Development of a bioanalytical method for the quantitative analysis of cannabinoids and their metabolites in plasma

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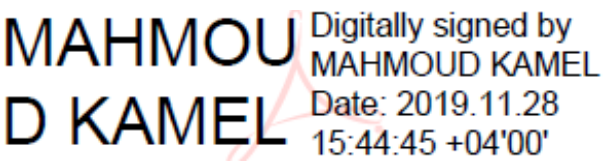
DECLARATION AND PREFACE

I, **Mahmoud Mohamed Kamel Mohamed**, hereby declare that this dissertation is original work and has not been previously submitted to another university.

Signature



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Mahmoud Mohamed Kamel Mohamed

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The emerging discoveries of the medicinal benefits of cannabinoids, the need to understand their pharmacokinetics and the forensic requirements to detect *Cannabis* exposure inspired the development of sensitive analytical methods. Motivated by such inspiration, this study was conducted utilizing the state of art high resolution mass spectrometry to develop sensitive and specific analytical method for the analysis of cannabinoids in human plasma.

I would like to express my appreciation for the great support and guidance received from my supervisor Dr John Takyi-Williams, Professor Anne Grobler and Mr. Bertrand Baudot. I benefited greatly from their mentorship and patient teaching. I express my sincere gratitude for their motivation, encouragement and immense knowledge.

I would like to thank my family for their support and encouragement.

ABSTRACT

There is a continuous need to develop sensitive analytical methods for detection of cannabinoids and their metabolites in human plasma for forensic purposes as well as for pharmacokinetics studies. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been the technique of choice due to its sensitivity and rapid sample preparation. High resolution mass spectrometry offers more selectivity due to accurate mass measurement of the targeted compounds and, therefore, better signal-to-noise ratio. The aim of the study was to develop and validate a sensitive liquid chromatography high resolution mass spectrometry (LC-HRMS) method for the quantitative analysis of cannabidiol (CBD), cannabinol (CBN), Δ9-tetrahydrocannabinol (Δ9-THC) and its major metabolites 11-OH-Δ9-THC and 11-Nor-Δ9-THC-9-COOH in human plasma. The method utilized a simple liquid-liquid extraction of the cannabinoids from plasma samples followed by an isocratic chromatographic separation on Zorbax Eclipse reverse phase C18 column (1.8 µm, 50 x 2.1 mm). The aqueous mobile phase (Phase A) consisted of 0.2 % acetic acid in pure HPLC water while the organic mobile phase (Phase B) was acetonitrile. An isocratic program with a composition of 35 % phase A and 65 % phase B at a flow rate of 0.35 mL/min for 10 minutes was used. Detection was performed by electron spray ionization (ESI) HRMS Q-Exactive plus platform in parallel reaction monitoring mode (PRM). One quantitative product ion and one qualitative product ion were monitored for each cannabinoid. Validation was carried out according to FDA guidelines on validation of bioanalytical methods. The method was found to be selective for the target analytes as no interferences were found at the retention times of the cannabinoids in six different blank plasma samples. The specificity was tested by spiking plasma samples with possible concomitant medications, no interferences were found. The method was linear from 0.2 ng/mL to 100.0 ng/mL, having a lower limit of quantitation (LLOQ) of 0.2 ng/mL for the targeted cannabinoids. The average coefficient of determination (r^2) was higher than 0.995 for all the analytes. The accuracy was within 15 % at three different concentration levels and within 20 % at LLOQ. The method's intra-day and inter-day precision were < 11 %. Extraction recovery ranged from 60.4 % to 85.4 % for the target analytes. Matrix effect (ME) was reduced due to high resolution mass separation from backgrounds noise; however, there was still significant ion suppression for some of the analytes originating from the competition of co-eluting compounds for ionization in electrospray ionization source. The lowest ME was observed for 11-OH-THC and ranged from 1.1 % to 7.4 % while for cannabinol, cannabidiol and THC-COOH, ME ranged from 21.3% to 37.5%. THC showed the highest ME of 49.8 % at the low concentration level and 48.9 % at the high concentration level. There was no carry over of the analytes in the blank samples injected after the higher limit of quantitation.

Keywords: Cannabinoids; THC; CBD; CBN; LC-HRMS; Orbitrap; Human Plasma; method validation

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DISSERTATION LAYOUT

This dissertation consists of four chapters. Chapter 1 is an introduction to the study with emphasis on its scope, aims and objectives. Chapter 2 is a comprehensive literature review about the cannabinoids, their biosynthesis and medical applications. It also provides a review into the analytical methods used for analysis of cannabinoids in various matrices, the sample preparation involved and the technology used for this purpose. Method validation parameters are also discussed in this chapter. Chapter 3 is the research manuscript which comprise the method development information, reagents and chemicals used, sample preparation, instrumentation and the method validation results. Chapter 4 discusses the research outcome, potential method applications, study limitations and recommendations. Author guidelines, certificates of analysis and journals' copyright permission are included in the **Annexures**.

CHAPTER 1 INTRODUCTION

1.1 Study rational and scope

There is a continuous need to develop sensitive analytical methods for the detection of cannabinoids and their metabolites in human plasma, mainly for forensic purposes, as *Cannabis* is widely abused and considered an illicit drug in many countries. Another application is to study the pharmacokinetics and pharmacodynamics of the constituents of the extract and their metabolites in order to understand the therapeutic effects associated with each of these components.

There are many reported analytical methods for the detection and quantification of cannabinoids in plant extracts and biological fluids using gas chromatography or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS and GC-MS/MS). However; there is limited data on the use of high resolution mass spectrometry (HRMS) for cannabinoid analysis. HRMS gives the advantage of measuring the exact mass of the compounds to several decimal points, thus allows discrimination between compounds that have the same nominal mass. Such advantage enables accurate separation of the signal of interest from the background and thus reduces the matrix effect significantly.

In this study, HRMS in combination with MS/MS fragmentation was utilized to detect and quantify cannabidiol (CBD), cannabinol (CBN), Δ9-tetrahydrocannabinol (THC) and its major metabolites 11-OH-Δ9-THC (11-OH-THC) and 11-Nor-Δ9-THC-9-COOH (THC-COOH) in human plasma. The developed method was validated according to FDA guidelines on bioanalytical methods.

1.2 Study aims and objectives

The aim of the study was to develop and validate a sensitive LC-HRMS/MS method for the quantitative analysis of CBD, CBN, THC and its major metabolites 11-OH-THC and THC-COOH in human plasma.

Study objectives:

A- To develop and optimise a LC-HRMS/MS method for THC, CBD, CBN, 11-OH-THC and THC-COOH using reference standards.

B- To develop a method for the extraction of THC, CBD, CBN, 11-OH-THC and THC-COOH from human plasma.

C- To fully validate the proposed method.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Cannabinoids are phytochemicals produced as secondary metabolites of the *Cannabis sativa* plant. They are a group of terpenophenloic compounds formed mainly by decarboxylation of the corresponding acids in plant (Isvett Josefina & Robert, 2008). Chemical characterization of 104 cannabinoids as well as 22 non-cannabinoid constituents exits (ElSohly & Gul, 2014). According to the aforementioned research, cannabinoids can be divided into 11 classes of which the major types are the cannabigerol type (CBG), the cannabichromene type (CBC), cannabidiol type (CBD), tetrahydrocannabinol type (THC), cannabinol type (CBN) and the cannabielsoin type (CBE). Tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) are biosynthesized from cannabigerolic acid as products of an oxidative cyclization reaction with the aid of THCA synthase and CBDA synthase, respectively. CBD and THC are then formed by decarboxylation under light and/or heat. THC is then oxidized further to CBN (**Figure 2-1**). Different products of *Cannabis* are available; including *Cannabis* dried leaves (Marijuana), *Cannabis* resin (Hashish) and *Cannabis* oil, which is produced by distillation of the resins.

There are two identified cannabinoid receptors in humans: CB1 and CB2 receptors. CB1 receptors are mainly expressed in the central nervous system while CB2 receptors are peripheral receptors; however, functional CB2 receptors were also found throughout the central nervous system. The effects of THC are mediated mainly by CB1 receptors and results in the psychoactive symptoms of THC while CBD acts as negative modulator of CB1 receptors. CB2 receptors are expressed postsynaptic, thus they have opposite functions in neuronal firing to CB1 receptors, which are expressed in presynaptic terminals (Wu, 2019).

The metabolism of THC has been previously characterised with the identification of two major metabolites in human plasma, namely 11-Hydroxy-Δ9-THC and 11-Nor-Δ9-THC-9-Carboxylic acid (**Figure 2-2**). The most abundant metabolite of CBD in human plasma is CBD-carboxylic acid (7-COOH-CBD) with hydroxy-CBD (7-OH-CBD) as a minor metabolite (Wall & Perez-Reyes, 1981)

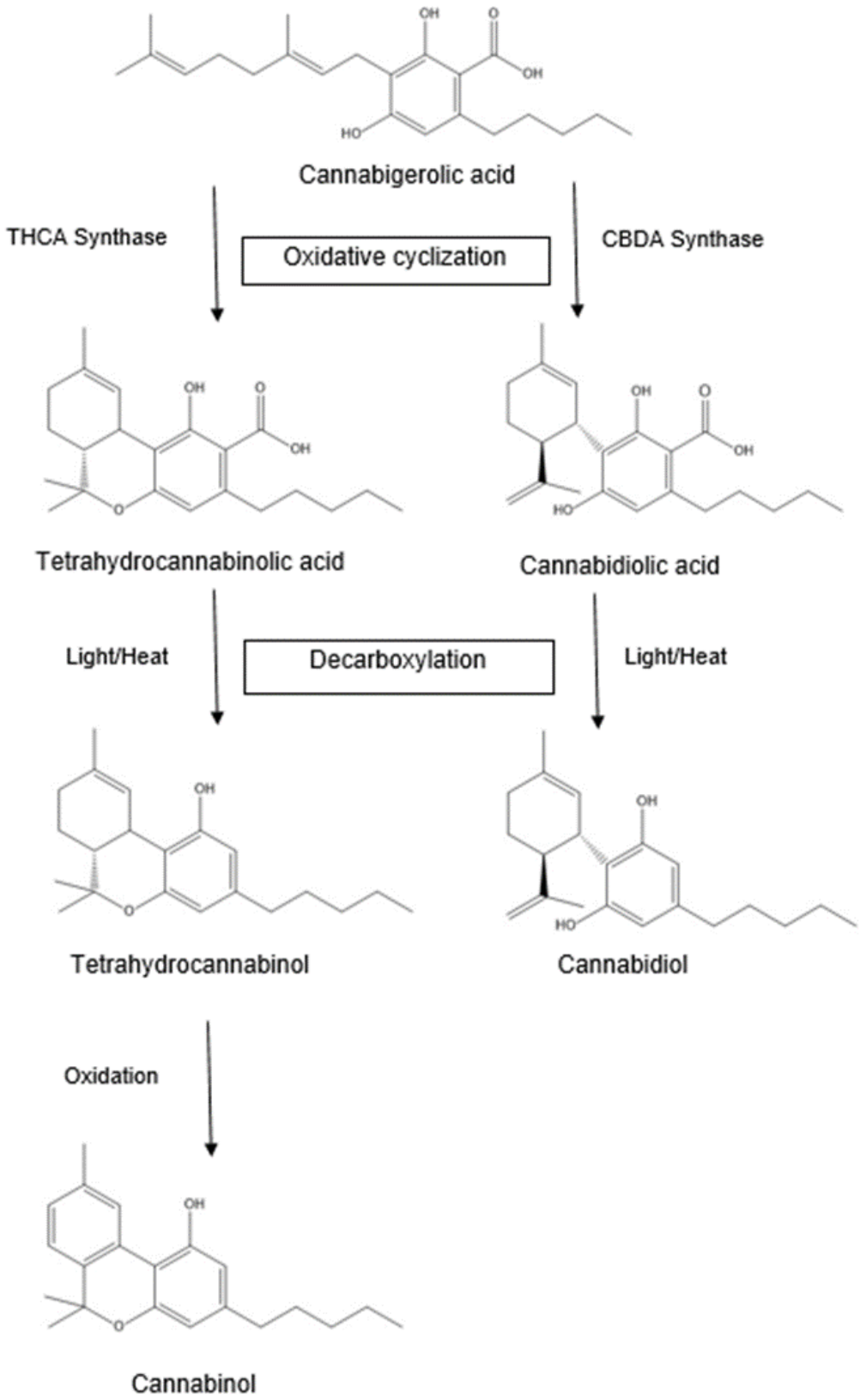

Figure 2-1. Biosynthesis of cannabidiol, tetrahydrocannabinol and cannabinol from cannabigerolic acid.

Adapted and modified from (Citti et al., 2018)

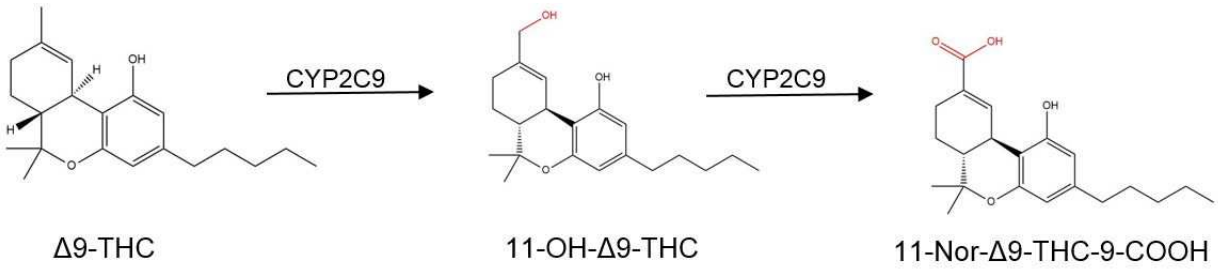

Figure 2-2. The Δ9-THC metabolism.
Adapted and modified from (Joana *et al.*, 2019)

2.2 Therapeutic indications of Cannabinoids

THC and CBD are the major pharmacologically active compounds present in *Cannabis*. While THC is responsible for the psychoactive effects and acts as sedative (A.W. Zuardi, 2003), antiemetic (Parker et al., 2002) and antiepileptic (Karler & Turkanis, 1981) CBD is devoid of psychotropic effect (Zuardi et al., 1982). CBD was also found to reduce the anxiogenic effects of THC (Zuardi *et al.*, 1981) in addition to its anti-inflammatory activity (Costa *et al.*, 2004). CBD is effective in treatment of autism spectrum disorder (Poleg *et al.*, 2019), neuroprotective (Schröder *et al.*, 2017) and has antitumor properties in different kinds of cancer (Xin *et al.*, 2019; Elbaz *et al.*, 2015). On the other hand, CBN acts as an anticonvulsant (Karler *et al.*, 1973). Sativex® is a *Cannabis*-based medicine indicated for controlling pain related to rheumatoid arthritis (D. R *et al.*, 2006), as well as management of multiple sclerosis symptoms (Giacoppo *et al.*, 2017).

2.3 Analysis of cannabinoids

Cannabis Sativa constituents are mainly analysed for forensic purposes or to study the pharmacokinetics and pharmacodynamics of the constituents of the extract and their metabolites (Newmeyer *et al.*, 2016). They are also monitored in plasma or serum to follow up medical *Cannabis* therapy.

2.3.1 Matrices used

Many analytical methods have been developed for the analysis and quantification of *Cannabis sativa* constituents, either in the plant extract itself (Mei *et al.*, 2017) or in biological matrices e.g. urine (Dong *et al.*, 2016), plasma (Grauwiler *et al.*, 2007), whole blood (Jagerdeo *et al.*, 2009; Scheidweiler *et al.*, 2016), oral fluids (Sobolesky *et al.*, 2019) and hair (Salomone *et al.*, 2012).

The analysis of plant material is mainly for the purpose of quality control and characterization of plant phenotype (Tsatsakis *et al.*, 2000). Different biological matrices have been used for forensic purposes; mainly whole blood, plasma and urine. For pharmacokinetic studies, plasma is the

matrix of choice. While analysis of whole blood and plasma offers detection time of several hours to a few days, urine analysis can detect cannabinoid exposure for months in chronic users (Ellis Jr *et al.*, 1985). Whole blood is used more frequently than plasma because of the difficulty of obtaining good quality plasma in forensic cases. Plasma concentrations of cannabinoids are considerably higher than corresponding whole blood concentrations (Giroud *et al.*, 2001).

Ethylenediaminetetraacetic acid (EDTA) is added to the blood tubes as an anti-coagulant and resulting separated plasma is therefore called EDTA plasma. Oral fluids are simple to collect with a non-invasive procedures. High concentrations of CBD and CBN in oral fluids indicate recent *Cannabis* exposure, while THC and THC-COOH are detected for up to 22 hours (Milman *et al.*, 2012). Hair is another interesting matrix due to its long detection window, ease of collection and stability (Pragst & Balikova, 2006); however, external contamination and low concentration of cannabinoid metabolites distributed into hair are two main critical challenges (Minoli *et al.*, 2012).

2.3.2 Extraction methods

Sample preparation is critical to establish effective, sensitive and robust extraction of cannabinoids from the matrix. Different extraction approaches are adopted according to the matrix. As the focus of this study is human plasma a summary of different extraction methods reported in the literature for cannabinoids is presented below.

2.3.2.1 Solid phase extraction

Solid Phase Extraction (SPE) is an extraction technique where the matrix sample (e.g. plasma), is passed through a preconditioned cartridge with a polymer that binds the compounds of interest according to their physical or chemical properties. The cartridge is then washed, the compounds of interest eluted using an appropriate solvent, then evaporated to dryness and reconstituted in the mobile phase. The four basic steps of SPE are illustrated in **Figure 2-3**.

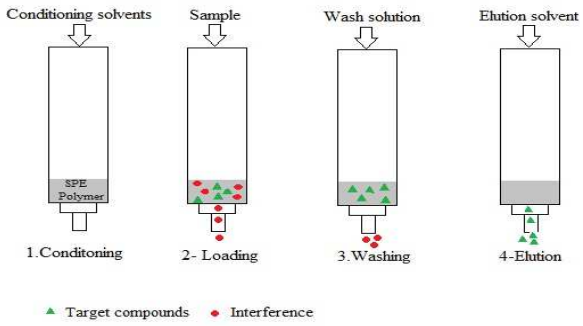

Figure 2-3. Solid phase extraction steps.
Adapted and modified from (Abo *et al.*, 2016).

Different polymers are utilized for the extraction of cannabinoids from plasma samples. C18 SPE is an octadecyl silica polymer which forms strong hydrophobic interactions with non-polar compounds. It was utilized by Nadulski et al. (2005) for the extraction of cannabinoids followed by trimethylsilylation (TMS) derivatization and analysis by GC-MS in single ion monitoring (SIM) mode. Agilent Bond Elute Certify II™ SPE is a mixed mode SPE cartridge that utilizes two different polymers; non polar C8 polymer and strong anion exchange sorbent, to obtain more specificity to acidic and neutral compounds. Agilent Bond elute certify II SPE extraction of cannabinoids from plasma has been reported followed by LC-MSMS analysis (Grauwiler et al., 2007). Online extraction using a C8 column was utlized to extract 11 cannabinoids from plasma and urine prior to LC-MS/MS analysis (Jelena et al., 2017). Phree™ extraction is similar to the SPE concept; however, it uses a sorbent that retains lysophosphatidylcholines and phosphatidylcholine phospholipids while allowing the analytes to pass through. Phospholipids are a major concern when extracting and analysing biological matrices due to signal suppression in electrospray ionization (ESI), a commonly used ionization source in LC-MS. An interesting research publication suggested the application of Phree™ extraction prior to cannabinoids analysis, which lead to enhanced sensitivity (Palazzoli et al., 2018).
The main advantages of SPE methods are the ease of automation, cleanliness of the extract and little amount of solvent used during the extraction process; however, it is costly compared to other extraction techniques.

2.3.2.2 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a sample extraction approach where an organic solvent is added to the plasma samples, and vortexed or rotated to allow compounds to partition into the organic and aquoeus phase according to their solubility. After centrifugation the supernatant is transferred to another tube, evaporated to dryness and reconstituted in the mobile phase. Manual and automated LLE from human serum using n-hexane/ethyl acetate (9/1, v/v) and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) for silylation was applied by (Purschke et al., 2016). The same LLE solvent was utilized after protein precipitation by (Andrenyak et al., 2017). LLE is a cost-effective techqniue, simple and straight forward. The major drawbacks of this extraction method are the amount of solvent used and matrix effects associated with the coextracted substances.

2.3.2.3 Protein precipitation

Protein precipitation is achieved by addition of methanol, acetonitrie or an acid e.g. triflouroacetic acid to remove plasma proteins and release the bounded analytes. Simple protein precipitation was reported for quick extraction of cannabinoids from human serum using methanol (Dziadosz *et al.*, 2017). One percent formic acid in acetonitrile was also reported for extraction of

cannabinoids from human plasma by protein precipitation (Jamwal *et al.*, 2017). Direct protein preciptation with acetonitrile was applied to a micro volume of blood/plasma followed by dabsylation (Lacroix & Saussereau, 2012). Protein precipitation extracts may contaminate the ion source rapidly.

2.3.3 Analytical instrumentation

2.3.3.1 Gas chromatography (GC)

Gas chromatography is a technique used to separate thermo-stable compounds prior to their detection by various detectors. In GC, the mobile phase is the carrier gas (mostly helium) and a stationary phase, which consists of a layer of a polymer on an inert solid support. The concept of GC is to evaporate the sample during injection and the carrier gas transfers the evaporated sample through the stationary phase, which is located in a controlled temperature oven. The different components of the sample are separated by their binding affinity to the stationary phase "column". Compounds which do not bind or have a low affinity to the stationary phase elute quickly, thus reaching the detector faster, resulting in a shorter retention time. The separation of the compounds according to their retention times on the stationary phase allows the detector to identify and quantify each compound in a complex matrix. Gas chromatography is suitable for thermo-stable compounds that can withstand the high temperature during sample injection and elution through the column. A simple diagram of gas chromatography is presented in **Figure 2-4** (Poole, 2012).

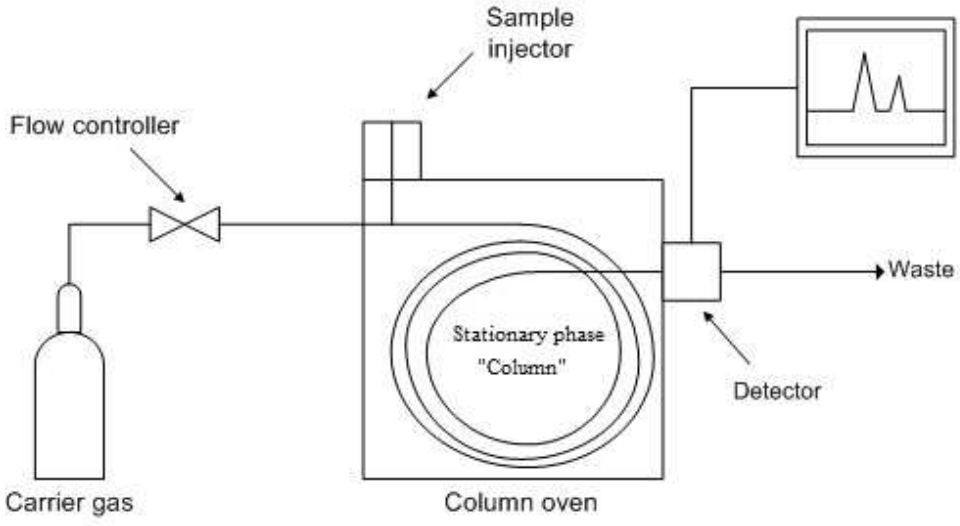

Figure 2-4 Diagram depicting different components of gas chromatography. Reprinted from (Science.oregonstate.edu, 2019).

2.3.3.2 Liquid chromatography (LC)

Liquid chromatography is similar to gas chromatography, the difference being that the mobile phase is liquid instead of gas. The concept is still the same; compounds in the sample elute at different retention times according to their affinity to the stationary phase (**Figure 2-5**). Liquid chromatography does not require evaporation of the sample or application of high temperatures during the separation process, thus it is suitable for thermolabile compounds. During the last decades, liquid chromatography technology has improved tremendously in areas of different stationary phases, the dimensions of the columns and the pressure that the system can withstand. High performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UHPLC) are now used for different analytical applications (Fanali, 2013).

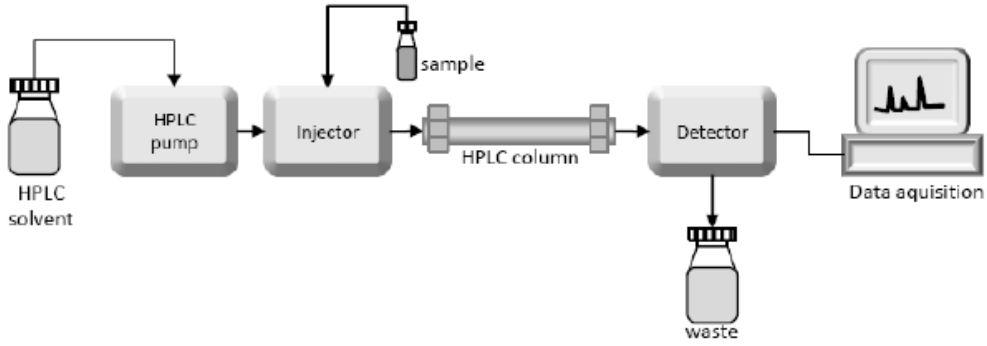

Figure 2-5 Schematic diagram showing elements of liquid chromatography. Reprinted from (Laboratoryinfo.com, 2019).

2.3.3.3 Mass spectrometry (MS)

Mass spectrometry is a detection technique that made huge leaps in analysis of compounds within a complex matrix. The concept of mass spectrometry is to measure mass-to-charge (*m/z*) ratios of ions generated from their respective molecules. The ions of the molecules are produced in the ion source by different techniques: either by electron impact ionization (EI) or chemical ionization (CI) for gas chromatography coupled mass spectrometry (GC-MS) or electrospray ionization (ESI) or atmospheric-pressure chemical ionization (APCI) for liquid chromatography coupled mass spectrometry (LC-MS). The generated ions are then guided and focused through different electric lenses until reaching the mass analyser and the detector. Mass analysers have evolved greatly in recent years from single quadruple to triple quadruple (QQQ) to ion traps (IT), and then high resolution mass spectrometers; Time-of-flight (TOF) and Orbitraps. This continuous development increased the sensitivity and specificity of the instruments to target analytes, which enabled accurate and precise identification and quantification of various classes of compounds in very complex matrices (Griffiths, 2008).

2.3.3.3.1 Ionization sources

In mass spectrometry, gas phase ion formation from molecules is critical to guide the ions of interest through the different components of the mass spectrometer. Hence, depending on the mode of analysis, positive or negative ions are produced (Hoffmann & Stroobant, 2007)

2.3.3.3.1.1 Electron impact

As the name indicates, electron impact (EI) ionization is achieved by bombarding the molecules with electrons generated from the filament. This process results in shooting out an electron from the molecule leaving a positively charged molecule. It also fragments the molecule to a unique set of fragment ions that can be used as a fingerprint of the molecule at a certain value of electron volt (70 eV). EI is used solely in GC-MS.

2.3.3.3.1.2 Chemical ionization

Chemical ionization (CI) is also unique to GC-MS. In this technique, there is an additional gas in the ion source (usually methane). The gas is ionized by the filament electron and in turn it ionizes the molecules. It is considered a “softer “technique as it does not break the molecule completely and thus more information about the parent molecule is available.

2.3.3.3.1.3 Electrospray ionization

Electrospray ionization (ESI) is used in LC-MS. The negative or positive ionization is achieved by spraying the sample carried by the mobile phase into the ion source through a capillary needle while simultaneously applying high voltage. The spray and thus droplets formation is stabilized with the aid of gas (mostly nitrogen) and heat. Ion formation by a heated ESI source in Thermo Q-Exactive plus is illustrated in **Figure 2-6**.

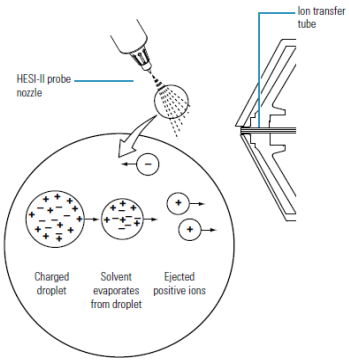

Figure 2-6 Ion formation by a heated electrospray ionization source in positive mode. Reprinted from (Tools.thermofisher.com, 2019).

2.3.3.3.1.4 Atmospheric pressure chemical ionization

Similar to chemical ionization in GC-MS, atmospheric pressure chemical ionization (APCI) is the equivalent in LC-MS. Ionization is achieved with the aid of an electrical discharge applied to the sprayed molecules in the presence of nitrogen under atmospheric pressure. This discharge starts a chemical reaction, which ends up with ionized species of the target molecules.

2.3.3.3.2 Mass analysers

The mass analyser is the part of the mass spectrometer responsible for selection and filtration of ions received from the ion source based on their mass-to-charge ratio (*m/z*). (Hoffmann & Stroobant, 2007)

2.3.3.3.2.1 Single quadrupole

The quadrupole is a mass analyser which consists of four circular metallic rods. It filters target ions with a certain *m/z* ratio with the aid of radio frequency voltage and a direct current (DC) offset voltage. The specificity of such analysers is limited.

2.3.3.3.2.2 Triple quadrupole

Triple quadrupole (QQQ) mass analysers have been developed to overcome the specificity issue of the single quadrupole. QQQ consists of two quadrupoles separated by a collision cell. The precursor ion is filtered in the first quadrupole and then fragmented in the collision cell with the aid of an inert gas. The product ions are then selected in the second quadrupole and passed to the detector. This mode of acquisition is called selected reaction monitoring (SRM). In the case of many transitions monitored in the same time, it is called multiple reaction monitoring (MRM). QQQ mass analysers are very sensitive and specific.

2.3.3.3.2.3 Ion trap

The ion trap mass analyser operates on a similar concept as the quadrupole mass analyser using DC current and radio frequency (RF) oscillating electric field. The 3D ion trap consists of two hyperbolic metal electrodes (endcap electrodes) and two ring electrodes. Ions are trapped by the applied electric field and analysed. The linear ion trap utilizes a set of quadrupoles which are connected by the electrodes at each end to trap the ions. Fragmentation of the precursor ions is also performed inside the ion traps.

2.3.3.3.2.4 Time-of-flight (TOF) high resolution mass spectrometer

The time-of-flight mass analyser is a type of high resolution mass spectrometer (HRMS) which determines the *m/z* ratio of ions through measuring the time the accelerated ions take to reach the detector through a flight tube under electric field. Low *m/z* ions reach the detector faster than higher *m/z* ions. The advantages of TOF mass analysers are the high speed acquisition, unlimited *m/z* range, high mass accuracy and high resolution. The mass accuracy and the high resolution allow the measurement of *m/z* to several decimal points (accurate mass) instead of just the nominal mass, which give more information about the elemental compositions of analysed molecules and separate the target molecules from the co-eluted compounds.

2.3.3.3.2.5 Orbitrap high resolution mass spectrometer

Orbitrap is an ion trap mass analyser where the ions are trapped in orbital motion around a spindle-like electrode surrounded by two bell shaped outer electrodes. Orbitrap analysers offer very high resolution, which reaches up to 450,000 full width at half maximum (FWHM) at *m/z* = 200. It also offers high mass accuracy and sensitivity. The curved linear trap (C-trap) is utilized to store the ions before being injected into the Orbitrap. The hybrid design of Orbitrap is integrated with quadrupole and a collision cell to offer precursor selection and fragmentation.

Mass accuracy

Mass accuracy is the difference between the measured accurate mass and the theoretical accurate mass of a certain compound. It is calculated according to the following equation and expressed as delta parts per million (Δ ppm).

$$Mass\ accuracy = \frac{(measured\ mass - theoretical\ mass) X\ 10^6}{theoretical\ mass}$$

Mass resolution

Mass resolution is the ability to separate two peaks with slightly different *m/z* in a mass spectrum. The resolving power is usually expressed as full width at half maximum (FWHM).

A schematic of QQQ, Q-TOF and Q-Orbitrap is illustrated in **Figure 2-7.**

The advantage of HRMS analysers over low resolution mass spectrometry (LRMS) analysers e.g. triple quadrupole lies in the ability of resolving compounds with similar molecular formula, thus the same nominal mass (**Figure 2-8**). Furthermore, while QQQ is used for screening for only targeted compounds, HRMS is capable of untargeted screening and obtaining information-rich data that can be analysed retrospectively.

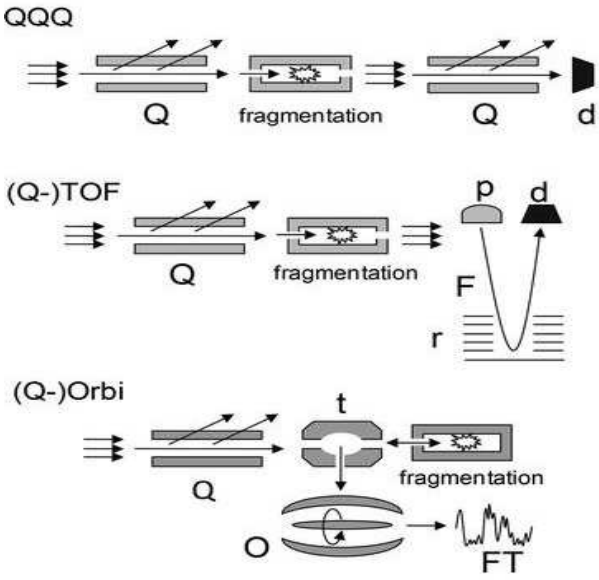

Figure 2-7 Schematic of triple quadrupole (QQQ), quadrupole time-offlight (Q-TOF) and quadrupole orbitrap mass analysers.

Adapted and modified from (Rochat, 2019)

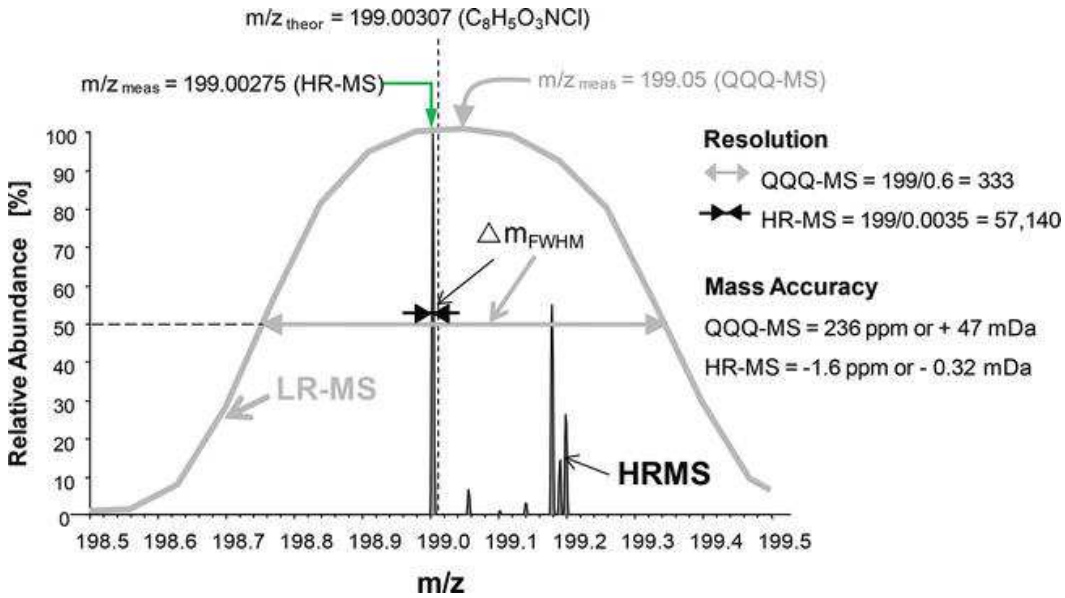

Figure 2-8 Difference between low resolution and high resolution mass spectrometry

QQQ-MS: Triple quadrupole mass spectrometer, LR-MS: low resolution mass spectrometer, HR-MS: High resolution mass spectrometer, ppm: part per million, FWHM: Full width at half maximum.

Adapted from (Rochat, 2019)

2.3.3.4 Analysis of cannabinoids by gas chromatography mass spectrometry (GC-MS)

Gas chromatography (GC) coupled to various detectors; electron capture detector (ECD), flame ionization detector (FID) and nitrogen-phosphorus detector (NPD) have been reported in the literature for the analysis of cannabinoids. Those methods lacked either the sensitivity or

specificity required for such analysis (McBurney *et al.*, 1986). Gas chromatography coupled to mass spectrometry in single ion monitoring mode (GC-MS-SIM) with electron impact ionization (EI) has been successfully used for the analysis of silylated THC, 11-OH-THC and THC-COOH in human plasma (Nadulski *et al.*, 2005), (Purschke *et al.*, 2016). GC-MS-SIM with chemical ionization (CI) in positive mode was also applied to achieve better selectivity and sensitivity (Gustafson *et al.*, 2003). For the same aforementioned reason two dimensional GC-MS-SIM with cryogenic focusing was utilized (Lowe *et al.*, 2007). GC-MS-MS methods based on ion trap analysers (Weller *et al.*, 2000) or triple quad technology (Andrenyak *et al.*, 2017) were developed for enhanced selectivity and higher signal-to-noise ratio. However, one major disadvantage of GC-MS is the time consuming sample preparation due to the derivatization step for thermolabile compounds.

2.3.3.5 Analysis of cannabinoids by liquid chromatography mass spectrometry (LC-MS)

Recently, liquid chromatography coupled to mass spectrometry (LC-MS) has become the instrument of choice for analysis of drugs of abuse including THC, CBD and their metabolites. It provides the required sensitivity and selectivity for detection and quantification of compounds of interest with less sample preparation. An LC-MS/MS method with atmospheric-pressure chemical ionization (APCI) has been developed for the same target analytes mentioned above with significantly less sample preparation (Grauwiler *et al.*, 2007). LC-MS/MS with APCI source was also reported for the purpose of therapeutic monitoring of CBD and THC in plasma as well as in decoctions (Barco *et al.*, 2018). Another method described the use of LC-MS/MS with electrospray ionization (ESI) for analysis of THC, THC-COOH and 11-OH THC in human plasma (Maralikova & Weinmann, 2004). Detection of cannabinoids in a micro volume (50 µL) of blood, serum or plasma has been achieved with the aid of online or offline dabsyl derivatization followed by positive ESI LC-MS/MS analysis (Lacroix & Saussereau, 2012).

2.3.3.6 Analysis of cannabinoids by liquid chromatography high resolution mass spectrometry (LC-HRMS)

Analysis of cannabinoids by high resolution mass spectrometry has been reported in oral fluids (Concheiro *et al.*, 2013) and in hair (Montesano *et al.*, 2015) using Q-Exactive Orbitrap and in plant extracts using Q-TOF (Aizpurua-Olaizola *et al.*, 2014). HRMS gives the advantage of measuring the exact mass of the compounds to several decimal points. This allows discrimination between compounds that have the same nominal mass. This advantage enables researchers to accurately separate the signal of interest from the background, thus reducing the matrix effects significantly. To the best of our knowledge, no available publications utilizing HRMS for quantitative analysis of cannabinoids in human plasma exist.

2.4 Method validation

Developed analytical methods require rigorous validation to prove that the method is fit for its purpose. Method validation procedures challenge the analytical method in many aspects to determine the factors that affect the quality of the results. Main validation parameters are explained below (Shah *et al.*, 2000; FDA, 2018).

2.4.1 Selectivity and specificity

The selectivity of an analytical method is the ability of the method to measure the target analytes in the presence of different matrix components that may cause interferences with the analytical results. Specificity is similar to selectivity; however, specificity takes into the account the external components that may be present in a certain matrix. For analysis of certain analytes in human plasma for example, selectivity will be evaluated by analysing blank plasma samples from different individuals, while specificity will be tested by analysing plasma samples spiked with expected concomitant medications or metabolites. Interference is then investigated.

2.4.2 Linearity

When a method is intended for quantitative analysis, linearity of the method along the expected concentration range has to be investigated. Linearity can be demonstrated with coefficient of determination (r^2), where r^2 values higher than 0.995 indicate acceptable linearity. The difference between the theoretical and the measured concentrations in a calibration curve is also a good indication of linearity.

2.4.3 Accuracy and precision

Accuracy of the analytical results is the closeness of the results to the true value. Precision evaluates the closeness of the repeated results. In bioanalytical analysis, accuracy and precision are evaluated by spiking known concentrations in the intended matrix at different concentration levels. The analysis is performed in replicates and on different days. Inter-day and intra-day accuracy are calculated based on percentage difference from the theoretical spiked value. Coefficient of variance (CV %) is used for evaluating precision in inter-day and intra-day batches.

2.4.4 Lower limit of detection and lower limit of quantitation

The lower limit of detection (LLOD) is the lowest concentration at which the analytical method can detect the target analytes. The lower limit of quantitation (LLOQ) is the lowest concentration the analytical method can quantify reliably. The LLOD is generally estimated at the concentration which results in a response with signal to noise ratio (S/N) higher than 3. The LLOQ is estimated at S/N equal to or higher than 10.

2.4.5 Carry over

Carry over is the interference between samples that are analysed sequentially in a batch. Carry over is caused by the auto-sampler because of improper wash of the needle between the injections. Incomplete elution of the analytes from the analytical column can also result in carry over. The analytical method should eliminate carry over or prove it is insignificant to the test results.

2.4.6 Dilution integrity

Dilution integrity tests the possibility of dilution of samples at a concentration exceeding the higher limit of quantitation. It ensures that applying a dilution factor will not affect the accuracy of the results.

2.4.7 Matrix effect

Complex matrices can affect the accuracy of the results by interfering with the analytical response. The matrix effect is evaluated by comparing the analytical response of the analytes in their neat solutions to the response in the matrix. In mass spectrometry, ionization suppression and ionization enhancement are often reported due to co-eluted matrix components. The analytical method should evaluate such effects and minimize them.

2.4.8 Recovery

Recovery is the ability of the analytical method to extract the analytes from complex matrices. Recovery is estimated by comparing the analytical response of the analytes spiked before and after the extraction. The analytical method should investigate the recovery percentage and prove it is consistent and reproducible.

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CHAPTER 3:

This chapter contains a research manuscript, to be submitted to The Journal of Pharmaceutical and Biomedical Analysis, and prepared according to the author guidelines of this journal. The author guidelines can be found in Annex 1.

RESEARCH MANUSCRIPT

Development and validation of an LC-HRMS method for the quantification of cannabinoids in human plasma

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3.1 Abstract

There is a continuous need to develop sensitive analytical methods for detection of cannabinoids and their metabolites in human plasma for forensic purposes as well as for pharmacokinetic studies. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been the technique of choice due to its sensitivity and rapid sample preparation. High resolution mass spectrometry (HRMS) offers more selectivity due to its accurate mass measurement of the targeted compounds and therefore better signal to noise ratios. The aim of this study was to develop and validate a sensitive LC-HRMS method for the quantitative analysis of cannabidiol (CBD), cannabinol (CBN), Δ9-tetrahydrocannabinol (Δ9-THC) and its major metabolites 11-OH-Δ9-THC and 11-Nor-Δ9-THC-9-COOH in human plasma. The method utilized a simple liquid-liquid extraction of the cannabinoids from plasma samples followed by an isocratic chromatographic separation and detection by the ESI-HRMS Q-Exactive plus platform. Validation was carried out according to FDA guidelines and the method was found to be specific, linear from 0.2 ng/mL to 100.0 ng/mL, having an LLOQ of 0.2 ng/mL for the targeted cannabinoids, accuracy within 15 % at three different concentration levels and within 20 % at LLOQ. The method's intra-day and inter-day precision expressed as CV % were < 11 %. Extraction recovery ranged from 60.4 % to 85.4 %. Matrix effects were reduced due to high resolution mass separation from background noise; however, there was still significant ion suppression, which ranged from 1.1 % to 49.8 % due to competition for ionization in the electrospray ion source.

Keywords: Cannabinoids; THC; CBD; CBN; LC-HRMS; Orbitrap; Human Plasma; method validation

3.2 Introduction

Cannabinoids are phytochemicals produced as secondary metabolites in the *Cannabis Sativa* plant. They are a group of around 70 terpenophenloic compounds formed mainly by decarboxylation of the corresponding acids in the plant [1]. *Cannabis Sativa* constituents are mainly analyzed for forensic purposes as *Cannabis* is widely abused and considered an illicit drug in many countries. Another purpose of their analysis is to study the pharmacokinetics and pharmacodynamics of the constituents of the extract and their metabolites to understand the therapeutic effects associated with each of these components. Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the major pharmacologically active compounds present in *Cannabis*. While THC is responsible for the psychoactive effects and acts as sedative [2], antiemetic [3] and antiepileptic [4], CBD is devoid of psychotropic effect [5]. On the other hand, Cannabinol (CBN) also has anti-convulsion properties [6]. The metabolism of THC has been previously characterized and two major metabolites were identified in human plasma, namely 11-hydroxy-Δ9-THC and 11-nor-9-carboxy- Δ 9-THC [7]. Many analytical methods have been developed for the analysis and quantification of cannabinoids, either in the plant extract [8] or in biological matrices e.g. urine, plasma [9], whole blood [10-12], oral fluids [13] and hair [14].

Researchers face many challenges while developing analytical methods for the analysis of cannabinoids and their related metabolites in plasma and urine. Sensitivity of the method is a major limitation as well as selectivity and elimination of matrix effects associated with complex biological matrices. As human plasma is the matrix in focus for this study, various sample preparations, analytical methods and instrumentations previously used for this purpose will be discussed.

Gas chromatography (GC) coupled to various detectors; electron capture detector (ECD), flame ionization detector (FID) and nitrogen-phosphorus detector (NPD) have been reported in the literature for the analysis of cannabinoids but those methods lacked either the sensitivity or specificity required for such analysis [15]. Gas chromatography coupled to mass spectrometry (GC-MS) has been successfully used for the analysis of THC, 11-OH-THC, THC-COOH, CBD, and cannabinol (CBN) in human plasma after C18 solid phase extraction (SPE) and trimethylsilyl derivatization [16]. Manual and automated liquid-liquid extraction with n-hexane / ethyl acetate mixture followed by silylation derivatization and GC-MS analysis were also reported [17]. However, one major disadvantage of GC-MS is the time consuming sample preparation process due to the required derivatization step for thermolabile compounds.

Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has become the method of choice for the analysis of drugs of abuse including cannabinoids and their metabolites. It provides the required sensitivity and selectivity for detection and quantification of the

compounds with less sample preparation. An LC-MS/MS method with atmospheric-pressure chemical ionization (APCI) has been developed for the same target analytes mentioned above while using mixed mode SPE Agilent Bond Elute Certify II™ with significantly less sample preparation [9]. Another method described the use of LC-MS/MS with electrospray ionization (ESI) for analysis of THC, THC-COOH and 11-OH THC in human plasma after C18 SPE [18]. Phospholipids are a major concern when extracting and analysing biological matrices due to signal suppression in ESI, commonly used as ionization source in LC-MS. An interesting research article suggested the application of Phree™ clean up extraction to eliminate phospholipids during sample preparation, which leads to enhanced sensitivity [19]. A comparison between the reported analytical methods and this research in terms of analytical instrument, extraction methods, plasma volume, sensitivity (LLOQ) and recovery % is presented in **Table 3-1.**

Table 3-1: Analytical instruments, matrix, extraction methods, LLOQ and recovery %

Analytical instruments	Matrix	Extraction method		Target cannabinoids					Reference
				THC	THC-COOH	THC-OH	CBD	CBN	
LC-HRMS	Human plasma 0.5 mL	LLE	LLOQ (ng/mL)	0.2	0.2	0.2	0.2	0.2	This work
			Recovery %	66.3	78.7	69.9	75	70.3	
GC-MS-SIM	Human plasma 1.0 mL	C18 SPE + TMS derivatization	LLOQ (ng/mL)	0.8	0.88	0.51	0.95	3.9	(Nadulski et al., 2005)
			Recovery %	50	85	95	90	43	
GC-MS-SIM	Human serum 1.0 mL	Automated and Manual LLE + MSTFA derivatization	LLOQ (ng/mL)	0.6	1.1	0.8	N/A	N/A	(Purschke et al., 2016)
			Recovery %	N/A	N/A	N/A	N/A	N/A	
LC-APCI-MS/MS	Bovine serum 1.0 mL	Bond elute certify II SPE	LLOQ (ng/mL)	0.2	0.2	0.2	0.2	0.2	(Grauwiler et al., 2007)
			Recovery %	77.5	50	77.6	71.4	47.7	
LC-ESI-MS/MS	Human plasma 1.0 mL	C18 SPE	LLOQ (ng/mL)	0.8	4.3	0.8	N/A	N/A	(Maralikova & Weinmann, 2004)
			Recovery %	N/A	N/A	N/A	N/A	N/A	

Analysis of cannabinoids by high resolution mass spectrometry (HRMS) have been reported in oral fluids [13] and in hair [20] using Q-Exactive Orbitrap and in plant extracts using Q-TOF [21]. The lower limit of quantitation (LLOQ) in oral fluids was 0.5 ng/mL for CBD, CBN, THC and 0.015 ng/mL for THC-COOH while in hair analysis the LLOQ was 0.1 pg/mg for THC-COOH, 1 pg/mg for THC and 2 pg/mg for CBD and CBN, respectively. The use of HRMS has the advantage of measuring the exact mass of the compounds to several decimal points. This allows discrimination between compounds that have the same nominal mass and enables researchers to accurately separate the signal of interest from the background, thus reducing the matrix effect and improving selectivity.

To date, there is no available report on the use of HRMS for the quantitative analysis of cannabinoids in human plasma. Here, extraction of cannabinoids from human plasma was investigated and HRMS was utilized to develop a sensitive method able to quantitate cannabinoids in human plasma with a high degree of accuracy and precision. Chemical structures of the target analytes are presented in **Figure 3-1**.

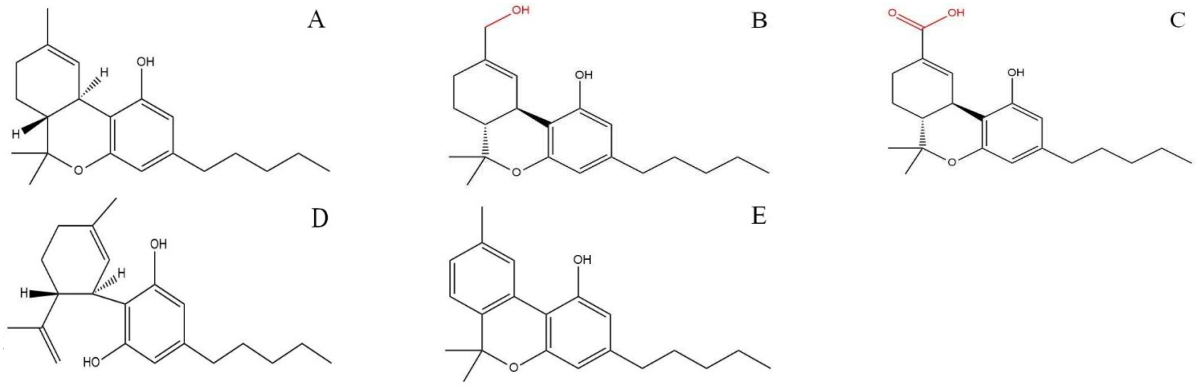

Figure 3-1 Chemical structure of target analytes.
A) THC and its two main metabolites (B) 11-OH-THC and (C) THC-COOH (D) CBD (E) CBN.

3.3.1 Chemicals and reagents

Certified reference materials (CRMs) for cannabidiol (99.85 %), cannabinol (99.12%), Δ9-THC (97.81 %), 11-Hydroxy-Δ9-THC (95.47%), 11-Nor-9-carboxy- Δ 9-THC (98.08 %) and Δ9-THC-D3 (97.93%) (1 mg/mL) were purchased from Cayman Chemicals (USA). HPLC grade acetonitrile and ethyl acetate were purchased from Sigma-Aldrich (Germany). HPLC grade methanol, analytical grade n-hexane, pure HPLC water and potassium dihydrogen orthophosphate were purchased from Loba (India). LC-MS grade acetic acid was purchased from Fisher Chemicals (UK) and blank EDTA human plasma was sourced from Divbio Science (Netherland).

3.3.2 Preparation of standard and quality control (QC) samples

Stock solutions of cannabidiol, cannabinol, Δ9-THC, 11-Hydroxy-Δ9-THC, 11-Nor-Δ9-THC-9-Carboxylic acid and Δ9-THC-D3 were prepared at 10 µg/mL in methanol. Working concentrations of 1000 ng/mL, 100 ng/mL and 10 ng/mL were prepared for target cannabinoids in methanol for spiking of plasma calibrations and quality controls. Internal standard Δ9-THC-D3 was prepared at 100 ng/mL in methanol.

Plasma calibrators were spiked at 8 concentration levels; 0.2, 0.5, 1, 5, 10, 20, 50,100 ng/mL. Quality controls (low, medium and high) were prepared at concentrations of 0.6, 50, 80 ng/mL, respectively.

3.3.3 Human plasma extraction

Five hundred microliter human EDTA plasma was transferred into a 15 mL polypropylene centrifuge tube. Then 100 µl of Δ9-THC-D3 internal standard solution was added followed by the addition of 0.5 mL phosphate buffer (1.5 M potassium dihydrogen phosphate, pH 4.5) and the sample was vortex-mixed for 10 seconds. Liquid-liquid extraction was performed by adding 5 mL n-hexane/ethyl acetate 8: 2 (v/v) followed by mixing by roller mixer for 30 minutes. The sample was then centrifuged for 10 minutes at 3,506 ×g. The organic phase was transferred into a glass reaction vial and evaporated to dryness under a stream of nitrogen at 40˚C in a Ratek™ dry block heater. The dried sample was reconstituted in 100 µl mobile phase (35 % 0.2 % acetic acid in pure HPLC water and 65 % acetonitrile), transferred to an auto-sampler vial with glass insert and 20 µl was injected for LC-HRMS/MS analysis.

3.3.4 LC-ESI-HRMS-MS

3.3.4.1 Liquid chromatography

A robust and rapid chromatographic separation was established using Ultimate 3000 UPLC (Thermo Scientific, Germering, Germany) on a Agilent Zorbax Eclipse™ reverse phase C18 column (1.8 µm, 50 x 2.1 mm) fitted with a Phenomenex C18 pre-column (4 x 3.0 mm). The column oven was maintained at 40˚C. The aqueous mobile phase (Phase A) consisted of 0.2 % acetic acid in pure HPLC water while the organic mobile phase (Phase B) was acetonitrile. An isocratic program with a composition of 35 % phase A and 65 % phase B at a flow rate of 0.35 mL/min for 10 minutes was used. This chromatographic program allowed the essential separation of CBD and THC at retention times of 3.43 and 6.90 minutes, respectively. This separation is critical to differentiate between THC and CBD as they have the same *m/z* and the same product ions and thus, can't be distinguished in the mass spectrometer.

3.3.4.2 Mass spectrometry

For mass spectrometry detection, Q-Exactive plus (Thermo Scientific, Bremen, Germany) with a high resolution hybrid quadrupole orbitrap mass analyser with a higher energy collisional dissociation (HCD) collision cell was utilized. The MS acquired targeted-MS/MS data in parallel reaction monitoring mode at 17,500 full width at half maximum (FWHM) resolution at m/z 200. Single charged ions were generated using heated electrospray ionization (ESI) with negative/positive polarity switching. The target compounds were infused directly into the mass spectrometer while maintaining the mobile phase flow from the column through a T connection. The optimized ESI and front end parameters was as follows; sheath gas and auxiliary gas flow rates were 55 and 10 arbitrary units respectively, spray voltage was 3.5 kV in both negative and positive mode, capillary temperature at 320 °C, heater temperature at 300 °C and S-lens RF level was 60. Nitrogen was used as auxiliary and sheath gas as well as in the HCD and the C-Trap. During method development, a full scan of all product ions was performed at different collision energies for each compound and optimized HCD values were selected. The mass spectra are shown below in **Figure 3-2** and **Figure 3-3**. Mass calibration was performed in positive and negative mode, mass accuracy was lower than 5 ppm. Ionization polarity, precursor ions, quantitation ions, qualifier ions, normalized collision energy (NCE) and retention time for each analyte are summarized in **Table 3-2**. The software used for instrument control, data acquisition and quantitation was XCalibur ™ 4.0 and Tracefinder ™ 4.1.

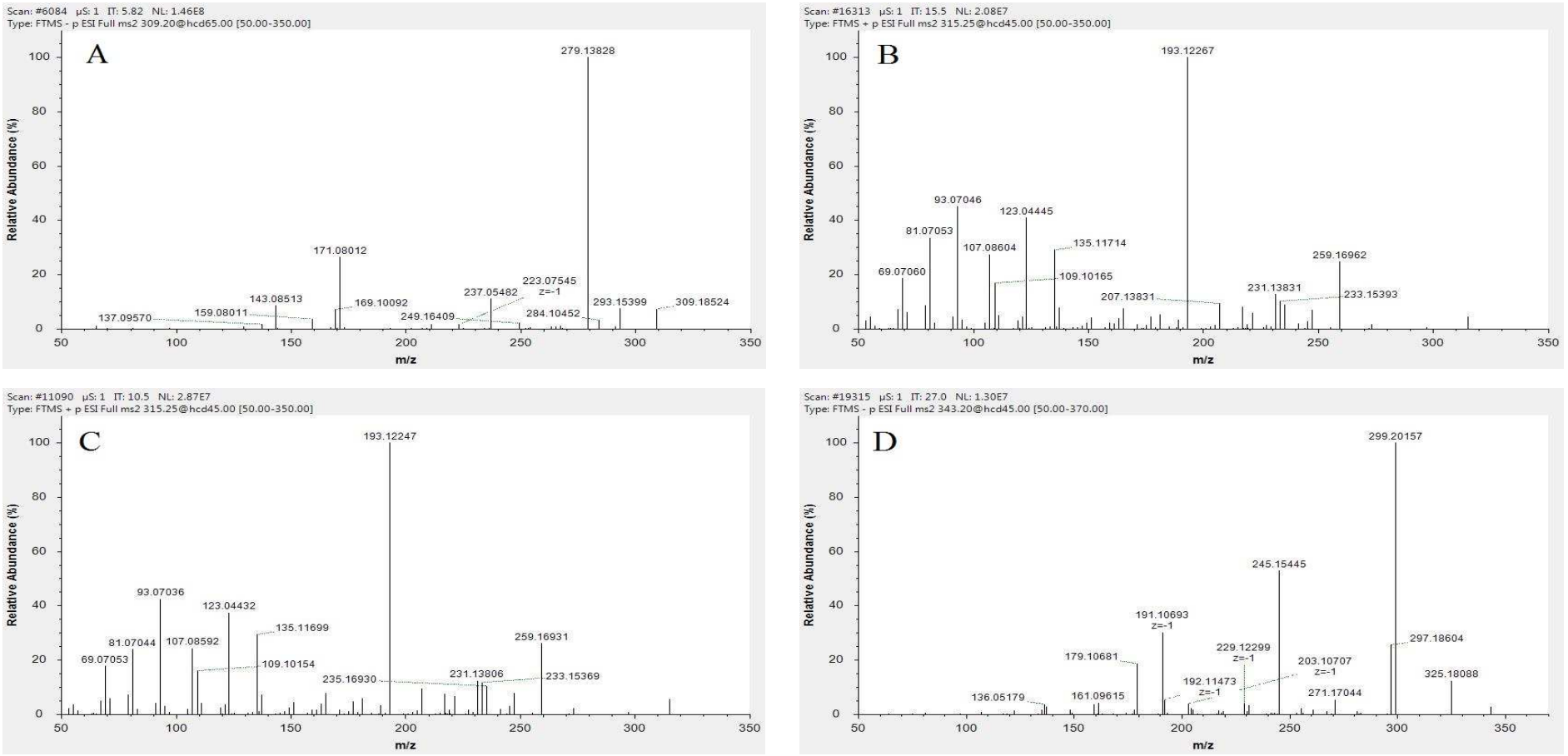

Figure 3-2. Mass spectra of the cannabinoids and THC metabolites

(A) Cannabinol MS spectrum at 65 HCD in negative mode (B) Cannabidiol MS spectrum at 45 HCD in positive mode (C) THC MS spectrum at 45 HCD in positive mode (D) THC-COOH MS spectrum at 45 HCD in negative mode.

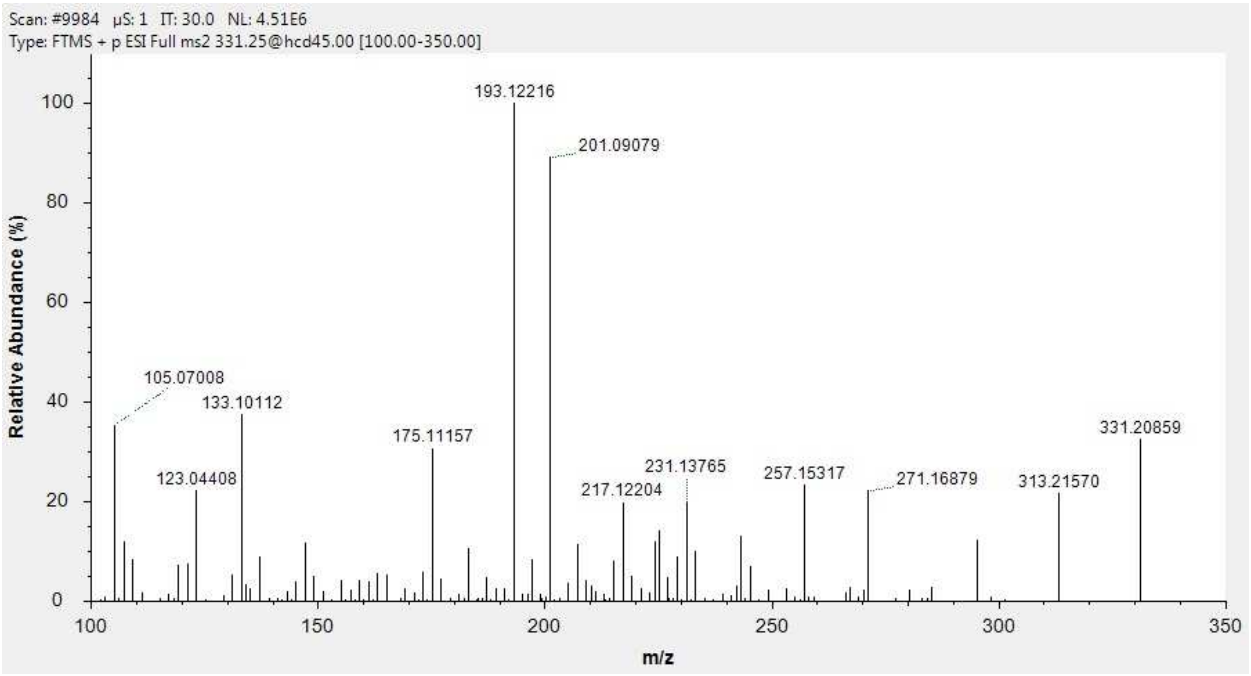

Figure 3-3 11-OH-THC MS spectrum at 45 HCD in positive mode.

Table 3-2: Ionization polarity, precursor ions, quantitation ions, qualifier ions, normalized collision energy and retention time for each analyte.

Analyte	**Ionization polarity**	**Precursor ion (m/z)**	**Quantitative product ion (*m/z*)**	**Qualitative product ion (m/z)**	**NCE (%)**	**RT (min)**
CBN	-VE $[M-H]^-$	309.18600	279.13828	171.08012	65	5.25
CBD	+VE $[M+H]^+$	315.23185	193.12177	259.16962	45	3.43
THC-COOH	-VE $[M-H]^-$	343.19148	299.20097	245.15370	45	1.8
11-OH-THC	+VE $[M+H]^+$	331.22677	193.12241	201.09079	45	1.79
THC	+VE $[M+H]^+$	315.23185	193.12177	259.16931	45	6.9
THC-D3	+VE $[M+H]^+$	318.25068	196.14128	262.18683	45	6.8

NCE: Normalized collision energy, RT: Retention time.

3.3.5 Method validation

Validation was performed according to Food and Drug Administration guidelines on bioanalytical method validation [22]. Validation parameters: selectivity, specificity, linearity, accuracy and precision, LLOD, LLOQ, carry over, dilution integrity, matrix effect and recovery were evaluated for the target analytes.

3.3.5.1 Selectivity and specificity

Selectivity was assessed by analysing six different human EDTA blank plasma samples. Specificity was evaluated by spiking the blank plasma with caffeine, ibuprofen, diclofenac, paracetamol, aspirin and tramadol as examples of possible concomitant medications.

Chromatograms at the retention times of the target compounds and internal standard were assessed for the presence of interference from these medications.

3.3.5.2 Linearity

Linearity of the method was assessed by spiking all the target cannabinoids at 8 concentration levels: 0.2, 0.5, 1.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL in human EDTA plasma. Non-zero calibration levels accuracy should be ± 15% of nominal concentrations, except at the LLOQ where the calibrator should be ± 20% of the nominal concentration. Seventy five percent and a minimum of 6 non-zero calibration levels should pass the aforementioned criteria. Moreover, the concentration – response relationship should fit with a simple regression model and kept uniform all over the study.

3.3.5.3 Accuracy and precision

Accuracy and precision of the method were evaluated by running 3 different sample batches on 3 different days. Each batch consisted of a calibration curve and 4 different quality control levels in 5 replicates: LLOQ (lower limit of quantitation) at 0.2 ng/mL, QCL (low quality control) at 0.6 ng/mL, QCM (medium quality control) at 50.0 ng/mL, QCH (high quality control) at 80.0 ng/mL. All calibrator and quality controls were spiked in human EDTA plasma. The coefficient of variance percentage (CV %) was calculated for intra-day and inter-day precision. The accuracy of the quality controls to the nominal concentration expressed as % was assessed. Accuracy of the QCL, QCM and QCH should be ± 15% of nominal concentrations while a LLOQ ± 20% is acceptable. Similarly for precision, CV% within inter-day runs and intra-day runs should be ± 15% for QCL, QCM and QCH, ± 15% for LLOQ.

3.3.5.4 Lower limit of detection and lower limit of quantitation

The sensitivity of the method is expressed as its lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). The LLOD is the concentration where the signal-to-noise ratio is ≥ 3, while the LLOQ is when the signal-to-noise ratio is ≥ 10. The accuracy of the LLOQ should be within 20% of the nominal concentration and the CV% for precision runs should be ± 20 %.

3.3.5.5 Carry-over

Carry over was assessed by injecting the upper limit of quantitation, followed by 5 injections of blank samples. Chromatograms were then investigated for possible carry-over.

3.3.5.6 Dilution Integrity

Dilution integrity tests the possibility of diluting samples which exceed the upper limit of quantitation while maintaining accuracy and precision. A dilution factor of 3 was chosen and samples were spiked at 150 ng/mL and subsequently diluted 3 times with blank plasma. Five replicates were prepared and injected to evaluate accuracy and precision.

3.3.5.7 Matrix effect

Matrix effects were evaluated at low and high levels. Blank plasma samples were extracted and spiked after extraction with the reference standards of target compounds and the internal standard. The absolute peak areas were compared against those of pure reference standards at the same concentration levels. The neat standards were prepared in n-hexane/ethyl acetate 8: 2 (v/v), then evaporated and reconstituted in mobile phase.

The matrix effect was then estimated by calculating the percentage of the average absolute peak area of the spiked samples to the average absolute peak area of the corresponding pure standard. The percentage suppression was calculated by subtracting the matrix effect from 100.

$$\text{Matrix effect} = \frac{\text{average absolute area of plasma spiked after extraction}}{\text{average absolute area of pure standard}} \times \%$$

3.3.5.8 Recovery

Recovery of target analytes from plasma was estimated by comparing the QCL, QCM, QCH plasma quality controls to the extracted blank plasma samples spiked post-extraction with the corresponding concentration as per below equation.

$$\text{Recovery} = \frac{\text{average absolute area of plasma spiked before extraction}}{\text{average absolute area of plasma spiked after extraction}} \times \%$$

Process efficiency is calculated as proposed by Matuszewski *et al.* (2003), where recovery is multiplied by the matrix effect and then divided by 100 [23].

3.3.6 Results and discussion

3.3.6.1 Chromatographic and mass spectrometric optimization

Firstly, separation of the compounds using different columns and mobile phases was studied. Initially, a Phenomenex-Gemini-NX ™ reverse phase C18 3µ 110 A 150 x 2.0 mm column was used with a gradient program. Methanol and acetonitrile were tested as organic mobile phase, whilst HPLC grade water as the aqueous mobile phase with either formic acid or acetic acid as additive. Both acetonitrile and methanol showed efficient separation of the cannabinoids; however, acetonitrile showed higher signal intensity. Acetic acid as an additive to aqueous phase gave superior signal intensity at a concentration of 0.2 %. Retention time of the target compounds ranged from 12 to 14 minutes with a total run time of 18 minutes. A Zorbax Eclipse™ reverse phase C18 column (1.8µm, 50 x 2.1 mm) was then evaluated with a simple isocratic program. The optimized mobile phase composition was 35 % of 0.2 % acetic acid in water and 65 % acetonitrile at a flow rate of 0.35 mL/min. This chromatography program with the Zorbax Eclipse column allowed the efficient separation of the cannabinoids within a runtime of 10 minutes with the optimum signal intensity, and was thus chosen for the study.

Secondly, the cannabinoids reference standard solution was infused directly into the mass spectrometer through syringe infusion while maintaining mobile phase flow through a T-connection. This process enabled the optimization of the front end parameters of the mass spectrometer, including electrospray ionization (ESI) parameters, the heated capillary and S-Lens RF level. ESI parameters are responsible for the spraying of the sample carried in the mobile phase followed by either negative or positive ionization of the molecules. As those parameters are dependent on flow rate and mobile phase composition, T-connection was required to maintain the flow from the column. Sheath gas is the inner coaxial nitrogen that nebulizes the sample flowing from the needle into fine droplets, whilst auxiliary gas is the heated nitrogen flowing through the outer needle which aids the sheath gas in desolvation of the sample. The flow of those gases is optimized along with the heater temperature to ensure optimum desolvation of the sample. Sheath gas and auxiliary gas flow was adjusted to 55 and 10 arbitrary units respectively. Heater temperature was kept at 300˚C. The ionization was achieved by applying a spray voltage of 3.5 Kv in negative and positive mode. The ion transfer capillary is responsible for transferring the ionized species produced by the ESI source to the S-lens while ensuring the remaining solvent is evaporated. The capillary temperature was maintained at 320˚C. Ions are then focused by the S-lens through the application of RF voltage. The RF amplitude level is mass dependent and was optimized for the cannabinoids masses at 60 %.

Negative and positive polarities as well as different collision energies were investigated. Negative ionization was chosen for CBN and THC-COOH while positive ionization was selected for THC,

CBD, 11-OH-THC and THC-D3. Collision energies were varied to yield good intensity of the quantitative and qualitative fragment ions. Optimal parameters are presented in **Table 3-2.**

An attempt to add the full scan negative and positive experiments in addition to the PRM experiments was evaluated; however, the acquired data points per peak were not enough for quantitation. To utilize the information-rich full scan feature of HRMS during method application to clinical or forensic cases, samples can be re-injected using a modified method where only the full scan positive and negative experiments at 70,000 (FWHM at m/z 200) resolution are present. Different HRMS scan modes can be also utilized e.g. data-dependent or data independent acquisitions. This will allow retrospective analysis of the data in the case of discovery of new metabolites or other research purposes.

3.3.6.2 Sample preparation optimization

Three different sample preparation approaches were evaluated: liquid-liquid extraction (LLE), solid phase extraction (SPE) and direct protein precipitation, followed by clean up with PhreeTM columns.

LLE was performed using different solvents; diethyl ether, tertiary butyl methyl ether and hexane: ethyl acetate 8:2 (v/v). Addition of phosphate buffer to the plasma prior to extraction was also evaluated. For SPE sample extraction, C18 and HLB SPE cartridges were tested. Protein precipitation using acetonitrile followed by Phree^{TM} cleanup was also performed.

Both hexane/acetate (8:2, v/v) LLE and HLB SPE extraction methods achieved very high extraction recoveries. As the recovery was comparably similar, LLE was selected due to cost-effectiveness and the advantage of less sample preparation time. The addition of phosphate buffer (1.5 M potassium dihydrogen phosphate, pH 4.5) to the plasma sample prior to LLE improved the extraction recovery of the analytes.

3.3.6.3 Validation

3.3.6.3.1 Selectivity and Specificity

The method was found to be selective and specific, with an absence of interference peaks at the retention time of the target compounds and internal standard in extracted blank plasma samples and blank plasma samples spiked with concomitant medications. Chromatograms are shown in **Figure 3-4** and **Figure 3-5.**

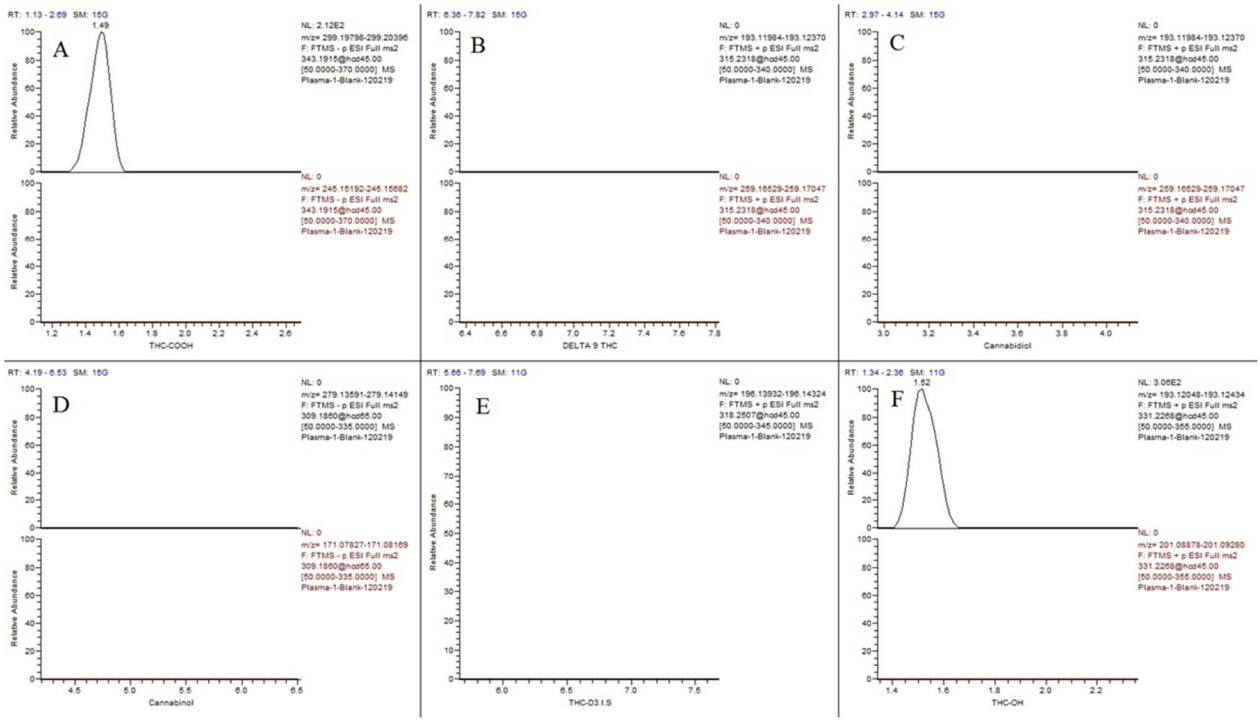

Figure 3-4 Extracted ion chromatograms for cannabinoids quantitative and qualitative product ions in blank plasma from plasma lot1.

(A) 11-Nor-Δ9-THC-9-COOH (B) Δ9-THC (C) Cannabidiol (D) Cannabinol (E) Internal standard Δ9-THC-D3 (F) 11-OH-Δ9-THC.

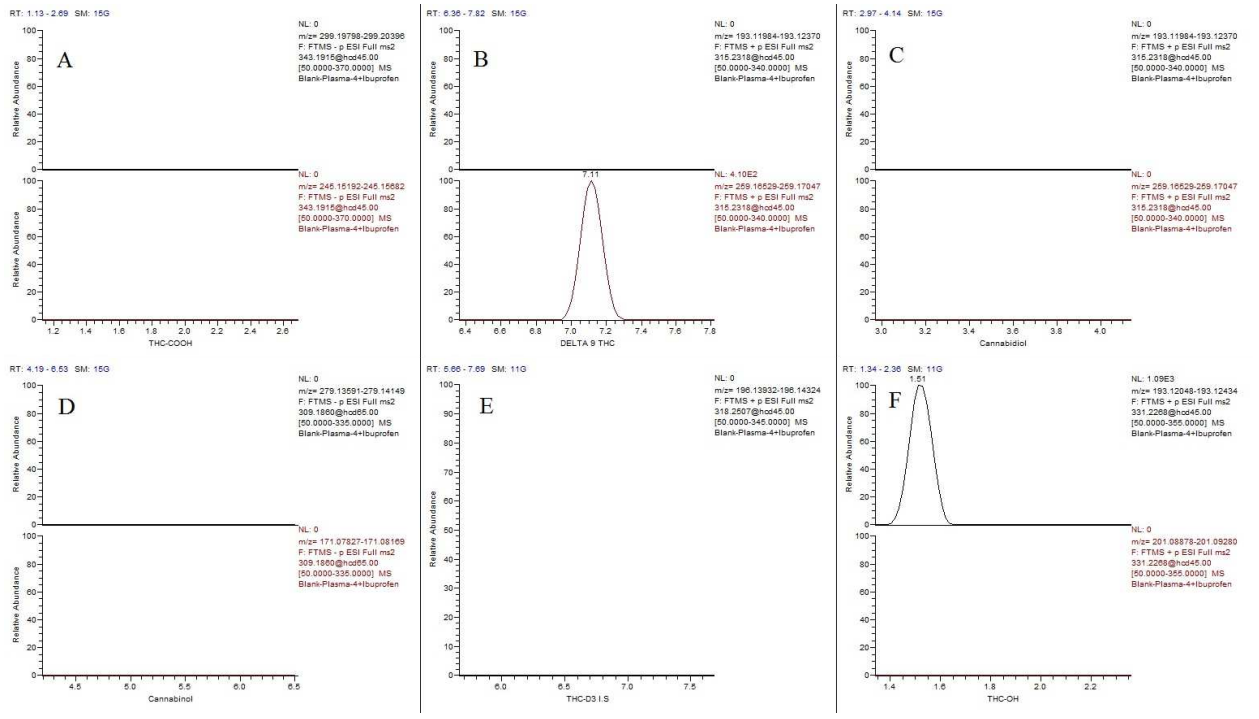

Figure 3-5 Extracted ion chromatograms for cannabinoids quantitative and qualitative product ions in blank plasma spiked with ibuprofen.

(A) 11-Nor-Δ9-THC-9-COOH (B) Δ9-THC (C) Cannabidiol (D) Cannabinol (E) Internal standard Δ9-THC-D3 (F) 11-OH-Δ9-THC.

3.3.6.3.2 Linearity

Linearity was established in a concentration range from 0.2 ng/mL to 100.0 ng/mL by spiking pooled blank human plasma with target analytes. Eight concertation levels were used; 0.2, 0.5, 1.0, 5.0,10, 20, 50 and 100 ng/mL. Response ratios of the peak area of the analyte to the peak area of the internal standard were plotted against nominal concentrations to establish a linear calibration curve. Calibration level accuracy of all analytes were within ± 15 % of the nominal concentrations except for the LLOQ, which was ±20 %. Weighting of 1/x was used for all the analytes. The average coefficient of determination (r^2) was higher than 0.995 for all target compounds. Regression data for each analyte is presented in **Table 3-3**.

Table 3-3: Linearity regression data for the target analytes.

Analyte	**r^2 ± SD (*n* = 4)**	**Slope ± SD (*n* = 4)**	**Intercept ± SD (*n* = 4)**
CBN	0.9974 ± 0.0027	0.0973 ± 0.0394	-0.0016 ± 0.0012
CBD	0.9987 ± 0.0007	0.0420 ± 0.0158	- 0.0018 ± 0.0008
THC-COOH	0.9977 ± 0.0010	0.0616 ± 0.0186	- 0.0016 ± 0.0014
11-OH-THC	0.9967 ± 0.0028	0.0293 ± 0.0229	0.0001 ± 0.0025
THC	0.9983 ± 0.0003	0.0484 ± 0.0108	-0.0011 ± 0.0019

Coefficient of determination (r^2); number of replicate (*n*); standard deviation (SD)

3.3.6.3.3 Accuracy and precision

Quality control samples were used to evaluate accuracy and precision of the method. Intra-day and inter-day precision for the target analytes were determined at four different quality control levels and expressed as % CV. As shown in **Table 3-3**, intra-day precision ranged from 2.3 % to 9.5 % while inter-day precision ranged from 2.9 % to 10.8 %. Intra-day and inter-day accuracy runs were within ±15 % of the quality control nominal concentrations, except the LLOQ, which was ± 20 %. The comprehensive results of the precision and accuracy data are presented in **Tables 3-4** and **3-5**.

Table 3-4: Intra-day and inter-day precision of cannabinoids in human plasma.

	Intra-day precision (% CV) (*n* = 5)				**Inter-day precision (% CV) (*n* = 15)**			
Analyte	**LLOQ**	**QCL**	**QCM**	**QCH**	**LLOQ**	**QCL**	**QCM**	**QCH**
CBN	7.5	4.1	5.2	3.0	10.1	2.9	7.1	6.3
CBD	6.5	3.1	4.6	7.1	7.5	3.4	4.8	4.6
THC-COOH	7.2	3.8	4.4	5.3	6.2	5.0	4.6	5.2
11-OH-THC	7.2	4.9	3.5	2.9	9.0	5.9	4.5	4.3
THC	9.5	2.3	4.7	4.5	10.8	6.8	3.9	2.9

% CV: Percent coefficient of variation, *n*: number of measurements

Table 3-5: Accuracy of the assay for cannabinoids in human plasma with quality control samples at four concentration levels.

	Intra-day accuracy (%, *n* = 5)				Inter-day accuracy (%, *n* = 15)			
Analyte	**LLOQ**	**QCL**	**QCM**	**QCH**	**LLOQ**	**QCL**	**QCM**	**QCH**
CBN	94.1	107.3	93.9	89.8	95.4	109.2	100.8	97.0
CBD	97.4	108.0	100.9	99.0	93.0	106.8	105.2	98.8
THC-COOH	98.4	106.6	96.2	93.2	99.7	104.4	99.3	94.2
11-OH-THC	98.4	99.6	98.9	98.7	95.6	99.5	100.8	96.9
THC	99.1	106.7	103.7	98.8	97.0	105.1	105.7	99.7

n: number of measurements

3.3.6.3.4 Lower limit of detection and lower limit of quantitation

The lower limit of detection (LLOD) was estimated to be 0.07 ng/mL for all target analytes with S/N ratio ≥ 3, while the LLOQ was 0.2 ng/mL with S/N ratio ≥ 5. Moreover, the acceptance criteria were met for the LLOQ, where the accuracy was ±20 % of the nominal concentration and precision was within ±20 %. Extracted ion chromatograms of target compounds spiked at the LLOQ (0.2 ng/mL) with the internal standard in plasma are shown in **Figure 3-6.**

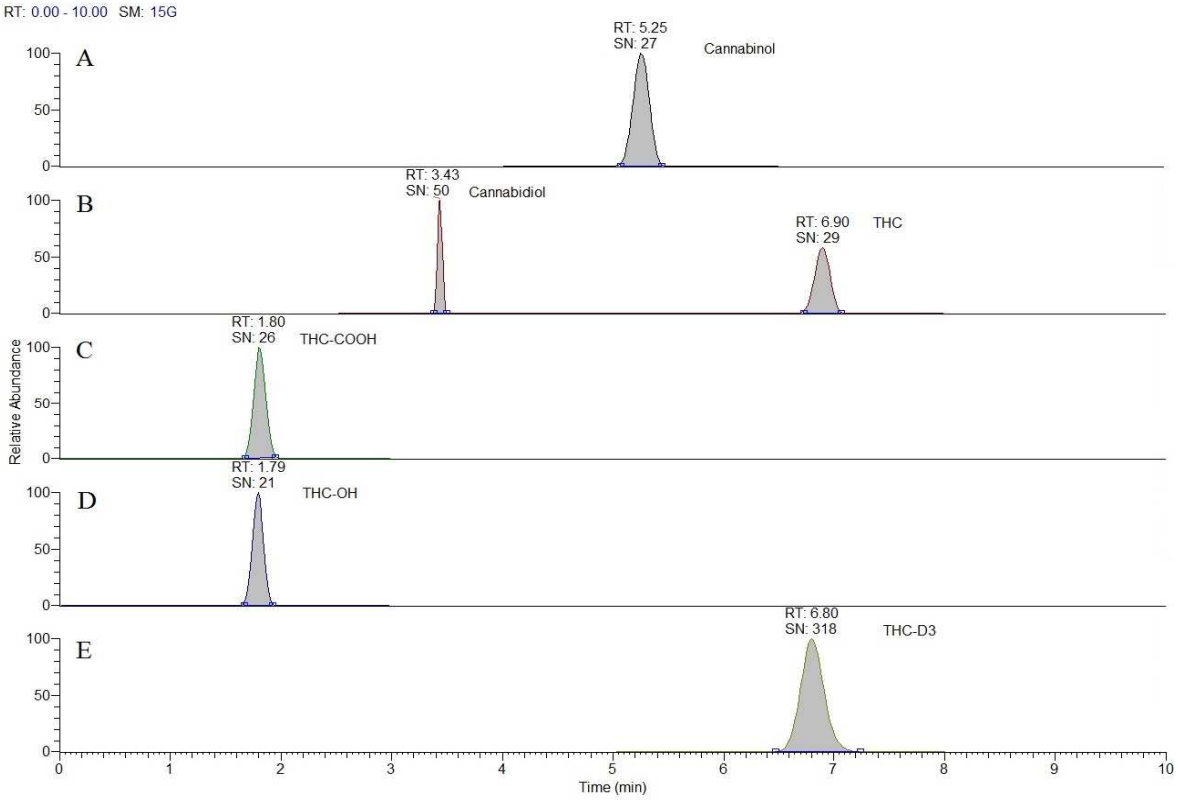

Figure 3-6 Extracted ion chromatogram for target compounds spiked at lower limit of quantification and internal standard.

(A) Cannabinol (B) Cannabidiol and Δ9-THC (C) 11-Nor-Δ9-THC-9-COOH (D) 11-OH-Δ9-THC (E) Internal standard Δ9-THC-D3.

3.3.6.3.5 Carry-over

Chromatograms of blanks injected after high concentrations of the target analytes showed no significant peaks at the retention times of the target analytes and the internal standard. Chromatograms are shown in **Figure 3-7 to Figure 3-12**.

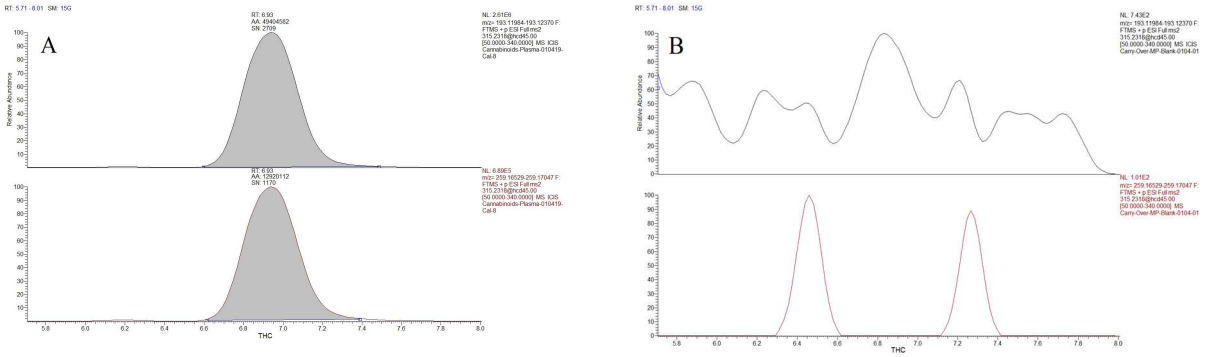

Figure 3-7 Carry over experiments for THC.

Extracted ion chromatograms of THC quantitative and qualitative product ions higher limit of quantitation 100.0 ng/mL spiked in plasma (A) followed by blank injection (B).

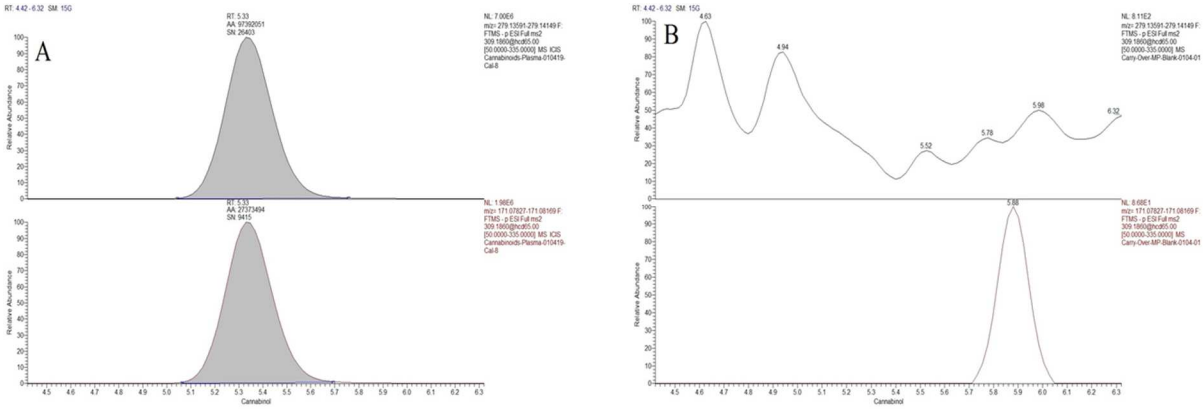

Figure 3-8 Carry over experiments for cannabinol.

Extracted ion chromatograms of cannabinol quantitative and qualitative product ions in higher limit of quantitation 100.0 ng/mL spiked in plasma (A) followed by blank injection (B).

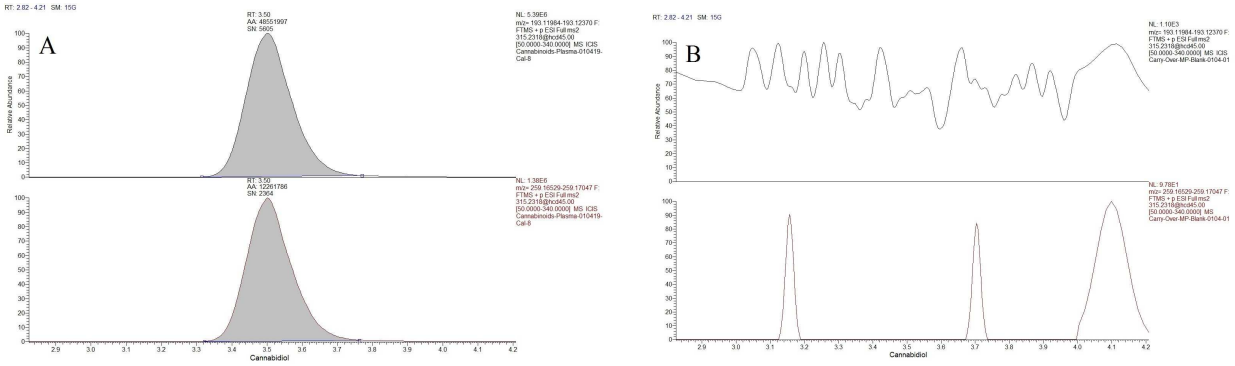

Figure 3-9 Carry over experiments for cannabidiol.

Extracted ion chromatograms of cannabidiol quantitative and qualitative product ions in higher limit of quantitation 100.0 ng/mL spiked in plasma (A) followed by blank injection (B).

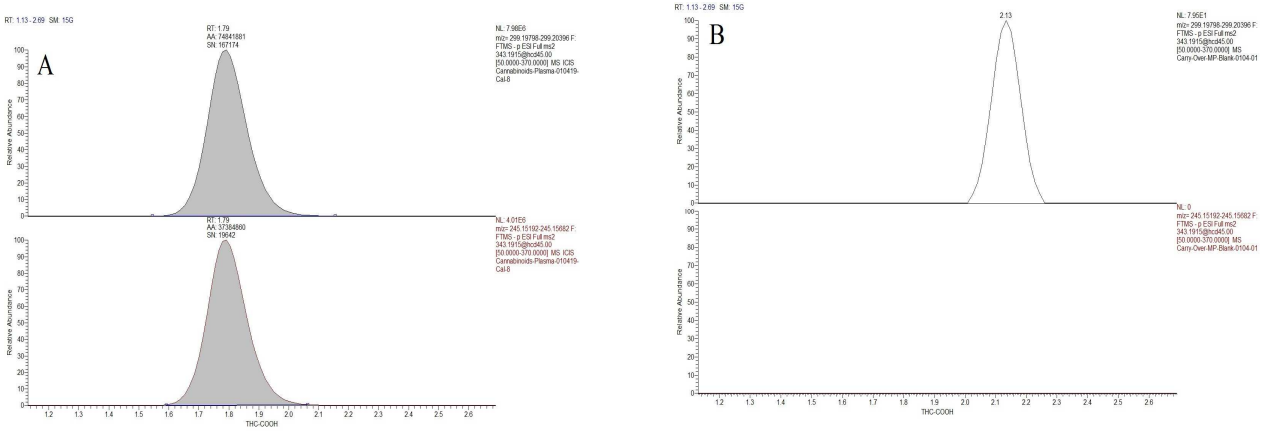

Figure 3-10 Carry over experiments for THC-COOH.

Extracted ion chromatograms of THC-COOH quantitative and qualitative product ions in higher limit of quantitation 100.0 ng/mL spiked in plasma (A) followed by blank injection (B).

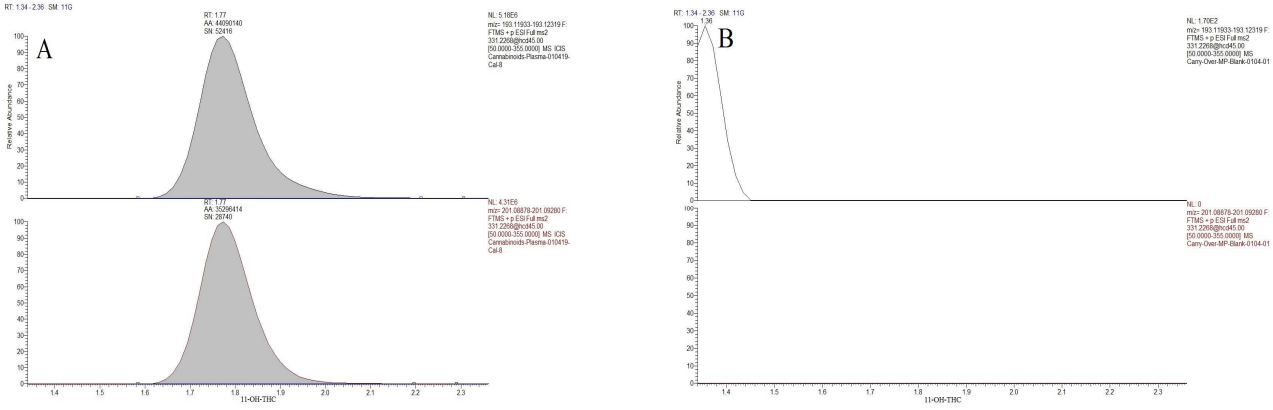

Figure 3-11 Carry over experiments for 11-OH-THC.

Extracted ion chromatograms of 11-OH-THC quantitative and qualitative product ions in higher limit of quantitation 100.0 ng/mL spiked in plasma (A) followed by blank injection (B).

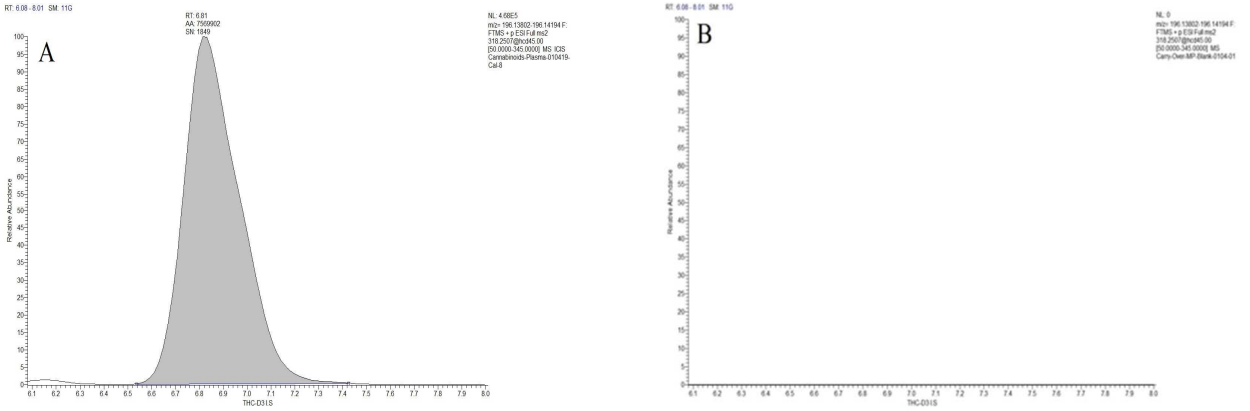

Figure 3-12 Carry over experiments for THC-D3.
Extracted ion chromatograms of internal standard THC-D3 quantitative product ion spiked in plasma (A) followed by blank injection (B).

3.3.6.3.6 Dilution Integrity

Dilution integrity tested at a dilution factor of 3 times in five replicates showed that integrity was maintained for all the target analytes as accuracy and precision of the replicates were within 15 % (**Table 3-6**).

Table 3-6: Dilution integrity accuracy and precision

Analyte	**Accuracy (%)**	**CV% (*n*=5)**
CBN	106.9	2.8
CBD	100.6	7.5
THC-COOH	93.2	11.7
11-OH-THC	109.6	3.5
THC	97.9	2.4

n: number of measurements

3.3.6.3.7 Matrix effect, recovery and process efficiency

The use of LCHRMS allowed a reduction of the matrix effect caused by co-eluting matrix peaks with the same nominal masses as the analytes; however, there is still a matrix effect due to ion suppression in the ESI source. Analysis showed that the ion suppression matrix effect ranged from 1.1 % for 11-OH-THC to 49.8 % for THC. Recovery ranged from 60.4 % to 85.4 % with a CV% less than 15% across the concentration range for all the cannabinoids which indicates good consistency of the recovery at different concentration levels. The overall process efficiency is a good indication to evaluate the methods' sample preparation and analysis by combining both matrix effect and recovery. The data for recovery is presented along with matrix effect and process efficiency data in **Table 3-7**.

Table 3-7: Recovery, matrix effect and process efficiency

	Recovery (%, *n*=3)				Matrix effect (%, *n* = 4)		Process efficiency (%)	
Analyte	**QCL**	**QCM**	**QCH**	**CV%**	**QCL**	**QCH**	**QCL**	**QCH**
CBN	69.7	74.5	66.8	5.53	21.3	29.9	54.9	46.8
CBD	73.2	74.5	77.3	2.79	25.7	26.4	54.3	56.9
THC-COOH	83.7	85.4	67	12.92	23.5	37.5	64	41.8
11-OH-THC	60.4	73.5	75.9	11.93	7.4	1.1	55.9	75.1
THC	65.9	69.4	63.7	4.33	49.8	48.9	33	32.6

n: number of measurements

LC-HRMS offered high selectivity and specificity to the developed method. The LLOQ achieved in plasma (0.2 ng/mL) was previously only reported by Grauwiler *et al*. (2007) [9] for the targeted cannabinoids using LC-MS-APCI in combination with cost-effective SPE and a larger sample volume of 1.0 mL plasma. Other reported methods for analysis of cannabinoids by LC-MS/MS in human plasma showed significantly higher LLOQs ≥ 0.8 ng/mL [18]. This method offered high sensitivity (0.2 ng/mL) with 0.5 mL plasma and simple chromatographic separation using an isocratic flow in combination with commonly used electrospray ionization and cost effective LLE extraction. Although the use of LC-HRMS for the analysis of cannabinoids in plasma is absent in literature, it has been reported for the analysis of cannabinoids in oral fluids with lower sensitivity than reported in this current study for THC, CBD and CBN (Concheiro *et al.*, 2013). Furthermore, analysis of plasma samples are more complex than that of oral fluids **Table 3-8.**

The use of tandem mass spectrometry (QQQ) after chromatographic separation has been the gold standard for the quantitation of small molecules in biological samples. Now LC-HRMS can offer similar linearity over a high dynamic range [24]. The method showed excellent linearity for the cannabinoids in the concentration range assessed, as well as satisfactory accuracy and precision. An advantage of LC-HRMS over QQQ is the ability to obtain information-rich full scan data that can be investigated retrospectively. This allows for re-analysing the data in case of the discovery of a new metabolite.

During the study, while investigating different aspects of the stability of extracts e.g. long term temperature stability, it was observed that THC showed higher accuracy and less variation in the quality control than other cannabinoids. It was concluded that the superior accuracy of THC is due to the closely related deuterated internal standard. Using only one internal standard achieved.excellent linearity and acceptable accuracy and precision. However, utilizing additional internal standards closer to the structure of the other targeted cannabinoids will certainly improve the method quantitation and may allow to control for individual molecule instability when storing for longer terms after extraction.

Table 3-8: Comparison of this LC-HRMS method with other reported LC-HRMS method to determine cannabinoids in biological samples

Matrix	**Oral fluid**	**Human plasma**
Targets	Δ 9-THC 11-nor-9-carboxy THC Cannabidiol Cannabinol	Δ 9-THC (THC) 11-nor-9-carboxy THC (THCCOOH) 11-Hydroxy-Δ9-THC (11-OH-THC) Cannabidiol (CBD) Cannabinol (CBN)
Internal standards	THC-d3 THCCOOH-d3 CBD-d3 CBN-d3	THC-d3
LLOQ	THC, CBD and CBN: 0.5 ng/mL THCCOOH: 0.015 ng/mL	THC, CBD, CBN, THCCOOH and 11-OH-THC: 0.2 ng/mL
Linearity range	THC, CBD and CBN: 0.5 - 50 ng/mL THCCOOH: 0.015 to 0.5 ng/mL	THC, CBD, CBN, THCCOOH and 11-OH-THC: 0.2 - 100 ng/mL
Elution mode and total run time	Gradient program Total run time 10 mins	Isocratic program Total run time 10 mins
Mass spectrometry	Negative ionization	Negative and positive ionization-Polarity switching
Sample preparation	Acetonitrile protein precipitation and SPE	Liquid-liquid extraction (n-hexane /ethyl acetate 8: 2 (v/v)) with addition of buffer (1.5 M potassium dihydrogen phosphate, pH 4.5)
Ref.	[Concheiro, 2013 #13]	This work

3.4 Conclusions

A LC-HRMS/MS method was developed for the analysis of cannabinoids in human plasma. The method employed LLE followed by a simple isocratic separation method coupled to electrospray ionization with positive-negative polarity switching and high resolution mass spectrometry detection in addition to MS fragmentation. Full validation was conducted and indicated that the method is selective, accurate, precise, sensitive and linear from 0.2 ng/mL to 100 ng/mL, and can be applicable to cannabinoid pharmacokinetic studies.

Conflicts of interest

The authors declare no conflicts of interest

Acknowledgement

We would like to thank Quantilab and DST/NWU PCDDP for funding the research.

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CHAPTER 4 RESEARCH OUTCOMES, POTENTIAL METHOD APPLICATIONS, STUDY LIMITATIONS AND FUTURE RECOMMENDATIONS

4.1 Research outcome

Comprehensive method development was performed using LC-HRMS to develop a sensitive analytical method for detection and quantitation of cannabinoids in human plasma. The method was able to quantify CBN, CBD, THC, and its two major metabolites; 11-OH-THC and THC-COOH from 0.2 ng/mL to 100 ng/mL. The method was then challenged with a rigours validation protocol which tested the methods' selectivity, specificity, linearity, recovery, matrix effect, accuracy and precision. The validation parameters met acceptance criteria as per FDA guidelines for bioanalytical methods.

The superior sensitivity of the method and the simple sample preparation will allow the method to be utilized in many applications.

4.2 Potential Method applications

The ongoing discoveries of the therapeutic indications of *Cannabis* motivated researchers to understand the pharmacokinetics and pharmacodynamics of the plants' cannabinoids. Many pharmaceutical preparations have been developed and approved e.g. Sativex® which is a mixture of CBD and THC and Epidyolex®; a CBD based medication. This developed and validated analytical method can be used to quantify the cannabinoids with excellent sensitivity in the controlled clinical trials aiming at providing more information about the pharmacokinetics and pharmacodynamics of *Cannabis* in humans. It can also be used in the bioequivalence studies which compare the generic product to the brand product as a requirement for a generic drug registration.

Another possible application of this method is therapeutic drug monitoring (TDM). As there are possible side effects of *Cannabis* based medications and preparations, it is important to monitor the plasma concentration of the cannabinoids and their metabolites in patients receiving the treatment. This will allow customization of the dose to each individual patient for an optimum pharmacological effect and better control of the side effects.

Cannabis is still considered a drug of abuse in many countries thus there is a forensic requirement for monitoring its abuse. Furthermore, it is considered a prohibited substance during sports competitions except for cannabidiol. This method can be used for detection of *Cannabis* abuse in forensic cases and doping control cases.

4.3 Study limitations

While investigating long term stability of the extracts, it was found that the accuracy and precision for THC is superior to those of the other targets. This is due to the structure of the internal standard which is closely related to THC. This limitation can be solved by incorporating deuterated internal standards closer in structure to the rest the of the target cannabinoids.

The effect of light and temperature on the recovery of the target cannabinoids during sample preparation wasn't studied in this work. Optimizing the environmental conditions may results in better and consistent recovery of the target analyst.

4.4 Future recommendations

To improve the methods' accuracy and precision by the incorporation of different deuterated internal standards closely related to the structure of cannabinol, cannabidiol and THC metabolites.

To adapt this method for different matrices such as oral fluids, whole blood and hair with the necessary changes to the sample preparation while utilizing the optimized LC-HRMS instrument method.

To investigate the acquisition modes of HRMS to simultaneously quantify cannabinoids while obtaining full scan data for future retrospective data analysis.

ANNEX 1 AUTHOR GUIDELINES

Author guidelines for journal of pharmaceutical and biomedical analysis can be found in the website below:

https://www.elsevier.com/journals/journal-of-pharmaceutical-and-biomedical-analysis/0731-7085/guide-for-authors

ANNEX 2 CERTIFICATES OF ANALYSIS

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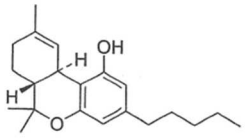
CERTIFICATE OF ANALYSIS

ACCREDITED
ISO/IEC 17025
#AT-1773
ISO Guide 34
#AR-1774

Δ9-THC CRM

6aR,7,8,10aR-tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol

Item #: ISO60157
Batch #: 0530060
CAS Registry Number: 1972-08-03



Expiry: 03MAY2021 (valid from date of certification)
Provided as: 1mg/mL (nominal) solution in methanol
Volume per ampule: not less than 1mL
***Ampules are overfilled. It is advised that labs use measured volumes.**
Storage and Handling: Store unopened at -20°C. Warm to RT prior to opening.
Safety: Flammable, Poison

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ANAB ACCREDITED ISO/IEC 17025 TESTING LABORATORY

Compound Information

Chemical Formula	$C_{21}H_{30}O_2$
Formula Weight	314.50 amu

Certified Concentration

Certified Concentration	1.000 mg/mL ± 0.025 mg/mL

Concentration is calculated based on product mass, solution mass, corrected purity and density at 20°C and is traceable to SI units through an unbroken chain of measurements.
Uncertainty of concentration is expressed as and expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2 and incorporates uncertainties from the corrected purity, solution preparation, homogeneity, long and short term stability.
Concentration was verified by comparison to an independently prepared calibration standard.

Neat Material Quality Information (Item: ISO00157 Batch: 0524244)

Qualifier	Method	Limit	Result	Meets Specification
Chromatographic Purity, HPLC	Cayman Method TST SD151	≥95.00%	97.81% ± 0.18%	Y
Identity, LCMS	Cayman Method TST SD13, +ESI	315.2 ± 0.5 amu	315.4 amu	Y
Identity, GCMS	Cayman Method TST SD12	Conforms	Conforms	Y
FTIR	USP<851> (diamond ATR)	Conforms	Conforms	Y
% LOD	Cayman Method TST SD24	≤5.00%	2.71% ± 0.49%	Y
% ROI	Cayman Method TST SD06	≤3.00%	<0.10% ± 0.21%	Y
*Identity, NMR	^{1}H NMR	Conforms	Conforms	Y
**Corrected Purity			95.06% ± 0.57%	

*NMR is provided as supplemental info but is not within scope of ISO accreditation
**Corrected purity is determined as follows: Corrected Purity = [(100 - % LOD - % ROI)*Chromatographic Purity/100]

Measurement Uncertainty
All measurement uncertainties are expressed as expanded uncertainties in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2.

Cayman Chemical certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration date when stored unopened as recommended.

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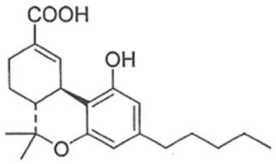
CERTIFICATE OF ANALYSIS

(±)-11-nor-9-carboxy-Δ9-THC CRM

(6aR,10aR)-rel-6a,7,8,10a-tetrahydro-1-hydroxy-6,6-dimethyl-3-pentyl-6H-dibenzo[b,d]pyran-9-carboxylic acid



Item #: 20754
Batch #: 0497428
CAS Registry Number: 104874-50-2

Expiry 08FEB2019 (valid from date of certification)
Provided as: 1 mg/mL (nominal) solution in Methanol
Volume per ampule: not less than 1 mL
***Ampules are overfilled. It is advised that labs use measured volumes.**
Storage and Handling: Store unopened at -20˚C. Warm to RT prior to opening.
Safety: Flammable, Poison

Compound Information

Chemical Formula	$C_{21}H_{28}O_4$
Formula Weight	344.40 amu

Certified Concentration

Certified Concentration	1.000 mg/mL ± 0.007 mg/mL

Concentration is calculated based on product mass, solution mass, corrected purity and density at 20˚C and is traceable to SI units through an unbroken chain of measurements.
Uncertainty of concentration is expressed as and expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2 and incorporates uncertainties from the corrected purity, solution preparation, homogeneity, long and short term stability.
Concentration was verified by comparison to an independently prepared calibration standard.

Neat Material Quality Information (Item: 14241 Batch: 0492461)

Qualifier	Method	Limit	Result	Meets Specification
Chromatographic Purity, HPLC	Cayman Method TST SD151	≥98.00%	98.08% ± 0.18%	Y
Identity, LCMS	Cayman Method TST SD13, +ESI	345.2 ± 0.5 amu	345.2 amu	Y
Identity, GCMS	Cayman Method TST SD12	Conforms	Conforms	Y
FTIR	USP<851> (diamond ATR)	Conforms	Conforms	Y
% LOD	Cayman Method TST SD24	≤5.00%	0.43% ± 0.50%	Y
% ROI	Cayman Method TST SD06	≤3.00%	0.12% ± 0.24%	Y
*Identity, NMR	^{1}H NMR	Conforms	Conforms	Y
**Corrected Purity			97.54% ± 0.58%	

*NMR is provided as supplemental info but is not within scope of ISO accreditation
**Corrected purity is determined as follows: Corrected Purity = [(100 - % LOD - % ROI)*Chromatographic Purity/100]

Measurement Uncertainty
All measurement uncertainties are expressed as expanded uncertainties in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2.

Cayman Chemical certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration date when stored unopened as recommended.

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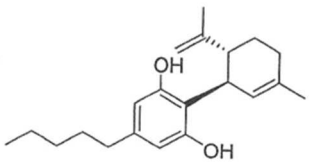
ACCREDITED
ISO/IEC 17025
#AT-1773
ISO Guide 34
#AR-1774

Cannabidiol CRM

2-[1R-3-methyl-6R-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol



Item #: ISO60156
Batch #: 0518548
CAS Registry Number: 13956-29-1

Expiry: 22NOV2020 (valid from date of certification)
Provided as: 1mg/mL (nominal) solution in methanol
Volume per ampule: not less than 1mL
***Ampules are overfilled. It is advised that labs use measured volumes.**
Storage and Handling: Store unopened at -20°C. Warm to RT prior to opening.
Safety: Flammable, Poison

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ANAB ACCREDITED ISO/IEC 17025 TESTING LABORATORY

Compound Information

Chemical Formula	$C_{21}H_{30}O_2$
Formula Weight	314.50 amu

Certified Concentration

Certified Concentration	1.000 mg/mL ± 0.009 mg/mL

Concentration is calculated based on product mass, solution mass, corrected purity and density at 20°C and is traceable to SI units through an unbroken chain of measurements.
Uncertainty of concentration is expressed as and expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2 and incorporates uncertainties from the corrected purity, solution preparation, homogeneity, long and short term stability.
Concentration was verified by comparison to an independently prepared calibration standard.

Neat Material Quality Information (Item: ISO00156 Batch: 0472958)

Qualifier	Method	Limit	Result	Meets Specification
Chromatographic Purity, HPLC	Cayman Method TST SD90	≥98.00%	99.85% ± 0.18%	Y
Identity, LCMS	Cayman Method TST SD13, +ESI	315.2 ± 0.5 amu	315.4 amu	Y
Identity, GCMS	Cayman Method TST SD12	Conforms	Conforms	Y
FTIR	USP<851> (diamond ATR)	Conforms	Conforms	Y
Loss on Drying	Cayman Method TST SD24	≤5.00%	0.57% ± 0.49%	Y
Residue on Ignition	Cayman Method TST SD06	≤2.00%	<0.10% ± 0.59%	Y
*Identity, NMR	^{1}H NMR	Conforms	Conforms	Y
**Corrected Purity			99.18% ± 0.77%	

*NMR is provided as supplemental info but is not within scope of ISO accreditation
**Corrected purity is determined as follows: Corrected Purity = [(100 - % LOD - % ROI)*Chromatographic Purity/100]

Measurement Uncertainty
All measurement uncertainties are expressed as expanded uncertainties in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2.

Cayman Chemical certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration date when stored unopened as recommended.

Approval: [signature] ***Title: Cayman Chemical ISO Quality Manager*** ***Certification Date: 27NOV2017***

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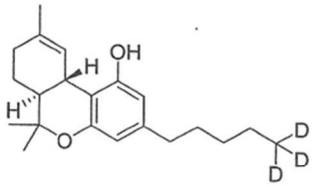
Email: crmquality@caymanchem.com

CERTIFICATE OF ANALYSIS

Δ9-THC-d_3 CRM

(6aR,10aR)-6a,7,8,10a-tetrahydro-6,6,9-trimethyl-3-(pentyl-d3)-6H-dibenzo[b,d]pyran-1-ol

Item #: 19332
Batch #: 0481951
CAS Registry Number: 81586-39-2



Expiry: 27APR2019 (valid from date of certification)
Provided as: 1 mg/mL (nominal) solution in methanol
Volume per ampule: not less than 1 ml
***Ampules are overfilled. It is advised that labs use measured volumes.**
Storage and Handling: Store unopened at -20°C. Warm to RT prior to opening.
Safety: Flammable, Poison

Compound Information

Chemical Formula	$C_{21}H_{27}D_3O_2$
Formula Weight	317.50 amu

Certified Concentration

Certified Concentration	1.000 mg/mL ± 0.012 mg/mL

Concentration is calculated based on product mass, solution mass, corrected purity and density at 20°C and is traceable to SI units through an unbroken chain of measurements.
Uncertainty of concentration is expressed as and expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2 and incorporates uncertainties from the corrected purity, solution preparation, homogeneity, long and short term stability.
Concentration was verified by comparison to an independently prepared calibration standard.

Neat Material Quality Information (Item #19328 Batch #0480455)

Qualifier	Method	Limit	Result	Meets Specification
Chromatographic Purity, HPLC	Cayman Method TST SD91	≥95.00%	97.93% ± 0.18%	Y
Identity, LCMS	Cayman Method TST SD13, +ESI	318.3 ± 0.5 amu	318.4 amu	Y
Identity, GCMS	Cayman Method TST SD12	Conforms	Conforms	Y
FTIR	USP<851> (diamond ATR)	Conforms	Conforms	Y
% LOD	Cayman Method TST SD24	≤5.00%	0.62% ± 0.25%	Y
% ROI	Cayman Method TST SD06	≤3.00%	<0.10% ± 0.23%	Y
*Identity, NMR	^{1}H NMR	Conforms	Conforms	Y
**Corrected Purity			97.22% ± 0.38%	

*NMR is provided as supplemental info but is not within scope of ISO accreditation
**Corrected purity is determined as follows: Corrected Purity = [(100 - % LOD - % ROI)*Chromatographic Purity/100]

Measurement Uncertainty
All measurement uncertainties are expressed as expanded uncertainties in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2.

Cayman Chemical certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration date when stored unopened as recommended.

Approval: *Title: Cayman Chemical ISO Quality Manager* ***Certification Date: 27APR2016***

Cayman Chemical Co.
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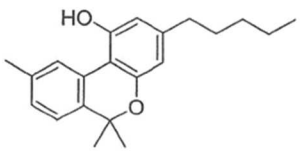
CERTIFICATE OF ANALYSIS

Cannabinol CRM

6,6,9-Trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol

Item #: ISO60183
Batch #: 0533416
CAS Registry Number: 521-35-7



Expiry: 20JUN2021 (valid from date of certification)
Provided as: 1 mg/mL (nominal) solution in methanol
Volume per ampule: not less than 1 mL
***Ampules are overfilled. It is advised that labs use measured volumes.**
Storage and Handling: Store unopened at -20˚C. Warm to RT prior to opening.
Safety: Flammable, Poison

Compound Information

Chemical Formula	$C_{21}H_{26}O_2$
Formula Weight	310.40 amu

Certified Concentration

Certified Concentration	1.000 mg/mL ± 0.011 mg/mL

Concentration is calculated based on product mass, solution mass, corrected purity and density at 20˚C and is traceable to SI units through an unbroken chain of measurements.
Uncertainty of concentration is expressed as and expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2 and incorporates uncertainties from the corrected purity, solution preparation, homogeneity, long and short term stability.
Concentration was verified by comparison to an independently prepared calibration standard.

Neat Material Quality Information (Item: 12066 Batch: 0531542)

Qualifier	Method	Limit	Result	Meets Specification
Chromatographic Purity, HPLC	Cayman Method TST SD114	≥98.00%	99.12% ± 0.18%	Y
Identity, LCMS	Cayman Method TST SD13, +ESI	311.4 ± 0.5 amu	311.2 amu	Y
Identity, GCMS	Cayman Method TST SD12	Conforms	Conforms	Y
FTIR	USP<851> (diamond ATR)	Conforms	Conforms	Y
% LOD	Cayman Method TST SD24	≤5.00%	3.10% ± 0.49%	Y
% ROI	Cayman Method TST SD06	≤3.00%	0.10% ± 0.22%	Y
*Identity, NMR	^{1}H NMR	Conforms	Conforms	Y
**Corrected Purity			95.95% ± 0.57%	

*NMR is provided as supplemental info but is not within scope of ISO accreditation
**Corrected purity is determined as follows: Corrected Purity = [(100 - % LOD - % ROI)*Chromatographic Purity/100]

Measurement Uncertainty
All measurement uncertainties are expressed as expanded uncertainties in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2.

Cayman Chemical certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration date when stored unopened as recommended.

Approval: *Title: Cayman Chemical ISO Quality Manager* ***Certification Date: 20JUN2018***

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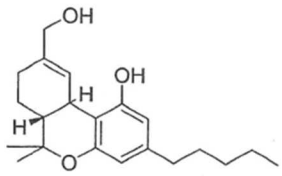
CERTIFICATE OF ANALYSIS

(±)-11-hydroxy-Δ9-THC CRM

rel-6aR,7,8,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-3-pentyl-6H-dibenzo[b,d]pyran-9-methanol

Item #: 21667
Batch #: 0504398
CAS Registry Number: 34675-49-5



Expiry: 14APR2019 (valid from date of certification)
Provided as: 1mg/mL (nominal) solution in methanol
Volume per ampule: not less than 1mL
***Ampules are overfilled. It is advised that labs use measured volumes.**
Storage and Handling: Store unopened at -20˚C. Warm to RT prior to opening.
Safety: Flammable, Poison

Compound Information

Chemical Formula	$C_{21}H_{30}O_3$
Formula Weight	330.50 amu

Certified Concentration

Certified Concentration	0.999 mg/mL ± 0.007 mg/mL

Concentration is calculated based on product mass, solution mass, corrected purity and density at 20˚C and is traceable to SI units through an unbroken chain of measurements.
Uncertainty of concentration is expressed as and expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2 and incorporates uncertainties from the corrected purity, solution preparation, homogeneity, long and short term stability.
Concentration was verified by comparison to an independently prepared calibration standard.

Neat Material Quality Information (Item: 19780 Batch: 0500954)

Qualifier	Method	Limit	Result	Meets Specification
Chromatographic Purity, HPLC	Cayman Method TST SD151	≥95.00%	95.47% ± 0.18%	Y
Identity, LCMS	Cayman Method TST SD13, +ESI	331.2 ± 0.5 amu	331.4 amu	Y
Identity, GCMS	Cayman Method TST SD12	Conforms	Conforms	Y
FTIR	USP<851> (diamond ATR)	Conforms	Conforms	Y
% LOD	Cayman Method TST SD24	≤5.00%	2.17% ± 0.53%	Y
% ROI	Cayman Method TST SD06	≤3.00%	1.13% ± 0.23%	Y
*Identity, NMR	^{1}H NMR	Conforms	Conforms	Y
**Corrected Purity			92.32% ± 0.60%	

*NMR is provided as supplemental info but is not within scope of ISO accreditation
**Corrected purity is determined as follows: Corrected Purity = [(100 - % LOD - % ROI)*Chromatographic Purity/100]

Measurement Uncertainty
All measurement uncertainties are expressed as expanded uncertainties in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2.

Cayman Chemical certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration date when stored unopened as recommended.

Approval: ***Title: Cayman Chemical ISO Quality Manager*** ***Certification Date: 14APR2017***

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Nov 25, 2019

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