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Method development and validation for synthetic and natural cannabinoids on LC-MS/MS.

The thesis is submitted in partial fulfilment of the requirements of Kingston University for the degree of:

Masters by research in Chemistry

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Abstract

A method was developed for analysis of natural and synthetic cannabinoids: THC, THC-COOH, AM-2201, JWH-018 N4-hydroxypentyl and AB-CHMINACA. The method was validated based on UKIAFT, SWGTOX and ISO 17025 guidelines. The parameters that required validation were accuracy, precision, linearity, range, carryover, LOD, LOQ, recovery, matrix effect, repeatability, reproducibility, dilution integrity, selectivity and specificity, stability and robustness. Moreover, the method was validated based on Forensic Science Regulators guidelines and criteria, so it could be utilised in road traffic toxicology. The validation was successful for all five analytes, all analytes passed tested parameters. This LC-MS/MS technique can be utilised for the detection of THC in road traffic casework as it is in line with the Forensic Science Regulators guidelines for quantifying drugs under Section 5A of the Road Traffic Act 1988. This method also facilitates the analysis of SC, at concentrations typically detected in users and coronial cases associated with SC overdose. There is also the possibility to expand the method, to detect a wider range of drugs, by adding new SC to LC-MS/MS library, including new and emerging SC appearing in market. The validated method can be beneficial to analyse road traffic casework samples and SC concurrently, which can help to find out the prevalence and popularity of SC with the general population. In addition, the method was tested using clotted versus non-clotted blood, to test methods robustness. The study looked at twenty clotted samples and 10 non-clotted samples, to find out what effects clotting has on THC and THC-COOH measured concentrations. The research seems to indicate that clotting of blood alters the measured concentrations of THC and THC-COOH. The results also indicate that the method is robust and can be utilised to analyse whole blood and clotted blood.

Contents

Abbreviations

A&E – Accident and emergency

ACN – Acetonitrile

Amu – Atomic mass unit

CAD – Collisionally activated dissociation

CB_1 – Cannabinoid receptor type one

CB_2 – Cannabinoid receptor type two

CBD – Cannabidiol

CBGA – Cannabigerolic acid

CBN – Cannabinol

CES – Carboxylesterase

CID – Collision induced dissociation

dMRM – Dynamic multiple reaction monitoring

EDTA – Ethylenediaminetetraacetic acid

EMCDDA – European Monitoring Centre for Drugs and Drug Addiction

ESI – Electrospray ionisation

FEWS – Forensic early warning system

GABA – gamma aminobutyric acid

GC-MS – Gas chromatography-mass spectrometry

HCl – Hydrochloric acid

ISO 17025 – International standard for testing and calibration laboratories

LC – Liquid chromatography

LC-MS – Liquid chromatography-mass spectrometry

LC-MS/MS – Liquid chromatography-tandem mass spectrometry

LOD – Limit of detection

LOQ – Limit of quantitation

ME – Matrix effect

MeOH – Methanol

MS – Mass spectrometry

NaOH – Sodium hydroxide

NHTSA – National Highway Traffic Safety Administration

NIDA – National Institute of Drug Abuse

NPS – New psychoactive substances

QC – Quality control

QqQ – Triple quadrupole

RE – Recovery

RF – Radio frequency

RTT – Road traffic toxicology

SC – Synthetic cannabinoid(s)

SPE – Solid phase extraction

SWGTOX – Scientific Working Group for Forensic Toxicology

TIAFT – International Association of Forensic Toxicologists

THC – Δ9-tetrahydrocannabinol

THCA – Tetrahydrocannabinolic acid

THC-COOH – 11-nor-9-carboxy-THC (non psychoactive metabolite of Δ9 tetrahydrocannabinol)

UGT – Uridine 5`-diphosphoglucuronosyltransferase

UKIAFT – United Kingdom and Ireland Association of Forensic Toxicologists

UNODC – United Nations Office on Drugs and Crime

UV – Ultraviolet

V – Voltage

WEDINOS – Welsh emerging drugs & identification of novel substances project

WHO – World Health Organization

WSA – Working solution a

WSB – Working solution b

WSC – Working solution c

WSD – Working solution d

WSF – Working solution f

%CV – Coefficient of variance

11-OH-THC – 11-hydroxy-THC (psychoactive metabolite of Δ9-tetrahydrocannabinol)

1. Introduction

1.1 Cannabis plant and history

Cannabis sativa L. also known as marijuana, weed and pot has been used for cultural, medicinal and recreational purposes for centuries (Bonini *et al.* 2018). It first originated and was domesticated in central Asia (Bonini *et al.* 2018), where it was originally used as a fibre, food and for medicinal purposes (Antonio 2006). Only in recent centuries has cannabis become synonymous with recreational use (Chandra *et al.* 2017). Cannabis can be grown in almost all parts of the world and the plant can be male, female (Figure 1), as well as a hermaphrodite, which is bred for fibre production (Chandra *et al.* 2017).

Figure 1: Male (A) and female (B) *cannabis sativa* plants in the flowering stage. During the vegetative stage the sex of the plant cannot be determined. The male grows pollen sacs, which eventually burst and release pollen for fertilisation of female plant. The female flowers produce tiny white or orange hairs, called pistils, which are the female plant sex organ. When pistils come into contact with pollen, fertilisation occurs, seeds begin to develop. The unfertilised female flower is an area of interest for cannabis growers, it is where most resin with cannabinoids is made *(*Bonini *et al.* 2018*).*

For industrial farming of cannabis females' plants are preferred, as they produce significantly higher quantities of the psychoactive cannabinoids than the male plant (Beckett *el al.* 2017). To ensure higher cannabinoid quantities in female plants they are often separated from male plants to avoid pollination (Chandra *et al.* 2017). Cannabinoids are made as a defence mechanism by the plant to protect against insects and predators (Bonini *et al.* 2018). The leaves, bract and stem of the plant is covered with trichomes, with female flowers having the highest amount of trichomes (Chandra *et al.* 2017). Trichomes produce cannabinoids and excrete them in resin (Figure 2). The main cannabinoid of interest is Δ9-tetrahydrocannabinol (THC), which is the psychoactive substance in cannabis (Chandra *et al.* 2017). Cannabis contains THC and cannabidiol (CBD) as a mixture of mono-carboxylic acids, which become decarboxylated upon heating (Sharma *et al.* 2012). Figure 3 describes the conversion of THC and CBD from Cannabigerolic acid (CBGA) (Citti *et al.* 2018). The main forms and routes of administration for cannabis are summarised in Figure 4 and Table 1.

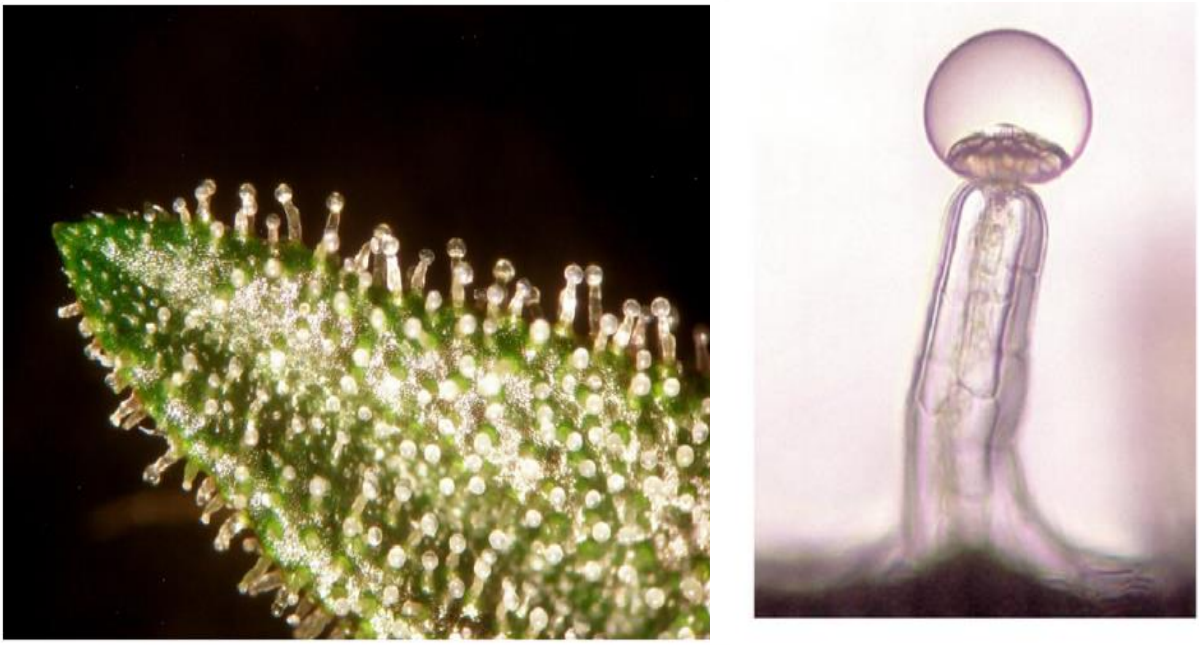


Figure 2: Trichomes on cannabis surface (left picture), from the single heads of trichomes (right picture) cannabinoids are secreted in resin like material. Resin plays a protective function as is viscous, hydrophobic and has low volatility. It prevents water loss, deters animals, protects the plant from environmental damage (Chandra *et al.* 2017).

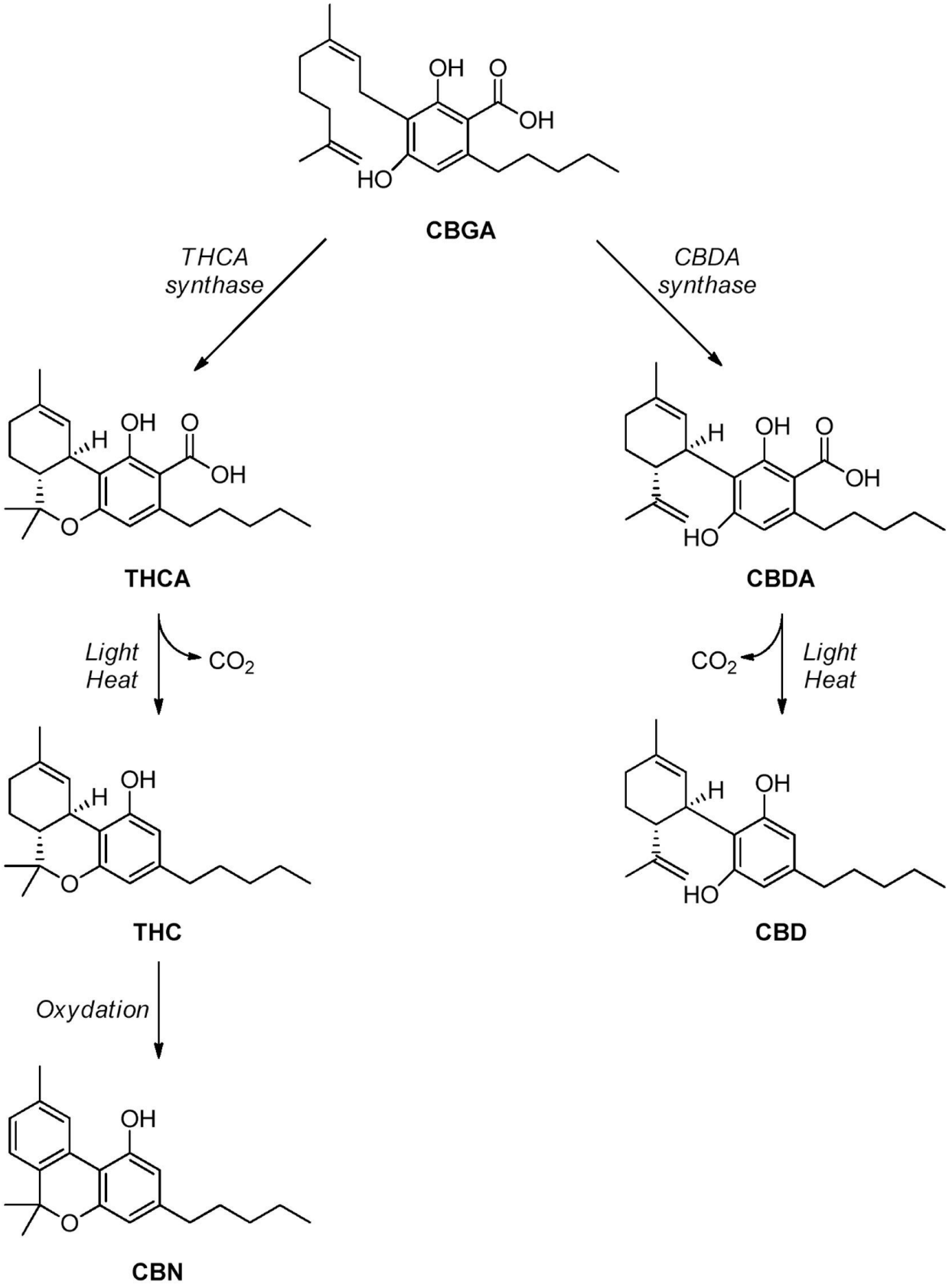


Figure 3: Biosynthetic formation of THC and CBD. CBGA is converted into tetrahydrocannabinolic acid (THCA) by THCA synthase enzyme or to Cannabidiolic Acid (CBDA) by CBDA synthase enzyme. Heat triggers a chemical reaction of THCA and CBDA and leads to decarboxylation of THCA and CBDA and formation of corresponding decarboxylated (neutral) species THC and CBD. Cannabinol (CBN) is formed from the oxidation of THC (Citti *et al*. 2018).

Figure 4: Physical representations of A - herbal cannabis, B - hashish, C - hash oil. The typical amount of THC in herbal cannabis is 10%, in hashish 20% and in hash oil up to 90% (Dotdash 2019)

Table 1: Differences of preparation, concentration and route of administration for herbal, resin and oil forms of cannabis (Afsahi *et al.* 2016, Sharma *et al*. 2012).

Form of cannabis	**Herbal cannabis (marijuana, ganja, charas)**	**Resin cannabis (hashish, bhang)**	**Cannabis (hash) oil**
Preparation	To prepare herbal cannabis the plant leaves, stalks, seeds and flowering tops have to be dried.	A traditional method of preparation is to rub fresh cannabis flower to gather resin by hands.	Solvent extraction on cannabis resin is utilised. The extract (oil) looks like a dark viscous liquid.

Table 1: (Continued)

Form of cannabis	Herbal cannabis (marijuana, ganja and charas)	Resin cannabis (hashish, bhang)	Cannabis (hash) oil
THC concentration	Herbal cannabis has the lowest levels of THC (5-10%).	The THC concentrations in hashish are between herbal and hash oil concentrations (20%).	Oil the highest amount of THC and is the most potent form of cannabis (80-90%).
Route of administration	Inhalation and smoked as joints.	Consumed orally and infused in foods.	Inhalation or oral consumption.

1.2 Cannabis prevalence in the UK

Cannabis is the most frequently used illicit drug worldwide and the most cultivated illegal drug in numerous countries (United Nations Office on Drugs and Crime (UNODC) 2019). It has been observed that the use of cannabis has increased globally in the decade from 2007 to 2017, from 162 million users to 188 million users (UNODC 2019). Moreover, cannabis has become legal to use in several countries of South America, North America and Europe.

Last year cannabis was the most used illicit drug in England and Wales (Home Office 2019a). It is also the drug that was more likely to be repetitively used (Home Office 2019a). Cannabis was also the most confiscated drug by English and Welsh law enforcement in 2018 (Home Office 2019b). In 2017 the majority of first-time entrants for drug treatment was cannabis related (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) 2019). For motorists in England and Wales the most commonly detected illicit drug was THC in the years 2010 to 2012 and 2015-2016 (Risk Solutions 2017, Rooney *et al.* 2017). The most frequent concentration of THC detected in blood in the period of 2010-2012 in motorists was 2-4ng/mL in 36.1% of 926 cases, followed by 1-2ng/mL in 28.4% of cases (Rooney *et al.* 2017). For over 60% of cases the THC concentration was above 2ng/mL, the Section 5A *per se* limit for drug driving in England and Wales (Rooney *et al.* 2017).

In United Kingdom under The Misuse of Drugs Act 1971 cannabis is classified as a class B and Schedule I drug, therefore a licence is required to possess or distribute it (Home Office 2019c). In 2019, Sativex (nabiximols), a cannabis-based medicine, which contains THC and CBD, was licenced for the treatment of multiple sclerosis related spasticity in England (Home Office 2019c).

1.3 Pharmacokinetics of THC

The principle constituent and the psychoactive component of cannabis is THC (Baselt 2015). Since the discovery of this compound in 1964 by Mechoulam and Gaoni there has been an ongoing interest in its pharmacology and potential medicinal use (Englund *et al* 2012).

THC is highly lipophilic, has a pK_a value of 10.6 (acid) and binds strongly to lipoproteins in blood with approximately 90% of THC concentrated in the plasma (Baselt 2015). The main routes of administration for cannabis are either oral ingestion or smoking/inhalation (Baselt 2015). The doses for smoking are typically 50mg of THC in a single cigarette and approximately 20-50mg in edibles, with an average THC concentration of 3.5-5% (Barrus *et al.* 2016, Hartman *et al.* 2016a, Vandrey *et al.* 2017).

The bioavailability of THC is largely determined by the route of administration, this effects the blood concentration peak times and the onset of psychoactive effects. Smoking of cannabis results in an immediate sensation of euphoria colloquially known as a “high”. These effects are felt in within 5 minutes of smoking cannabis and taper off after 2-3 hours, though the overall the psychosomatic effects can last up to 4-6 hours (Huestis 2007, Grotenhermen 2003, Wolff *et al.* 2013). The bioavailability of THC via smoking can range from 2% to 56%, this is dependent on dosage, user’s technique of smoking (Huestis 2007). The peak concentration of THC if administered by inhalation is reached within 10 minutes, while the metabolites of THC are higher in blood concentration than the parent drug 30-45 minutes after smoking (Huestis 2007, Hartman *et al.* 2016a). The detection window for THC, with a cut off limit of 0.5ng/mL, in plasma can range from 3-12 hours for lower doses (1.75% THC) and 6-27 hours for higher doses (3.55% THC) (Huestis 2007). Hartman *et al.* (2016a) found that the decrease in THC concentration relative to the maximum dose exceeds 90% in 4-8 hours when smoking. Schwope *et al.* (2011) found that peak THC concentrations are 50ng/mL in whole blood after smoking 54mg of THC (Figure 5). Orally ingesting cannabis results in the initial euphoria and sedative effects occurring within 30-90 minutes. The peak psychoactive effect is felt after 1.5-3 hours, with the psychosomatic symptoms lasting up to 6-8 hours (Vandrey *et al.* 2017). The bioavailability of the drug if taken orally is 10-20% (Huestis 2007). Administering cannabis orally delays the peak THC blood concentration, from 1-6 hours. In 1.5-6 hours the metabolites are higher in concentrations than THC (Vandrey *et al.* 2017). Vandrey *et al. (*2017) investigated orally consumed cannabis and a detection window of up to 8 hours in whole blood was demonstrated for THC, with a concentration cut off of 2ng/mL. This research also indicated that peak THC concentration did not exceed 5ng/mL in whole blood after oral use of cannabis at doses up to 50mg of THC (Figure 5). However, the volume of personal use needs to be considered when interpreting THC concentrations, as frequent users often have a baseline THC, ranging from

2.1-4.5 ng/mL and achieve higher peak THC concentrations with longer detection windows of up to 30 hours (Desrosiers *et al.* 2014).

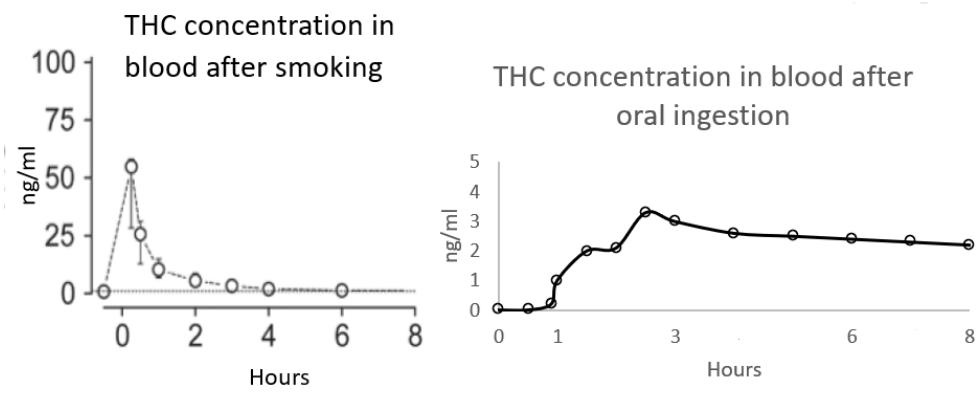


Figure 5: Differences in THC concentrations after smoking 54mg of THC (left) and oral ingestion of 50mg of THC (right). If smoked the peak THC concentrations were 50ng/mL in 15 minutes. With a concentration cut of 1ng/mL THC was detected for up to 6 hours and also at 22 hour point (Schwope et al. 2011). After oral ingestion the peak THC concentration did not exceed 5ng/mL (highest concentration measured in the research by Vandrey *et al*. 2017 for participants was 5ng/mL). With the concentration cut of 2ng/mL THC was detected for up to 8 hours (Vandrey *et al.* 2017).

The half-life of THC is 20-57 hours for infrequent users and 3-13 days for frequent users (Baselt 2015). Such large variation is due to different study designs, their monitoring time, doses of THC and different cut off limits for detection. THC first distributes to perfused organs such as lungs, heart, brain, liver and afterwards to less vascularised organs and into fats. THC is highly lipophilic and accumulates in fat, from which it is later slowly released back into the bloodstream (Huestis 2007). This adds a further variable when trying to estimate THC concentrations in blood.

THC is metabolised into over 100 metabolites (Grotenherman 2003). However, the primary metabolites are 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (THC-COOH), (Figure 6) (Huestis 2007). Phase I metabolism of THC is hydroxylation by the hepatic cytochrome P450 enzyme family, mainly cytochrome P450 2C9 and cytochrome P450 34A (Huestis 2007). The product of THC hydroxylation is 11-OH-THC, which is a psychoactive metabolite and more psychoactively potent than THC. The concentration of 11-OH-THC is greater if cannabis is taken orally due to the more significant first past metabolism in the liver and degradation of THC in the stomach (Huestis 2007). 11-OH-THC is converted to a non psychoactive metabolite THC-COOH (Huestis 2007). Phase II metabolism of THC is the glucuronic acid addition to THC-COOH via the C11 carboxyl group (or phenolic hydroxyl group) by uridine 5`-diphosphoglucuronosyltransferase (UGT) (Figure 7) (Huestis 2007). THC is mainly metabolised in the liver, but metabolism may also occur in the brain, lungs, and intestine (Huestis 2007).

About 70% of THC and its metabolites are removed from the body within 72 hours, they are mostly excreted in faeces (40%) and urine (30%) (Baselt 2015). The main metabolite of THC that is found in urine is THC-COOH-glucuronide and THC-COOH, with low amounts of THC and 11-OH-THC present. By contrast in faeces 11-OH-THC is the predominant compound (Huestis 2007). The clearance rates from plasma have been reported to be 15L/hour for men and 12L/hour for women, it has also been suggested that clearance rates are 36L/hour for infrequent users and 60L/hour for frequent users (Sharma *et al.* 2012).

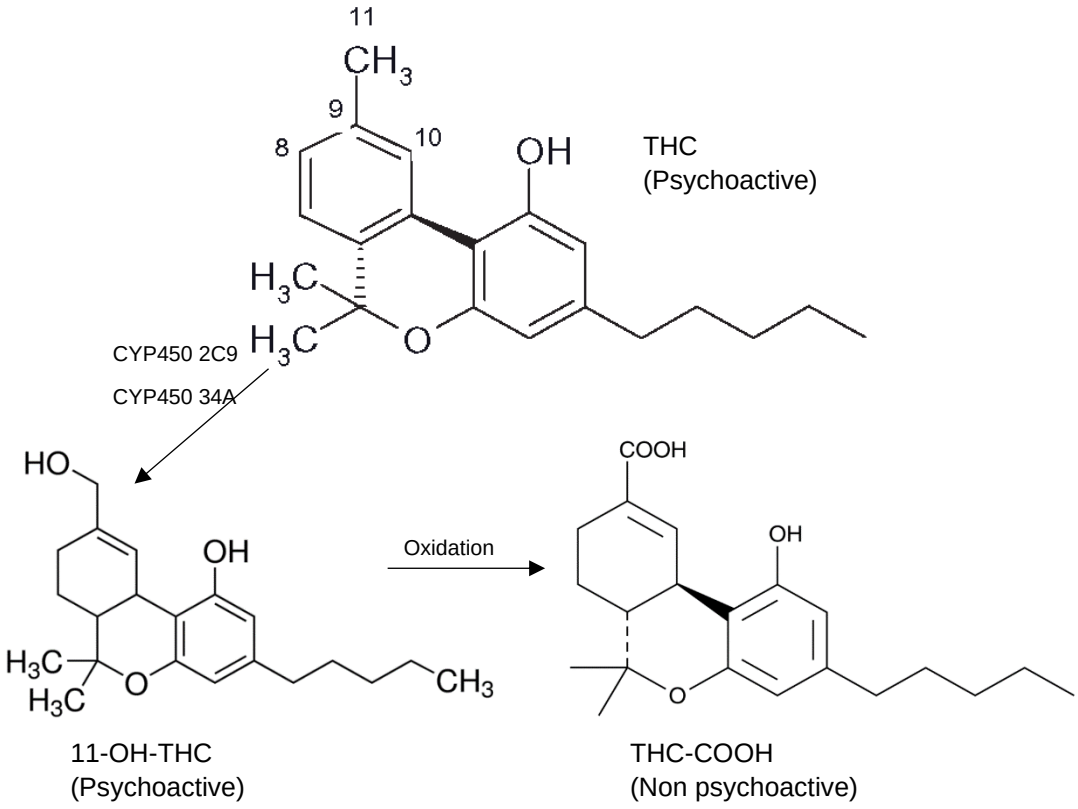


Figure 6: Phase I metabolism of THC. THC is hydroxylated by cytochrome P450 enzymes. A hydroxyl group is added to the 11th carbon of THC converting it to 11-OH-THC. 11-OH-THC is a psychoactive metabolite that is further metabolised by oxidation. An oxygen is added to the 11th carbon of 11-OH-THC making it THC-COOH, which is an inactive metabolite.

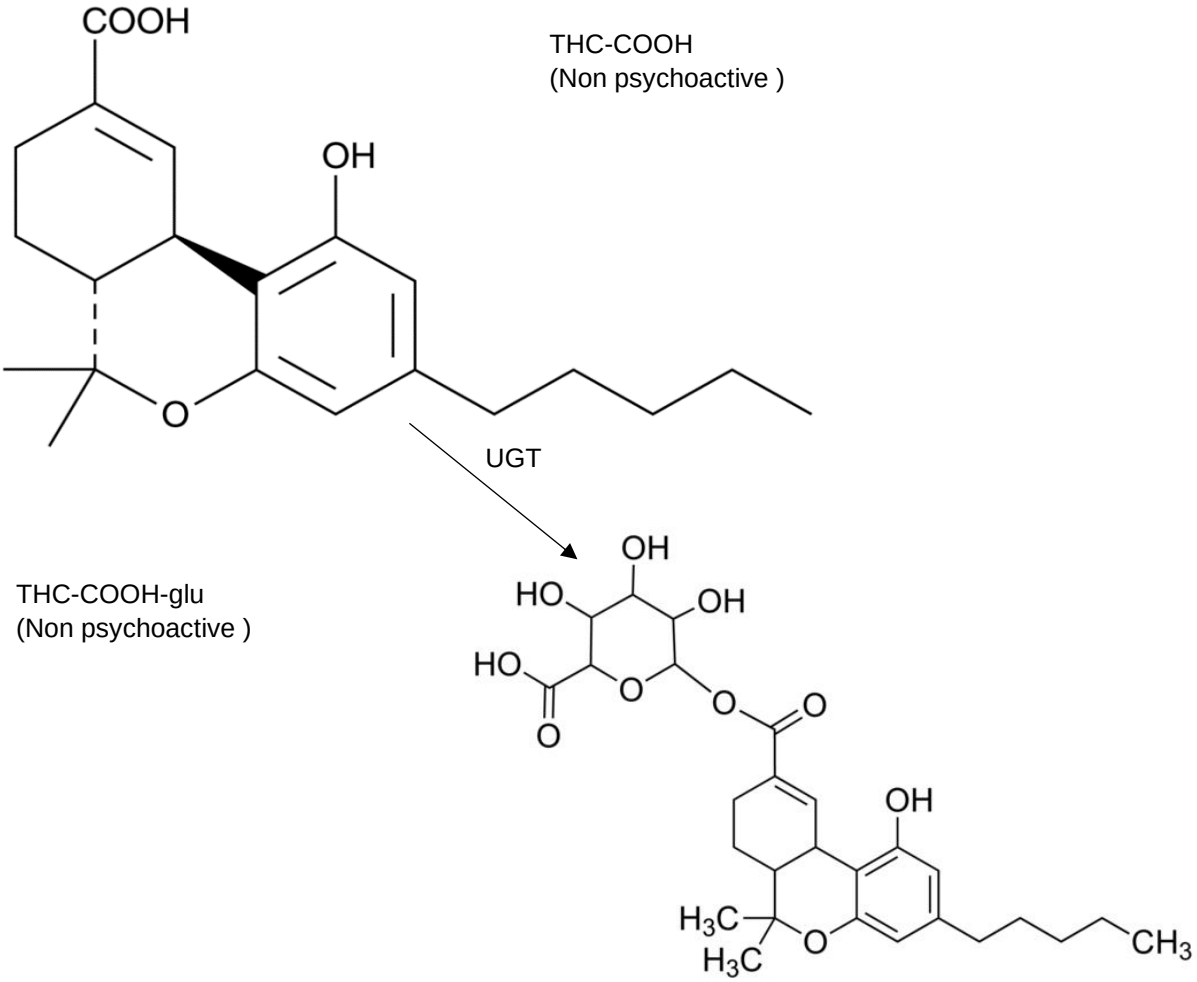


Figure 7: Phase II metabolism of THC. The second phase of metabolism is glucuronidation, this is a biotransformation reaction in which a glucuronic acid is added to the compound, increasing hydrophilicity. This allows easier elimination from the body via urine or faeces. THC-COOH interacts with UGT which transfers glucuronic acid to THC-COOH. The product of the reaction is glucuronide THC-COOH-glu.

1.4 Pharmacodynamics of THC

THC is a partial agonist that binds to G-coupled-protein receptors cannabinoid receptor type one (CB_1) and cannabinoid receptor type two (CB_2), CB_1 is primarily located in the central nervous system and CB_2 on immune cells (Grotenhermen 2004). THC has the same affinity for CB_1 and CB_2 but higher efficacy for the CB_1 receptor (Pertwee 2008). The exact mechanism of action that underlies THC's interaction with the CB_1 and CB_2 receptors are unclear but it appears to affect a wide range of neurotransmitters. It has been postulated that THC's interaction with CB_1 and CB_2 receptors results in inhibition of adenylate-cyclase, this in turn inhibits the release of the neurotransmitters, acetylcholine and glutamate while indirectly affecting opioid and serotonin receptors (Oberbarnscheidt and Miller 2017). Activation of the CB_1 receptors also modulates the transmission of other neurotransmitters

such as dopamine, gamma aminobutyric acid (GABA), histamine, glycine, noradrenaline and neuropeptides (Grotenhermen 2004).

CB_1 and CB_2 are known as the endocannabinoid receptors and together with endogenous ligands make up the endocannabinoid system (Grotenhermen 2004). THC`s chemical structure is similar to anandamide, a natural CB_1 ligand. The endocannabinoid system has several functions, modulating cardiovascular effects, smooth muscle contraction, liver, gastrointestinal tract, the immune system and the reproductive system. Recent research has also indicated that CB_1 and CB_2 receptors may be present in the respiratory tract and urinary system (Maccaroone *et al.* 2015).

Activation of CB_1 by THC induces feelings of euphoria and relaxation, in addition to sensory intensification (Bloomfield *et al.* 2019). At high doses THC can induce anxiety, panic attacks, increased heart rate/blood pressure, a decrease in intraocular pressure, enhanced appetite, impairment of memory, learning and motor coordination (Bloomfield *et al.* 2019). A summary of commonly observed THC effects in pharmacological studies is presented in Table 2 with the different brain regions affected by THC displayed in Figure 8. Chronic use of THC is a risk factor for schizophrenia, psychotic disorders, depression and dependency (Bloomfield *et al.* 2019). Cannabis use during adolescence, a key period in CNS development, may have long term effects on emotion, cognitive function, memory and addiction (Bloomfield *et al.* 2019, National Institute of Drug Abuse (NIDA) 2019). Drummer *et al.* (2019) suggests THC may cause increased risk of myocardial infarction, strokes and may also be a contributory factor or a direct cause of death, although, it appears that this phenomenon is rare (EMCDDA 2019).

Table 2: Observed THC effects in pharmacology studies (Grotenhermen 2004, Bloomfield *et al*. 2019, Colizzi and Bhattacharyya 2018).

Body systems, organs	**THC effect**
Mental state and behaviour	Relaxation, anxiety, panic, impairment of learning and memory, reduced reaction time, fatigue, euphoria, dysphoria, reduced anxiety, hallucinations, alteration of time perception, fragmented thinking, ataxia, weakness, unsteady gait and paranoia.
Nervous system	Increase of appetite, neuroprotection in ischemia and hypoxia, analgesia, vomiting, anti-emetic effects and psychosis.

Table 2: (Continued)

Body systems, organs	THC effect
Cardiovascular system	Increased heart rate, decrease or increase in blood pressure, enhanced heart activity, inhibition of platelet aggregation, myocardial infarction and stroke.
Eye	Decrease of intraocular pressure, reduced tear flow and reddened conjunctivae.
Body temp	Decrease or increase in body temperature.
Immune system	Anti-inflammatory, anti-allergic effects and decreased resistance towards pathogens and carcinogens.
Genetic material	Inhibition of DNA, RNA and protein synthesis, influence cell cycle and antineoplastic activity.
Reproductive system	Reduced sperm count, motility, suppressed ovulation and disturbed menstrual cycle.
Respiratory system	Bronchodilation and dry mouth.

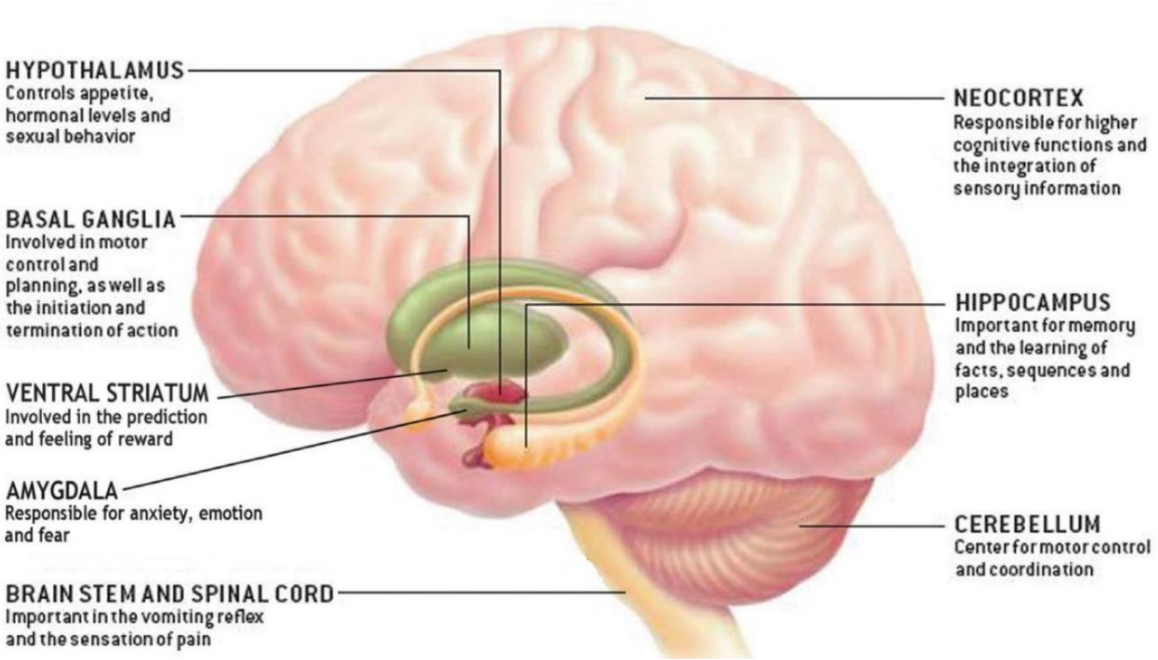


Figure 8: Brain areas and their functions. Cannabinoid receptors CB_1 are found in several brain areas and are activated by THC. Different brain regions produce different effects when interacting with THC. The following are common effects after receptor activation with THC. Cerebellum, loss of balance and motor coordination. Hippocampus, impairment of memory and learning. Neocortex, fragmented thinking, difficulty on focusing. Brain stem and spinal cord, prevention of nausea. Amygdala, elevation or reduction of anxiety. Ventral striatum, feeling of "high". Basal ganglia, relaxation, calmness. Hypothalamus, increased appetite (NIDA 2019).

Tolerance for THC can develop rapidly following during chronic use, thereafter acute effects of THC are less prominent. The current research suggests that cognitive functions of verbal memory, divided attention and reaction time show the highest tolerance levels (Colizzi and Bhattacharyya 2018, Ramaekers *et al.* 2016). The dependency/addiction to cannabis is known as cannabis use disorder (Bloomfield *et al.* 2019). Studies suggest that approximately 9% of people using cannabis can become dependent on it, while 17% of those who begin usage in adolescence can develop cannabis use disorder (NIDA 2019). THC withdrawal symptoms are relatively mild with most severe effects being anxiety, headaches, nausea, nightmares, insomnia, reduced appetite and depression lasting to around 4-14 days (Oberbarnscheidt and Miller 2017).

1.5 Synthetic cannabinoids

1.5.1 History and legal status in the UK

Synthetic cannabinoids (SC) also colloquially known as “Spice” in Europe or “K2” in the US, are CB_1 and/or CB_2 agonists. In the 1990s research was being carried out to develop THC analogues for pharmaceutical therapies by Professor John W. Huffman in Clemson University and at the same time by Professor Alexandros Makriyannis in Norheastern University (Wiley *et al.* 2011).The focus of their research was into the development of CB_1 and CB_2 agonists for medicinal purposes. However, the developed drugs were often more psychoactive than THC and not safe to use therapeutically (De Luca and Fattore 2018). It is likely that the research published during this time was the basis for illicit manufacturers to develop SC (Wiley *et al.* 2011).

The first SC appeared in 2004 in Europe and the first detected SC was JWH-018 (AM678) in Germany in 2008 (Wiley *et al.* 2011, UNODC 2013). JWH-018 was one of the chemicals synthetized by Professor John W. Huffman in 1990s, and derived its name from his initials, as did the AM chemicals, which were synthesised by Professsor Alexandros Makriyannis. SC popularity was originally due to its legal status, cheapness and ease of purchase (Rojek *et al.* 2017). SC could be purchased via the internet or in “head shops”, where it was typically sold as a product marked “not for human consumption”, with the actual contents not known (EMCDDA 2017a). In 2016 the Psychoactive Substances Act 2016 came into effect to control all new psychoactive substances. Prior to this, succeeding amendments to Misuse of Drugs Act 1971 were passed by UK government to regulate SC. In 2009 the first amendment was passed for “first generation” SC, in 2013 a second amendment for “second generation” SC and in 2016 third amendment for “third generation” SC. The succeeding amendments were needed as the manufacturers could circumvent the legislation by simple alterations to the SC structure.

Previously under the UK law drugs were under “generic” control, their core structure and specific modifications, and substitutions were controlled by law. By altering and adding a

substitute that was not illegal, a new compound could be made without contravening the existing drug legislation. JWH-018 was made illegal in 2009, as a result the manufacturers added halogen atoms to the side chain attached to the indole nitrogen atom. This new compound was not JWH-018 and therefore not controlled under the drug legislation, this resulted in the synthesis of a new compound, AM-2201 (fluorinated JWH-018) which was legal until 2013 (Home Office 2015). Retrospectively, it can be argued that this strategy of legislating SC in this fashion led directly to the development of more potent second and third generation SC as manufacturers altered the structure to avoid legal controls. Conversely these alterations led to an increased binding affinity for the new generation SC and the CB_1 and CB_2 receptors, this in turn increased the psychoactive and addiction potential of the new generation SC's.

1.5.2 SC recreational use

Like cannabis the most common route of administration for SC is inhalation. Plant material is infused by spraying or soaking it with an organic solution of SC and evaporating the solvent (UNODC 2013). The distribution around the plant material is not always homogenous and "hot pockets" may be present, these are areas where the SC solution are more concentrated (EMCDDA 2017a). SC can also be present in the solid, crystalline powder (UNODC 2013), while in recent years SC have been developed in e-liquids for vaping. Moreover, in UK prisons SC are frequently smuggled in by letters, pictures or drawings with the paper infused with the psychoactive chemicals (Ford and Berg 2018). Figure 9 demonstrates the physical forms, in which SC are routinely found in.

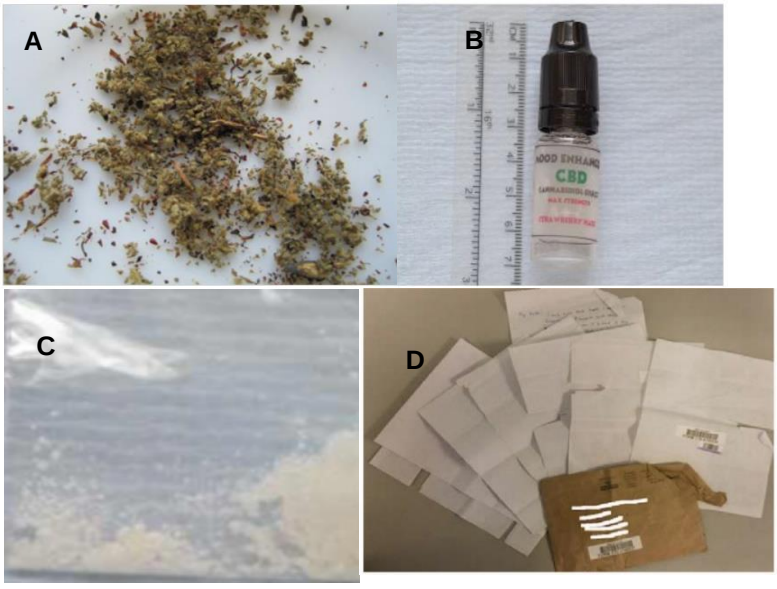


Figure 9: Physical forms of SC. A – Infused plant material, it is used by smoking. B - Adulterated e-liquid, it is used by inhalation. C - Powder form, it is snorted or swallowed. D – Letters infused with SC, to avoid detection in prisons, they are used by smoking (EMCDDA 2017a, Ford and Berg, 2018).

1.5.3 SC prevalence and most common SC's in the UK

The usage of new psychoactive substances (NPS) seems to have decreased since the passing of the Psychoactive Substances Act 2016 (Figure 10) (Home Office 2018b). However, there are still ongoing issues with SC in the UK particularly in economically vulnerable groups such as prisoners, young people, homeless and individuals from low income backgrounds (Her Majesty`s Inspectorate of Prisons 2018, EMCDDA 2017a). Two of the major incentive for SC as drugs of abuse is that they are frequently not detected in standard toxicological screenings and are more potent than THC (Gurney *et al*. 2014, Rojek *et al.* 2017, Tai and Fantegrossi 2014). It was reported that in 2018/2019 only 0.5% of adults (approximately 152,000 people) used any NPS, with the major group being SC (Home Office 2019a). It is most likely this number is an under estimation, as these surveys rely on self-reporting, in addition the majority of forensic laboratories do not screen for SC. Currently in the EU there is over 160 SC that have been detected and are present in circulation, with the number increasing every year (EMCDDA 2017a).

The last forensic early warning system (FEWS) report in the UK for 2016/17 found that the most common SC were 5F-ADB(5F-MDMB-PINACA) and MDMB-CHMICA (Home Office 2018a). The Welsh emerging drugs & identification of novel substances project (WEDINOS) monitors novel psychoactive substances, submitted from across the UK. A report by WEDINOS for the period of April 2018 to 2019 March found that 5F-ADB and AMB-FUBINACA were the most common SC identified in samples (Public Health Wales 2019). It was noted that there was an increase in the prevalence of 4F-MDMB-BINACA from December 2018 to March 2019 (Public Health Wales 2019).

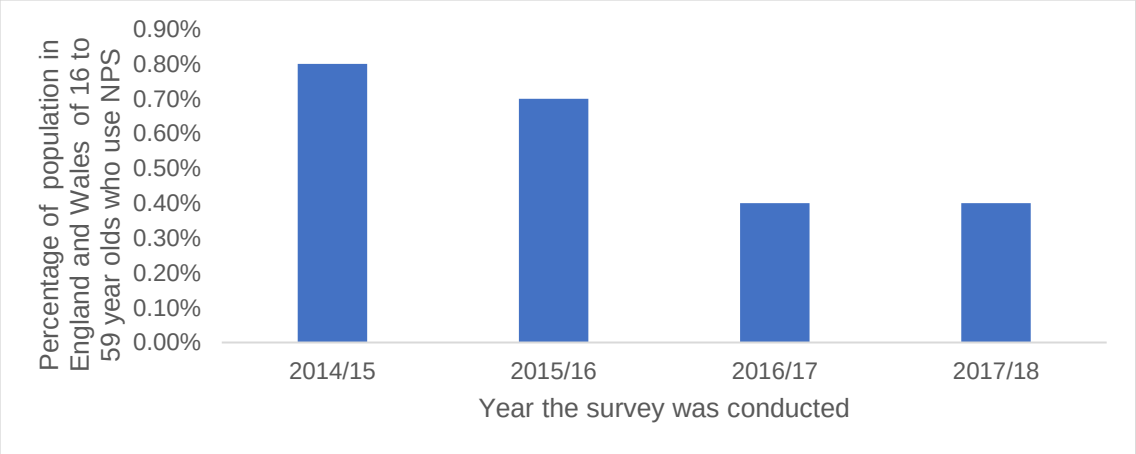


Figure 10: The Crime Survey for England and Wales for 16 to 59-year olds report of use of NPS. In 2015/16 approximately 244000 people were using NPS. After the Psychoactive Substance Act 2016 the number of people using NPS decreased in 2016/17 to approximately 143000 people, which was a significant decrease. The following year 2017/18 approximately 121000 were using NPS similar to previous year, no significant change was seen (Home Office 2018b).

1.5.4 General pharmacology for SC

Unlike THC, SC are relatively new and do not have extensive research, on their pharmacological and toxicological effects. (Presley *et al.* 2016). In addition, there are over 160 SC, with new drug variants of evolving chemical structure constantly emerging. These new SC compounds can have a lifespan of as little as 12 months in the market (Presley *et al.* 2016, Marusich *et al.* 2018). As a result most of the information regarding SC is established by *in vitro* cell culture studies and *in vivo* animal models. The majority of toxicological data in humans concerning SC is derived from accident and emergency (A&E), case studies, which are summarised in Table 3 (Gurney *et a*l. 2014).

Table 3: Observed effects of SC on different systems (Gurney *et al.* 2014, Behonick *et al*. 2014, Rojek *et al*. 2017, Tai and Fantegrossi 2014 and EMCDDA 2017a*)*

Parameter	SC effects
Kidney	Acute kidney injury.
Gastrointestinal	Vomiting, nausea and abdominal pain.
Cardiovascular	Increased heart rate, high blood pressure, chest pain, cardiovascular toxicity, stroke and heart attack.
Dermal	Rash and itchy skin.
Neurological	Agitation, confusion, dizziness, drowsiness, hallucinations, seizures, tremors, blurred vision, dilated pupils, paranoia,psychosis, suicidality, anger, sadness, blackouts, restlessness, numbness, altered mood and time perception, bloodshot eyes, dryness of mouth, panic attacks, short term memory defects, delusions, excessive sweating, slurred speech, delayed reactions and loss of memory.

The user desired effects of SC are mood elevation, euphoria, relaxation (Gurney *et al*. 2014). Unlike THC, SC are full agonists at CB_1 and the euphoria associated with the drug, is more potent, however the overall effects is shorter and typically only last for up to 2 hours (Behonick *et al.* 2014). Based on *in vitro* assays SC have higher binding affinity to CB_1 and CB_2 compared to THC. (Gurney *et al*. 2014). It has been proposed that the first generation SC have approximately 10 times the potency of THC for the CB_1 receptor, while the latest generation products are over 100 times more potent than THC.

Due to this increased potency dependence syndrome is more likely to develop for users of SC (Fantegrossi *et al.* 2014, Tai and Fantegrossi 2014). Moreover, the withdrawal

symptoms for SC are far more severe than those of THC. Users have been reported to experience tremors nausea, vomiting, insomnia, headache, seizures, hallucinations (Gurney *et al*. 2014, Tai and Fantegrossi 2014). Unlike natural cannabinoids SC have been more closely linked to fatalities. Deaths associated with SC have been reported in the USA, Poland, Russia and UK (EMCDAA 2017). AB-CHMINACA has recently been the SC linked to fatalities in UK, with the parent structure or its metabolites confirmed in post-mortem samples (EMCDAA 2017b).

Due to the high volume of SC available and the limited toxicological data, the metabolism of these drugs is not widely understood, especially for the new generation SC. Despite this the general metabolism of SC involves the hydroxylation, keto-oxidation, carboxylation, dehalogenation and hydrolysis of the parent compound by cytochrome P450 isoenzymes, (Presley *et al.* 2016). The main isoenzymes, that produces the highest amount of metabolites by oxidation, are CYP1A2, CYP2C9, CYP3A4, CYP2B6 (Presley *et al.* 2016). The defluorination is attributed to cytochrome P450 2E1. Carboxylesterase (CES) enzymes perform the hydrolysis, with CES1 being the major enzyme responsible. UGT is responsible for the glucuronic acid conjugation, with UGT1A1, UGT1A9, UGT2B7, UGT1A3, UGT1A10, UGT1A7 being major enzymes involved in the metabolism of SC (Fantegrossi *et al.* 2014, Presley *et al.* 2016). The metabolites of SC can be full or partial agonists at CB_1 receptor and produce synergetic effects with the parent compound, thereby increasing the potency of the user desired effects (Presley *et al*. 2016). Moreover, it was reported that SC MAM-2201 was found in high concentrations in adipose tissue, suggesting that SC may be able to accumulate in fat of the body, like THC (Presley *et al.* 2016).

1.5.5 Pharmacology of JWH-018, AM-2201 and AB-CHMINACA

JWH-018, a first generation SC, is metabolised by CYP1A2 and CYP2C9 enzymes. JWH-018 produces several metabolites, the main metabolites are JWH-018 N-(3-OH-pentyl), JWH-018 N-(4-OH-pentyl), JWH-018 N-(5-OH-pentyl), JWH-018 pentanoic acid, JWH-018 (5-OH-indole), JWH-018 (6-OH-indole) (World Health Organization (WHO) 2014). The second phase of metabolism is glucuronidation via the UDP enzymes UGT1A1, UGT1A9 and UGT2B7. The glucuronic conjugates are afterwards excreted by urination. The metabolites of JWH-018 are psychoactive and can produce synergistic effect with the parent drug (WHO 2014). The maximum concentrations of JWH-018 detected in blood are less than 5ng/mL (Kacinko *et al*. 2011, Öztürk *et al.* 2015).

AM-2201 is lipophilic second generation SC and is also a full agonist at CB_1 receptor (Figure 11). Like JWH-018 the same issues of toxicity are associated with AM-2201, the pharmacology of AM-2201 is also similar to JWH-018. The metabolism for AM-2201 is almost identical as for JWH-018, with the same enzymes being involved in the breakdown of the parent compound and related metabolites. The major AM-2201 metabolites are AM-2201 N-(4-hydroxypentyl), AM-2201 6`-hydroxyindole, JWH-018 N-(5-OH-pentyl), JWH-

018 pentanoic acid JWH-073 N-(4-OH-butyl), JWH-073 butanoic acid (Figure 12). Typically, the concentrations of AM-2201 detected in blood does not exceed 5ng/mL and for metabolites it ranges from 0.1 to 12ng/mL (Carlier *et al.* 2018), while the dependence risk and withdrawal symptoms of AM-2201 is similar to that of JWH-018.

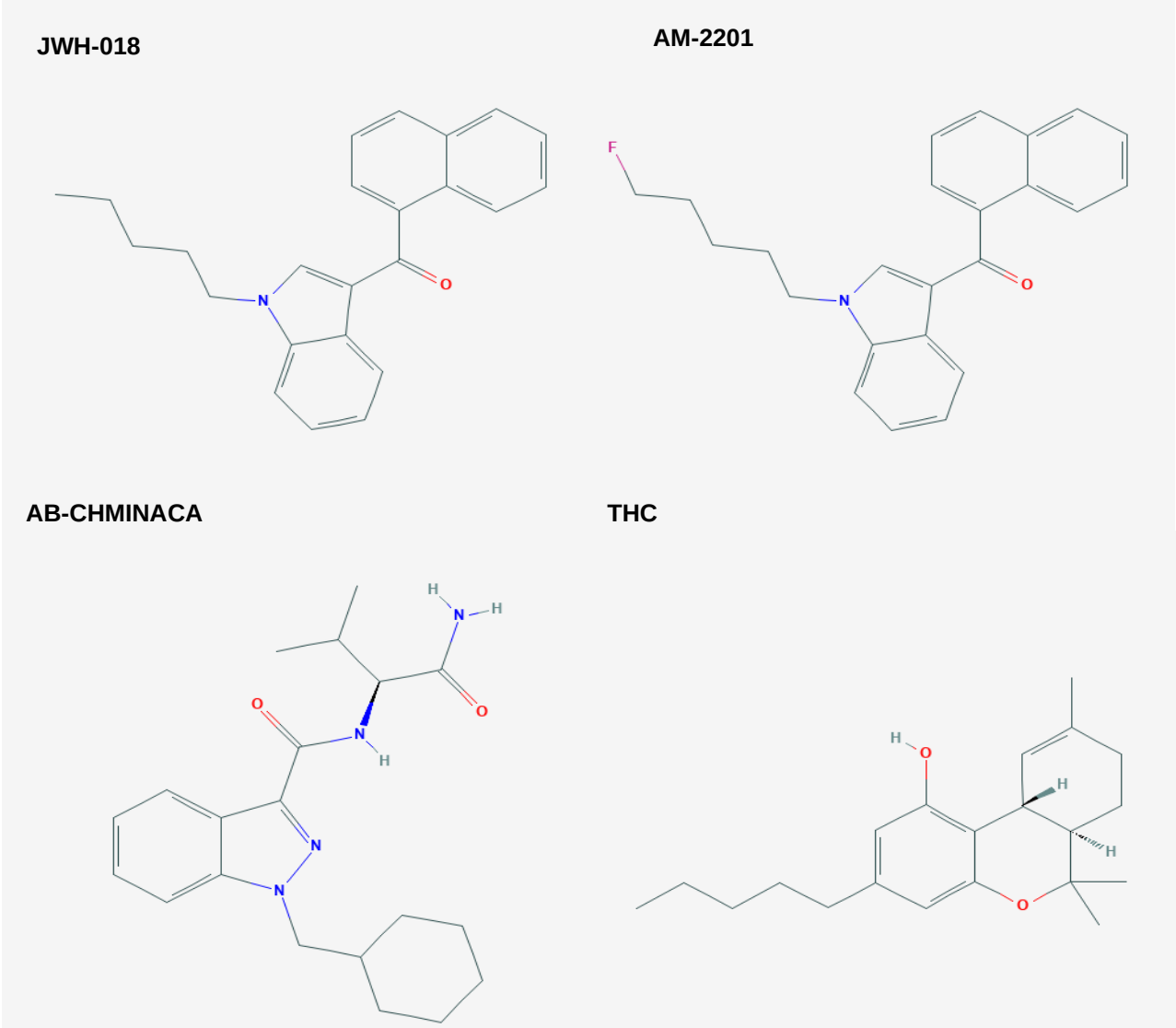


Figure 11: Structures of different generations of SC and of THC. JWH-018 is a "first generation" SC and is an aminoalkylindole (the core of it is an indole, has a pentyl tail, methanone link section and a naphthyl ring). It has 8 times higher binding affinity at CB_1 receptor than THC. AM-2201 is a "second generation" SC and is a fluorinated aminoalkylindole (the core of it is an indole, methanone link section, naphthyl ring and a 5-fluoropentyl tail). AM-2201 is the fluorinated version of JWH-018 and has higher binding affinity to CB_1 receptor than JWH-018. AB-CHMINACA is a "third generation" SC and an indazolecarboxamide (the core of it is an indazole, carboxamide link section, carbamoyl substitution and a cyclohexylmethyl tail). AB-CHMINACA is reported to have 4 times higher binding affinity to CB_1 receptor than JWH-018. THC is a natural cannabinoid (made of three rings, phenol, pyran, cyclohexane, an alkyl tail), whose effects the SC cannabinoids mimic, when they interact with CB_1 receptors. THC is a partial agonist at CB_1 receptor, while all of the mentioned SC are full agonists at CB_1.

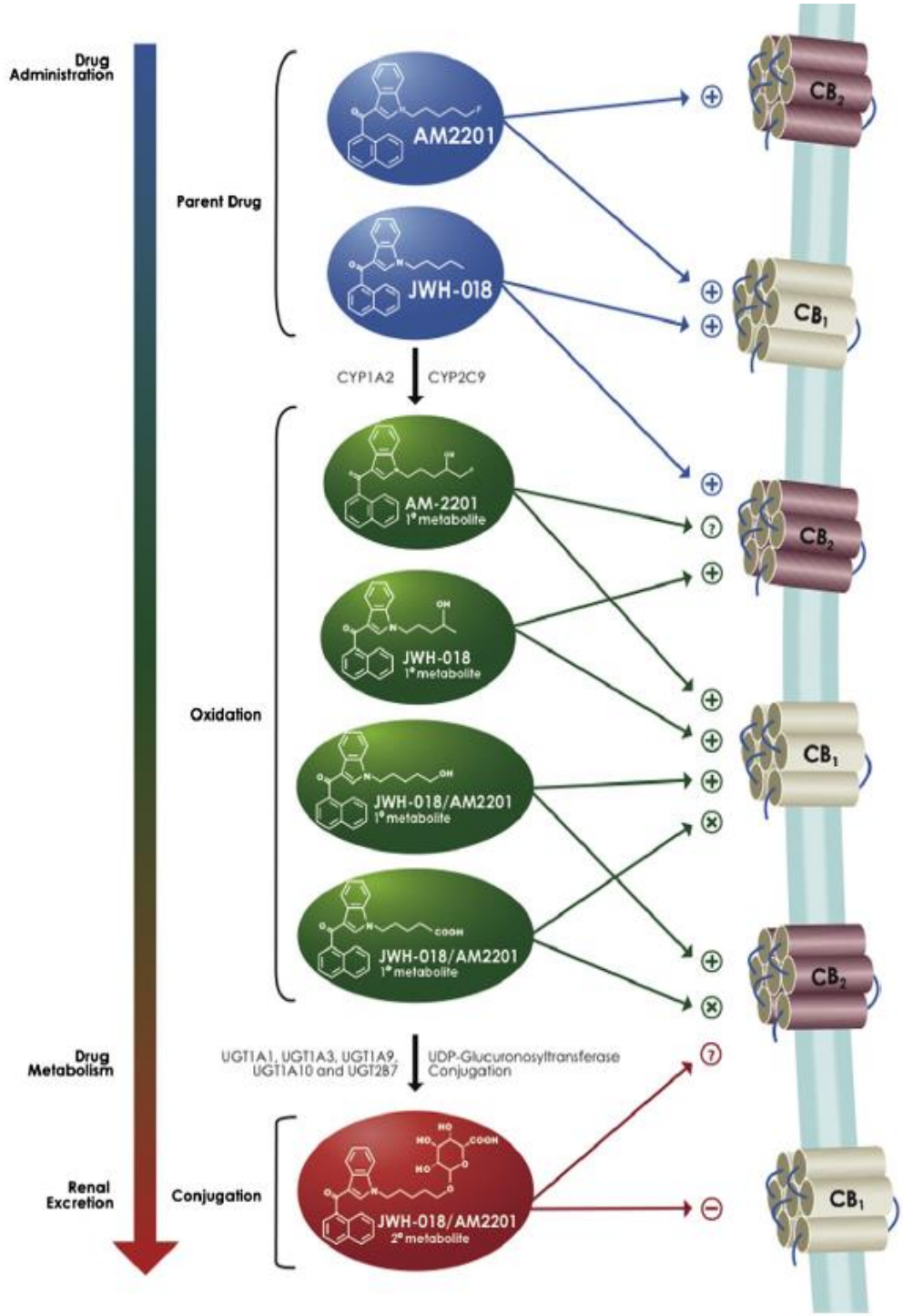


Figure 12: The metabolic pathway of AM-2201 and JWH-018. The notations + (agonist), - (antagonist), x (no binding affinity), ? (unknown). AM-2201 and JWH-018 are metabolised similarly, as AM-2201 is a fluorinated version of JWH-018. JWH-018 and AM-2201 are both full agonists at CB_1 and CB_2, several metabolites are also agonists at the cannabinoid receptors. The enzymes responsible for metabolism are mainly CYP1A2 and CYP2C9. Examples of metabolites produced only by AM-2201 are AM-2201 N-(4-hydroxypentyl), AM-2201 6`-hydroxyindole, by only JWH-018 is JWH-018 N-(3-OH-pentyl. Both SC have same metabolites JWH-018 N-(5-OH-pentyl), JWH-018 pentanoic acid JWH-073 N-(4-OH-butyl), JWH-073 butanoic acid. Second phase of metabolism is the glucuronidation followed by excretion of the metabolites in urine (Fangetrossi et al 2014).

AB-CHMINACA is a lipophilic third generation SC and is a full agonist at CB_1 receptor (Figure 11). AB-CHMINACA is 16 times more potent than THC. There is a high risk of acute toxicity with AB-CHMINACA, with even less toxicity data relating to this SC compared to JWH-018 and AM-2201. The reported adverse effects for AB-CHMINACA are depersonalisation, distorted perception of time, impaired motor performance, hallucinations, paranoia, confusion, fear, anxiety, dry mouth, “red eyes”, tachycardia, nausea, hyperemesis, delirium and violent behaviour. More severe effects include rapid loss of consciousness/coma, myocardial infarction, stroke, psychosis seizures and convulsions. At least 31 fatalities have been associated with AB-CHMINACA from the period of 2014 and 2017 (WHO 2017).

In vitro studies of AB-CHMINACA metabolism suggested there is up to 26 metabolites produced and the majority of these were confirmed in urine samples from AB-CHMINACA users (Erratico *et al.* 2015).The metabolites observed in urine samples were M1-M7, M9M M11, M21, M25 and M26 (Erratico *et al.* 2015, WHO 2017). Six mono-hydroxylated metabolites (M9-14) and six di-hydroxylated metabolites (M2-M7) are produced by CYP enzymes. The major enzyme of CYPs involved in the metabolism is CYP3A4, with minor contribution from CYP2D6, 2C9, 2C19, 2B6, 1A2. N-dealkylation of AB-CHMINACA by CYPs enzymes produce the M8 metabolite, which is further hydroxylated into M1 by CYPs. Two deaminated metabolites (M20, M21) are produced by amidase enzymes. M21 undergoes further metabolization by CYPs enzymes and produces mono hydroxylated metabolites (M15-M19). Glucuronidation for AB-CHMINACA metabolites by UGT enzymes results in 5 glucurinidated metabolites: M24 formed from M20, M25 and M26 formed from M21, and M22 and M23 formed from M14-M19. The typical concentrations of AB-CHMINACA in blood are low, with the highest reported concentrations to be below 5ng/mL for antemortem samples and for post-mortem samples the concentration ranged between 0.32 and 12ng/mL (median 3.7 ng/mL) (EMCDDA 2017b). The dependence risk of AB-CHMINACA is high, due to its short-lasting effects and potency with withdrawal symptoms being similar to those as described for JWH-018 (WHO 2017).

1.6 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Liquid Chromatography (LC) is a separation technique in which liquid mobile phase flows through solid/liquid stationary phase. The separation occurs based on partition and adsorption principles with the former being liquid-liquid chromatography and the latter liquid-solid chromatography (Agilent, 2016). The partition separation occurs as different analytes partition separately between mobile and stationary phases, which leads to different rates of migration. In adsorption separation, analytes adhere to the stationary phase at different strengths, which means those that adhere more strongly take longer to elute, and hence have a longer retention time (Bayne 2010). The schematic of a typical LC system and is

workflow is shown in Figure 13. Compounds can be detected by utilising ultraviolet (UV) detector coupled with a known retention time or for more quantitative and definitive analysis LC units can be coupled with mass spectrometer (MS) systems. MS is a powerful analytical technique used for quantitative and qualitative applications. The basic principle, of the mass spectrometry is illustrated in Figure 14.

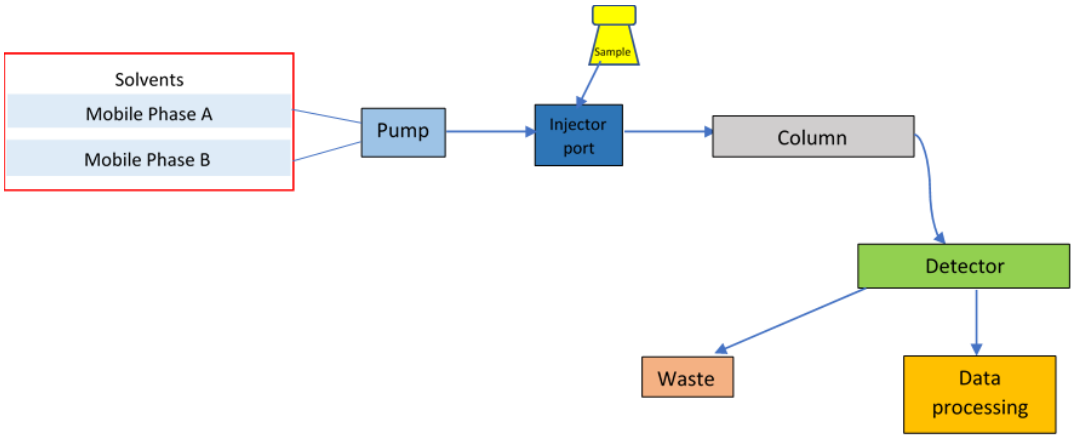


Figure 13: A basic workflow of a LC system. The mobile phase A is typically water with or without buffers and B is an organic solvent, for example methanol, with or without buffer. The mobile phases are pushed throughout the system by the pump. The pistons in the pump draw solvents from bottles to pulse damper, to the mixing chamber, purge valve and finally column. Sample is injected via a rotating valve system and is pushed through the column by solvents. The column sits in a thermostatted temperature compartment, the sample components interact with stationary phase inside the column and are separated based on their affinity to the stationary phase. From the column the sample components go to the detector, for example the mass analyser or UV and signals obtained are delivered to the computer for data analysis (Agilent 2016).

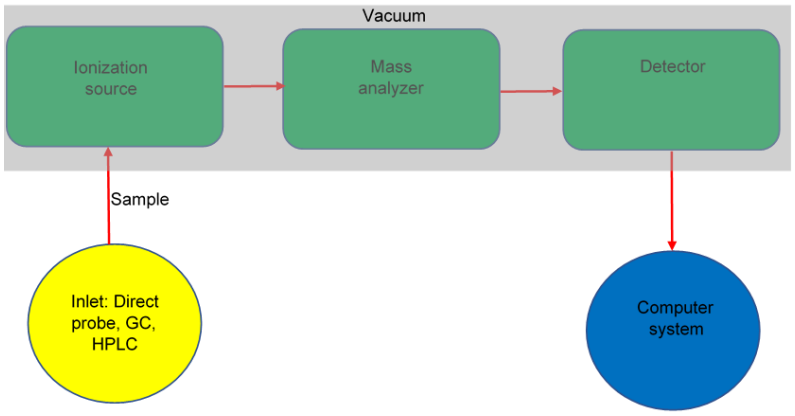


Figure 14: Basic overlook of mass spectrometer instrument. Sample is ionized in the ionisation source. Ions are separated in mass analyser according to their mass-to-charge ratio. Ions that emerge from the mass analyser are measured in the detector that converts the ions into electrical signals. The computer processes the electrical signals from the detector, data is produced. The system works under vacuum to allow ions to reach the detector, prevent ion collisions with other molecules (Cooper 2013).

The first step in MS is the production of gaseous ions of the compound. Ions are produced in the ionisation source. There are variety of ion sources, which can be utilised depending on the physico-chemical properties of analyte and the energy required during the ionisation process (Hoffmann 2007). Some ionisation techniques are soft, and transfer minimum energy during ionisation, while other techniques are highly energetic and cause extensive fragmentation of the drug (Hoffmann 2007). A common ionisation technique utilised in forensic toxicology is electrospray Ionisation (ESI). ESI is a soft ionisation technique that works under atmospheric pressure and can easily be coupled to a LC system (Hoffmann 2007). The sample from the LC comes to the ESI source through a thin needle. As the sample is constantly sprayed a high electrical potential is applied to the needle, resulting in the formation of highly charged droplets. These droplets are driven electrically and the solvent molecules in the sample are vaporised with aid of warm neutral gas, usually nitrogen. As the solvent contained in droplets is evaporated it causes them to shrink and their charge per unit volume to increase. There are three theories what happens afterwards, ion evaporation theory, charge residue theory and the chain ejection model. Ion evaporation theory states that as the droplet is decreasing in volume and is increasing in charge the repulsive forces (coulomb forces) increase. These forces will eventually exceed the surface tension of the solvent, causing what is known as the coulomb explosion, resulting in ions desorbing into the gas phase. The evaporation theory is associated with low molecular weight analytes (Konermann *et al.* 2013), while the charge residue model is thought to apply for large molecular weight analytes, such as proteins. The evaporation theory involves suggest that the solvent is evaporated and as the last solvent shell evaporates the charge of the vanishing droplet is transferred to the compound (Konermann *et al.* 2013). In the chain ejection model, which typically occurs for unfolded proteins, as the solvent is evaporated the chains of protein migrate to the solvent surface. Afterwards one chain terminus gets expelled into the vapor phase, which is followed by step wise ejection of remaining protein chains (Konermann *et al.* 2013). These models apply in both positive and negative ionisation modes (Konermann *et al.* 2013). The purpose of the ionisation step is to produce high enough energy to knock an electron off the drug molecule to form a positive ion. This ion is called the molecular ion or sometimes the precursor/parent ion.

Once a sample has cleared the ionisation stage it then enters the vacuum of the mass spectrometer wherein it is analysed for molecular weight and fragmentation pattern. The mass analyser is the section of the mass spectrometer where the ions are separated based on their mass-to-charge ratio. There is a variety of mass analysers, all of which use magnetic and electric fields to achieve separation. Mass analysers can be combined for better results and multiple experiments. A common mass analyser system used is the triple-quadrupole (QqQ), this is a hybrid system consisting of three quadrupole mass analysers. QqQ is a tandem system, as the analysis is performed in different mass analysers. QqQ

system can be broadly separated into three stages Q1, Q2 and Q3. In Q1, the parent ions are identified. Q1 filters the parent using the stability of the trajectories in oscillating electric fields to separate ions according to their mass-to-charge ratio (Hoffmann, 2007). Q1 is composed of four parallel electrical rods with varying direct current and alternating radio-frequency potentials. Ions formed in the ionisation source are pulsed towards the Q1 by an electric field. A positive ion will move in the direction of a negatively charged rod. If the potential is changed the ion will switch is movement path before striking the rod. If this occurs, they undergo oscillation (trajectory) and only ions with specific mass-to-charge range will survive the path to the collision cell (Q2). (Hoffmann 2007). Q2 consists of quadrupoles operating in only radio frequency (RF) mode and are used for focusing ions as collision cells. It is in this portion of the mass spectrometer, Q2, that ion fragmentation occurs and daughter ions are produced. Inside Q2 an ion collision with an inert gas (helium or nitrogen) occurs. This process is called collisionally activated dissociation (CAD) or collision-induced dissociation (CID). The result is the production of product (daughter) ions from the ions selected from first quadrupole, (precursor/parent ions). From the collision cell ions migrate to the last quadruple (Q3), where they are filtered and sent to the detector for quantitative analysis. (El-Aneed *et al.* 2009). The detector measures the current produced by the ions, a current which is then converted into a digital value and sent to the computer the system.

1.6.1 SC and natural cannabinoids analysis

The analysis of SC presents various challenges there are over 160 SC drugs in circulation, with a multitude of chemical structures (EMCDDA 2017a). Analytical methods need to be developed and constantly updated to keep up with the ever changing structures of the new analogues, for forensic toxicology laboratories this is time consuming and costly. In addition, the reference materials are not always available, are expensive and in many cases non-existent for the latest SC compounds (Favretto *et al.* 2013). For the new generation of SC a conventional method like LC-MS/MS is not always possible (Favretto *et al.* 2013). As new compounds have no reference materials and no defined fragmentation patterns frequently LC with high resolution mass spectrometry is needed to detect compounds based on their accurate mass (Aldlgan and Torrance 2016, Favretto *et al.* 2013). The detection window for compounds in blood for SC can be as short as 2-3 hours (Aldlgan and Torrance 2016). As a result there is a requirement for highly sensitive methods as these drugs are usually present at low concentrations in human matrices and frequently detected in blood in concentrations below 1ng/mL (Langford and Bolton 2018, Rojek *et al.* 2017). The stability of SC in biological matrices is also not known (Langford and Bolton 2018). Research to date has suggested that frequently no parent compounds are present in urine samples of SC users (Favretto *et al.* 2013). This coupled with no reliable knowledge of the metabolites results in many samples that might have SC present producing false negatives. Moreover,

some SC have similar metabolites, which poses problems for interpretation of the analytical data (Presley *et al.* 2016). Therefore, due to the cost, the required sensitivity of the methods, difficulty of obtaining reference materials and the supposed infrequent use of SC, they are frequently not part of the routine toxicological testing panel in numerous forensic toxicology laboratories in the UK.

Depending on sensitivity requirements different methods may be utilised, with MS based methods being the more sensitive (Citti *et al*. 2018). Screening analysis is usually carried out by gas chromatography with mass spectrometry (GC-MS) or LC-MS/MS, with latter being more sensitive and then used for quantitative analysis with additional ion transitions used to confirm the presence of the drug. The method of choice for analysis of cannabinoids in recent years has become LC-MS/MS (Citti *et al*. 2018).

1.7 Method development and validation

Method development for LC-MS/MS is task specific, dependent on what are the requirements of the customer or research that is being carried out. In general method development involves three processes: sample preparation, chromatography and mass spectrometric detection. The start of method development is the consideration of what analytes need to be analysed, concentration range of the analytes of interest and the matrix that will be analysed (Polettini 2006) Once the mentioned parameters are known, literature search about the analyte, previous methods should be carried out. The undertaken research helps to find out what are the best starting conditions for MS, what column to use for analysis and to select most efficient sample preparation technique. The first experiments that need to be carried out is the infusion of analytes into the MS. The next set of experiments is the development of chromatography method. Finally, a sample preparation technique needs to be developed. In forensic toxicology solid phase extraction (SPE) is considered the most reliable and sensitive technique for sample preparation and extraction (Bayne 2010).

After a method is developed it needs to be validated to assure reliability, applicability of the method for the required purposes and to establish method`s limitations. In forensic toxicology the governing bodies that give guidelines for method validation are: Scientific Working Group for Forensic Toxicology (SWGTOX), United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT), international standard for testing and calibration laboratories (ISO 17025) and also in the UK the Forensic Science Regulator (Elliott *et al.* 2018). The parameters that need to be considered and tested based on governing body guidelines are: accuracy (bias), precision, carryover, selectivity and specificity, matrix effect (ME), limit of detection (LOD), limit of quantitation (LOQ), linearity (calibration model), range, recovery (RE), reproducibility, repeatability, ruggedness, stability and dilution integrity. The general definitions for these parameters are presented in Table 4. Prior to starting any experiments, a validation plan needs to be created (SWGTOX 2013).

The plan sets out criteria for each parameter, experiments that will be performed and limits of the method to allow it to fit for use. All the experiments need to be carried out in the matrix, for which the method is intended to be used (SWGTOX 2013, Polettini 2006). In addition, some minimum criteria for parameters is set by UKIAFT, SWGTOX and ISO 17025. Moreover, criteria is set for methods, that are employed for analysis of samples related to Road Traffic Act 1988 Section 5A, by Forensic Science Regulator in FSR-C-133 (Forensic Science Regulator 2020).

Table 4: General definitions of parameters that need to be tested for method validation (SWGTOX 2013)

Parameter	Definition
Accuracy (Bias)	Measurement of closeness of the calculated value for the measurand and to the true value of a measurand.
Precision	The closeness of agreement of repeated measurements from multiple samples of same homogenous sample.
Linearity (calibration model)	A mathematical model that demonstrates relationship between the analyte signal and its concentration.
Range	The concentration that can be adequately determined.
Carryover	The appearance of analyte signal in a subsequent sample after analysis of positive sample.
Matrix effect	Suppression or enhancement of analyte signal due to interferences from the matrix.
LOD	The lowest concentration of the analyte that can be reliably differentiated from background noise.
LOQ	The lowest concentration that can be reliably measured.
Selectivity and specificity	The ability to detect, differentiate the analyte of interest, when there are other non-targeted analytes present, other drugs, impurities.
Recovery	The percentage of analyte response after sample preparation compared to solution of neat analyte of same concentration.
Ruggedness	The susceptibility of a method to small changes that might occur during day to day analysis, for example small temperature or pH variations.
Reproducibility	The preparation of samples by more than one analyst on separate days in same laboratory.

Table 4: (Continued)

Parameter	Definition
Repeatability	Testing the sample on different days to test the closeness of the values to one another on different days.
Dilution integrity	Testing the sample accuracy when using smaller volumes of sample than in the method.
Stability	Testing the variability of sample concentration when they are kept frozen, are not analysed on same day when they are prepared.

1.8 Aim and objectives of the project

Currently there is a lack of methods that analyse both natural and synthetic cannabinoids. Therefore, the aim of the research is to develop and validate a method that can quantify natural cannabinoids, THC and THC-COOH and several synthetic cannabinoids in a single run using LC-MS/MS.

The research objectives:

- Assess matrix effects utilising the techniques recommended by Matuszewski *et al.* (2003).
- Develop and validate method suitable for analysis of JWH-018, one metabolite of JWH-018, AM-2201, AB-CHMINACA. These SC have been chosen as they have reliable reference materials and are among the most commonly abused SC in the UK
- Validate the method based on the requirements of SWGTOX, UKIAFT and ISO 17025 standards.
- Develop and validate a method that meets the quality guidelines of road traffic toxicology casework samples in the UK.
- Develop and validate a method with sensitivity parameters that will allow effective detection of SC in real world samples.
- Perform robustness testing on the quantitation of THC samples in blood under conditions of dilutions and physiological changes to the sample matrix due to blood coagulation.

2. Methods and materials

2.1 Reagents and chemicals

The following reagents and chemicals were utilised in this project: liquid chromatography mass spectrometry (LC-MS) grade acetonitrile (ACN) (Fisher Scientific, Loughborough, UK), LC-MS grade water (Fisher Scientific), formic acid (Sigma Aldrich, Gillingham, UK), acetic acid (Fisher Scientific), defibrinated horse blood (TCS bioscience, Buckingham, UK), distilled water, LC grade methanol (MeOH) (Fisher Scientific), hexane (Fisher Scientific), ethyl acetate (Fisher Scientific), sodium acetate (Sigma Aldrich), sodium hydroxide (NaOH) solution (Fisher Scientific), hydrochloric acid (HCl) (Fisher Scientific) and potassium dihydrogen orthophosphate (Fisher Scientific).

The following Cerilliant (Gillingham, UK) solutions were used for calibration curve: THC 1.0 mg/mL, THC-COOH 100µg/mL, THC-COOH-D_3 100 µg/mL, THC-D_3 100µg/mL, JWH-018 100µg/mL, AB-CHMINACA 100µg/mL, "Spice Cannabinoid Mix 2" 100µg/mL, JWH-018 N4-hydroxypentyl 100µg/mL and JWH-018 N4-hydroxypentyl-D_5 100µg/mL. Cayman chemicals (Michigan, USA) solution AM-2201-D_5 5.0mg/mL was used for calibration curve. LGC (Teddington, UK) solution THC-COOH 100µg/mL was used for quality control (QC) samples. Sigma Aldrich solution THC 1.0mg/mL was used for QC samples.

Strata C18-E (55 µm, 70 A) 200 mg/3 mL cartridges from Phenomenex (Macclesfield, UK) were used for extraction procedure. 15mL conical centrifuge tubes from Fisher Scientific were employed for sample preparation. 5mL LABCO (Lampeter, UK) vials with 1% sodium fluoride/potassium oxalate were utilised for collection of whole blood. 6mL Vacutest (Arzergrande, ITL) clot activator tubes were utilised for collection of whole blood for clotting.

2.2 Reagent preparation

The following reagents were made and used for sample extraction and preparation.

2.2.1 Internal standard solution

To a 2.5mL volumetric flask the following solutions were added THC-COOH-D_3 100µg/mL (125µL), THC-D_3 100µg/mL (25µL), AM-2201-D_5 (100µL) (after diluting 5µL of the original AM-2201-D_5 5.0mg/mL solution in 995µL of MeOH), JWH-018 N4-hydroxypentyl-D_5 100µg/mL (25µL) and made up to the volume with MeOH, to get working solution e.

2.2.2 Solution for calibrators

To a 2.5mL volumetric flask the following solutions were added THC 1.0mg/mL (5µL), THC-COOH 100µg/mL (250µL), JWH-018 100µg/mL (25µL), "Spice Cannabinoid Mix 2" 100µg/mL (25µL), AB-CHMINACA 100µg/mL (25µL), JWH-018 N4-hydroxypentyl 100µg/mL (25µL) and made up to volume with MeOH to get working solution a (WSA). 100µL of WSA was added to a 1mL volumetric flask and made up to the volume with MeOH

to get working solution b (WSB). 100µL of WSB was added to a 1mL volumetric flask and made up to the volume with MeOH and labeled as working solution c (WSC).

2.2.3 Solution for QC

To a 5mL volumetric flask the following solutions were added THC 1.0mg/mL (5µL), THC-COOH 100µg/mL (250µL), JWH-018 100µg/mL (25µL), "Spice Cannabinoid Mix 2" 100µg/mL (25µL), AB-CHMINACA 100µg/mL (25µL), JWH-018 N4-hydroxypentyl 100µg/mL (25µL) and made up to the mark with MeOH, this solution was labelled as working solution d (WSD). 50µL of WSD was added to 200µL of MeOH in a 1mL volumetric flask and labelled as working solution f (WSF).

2.2.4 2M sodium acetate buffer (pH 4)

Distilled water (80mL) was added in a 0.1L volumetric flask to which sodium acetate (4.9g) and acetic acid (14.07mL) was added. The buffer was adjusted to final desired pH using HCl or NaOH. The solution was made up to the volume.

2.2.5 0.1M hydrochloric acid

To a 0.1L volumetric flask 4M HCl (2.5mL) was added and made up to the volume with distilled water.

2.2.6 0.1M phosphate buffer (pH 7)

Potassium dihydrogen orthophosphate (13.61g) was dissolved in distilled water (900mL). The pH was adjusted to pH 7.0 with NaOH or HCl and made up to 1L using distilled water.

2.2.7 Elution mixture

To a 20mL volumetric flask hexane (14mL) and ethyl acetate (6mL) was added. The ratio of hexane to ethyl acetate was 7:3 in the mixture.

2.3 Preparation of calibration line and QC samples

Table 5 outlines the volumes of working solutions utilised to prepare calibration curve and QC samples in blood matrix.

Table 5: Volumes of working solutions used for calibrators and QC to spike blood for extraction

Calibrator/QC	**Volume**	**Solution to use**	**Concentration THC/THC-COOH/SC (ng/µL)**	**Final volume**	**Final Concentration THC/THC-COOH/SC (ng/µL)**
Cal 1	50µL	WSC	0.01/0.05/0.005	1mL	0.5/2.5/0.25
Cal 2	10µL	WSB	0.1/0.5/0.05	1mL	1/5/0.5
Cal 3	40µL	WSB	0.1/0.5/0.05	1mL	4/20/2
Cal 4	10µL	WSA	1/5/0.5	1mL	10/50/5

Table 5: (Continued)

Calibrator/QC	Volume	Solution to use	Concentration THC/THC-COOH/SC (ng/µL)	Final volume	Final Concentration THC/THC-COOH/SC (ng/µL)
Cal 5	20µL	WSA	1/5/0.5	1mL	20/100/10
QC1 (low)	20µL	WSF	0.1/0.5/0.05	1mL	2/10/1
QC2 (high)	30µL	WSD	0.5/2.5/0.25	1mL	15/75/7.5

2.4 Sample preparation and extraction

2.4.1 Sample preparation

Samples, calibrators, blanks and QC standards had a starting volume of 1mL of whole blood in 15mL conical centrifuge tubes. It should be noted that, later experiments indicated that the sample volume can be reduced to 0.5mL. Internal standard (10ng/mL, 50ng/mL and 7.5ng/mL for THC, THC-COOH and SC respectively) was added to all samples, calibrators and QC, and vortexed thoroughly for ≈10s. Following this ACN (2mL) was added to all samples, calibrators, blanks and QCs, and vortexed for ≈10s, and centrifuged at 3500g at 20°C for 15 minutes. The supernatants were transferred to a new 15mL conical centrifuge tubes and diluted with 2M sodium acetate buffer (pH 4) (1mL) and vortexed for ≈10s. After sample preparation SPE extraction was performed as described below.

2.4.2 SPE extraction

The extraction process selected was the SPE method which was adapted from Aizpurua-Olaizola *et al.* (2017). SPE was selected as it achieves highest sensitivity, robustness of the extraction methods used in forensic toxicology. An additional vacuum step was added, after the wash steps to the method, to remove the matrix interferences, have a cleaner sample that will not overload column with interfering substances. Buffering solutions were tested to optimise SPE extraction. The solutions were tested in blood matrix in duplicates.

The SPE procedure was utilizing strata C18-E (55 µm, 70 A) 200 mg/3 mL cartridges. The cartridge was conditioned by sequential elution of: methanol (3mL), water (3mL), 0.1M phosphate buffer pH 7 (1mL). Afterwards the samples were applied to appropriate cartridges. The column was washed sequentially with water (2mL), 0.1M hydrochloric acid (2mL), methanol (200µL). The cartridges were dried under vacuum for five minutes. The elution solvent (hexane/ethyl acetate mixture) (1.5mL) was applied to each column and collected into LC vials. The eluted solvent was evaporated under nitrogen flow at 40°C. Samples were reconstituted in 160µL of 50/50 of mobile phase A (water 0.1% formic acid) and mobile phase B (ACN 0.1% formic acid) and vortexed for ≈20s.

2.5 Preparation of clotted blood samples

The study protocol was approved by The Faculty Research Ethics Committee (FREC) of Kingston University London, Ethics code: 1819063.1. Venous blood was taken from one male participant from the antecubital vein into 5mL LABCO vials and into 6mL Vacutest clot activator vials. Twenty clotted samples were made and ten whole venous blood samples that were used as a control for the variability of spiking, extraction. Both vials were spiked 2-3 minutes after blood collection (before blood clots in clot activator vials) with the same concentrations of analytes, vortexed and kept to homogenise for one hour in 4°C in refrigerator. Afterwards the samples were extracted and analysed as duplicates with a calibration line and QC samples alongside it. All QC samples passed the required criteria. Once sample analysis was complete samples were destroyed as per HTA guidelines.

2.6 Method validation experiments

2.6.1 Accuracy (bias)

Accuracy was assessed by using QC results obtained from repeatability and reproducibility batches, containing QC samples at two levels, low and high with two replicates. Five runs were examined in total. The accuracy was determined as a percentage by comparing the calculated value to the expected concentration. Accuracy for natural cannabinoids should not exceed 20%, as it is the minimum requirement proposed by FSR-C-133 (Forensic Science Regulator 2020). The accuracy for synthetic cannabinoids should not exceed 30%, as this is the common percentage utilised by other researchers (Borg *et al.* 2017, Freijo *et al.* 2014, Kneisel *et al.* 2013).

2.6.2 Precision

Precision was assessed using the QC results obtained from repeatability and reproducibility batches. Five different runs were looked at containing QC samples at 2 levels low and high with 2 replicates. Coefficient of variance (%CV) value was looked within run and within days. The within day precision was calculated by finding the standard deviation of duplicates, dividing it by the mean value and multiplying by 100. The between day precision was calculated by looking at all values for the QC (n=10), finding the standard deviation for them, dividing it by the mean of QC (n=10) and multiplying by 100. The precision for all analytes should not exceed 20% (SWGTOX 2013).

2.6.3 Linearity (calibration model)

Linearity was demonstrated by running a five point calibration curve five times. The concentrations for THC was in the range of 0.5-20ng/mL (0.5, 1, 4, 10 and 20ng/mL), concentrations for THC-COOH was 2.5-100ng/mL, (2.5, 5, 20, 50 and 100ng/mL), concentration for AM-2201, JWH-018, AB-CHMINACA, JWH-018 N4-hydroxypentyl 0.25-10ng/mL (0.25, 0.5, 2, 5 and 10ng/mL) to cover the expected working range of the analysis.

For linearity to pass the R^2 must be equal or greater than 0.99, as recommended by SWGTOX, UKIAFT and ISO 17025.

2.6.4 Range

For THC 5-20ng/mL, for THC-COOH 2.5-100ng/mL and for all SC 0.25-10ng/mL was the chosen range of the method. QC samples were run at concentrations covering the range of calibration curve to demonstrate acceptable accuracy and precision at low and high concentrations. The QC values had to achieve desired accuracy and precision for the analytes for the parameter to pass.

2.6.5 Carryover

Blank matrix samples were analysed immediately after the highest calibrator. The area counts in blank matrix sample was required to be less than 25% of the area counts of the lowest calibrator to be accepted free of carryover. However, if the area was above 25% it was expected that then ion ratio for the analyte fails for there not to be carryover (SWGTOX 2013).

2.6.6 LOD

Blank matrix spiked with analytes at (THC 0.25ng/mL, THC-COOH 1.25ng/mL, AM-2201, JWH-018, AB-CHMINACA, JWH-018 N4-hydroxypentyl 0.125ng/mL) was analysed alongside the calibration curve and QCs samples. The LOD was the lowest concentration at which the S/N ratio is greater or equal to 3:1 and achieves identification criteria (retention time, mass spectral ion ratios based on calibration curve of the run) (SWGTOX 2013).

2.6.7 LOQ

The LOQ for the analytes was the lowest calibrator. For LOQ it was expected that no significant difference between calculated value and expected value will be found and signal to noise ratio will be above or equal to 10:1. Also, the identification criteria (retention time, mass spectral ion ratios based on calibration curve of the run) had to be met.

2.6.8 Selectivity and specificity

Selectivity and specificity was tested by making independent solution containing analytes of interest, their internal standards and common drugs of abuse found in blood: alcohol, cocaine, diazepam and benzoylecgonine. Analytes were expected to meet the criteria of identification (retention time, mass spectral ion ratio based on calibration curve),

Also, a solution of blank blood with no analytes was analysed. Carryover criteria had to pass for this parameter to be valid.

2.6.9 Repeatability

A sample was spiked with analytes and analysed on two different occasions, alongside the calibration curves. No significant difference was expected to be found for the concentrations and the difference should not be more than 20%.

2.6.10 Reproducibility

This was assessed by preparing three batches on three different days by three different analysts but run on the same instrument, as recommended by FSR-C-133 and ISO 17025. Each batch contained a calibration curve consisting of five points and QC`s at two different concentrations. The QC samples had to pass.

2.6.11 Ruggedness

Results from different analysts was looked at. Different pH buffers (made on different days, by different people) was tested. Mass spectrometry source plate was cleaned before analysis, temperature change in the room and its effect on analysis monitored. Day to day variability should not affect the assay accuracy.

2.6.12 Matrix effect

The matrix effect was analysed based on Matuszewski *et al.* (2003) recommendation. Post extraction addition approach was used in which two sets of samples were compared. Set 1(A) consisted of samples spiked with analytes and their internal standards in 1mL of LC-MS grade methanol and afterwards reconstituted. Set 2(B) consisted of samples spiked with analytes and their internal standards before the evaporation of the elution solution following extraction of blank blood. The ion suppression or enhancement was established at low and high concentrations. Formula for calculating matrix effect was: ME%=(B/Ax100)-100. The values for ME should not exceed 25%, however if the values did exceed 25% an impact on LOD, LOQ and bias was looked and evaluated, to see if all 3 values are suitable, pass their required criteria.

2.6.13 Recovery

Recovery was established based on recommendations from Matuszewski *et al. (*2003). Pre spiked and neat sample sets were compared. Set 1(A) consisted of samples spiked with analytes and their internal standards in 1mL of LC-MS grade methanol and afterwards reconstituted. Set 3 (C) consisted of blank blood samples spiked with analytes and their internal standards followed by extraction procedure. Recovery was established at low and high concentrations. The formula for calculating recovery was: RE%=C/Ax100. It was expected that the recovery will be above 80% if not it should not affect the bias and LOQs.

2.6.14 Stability

Stability was tested by analysing the same blood sample, spiked with analytes, on two different occasions, on day one and then after a week (condition 1). The blood sample concentrations were expected to be similar on both days when analysed (not different by more than 20%).

Stability was also tested by extracting samples and keeping them in the refrigerator (4°C) for two weeks (condition 2). The extracted samples after being kept in the refrigerator were expected to pass bias and linearity criteria.

2.6.15 Dilution Integrity

Sample dilution of 1:2 was tested. Into 0.5mL of fortified blood 0.5mL of blank blood was added to dilute it by 1:2. Sample afterwards was spiked with internal standard and standard procedure for extraction was followed (method 1 of dilution). Also, dilution integrity was tested by extracting only 0.5mL of fortified blood instead of 1mL (method 2 of dilution). Samples bias was expected not exceed 20% from the expected values, identification criteria to be achieved (retention time, ion ratios, based on the calibration curve of the run).

2.7 Instruments and laboratory equipment

LC used was Agilent (Cheshire, UK) 1260 Infinity Binary Pump. MS used was Agilent Triple Quad LC/MS 6430. Two columns were employed for method analysis Agilent InfinityLabPoroshell 120 EC-C18 2.1 x 75 mm, 2.7 µm, narrow bore LC column (Agilent 2013) and Phenomenex Kinetex 2.6 µm polar C18 100A, 150 x 3.0mm column. Pipettes utilised were VWR (Lutterworth, UK) positive displacement pipette 1 -10 µL, VWR positive displacement pipette 10 -100 µL and VWR positive displacement pipette 100 -1000 µL.

2.8 Instrument parameters

The parameters for ion source were: positive electrospray, gas temperature at 350°C, gas flow at 12L/min, nebulizer 25psi, capillary +4000 voltage(V) and EMV+ 400.

The parameters for LC were: mobile phase A water 0.1% formic acid, mobile phase B ACN 0.1% formic acid, temperature 20°C, stop time for LC pump 10.6 min, injection with needle wash (ACN as wash solvent), injection volume 20µl, injection draw position -1.6mm, injection draw speed and eject speed 200µl/min and auto sampler temperature 20°C. The gradient utilised for the method is described in Table 6.

Table 6: LC Gradient Elution Method Parameters

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (mL/min)	Max pressure (bar)
0	60	40	0.35	500
1	60	40	0.35	500
2.5	10	90	0.35	500
8	10	90	0.35	500
8.5	60	40	0.35	500
10.5	60	40	0.35	500

3. Results

3.1 Method development

3.1.1 Infusion

Post column infusion with a syringe pump at the speed of 20µL/min and MassHunter Optimiser software were used to construct the analyte database. The settings for the ion source were the same for all analytes, as described in the method section 2.7. All analytes were infused individually with the optimiser software settings as described in Table 7. All samples were made in 50:50 (mobile phase A, water 0.1% formic acid and mobile phase B, ACN 0.1% formic acid mix) for infusion with a concentration of 50ng/mL for all compounds. During the infusion a continuous flow of solvent is pumped into the ion source. The optimiser software identifies the parent ion and the optimum fragmentor energy and collision energy for the parent ion to target and quantifier ion transitions. The results of all compounds precursor ions and product ions, energies are presented in Table 8.

Table 7: Optimiser parameters used for infusion of natural and synthetic cannabinoids

Parameter	Value
Optimisation dwell time	20ms
Fragmentor course range	60-180V
Collision energy range	10-70V
Low mass cut-off	50m/z

Table 8: Dynamic multiple reaction monitoring (dMRM) transitions of natural and synthetic cannabinoids.

Analyte name	Precursor ion (amu)	Retention time (min)	Retention time window (min)	Fragmentor (V)	Product ions (amu)	Collision energy (V)	Dwell time (ms)
THC	315.2	6.340	1.19	84	**193.2** 123.0 259.2	21 32 18	78.64 78.64 78.64
THC-D_3	318.2	6.367	1.19	122	196.2	21	78.64
THC-COOH	345.2	4.960	0.94	96	**327.2** 299.2 193.1	13 17 25	35.63 35.63 35.63

Table 8: (Continued)

Analyte name	Precursor ion (amu)	Retention time (min)	Retention time window (min)	Fragmentor (V)	Product ions (amu)	Collision energy (V)	Dwell time (ms)
THC-COOH-D_3	348.2	4.940	1.19	76	330.2	12	40.52
JWH-018	342.2	5.580	1.01	94	**155.1** 214.2 127.0	21 24 44	49.40 49.40 49.40
AM-2201	360.2	4.960	0.97	122	**155.1** 127.1 232.2	26 54 22	37.45 37.45 37.45
AM-2201-D_5	365.2	4.950	0.96	122	155.0	25	35.63
AB-CHMINACA	357.2	4.480	1.17	98	**241.3** 312.4 340.3	26 14 10	46.19 46.19 46.19
JWH-018 N4-hydroxypentyl	358.2	4.390	1.19	122	**155.1** 127.1	22 54	62.10 62.10
JWH-018 N4-hydroxypentyl-D_5	363.2	4.370	1.15	98	155.0	22	69.57

Amu - atomic mass unit, in bold the quantifier ion

3.1.2 LC gradient development

The development of the LC method was based on a literature search of methods that previously analysed cannabinoids. At the beginning of method development Kinetex polar C18 column was selected as well as the solvents to be used, water 0.1% formic acid and ACN 0.1% formic acid, initial conditions for the gradient (Table 9). A method was developed that could detect THC and THC-COOH for the column, with the prospect of adding SC at a later stage the method development and validation process. Calibration and with QCs samples were run on the Kinetex polar C18 column for THC and THC-COOH. Both calibration lines and QCs run on the column passed their required criteria. However, it later emerged that repeated experiments and analysis of sample extracts led to a significant decline in the column's performance. This resulted in instability in the retention time of THC and THC-COOH and a corresponding loss of sensitivity due poor analyte resolution. Modifications of the chromatography and extension of the gradient runtime by 10 minutes were carried out. These alterations indicated that the analytes shifted by six minutes to the right and changed the order in which they were eluting. Moreover, the sensitivity for the

analytes decreased as well, previously 0.5ng/mL of THC could be detected, but after the column problem the lowest concentration that could be quantified was approximately 2ng/mL. The data relating to these method development experiments is detailed in the appendices (Column issue section). Due to the issues another column was selected Agilent InfinityLabPoroshell 120 EC-C18, this was utilised with same solvents and gradient (Table 9). Initial conditions for the method were not achieving separation, THC was not eluting during the run, only THC-COOH peak was detected. The gradient was changed from 70% B to 90% B and the run extended to 13 minutes. The new gradient achieved adequate separation for the compounds. Later the run time was optimised. The final method chosen is described in method section 2.7 (Table 6).

Table 9: Gradient of method 1, the initial method used for method development for, which did not elute THC in the run

Time (min)	Mobile Phase A (%)	Mobile phase B (%)	Flow rate (mL/min)	Max pressure (bar)
0	60	40	0.35	500
1	60	40	0.35	500
2.5	30	70	0.35	500
9	30	70	0.35	500
9.5	60	40	0.35	500
11	60	40	0.35	500

3.1.3 LC and MS optimisation

Once an acceptable gradient was found the method was optimised to achieve good sensitivity, robustness.

3.1.3.1 Ion source parameters

Optimisation of the ion source was carried out by testing different flow rates and nebulizer pressures, which have an influence on response sensitivity (Table 10). The results indicated that the initial conditions selected for infusion, should be used (underlined parameters), as these parameters achieved best signal to noise ratio for the analytes.

3.1.3.2 Injection volume

The injection volume of 20µL was chosen to achieve the required sensitivity of below 1ng/mL.

Table 10: Different ion source parameters and their effect on response of the analytes

Parameters tested for the ion source	THC signal to noise ratio (2ng/mL sample)	THC-COOH signal to noise ratio (10ng/mL sample)
Nebulizer 25psi, flow 12L/min	488.6	150.8
Nebulizer 40psi, flow 12L/min	420.6	135.2
Nebulizer 40psi and flow 10L/min	320.2	101.5

Underlined text denotes the parameters that were most sensitive and chosen for the method

3.1.3.3 Ion scan type

dMRM scan type was selected, as this enables the software to optimise and select dwell times for each ion, thereby achieving a higher sensitivity.

3.1.3.4 Reconstitution solvent

Different reconstitution volumes for samples were tested (Figure 15). A volume of 160µL was chosen, as it achieved better results than higher volumes and allowed sufficient sample volume for a reinjection.

Multiple reconstitute solvents were tested (Figure 16). A 50:50 mix of mobile phase A (water 0.1% formic acid) and mobile phase B (ACN 0.1%formic acid) was chosen, as it achieved higher sensitivity (Figure 16).

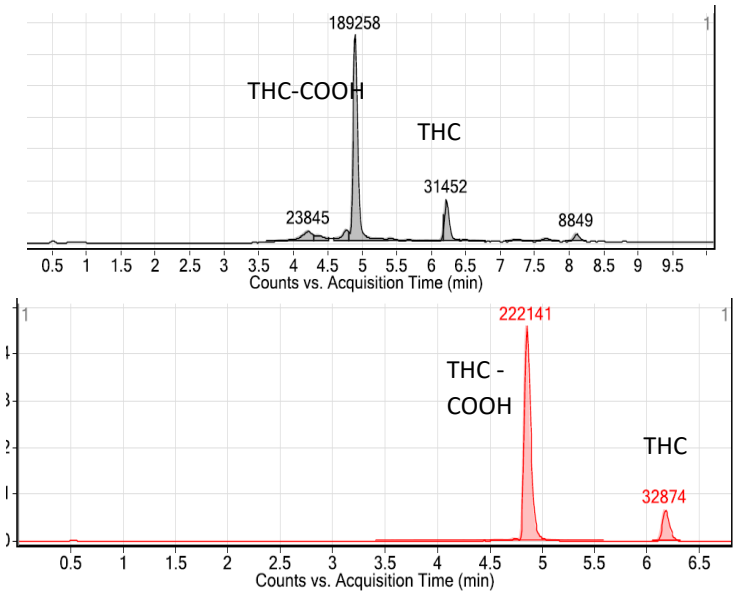


Figure 15: Different reconstitute volumes for same concentration sample, upper 200µL, lower 160µL. With the lower reconstitute volume, higher response for the analytes was achieved.

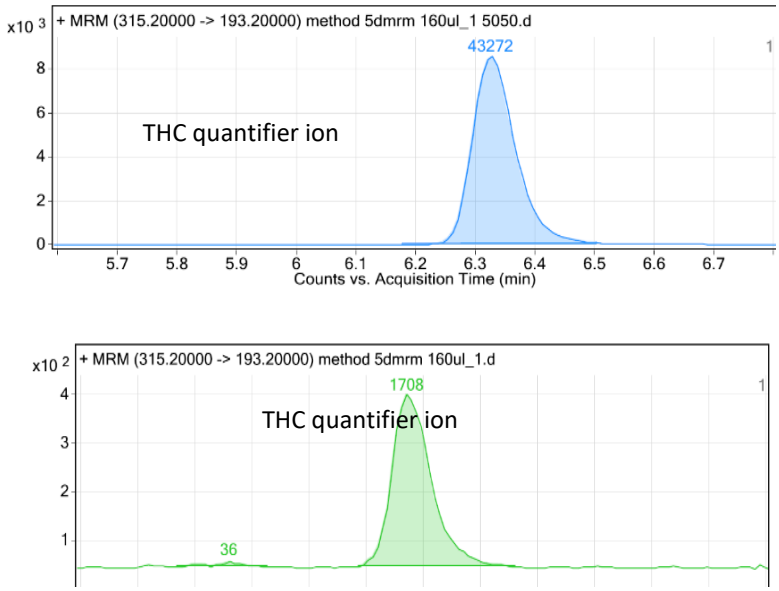


Figure 16: Different reconstitute solvents test. Upper result is for 50:50 of mobile phase A and B at 160µL volume, bottom one is mobile phase A at 160µLvolume. The results clearly show that using A:B mix for reconstitution is the better option, as it achieves higher response.

3.1.4 Extraction optimisation

Experiments were carried out to optimise extraction (Table 11). Different buffering solutions were tested in blood matrix in duplicates. The recovery percentage was calculated based on Matuszewski *et al. (*2003). The final method chosen was with pH4 and pH7 buffering conditions in place of pH4 and pH3 buffers described by Aizpurua-Olaizola *et al*. (2017). The pH4 and pH7 buffering conditions were chosen as they achieved the highest recovery percentages for THC and THC-COOH. It should be noted that these experiments were carried out only with THC and THC-COOH in the matrix, therefore the final recovery was different, when more analytes were present in the matrix.

Table 11: Extraction experiment results for THC and THC-COOH

Preparation method	**THC recovery (%)**	**THC-COOH recovery (%)**
Sample diluted with pH 4, buffer used pH3, first wash HCl then with water	58.77	36.91
<u>Sample diluted with pH 4, buffer used pH7,</u>	102.76	51.11
Sample diluted with pH 7, buffer used pH7,	151.08	23.80
Sample diluted with pH 4, buffer used pH3 (original method from the paper)	166.16	25.17

Underlined text denotes the parameters that were chosen, achieved best results

3.2 Method validation results

The summarised validation results are presented in Table 12.

Table 12: Summary of method validation results

Criteria	Result
Accuracy	All analytes with the exception of JWH-018 passed required criteria.
Precision	All analytes with the exception of JWH-018 passed the required criteria.
Linearity (Calibration model)	R^2 was above 0.99 for all analytes.
Range	All analytes passed the required criteria.
Carryover	No carryover was observed after the highest calibrator for any of the analytes.
LOD	THC, THC-COOH and AB-CHMINACA LOD=LOQ. JWH-018, AM-2201 and JWH-018 N4-hydroxypentyl LOD=0.125ng/mL.
LOQ	All LOQs, with exception JWH-018 passed.
Selectivity and specificity	All analytes passed the required criteria
Repeatability	All analytes passed the required criteria with exception of JWH-018
Reproducibility	All analytes passed the required criteria with exception of JWH-018
Ruggedness	All analytes passed the required criteria with exception of JWH-018
Matrix effect	All analytes have matrix effect higher than 25%, however the LOQs, bias was not affected for all analytes with exception of JWH-018
Recovery	All analytes have a recovery below 80%, however the LOQs, bias was not affected for all analytes with exception ofJWH-018
Stability	Extracted samples were stable for two weeks in the refrigerator and sample spiked with analytes and stored in the refrigerator was stable for a week, for all analytes with exception of JWH-018
Dilution integrity	All analytes passed the required criteria

3.2.1 Accuracy

The accuracy results for QCs are presented in Table 13 and Table 14. QC1 was for THC 2ng/mL, THC-COOH 10ng/mL, SC 1ng/mL, while QC2 was for THC 15ng/mL, THC-COOH 75ng/mL, SC 7.5ng/mL. The accuracy of analytes, which had respective internal standards (THC, THC-COOH, AM-2201, JWH-018 N4-hydroxypentyl) was in the range of 20%. The accuracy of AB-CHMINACA, which did not have a respective internal standard was 30%. For JWH-018, which also did not have a respective internal standard, QCs were outside the 30% range, however JWH-018 fail rate for QC 1 was 2 in 10, while for QC2 1 in 10. The method can quantify all analytes with an acceptable accuracy, with exception of JWH-018.

Table 13: Accuracy results. The mean of 10 results for QC1 and QC2

Analyte name	Mean accuracy (%) QC1	Mean accuracy (%) QC2
THC	96.94 (86.59-108.54)	94.54 (82.75-105.57)
THC-COOH	103.75 (95.58-117.87)	97.68 (87.40-103.71)
JWH-018	109.24 (79.18-150.9)	107.45 (84.41-132.16)
AM-2201	101.06 (89.89-108.63)	100.13 (86.52-108.55)
AB-CHMINACA	101.25 (78.55-112.81)	103.65 (95.03-112.03)
JWH-018 N4-hydroxypentyl	100.04 (87.16-108.79)	101.21 (92.31-108.03)

Table 14: Accuracy results. Calculated concentrations range and pass rate of QC1 and QC2 in five calibration runs

Analyte name	Calculated concentration QC1 range (ng/mL) [pass range]	Pass rate for QC1 (%) (n=10)	Calculated concentration QC2 range (ng/mL) [pass range]	Pass rate for QC2 (%) (n=10)
THC	1.73-2.17 [1.6-2.4]*	100	12.49-15.84 [12-18]*	100
THC-COOH	9.56-11.79 [8-12]*	100	65.55-77.79 [60-90]*	100
JWH-018	0.79-1.51 [0.7-1.3]^	80	6.33-9.91 [5.25-9.75]^	90
AM-2201	0.89-1.09 [0.8-1.2]*	100	6.49-8.14 [6-9]*	100

*20% range from expected value; ^30% range from expected value

Table 14: (Continued)

Analyte name	Calculated concentration QC1 range (ng/mL) [pass range]	Pass rate for QC1 (%) (n=10)	Calculated concentration QC2 range (ng/mL) [pass range]	Pass rate for QC2 (%) (n=10)
AB-CHMINACA	0.78-1.12 [0.7-1.3]^	100	7.13-8.27 [5.25-9.75]^	100
JWH-018 N4-hydroxypentyl	0.87-1.09 [0.8-1.2]*	100	6.92-8.10 [6-9]*	100

*20% range from expected value; ^30% range from expected value

3.2.2 Precision

The results for precision experiments is presented in Table 15. The %CV value was below 20% for all analytes with exception of JWH-018, which had %CV value above 20% but below 30%. The results show methods applicability to be used repeatedly for all analytes, with exception of JWH-018.

Table 15: Precision results for the analytes. Within day precision, looking at five different days and between day, overall precision

Analyte name	%CV QC1 within day	%CV QC2 within day	%CV QC1 between day	%CV QC2 between day
THC	1- 8.22 2- 2.91 3- 5.18 4- 0.88 5- 6.77	1- 0.36 2- 2.48 3- 0.60 4- 0.88 5- 1.66	6.08	9.50
THC-COOH	1-1.61 2-1.57 3-7.67 4-0.19 5-5.51	1-1.48 2-1.25 3-2.92 4-0.97 5-1.60	6.63	5.05
JWH-018	1-4.42 2-19.50 3-13.98 4-25.25 5-3.00	1-9.70 2-22.05 3-8.91 4-3.66 5-11.08	19.83	13.49

Table 15: (Continued)

Analyte name	%CV QC1 within day	%CV QC2 within day	%CV QC1 between day	%CV QC2 between day
AM-2201	1-0.28 2-0.87 3-9.44 4-0.40 5-4.66	1-1.56 2-2.01 3-2.90 4-0.72 5-1.66	4.99	8.30
AB-CHMINACA	1-10.90 2-7.18 3-7.19 4-8.98 5-1.36	1-3.50 2-4.92 3-1.15 4-1.98 5-0.89	10.21	5.07
JWH-018 N4-hydroxypentyl	1-3.02 2-2.45 3-9.81 4-0.85 5-4.43	1-0.01 2-1.57 3-2.73 4-0.46 5-1.61	5.59	4.77

1,2,3,4,5 represents different days, different batches were analysed

3.2.3 Linearity (Calibration model)

Linearity results for five batches is presented in Table 16 and Figures 17-22. The method has passed the required linearity criteria, as R^2 value was above or equal 0.99 for all of the analytes. The method can reliably quantify analytes, in the calibration range specific for analytes.

Table 16: Linearity data obtained at the method validation stage

Analyte name	Linearity R^2 day 1	Linearity R^2 day 2	Linearity R^2 day 3	Linearity R^2 day 4	Linearity R^2 day 5
THC	0.9908	0.9967	0.9951	0.9930	0.9951
THC-COOH	0.9962	0.9980	0.9925	0.9948	0.9925
JWH-018	0.9907	0.9923	0.9952	0.9969	0.9952
AM-2201	0.9965	0.9972	0.9940	0.9905	0.9940
AB-CHMINACA	0.9978	0.9987	0.9954	0.9936	0.9954
JWH-018 N4-hydroxypentyl	0.9973	0.9975	0.9948	0.9953	0.9948

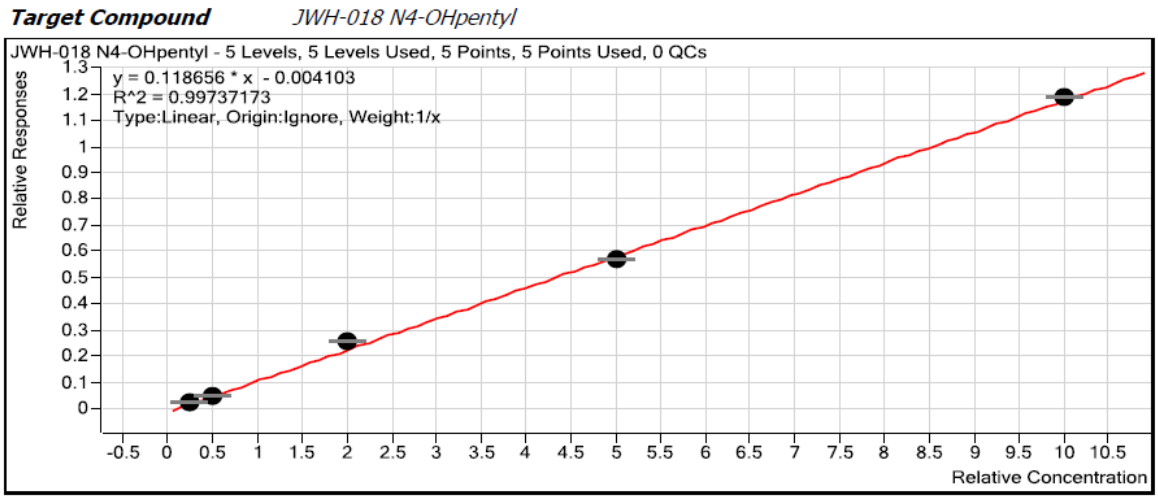


Figure 17: Representative calibration line of JWH-018 N4-hydroxypentyl, run on 10th February 2020

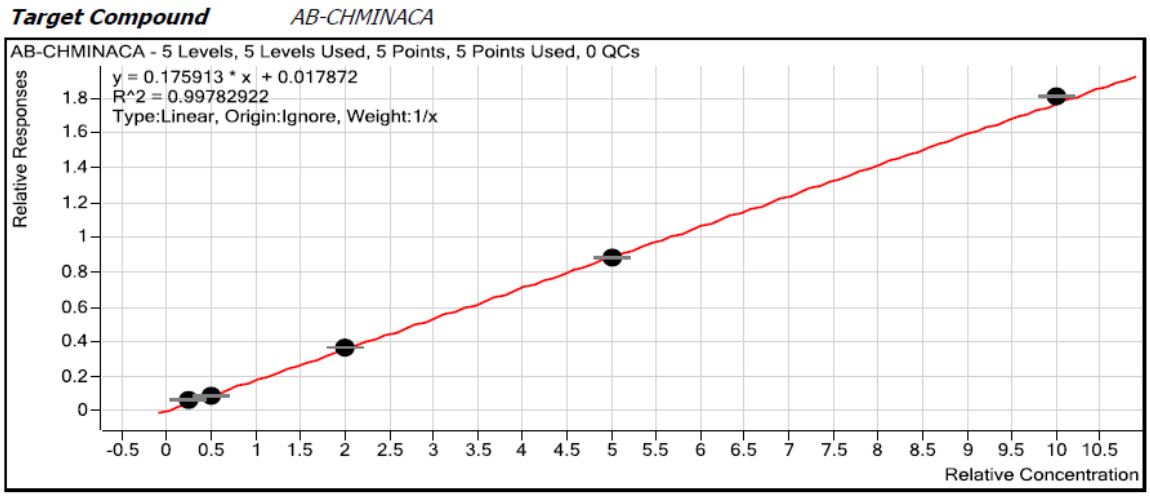


Figure 18: Representative calibration line of AB-CHMINACA, run on 10th February 2020

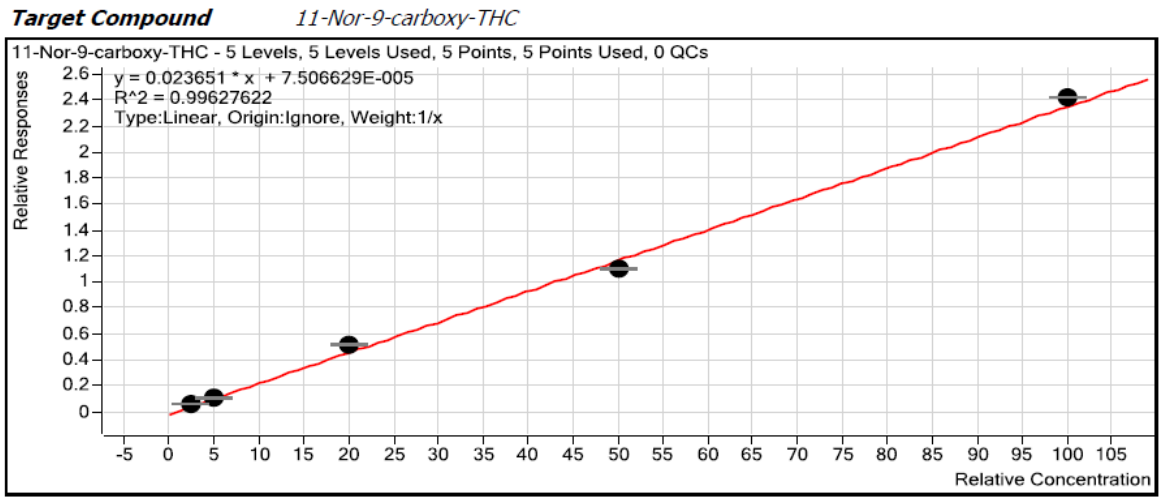


Figure 19: Representative calibration line of THC-COOH, run on 10th February 2020

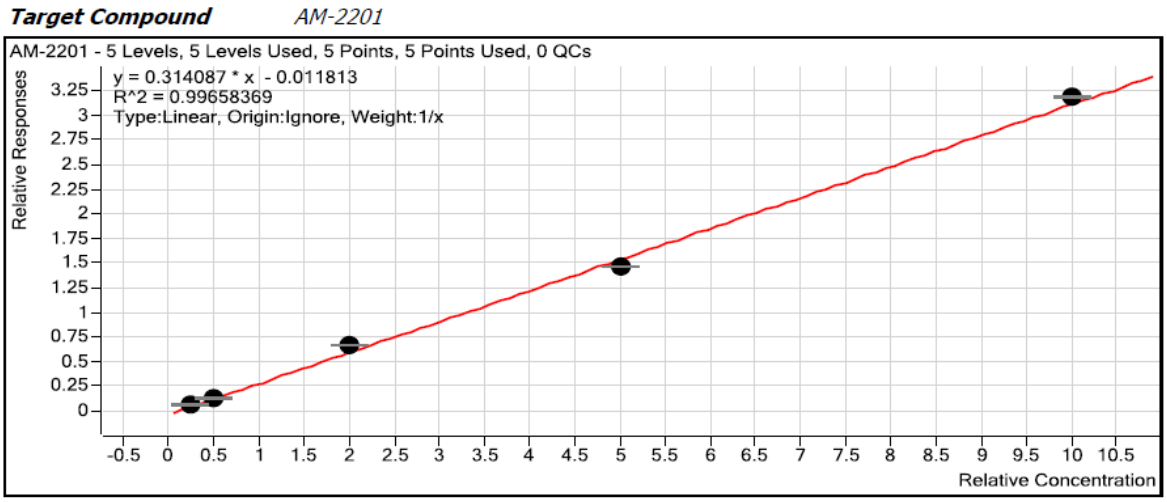


Figure 20: Representative calibration line of AM-2201, run on 10th February 2020

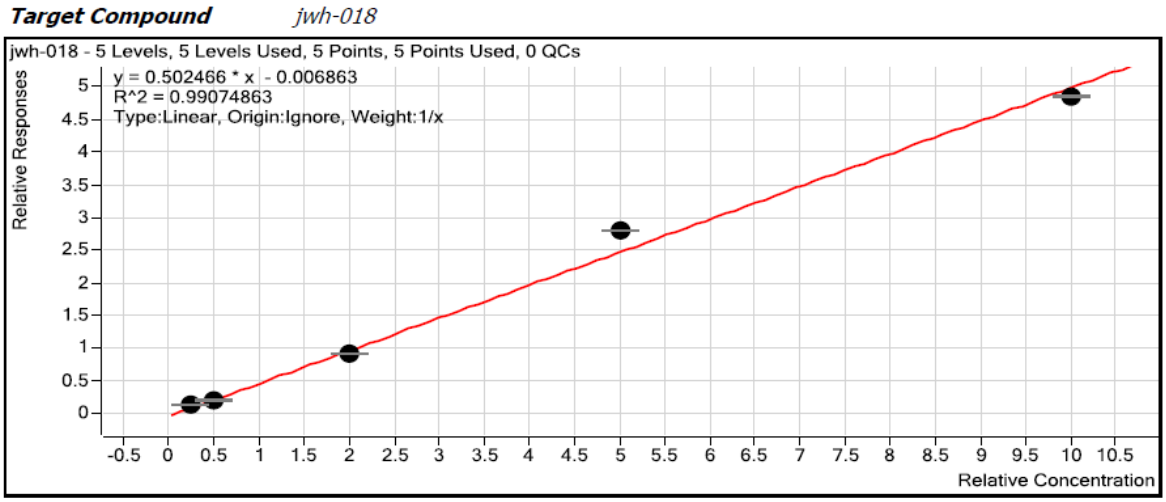


Figure 21: Representative calibration line of JWH-018, run on 10th February 2020

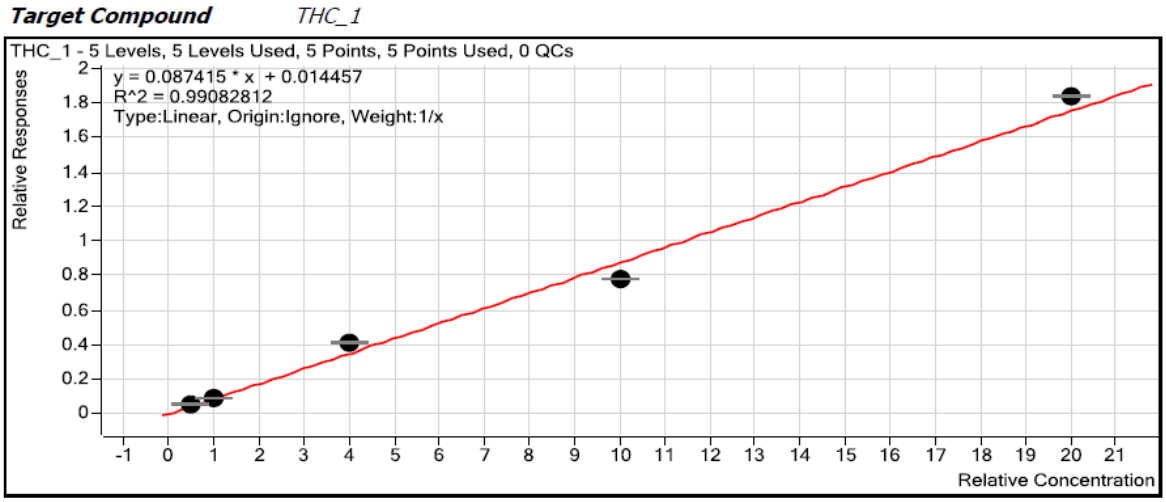


Figure 22: *Representative calibration line of THC, run on 10th February 2020*

3.2.4 Range

All analytes with exception of JWH-018 passed the required criteria (accuracy and precision section), with JWH-018 having a fail rate of 2 in 10 for QC1 (low concentrations) and 1 in 10 for QC2 (high concentrations).

3.2.5 Carryover

No carryover was observed for any of the analytes after the highest calibrator (Table 17).

Table 17*:* Carryover after the highest calibrator was tested on three different days

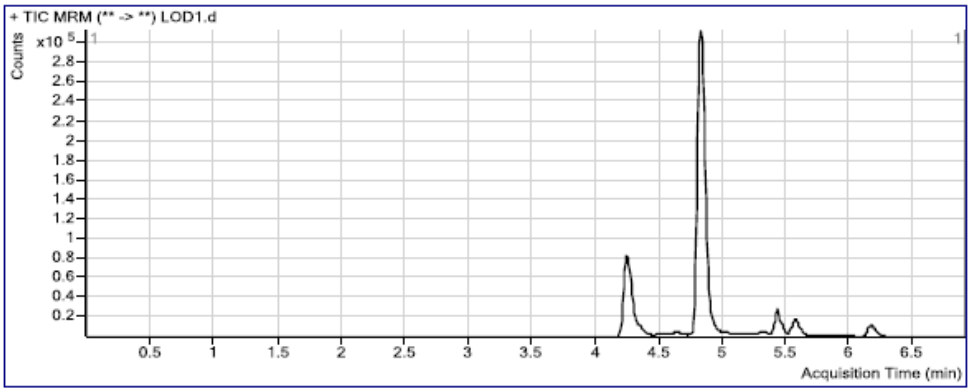
Analyte name	Signal less than 25% of LOQ	Ion ratio
THC	No	Fail
	Yes	Fail
	Yes	Fail
THC-COOH	Yes	Fail
	Yes	Fail
	Yes	Fail
JWH-018	Yes	Pass
	Yes	Pass
	Yes	Pass
AM-2201	Yes	Pass
	Yes	Pass
	Yes	Pass
AB-CHMINACA	Yes	Fail
	Yes	Fail
	Yes	Fail
JWH-018 N4-hydroxypentyl	Yes	Fail
	Yes	Fail
	Yes	Fail

3.2.6 LOD

The results for LODs is presented in Table 18, it should be noted as quantitative results were below calibration curve the accuracy for them cannot be assured. The LODs for JWH-018, AM-2201, JWH-018 N4-hydroxypentyl were determined to be 0.125ng/mL, as it this concentration signal to noise ratio, ion ratios, and retention time passed the required criteria. The LODs for THC, THC-COOH, AB-CHMINACA were found to be the same as LOQs, as it can be seen in Table 18, the ion ratios for these analytes fail at concentrations lower than LOQ. Figures 23-26, present the representative results, ion ratios of the LODs for analytes.

Table 18: LOD result summary. Three LODs were analysed

Analyte name	Accuracy (%)	Signal to noise ratio	Ion ratio	Retention time (min)
THC	120.20	37	Fail	6.16
	119.20	35	Pass	6.22
	146.04	7	Fail	6.27
THC-COOH	114.64	6	Fail	4.82
	107.35	11	Fail	4.84
	114.96	11	Fail	4.89
JWH-018	122.48	266	Pass	5.43
	119.92	36	Pass	5.46
	96.48	160	Pass	5.51
AM-2201	135.44	359	Pass	4.83
	137.76	496	Pass	4.86
	130.72	231	Pass	4.89
AB-CHMINACA	172.08	215	Pass	4.34
	172.72	224	Fail	4.36
	168.80	384	Fail	4.41
JWH-018 N4-hydroxypentyl	113.28	96	Pass	4.25
	108.88	78	Pass	4.28
	102.72	76	Pass	4.32



Compound	ISTD	RT	S/N	Final Conc	
JWH-018 N4-OHpentyl	JWH-018 N4-OHpentyl D5	4.256	96.817	0.1416	ng/ml
AB-CHMINACA	JWH-018 N4-OHpentyl D5	4.342	215.375	0.2151	ng/ml
11-Nor-9-carboxy-THC	11-Nor-9-carboxy-THC-D3	4.827	6.713	1.4330	ng/ml
AM-2201	AM2201-D5	4.838	359.299	0.1693	ng/ml
jwh-018	JWH-018 N4-OHpentyl D5	5.436	266.035	0.1531	ng/ml
JWH-081	JWH-018 N4-OHpentyl D5	5.563	451.822	0.1488	ng/ml
THC_1	THC-d3	6.169	37.095	0.3005	ng/ml

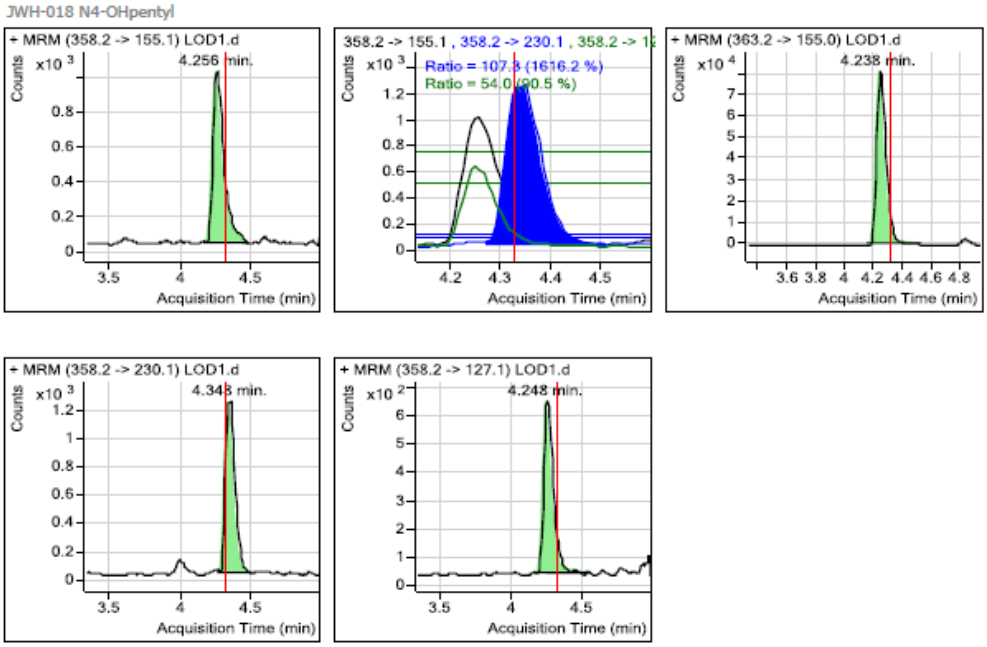


Figure 23: Representative chromatogram of LOD and ion ratios of JWH-018 N4-hydroxypentyl. The ion ratio for JWH-018 N4-hydroxypentyl passed, as only one ion is looked for this compound as qualifier (127.1), due to unreliability, instability of all other tested second qualifier ions.

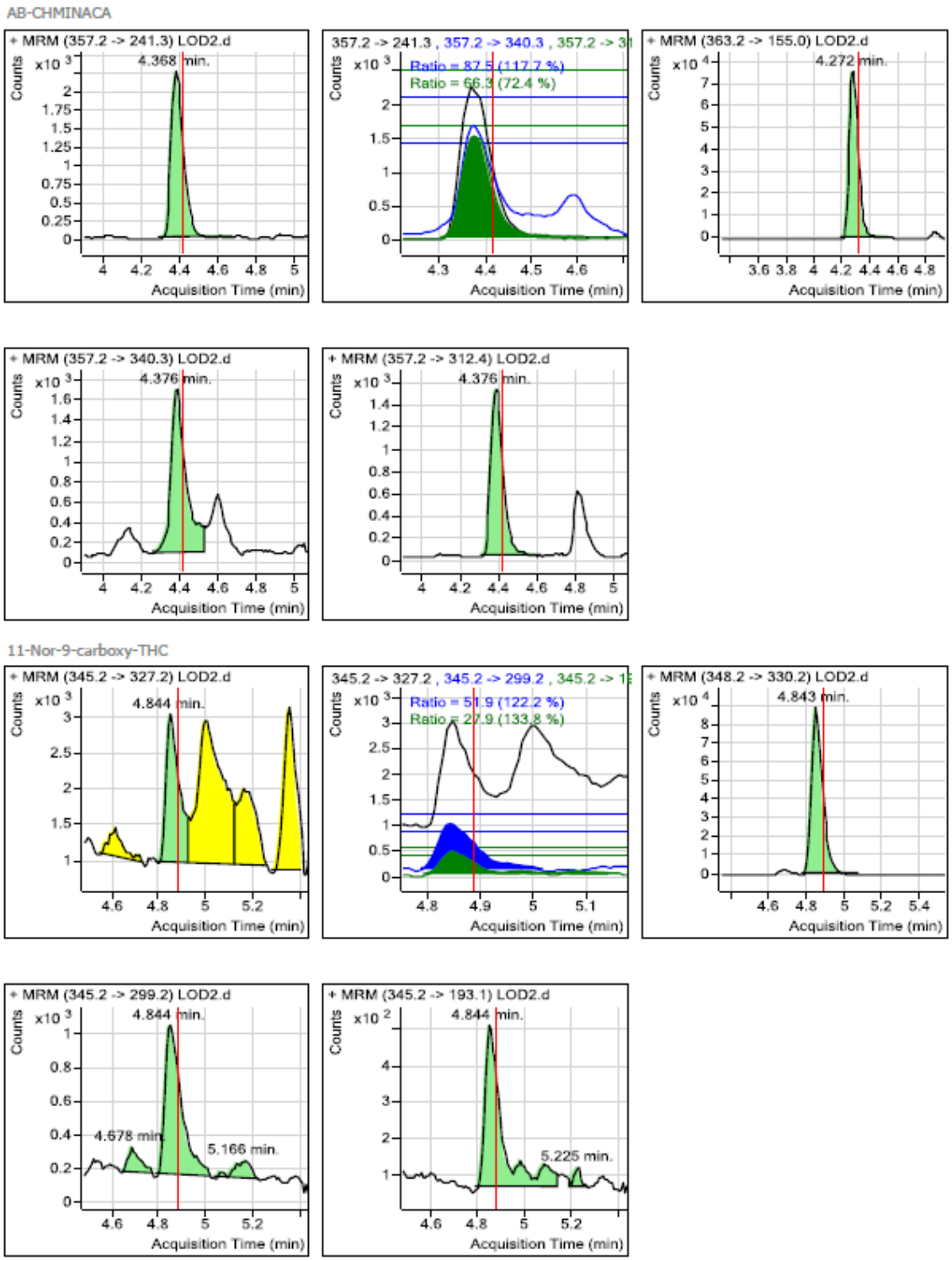


Figure 24: Representative ion ratios for AB-CHMINACA and THC-COOH in the LOD. Both analytes ion ratios failed at LOD concentration.

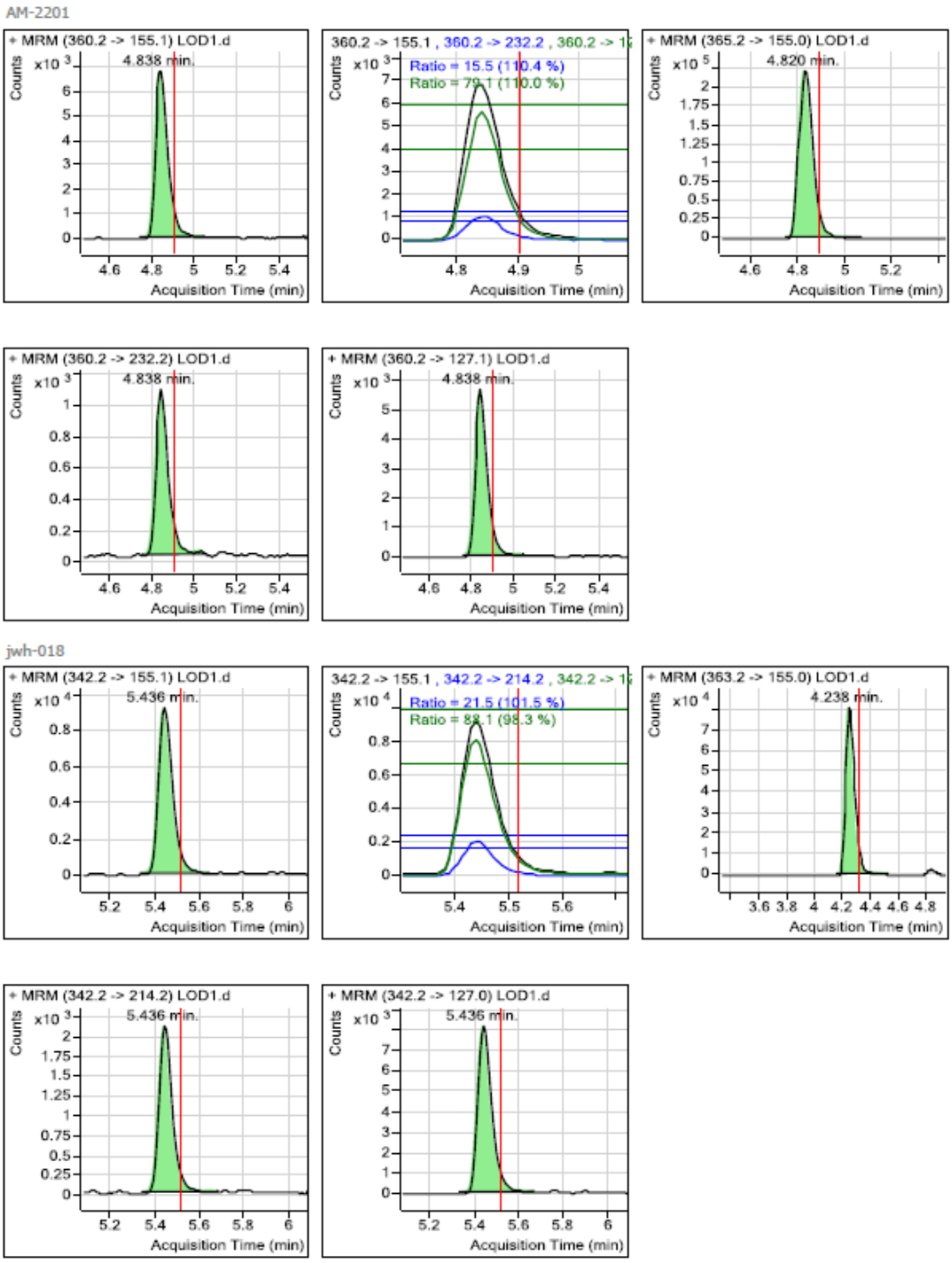


Figure 25: Representative ion ratios of AM-2201 and JWH-018 in the LOD. Both analytes ion ratios passed at LOD concentrations

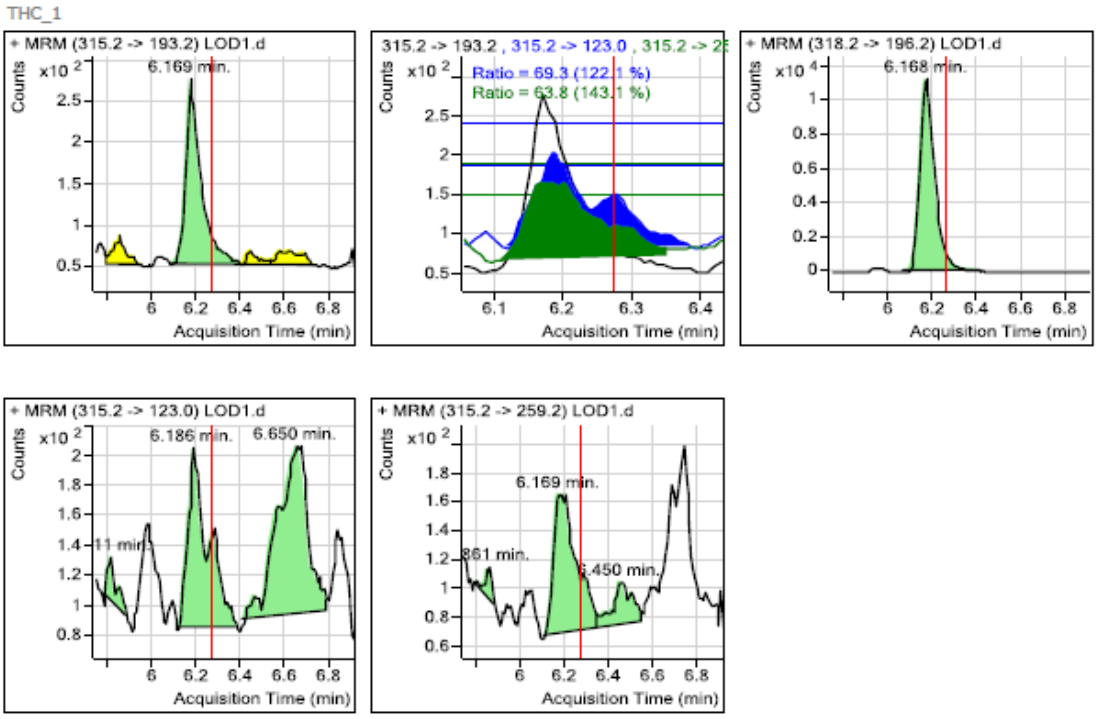


Figure 26: Representative ion ratio for THC in the LOD. The ion ratio failed for THC in the concentration of LOD

3.2.7 LOQ

All analytes passed the required criteria, with the exception of JWH-018, as for this analyte the accuracy was above the range of 30% (Table 19). The method for all analytes, with exception of JWH-018, can reliably quantify concentrations from 0.5ng/mL for THC, 2.5ng/mL for THC-COOH and 0.25ng/mL for SC.

Table 19*:* LOQ result summary. Three LOQs were looked at different days

Analyte name	Accuracy (%)	Signal to noise ratio	Ion ratio
THC	101.68	52	Pass
	117.52	12	Pass
	119.60	27	Pass
THC-COOH	108.59	29	Pass
	115.60	53	Pass
	110.47	22	Pass
JWH-018	123.00	1828	Pass
	130.52	391	Pass
	112.52	349	Pass

Table 19: (Continued)

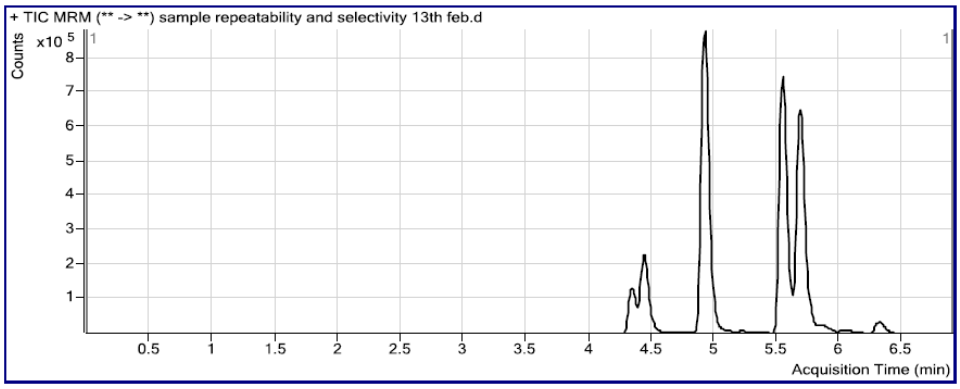
Analyte name	Accuracy (%)	Signal to noise ratio	Ion ratio
AM-2201	107.92	571	Pass
	119.72	114	Pass
	113.16	514	Pass
AB-CHMINACA	120.08	1002	Pass
	112.56	190	Pass
	108.72	193	Pass
JWH-018 N4-hydroxypentyl	105.40	158	Pass
	119.36	373	Pass
	112.56	195	Pass

3.2.8 Selectivity and specificity

All analytes passed their ion ratios and accuracy criteria, no additional peaks or interference was seen in the chromatograph from the additional compounds added (Table 20). Figure 27 demonstrates that there were no interfering peaks present in the chromatogram for the sample.

Table 20*:* Results for selectivity and specificity sample. The sample was spiked with analytes of interest and additional analytes (alcohol, cocaine and diazepam)

Analyte name	Sample concentration accuracy (%)	Ion ratio
THC	97.29	Pass
THC-COOH	93.65	Pass
JWH-018	103.25	Pass
AM-2201	91.67	Pass
AB-CHMINACA	108.76	Pass
JWH-018 N4-hydroxypentyl	95.21	Pass



Compound	ISTD	RT	S/N	Final Conc	
JWH-018 N4-OHpentyl	JWH-018 N4-OHpentyl D5	4.348	7328.958	4.7606	ng/ml
AB-CHMINACA	JWH-018 N4-OHpentyl D5	4.443	7908.693	5.4378	ng/ml
11-Nor-9-carboxy-THC	11-Nor-9-carboxy-THC-D3	4.910	631.938	46.8252	ng/ml
AM-2201	AM2201-D5	4.921	5855.660	4.5984	ng/ml
jwh-018	JWH-018 N4-OHpentyl D5	5.542	12945.683	5.1623	ng/ml
JWH-081	JWH-018 N4-OHpentyl D5	5.680	4896.275	5.0858	ng/ml
THC_1	THC-d3	6.316	1454.471	9.7288	ng/ml

Figure 27: Selectivity and specificity sample chromatogram and concentration results. No interfering samples were detected, only target analytes were detected

3.2.9 Repeatability

All analytes, with exception of JWH-018, passed the repeatability criteria, showing that samples can be reanalysed after a week, method is reliable, can quantify analytes accurately (Table 21 and Table 22).

Table 21: Repeatability sample values on two different days of analysis

Analyte name	Sample value on 13 02 2020 (ng/mL)	Sample value on 20 02 2020 (ng/mL)	Difference %
THC	9.72	8.60	13.04
THC-COOH	46.82	50.64	7.56
JWH-018	5.16	3.71	38.81
AM-2201	4.59	4.65	1.24
AB-CHMINACA	5.43	6.10	10.96
JWH-018 N4-hydroxypentyl	4.76	5.27	9.77

Table 22: Repeatability batches QC concentration values

Analyte name	QC1 values on 13 02 2020 (ng/mL)	QC2 values on 13 02 2020 (ng/mL)	QC1 values on 20 02 2020 (ng/mL)	QC2 values on 20 02 2020 (ng/mL)
THC	1.81&2.00 Pass	12.56&12.41 Pass	2.12&2.01 Pass	16.20&14.51 Pass
THC-COOH	9.84&11.47 Pass	69.50&65.55 Pass	9.69&11.84 Pass	84.73&87.76 Pass
JWH-018	0.80&1.06 Pass	8.33&6.97 Pass	1.37&1.01 Fail	5.36&7.29 Pass
AM-2201	0.89&1.08 Pass	6.87&6.48 Pass	1.02&1.05 Pass	8.16&7.55 Pass
AB-CHMINACA	0.97&1.12 Pass	8.21&8.40 Pass	1.14&1.14 Pass	8.98&8.88 Pass
JWH-018 N4-hydroxypentyl	0.87&1.06 Pass	7.31&6.92 Pass	1.10&112 Pass	8.89&8.32 Pass

3.2.10 Reproducibility

The results for reproducibility are presented in Table 23 to Table 25. The method passed the reproducibility criteria for all analytes with exception of JWH-018. The method is easy to use, can be employed by different analysts.

Table 23: Analyst one results

Analyte name	Linearity R^2	QC1 Accuracy (%)	QC1 duplicate Accuracy (%)	QC2 Accuracy (%)	QC2 duplicate Accuracy (%)
THC	0.9951	99.16	86.59	86.07	83.25
THC-COOH	0.9925	117.87	105.55	96.18	93.15
JWH-018	0.9952	110.16	103.75	112.78	90.28
AM-2201	0.9940	104.38	95.09	92.95	89.91
AB-CHMINACA	0.9954	107.01	109.95	99.07	100.84
JWH-018 N4-hydroxypentyl	0.9948	108.79	99.57	98.51	95.39

Table 24: Analyst two results

Analyte name	Linearity R^2	QC1 Accuracy (%)	QC1 duplicate Accuracy (%)	QC2 Accuracy (%)	QC2 duplicate Accuracy (%)
THC	0.9930	90.58	100.49	83.75	82.75
THC-COOH	0.9948	98.43	114.77	92.67	87.40
JWH-018	0.9969	80.03	106.05	111.15	92.96
AM-2201	0.9905	89.89	108.63	91.68	86.52
AB-CHMINACA	0.9936	97.68	112.81	109.48	112.03
JWH-018 N4-hydroxypentyl	0.9953	87.16	106.12	97.48	92.31

Table 25: Analyst three results

Analyte name	Linearity R^2	QC1 Accuracy (%)	QC1 duplicate Accuracy (%)	QC2 Accuracy (%)	QC2 duplicate Accuracy (%)
THC	0.9957	106.08	100.79	108.02	96.74
THC-COOH	0.9951	97.00	118.43	112.98	117.02
JWH-018	0.8925	137.07	101.68	71.54	97.30
AM-2201	0.9968	102.92	105.96	108.80	100.72
AB-CHMINACA	0.9958	114.39	114.67	119.82	118.46
JWH-018 N4-hydroxypentyl	0.9960	110.25	112.29	118.66	111.06

3.2.11 Dilution integrity

Dilution integrity results are presented in Table 26. Both dilution methods (method 1 adding 0.5mL of blank blood to 0.5mL fortified blood, method 2 analysing only 0.5mL of fortified blood), are applicable for all analytes and 0.5mL of sample can be used for analysis, as after dilution samples are still accurately quantified and ion ratios pass.

Table 26: Dilution integrity results for QC1 sample

Analyte name	Method 1 Accuracy (%)	Method 1 ion ratios	Method 2 Accuracy (%)	Method 2 ion ratios
THC	118.94	Pass	102.69	Pass

Table 26: (Continued)

Analyte name	Method 1 Accuracy (%)	Method 1 ion ratios	Method 2 Accuracy (%)	Method 2 ion ratios
THC-COOH	99.37	Pass	96.26	Pass
JWH-018	114.12	Pass	115.52	Pass
AM-2201	97.16	Pass	98.18	Pass
AB-CHMINACA	121.38	Pass	125.04	Pass
JWH-018 N4-hydroxypentyl	99.64	Pass	105.02	Pass

3.2.12 Ruggedness

The ruggedness of method was investigated by different analysts performing the extraction technique and assessing the performance of the calibration curve and QC samples. The results are presented in reproducibility section (Tables 23-25). The extraction of samples by different analysts showed that the method is reproducible and easy to use for different analysts.

Ruggedness was also investigated by having different analysts prepare pH buffers, mobile phases during different days of analysis. This did not influence the results, accuracy of the batches.

Moreover, tests for dilution integrity were carried out (Table 26). The results show that 0.5 or 1mL of sample can be used for analysis to produce reliable results. The method is efficient to use smaller sample volumes, it does not affect the accuracy or ion ratios for analytes.

3.2.13 Stability

The stability of samples was tested by examining two storage conditions. The results for condition 1 (analysing same sample one week apart) is presented in repeatability section (Table 21). The results for second storage condition (extracting samples and keeping two weeks in refrigerator) , is presented reproducibility subheading, results for analyst 1 (Table 23). Both storage conditions passed the assigned criteria parameters. This shows that the method can be used to analyse samples, that were extracted and kept in the refrigerator for up to 2 weeks and sample can be analysed if it was kept in the refrigerator for up to one week. This is an important advantage of the method, as often real-life samples cannot be analysed during the same day as they were received, the analysis may need to be delayed by few days. Based on the tests it seems the delay would not affect the results and analysis would still be valid and reliable.

3.2.14 Recovery

Triplicates of low and high concentration samples of set A were made. The average value of low concentration and average value of high concentration were used to compare to set C results (Table 27). Low concentration was QC1 and high concentration was QC2.

The recovery for all analytes was below 50%. However, the sensitivity and LOQs for all analytes, with exception of JWH-018, passed the required criteria of the method.

Table 27: Recovery for analytes at low concentration (triplicates) and high concentration (triplicates)

Analyte name	Average RE at low concentration (%)(range %)	Average RE at high concentration (%) (range %)	Average RE (%)
THC	58.87 (26.20 to 79.79)	38.82 (24.44 to 86.36)	48.85
THC-COOH	48.06 (23.96 to 66.04)	37.47 (26.03 to 46.38)	42.76
JWH-018	26.63 (20.53 to 31.70)	45.44 (34.44 to 62.23)	36.03
AM-2201	38.18 (27.43 to 48.07)	61.95 (46.41 to 81.76)	50.06
AB-CHMINACA	38.14 (23.27 to 50.81)	35.81 (24.55 to 50.18)	36.97
JWH-018 N4-hydroxypentyl	13.01 (9.17 to 16.16)	13.73 (10.64 to 17.03)	13.37

3.2.15 Matrix effect

Triplicates of low and high concentration samples of set A were made. The average value of low concentration and average value of high concentration were used to compare to set B results (Table 28). Low concentration was QC1 and high concentration QC2.

Overall, all analytes experienced ion suppression. However, the methods sensitivity, LOQ still passed the required criteria for all analytes, with exception of JWH-018.

Table 28: Matrix effects for analytes at low concentration (triplicates) and high concentration (triplicates)

Analyte name	Average ME at low concentration (%) (range %)	Average ME at high concentration (%) (range %)	Average ME (%)
THC	-25.40 (-48.72 to 2.71)	-44.74 (-37.24 to -48.70)	-35.07
THC-COOH	-51.58 (-44.89 to -59.35)	-44.49 (-40.66 to -48.85)	-48.04

Table 28: (Continued)

Analyte name	Average ME at low concentration (%) (range %)	Average ME at high concentration (%) (range %)	Average ME (%)
JWH-018	-67.97 (-65.87 to -71.47)	-48.63 (-28.75 to -62.01)	-58.30
AM-2201	-58.01 (-54.35 to -62.55)	-28.59 (-3.63 to -45.88)	-43.30
AB-CHMINACA	-29.19 (-23.97 to -38.52)	-24.25 (-44.56 to 8.01)	-26.72
JWH-018 N4-hydroxypentyl	-62.12 (-58.61 to -65.91)	-56.38 (-42.08 to -65.46	-59.25

3.3 THC and THC-COOH concentrations in clotted blood

Clotted blood was compared to non-clotted blood samples, spiked at same concentration. Duplicates of controls and clotted blood samples were analysed, with results of duplicates being varied by less than 10% The results for THC and THC-COOH concentration differences in venous blood and venous clotted blood is presented in Table 29 and Table 30. The results for THC seem to indicate that on average THC concentration is higher in clotted blood by approximately 1ng/mL compared to non-clotted blood. Moreover, the majority of clotted samples had higher THC concentration compared to non-clotted samples. THC-COOH, shows a similar pattern to THC, with THC-COOH clotted blood samples having on average higher concentration by approximately 5ng/mL compared to non-clotted samples. Also, the majority of THC-COOH clotted samples have higher concentration than the non-clotted samples. The % difference between clotted and non clotted samples for THC is on average approximately 6%, while for THC-COOH is approximately 12%. However, there was seven clotted samples for THC and nine clotted samples for THC-COOH that had lower concentration compared to their respective controls.

Table 29: Results of THC concentrations difference in clotted venous and venous (control) blood. – difference the clotted blood is lower, + difference clotted blood is higher in concentration

Sample	THC clot (ng/mL)	THC control (ng/mL)	Difference (ng/mL)	Difference by (%)
1	1.78	2.02	-0.24	-11.77
2	10.37	10.08	0.29	2.86
3	1.89	2.01	-0.12	-5.97
4	12.01	9.07	2.95	32.50
5	1.94	2.20	-0.26	-11.92
6	2.21	2.20	0.01	0.33
7	2.38	2.20	0.17	7.82
8	2.19	2.12	0.06	2.92
9	14.56	11.61	2.95	25.38
10	13.92	11.61	2.31	19.88
11	13.52	11.61	1.91	16.45
12	2.23	2.54	-0.30	-11.89
13	2.15	2.54	-0.39	-15.37
14	10.01	9.64	0.37	3.86
15	11.05	9.64	1.41	14.66
16	17.03	19.06	-2.04	-10.69
17	17.44	19.06	-1.62	-8.50
18	19.47	19.06	0.40	2.12
19	29.80	19.06	10.74	56.32
20	22.27	19.06	3.21	16.83
		Average	1.09	6.29

Table 30: Results of THC-COOH concentrations difference in clotted venous and venous (control) blood. –difference the clotted blood is lower, + difference clotted blood is higher in concentration

Sample	THC-COOH clot (ng/mL)	THC-COOH control (ng/mL)	Difference (ng/mL)	Difference by (%)
1	5.49	6.47	-0.98	-15.15
2	45.53	47.64	-2.11	-4.42
3	6.46	7.13	-0.67	-9.37
4	51.76	39.36	12.39	31.49
5	6.19	4.25	1.95	45.79
6	6.26	4.25	2.01	47.25
7	6.33	4.25	2.08	49.01
8	5.64	4.83	0.81	16.76
9	63.61	45.96	17.65	38.40
10	55.69	45.96	9.73	21.16
11	57.93	45.96	11.97	26.03
12	5.63	7.86	-2.23	-28.36
13	5.71	7.86	-2.16	-27.42
14	43.49	45.75	-2.25	-4.93
15	50.66	45.75	4.91	10.74
16	78.98	90.64	-11.66	-12.86
17	87.05	90.64	-3.59	-3.96
18	89.94	90.64	-0.69	-0.77
19	135.33	90.64	44.69	49.31
20	109.79	90.64	19.15	21.13
		Average	5.05	12.49

4. Discussion

This research describes the development and validation of LC-MS/MS technique that can quantify two natural and four SC in blood. Following method development experiments, the method was validated to assure reliability, in line with ISO 17025 guidelines and UKAS recommendations. Moreover, the validated method was utilised to study the affects of clotting blood on THC and THC-COOH concentrations, with results indicating that clotting alters measured concentrations of analytes.

Pre-method development literature was researched to identify potential ion fragmentation pathways relating to THC, THC-COOH and SC. Numerous studies researching THC or SC have utilised the same ions fragmentation pathways, as described in this study (Krotulski *et al*. 2018, Shah *et al*. 2014, Agilent 2017, Borg *et al. 2017*). The sample preparation, extraction and separation procedure were based on research by Aizpurua-Olaizola *et al.* (2017) but with several modifications to improve recovery and efficiency. The research by Aizpurua-Olaizola *et al.* (2017) provided the starting point for this research. The method published in their study utilised an Agilent 1260 LC coupled to an Agilent 6430 MS/MS. This method had excellent precision, accuracy and sensitivity. Despite this the column that was associated with the Aizpurua-Olaizola *et al.* (2017) method, Kinetex polar C18, was shown to have extremely poor robustness and reproducibility. As a result of this lack of robustness, sensitivity and subsequent elongation of the method runtime it was decided that this column would not be suitable to complete a validation programme designed to test casework samples or SC samples.

Following these results and using the Phenomenex C18 column, an Agilent Infinity Lab Poroshell 120 EC-C18 column was chosen, based on a method developed for analysis of THC and THC-COOH by Agilent (2013).This method utilised an Agilent 6430 system and achieved a sensitivity of 1ng/mL of THC in whole blood. This technique was further optimised into a method that could analyse natural and SC in a single chromatographic run at concentrations in blood below 1ng/mL. Following the successful method development, using the Agilent Infinity Lab Poroshell column, the validation process was started. The parameters that were tested for the quantitative method were accuracy (bias), precision, carryover, selectivity and specificity, matrix effect, LOD, LOQ, linearity (calibration model), range, recovery, reproducibility, repeatability and ruggedness. These are the parameters that need validation to be in line with SWGTOX, UKIAFT and ISO17025 recommendations for casework sample analysis. However, there are no universally defined standards or exact criteria for all parameters when utilising analytical methods in forensic toxicology in the UK. The criteria for the parameters are decided by individual laboratories and should broadly be in line with UKAS (United Kingdom Accreditation service) guidelines for best practice in ISO 17025 accreditation. Prior to the laboratory examination of the method parameters, a validation outline was proposed, which included the criteria for each method specification.

All the experiments were to be performed on whole blood matrix for which the method is intended to be used (SWGTOX 2013, Polettini 2006).

The data from the validation experiments indicated that the accuracy results for five out of six analytes passed the validation criteria, with a failure rate of 0%, while one analyte, JWH-018, had a fail rate of 2 in 10 for the low QC samples and 1 in 10 for the higher QC concentration. Moreover, the precision for all analytes, with exception of JWH-018 passed the validation requirements. These results showcase the methods quantitation efficiency, and repeatability. The calibration range for all analytes was validated, with a focus on drug concentrations of analytes being similar to those encountered in casework settings (Ambroziak and Adomowicz 2016, Vaiano *et al. 2016*). An optimisation of this method was that the LOQ validated for analytes was in lower concentrations than methods developed by Aizpurua-Olaizola *et al.* (2017) and Agilent (2013). However, both of the mentioned methods were able to analyse 11-OH-THC, alongside THC and THC-COOH, which this method cannot. Moreover, the LOQ of this method for THC and several SC was lower and more sensitive than numerous studies described in the literature utilising similar instrumentation (Hackett and Elian 2009, Krotulski *et al.* 2018). Moreover, Hackett and Elian (2009) and Krotulski *et al.* (2018) were only looking at THC and THC-COOH, while this method also looks at SC. The validation of dilution integrity demonstrated that lower volumes of sample can be utilised for analysis, this is useful in cases requiring repeat analysis of sample or instances of low sample availability. The robustness and repeatability parameters validated in this method demonstrates its applicability for different analysts, and potential use in research or commercial settings. Overall, five out of six analytes (THC, THC-COOH, AM-2201, AB-CHMINACA, JWH-018 N4-hydroxypentyl) were fully validated, passed all validation criteria. One analyte (JWH-018) did not meet the validation requirements for repeatability, LOQ and reproducibility but passed all other criteria, was considered only semi-validated.

One of the major challenges of incorporating research based methods into casework settings is the lack of applicability of certain methods to identify all drugs in a polydrug sample specifically. Often methods developed in research and applied in real cases only quantify ion ratio's using one qualifier ion (Aldlgan and Torrance, 2016). This can cause specificity issues for the analysis of certain analytes, in particular SC, which have similar structures, chemistry and fragmentation patterns. Identifying only one qualifier ion for an analyte is not reliable as certain analytes can have the same parent and daughter ions, for example JWH-122 and JWH-019. Moreover, recently Huestis and LeBeau have proposed a point-based scoring system to be used for the identification of analytes in forensic toxicology laboratories (ASB 2019). The recommendations from Huestis and LeBeau state that a minimum of 3 ions need to be used for confirmation of an analyte. Two of the analytes as qualifiers and one analyte as the quantifier (International Association of Forensic

Toxicologists (TIAFT) 2019). The point-based scoring system is proposed to make the identification process of analytes more objective in the forensic toxicology field. A minimum of 4 points is required to state that an analyte was identified utilising no more than 3 analytical techniques working in tandem. For example, analysis of compounds with LC-MS/MS using dMRM of 3 ions transitions, two qualifiers and one quantifier, receives 4 points. One point is received for LC separation and a point is received for each ion (TIAFT 2019, ASB 2019). The risk of utilising fewer ions in identification process is the possibility of utilising ions, that belong to another compound other than the target analyte, thus mistakenly confirming the analyte. This risk is increased in cases whereby the sample has multiple drugs present and more so if these drugs and their metabolites share similar structures, chemistry and ion fragmentation pathways. A greater number of ion transitions used and more strict criteria employed enables confirmation of an analyte with a higher degree of certainty. The method developed and validated in this research confirms an analyte using two qualifier ions, while the majority methods for SC and natural cannabinoids described in the literature only use one qualifier ion. Moreover, the developed and validated method follows the recommendations from Huestis and LeBeau, which are likely to circulated to US and European laboratories in the next 12 months.

The described method can be used for road traffic toxicology (RTT) casework to detect and quantify THC for Road Traffic Act 1988 Section 5A. THC is the most commonly detected illicit drug in motorist in European countries, and in the United States of America (EMCDDA 2018, Hartman *et al.* 2016b, Rooney *et al.* 2017). The limits for THC in blood vary in several countries across Europe, with typical limits ranging from 1-3ng/mL. In the United States the majority of limits in each state are set at 5ng/mL. In England and Wales Section 5A of the Road Traffic Act 1988 states the limit for THC in blood is 2ng/mL (Department of Transport 2017, EMCDDA 2018). The developed method can reliably quantify THC concentrations between ranges of 0.5-20ng/mL in blood and therefore determine if the THC concentration was above or below the Section 5A *per se* limit. The method can detect and quantify THC-COOH, the main metabolite of THC. The quantitation and confirmation of THC-COOH in a sample, which has THC present, demonstrates that THC was not due to passive exposure, confirms the use of THC. This is due to fact that THC-COOH is a metabolite of THC can be found only after cannabis consumption and metabolism in the body. The method is also relatively cheap and has more efficient sample preparation procedure than methods that use GC-MS. One the key steps in sample preparation for GC-MS is derivatisation Derivatization is a process by which a compound is chemically changed, producing a new compound that has properties applicable to an analytical method. For GC-MS an analyte of interest is modified with a derivatization reagent to produce a new product that is more volatile, thermally stable and can produce reproducible results. The process of

derivatization is time consuming, expensive and the analyte of interest can be lost due to contamination from the reagent.

In the UK, specifications for methods used to quantify blood drug concentrations for the purpose of Road Traffic Act 1988 Section 5A analysis are detailed in FSR-C-133. FSR-C-133 is a guideline proposed by the Forensic Science Regulator in collaboration with UKIAFT. It should be noted at that these recommendations are not legally enforceable and are at this point still guidelines (Forensic Science Regulator 2020). This method was developed with the FSR-C-133 guidelines in mind and it is these requirements that most forensic science providers adhere to when testing RTT samples in the UK. These recommendations state that isotopically labelled deuterated standard should be used for analytes, which this method follows. The parameters that need to be validated and tested are the same ones, as in this methods validation plan. Moreover, it is required that the upper quantitation limit be at least 5 times that of the *per se* limit. Therefore, the minimum requirement for the LOQ is 1ng/mL and for the upper concentration limit it is 10ng/mL for THC. The validated method has a lower LOQ of 0.5ng/mL with upper concentration limit of 20ng/mL for THC. This is in line with the requirements stated in the FSR-C-133. In addition, QC at legal limit is required to be used and the developed method is utilising a QC of THC at the legal limit of 2ng/ml. The only criteria of FSR-C-133 that this method does not meet is the requirement that the method be accredited by UKAS to ISO 17025 standard. Due to the costs associated with accreditation, the developed method has not accredited to ISO 17025 standards but has been validated based on ISO 17025 and UKIAFT guidelines.

A method that analyses THC for RTT is important not only due to widespread use of THC in society but also due to the risks associated with cannabis use and driving. THC produces impairment on driving by affecting cognitive and psychomotor performance (Bondallaz *et al.* 2016, EMCDDA 2018, Hartman and Huestis 2013). Motorists under the influence of cannabis drive slower, have difficulty in driving exercises such as tracking and lane position variability (Hartman and Huestis 2013, Lenné *et al.* 2010, Ronen *et al.* 2008,). Moreover, the ability to perform divided attention tasks, a fundamental skill in driving, declines when under the influence of cannabis as does concentration span and reaction time (Battistella *et al*. 2013, Bondallaz *et al*. 2016, Hartman and Huestis 2013, National Highway Traffic Safety Administration (NHTSA) 2017,).

The developed and validated method was also utilised to research the effects of blood clotting on concentrations of THC and THC-COOH and test methods robustness. These two analytes were chosen as THC is highly prominent in motorists, where the exact concentration of the drug is vital to know and has legal ramifications in light of Road Traffic Act 1988 Section 5A limits. The concentrations, which were analysed for THC were at the legal limit and above it (2, 10 and 20ng/mL). In the UK blood collected for analysis of drugs, including THC, is preserved in vials with preservatives of sodium fluoride and potassium

oxalate or ethylenediaminetetraacetic acid (EDTA) (Faculty of Forensic and Legal Medicine 2020). Potassium oxalate and EDTA are anticoagulants, however if the blood sample is taken improperly or not sufficiently mixed with the preservative and anticoagulant then blood clotting can occur. Blood clotting is a complicated process, with many reactions taking place simultaneously (appendices, clotting cascade section). In short, when blood clots a solid mesh is formed that is composed of platelets and other blood cells, mainly red blood cells, to prevent excess loss of blood. It has been shown by Huestis *et al.* (1992a, 1992b) that THC concentration is significantly different in plasma compared to whole blood. Huestis *et al.* (1992a, 1992b) conducted research, whereby participants consumed cannabis and after cessation of smoking, blood samples were collected for analysis. Whole blood as well as plasma was analysed for pharmacological data, which demonstrated concentration differences for plasma and whole blood. THC and THC-COOH concentrations in whole blood are lower by approximately half compared to plasma, due to high plasma protein binding and poor cannabinoid distribution into erythrocytes (Schwilke *et al.* 2008). This suggest that during sample partition THC will favourably dissolve into the water rich solution as opposed to the protein portion of a blood sample. Moreover, the haematocrit value influences how much of THC, THC-COOH is present in whole blood (Giroud *et al.* 2001). The analysis of venous clotted blood compared to venous non-clotted blood seem to indicate, that THC and THC-COOH concentrations are artificially altered and on average raised by the clotting process, with THC-COOH being affected more than THC. The exact mechanism of why it is happening is not clear. One possible explanation is THC and THC-COOH are unable to bind to proteins with a high iron content. It has previously been suggested that the heme protein in red blood cells prevents cannabinoid binding. The research by Huestis *et al.* (1992a, 1992b) and pharmacological data in literature does demonstrate universally that THC and THC-COOH have high affinity to plasma proteins but not to red blood cells. It is only in settings whereby the blood plasma ratio is higher that THC does not remain protein bound, by contrast non iron containing protein such as albumin demonstrate strong binding affinity for THC.

Overall, the clotting study indicates that clotted blood samples analysed for THC and THC-COOH alters measured concentration and typically tends to overestimate the concentration of drug analyte in clotted blood compared to concentrations in whole blood. This is an important in RTT, as analysis of clotted blood samples for THC may lead to unreliable results, and result in a miscarriage of justice or an incorrect conviction. For THC on average by approximately 6% clotted samples are higher in concentration compared to non clotted samples, while for THC-COOH clotted samples are 12% higher compared to non clotted. Moreover, a further study with cannabis users is needed to confirm the findings of this study and to find an average percentage difference of venous clotted and venous non clotted blood, so it could be used in road traffic casework.

The concentrations for SC that can be reliably quantified by the method are in the range of 0.25-10ng/mL in whole blood. This concentration range can be applicable to analyse samples of SC users, as the SC concentrations are often below 1ng/mL in whole blood. The case studies of SC users, who are treated in emergency departments, commonly have low concentrations of SC present in blood, typically below 10ng/mL, as the drugs are more potent, so less drug dosage is needed for adverse side effects to occur (WHO 2017). The designed method could analyse blood samples from users, who had experienced adverse side effects of SC. The low detection limits of the method would allow to analyse samples with a relatively long detection window of 4-6 hours. After the first few hours SC usually are below 1ng/mL, and the method can detect and accurately quantify SC to 0.25ng/mL. Moreover, the method detects different generation SC and often the products consumed by users have mix of SC, is rare for them to have only one SC present (Presley *et al.* 2016, Marusich *et al.* 2018).

The method has its limitations, it cannot accurately quantify JWH-018 all the time, with a failure rate of 1 in 10 for high concentrations and 2 in 10 for low concentrations. It should however be noted that must casework laboratories operate methods with a QC failure rate of approximately 10% (SWGTOX 2013, Elliott *et al.* 2018). The performance of the QC's in relation to JWH-018 could be due to variation in extraction and not using deuterated internal standards for this compound, to control for the differences in extraction. Another drawback of the method is only one qualifier ion can be used for JWH-018 N4-hydoxypentyl, none of the second qualifiers tested were stable, with all qualifying ions displaying different ion ratios in different concentrations. This is an issue that has been widely described for SC, as some analytes have the same parent and daughter ions and only the ion ratio and chromatography can differentiate them. For instance, JWH-122 and JWH-019 can only be identified by chromatography (Scheidweiler and Huestis 2014). Having two qualifiers for JWH-018 N4-hydroxypentyl could yield a more reliable confirmation of the compound, address the issue of some SC and their metabolites having identical ion transitions.

Further optimisation of the extraction procedure could improve the method. Currently, the method has high matrix effects for all analytes, which is compensated by their respective internal standards. The optimisation of the solvents and pH buffer for the extraction procedure might yield better recovery and lower ion suppression. Also, a lower sample volume than 0.5mL would be beneficial. The samples taken for RTT cases are approximately 10mL of whole blood, which is divided into two samples A and sample B (Faculty of Forensic and Legal Medicine 2020). In theory the volume received by the laboratory should be approximately 5mL of whole blood per sample, but in practice this is not always the case as there may be issues sampling blood from the subject. A lower volume of sample used for extraction would mean the analysis could be repeated and that more sample volume could be devoted to the screening and analysis of other drugs if

needed. The addition of more SC, including ones that are most prevalent in the UK in last few years would make the method more beneficial to the UK toxicology market. In the UK there is no national forensic body, independent companies analyse samples for toxicological results. The major toxicological companies have no incentive to analyse SC, as is not widely prominent, not financially beneficial. Only government bodies like FEWS, WEDINOS and work carried out by researchers in universities, indicate what SC are currently used and how widespread they are. A method that can analyse THC for road traffic toxicology and popular SC would address this issue and help to further determine what the true usage of SC is among the general populace.

5. Conclusion

A method for analysis of natural and synthetic cannabinoids has been developed for LC-MS/MS. It has been validated for THC, THC-COOH, AM-2201, JWH-018 N4-hydroxypentyl and AB-CHMINACA. The method was validated based on guidelines from UKIAFT, SWGTOX and ISO 17025. Moreover, the method follows the guidelines of FSR-C-133, with exception of being accredited. This method can be used for analysis of RTT casework samples, for *per se* limits of THC for Road Traffic Act 1988 Section 5A. In addition, SC can be analysed at levels usually found in users and coroners' cases. The SC can be analysed alongside THC in RTT, to show prevalence of SC in general population. Lastly, a clotting side study for THC and THC-COOH was carried out, which indicates that clotting alters the concentrations of these analytes, clotted samples are not reliable to use, where accurate quantitation is required. The side study showcases the utility of the method to be used for whole blood as well as clotted blood.

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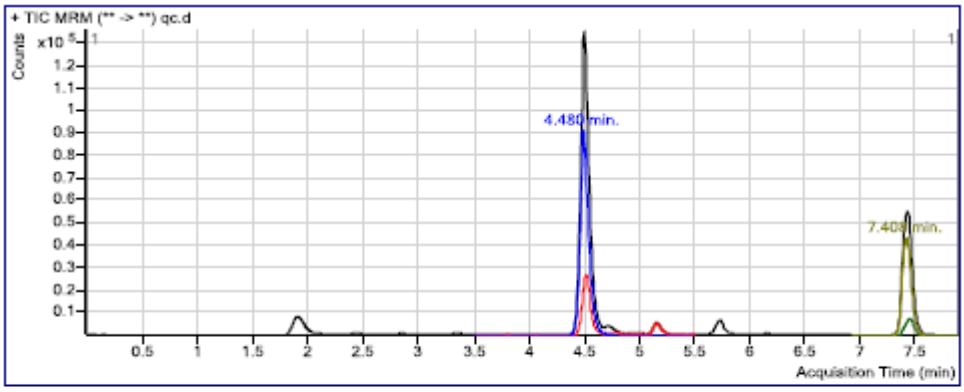
Appendices

1. Column issue

Kinetex polar C18 column robustness issues. The column at first performed well (for almost a month), the extracted samples for THC and THC-COOH eluted in less than 10 minutes (Figure 28). However, after few calibration lines run on the column, the column lost its robustness. The retention times shifted by over 6 minutes for both compounds, for compounds to elute the gradient had to be changed and run extended to atleast 17 minutes from previously 11 minutes (Figure 29). Moreover, the sensitivity has decreased for the analytes, 0.5ng/mL of THC could not be detected anymore (Figure 30). The reason for the issue is not clear as following recommendations by the manufacturer (Phenomenex), how to clean the column nothing changed, they could not comment how come such a drastic change in retention time occurred. As the issue was not resolved and because the column was not reliable, sensitivity decreased a new column was chosen for the method.



Compound	ISTD	RT	S/N	Final Conc	
11-Nor-9-carboxy-THC	11-Nor-9-carboxy-THC-D3	4.499	245.215	12.0041	ng/ml
THC_1	THC-d3	7.435	759.071	2.1563	ng/ml

Figure 28: Result from Kinetex polar C18 column at the beginning of method development. THC and THC-COOH elute in less than 10 minutes.

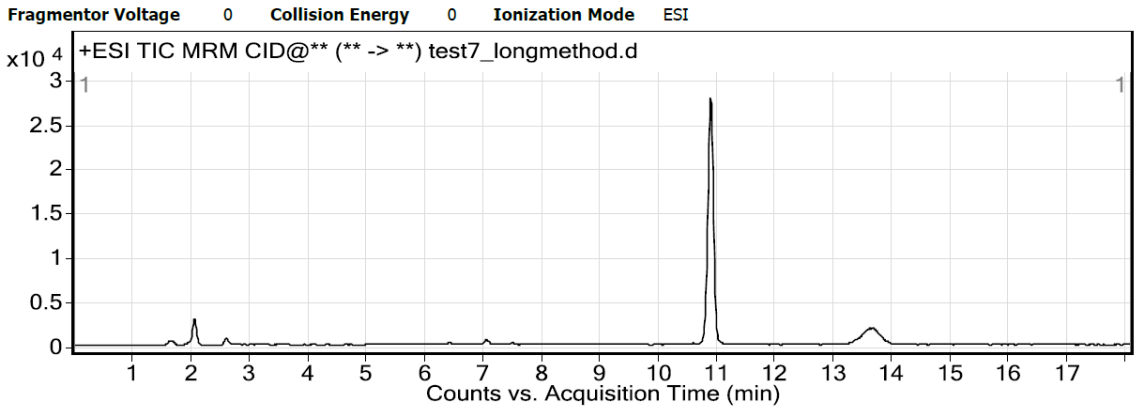
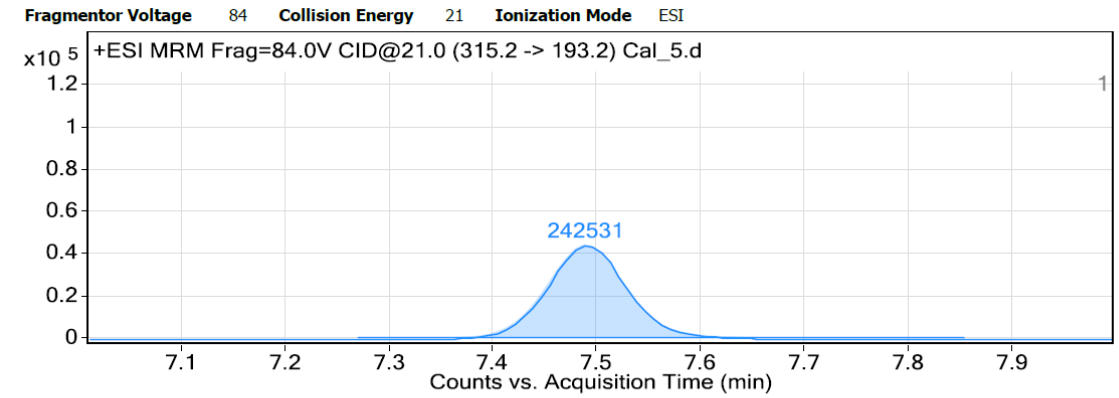
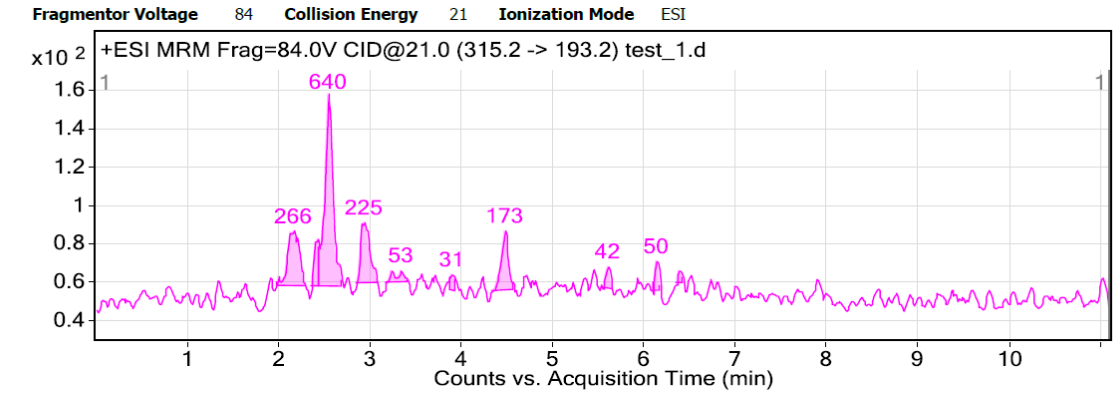


Figure 29: Results after less than a month of using the Kinetex polar C18 column. The retention time has shifted for both analytes, THC-COOH elutes at 11min, while THC at 14min. The mobile phase that was used was not changed, the extraction procedure was the same as before, no changes were made to the method or solvents that were used.



Integration Peak List

Start	RT	End	Height	Area
7.271	7.49	7.854	44113	242531



Integration Peak List

Start	RT	End	Height	Area
1.985	2.161	2.298	29	266
2.359	2.424	2.442	25	86
2.442	2.542	2.689	101	640
2.852	2.934	3.084	32	225
3.172	3.341	3.408	7	53
3.878	3.897	3.933	9	31
4.337	4.475	4.569	31	173
5.55	5.604	5.652	12	42
6.102	6.135	6.172	16	50

Figure 30: Results for 20ng/mL of THC. The top picture demonstrates the response area for THC quantifier ion and retention time, when the method was working with Kinetex polar C18. The bottom picture presents what response was for THC quantifier ion after unknown column issues occurred.

2. Clotting cascade

Figure 31 describes the clotting cascade process.

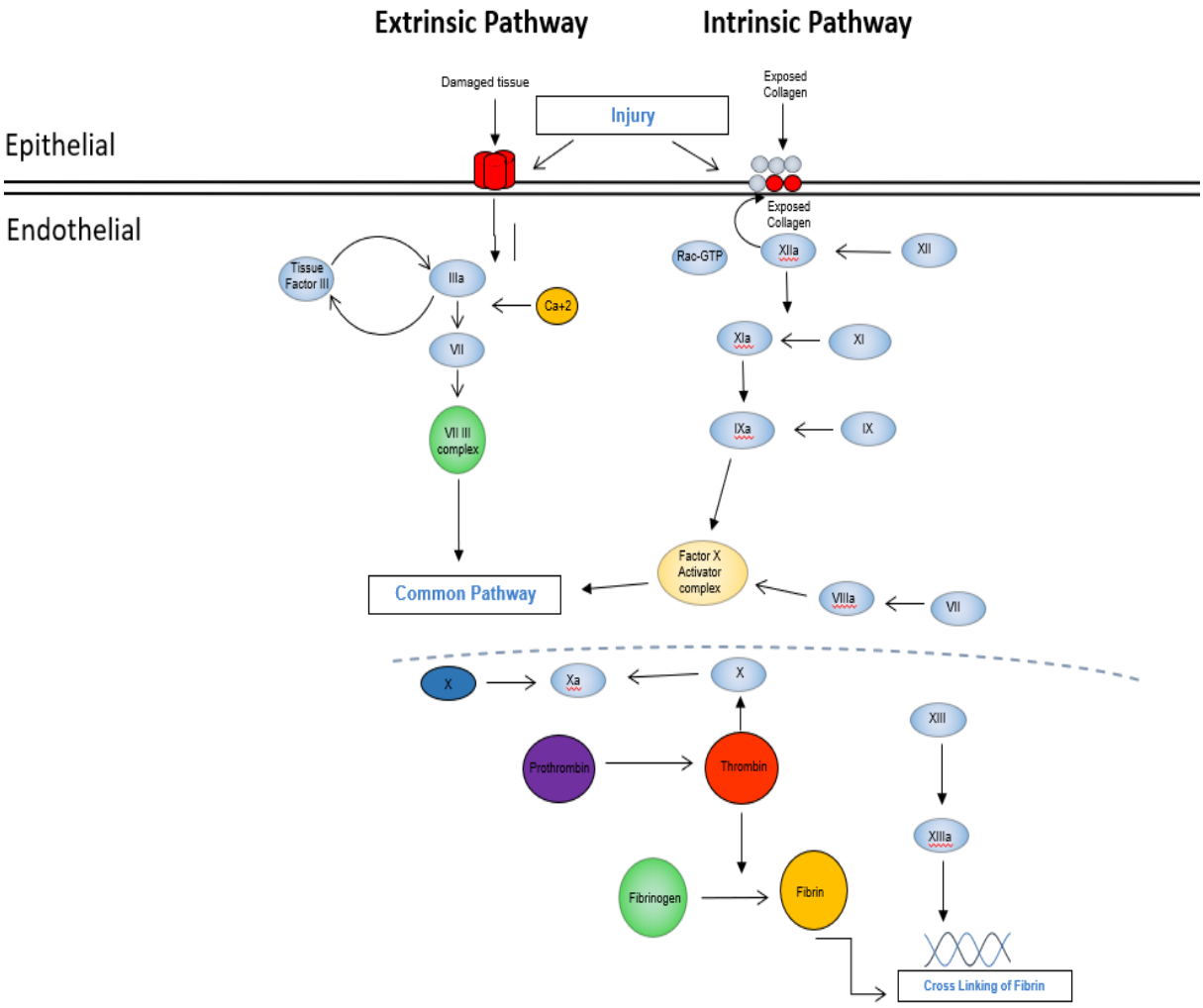


Figure 31: Clotting cascade process. The intrinsic pathway, it is activated when blood is exposed to collagen, or other damaged surfaces. The end result is factor IXa (with PF3) will join to factor VIIIa to form a complex, which activates factor X. The extrinsic pathway is activated as the damaged endothelial cells release factor III, greater the damage more of it is released. Factor III and factor VIIa form a complex, which activates factor X. The convergence of pathways to activate factor X begins the common pathway. In the end fibrinogen is converted into fibrin strands, which help secure platelets, erythrocytes and form a clot.