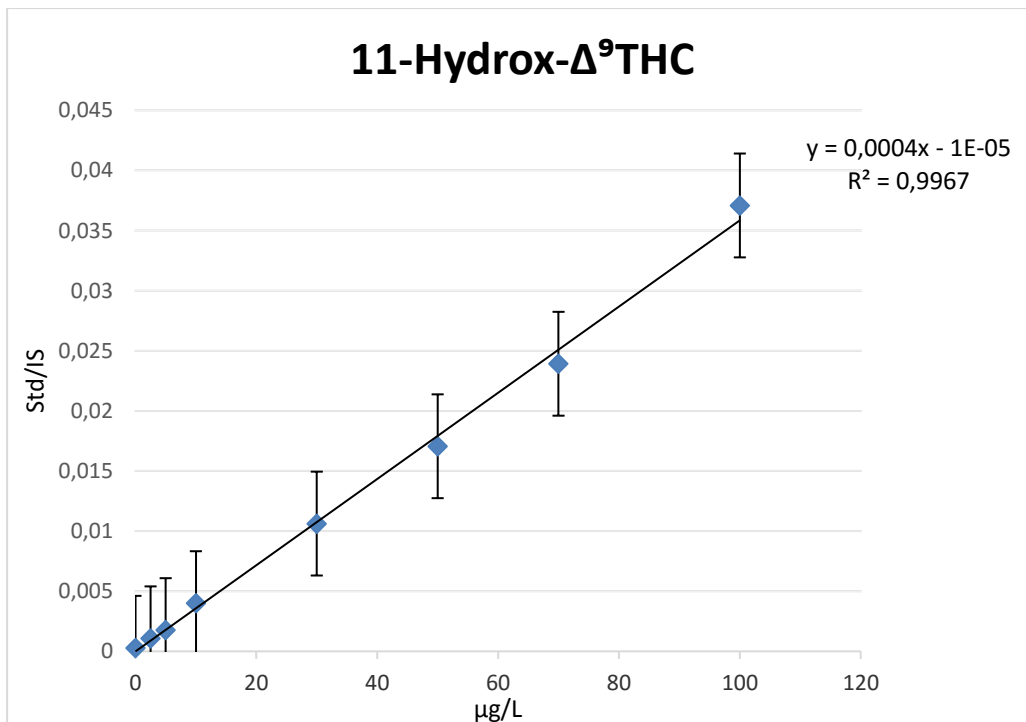


Fig 23: Calibration curve: 11-OH- Δ^9 -THC metabolite concentration range of 0 – 100 µg/L.
n = 3

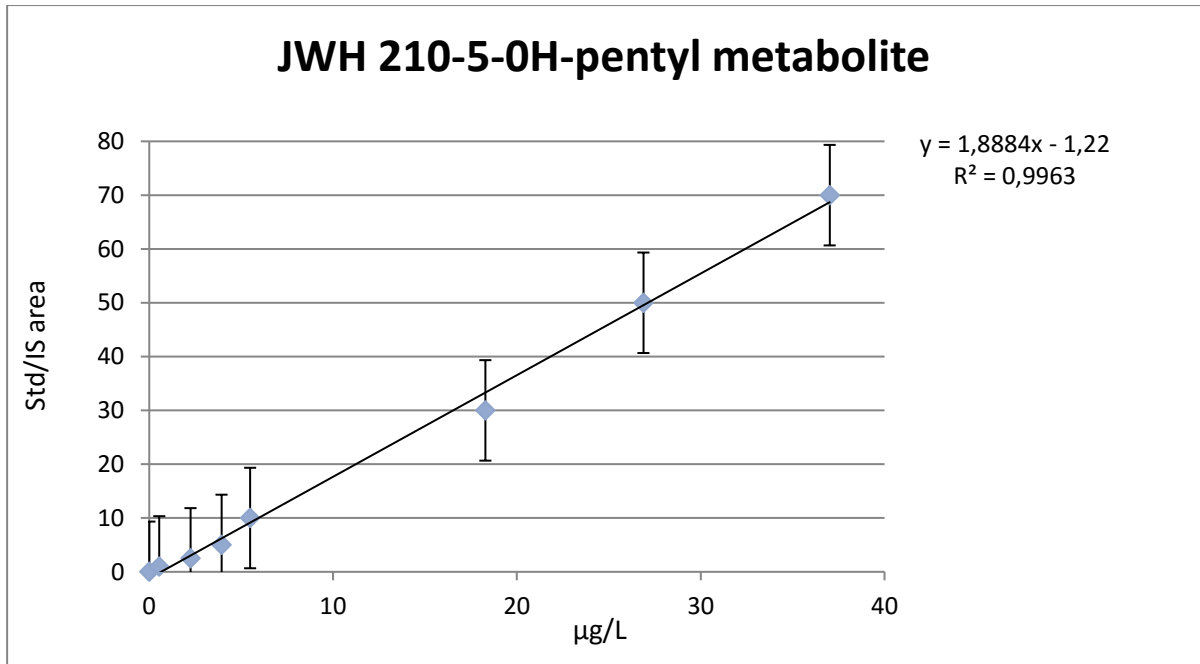


Fig 24: Calibration curve: JWH-210-5-Hydroxy-pentyl metabolite concentration range of 0 – 100 µg/L. n = 3

Table 6: Summary of regression analysis of the synthetic and natural cannabinoids in plasma.

Component	Slope b	Intercept a	r ²	Calibration Uncertainty (Sy/x)	Slope Uncertainty Sb	Intercept Uncertainty Sa	Sa/Sb	LOD	LOQ
Cannabinol	1.102	4.366	0.990	4.66	0.049	2.49	51.14	6.78	22.61
Cannabidiol	0.006	0.015	0.991	0.025	0.0002	0.012	47.87	5.56	18.52
Δ⁹-THC	0.006	0.007	0.998	0.011	0.0001	0.005	45.26	2.40	8.01
JWH-073	15.50	-0.87	0.995	1.538	0.516	0.765	1.483	0.15	0.49
JWH-018	0.0655	0.276	0.977	0.415	0.0044	0.190	42.48	8.71	29.0
HU 210	0.001	0.001	0.999	0.0008	0.00001	0.0004	32.47	1.03	3.46

If Sa and Sb < Sy/x indications of good precision.

Table 7: Significantly linear if: $F_{calc} > F_{crit}$. If $T_{calc} > T_{crit}$ correlation significant linear

Component	F calc	F- crit	T-cal	T- crit
Cannabinol	511.44	5.99	20.23	2.447
Cannabidiol	668.18		23.59	
Δ^9 -THC	3195.17		52.33	
JWH-073	902.06		26.86	
JWH-018	213.66		13.07	
HU 210	8793.1		85.60	

Table 8: 95 % confidence level of intercept (a) and slope (b) of the major components in plasma.

95% CL	Cannabinol		Cannabidiol		Delta 9 THC	
	Intercept (a)	4.36±6.40	-2.04<a<10.77	0.015±0.029	-0.014<a<0.044	0.007±0.013
Slope (b)	1.10±0.12	0.97<b<1.227	0.006±0.0007	0.006<b<0.007	0.006±0.0003	0.006<b<0.006
	JWH-073		JWH-018		HU-210	
Intercept(a)	-0.87±1.967	-2.83<a<1.096	0.277±0.489	-0.213<a<0.766	0.001±0.00	0.0005<a<0.002
Slope(b)	15.50±1.32	14.17<b<16.83	0.066±0.011	0.054<b<0.077	0.001±0.00003	0.001<b<0.001

Table 9: Slope and Y intercept parameters for the natural and synthetic cannabinoids using the optimised method

Component name	Regression line
Cannabidiol	$y=679.8x+1169.4$
JWH-210-4-hydroxy-pentyl	$y=0.4487x+1.3055$
Δ^9 -THC	$y=0.9941x+0.1773$
Cannabinol	$y=0.0872x-0.1037$
JWH-018	$y=0.9837x+0.4861$
JWH-073	$y=3.5722x+4.4138$
HU-210	$y=0.0036x+0.005$
11- Hydroxy-THC	$y=0,0004x-1E-05$
JWH- 073-4-hydroxy-pentyl	$y=2.9972x+10.392$
JWH-210-5-hydroxy-pentyl	$y=1.8884x-1.22$
11-Nor-9-carboxy-9-delta THC	$y=0.060x-0.0211$

4.9 PERCENTAGE RECOVERY AND MATRIX EFFECTS

Determination of percentage recovery was obtained by preparing a calibration set of standards in pure mass spectrometer grade methanol and comparing this curve to the values obtained from a matrix-matched calibration set where the complete process of extraction, drying down and reconstitution is followed.

Spiked calibration sets with the same concentrations were prepared using pooled urine and canine plasma and extracted as described in the relevant methods in Sections 4.5 and 4.6.

The clean methanol-based calibration set was injected in sequence from lowest to highest concentration as a series of “standards”. Both the spiked urine and plasma matrix based series of standards with the same concentrations as the solvent based curve were run as “unknown” samples. Exactly the same amount of Internal standard was added to all three the series of standards to minimize possible injection volume variance and to compensate for recovery losses.

For assessing matrix dependant ion suppression or enhancement during analysis, samples of blank urine or blank serum were extracted as described above and injected and eluted using the optimised chromatographic program while infusing a constant flow of a combination of all the analytes into the flow path post-column but before the mass spectrometer.

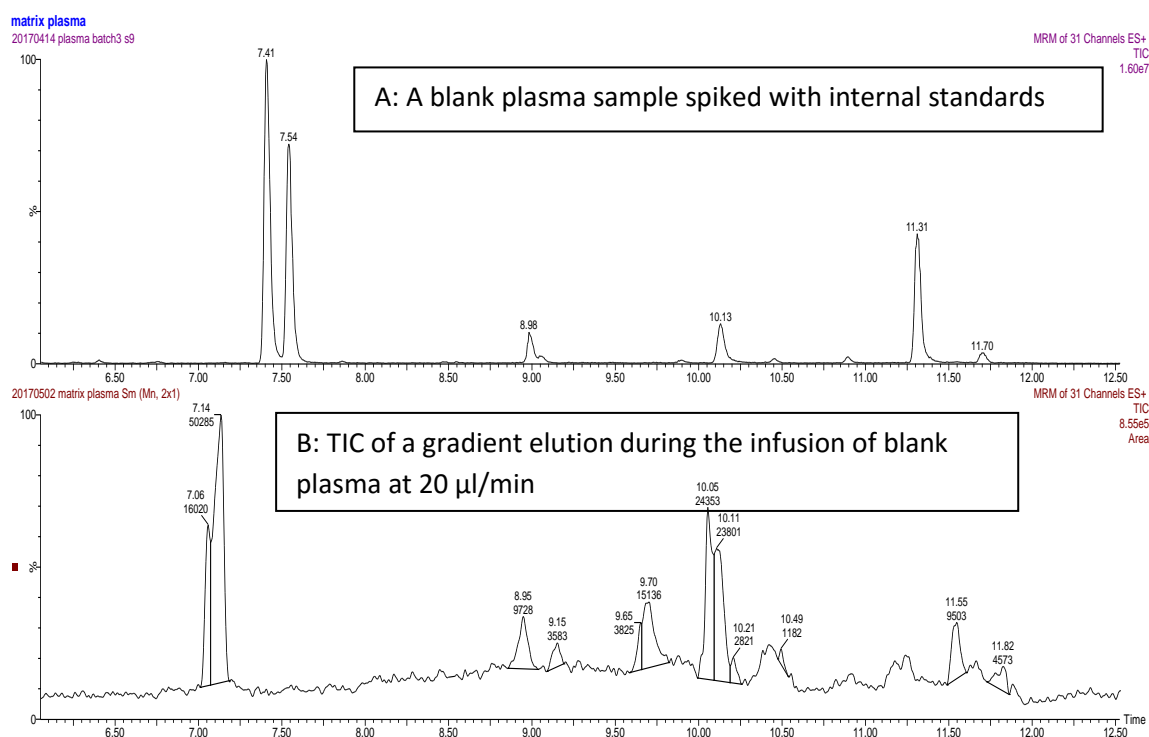


Fig 25: Blank plasma matrix infused during the total run of the gradient shown in Table 1.

Table 10: Summary of the percentages recovery and influence of matrix effects

Component	% Recovery Plasma	% Matrix Suppression Plasma	% Matrix enhancement Plasma	% recovery Urine	% Matrix Suppression Urine	% Matrix enhancement Urine
Cannabinol	58.7	No Matrix effect	No Matrix effect	96.5	No Matrix effect	No Matrix effect
Cannabidiol	113.6	No Matrix effect	No Matrix effect	89.2	No Matrix effect	No Matrix effect
Δ^9 -THC	110.58	No Matrix effect	No Matrix effect	87.3	No Matrix effect	No Matrix effect
JWH-073	82.3	No Matrix effect	No Matrix effect	90.9	No Matrix effect	No Matrix effect
JWH-073-4-hydroxy butyl metabolite	133.8	No Matrix effect	No Matrix effect	88.4	No Matrix effect	No Matrix effect
11-Hydroxy-THC	104.8	No Matrix effect	No Matrix effect	80.0	No Matrix effect	No Matrix effect
11-Nor-9-carboxy-9-delta THC		No Matrix effect	No Matrix effect		No Matrix effect	No Matrix effect
JWH-018	108.5	No Matrix effect	No Matrix effect	86.2	No Matrix effect	No Matrix effect
JWH-210-4-hydroxy-pentyl metabolite	147.3	No Matrix effect	36.1	98.9	No Matrix effect	No Matrix effect
JWH-210-5-hydroxy-pentyl metabolite	107.7	No Matrix effect	7.7	93.9	No Matrix effect	No Matrix effect
HU-210	99.2	No Matrix effect	No Matrix effect	98.2	No Matrix effect	No Matrix effect

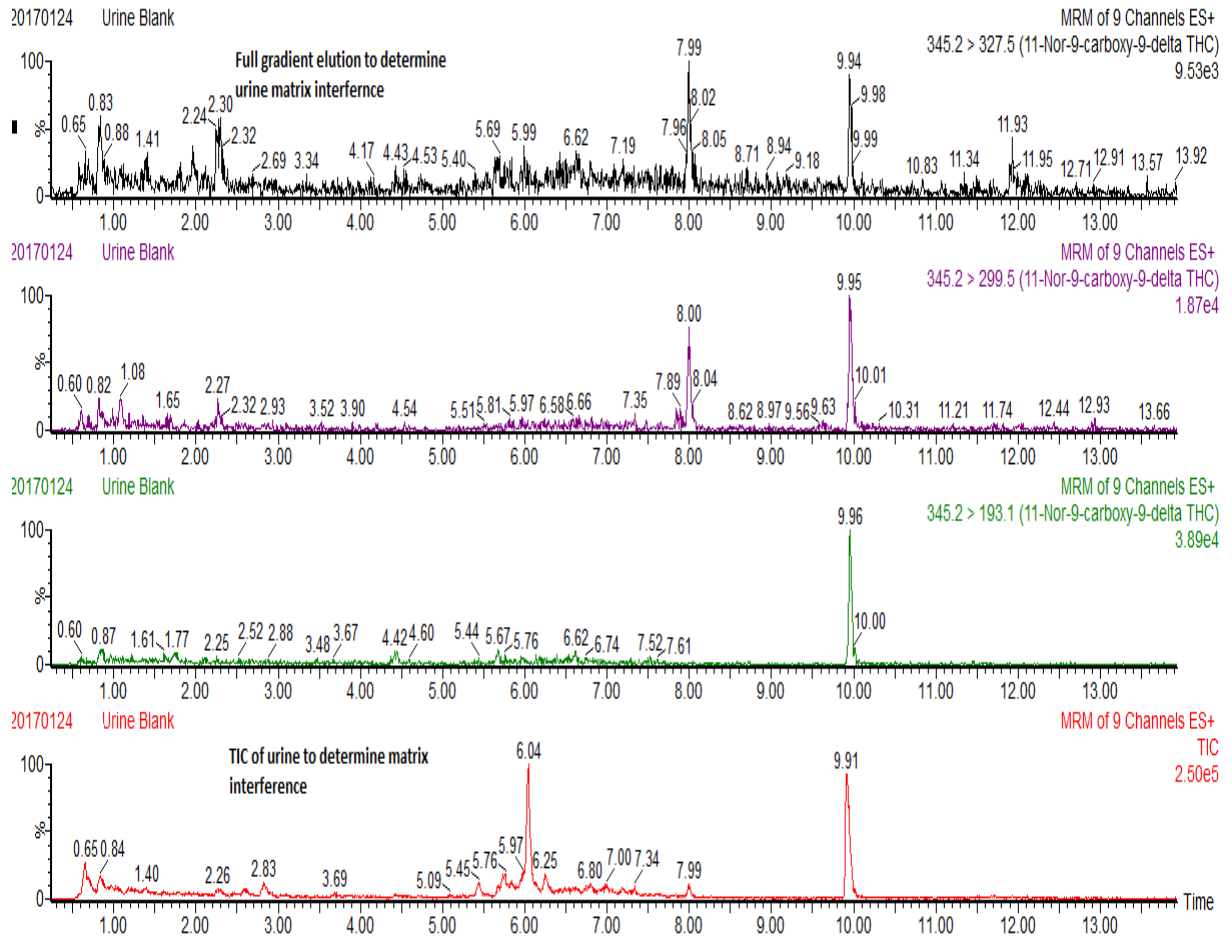


Fig 26: TIC of blank urine matrix infused during the total gradient run with an infusion rate of 20 $\mu\text{L}/\text{min}$.

Table 11: Inter batch accuracy CRM repeatability: Quality control samples, QC low (10.0 µg/L); QC medium (50.0 µg/L) were prepared weekly calibration curves were used to calculate the concentration of the QC's.

Component	Mean	Std error	Min	Max	95% Confidence limit 10 µg/L	95% Confidence limit 50 µg/L
Cannabinol	11.4	0.41	11.11	15.4	2.43	8.72
Cannabidiol	9.85	0.28	8.9	11.8	1.54	3.79
Δ ⁹ -THC	13.66	0.86	8.3	18.7	1.96	3.03
JWH-073	10.43	0.99	6.1	15.9	2.25	3.57
JWH-073-4-hydroxybutyl metabolite	10.24	0.41	11.4	15.4	1.32	1.57
11-Hydroxy-THC	15.79	1.33	8.0	19.7	3.02	4.02
11-Nor-9-carboxy-Δ ⁹ -THC	10.52	2.01	9.0	11.4	2.17	4.55
JWH-018	13.27	1.16	6.6	20.0	2.64	2.83
JWH-210-4-Hydroxypentyl metabolite	12.8	0.98	7.3	17.7	2.21	1.93
JWH-210-5-Hydroxypentyl metabolite	12.8	0.97	7.3	17.7	2.00	2.65
HU 210	19.95	2.42	9.9	35.7	5.49	5.64

4.10 SAMPLE STABILITY

A set of QC standards of 10.0; 50.0 and 100.0 µg/L were analysed at seven day intervals. QC standards were prepared in-house and stored in batches at -20°C, 2-8°C and ambient temperature (22°C) for four weeks which was the duration of the stability assessment.

Extraction efficiencies from samples stored at different temperatures:

The extraction efficiencies and matrix effects were estimated with a set of ten different samples spiked at concentrations of 10.0; 50.0 and 100.0 µg/L without addition of internal standards. Batch A was neat standards made up in 20% methanol in water. Batch B and C were prepared in blank urine and blank plasma respectively. The plasma based samples (200µL) were extracted as described in the methods section. A fixed volume of 50 µL of the mixed internal standards in methanol was added to each sample just prior to extraction.

Table 11: Spiked plasma for stability test at different temperatures. Values are reported in terms of µg/L

	JWH-073.			JWH-018			HU 210		
	10 µg/L	50 µg/L	100 µg/L	10 µg/L	50 µg/L	100 µg/L	10 µg/L	50 µg/L	100 µg/L
	Week1			Week1			Week1		
	-20°C	2-8°C	22°C	-20°C	2-8°C	22°C	-20°C	2-8°C	22°C
Mean	7.33	44.71	77.89	13.66	51.15	86.79	11.65	48.3	99.7
Stdev	2.05	4.24	4.57	2.93	4.13	8.76	2.16	4.28	7.24
95% CI	1.46	3.04	3.27	2.09	2.96	6.27	1.54	3.06	5.18
	Week 2			Week 2			Week 2		
Mean	11.7	52.8	106.7	11.56	52.66	100.58	13.61	53.95	99.94
Stdev	2.05	3.94	9.45	1.89	4.76	11.74	3.31	6.53	8.59
95% CI	1.47	2.82	6.77	1.54	3.10	8.40	2.37	4.68	6.15
	Week 3			Week 3			Week 3		
Mean	9.57	49.34	86.09	11.26	50.38	90.19	16.32	54.16	97.59
Stdev	1.52	5.53	10.95	1.87	3.85	12.45	5.56	6.63	15.73
95% CI	1.24	3.06	7.84	1.33	2.67	9.38	3.98	4.75	11.25
	Week 4			Week 4			Week 4		
Mean	10.49	50.56	100.08	10.21	52.35	93.79	16.1	49.83	103.27
Stdev	2.21	3.57	4.96	1.83	3.83	6.48	4.32	6.04	12.39
95% CI	1.59	2.55	3.55	1.32	2.74	4.64	3.09	4.32	8.86

Table 12: Spiked urine for stability test at different temperatures.

	11-Nor-9-carboxy-9-delta THC			JWH-073-4-OH-butyl metabolite			JWH-210-4-OH-pentyl metabolite		
	10 µg/L	50 µg/L	100 µg/L	10 µg/L	50 µg/L	100 µg/L	10 µg/L	50 µg/L	100 µg/L
	Week1			Week1			Week1		
	-20°C	2-8°C	22°C	-20°C	2-8°C	22°C	-20°C	2-8°C	22°C
Mean	7.33	42.1	77.89	13.66	53.41	83.32	11.01	48.3	99.7
Stdev	2.04	6.84	4.57	2.92	8.36	9.15	1.61	4.28	7.24
95% CI	1.46	4.89	3.27	2.09	5.98	6.55	1.15	3.06	5.18
	Week 2			Week 2			Week 2		
Mean	11.86	48.32	97.67	11.23	50.3	99.43	11.86	54.68	99.23
Stdev	2.61	4.24	9.33	1.13	2.16	5.37	1.85	5.89	14.50
95% CI	1.87	3.04	6.67	0.81	1.55	3.85	1.33	4.22	10.38
	Week 3			Week 3			Week 3		
Mean	14.91	49.26	99.28	11.63	51.31	99.55	12.02	46.49	88.68
Stdev	4.29	4.28	9.45	1.18	2.12	5.27	2.06	2.96	8.07
95% CI	3.07	3.07	6.76	0.85	1.52	3.77	1.48	2.12	5.77
	Week 4			Week 4			Week 4		
Mean	15.75	48.3	99.7	18.8	51.92	101.88	8.52	48.33	80.82
Stdev	4.87	4.28	7.24	29.59	2.18	5.45	0.87	2.18	8.18
95% CI	3.49	3.06	5.18	24.01	1.77	3.90	0.71	2.06	5.85

Table 13: Spiked urine for stability test at different temperatures -20, 2-8, 22°C

	JWH-210-5-Hydroxypentyl			11-Hydroxy THC		
	10 µg/L	50 µg/L	100 µg/L	10 µg/L	50 µg/L	100 µg/L
	Week 1			Week 1		
	-20°C	2-8°C	22°C	-20°C	2-8°C	22°C
Mean	12.39	48.76	82.36	13.35	51.72	106.44
Stdev	2.64	2.87	6.40	2.90	3.28	5.62
95% CI	1.99	1.97	4.31	2.14	2.44	3.78
	Week 2			Week 2		
Mean	12.33	51.53	86.91	12.8	55.78	97.02
Stdev	2.76	4.04	6.29	2.98	4.67	12.37
95% CI	2.07	3.00	4.23	2.24	3.33	8.31
	Week 3			Week 3		
Mean	11.01	52.33	99.7	12.78	51.45	106.44
Stdev	1.99	2.50	8.81	2.50	3.63	5.75
95% CI	1.30	1.74	5.92	1.73	2.26	3.86
	Week 4			Week 4		
Mean	10.12	52.33	99.48	12.24	54.23	103.08
Stdev	1.52	2.29	8.16	2.88	5.33	2.50
95% CI	1.08	1.49	5.48	2.14	3.97	1.68

5 DISCUSSION

LC-MS/MS has proved itself to being superior to GC-MS for analysis of temperature sensitive and polar compounds that show poor volatility and for compounds not easily extracted into water non-miscible organic solvents. LC-MS/MS sample preparation and run times are generally faster, more sensitive and specific for polar compounds that have the ability to ionise easily. This type of compound accounts for many exogenous compounds that are excreted in the urine due to the phase I and phase II metabolism that takes place in the liver. Cleaning of the source and general maintenance for a LC-MS/MS system is more user friendly than for GC-MS systems. If a decrease in sensitivity is observed, cleaning the LC-MS/MS system is generally a routine user maintenance and requires minimum down time. Maintaining an LC-MS/MS system to deliver good and precise results does depend on the user training, skills and the knowledge of how the instrument responds when problems should arise. One drawback for LC-MS/MS is that there are very few compound libraries showing the ionized precursor masses that could also differ when using different buffers due to alternative adduct formation. Small changes in collision energy can alter the ratios of product masses, which could then change the sensitivity of the method. Solvents used in the chromatography mobile phase and source conditions can affect the formation of ion clusters and charge exchange under the atmospheric conditions of ionisation. Other disadvantages are that highly lipophilic and fully saturated carbon compounds are not easily ionised under typical atmospheric ionisation conditions resulting in poor sensitivity for these classes of compounds. In addition, there are several potential matrix effects resulting from the sample matrix and the mobile phase composition. Negative ES ionisation is often used for lipophilic compounds but atmospheric pressure chemical ionization is generally more successful. The sensitivity of negative mode ionization is generally a factor of 5 – 10 less sensitive than positive mode ionization. The combination of analytes eluting together or in combination with interfering contaminants can influence the charge stability of the analytes and influence the sensitivity of the method for different analytes. Despite these limitations, the analyst can build a dedicated library for different compounds using a specific instrument with selected conditions to provide a searchable database of compounds that have specific groups of pharmaceutical compounds when using the optimised separating conditions, and this can be generated using standards where the retention time, precursor ion mass, the ratio of fragment ions collected after different collision energies are used to induced dissociation under defined conditions are collated and used for later analysis. This function database generating function has been incorporated into most LC-MS/MS programs to enable self-generated libraries to be created for later use.

The optimised method developed during this study for identifying and quantitating the synthetic cannabinoids JWH-018, JWH-073 and HU-210 as found in Spice and K2 had an LOD of 8.71 µg/L, 0.15 µg/L and 1.03 µg/L respectively. This is a relatively low LOD and acceptable for raw products.

In the application of the optimised method to real samples, the JWH-018 content of different packets of Spice sourced from local suppliers was tested and found to vary widely, from as little as 0.2% to as much as 3% of the total methanol extractable components. For this testing 5 g of “Spice” samples or adulterated tobacco products were finely ground and extracted with 50 ml 100% methanol filtered through 0.22 µm syringe filter, and 0.5 µl injected. The same ions and retention time gave a positive result. Only positive or negative were reported. No official report was issued

In the experience of the analyst the LC-MS/MS method as optimised in this study was superior to the existing GC-MS methods in terms of time for complete analysis, cost, robustness and ease of use. The same hydrolysis and extraction of urine or plasma as described in the methods section of this study were applied in the analysis of the same cannabinoid analytes by GC-MS, but these methods required further derivatization and sample clean-up steps that were both time consuming and equipment intensive, proving to be a longer and more complicated process. The derivatization is both labour intensive and prone to variability, which also appears to be dependent on the extent of drying after extraction. The method for the GC-MS is also more costly in terms of reagents. The derivatization procedure leaves reactive residues in the sample that are corrosive on the instrument inlet and column. Using the same GC column for other analytes that are thermally stable gives a high background signal following cannabinoid analysis and the derivatization reagents can have negative influence on the stability of different analytes. The GC column needs to be trimmed back or replaced often. The regular cleaning required for the GC inlet and MS interface of the GC-MS means longer down-times are required especially when system venting is required.

The LC-MS/MS instrumentation requires mass calibration at fairly regular intervals and each analyte needs to be “tuned” to find the optimal ionization source conditions and fragmentation conditions to achieve the best sensitivity and selectivity, implying that the analyst needs standards of each drug to be quantitated and must perform initial method development to optimise the instrument for a particular analyte.

Certified reference standards are required for LC-MS/MS tuning to achieve optimal sensitivity and to determine the best operating parameters of the mass spectrometer during the analysis. The

mass spectrometer needs to be mass calibrated at regular intervals. This is a critical step that a ISO-15189 certified laboratory must adhere to. The expiry date, molecular weight and date of calibration must be recorded together with the analyst name. The molecular characteristics and mass of the analytes gives the analyst an indication as to what type of ionization will work best to obtain the maximum sensitivity. Most drugs make use of ES+ or ES- mode. The ES- mode is recommended for compounds that easily lose a proton such as acids and steroids. With nitrogen structures that can easily accept a proton under relatively mild conditions ES+ ionisation is recommended. An example is that cortisol will work well in ES- and chlorpropanamide, a diabetic drug in both ES+ and ES-. Most LC-MS/MS systems are equipped with an ionisation source where the mobile phase is evaporated into small droplets. The inclusion of 0.1% formic acid in the mobile phase (water or acetonitrile) enables effective ionisation within the source. This depends on the capillary voltage, which can be tuned to some extent, in order to influence selective ionisation of the analyte of interest. The analyte most commonly ionizes to form a pseudo-molecular ion structure of $[M+H]^+$ for which a signal can be detected at the precursor analyte mass plus one Da. The degree of ionisation can be optimised by adjusting the cone and the capillary voltages to maximise the signal detected under the elution conditions of the chromatographic system. The chromatographic system elutes the different analytes in the sample from the column at different retention times depending on the mobile phase composition. For separation of a complex mixture of structurally similar compounds like the synthetic cannabinoids it can become difficult to identify and quantitate analytes if different retention times are the only method of identification where non-selective detection techniques are used. When using such a non-selective detection system different types of columns and mobile phases should be tested to determine the best separation to ensure complete resolution of the individual analytes within the mixture. With LC-MS/MS where high analyte selectivity can be achieved, baseline separation of all the individual analytes is not required due to the ability to identify the individual analytes based on their unique mass and fragmentation pattern. However, the effect of co-elution on the ionisation efficiency and the possibility of isobaric analytes must be considered. The LC system is optimised to separate the individual compounds with an optimised gradient to avoid co-elution of analytes as far as possible, yet if this separation cannot be obtained the individual masses can be quantitated based on the mass $[M+H]^+$ of the individual compounds. It is not uncommon that two or more peaks co-elute during complex mixture analysis. Selecting particular masses of interest, the software can extract specific analytes. In LC-MS/MS methods, care should be taken with potential matrix interference that could either suppress or enhance analyte signals for particular analytes. Matrix interferences

can be ignored when samples are run together with stable isotope labelled standards, as the same effect is expected for both the analyte and standard so the effect is compensated for despite running unknown samples from various origins. Many of the matrix effects are compensated for and overcome with the use of multiply deuterated internal standards for each of the different analytes. If these standards are added into the sample at an early stage of sample preparation, they compensate for extraction and sample processing variability and source related matrix effects. For example, for the analysis of cannabidiol the sample is spiked with the stable isotope labelled cannabidiol-D₃.

Plasma and urine are common complex biological matrices where the screening, identification and quantitation of analytes is required and almost always requires extraction or other clean-up procedures to remove interferences and to enrich the analytes of interest. Because of the many different compounds normally found in the urine, even after clean-up using SPE there can still be many interferences present that exhibit extensive matrix effects during the ionisation within the mass spectrometer ionisation source. Matrix interference can increase or decrease the signal for a particular analyte and this can be compensated for by using deuterated internal standards. Although these standards are expensive, only a small amount is used and it is possible to maintain a low price for the overall analysis.

Good laboratory practice (GLP) recommends the use of stable isotope labelled internal standards as surrogate standards which must be added to the sample at the earliest stage possible before initiating the extraction step to compensate for analyte losses during sample preparation.

Poor analyte recoveries or the effect of any interference in the sample are compensated for by the addition of stable isotope labelled internal standard to a certain degree. The internal standard extraction, potential derivatization and instability are equivalent and the ionization is exactly the same as the analyte under the identical conditions within the ionisation source. Calibration curves are thus set up by plotting the peak area ratio of the analyte $[M+H]^+$ relative to the internal standard response on the Y axis versus the analyte concentration on the X axis.

During the separation of the plant derived cannabinoids two of the major constituents cannabidiol and cannabinol, were commonly observed. The cannabinoids in cannabis are generally large non-polar molecules or only slightly polar molecules that have poor ionization under the mild ionization conditions of the LC-MS/MS system. The synthetic constituents including the synthetic cannabinoid HU-210 are generally slightly more polar. The analytical column used in this study,

retained all the non-polar cannabinoid compounds effectively except for the carboxy metabolites of Δ^9 -THC that co-eluted at the mid-gradient region of the elution gradient but still allowed good chromatography.

The chromatographic separation achieved shows that the natural cannabinoids elute slightly later than the synthetic cannabinoids from the column giving an indication of their highly lipophilic nature.

In the TIC when injecting the same concentrations of the natural and synthetic cannabinoids the response for the natural cannabinoids were lower showing poor sensitivity which is related to the lipophilic nature of these compounds. In this study it was observed that the optimal capillary voltage for the natural cannabinoids was 3.5 kV. For all the compounds separated, the optimal capillary voltage was between 2.9-3.5 kV. An average value of 3.2 kV was chosen for the analysis to avoid the complexity of changing the voltages during a method.

Sample preparation for the urine samples followed a simple routine enzymatic hydrolysis following addition of the internal standards, with a final methanol dilution after hydrolysis and centrifuging as clarification step. This resulted in good recovery of cannabinoid metabolites.

The lowest recovery for the natural and synthetic cannabinoids from urine was for 11-hydroxy THC at 80.0%, with a maximum recovery of 98.9% for the JWH-210-4-hydroxypentyl metabolite. The matrix effect of the urine was avoided to a large extent by diluting the matrix with 3 times the volume of 100% methanol containing 0.1% formic acid This was a 5 times dilution of all the samples including standards. In theory 100% could be recovered but in practice this is seldom achieved, and in most routine pathology laboratories the instruments are used daily and are not cleaned after each batch of samples which adds to instrument based technical variability. Human error is also always present, but reducing this error as far as possible always remains a priority of an accredited laboratory.

Sample preparation for the plasma samples required optimisation as the concentrations of the non-metabolised parent drug analytes in plasma are lower than the concentrations found in the urine due to the lack of an accumulation effect in plasma. The analyte extraction was based on a "Hydrophilic-Lipophilic-Balanced" solid phase extraction cartridge. This type of extraction phase was the most efficient choice as it allows for cartridge conditioning, sample loading and washing which is required for plasma sample clean up. This extraction was performed using gravity only, to

allow for effective time for equilibration during extraction. The most important step in the clean-up was optimising the conditions of the washing step. Five different wash solutions were assessed although the activation and conditioning were the same in all cases and a volume of 200 μ L of spiked plasma was loaded onto each cartridge. These wash solutions included 10%, 20%, 30%, 40% and 50% methanol in water solutions and were used to confirm at which methanol concentration the cannabinoid analytes would start eluting from the cartridges. Each wash step was separately collected and analysed using the optimised LC-MS/MS method developed during the study.

This gave an indication of the concentration of methanol in water that eluted the more polar interferences without eluting significant amounts of the cannabinoid analytes to be able to remove most of the lipophilic interferences. A 40% methanol wash step did not elute significant amounts of the cannabinoid analytes but the 50% methanol wash did contain small amounts of the analytes. A slow gravity induced flow of a 1.0 mL wash with 40% methanol was used in the final SPE extraction and wash method. This was followed by an analyte elution step using a small volume of 100% methanol that was found to be most effective if the cartridge was dried completely of the wash solution before attempting to elute the cannabinoids.

The lowest recovery from plasma was 82.3% for JWH-073 and a maximum recovery of 147.3% for the JWH-210-4-hydroxy-pentyl metabolite was observed which indicates a matrix effect that enhances the signal.

The matrix effect for a blank plasma extraction for JWH-210-4-hydroxy-pentyl metabolite was estimated to be an increase in signal intensity of 36.1%.

The synthetic cannabinoid, HU 210 has a proton adduct ionised mass $[M+H]^+$ of 387.3 and JWH-210-4-hydroxy-pentyl metabolite $[M+H]^+$ 386.3 which are only one mass unit different. During the initial optimisation of HU-210 using infusion, three product ions at m/z 201.0, 243.0 and 183.3 were present. The product ion at 183.3 m/z was later identified as not being a product of HU-210 due to retention time discrepancies.

A possible reason why the recovery of the JWH-210-4-hydroxy-pentyl metabolite was higher than 100% is that the calibration curve obtained using the 183.3 ion as a quantifier did not give a linear response. According to literature, HU-210 has 22 different possible metabolites in humans. ES- ionization for this compound was also possible. Using ES- will have a mass of $[M-H]^-$. Identification

of HU-210 was therefore made according to the combination of the precursor mass and the retention time of the standard and the alternative but less sensitive fragment ions.

When making the choice of analysing urine or plasma, the analyst needs to take into account which analytes will be analysed because of the wide range of metabolites that includes the phase II metabolites that would be found in urine only due to the very rapid excretion via the kidneys and includes the glucuronide and sulphate conjugates which are not detected in the plasma. Plasma analysis should give the unchanged precursor analyte in higher concentration for a reasonably short period after dosing.

Identifying the synthetic cannabinoid metabolites in the urine will be of value, not only for confirmation of drug abuse but also for research purposes as it is proposed that several of the synthetic cannabinoids could be used clinically for neurological disorders. Research with respect to the synthetic cannabinoid metabolites in urine will provide valuable information with respect to the drug metabolism and pharmacokinetics of these compounds.

6 CONCLUSION

6.1 CONCLUDING REMARKS

During this study, an LC-MS/MS method was developed and validated for simultaneous determination of the major natural phyto-cannabinoids, their main metabolites as well as three common synthetic cannabinoids and the main metabolites of these in urine samples. The method is based on a simple and efficient hydrolysis procedure and solvent dilution with the use of low sample volume of 0.5 mL for urine and only 200 μ L for plasma. The method shows satisfactory linearity and accuracy for the eight investigated analytes (HU 210; JWH-018; JWH-073; 11-Nor 9 Carboxy- Δ^9 -THC; JWH-073-4-OH-butyl; JWH-210-4-OH-pentyl; JWH-210-5-OH-pentyl and 11-Hydroxy-THC). The LOD values ranged from 1.18 μ g/L to 8.66 μ g/L based on the statistical validation method and could be applied to real case applications where the developed method meets the needs of toxicological analysis and could be applied to routine work flows where mixed use of natural and synthetic cannabinoids is suspected.

JWH-018 and JWH-073 are members of the naphthoylindole group of synthetic cannabinoids, which have been detected in various herbal incenses and smoking blends that can be readily purchased via convenience stores, liquor stores and the internet. The USA DEA has temporarily placed JWH-018 and JWH-073 into Schedule 1 (the highest schedule in USA) of the controlled

Substances Act due to the high potency and unknown long-term effects of these synthetic cannabinoids. These compounds are agonists at the CB1 and CB2 cannabinoid receptors in the body and can produce some severe side effects especially when used without less active competitive cannabinoids.

More research is needed in this area, as few studies for the analysis of these drugs in biological matrices have been published in peer-reviewed scientific journals, and this is the first study to detail quantitative data of whole blood analysis. An accurate, precise and reproducible method of detection of JWH-018 and JWH-073 in human blood by SPE extraction and UPLC-MS/MS has been developed and validated during this study.

Further investigation in the laboratory can include the possible addition of other relevant synthetic cannabinoid compounds, but their identification and quantitative analysis is at present limited by the availability of pure reference material.

6.2 COMMENTS WITH RESPECT TO THE USE OF LC-MS/MS METHODOLOGY

More and more analysts prefer to work with LC-MS/MS, because it is more versatile, can analyse many compounds that are polar, the analytical methods are cost effective and less time consuming than methods run on other instrumentation. The LC-MS/MS is very sensitive, has a wide dynamic range, can be extremely selective and is a robust quantifying system for highly diverse quantitative methods, where the high selectivity can minimize false positive and negative results. It can analyse a diverse range of chemical compounds from inorganic through small molecules through to large biological polymers making it a very broadly applicable technique. Compounds that are more polar than those that can be analysed by GC-MS can easily be analysed by LC-MS/MS. There are also more qualifier fragment ions to identify particular components, despite there not being compound libraries for LC-MS/MS methods.

The technique is more user friendly than many immunology-based systems and the sample preparation is easier to perform. The ability to use stable isotope labelled standards which are the ideal internal and surrogate standards makes this method far more robust than most existing quantitative methods available.

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8 APPENDIX

STANDARD OPERATING PROCEDURES FOR ANALYSIS OF CANNABINOIDS FROM PLASMA SAMPLES

PURPOSE

The major metabolites of THC and the synthetic cannabinoids in urine are 11-NOR- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THC-COOH), JWH-073-4-Hydroxy-Butyl metabolite, JWH-210-4-Hydroxy-Pentyl metabolite, JWH-210-5-Hydroxy-Pentyl metabolite, 11-Hydroxy-THC Total THC-COOH and the synthetic cannabinoid is determined as the free metabolites.

○ SCOPE

This applies to the Esoteric Science Laboratory.

○ RESPONSIBILITY

This applies to the scientist in the Esoteric Analytical laboratory performing the test.

○ REFERENCES

Clinical Chemistry February 2002 vol. 48 no. 2 301-306

W. Heil et al. Reference Ranges for adults and children. Pre-analytical considerations, 2004.

○ DEFINITIONS

JWH-073-4-OH-Butyl metabolite

JWH-073-3- OH-Butyl D5

JWH-018-4-OH-Pentyl D5

JWH-210-4-OH-Pentyl metabolite

JWH-210-5-OH-Pentyl metabolite

11-Hydroxy-THC

11-Hydroxy-THC D3

11- Nor- Δ^9 THC-COOH

11- Nor- Δ^9 THC-COOH D9

LOD Limit of detection

LOQ Limit of quantification

UPLC-MS/MS TQ-s Waters Ultra High performance liquid chromatography, with mass selective detector

DDH₂O Double Deionised water

Formic acid Sigma (Formic acid)

Acetonitrile (Romil Water's)

Formic acid Sigma Aldrich

RT Room temperature

○ DOCUMENTATION

MF-aaa-xxx

PROCEDURE AMENDMENT FORM.

MF-aaa-xxx
LF-aaa-xxx
MF-aaaM-xxx

DISTRIBUTION LIST.
DOCUMENT ACKNOWLEDGEMENT FORM.
AMENDMENT HISTORY FORM

○ ACTIONS AND PROCEDURE

○ PRINCIPLE:

The natural and synthetic cannabinoids are extracted from a plasma sample by a solid phase extraction step to reduce interferences and give a cleaner background with less matrix effect. Oasis HLB cartridges were activated with 1.0 mL methanol followed by conditioning with 1 mL deionized water then 1 mL 4% phosphoric acid. A sample volume of 200 µL plasma to which 50 µL internal standard solution was added was slowly percolated through the cartridges then washed with 1 mL 4% phosphoric acid followed by 1.0 ml of 40% methanol and drying before eluting the analytes with 100% methanol.

○ PERFORMANCE CHARACTERISTICS:

Refer to method validation.

○ SPECIMEN:

- Patient preparation: None
- Type: 2 X 5 mL EDTA blood tubes spun down within 30 minutes of collection.
- Handling condition: Once received samples are centrifuged and plasma is stored frozen at -18°C.
- Sample volume: Max volume of plasma not more than 2 mL
- Minimum volume: 1 mL

○ EQUIPMENT AND MATERIALS:

Waters UPLC MS/MS TQ-S

Analytical column: Waters Aquity Cortecs UPLC C18 2.1 X 100 mm 1.6µm, Part Nr 186007095

Eppendorf centrifuge tubes 1.5 mL

UPLC autosampler vials. 1.5 mL

Variable Pipette: 1 – 5 mL

Variable Pipette: 100 – 1000 µL and 20 – 200 µL

Vortex mixer

2 X 1 litre volumetric flask

○ MATERIALS AND STORAGE:

All certified reference standards are stored in freezer at -18 °C

β-Glucuronidase Catalogue Nr. G7017, Sigma–Aldrich, fridge 2-8°C

Deionised water, RT

Formic acid, UPLC grade, RT, Acid cabinet

Acetonitrile UPLC grade Ultra-pure reagent, RT

Methanol UPLC grade at RT

Blank plasma was purchased from Onderstepoort Veterinary Laboratories

○ PREPARATION:

Mobile buffer A: (1 Litre)

Double Deionised H₂O to a 1 L volumetric flask. Add 1 mL Formic acid and fill up to the mark with DDH₂O and mix. Stable for 1 month at room temperature.

Mobile buffer B: (1 Litre)

Add 900 mL 100% acetonitrile to a 1 L volumetric flask and add 1 mL Formic acid then fill to the mark with acetonitrile and mix. Stable for 1 month at room temperature.

○ **CALIBRATION:**

Preparation of standards

Standards:

The certified standards ampoules are purchased from Cerilliant, and kept in a freezer at -18 to -22°C. All CRM concentrations were 100.0 µg/mL

Internal standard (IS):

Take 100 µL of the internal standards: 11-Nor- Carboxy-Δ⁹ THC-D₉, JWH-073-3- OH-Butyl-D₅, JWH-018-4-OH-Pentyl-D₅ and 11-Hydroxy-THC-D₃ and make up with methanol in a 10 mL volumetric flask. Keep in the freezer at -18 to -22 °C prior to analysis.

Final concentration of IS= 1000 µg/L.

Preparation of standard solution A:

Pipette exactly 100 µL of the standard, 11-Nor-9-Carboxy-Δ⁹-THC, JWH-073-4-OH-Butyl metabolite, JWH-210-4-OH-Pentyl metabolite, JWH-210-5-OH-Pentyl metabolite, 11-Hydroxy-THC, into a 10 mL volumetric flask and make up with blank urine.

The final concentration of **A** is 1000 µg/L.

For each standard prepared as described above a series of calibration standards using blank urine was prepared.

Standard curve	Volume µl of working standards	Reconstituted volume (ml)	Final concentration µg/L
Blank	0	10.0	0
Std1	10	10.0	1.0
Std2	25	10.0	2.5
Std3	100	10.0	10
Std4	300	10.0	30
Std5	500	10.0	50
STD6	700	10.0	70
STD7	1000	10.0	100

Storage:

500 µL of each standard is aliquoted into Eppendorf tubes and kept frozen at -18 to -22°C prior to analysis

Stable for 3 months.

Calibration Procedure:

The instrument calculates a calibration curve with every run using the relative response of the analyte and the internal standard against the stipulated calibration range.

○ **Quality control:**

Quality is assured by means of internal quality control samples, two in-house and one commercial control Bio-Rad level 3 for the 11-Nor-9-Carboxy-Δ9 THC. No commercial control is available for the synthetic cannabinoid metabolites.

○ **In-house control: Blank urine is spiked with as follows:**

Controls	Volume µL of working standards	Reconstituted volume (mL:)	Final concentration µg/L
C1	100	10.0	8.0 < 10.0 < 12.0
C2	500	10.0	40 < 50.0 < 60

The lot number of the quality control sample must be noted on each worksheet as well as the relevant reference ranges. Standard deviation of 20%

○ **INSTRUMENTATION CONDITIONS:**

○ **Gradient elution on Waters Acquity UPLC Cortex C18 2.1x100 mm 1.6 µm column (Part no 186007095) according to the following gradient**

	Time (min)	Flow mL/min	% A	%B	Curve
1	0.0	0.250	80	20	1
2	2.0	0.250	80	20	1
3	3.0	0.250	40	60	6
4	8.0	0.250	0	100	1
5	10.0	0.250	0	100	1
	10.5	0.250	80	20	1
6	14.0	0.250	80	20	1

○ **Mass spectrometer settings**

Capillary voltage: 3.2 kV (ES+)

Source offset:	60 V
Source temperature:	150°C
Desolvation temperature:	350°C
Cone gas flow:	150 L/Hr
Desolvation gas flow:	600 L/Hr
Collision gas flow:	0.17 ml/Min
Nebulizer gas flow:	7.0 (bar).

○ **Safety:**

See safety precautions in Medical Safety Data Sheets

PPE: Lab coat and gloves, safety glasses.

Environmental impact: Chemicals are disposed of in a manner not to harm the environment as per LP-ESO-026 Handling, storage and discarding of potentially hazardous material.

○ **Procedure – stepwise:**

1. Draw worksheet: PETCANP
2. Condition the 60 mg SPE Oasis HBL with 1 ml of methanol followed by 1 mL deionized water. Close off the vacuum to the SPE columns
3. Add 1 mL of 4% phosphoric acid, 50 µL of internal standard and 200 µL plasma samples, plasma matched standards or plasma controls. Mix lightly in the top of the SPE using a pipette.
4. Open the valve to the SPE and let the liquid run through under gravity.
5. Wash the column with 1 mL of DD water. Add 1 mL of 4% phosphoric acid and allow to drain before adding a further 1 mL of 40% methanol as a solvent based wash step. Dry the columns under vacuum for ± 5 minutes.
6. Elute the analytes into a clean test-tube with 500 µL 100% methanol twice.
7. Dry the combined eluent under nitrogen. Reconstitute in 1000 µL of 20% methanol in water
8. Vortex mix for approximately 10 seconds
9. Transfer supernatant to a clean 1.5 mL UPLC autosampler vials, cap and inject 2.5 µL onto the UPLC-MS/MS system using method named “Endo syn” and inlet method file “Endo syn inlet”.

○ **Reporting results:**

Reporting Units Format: **µg/L**

Linearity range from 0 – 100.0 µg/L

If patient samples are above the calibration curve of 100 µg/L

Repeat analysis of sample after dilution with the appropriate dilution with 20% methanol in DDH₂O containing the same ratio of IS to sample volume.

Component	LOD µg/L	LOQ µg/L
JWH-073-4-Hydroxy-butyl metabolite	1.31	4.38
11-Hydroxy-THC	8.66	28.8
11-Nor-9-Carboxy-9-Delta-THC	2.66	8.86
JWH-210-4-Hydroxy-pentyl metabolite	1.18	3.93
JWH-210-5-Hydroxy-pentyl metabolite	2.53	8.43

○ **Management**

Reporting format: Above LOQ Positive
Between LOD and LOQ: Trace
Under LOD: Negative

Management of calculations on MassLynx Software platforms:

The software display of the sample set, processing- and report methods must be altered to display “User divisor function 1” and “User factor’s 1 to 3 (Multiplication)”.

Dilution calculations may be programmed into sample sets before a batch of samples.

Any dilutions performed on patient samples must be calculated manually and the calculation shown indicating the raw data, date and be signed off.

Typical chromatograms of the separation of the mixed standards, calibration curves and detail of extracted ion chromatograms for JWH-073 is given on the next two pages.

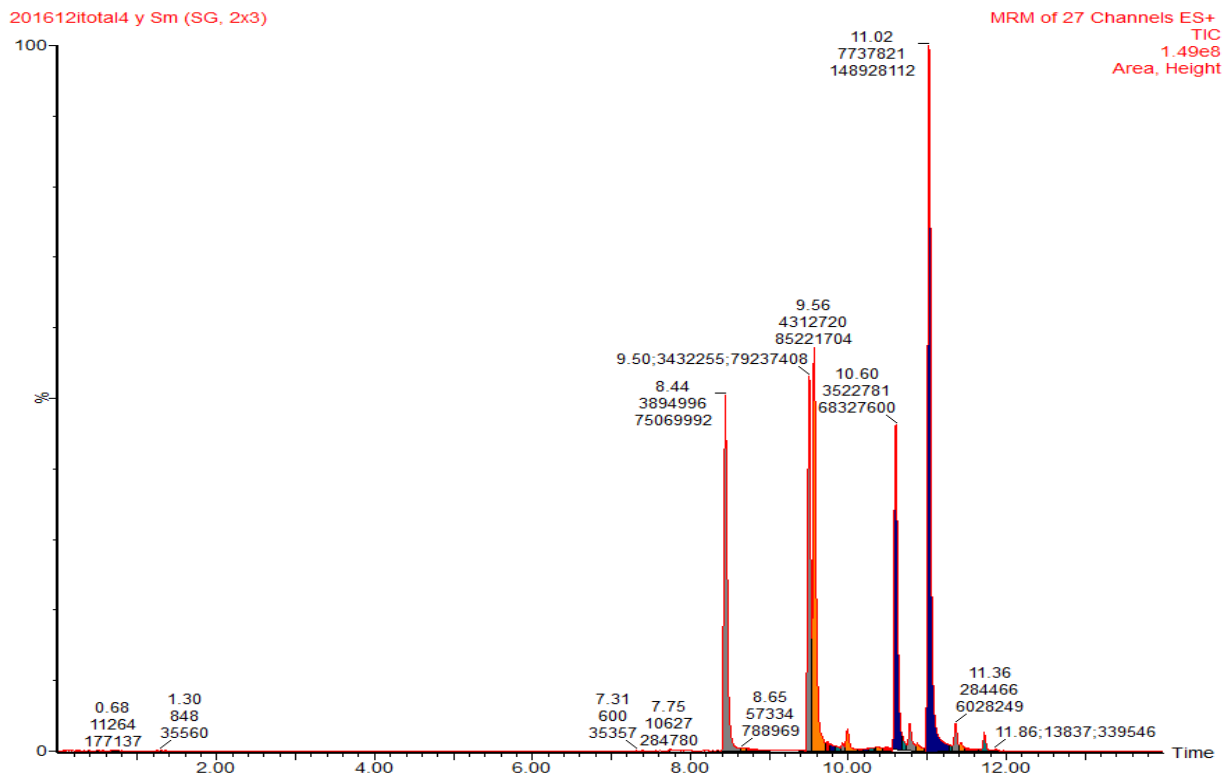


Figure A1-1: TIC of the natural and synthetic cannabinoid standards spiked into blank plasma at a concentration of 30 µg/L for each standard with an injection volume of 2.5 µl.

Compound name: JWH 073
 Correlation coefficient: $r = 0.997109$, $r^2 = 0.994227$
 Calibration curve: $23990.9 * x + 56995.4$
 Response type: External Std, Area
 Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

Calibration curve JWH-073

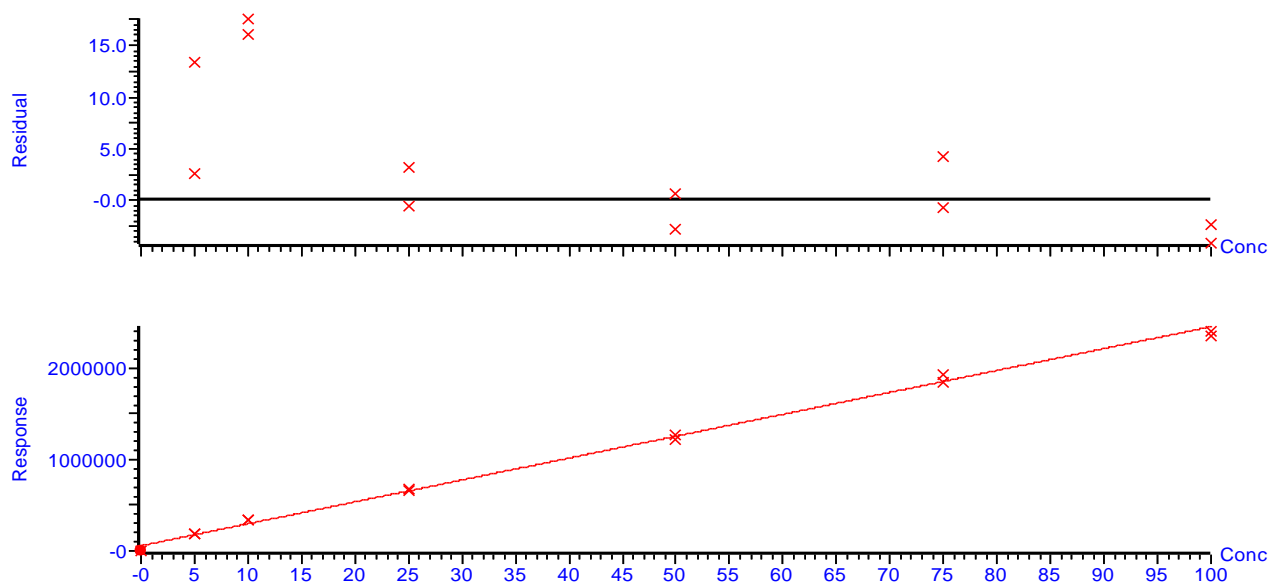


Figure A1-2: Typical percentage error residual calculation for duplicate runs and the calibration curve for JWH-073 spiked into plasma.

syn3

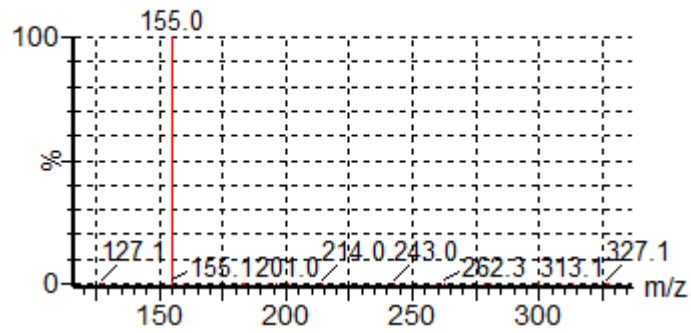


Figure A1-3: The product ion spectrum generated during the infusion of the JWH-073 standard showing the large product ion of m/z 155.

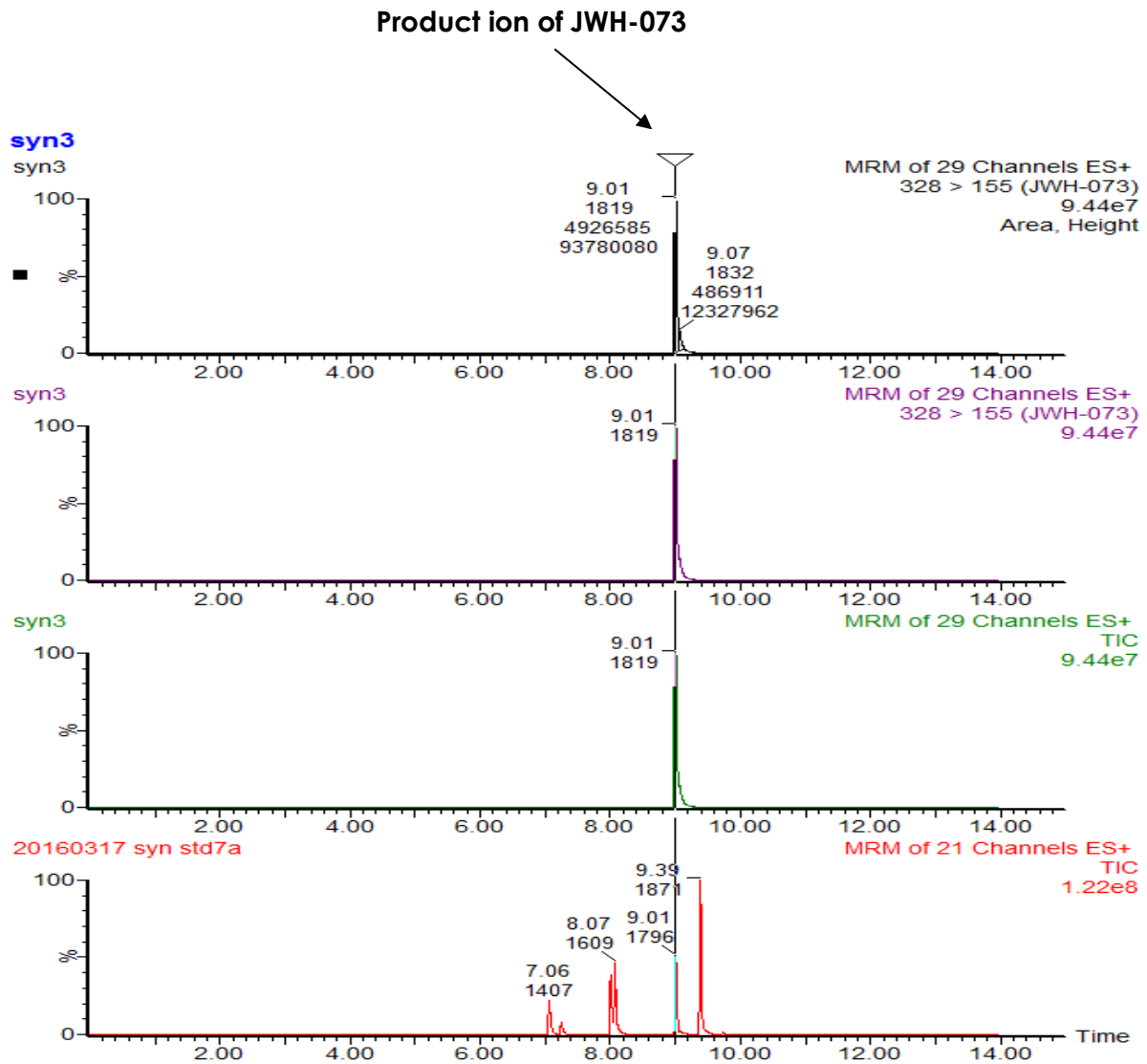


Figure A1-4: Extracted ion chromatogram showing retention time and coincidence of the different fragment peaks for a standard of JWH-073 in a spiked plasma sample

STANDARD OPERATING PROCEDURES FOR ANALYSIS OF CANNABINOIDS FROM URINE SAMPLES

○ PURPOSE

The major metabolites of THC and the synthetic cannabinoids in urine are 11-NOR- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THC-COOH), JWH-073-4-Hydroxy-Butyl metabolite, JWH-210-4-Hydroxy-Pentyl metabolite, JWH-210-5-Hydroxy-Pentyl metabolite, 11- Hydroxy-THC Total THC-COOH and the synthetic cannabinoid is determined as the free metabolites.

○ SCOPE

This applies to the Esoteric Science Laboratory.

○ RESPONSIBILITY

This applies to the scientist in the Esoteric Analytical laboratory performing the test.

○ REFERENCES

Clinical Chemistry February 2002 vol. 48 no. 2 301-306

W. Heil et al. Reference Ranges for adults and children. Pre-analytical considerations, 2004.

○ DEFINITIONS

JWH-073-4-OH-Butyl metabolite

JWH-073-3- OH-Butyl D5

JWH-018-4-OH-Pentyl D5

JWH-210-4-OH-Pentyl metabolite

JWH-210-5-OH-Pentyl metabolite

11-Hydroxy-THC

11-Hydroxy-THC D3

11- Nor- Δ^9 THC-COOH

11- Nor- Δ^9 THC-COOH D9

LOD Limit of detection

LOQ Limit of quantification

UPLC-MS/MS TQ-s Waters Ultra High performance liquid chromatography, with mass selective detector

DDH₂O Double Deionised water

Formic acid Sigma (Formic acid)

Acetonitrile (Romil Water's)

Formic acid Sigma Aldrich

RT Room temperature

- **DOCUMENTATION**

MF-aaa-xxx	PROCEDURE AMENDMENT FORM.
MF-aaa-xxx	DISTRIBUTION LIST.
LF-aaa-xxx	DOCUMENT ACKNOWLEDGEMENT FORM.
MF-aaaM-xxx	AMENDMENT HISTORY FORM

- **ACTIONS AND PROCEDURE**

- **PRINCIPLE:**

The natural phyto-cannabinoid and synthetic cannabinoid glucuronide metabolites that accumulate in urine after excretion by the kidney are hydrolysed to the free unconjugated form using the commercially available β -glucuronidase/arylsulfatase enzyme by incubation in a water bath at 37°C for 8 hours. Thereafter matrix dilution for the urine is done using 0.1% formic acid in methanol, the sample clarified by centrifugation and the supernatant is injected into the UPLC-MS/MS system.

- **PERFORMANCE CHARACTERISTICS:**

Refer to method validation.

- **SPECIMEN:**

Patient preparation:	None
Type:	Urine in glass container
Handling condition:	Once received samples are stored frozen.
Sample volume:	Max volume required is not more than 10 mL
Minimum volume:	1 mL

- **EQUIPMENT AND MATERIALS:**

Waters UPLC MS/MS TQ-S
Analytical column: Waters Aquity Cortecs UPLC C18 2.1 X 100 mm 1.6 μ m, Part Nr 186007095
Eppendorf centrifuge tubes 1.5 mL
UPLC autosampler vials. 1.5 mL
Variable Pipette: 1 – 5 mL
Variable Pipette: 100 – 1000 μ L and 20 – 200 μ L
Vortex mixer
2 X 1 litre volumetric flask

- **MATERIALS AND STORAGE:**

All certified reference standards are stored in freezer at -18 °C

β -Glucuronidase Catalogue Nr. G7017, Sigma–Aldrich, fridge 2-8 °C

Deionised water, RT

Formic acid, UPLC grade, RT, Acid cabinet

Acetonitrile UPLC grade Ultra-pure reagent, RT

Methanol UPLC grade at RT

Blank urine was purchased from Utak laboratories

○ **PREPARATION:**

Mobile buffer A: (1 Litre)

Double Deionised H₂O to a 1 L volumetric flask. Add 1 mL Formic acid and fill up to the mark with DDH₂O and mix. Stable for 1 month at room temperature.

Mobile buffer B: (1 Litre)

Add 900 mL 100% acetonitrile to a 1 L volumetric flask and add 1 mL Formic acid then fill to the mark with acetonitrile and mix. Stable for 1 month at room temperature.

○ **CALIBRATION:**

Preparation of standards

Standards:

The certified standards ampoules are purchased from Cerilliant, and kept in a freezer at -18 to -22°C. All CRM concentrations were 100.0 µg/mL

Internal standard (IS):

Take 100 µL of the internal standards: 11-Nor- Carboxy-Δ⁹ THC-D₉, JWH-073-3- OH-Butyl-D₅, JWH-018-4-OH-Pentyl-D₅ and 11-Hydroxy-THC-D₃ and make up with methanol in a 10 mL volumetric flask. Keep in the freezer at -18 to -22 °C prior to analysis.

Final concentration of IS= 1000 µg/L.

Preparation of standard solution A:

Pipette exactly 100 µL of the standard, 11-Nor-9-Carboxy-Δ⁹-THC, JWH-073-4-OH-Butyl metabolite , JWH-210-4-OH-Pentyl metabolite, JWH-210-5-OH-Pentyl metabolite, 11-Hydroxy-THC, into a 10 mL volumetric flask and make up with blank urine .

The final concentration of **A** is 1000 µg/L.

For each standard prepared as described above a series of calibration standards using blank urine was prepared.

Standard curve	Volume µl of working standards	Reconstituted volume (ml)	Final concentration µg/L
Blank	0	10.0	0
Std1	10	10.0	1.0
Std2	25	10.0	2.5
Std3	100	10.0	10
Std4	300	10.0	30
Std5	500	10.0	50
STD6	700	10.0	70
STD7	1000	10.0	100

Storage:

500 µL of each standard is aliquoted into Eppendorf tubes and kept frozen at -18 to -22°C prior to analysis

Stable for 3 months.

Calibration Procedure:

The instrument calculates a calibration curve with every run using the relative response of the analyte and the internal standard against the stipulated calibration range.

○ **QUALITY CONTROL:**

Quality is assured by means of internal quality control samples, two in-house and one commercial control Bio-Rad level 3 for the 11-Nor-9-Carboxy-Δ9 THC. No commercial control is available for the synthetic cannabinoid metabolites.

○ **In-house control: Blank urine is spiked with as follows:**

Controls	Volume µL of working standards	Reconstituted volume (mL:)	Final concentration µg/L
C1	100	10.0	8.0 < 10.0 < 12.0
C2	500	10.0	40 < 50.0 < 60

The lot number of the quality control sample must be noted on each worksheet as well as the relevant reference ranges. Standard deviation of 20%

○ **INSTRUMENTATION CONDITIONS:**

○ **Gradient elution on Waters Acquity UPLC Cortex C18 2.1x100 mm 1.6 µm column (Part no 186007095) according to the following gradient**

	Time (min)	Flow mL/min	% A	%B	Curve
1	0.0	0.250	80	20	1
2	2.0	0.250	80	20	1
3	3.0	0.250	40	60	6
4	8.0	0.250	0	100	1
5	10.0	0.250	0	100	1
	10.5	0.250	80	20	1
6	14.0	0.250	80	20	1

- **Mass spectrometer settings**

Capillary voltage:	3.2 kV (ES+)
Source offset:	60 V
Source temperature:	150°C
Desolvation temperature:	350°C
Cone gas flow:	150 L/Hr
Desolvation gas flow:	600 L/Hr
Collision gas flow:	0.17 ml/Min
Nebulizer gas flow:	7.0 (bar).

- **Safety:**

See safety precautions in Medical Safety Data Sheets

PPE: Lab coat and gloves, safety glasses.

Environmental impact: Chemicals are disposed of in a manner not to harm the environment as per LP-ESO-026 Handling, storage and discarding of potentially hazardous material.

- **Procedure – stepwise:**

1. Draw worksheet: PETCANU
2. Take 200 µl of each standard, control and blank into a 1.5 mL Eppendorf tube.
3. Add 50 µL β-Glucuronidase/Arylsulfatase to every Eppendorf tube.
4. Close the Eppendorf tube and incubate in a water bath for at least 12 hours (overnight) at 37°C. **Do not shake.**
5. Remove from water bath and aliquot 200 µl into clean Eppendorf tube
6. Add 50 µL internal standard and 700µL of (0.1 % formic acid in methanol) to each tube.
7. Vortex for approximately 10 seconds
8. Centrifuge for 10 min at 10500rpm. Immediately proceed to step 9 samples are not allowed to stand longer than 8 hours; keep refrigerated when not in use.
9. Transfer supernatant to a 1.5 mL UPLC vial, cap and inject 2.5 µL UPLC-MS/MS
10. If patient samples are above the calibration curve of 100 µg/L
11. Repeat with the appropriate dilution.

- **Reporting results:**

Reporting Units Format: µg/L

Linearity range from 0 – 100.0 µg/l

If patient samples are above the calibration curve of 100 µg/L

Repeat after appropriate dilution

Component	LOD µg/L	LOQ µg/L
JWH-073-4-Hydroxy-butyl metabolite	1.31	4.38
11-Hydroxy-THC	8.66	28.8
11-Nor-9-Carboxy-9-Delta-THC	2.66	8.86
JWH-210-4-Hydroxy-pentyl metabolite	1.18	3.93
JWH-210-5-Hydroxy-pentyl metabolite	2.53	8.43

○ **Management**

Reporting format: Above LOQ Positive
Between LOD and LOQ: Trace
Under LOD: Negative

Management of calculations on MassLynx Software platforms:

The software display of the sample set, processing- and report methods must be altered to display “User divisor function 1” and “User factor’s 1 to 3 (Multiplication)”.

Dilution calculations may be programmed into sample sets before a batch of samples.

Any dilutions performed on patient samples must be calculated manually and the calculation shown indicating the raw data, date and be signed off.

Typical chromatograms of the separation of the mixed standards, calibration curves and detail of extracted ion chromatograms for JWH-210-4-Hydroxy-Pentyl metabolite is given in the next three pages.

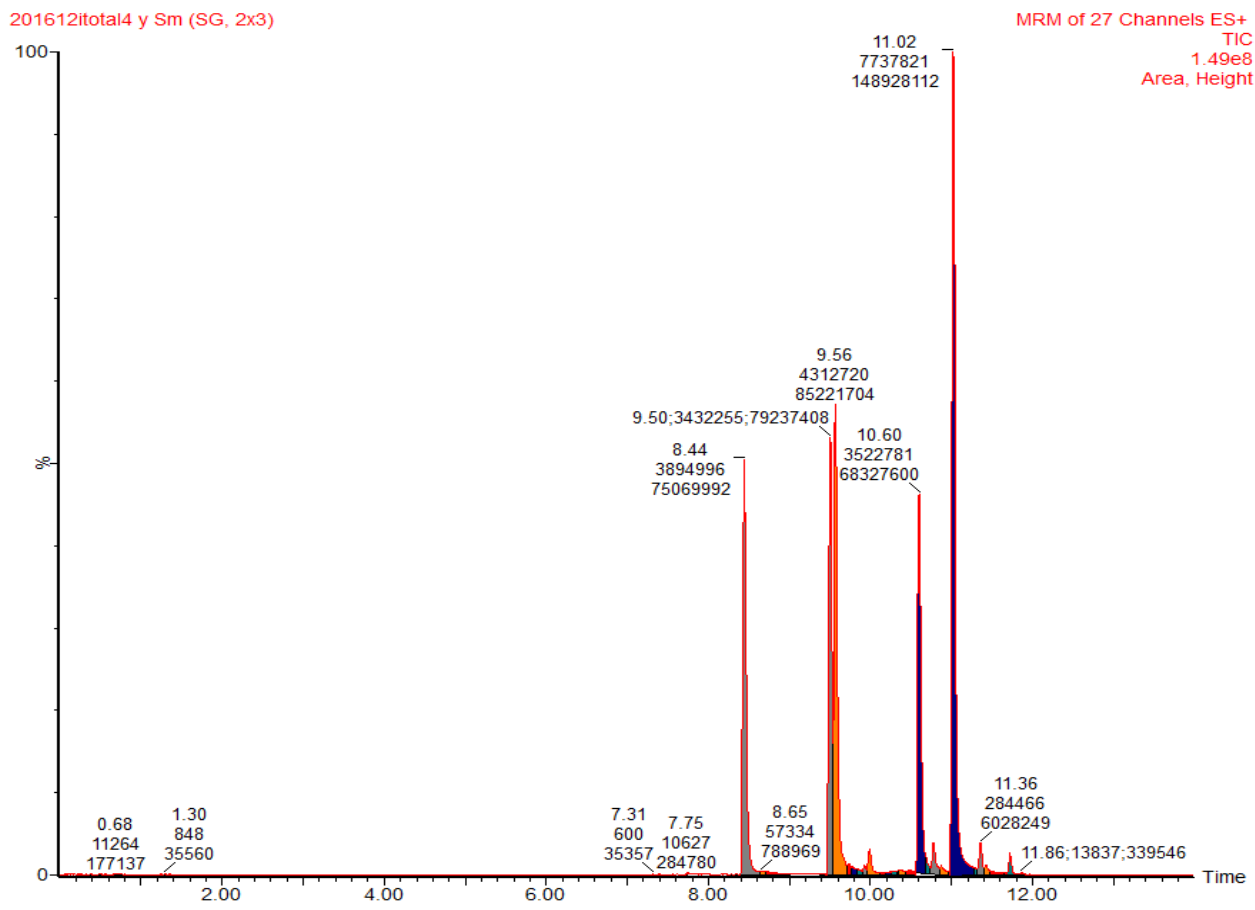


Figure A2-1: TIC of the natural and synthetic cannabinoid standards spiked into blank urine at a concentration of 30 µg/L for each standard with an injection volume of 2.5 µl.

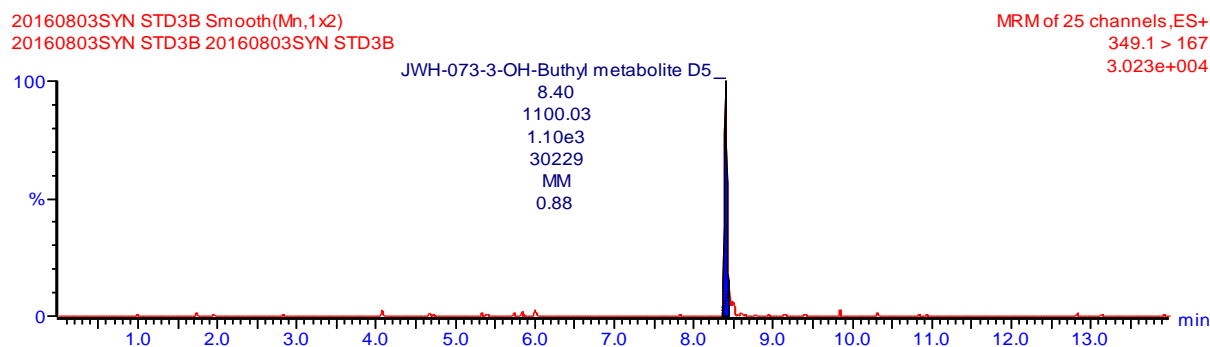
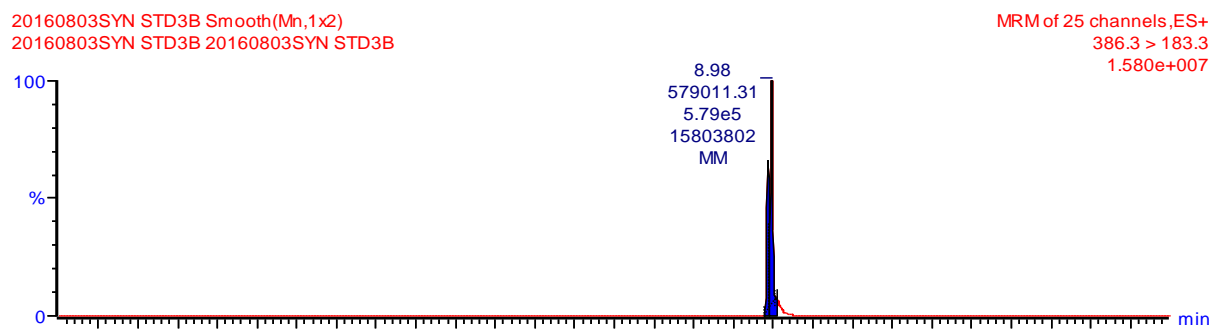


Figure A2-2 XIC of the natural and synthetic cannabinoids in spike urine. Concentration was 30 µg/L with an injection volume of 2.5 µl

Compound name: JWH-073-4-OH Butyl metabolite
Correlation coefficient: $r = 0.991710$, $r^2 = 0.983488$
Calibration curve: $7.82802 * x + 38.1185$
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Include, Weighting: Null, Axis trans: None

JWH-073-4-Hydroxy-butyl metabolite

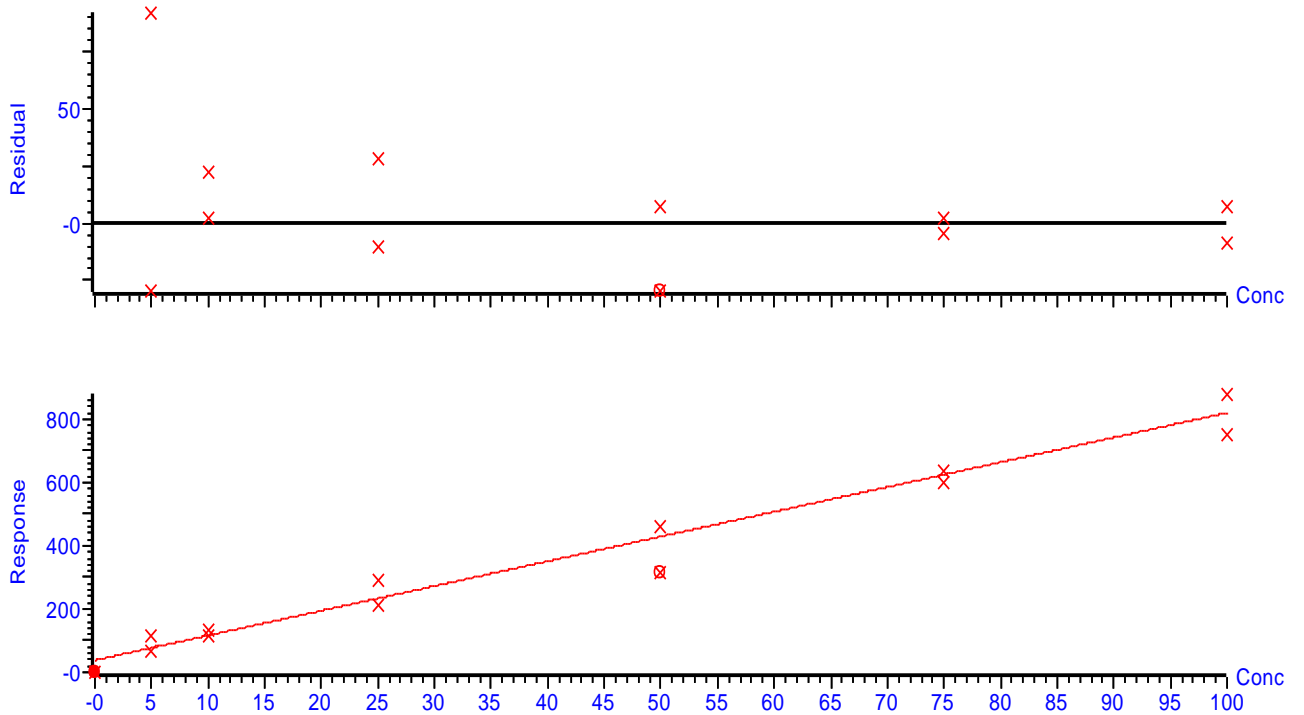


Figure A2-3: Typical percentage error residual calculation for duplicate runs and the calibration curve for the JWH-073-4-Hydroxy-butyl metabolite

syn8

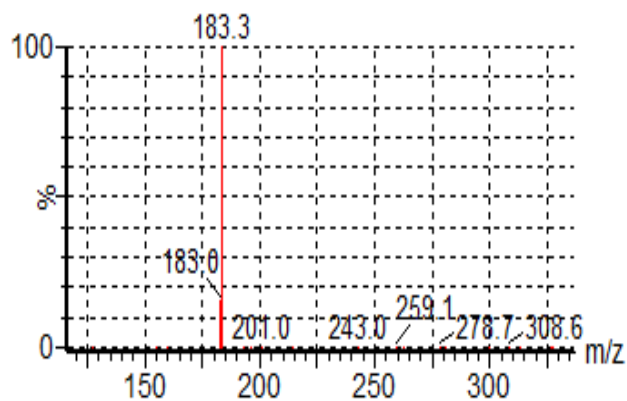


Figure A2-4 The product ion spectrum generated during the infusion of the JWH-073-4-Hydroxy-butyl metabolite standard showing the large interfering product ion of m/z 183 and 183.3. From the chromatographically separated standard this interference can conclusively be assigned to a contaminating compound.

Product ion of JWH-210-4-Hydroxy-pentyl metabolite

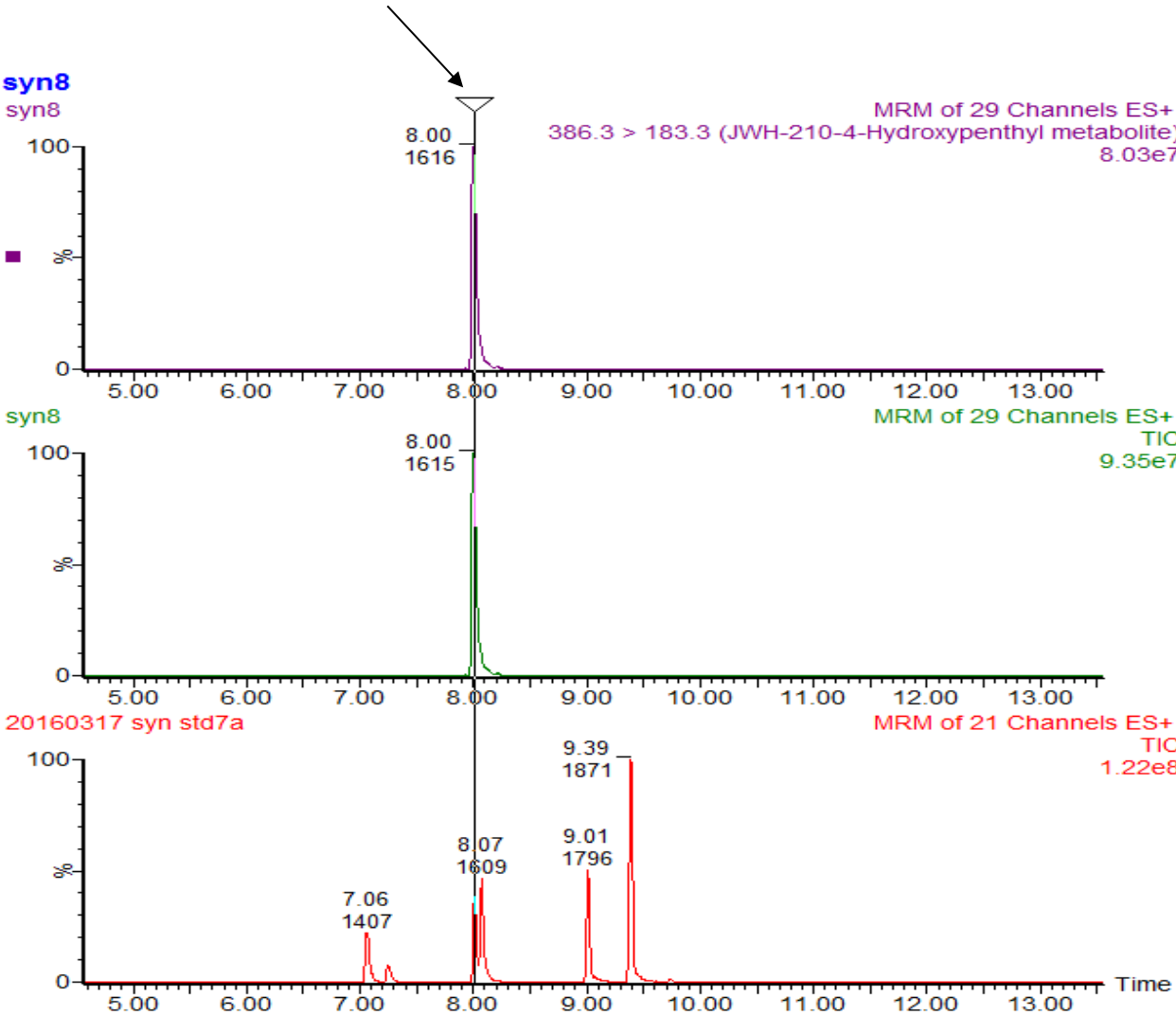


Figure A2-5: Extracted ion chromatogram showing retention time and coincidence of the different fragment peaks for JWH-073-4-Hydroxy-butyl metabolite