Development and validation of a UPLC-MS method for quantification of selected cannabinoids in canine plasma and its application to commercial cannabis products

by

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Abstract

Introduction. Cannabinoids are the important chemicals in cannabis plant with medicinal value [51]. However, effective and safe use is best based on studies that describe their behavior in the plasma of the species being treated. This requires a method for accurate and precise quantification of these closely chemically related compounds. Several LC-MS and GC-MS methods have been described in the literature that quantify cannabinoids in human plasma, rat urine, waste water, surface water, cannabis plant, and cannabis oil. However, the quantification of cannabinoids in canine plasma has not being described. This study describes the development and validation of a reverse phase ultra-performance liquid chromatographic (UPLC) method with mass spectrometry (MS) detection using solid phase extraction for the simultaneous determination of the major cannabinoids. (cannabidiol, tetrahydrocannabinol cannabigerol, cannabinol, and cannabichromene) in canine plasma.

Methods. Based on the chemical structures, physical properties, sample type (canine plasma), and previously reported methods, an analytical method was developed and validated using solid phase extraction to clean up the sample, liquid chromatography for separation and tandem mass spectrometry (LC-MS/MS) for detection.

Results. Cannabinoids were extracted from canine plasma by using Oasis HLB SPE cartridges. Cannabinoids detection, separation and quantification was accomplished using a C18

chromatographic column, a mobile phase consisting of formic acid in water and acetonitrile at a flow rate of 0.5 mL/min. LC-MS/MS with Electrospray ionization (ESI) in positive mode and multiple reaction monitoring (MRM) was used for quantification. The limit of detection (LOD) for the five major cannabinoids was 1.95 ng/mL. The lower limit of quantification (LLOQ) for cannabidiol, and tetrahydrocannabinol was 3.91 ng/mL. For cannabidiol the mean accuracy (% recovery) was 100% ± 18% with a 16% Precision. For tetrahydrocannabinol the mean accuracy (% recovery) was 105% ± 5% with a 5% Precision. Using this method, both cannabidiol and tetrahydrocannabinol were detected and quantified in the plasma of canine patients receiving commercial cannabis-based products. The analytical method for the analysis of cannabinoids in commercial products will require a future validation

Conclusions. We have successfully validated a cannabinoid LC-MS/MS method for quantitation of cannabidiol and tetrahydrocannabinol in canine plasma. This assay will support clinical trials and pharmacokinetic studies necessary to demonstrate safety and efficacy of these promising agents. Identification of cannabigerol, cannabinol, and cannabichromene in canine plasma can be performed with this method, but validation is still pending.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AJ ESI	Agilent Jet Electrospray Ionization
CBD	Cannabidiol
CBG	Cannabigerol
CBC	Cannabichromene
CBN	Cannabinol
FDA	The Food and Drug Administration
HPLC	High Performance Liquid Chromatography
HLB	Hydrophilic-Lipophilic Balance
ICH	The International Council for Harmonization
LOD	Limit of Detection
LLOQ	Lower Limit of Quantification
MS	Mass Spectrometry
MRM	Multiple Reaction Monitoring
RSD	Relative standard deviation (Coefficient of variation (CV))
SPE	Solid Phase Extraction
THF	Delta-9-tetrahydrocannabinol (Δ-9-THC)
UPLC	Ultra-Performance Liquid Chromatography

ULOQ	Upper Limit of Quantification
USP	United States Pharmacopeia

Chapter 1 Introduction to cannabinoids

1.1 Phytocannabinoids, Endocannabinoids, and Endocannabinoid system

The endocannabinoid system have many significant roles in the human body. It is a biological system and is responsible for the physical and psychological effects of the cannabinoids in cannabis. Figure 1.1 shows that cannabinoids can be defined based on their source. (1) Endocannabinoids, are produced naturally by the mammalian body. The "Endo" word means originating within the body. Although the endocannabinoids are different from those formed in marijuana, they have comparable properties and effects. The endocannabinoids interact with the cannabinoid receptors in the brain [1] and body similar as the phytocannabinoids. There are many different endocannabinoids accordingly with some researchers, but 2-AG and anandamide are the most investigated [2] [3] (2) Phytocannabinoids ("phyto" means plant) are exogenous cannabinoids which means produced outside the body, they come from cannabis plants [2]. (3) Finally there are synthetic cannabinoids, which are produced in the laboratory and are intended only for research or development uses. However, synthetic cannabinoids have also been abused for recreational use.

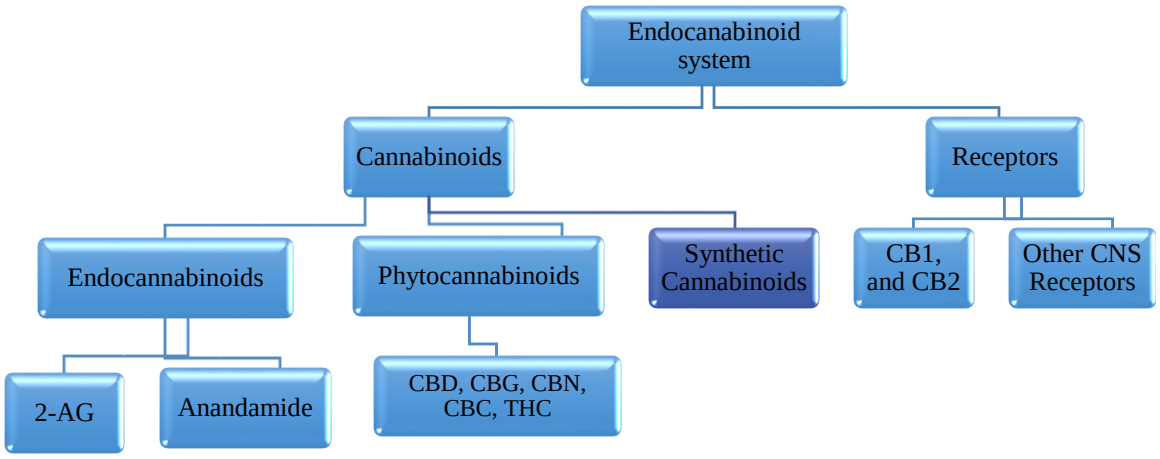


Figure 1.1 Flow chart of Endocannabinoid system

The endocannabinoid system involve cannabinoids and cannabinoid receptors. They can be understood of as a lock-and-key system. Cannabinoids bind to cannabinoid receptors like a key fits into a lock. Unlocking the receptor causes changes in how cells function, leading to different effects in the body [2].

Cannabinoids, whether endogenous or exogenous, have the potential to activate or antagonize these receptors. For example THC activates the endocannabinoid system by attaching to and activating cannabinoid receptors [2]. Cannabinoids, phytocannabinoids and synthetic cannabinoids work with receptors. The endocannabinoid system has two receptors: CB1 receptor was the first cannabinoid receptor identified. This receptor was found primarily in the brain, and secondarily in the spinal cord. It is concentrated in brain regions associated with cannabinoid-induced changes in behavior. CB2 receptor was found, mainly in the immune system, and also throughout the body. The endocannabinoid system is connected with many

biological activities and interact with many parts of the body. Cannabinoids may be effective for so many different diseases for this reason, Figure 1.2, [4][2].

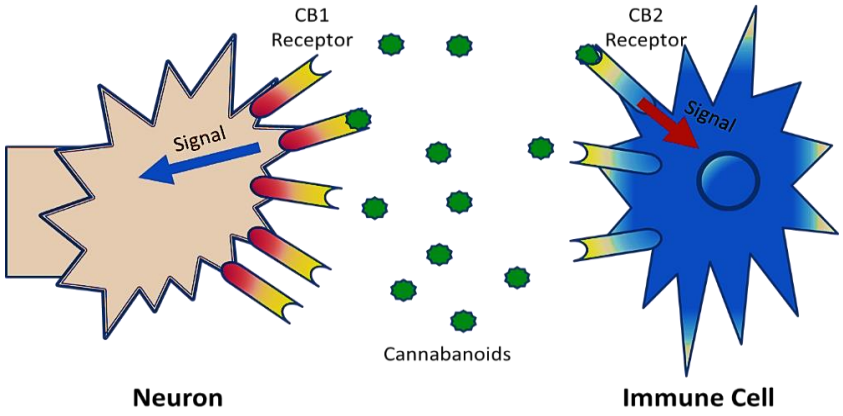


Figure 1.2. Endocannabinoid system: Cannabinoids are the chemical messengers for the endocannabinoid system, and receptors are message receivers. Messages come in the form of chemical messengers binding to the receptor. These messages produce a characteristic effect within the body [4]

The cannabis (*Cannabis sativa),* plant or "marijuana" plant, was used since centuries ago [5]. "Hemp" term usually refers to the use of cannabis as a source of fiber or as a medical use. Both hemp and cannabis come from the same plant, but different parts. The biologically active constituents of the cannabis plant are the cannabinoids and they are considered to be and they are the chemicals which give the cannabis plant and hemp commercial products its unique medical properties [2] [47].

All cannabis plant parts can contain cannabinoids, but not the seeds. The cannabinoid spectrum between plant parts do not present qualitative differences in terms of chemicals, only quantitative differences in the cannabinoid or other constituent ingredients. The major concentration (in % of dry weight plant material) of the cannabinoids is in the bracts of the flowers and fruits. The foliage leaves has a lower concentration of cannabinoids, and the stems and roots is even lower [6].

In 1963-1964 a chemist named Raphael Mechoulam (from Bulgaria), along with his colleagues discovered the chemical structure of tetrahydrocannabinol (THC) and cannabidiol (CBD), the main cannabinoids in the cannabis plant [7] [5]. They are the chemicals which give the cannabis plant and hemp commercial products its medical properties. The cannabis plant produces as many as 100 different cannabinoids. While THC and CBD are the most well-known cannabinoids, there are many other cannabinoids in cannabis plant that offer health benefits. Some of these include cannabigerol (CBG), cannabinol (CBN), and cannabichromene (CBC).

The highest level of THC in cannabis plant is comprised exclusively of the female flower heads ("buds") that remain unfertilized throughout maturation of the plant and which, consequently, contain no seeds [8]. Industrial cannabis (industrial hemp) comprises a number of varieties of the cannabis plant intended for agricultural and industrial purposes. Hemp is grown for seeds and fibers and as such, industrial cannabis is characterized by low THC content and high CBD content. The ratio of CBD to THC is greater than one [8]. In most European countries the current upper legal limit for cultivation is >0.2 percent THC, whereas in Canada 0.3 percent and in USA, it is 0.3 percent (The Farm Bill) [8][9] [10].

The THC content varies depending on the plant part: 10-12 percent in pistillate flowers, 1-2 percent in leaves, 0.1-0.3 percent in stalks, and < 0.03 percent in the roots [8].

THC, CBD, CBN, CBG and CBC are the main cannabinoids in cannabis plant detected in each breeding strain or cultivar of cannabis, Figure 1.3, shows the chemical behavior [6] [11].

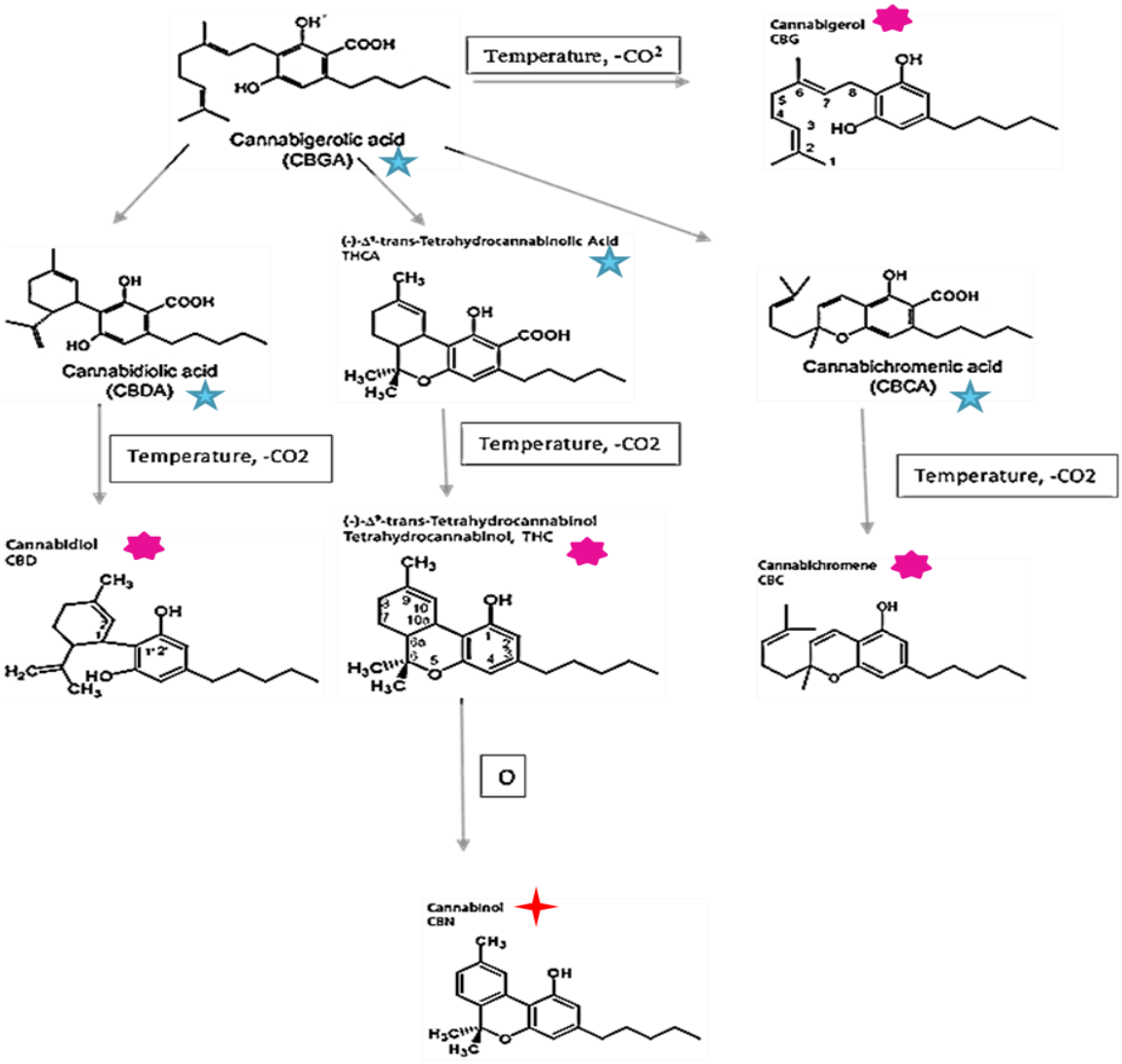


Figure 1.3. Chemical pathway for the production of cannabinoids: [O] = oxidation, -CO2 = decarboxylation [6] [11]. Those cannabinoids marked with pink stars are the main cannabinoids in cannabis plant

Basically, the phytocannabinoids can be classified in three main groups based on their chemistry [6].

- Acidic cannabinoids as a result of metabolism of the plant;
- Neutral cannabinoids resulting from decarboxylation
- Cannabinoids as a results of degradation (oxidation, isomerization, UV-light) [6].

How the cannabinoids are chemically related between them is an important key when each cannabinoid in the plant is studied. Changes or degradation in some of the cannabinoids might happen as a result of storage conditions.

For Cannabis plant, cannabinoids are biosynthesized to an acidic (carboxylated) form. The most common forms of acidic cannabinoids are Δ9-tetrahydrocannabinolic acid A (THCA-A), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA). THCA-A is the main form and will be later out mentioned to as THCA. CBGA is the direct precursor of THCA, CBDA and cannabichromenic acid (CBCA). The carboxyl group is unstable and is simply lost as CO2 under effect of heat or light, resulting in the corresponding neutral cannabinoids: THC, CBD, CBG and (CBC). When the cannabis plant is drying, these are formed during heating and or during storage. (Figure 1.3) [6].

The 5 main cannabinoids and their characteristics are as follow:

THC is the psychoactive substance in the cannabis plant, and is therefore either absent or present in a low concentrations in the hemp oils and extracts. It is also a scheduled 1 substance in many countries and therefore illegal. THC acts as analgesic, muscle relaxant, antispasmodic and anti-inflammatory [7] [12].

CBD is the most common cannabinoid in most hemp plants, and has demonstrated the widest variety of potential medicinal uses. Many scientific papers and anecdotal reports detail possible pain relieving, a neuroprotective antioxidant, anti-inflammatory, anticancer, antianxiety, anticonvulsant, anti-seizure and anti-nausea effects of THC [12].

CBN is a product of aged THC, and it has demonstrated possible anticonvulsant and anti-inflammatory effects [12]. THC in cannabis plant will degrade and change its molecular structure

becoming CBN if is exposed to light or heat. Scientists have found that CBN has strong antibacterial properties.

CBC may possess anti-inflammatory, pain relieving and antidepressant properties, maybe partially due to its ability to slow the breakdown of the cannabinoids of the body. CBC appears to have antifungal and anticonvulsant properties [12][7].

CBG concentration is usually found less than 1% in the cannabis plant, but in hemp can be found a little higher. The CBG has demonstrate to have therapeutic effects, including analgesic, modest antifungal, and antidepressant effects, also muscle relaxant and mildly anti-hypertensive effects [2] [7].

1.2 Why the need to measure the content of cannabinoids in canine plasma?

How important is it to be able to measure the content of cannabinoids in canine plasma or commercial products? To answer this question, one should start with historical information: Cannabis has been used as an agricultural crop for textile fibers for centuries, for recreational, religious and medicinal uses. Other legitimate cannabis products include cannabis seed, cannabis seed oil and the essential oil of cannabis. However, despite the popularity of cannabis today perhaps being the most widely used drug worldwide, for nearly 70 years the cannabis plant went into hiding, and medical research into its attributes largely stopped. In 1970 the federal government in USA made it even harder to study cannabis (marijuana) plant, classifying it as a Schedule I drug, which means is a dangerous substance with no valid medical purpose and a high potential for abuse. This Schedule I status, is likely to have contributed to the lack of medical research on cannabis in the United States [4].

As of 2009 around 100 compounds known as “cannabinoids” have been identified in cannabis extracts. The number of active chemicals in cannabis is one of the reasons why treatment with cannabis is difficult to classify and study.

Besides “Cannabinoids” there is an uncertain number of other compounds in the cannabis plant, which makes cannabis more difficult to be analyzed and classified. Some of these components can interfere with the identification and quantification of the cannabinoids, and here is why the need to develop a specific analytical methods for the identification and quantification of cannabinoids.

Most cannabinoid products that are in the market are formulated from industrial hemp that is obtained by pressing hemp seeds rather than marijuana plant to get the “hemp oil”. Hemp producers believe that “Hemp oil or hempseed oil” contain mainly CBD and a small amount of THC which make this a better option for cannabinoids to be approved as an alternative medicine [54].

Legalization of medical *Cannabis sativa* (marijuana) has been accompanied by the emergence of a growing market of *Cannabis sativa* as a medicinal plant around the world, and also here in USA. This rapidly expanding interest in medical cannabinoid has a potential therapeutic application in humans and animals to treat different illnesses [54]. Understanding the cannabis plant and hemp composition will allow the scientists to start finding the path for future research. This is one of the main reasons why it is necessary to develop specific analytical methods for the identification and quantification of cannabinoids. Also, the researchers will know the exact composition of the cannabinoids in the products to differentiate and classify them according to the concentration of its components. The approval of medical marijuana in many states has led to a plethora of internet cannabinoid products being marketed to pets and

people. However, there is no regulatory oversite regarding product quality, and identifying and quantifying the cannabinoids in *Cannabis sativa* (marijuana), industrial hemp (hemp oil) and plasma samples will be important, because the patients (humans and pets) are consuming those commercial products. Testing the amount of cannabinoids in blood stream (plasma) and the commercial products will support clinical trials, pharmacokinetic and toxicology studies necessary to demonstrate safety and efficacy of these promising agents.

During the past 30 years, multiple studies have reported methods for the detection and quantitation of cannabinoids in the tissue of animals (Table 1.1), a very limited number of which report the determination of CBD and THC in canine biological tissues. : In 1977 [13] an assay was published for the separation of THC in canine plasma by HPLC-UV/GC using liquid-liquid preparation and radioactivity-based method of detection. In 1987 [14] an HPLC-UV assay was developed for the intent of pharmacokinetic description of CBD canine in plasma. Later, in 2012 [15], in the plasma and brain pharmacokinetic profiles of CBD and other cannabinoids in rats and mice oral following intraperitoneal administration was published using LC/MS. In 2015 [5] a development of a simple and sensitive HPLC-UV method for a simultaneous determination of CBD and THC in rat plasma (See Table 1.1).

For humans several LC-MS/MS methods for the quantification of cannabinoids were developed for the detection and quantification of THC and CBD: In 2008 [16] a determination of cannabinoids in whole blood by UPCL-MS-MS, using ESI and positive ion mode. In 2015 [17] a pitfall in cannabinoids analysis detection in serum by LC-MS/MS. In 2016 [18] [19] a simultaneous quantification of the major cannabinoids in human plasma and urine by LC/MS/MS. Recently in 2017 [20] a protein precipitation analysis by LC/MS/MS in human serum.

Mass spectrometers (MS) have more sensitive and selective analysis than ultra-violet (UV) detectors. However, equipment and maintenance, as well as sample running costs are considerably expensive [21] [22]. For the detection of CBD and/or THC in human plasma, bioanalytical methods had been developed using radioactivity assays [23], high-performance liquid chromatography (HPLC)-UV detection [5]. Some LC-MS [17] [18][19][20] in human plasma, serum, and urine. However, issues of low percent recovery and sampling volumes needed for the analysis preclude the use of these methods to conduct the analysis with a good accuracy in dogs. The limit of detection (LOD) in these assays was from 1 ng/mL to 25 ng/mL, and the lower limit of quantification for CBD in dogs was from 1 ng/mL to 25 ng/mL, for mice and rats between 10 ng/mL to 25 ng/mL.

Some of these methods have a low recovery (55%) and requires derivatization with pentafluorobenzyl bromide, others they synthesized and purified the standards in their laboratories and radiolabel+ed quantitation. Radioimmunoassay for CBD has the disadvantage of misidentification and misquantitation because of cross-reactivity with other cannabinoid compounds and metabolites (Table 1.1). [13][14][15][5][16][17] [18][19][20].

For the UPLC-MS method developed there was no interference from canine plasma matrix. Calibration figures from the plasma extract showed no interference between the cannabinoids and the matrix.

Table 1.1. An extract of methods reported for detection and quantitation of cannabinoids in tissue of mammals

Article Name	Authors	Date	Species	Sample Type	Detector	Mobile Phase	Sample Preparation	Injection Vol. (µl)	Sample volume	Retention time (min)	Cal. Curve range ng/mL	LOD ng/mL	LLOQ ng/mL	% Recovery	%RSD
Separation and Analysis of Δ9-Tetrahydrocannabinol (THC) in biological fluids by HPLC and GLC	Garrett E.R. et al.	1977	Canine	Plasma	HPLC - UV, λ = 254 nm	51 %A acetonitrile in water at 1.5 ml/min.	Liquid Liquid Extraction (LLE)	10	5 mL blood, 2 mL plasma	THC	1-100	Fasting: 1 No Fasting: 5-10	1	97.57 ± 0.69%	Not reported
Rapid high-performance liquid chromatographic assay with pharmacokinetic applications for monitoring cannabidiol in plasma	Samara E., et al.	1987	Canine	Plasma	HPLC-UV, λ = 220 nm	Acetonitrile : Methanol : Water (v/v/v) 7: 1 : 2. Flow 2 ml/min.	Precipitation with acetonitrile, SPE (C18), evaporate solvent, reconstitute with methanol.	100	500 µL	CBD: 4.2 THC: 7.6	25-500	CBD: 25	25 ng/mL?	100.9 ± 2%	Mean 6.6%
Plasma and brain pharmacokinetic profile of CBD, CBDV, Δ9-THCV, and CBG in rats and mice following oral and intraperitoneal administration and CBD action on obsessive compulsive behviour	Deiana S., et al.	2012	Rat and Mice	Plasma	Quattro Micro MS, ESI, MRM. CBD negative mode. Cone voltage 26V, collision energy 25 eV, Dwell 300 ms, transition 313.3-245.2.	1 mM ammonium formate in water : acetonitrile. Gradient : 75:25, 0:100, 75:25. Flow 0.35 ml/min	Precipitation with acetonitrile, addition of 0.1% ascorbic acid before injection.	5	Not mentioned	Not mentioned	100-50,000 10-2000 for rat. 25-7500 for mice	CBD: 3	25 (mice) 10 (rat)	Not reported	Not reported
Development of a simple and sensitive HPLC–UV method for the simultaneous determination of cannabidiol and Δ9-tetrahydrocannabinol in rat plasma simultaneous determination of cannabidiol and delta 9-tetrahydrocannabinol in rat plasma	Zgair, A. et al.	2015	Rat	Plasma	HPLC-UV, (PDA), 220 nm	Was a mixture of acetonitrile and water in a ratio of 62:38 (v/v). 1 mL/min. Run time 20 min	Precipitation with acetonitrile, LLE, Dry under N2 35 °C, reconstituted with acetonitrile.	30	150 µL	CBD: 8.3 THC: 15 Run time: 20	7.5 - 10,000	Not reported	CBD: 10 THC 10	CBD 86.2 to 91.0 ±3.1% THC 96.7 to 94.6 ±1.9%	CBD: 8.02 %, THC 14.33%
Determination of Cannabinoids in Whole Blood by UPLC-MS-MS	Janeey C., et al.	2008	Human	Whole Blood	UPLC-MS/MS. ESI. MRM. Capillary voltage 2.5 kV, ion source temp 150°C, desolvation gas temp 450°C with flow of 1100 L/hr, cone gas 100 L/hr, collision gas pressure 4 mbar.	0.1% Formic Acid pH 2.6 : Acetonitrile. Gradient. Flow 0.7 ml/min.	Precipitation with acetonitrile, SPE (Bond Elute Certify), Dry at 45 °C, reconstitute with acetonitrile: 0.1% formic acid 50:50 v/v.	10	1 mL	THC: 10	0.05-50	THC: 0.02	THC: 0.05	47.1 to 58.9%	<7.3 %
Pitfall in cannabinoid analysis-detection of a previously unrecognized interfering compound in human serum	Toennes S.W., et al.	2015	Human	Serum	LC-MS/MS, ESI, MRM. Gas flow 13 L/min (250°C), nebulizer 20 psi, sheath gas 12 L/min (400°C), and capillary voltage 4000V.	0.01 % formic acid with 5 mM ammonium formate : acetonitrile with 0.1% formic acid. Gradient: 50:50, 0:100, 50:50. Flow 0.5 ml/min	Automated SPE (Bakebond), Dry at 25 °C, reconstitute with acetonitrile:methanol:water 6:6:4 v/v/v.	5	200 µL	THC: 3.97 Run time: 4.2	1-50 THC	No reported	1	>95 %	< 11%
Simultaneous quantification of major cannabinoids and metabolites in human urine and plasma by HPLC-MS/MS and enzyme-alkaline hydrolysis	Aizpurua-Olaizola O., et al.	2016	Humans	Plasma, Urine	HPLC-MS/MS, APCI. Corona discharge current 5 uA, capillary voltage of 3500V, heated vaporizer at 280°C, nitrogen flow 7 L/min, source temp of 210°C, nebulizer pressure of 32 psi. MRM.	0.1% formic acid in water : 0.1% formic acid in methanol. Gradient: 40:60, 20:80, 5:95, 40:60. Flow rate 0.3 ml/min.	Precipitation with methanol, Dry supernatant at 40 °C, addition of B-glucuronidase enzime solution, Incubation (16 h), precipitation with acetonitrile, SPE (Strata C18-E) for the supernatant, evaporate to dryness at 40 °C, recontitute with methanol.	10	1 mL	CBD: 12.8 THC:15.5	1-1000	CBD - 0.3 (plasma/urine) THC : 0.4 (urine) THC : 0.5 (plasma)	CBD:1 THC:1	CBD: 64% for urine 43% for plasma THC : 78% for urine 54 % for plasma	1 to 12%
Simultaneous quantification of eleven cannabinoids and metabolites in human urine by liquid chromatography tandem mass spectrometry using WAX-S tips	Andersson M., et al.	2016	Humans	Urine	UPLC-MS triple Quad, ESI, MRM. Nebulizer flow 2 L/min, heating gas flow 10 L/min, drying gas 6 L/min, interface temp 350°C, desolvation line temp 200°C, and heat block temp 450°C.	10 mM ammonium acetate in water : 15% methanol in acetonitrile. Gradient - 70:30, 50:50, 29.7:70.3, 2:98, 70:30. Flow rate 0.5 ml/min.	Precipitation with methanol and acetonitrile, WAX - tips extraction, supernatant diluted with mobile phase. Autosamples at 4°C.	30	200 µL	CBD: 5.7 CBG: 5.9 CBN: 6.92 THC: 7.6	THC : 0.5-100 CBD: 0.5-50 CBN: 0.5-50 CBG: 1-100	CBD: 0.5 THC:0.5	CBD: 0.5 THC: 0.5	CBD: 100-112% THC : 96.3-108%	CBD: 6.5-11.6% THC : 5-11.9%
Simple protein precipitation-based analysis of Δ9-THC and its metabolites in human serum by LC-MS/MS	Dziadosz M., et al.	2017	Human	Serum	LC-MS/MS- API 550 Qtrap. (-ESI). sMRM. Curtain gas 35 psi, collision gas medium, temp 350°C, ion source gas 1 60 psi, ion source gas 2 50 psi, ion spray voltage - 4500V.	A) 10 mM ammonium acetate and 0.1 % acetic acid in water/Methanol, 95:5, v/v and B) 10 mM ammonium acetate and 0.1 % acetic in water/Methanol, 3:97, v/v. Gradient: 70%B, ramping to 100%B from 0 to 1.0 min, holding 100%B from 1 to 4.5 min returning to 70%B from 4.5 to 5.0 min, and holding 70% B from 5 to 5.5 min. The total flow was: 0.4 ml/min, 0.5 ml/min, 0.4 ml/min. Colum temp 25°C, autosampled temp 15°C.	Protein precipitation with methanol and mobile phase.	40	15 µL	THC: 3.75	THC : 0.1- 20	THC : 0.11	THC : 0.25	THC : 79%	THC : ±15%

Chapter 2 Introduction to detection and quantification of compounds in biological matrices

Multiple steps must be implemented during the development of a method for detection and quantitation of compounds in biologic matrices, Figure 2.1.

Various analytical techniques have been described to detect drugs in a variety of biological matrices. The most relevant matrices used for analysis of drugs are serum, plasma, whole blood, urine and oral fluids. Among the targets of these methods are drugs of abuse, which commonly is urine the sample of choice for identification and quantitation of unknown drugs due to high concentration of drugs or their metabolites in urine. However, improvements in sample preparation and instrumentation techniques that included improvements in sensitivity and accuracy have let do blood being satisfactory as a screening matrix for drugs of abuse. In contrast to urine, and because physiological parameters can vary within only narrow limits, (to maintain life), blood as a matrix is relatively homogeneous and thus an easier tissue to test. Drugs in plasma or blood can be detected prior to metabolism [24].

Figure 2.1 shows a number of factors that must be considered immediately as a method is being developed because of their impact on methods development. The identification of the type of matrix in the sample is important for the method development because the nature of the sample determines the cleaning sample procedure. Examples include protein precipitation, solid phase extraction, liquid-liquid extraction or a combination of them can impact recovery and thus the concentration of the compound of interest. Ultimately, sample cleaning along with other factors, determines the minimum sample size that is necessary for the analysis.

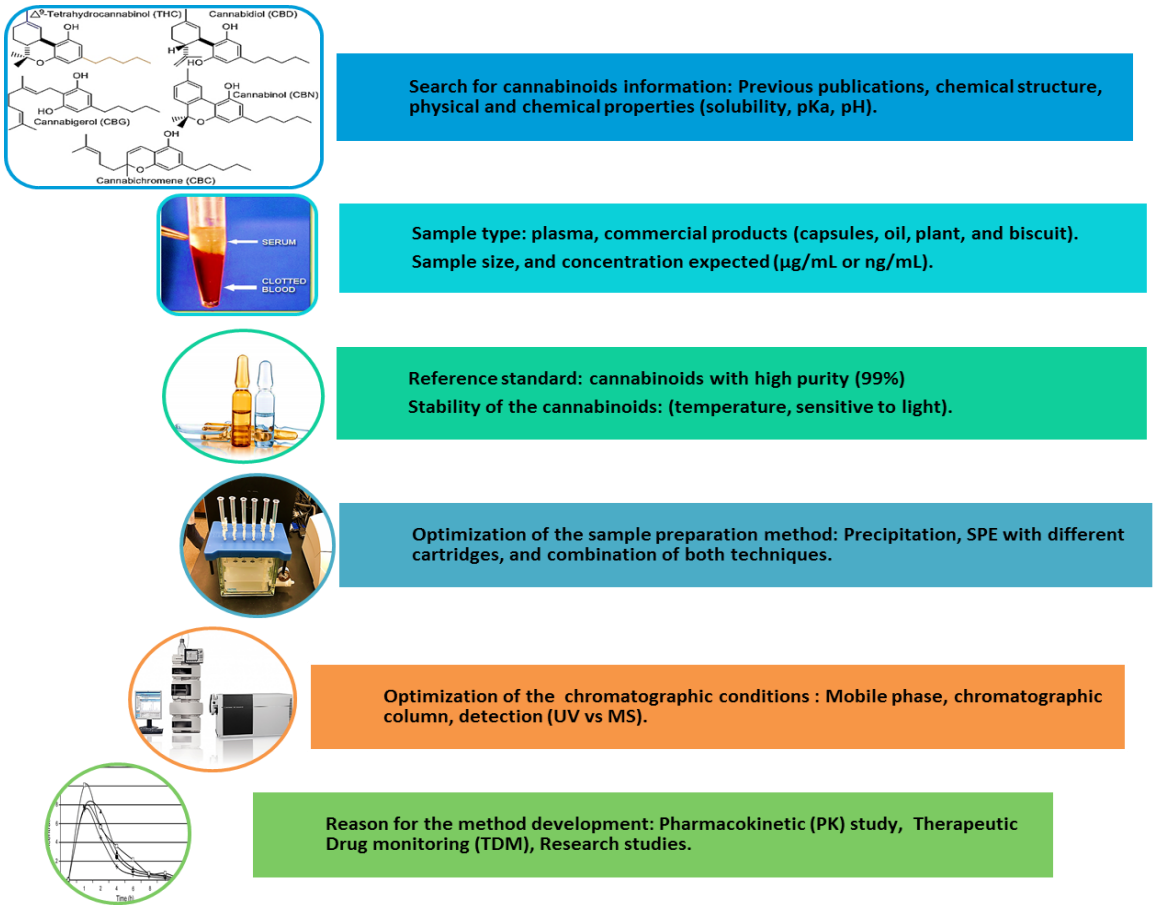


Figure 2.1. Steps for method development

The concentration of drug expected (µg, ng or pg/mL) and the physical and chemical properties of the drug are also important, and should be taken into account during development of the analytical technique and the selection of the analytical instrument and detection method. The drug extraction, separation, identification and quantification is more difficult if the chemical and physical characteristics of the drugs are similar to constituents in the matrix and, if the sample contains more than 2 analytes.

Sample preparation is an integral part of most bioanalytical methods. It consists of selective isolation of the analyte of interest from the matrix, minimization/elimination of matrix components in the processed sample and, if required, concentration of the analyte of interest. In a clinical

situation, the drug/metabolite/biomarker of interest is present in biological matrix, which has a complex biochemical nature and comprises numerous components (e.g. salts, acids, bases, proteins, cells, exogenous/endogenous small organic molecules like lipids and lipoproteins). However, because the biochemical complexity of the matrix may differ (e.g. tissue, whole blood, plasma/serum, urine, saliva, cerebral spinal fluid, etc.), effective sample preparation methods will also differ. This skill accounts for up to 80% of the total bioanalysis and as such is the most labor-intensive and error-prone process in overall bioanalytical methodology. For example, sample preparation is often the step of methods development in which various aspects of chromatography and mass spectrometry analyses are improved. The most commonly used techniques for sample preparation are: precipitation, centrifugation, solid phase extraction (SPE), liquid/liquid extraction (LLE), or their combination, Figure 2.2.

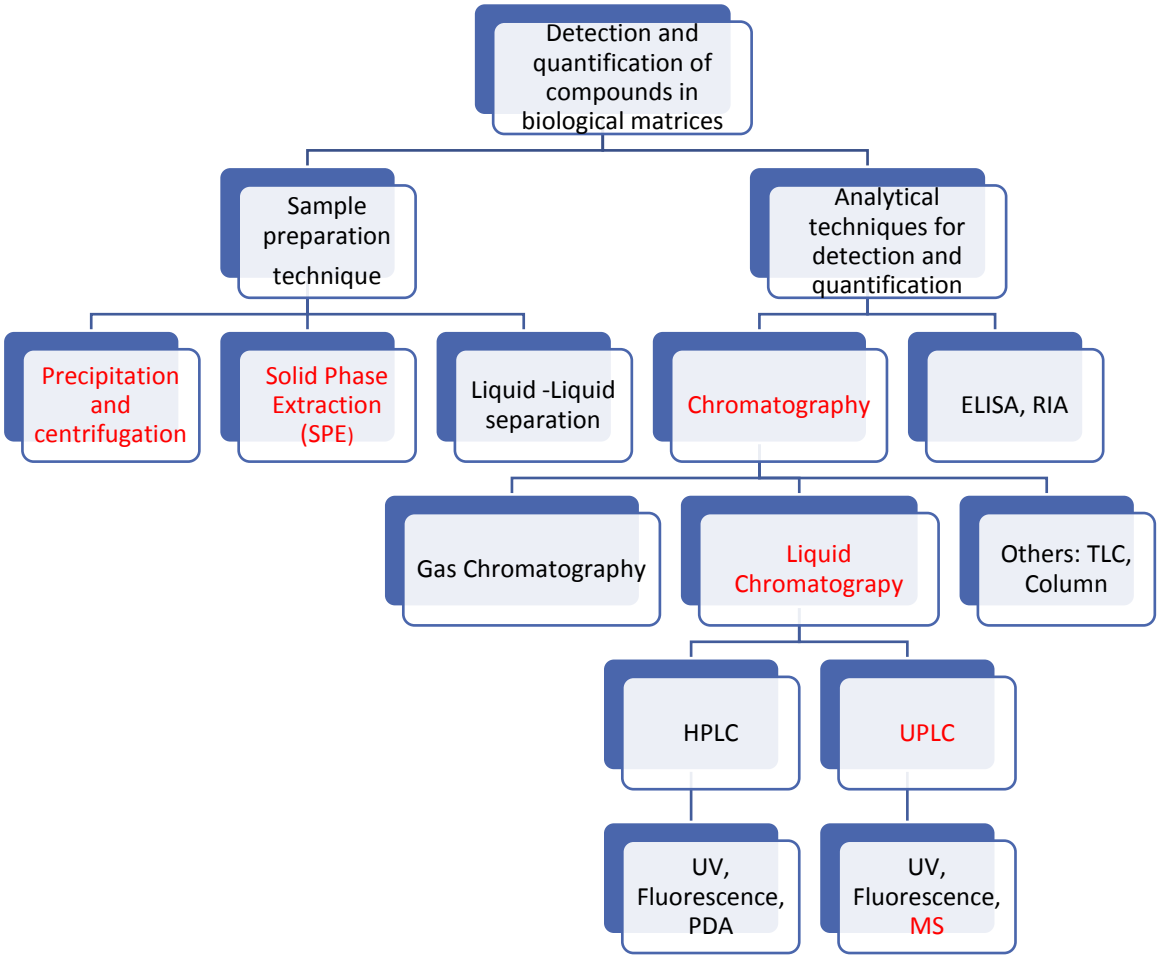


Figure 2.2. The detection of compounds (analytes) in biological fluids involves multiple steps. Sample preparation technique and the analytical techniques for detection and quantification are the bases for the path. In black refers to the general steps and the red that were follow for the purpose of this development and validation.

2.1 Solid Phase extraction sample preparation technique

SPE is a sample preparation technique that uses packing material to chemically separate different analytes and aid in removing the matrix components in a sample. One of the most common and useful purposes of SPE is to remove or reduce the interferences from matrix and concentrate the analyte. As such, SPE is an important sample preparation technique, and is one

of the more widely used for liquid or solid samples that have been put into a liquid form by dissolution or extraction.

The major benefits to using solid-phase extraction are:

- Removal or reduce the interferences from matrix (proteins, fat, oils) in the sample that overlap the analyte.
- Increased analyte concentration: a strong solvent elutes the analyte from the cartridge in a small concentrated volume. If taken to dryness, the sample residue can be dissolved (in a solvent compatible with the subsequent HPLC separation.
- Desalting: the inorganic salts to be washed from the cartridge with water.
- Sample storage and transportation: analytes have affinity to the solid sorbent of the cartridge and are stable enough to stay there until they are eluted. [25]

Reversed phase separation is a commonly used approach, and involves a polar (usually aqueous) or moderately polar sample matrix (mobile phase) and a nonpolar stationary adsorbant phase (column). The analyte of interest is typically mid- to nonpolar. For reverse phase extraction of non-polar to moderately polar compounds, the most used adsorbent is C18 (octadecyl bonded, endcapped silica) and C8 (octyl bonded, endcapped silica).

An example SPE cartridge (used for this study) is the Oasis HLB from Waters. The characteristic of this sorbent is that it has a strong hydrophilic, reversed-phase, water-wettable polymer with a unique Hydrophilic-Lipophilic Balance. This sorbent is ideal for acidic, basic and neutral analytes because it is stable from pH 0-14[26]

Pre-treatment of the SPE column may be necessary prior to adding sample to the SPE cartridge. Depending on the sample characteristics, these steps may include dilution of the sample to reduce viscosity, changing the pH to help the retention of the compound of interest

on the column versus the mobile phase, or precipitation of interfering proteins in the sample (plasma, serum or blood). For example, for this study, canine plasma samples were pre-treated prior to SPE by precipitation with organic solvents. Denatured protein is then removed by centrifugation (high g-force: 10,000-15,000 g) or filtration, leaving a clear supernatant containing the compound of interest.

The main steps of the solid phase extraction preparation technique are demonstrated in Figure 2.3.

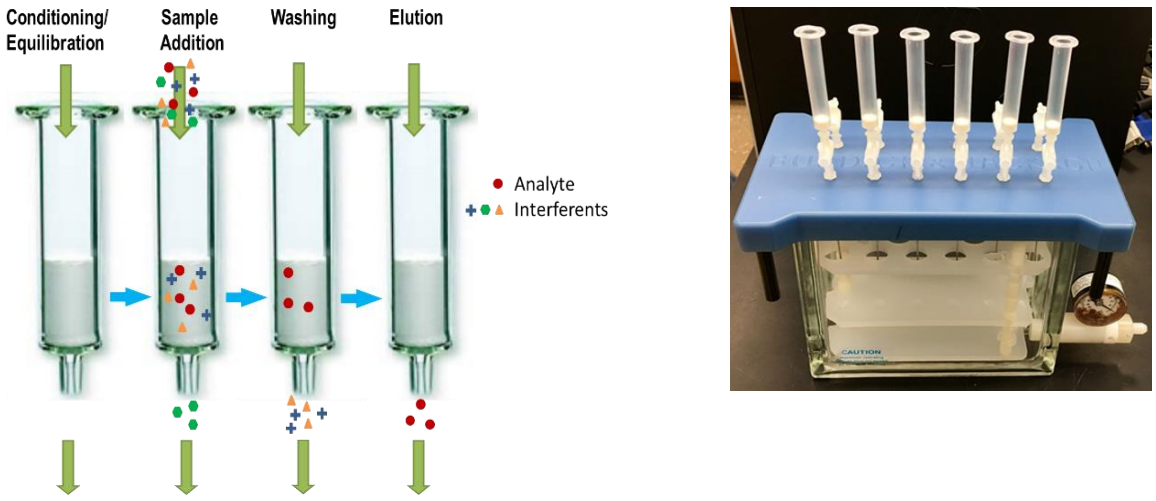
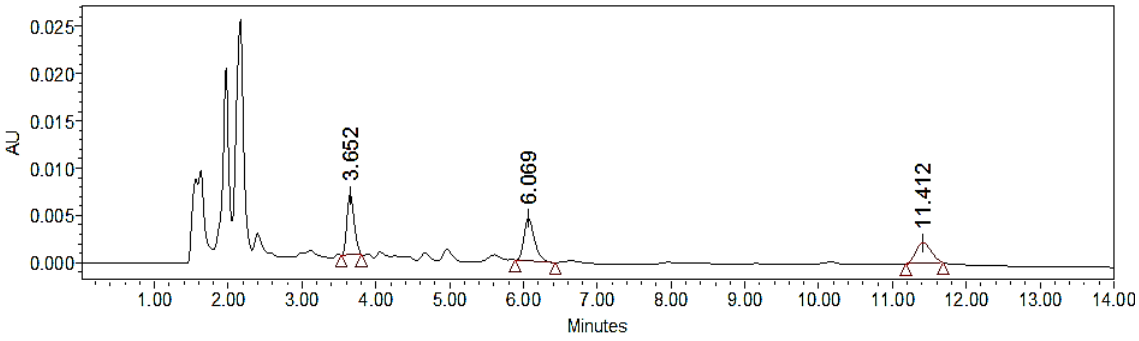


Figure 2.3. (a) Schematics of the main steps for solid phase extraction technique are: conditioning/equilibrating the SPE cartridge, sample addition, washing and elution. (b) Picture of device used for SPE

The impact of SPE on cleaning a canine plasma sample (removal or reduction of interferences from matrix and concentration of the analyte) is demonstrated in Figure 2.4:

a) Canine plasma sample after protein precipitation (1 µg/mL)



b) Canine plasma sample after protein precipitation followed by SPE (1 µg/mL)

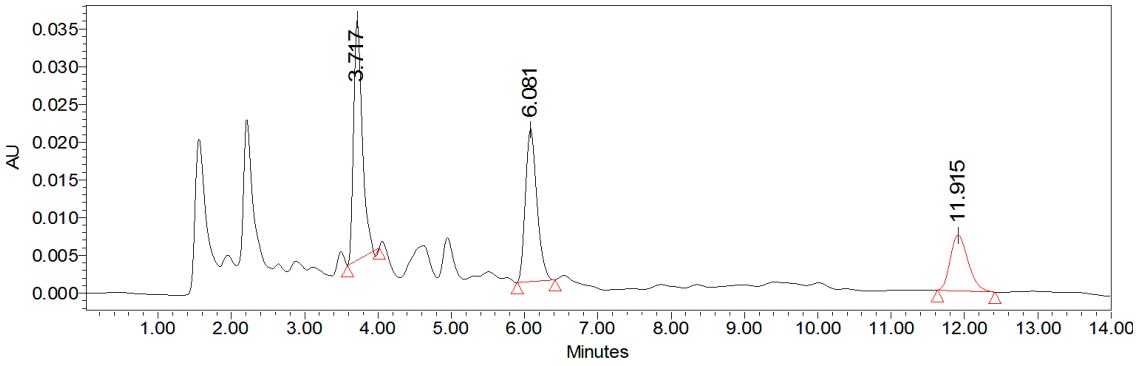


Figure 2.4. SPE is used to concentrate the analyte: a) The areas (response) are smaller after precipitation than the areas when b) precipitation followed by SPE technique is used

2.2Analytical techniques for detection and quantification

Once the sample has been cleaned of interfering compounds, the analyte of interest is ready for the development of methods for its detection and quantitation. However, first, the analyte of interest must be separated from other interfering compounds that remain even in the cleaned sample, including related analytes of interest. Recent improvements in technology have focused on increasing sensitivity and specificity (selectivity). Sensitivity referrers to detection of very small concentration of the drugs in the mixture. Specificity refers to being specific for a particular drug

in a mixture. Instruments capable of meeting these needs as liquid chromatography (LC) with UV, High protein content in biofluids precludes direct analysis by LC/MS or LC-MS/MS decreasing performance of the LC column, and ion source contamination in the mass spectrometer. Background from the matrix can suppress the drug signal. As such, sample preparation is critical to analyte detection and quantification by these methods.

2.2.1 High performance liquid chromatography (HPLC) and ultra- performance liquid chromatography (UPLC)

The Russian scientist Tswett first documented the concept and coined the term for chromatography (chroma: color, graphy: writing/study of) in 1903-1906 [27]. He subsequently introduced the column adsorption chromatography concept, based on the ability to separate pigments in plants [28]. His concept of the ‘‘chromatogram’’ and its advance by using different eluents [28]. By the end of the 1970’s chromatography played a fundamental role as an analytical technique for quality control and quantification of compounds. Table 2.1 lists the evolution of chromatography

Table 2.1 Chromatography evolution

Chromatographic technique	Years
Column chromatography	1900 -1930s
Thin layer and paper chromatography	1940
Gas chromatography (GC)	1950
High Performance Liquid Chromatography (HPLC)	1960 - 1970
Ultra-performance liquid chromatography (UPLC)	2004

The greatest extraordinary advances in chromatography have happened in the area of HPLC, regardless of the fact that the technique itself has only been present for about 50 years. Figure 2.5, shows the principal components of the HPLC and UPLC.

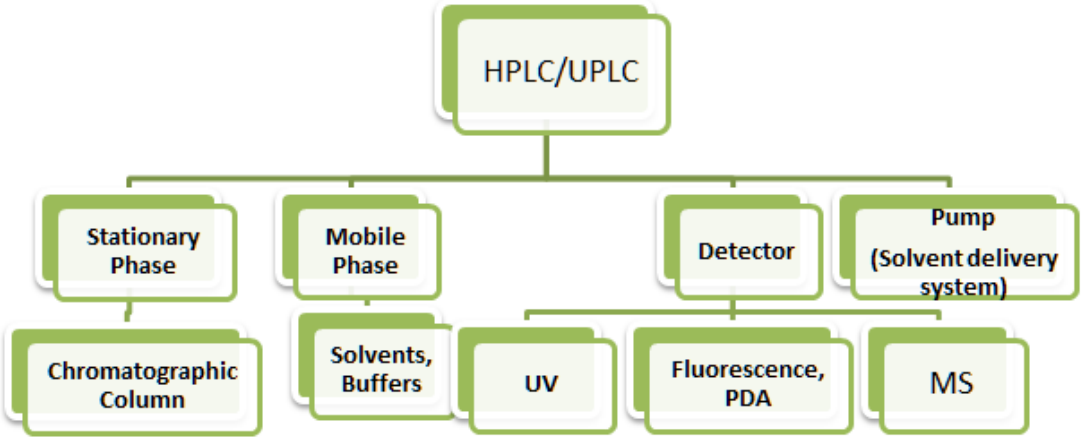


Figure 2.5. HPLC-UPLC System components: The main components on HPLC system are stationary phase (chromatographic column), mobile phase (solvent/liquid), the detector and the pump

HPLC and UPLC are column chromatography techniques, and are the most useful tools in analytical chemistry, with the capability to separate (from like compounds), identify and quantitate the analytes of interest present in the sample that are soluble in a liquid. The viscosity of liquids is higher than in the gases leading to therefore the necessity for pressure in the columns and the innovative name ‘‘high-pressure liquid chromatography’’. ‘‘Pressure’’ was replaced by ‘‘performance’’ as particles became smaller and columns also became shorter [28].

HPLC and UPLC are the most useful and widely applied analytical techniques for the separation, identification, and quantification of chemical mixtures.

The most useful packing materials used as stationary phases for the HPLC and UPLC are the reversed-phase (RP) octadecyl (RP-18) and octyl (RP-8)[28]. Once the proper column has been identified based on the chemistry of the compound of interest, the next step is to identify the proper eluant to carry the compound across the column. [29].

HPLC and UPLC are by far the most widely used chromatographic technique and have enjoyed the greatest revolution in analytical chemistry over the past 40 years. Among

the biggest change was the development of UPLC, reflecting changes in the column [49]: sphere-shaped particles has allows smaller particles (1.5 µm - 1.7 µm) and a reduction in length from 25 cm to 6 cm for 3 µm particles, and even or shorter for 1.5 µm. As such, the analytical time is shorter [28] and column efficiency (the sharpness of the peak) is improved leading to better separation [30]. Sensitivity and specificity are both improved. Normal HPLC equipment (Pumps, injectors, and detectors) did not have the required power to take full benefit of sub-2 µm particles. Small volume injections with minimal carryover were also necessary to comprehend the increased sensitivity benefits. Theoretically, the sensitivity increase for UPLC detection should be 2–3 times higher than with HPLC separations.

The main components for UPLC are in demonstrated in Figure 2.6. Both systems have a stationary phase (column) (1) that contains the chromatographic packing material needed to affect the separation. A mobile phase (2) which is the solvent (or mixture) that carries the sample into the column to separate the compounds. A high pressure pump (3) is required to force the mobile phase through the column at typical flow rates of 0.5 – 2 mL/min for HPLC and 0.5 to 1.0 mL/min for UPLC. Briefly, the sample to be separated is introduced into the system by an automatic injection (4). The mobile phase carries the sample to the detector (5) where the components are identified (UV, Fluorescence and PDA detector for HPLC, and UV, PDA, MS detector for UPLC). The UV or MS detector give the responses.

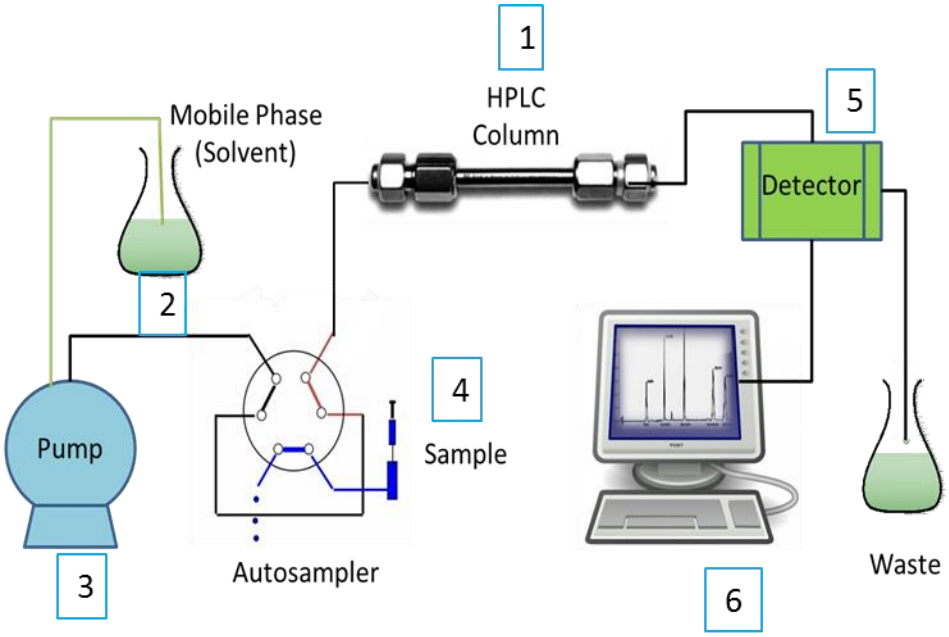


Figure 2.6. A diagram of HPLC and UPLC components

2.2.2. Ultraviolet Visible (UV) versus Mass Spectrometry (MS) detector

Once the compounds of interest are effectively separated from other compounds on a column, they must then be detected. The eluant containing the compound that has been retained and subsequently eluted from the compound then enters a detector that converts the presence of the compound to a quantifiable signal. The method of detection is chosen, again, based on the chemistry of the compound of interest. Among the most common methods of detection is absorption of ultraviolet (UV) light. A UV detector generates a signal whose magnitude reflects the concentration of the compound (which is recognized based on its retention time) in the eluant.

UV Detector

The UV detector gives a response in terms of millivolt, an electrical signal that is then processed by the computer to give a "chromatogram" (Figure 2.7).

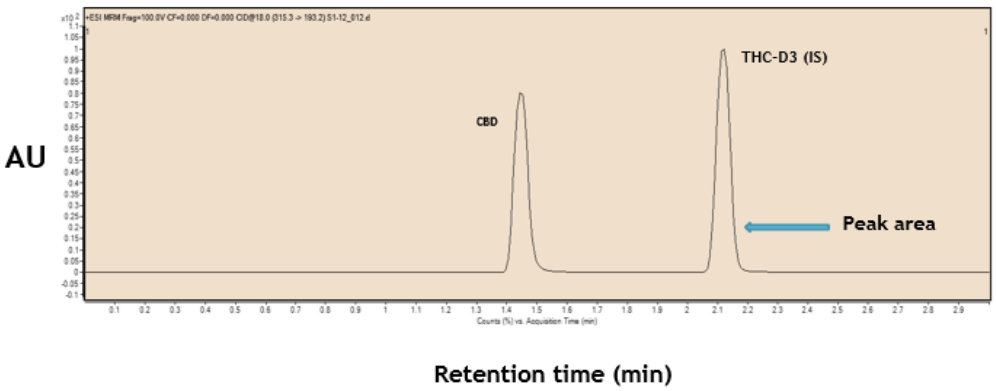


Figure 2.7. Chromatogram: a fingerprint of the compound

UV absorption detectors respond to those substances that absorb light in the range 180 to 350 nm. Many (but not all) substances absorb light in this wavelength range, including those substances having one or more double bonds (¶ electrons) and substances having unshared (unbonded) electrons, e.g. all olefins, all aromatics and compounds, for example, containing >C=O, >C=S, –N=N–groups.

MS Detector

Liquid chromatography–mass spectrometry (LC–MS) represents one of the most important tools in the characterization of all organic, inorganic and biological compounds and has gained extensive acceptance as analytical tool for identification and quantification of many types of compounds in small concentrations (ng, pcg, or fg). A mass spectrometer produces charged particles (ions) from the chemical substances that are to be analyzed. The mass spectrometer then uses electric and magnetic fields to measure the mass ("weight") of the charged particles. In simpler terms, a mass spectrum measures the masses within a sample.

A mass spectrometer generates a multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type.

The MS detector responses are called mass spectra, which are used as fingerprints (Figure 2.8), where the most abundance signal is registered and represent the precursor ion (MS1 or MS) and the product ion (MS2) which represent the daughter ion, also is the fragment of the precursor ion. MS/MS (MS2) confirms the structure of interest for quantification.

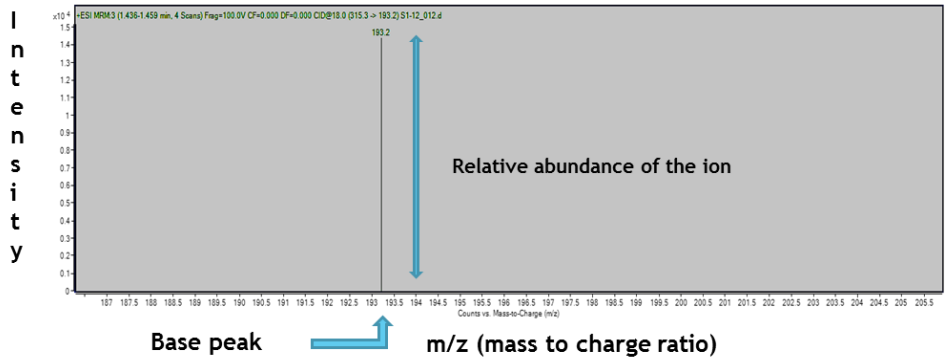


Figure 2.8. Mass Spectra (fragments of the compound of interest)

Ionization [48] is the major method by which a MS separates compounds. Electrically charged particles are affected by a magnetic field although electrically neutral ones are not affected. Atoms and molecules can be deflected by magnetic fields provided the atom or molecule is first turned into an ion. Electrically charged particles are affected by a magnetic field although electrically neutral ones are not affected. The atom or molecule ionized by knocking one or more electrons off to give a positive ion.

There are different “ionization sources” (device used to form ions) in MS, but for the identification and quantification of drugs the most common used is the electrospray ionization (ESI). ESI is used to produce ions using an electrospray. A sample solution is

sprayed from a small tube into a strong electric field in the presence of a flow of warm nitrogen to assist desolvation. The droplets formed evaporate in a region maintained at a vacuum of several Torr causing the charge in the droplets to increase. The multiply charged ions then enter the analyzer (Figure 2.9).

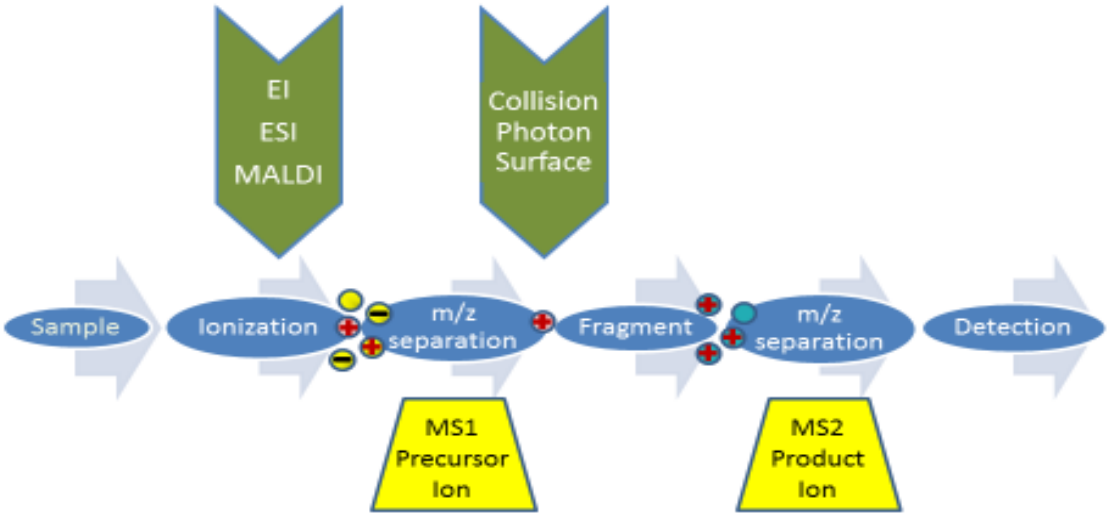


Figure 2.9. ESI is used to produce ions using an electrospray

Usually, the term LC-MS is used for this technique, but the terms UPLC-MS or LC-MS/MS also are used. In contrast, LC-MS/MS, or tandem mass spectrometry involves multiple steps of mass spectrometry selection. MS may involve one or two mass analyzers. The quadrupole mass analyzer (QMS) is also called a "single quadruple mass spectrometer". The quadrupole filters sample ions based on their mass-to-charge ratio (m/z). MS/MS uses two quadruple mass analyzers in series, and as such, it is referred to as "triple quadruple mass spectrometer (TQMS)".

Between the two analyzers (MS1 and MS2) is a cell for collision-induced dissociation (Figure 2.9). Precursor ions selected by MS1 collide with a high pressure gas (usually

helium) in the cell and undergo fragmentation. The fragments of a molecule cause a unique pattern in the mass spectrum which is used to determine structural information of the molecule.

The UPLC coupled with a triple quadruple mass spectrometry (LC-MS/MS) improves efficiency, enhances chromatography resolution and yields short analysis times. Probably the largest application area for a triple quadrupole mass spectrometer is when accurate quantitation is needed. For this, very accurate sampling of the chromatographic peak by the mass spectrometer must be achieved; otherwise, large variations in the detected peak area can be expected. Quantitation is usually done in MRM (multiple reaction monitoring) mode, in which the first quadrupole is set to transmit a characteristic precursor ion (ions of a particular mass to charge ratio) and the second to detect a product ion (selected and fragmented ions) resulting from a collision-induced fragmentation inside the collision (Figure 2.9).

In summary, the advantages of LC-MS/MS [45] are: narrower chromatographic peaks effectively increase concentration of analytes entering the MS source, increasing signal intensity and improving detection limits. [26].

2.2.3 Past and current methods for detection of cannabinoids in biological matrices.

The detection of cannabinoids in various biological matrices has been of interest since 1970's. Table 1.1 summarizes the various methods that have been used among the different tissues in different species. The major species of interest have been rodents and humans; a very little work has been performed in dogs Initial attempts focused on canine plasma using

HPLC-UV techniques. Several high performance liquid chromatography (HPLC) methods have been described in the literature, most of them for quantification of the main cannabinoids in *Cannabis sativa* plant [46] and human plasma, but just a few in canine plasma. All methods are based on chromatography. Several methods for plasma and urine were based on gas chromatography/mass spectrometry (GC/MS) [52] after liquid/liquid or solid-phase extraction (SPE) and derivatization [2][24]. Major disadvantages of these methods are the elaborate sample preparation and the need to use various derivatization techniques for non-volatile and thermolabile compounds, derivatization technique is mainly used for urine samples, it can also be used for plasma samples, but because of the poor recovery (43% for CBD and 54% in plasma) and long time (approx. 20 h) procedure reported with this technique it was not an option for our purpose. Recently liquid chromatography-mass spectrometry (LC-MS) and LC-MS-MS methods were developed for determination of cannabis with an improvement in selectivity and lower limits of quantification in human blood [25][2]

Recently liquid chromatography-mass spectrometry (LC-MS) and LC-MS-MS methods were developed for determination of cannabis with an improvement in selectivity and lower limits of quantification in human blood [25][2].

2.3 Challenges encountered in the detection and quantification of cannabinoids

The cannabis plant and its commercial products have an enormous variety of unique chemicals, including cannabinoids and other components as terpenes, hydrocarbons, nitrogen-containing compounds (carbohydrates, flavonoids, fatty acids, non-cannabinoids phenols, simple alcohols, aldehydes, ketones, acids, and esters, and others).

Interest in the detection of cannabinoids in the various biological matrices of animals can be found in the literature as early as1970's. At that time, the goal was to get an easy method on HPC with UV detection to target concentrations around ng/mL. The major species of interest were dogs. Methods at that time included a sample preparation using radiochemical analysis, protein precipitation and liquid-liquid separation technique. However, in contrast to today's methods, limitations in early methods included synthetization of their own standards, complex sample preparation technique, high LOD and LLOQ. As the interest increased, changes in detection and quantitation methods included HPLC-MS with a sample preparation including protein precipitation and solid phase extraction technique. A representative selection of different methodologies can be found in Table 1.1.

The quantitation of cannabinoids in dogs did not emerge in the literature until 1970s. Interest was largely as a model for understanding both the response of mammals to cannabinoids as well as concentrations achieved in the body after IV and/or oral administration. Following a review of these reports in dogs, the following challenges for a more accurate and precise measurement of cannabinoids in dogs were identified.

- The first major challenge was to find the optimal conditions for the chromatographic column and the mobile phase to get an optimal identification and separation of the cannabinoids. Because sensitivity was likely to be an issue with plasma samples, LC-MS was the chosen method. Detection of individual cannabinoids began in methanol in order to determine the initial chromatographic conditions. However, our ultimate goal was to simultaneously analyze all cannabinoids in canine plasma. As such, the next focus of development was separation of the cannabinoids when analyzed as a mixture. This in particular was challenging because the chemical formula, molecular weight, the physical

and chemical properties for the five main cannabinoids are very similar. Further, degradation products (for example, CBN from THC degradation) or precursors (eg, CBG which serves as a building block for THC and CBD) may be present in canine samples. Separation from these interfering chemicals was necessary.

- Once a preliminary method (in methanol) was determined, the next major challenge was optimal sample preparation method (clean up sample). This was particularly important for cannabinoids because we anticipated very low concentrations (most of the cannabinoids are metabolized after oral administration, before they enter circulation). Because of the need for a very sensitive assay, cleaning out matrix components and unwanted chemicals was critical. Selectivity (specificity) also would be impacted if the matrix was not well removed. Finally, poor sample preparation method can contaminate in the column (decreasing selectivity, specificity and prolonging retention time while decreasing column life span). However, even more critical is the impact of contamination factors on the MS detector, and particularly ion suppression.
- As such, the target optimal parameters for the MS detector and specifically the conditions for quantitation of cannabinoids in canine plasma were: the nitrogen gas (used as the dry, nebulizer, and collision gas), the capillary voltage, the selection of positive or negative ion mode, and the monitoring for the mass transitions (quantifier and qualifier ion) [16] for the five main cannabinoids in the mixture, was a challenge because the method has to have a balance between the sensitivity and selectivity of all of them.

Chapter 3 Description of development and validation of a UPLC-MS method for quantification of selected cannabinoids in canine plasma

Developing and validating analytical methods includes performing all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix (e.g., blood, plasma, serum, or urine) is reliable and reproducible for the intended use. Validated analytical methods for the quantitative evaluation of drugs (analytes) are critical for the successful conduct of nonclinical and clinical pharmacology studies. The purpose of this study was to develop and validate a method for simultaneous detection and quantification of selected cannabinoids in canine plasma using UPLC with MS detection.

3.1 Method development in canine plasma

The first step to develop the cannabinoids method was to search for LC-MS/MS related literature that was already published and review it to establish the basic chromatographic conditions for the method analysis [27] [31][16]. Some initial considerations from those publications were considered, but modifications were performed during the optimization of the chromatographic separation and detection conditions. The main chromatographic conditions considered during the development were: the chemical structure for each of the compounds to be analyzed, chemical and physical properties as solubility, pKa, pH in solution, stability, polarity. The sample type (serum, plasma), to select the matrix cleaning procedure (precipitation, SPE, liquid –liquid separation). The stationary phase (chromatographic column) chemistry, lengths and particle sizes. The mobile phase pH and composition. Also, range of the expected concentration, flow rate, temperature, injection volume, sample size, stability of the main compounds. And

finally, the purpose of the method development (to be used for pharmacokinetic study, stability test or therapeutic drug monitoring).

3.1.1 Materials and methods

The cannabinoid standards (CBD, THC, CBN, CBG, CBC) were purchased from Cerilliant® Analytical Reference Standards a Sigma-Aldrich® company (Round Rock, Texas, USA) [27][31]. THC-D3 was also purchased from Cerilliant® and used as the internal standard (IS) (Table 3.1). To assure blank canine plasma was free of drug, it was obtained from Animal Blood Resources International (formerly Animal Blood Bank and Midwest Animal Blood Services) (Dixon, CA, USA). HPLC and MS grade methanol, acetonitrile, water, ammonium formate, were purchased from VWR® (Radnor, PA, USA), formic acid was purchased from Sigma-Aldrich® (St. Louis, MO, USA). SPE C8, C18 cartridges were purchased from Phenomenex® (Torrance, CA, USA), SPE Oasis HLB cartridges were purchased from Waters® (Milford, MA, USA).

Table 3.1. Cannabinoids standards information

Provider	Cannabidiol	Cannabigerol	Cannabinol	Cannabichromene	delta-9-tetrahydrocannabinol	delta-9-tetrahydrocannabinol D3
	CBD	CBG	CBN	CBC	Δ-9-THC	Δ-9-THC-D3
Cerilliant	C-045	C-141	C-046	C-143	T-005	T-003
Formula	$C_{21}H_{30}O_2$	$C_{21}H_{32}O_2$	$C_{21}H_{26}O_2$	$C_{21}H_{30}O_2$	$C_{21}H_{30}O_2$	$C_{21}H_{27}D_3O_2$
Molecular weight	314.46	316.48	310.43	314.46	314.46	317.44
CAS Number	13956-29-1	25654-31-3	521-35-7	20675-51-8	01972-08-3	81586-39-2
Concentration	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL	100 ug/mL
Solvent	Methanol	Methanol	Methanol	Methanol	Methanol	Methanol
Storage	Freezer	Freezer	Freezer	Freezer	Freezer	Freezer
Long term stability	60 months	24 months	56 months	15 months	60 months	60 months
Regulatory	USDEA Exempt	USDEA Exempt	USDEA Exempt	USDEA Exempt	USDEA Exempt	USDEA Exempt
Lot. Number	FE01271601	FE08031502	FE06081502	FE10011502	FE09101501	FE03091602
Purity (HPLC/UV)	99.10%	99.00%	99.50%	97.70%	98.60%	96.70%
Ordered	6/15/2016	6/15/2016	6/15/2016	12/12/2014	6/15/2016	6/15/2016
Expiration	Feb-21	Oct-18	Jul-19	Mar-18	Nov-20	Mar-21

3.1.2 Preparation of cannabinoids standard working solutions

The working standard solutions CBD, THC, CBN, CBC and CBG were prepared by dilution of the stocks (1 mg/mL) in methanol to get a final concentration of 10 µg/mL [12]. The IS (THC-D3) working solution was prepared in methanol at a concentration of 1µg/mL. The stock solutions and the working solutions were stored at – 20 °C. The working solutions of the cannabinoids were diluted in methanol immediately before preparation of calibration curves.

3.1.3 Calibration curve preparation

Two calibration curves were prepared one for cannabinoids in methanol and the other in canine plasma. They were prepared by adding to methanol or drug-free canine plasma (blank) known concentrations of each cannabinoid followed by serial dilution. The end result was individual cannabinoid concentrations ranging from 1.91 ng/mL to 1000 ng/mL. Briefly, 200 µl of CBD work solution and 200 µl THC work solution were added to 1600 µl canine plasma or methanol to get a 1µg/ml solution. This concentration correspond to the standard canine plasma with the high concentration (1000 ng/mL). The calibration curve was prepared as a serial dilution starting from the high concentration to the low concentration. The final concentrations for the calibration curves in canine plasma and methanol were 1.95, 3.91, 7.81, 15.63, 31.25, 62.50, 125, 250, 500 and 1000 ng/mL.

Calibration curves were constructed by plotting the peak area ratios of the selected ion species (for the analyte and IS) versus analyte concentration ratios, using at least 7 calibration points.

The quality controls (QCs) were independently prepared by serial dilution at final concentrations of 1.95, 7.81, 62.5, and 250 ng/mL

3.1.4 Sample preparation

For the sample preparation method, the mixture of cannabinoids in canine plasma was a complex mixture (proteins), and for this reason had to involve a cleaning procedure to extract efficiently the cannabinoids from the matrix. Also, the expected concentrations in canine plasma were small (ng/mL), so it was crucial to select the best sample preparation method.

For cannabinoids method, precipitation and centrifugation with 2 different solvents (acetonitrile versus methanol) to precipitate most of the proteins was used. Solid phase extraction with different SPE cartridges was also performed (C18, C8 and HLB), and a combination of both techniques (precipitation, followed by solid phase extraction) was used to check the best extraction of the cannabinoids and reduction of the matrix. The analyte was concentrated after the precipitation and SPE, using a stream of nitrogen and temperature.

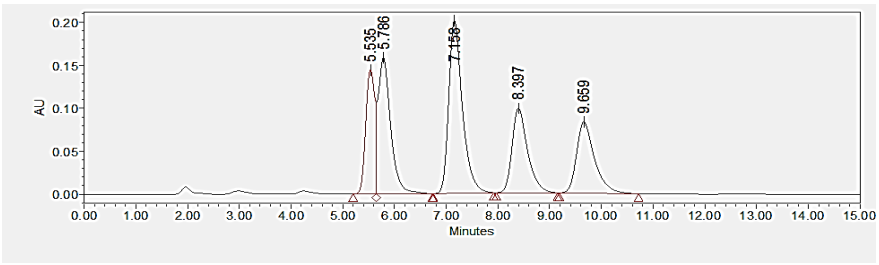
Results:

Pre-treatment: 500 µl of canine plasma sample was transferred to a clean tube, 50 µl of IS working solution and 500 µL acetonitrile were added and, the sample was vortex for 20 seconds. After centrifugation at 3000 rpm (1900 x g) for 15 min at 5 °C, the supernatant was follow the SPE cleaning procedure.

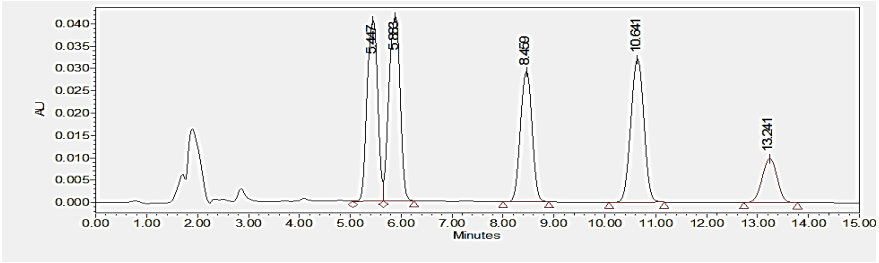
SPE procedure: the SPE was performed using vacuum. The SPE cartridge was conditioned with 2.0 mL methanol followed by equilibration with 2.0 mL distillated water. Then 800 µl of the supernatant was loaded into the cartridge followed by a washing step with 1 mL distilled water. The cartridge was dried under vacuum for 10 min, and then the cannabinoids were eluted with 1 mL of methanol. The eluted solution was evaporated to dryness under a gentle nitrogen stream at 40 °C for 20 min. The residue was dissolved in 60 µL of methanol and vortex for 20 seconds. The solution was centrifuged at 14,000 rpm for 10 min at room temperature. 45 µL of the clear supernatant was transferred to the vial for the LC-MS analysis. 1 µL of the clear solution was injected into the LC-MS system by duplicate.

3.1.5. Mobile phase preparation and chromatographic column selection

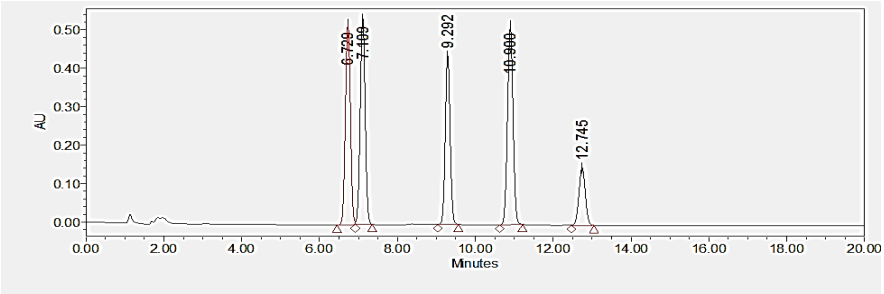
The selection of the stationary phase (chromatographic column) which is the packing material needed for the separation, and the solvents for the mobile phase which carries the sample into the column to separate the compounds was an important part of the development. For cannabinoids assay the goal was to separate and quantify selective cannabinoids simultaneously: two chromatographic columns were tested (C18 and C8 column), which varied in both length and particle size. Isocratic and gradient elution were tested, two different temperatures (40 °C, room temperature) and flow rate were adjusted. The mobile phase used was a mixture of 0.1% formic acid in water (A) with acetonitrile (B). Each part of the mobile phase was previously filtered (0.45 micron) and degassed under vacuum Figure 3.1.



(a)



(b)



(c)

Figure 3.1. Separation of the compounds:

a) C8, 150 mm x 4.6 mm, 5 µm column, ammonium formate : acetonitrile: mobile phase.
b) C18, 250 mm x 4.6 mm, 5 µm column, 35 °C, water :acetonitrile 30:70 v/v mobile phase.
c) C18, 250 mm x 4.6 mm, 5 µm column, 40 °C, water :acetonitrile 20:80 v/v mobile phase

3.1.6. LC/MS/MS chromatographic conditions

For the LC-MS/MS: two different columns (C8 and C18) at different lenghts and particle sizes (50 mm, and 100 mm x 2.1 mm, 1.7, and 1.8 µm), using temperature (40 °C and room temperature) were tested. Two different mobile phases (methanol:ammonium formate/formic acid (pH 3.8) and formic acid:acetonitrile) were tested at different flow

rates (0.2 -0.6 mL/min). Also for the SPE procedure three SPE cartridges were tested (Oasis HLB, C18 Strata and C18 Strata-X 33).

The ESI parameters as capillary voltage, nitrogen gas (as drying, nebulizer and collision gas) volume, temperature and pressure were optimized. Positive-ion mode [31] , and mass transitions were monitored using multiple-reaction monitoring (MRM). The transitions for each cannabinoid were found based on these conditions [16].

The cannabinoids standards stock solutions were prepared at 1 µg/mL in methanol [31], injected individualy and in a mixture to the LC/MS/MS system to optimize the separation and detection conditions. Also, blank canine plasma samples of different origin without IS, and blank plasma samples spiked with the internal standard, were extracted and analyzed. Finally, blank canine plasma samples spiked with the five main cannabinoids reference standards, were extracted and analyzed. These steps were performed to determine the extent to which matrix components may contribute to the interference at the retention time of cannabinoids and the internal standard. The chromatograms were evaluated and the retention times are reported on Table 3.2

Table 3.2 The retention times for the cannabinoids

CBG 1.28 to 1.31min	THC-D3 2.40 to 2.44 min
CBD 1.36 to 1.39 min	THC 2.41 to 2.45 min
CBN 2.02 to 2.09 min	CBC 2.68 to 2.72 min

Results:

Chromatographic separation was performed with an Agilent ZORBAX Eclipse Plus - C18 column (50 mm x 2.1 mm, 1.8 µm) (Santa Clara, CA, USA). An Agilent 1290 UPLC system was used for the chromatographic identification and separation of the cannabinoids.

Gradient elution was performed with (A) 0.1% formic acid in water and (B) acetonitrile as mobile phase starting at 30% A: 70% B (v/v) 1.5 min with a flow rate of 0.5 mL/min, and then increase to 90% at 1.5 min, then the column was re-equilibrated to the initial conditions. The mass spectrometric measurements were performed on the Agilent 6460 Triple Quad mass spectrometer detector equipped with an Agilent Jet stream Electrospray Ionization (AJ ESI) source. The MassHunter software from Agilent was used for system control, data acquisition and quantification.

Optimized source parameters were as follow: Capillary voltage was set at 4000 V. Nitrogen gas was used as the dry (10 L/min at 300 °C), nebulizer (45 psi), and collision gas. Mass spectra of the cannabinoids were acquired in positive-ion mode [14], and mass transitions were monitored using multiple-reaction monitoring (MRM). The transitions for each cannabinoid are listed in Table 3.3[16].

Table 3.3. Mass transitions used for quantification and qualification

Compound	Type of Transition	Mass Transition (MRM)	Fragmentor (V)	Collision Energy (V)
CBD	Quantifier ion	315.3 – 193.2	100	18
	Qualifier ion	315.3 – 259.0	100	15
CBC	Quantifier ion	315.1 – 193.0	100	16
	Qualifier ion	315.1 – 259.2	100	9
CBG	Quantifier ion	317.2 – 193.1	100	10
	Qualifier ion	317.2 – 123.0	100	34
CBN	Quantifier ion	311.2 – 223.0	120	17
	Qualifier ion	311.2 – 241.0	120	15
THC	Quantifier ion	315.2 – 193.1	110	20
	Qualifier ion	315.2 – 259.1	110	16
THC-D3	Quantifier ion	318.2 – 196.1	110	20
	Qualifier ion	318.2 – 262.1	110	16

3.2. **Method Validation**

Validation means assessment of validity or action of demonstrating efficiency. Method validation is the way of documented data which provides high degree of assurance that the method will meet the requirements for the intended analytical applications [11][32][33][34][35][36][37][38][39].

Based on the method development information, the optimal chromatographic conditions for a good identification, separation and quantification of the analytes (cannabinoids) were established. Each step in the method validation (Specificity, sensitivity, linearity, accuracy, precision, and robustness) as described below was performed to conform the validation process (Figure 3.2).

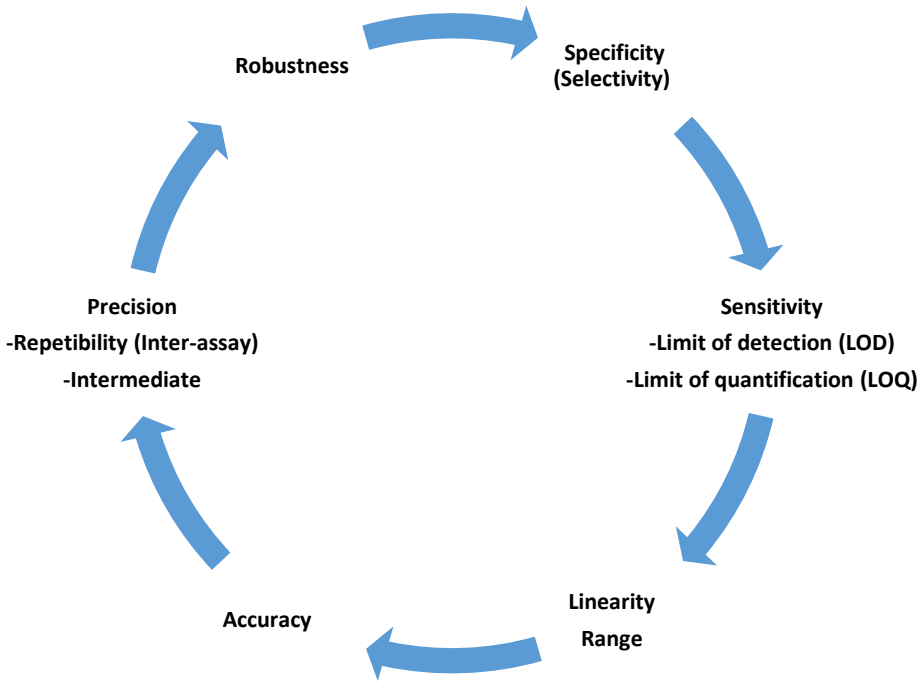


Figure 3.2. Method validation steps

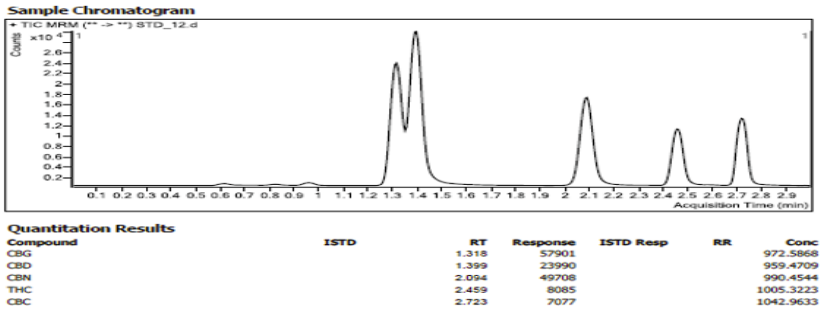
Specificity, sensitivity (limit of detection (LOD) and lower and upper limit of quantification (LLOQ, ULOQ)), linearity, accuracy (% recovery), repeatability, intra-assay precision, intermediate precision, matrix effect, robustness, and system suitability were determined to evaluate the cannabinoids LC-MS/MS analytical method.

3.2.1 Specificity (selectivity)

Specificity is the ability to measure accurately and specifically the analyte (drug) in the presence of components that may be expected to be present in the matrix as proteins, impurities, degradation products. Sensitivity is the ability of a test to detect a target analyte which is usually expressed as the minimum detectable concentration of the analyte or lower limit of detection (LLOD) [11][32][33][34][35][36][37][38][39].

Based on the retention time the method was tested by comparing the chromatograms of extracted blank canine plasma from different batches with samples spiked with the cannabinoids at the lower limit of quantification. The chromatograms in Figure 3.3 (a) (b), Figure 3.4 (a) (b) and Figure 3.5 demonstrate that there is no significant interference within the elution zone.

(a)



(b)

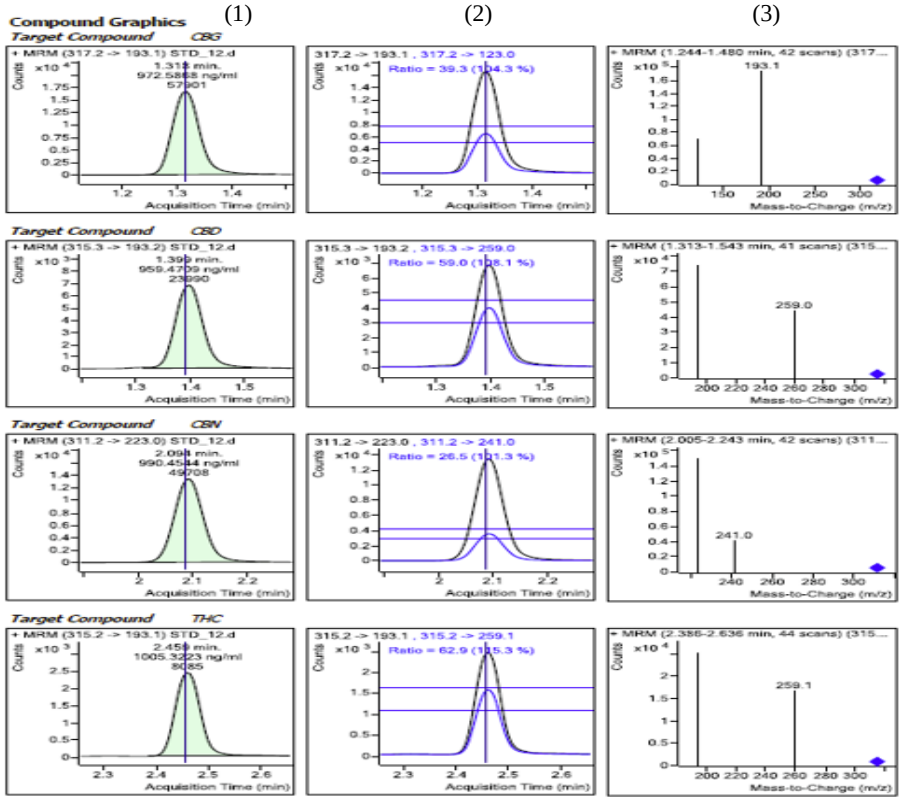
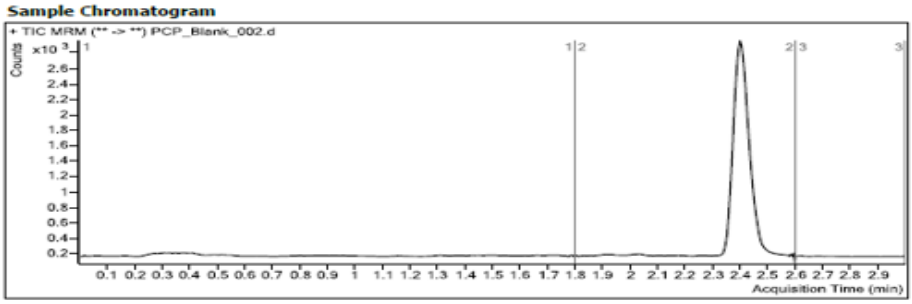


Figure 3.3. a) Chromatogram: mixture of cannabinoids in methanol ran under the optimal conditions (1µg/mL), good shape of the peak and no interferences between them. b) Mass spectrum: A mixture in methanol run under the optimal conditions. (1) Chromatogram with the quantifier ion and mass transitions, (2) Chromatogram with the quantifier ion vs qualifier ion and the mass transitions (3) mass spectrum for each cannabinoid with the mass to charge ratio (m/z), and the most abundant ion.

(a)



(b)

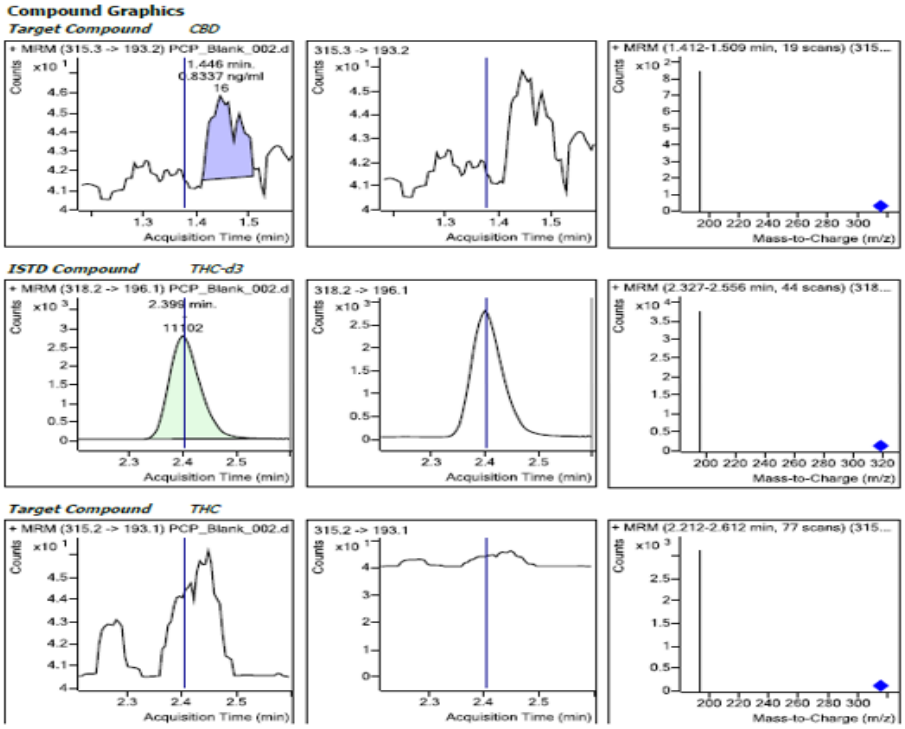


Figure 3.4. Chromatogram and mass spectra for the canine blank plasma (no cannabinoids) with the internal standard (THC-D3)

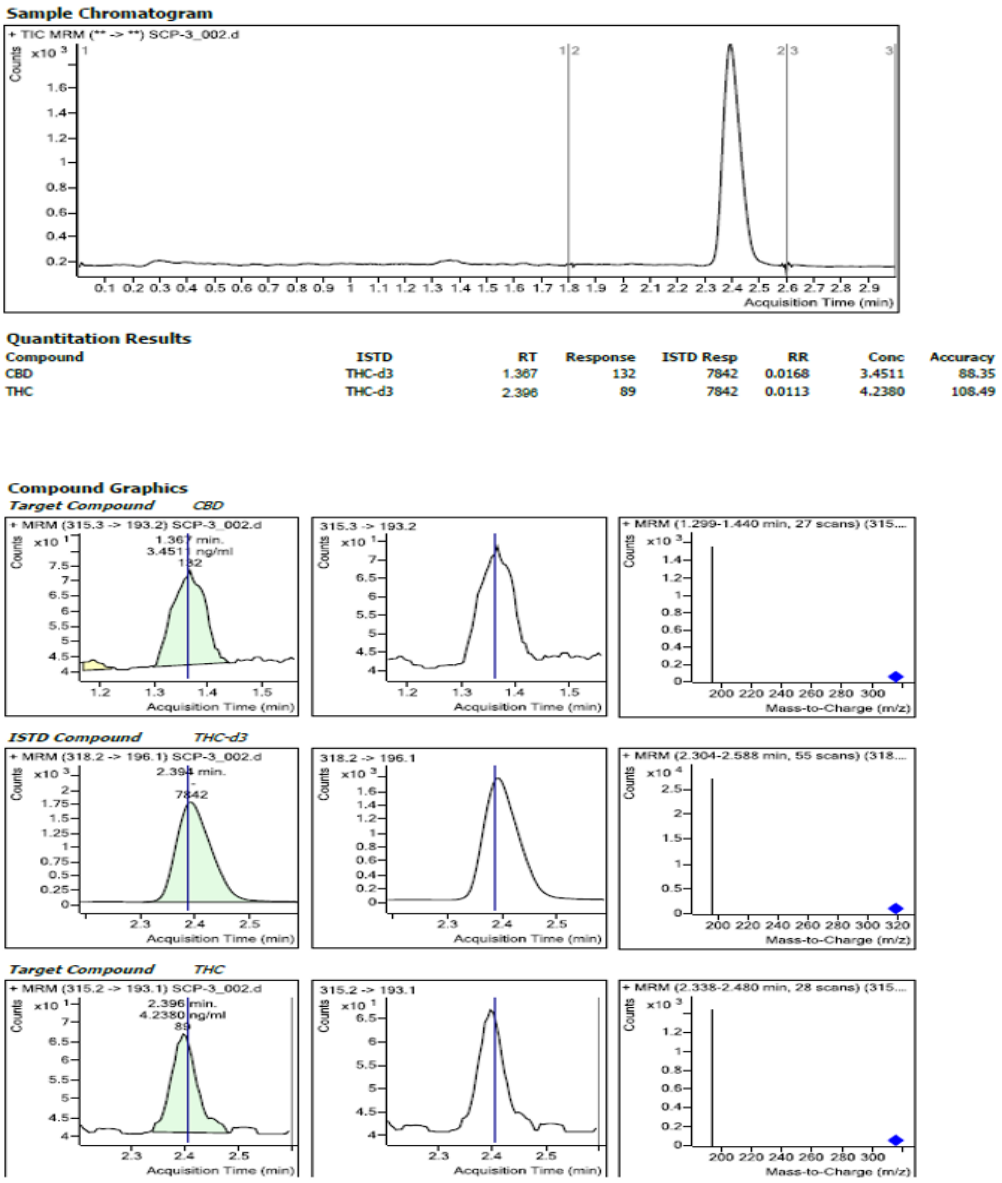


Figure 3.5. Chromatogram and mass spectra for the cannabinoids in methanol (1000 ng/mL)

3.2.2 Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The lower limit of detection is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified under the stated experimental conditions. It is usually expressed as the concentration of the analyte (%, µg, ng, and ppm) in the sample [11][32][33][34][35][36][37][38][39], estimated based on signal to noise ratio of 3:1.The lower limit of quantification is the lowest amount of analyte in a sample that can be quantified with acceptable precision and accuracy under the stated experimental conditions. It is expressed as the concentration of analyte (µg, ng, and ppm) in the sample [11][32][33][34][35][36][37][38][39], estimated based on signal to noise ratio of 10:1. Analyte peak (response) should be identifiable, discrete, and reproducible, and the calculated concentration should have precision that does not exceed 25% of the CV and accuracy within 20% of the nominal concentration.

The highest standard will define the upper limit of quantification (ULOQ) for the analytical method, this was selected based on the concentration range that was established.

Sensitivity was evaluated by the LOD and the LLOQ. Methanol and canine plasma preparations spiked with the low cannabinoid concentration were prepared and analized to determine the LOD and LLOQ. LOD was determined as the lowest concentration of the cannabinoids with a signal to noise ratio of 3:1 For all the main cannabinoids the **LOD was 1.95 ng/mL** (See Appendix A, Fig. A.1 to A.4).

The LLOQ was the lowest concentration of the cannabinoids that can be determined with acceptable precision and accuracy under the stated experimental conditions. Canine plasma preparations spiked with the low cannabinoid concentration were analyzed. The concentration was within at least ±20 % of target concentration, and relative standard deviation (RSD %)

within at least 25% of the mean concentration. For CBD and THC the **LOQ was 3.91 ng/mL**. For CBG, CBN was 3.91 ng/mL, and for CBC was 15.63 ng/mL (See Appendix-A Fig. A.5 to A.7). The Upper limit of quantification was 1000 ng/mL for CBD and THC. For CBG, CBN, and CBC was 250 ng/mL. For CBD and THC the final range for the validation was established from 3.91to 250 ng/mL because the concentrations expected for canine plasma will not be higher than this concentrations (See Appendix A, Fig. A.8 to A.10)

3.2.3 Linearity

Linearity is the ability of the method to elicit test results that are directly proportional to concentration of analyte in samples within a given range. This should be expressed as the variance of the slope of the regression line. Linearity should be established across the range of the analytical procedure. Linearity should be evaluated by appropriate statistical methods [11][32][33][34][35][36] [37][38][39].

Linearity for cannabinoids was determined by a series of two injections of nine standards whose concentrations range was from 1 to 200 % (3.91 to 1000 ng/mL) of the expected analytical concentration. If R2 was greater than 0.98 and the slope was 1.0 ±0.1, then the procedure was acceptable for this performance measure. The cannabinoids concentration range was changed later from 3.91 to 250 ng/mL (check the results section).

The range of an analytical assay is the interval between the upper and lower concentrations of analyte (including these levels) in the sample that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range is normally expressed on the same units as test results (example µg, ng, ppm) obtained by the analytical procedure [11][32][33][34][35][36][37][38][39].

The linearity test was performed in multiple samples over a specific range of concentration values, 1.95 ng/mL to 1000 ng/mL for CBD and THC and 1.95 ng/mL to 250 ng/ml for CBG, CBN, and CBC. The resultant data must verify that the detector response is directly proportional to the amount of analyte present in each sample. The linearity results are in Table 3.4, Table 3.5, and Table 3.6 (See Appendix A, Fig. A.11-A.13)

Table 3.4.Cannabinoids in Methanol no IS

Cannabinoids	**Internal Standard THC-D3**	**R^2**	**Curve Type**	**Linear range ng/mL**
CBD	No-IS	0.9979	Linear	1.95-1000
CBG	No-IS	0.9989	Linear	1.95-1000
CBN	No-IS	0.9996	Linear	1.95-1000
THC	No-IS	0.9964	Linear	1.95-1000
CBC	No-IS	0.9994	Linear	7.81-1000

Table 3.5. Cannabinoids in Methanol with IS

Cannabinoid	**Internal Standard THC-D3**	**R^2**	**Curve Type**	**Linear range ng/mL**
CBD	THC-D3	0.9959	Linear	3.91-1000
CBG	THC-D3	0.9869	Linear	1.95-500
CBN	THC-D3	0.9955	Linear	1.95-500
THC	THC-D3	0.9912	Linear	3.91-1000
CBC	THC-D3	0.9978	Linear	7.81-500

Table 3.6. Cannabinoids in canine plasma with IS

Cannabinoid	**Internal Standard**	**R^2**	**Type**	**Linear range**
CBD	THC-D3	0.9844	Linear	3.91-1000
CBG	THC-D3	0.9869	Linear	3.91-250
CBN	THC-D3	0.9904	Linear	3.91-250
THC	THC-D3	0.9981	Linear	3.91-1000
CBC	THC-D3	0.9984	Linear	15-250

3.2.4 Accuracy

The accuracy of the analytical method measures the deviation between the experimental and true values for a specified range. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte (QCs) [11][32][33][34][35][36][37][38][39]. Should be assessed using minimum of three concentrations and three replicates covering specified range of method. For cannabinoids, three concentration were used and three replicates for each concentration (2 injections each). Total of 6 determinations for each concentration. Reported as % recovery of known added amount of analyte in the sample, or as the difference between the mean and the accepted true value.

Criteria 1: The R2 value for the regression line should be ≥ 0.98, the Calibration curves for CBD and THC had values for the R2 of 0.9940 and 0.9984 for CBD and THC respectively, meeting the criteria (See Appendix A, Fig. A.14-A.17).

Criteria 2: The % recovery of each set of samples should be within the range of $100 \pm 20\%$. Four samples designated ACP (01-04) were prepared by serial dilution at different concentrations of the method range (1.95, 7.81, 62.50, 250 ng/mL) and analyzed in duplicate. The % recovery for each set is given in Table 3.7and Table 3.8for CBD and THC respectively.

Table 3.7. Accuracy results for CBD in canine plasma

Label	Cannabidiol (CBD) [Known] ng/mL	Mean Calculated Cannabidiol (CBD) Conc. (ng/mL)	s [Exp] n=6	%RSD n=6	% Recovery n=6
ACP-1	1.95	2.27	0.47	20.89	116.1
ACP-2	7.81	6.39	0.53	8.26	81.8
ACP-3	62.50	62.36	13.23	21.21	99.8
ACP-4	250.00	258.30	34.64	13.41	103.3
Mean			**12.2**	**15.9**	**100.2**

Table 3.8. Accuracy results for THC in canine plasma

Label	Theoretical Δ-9-THC Conc. (ng/mL)	Mean Calculated Δ-9-THC Conc. (ng/mL)	s [Exp] n=6	%RSD n=6	% Recovery n=6
ACP-1	1.95	2.50	0.19	7.62	128.14
ACP-2	7.81	7.55	0.50	6.67	96.70
ACP-3	62.50	60.26	1.70	2.82	96.41
ACP-4	250.00	241.91	8.13	3.36	96.76
Mean			**2.6**	**5.1**	**104.5**

Results:

The % recovery was 100.2% ± 15.9% for CBD, and 104.5% ± 5.1% for THC

3.2.5 Repeatability (Inter-assay precision)

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous sample. Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation, analyst, and analysis) under normal operating circumstances [11][32][33][34][35][36][37][38][39].

Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst, equipment, reagents, and laboratory.

Criteria 1: The $\mathbf{R^2}$ value for the regression line should be ≥ 0.98, the Calibration curves for CBD and THC had values for the $\mathbf{R^2}$ of **0.9878** and **0.9959 for CBD** and **THC** respectively (See Appendix A, Fig. A.18-A.21).

Four samples designated PCP (01-04) were prepared by serial dilution by triplicate at concentrations of 1.95, 7.81, 62.50, and 250 ng/mL and analyzed in duplicate.

Criteria 2: The %RSD for each sample should not be greater than 20%

The % recovery and the %RSD for each set are given in Table 3.9 for CBD and Table 3.10 for THC.

Table 3.9. Inter-assay precision results for CBD in canine plasma

Label	Cannabidiol (CBD) [Known] ng/mL	Mean Calculated Cannabidiol (CBD) Conc. (ng/mL)	s [Exp] n=6	%RSD n=6	% Recovery n=6
PCP-1	1.95	1.93	0.47	24.57	98.75
PCP-2	7.81	7.29	0.31	4.21	93.37
PCP-3	62.50	72.00	3.85	5.34	115.20
PCP-4	250.00	279.06	45.83	16.42	111.62
Average			**12.6**	**12.6**	**104.7**

Table 3.10. Inter-assay precision results for THC in canine plasma

Label	Theoretical Δ-9-THC Conc. (ng/mL)	Mean Calculated Δ-9-THC Conc. (ng/mL)	s [Exp] n=6	%RSD n=6	% Recovery n=6
PCP-1	1.95	2.21	0.56	25.20	113.28
PCP-2	7.81	7.66	1.08	14.06	98.13
PCP-3	62.50	62.65	5.64	9.00	100.25
PCP-4	250.00	272.09	17.73	6.52	108.84
Mean			**6.3**	**13.7**	**105.1**

Results:

The % recovery was 104.7% ±12.6% for CBD and 105.1% ±13.7% for THC

3.2.6 Intermediate Precision

Intermediate precision requires that the method be carried out by another analyst, on different days, and under normal operating conditions. Four samples designated PCP (01-04) were prepared by serial dilution by triplicate at concentrations of 1.95, 7.81, 62.50, and 250 ng/mL and analyzed in duplicate.

Criteria 1: The $\mathbf{R^2}$ value for the regression line should be ≥ 0.98, the Calibration curves for CBD and THC had values for the $\mathbf{R^2}$ of **0.980** and **0.9968** for CBD and THC respectively (See Appendix A, Fig. A.22-A.25).

Criteria 2: The % recovery of each set of spike samples should be within the range of 100 ± 20%. Criteria 3: The %RSD for a single analyst should not be greater than 20%.

The % recovery and the % RSD for each set is given in Table 3.11and Table 3.12 for CBD and THC respectively.

Table 3.11.Intermediate precision results for CBD in canine plasma

Label	Cannabidiol (CBD) [Known] ng/mL	Mean Calculated Cannabidiol (CBD) Conc. (ng/mL)	s [Exp] n=6	%RSD n=6	% Recovery n=6
PCP-1	1.95	2.68	0.23	8.48	137.40
PCP-2	7.81	8.50	1.91	22.50	108.81
PCP-3	62.50	61.83	5.96	9.64	98.93
PCP-4	250.00	295.17	35.70	12.09	118.07
Mean			**10.9**	**13.2**	**115.8**

Table 3.12.Intermediate precision results for THC in canine plasma

Label	Theoretical Δ-9-THC Conc. (ng/mL)	Mean Calculated Δ-9-THC Conc. (ng/mL)	s [Exp] n=6	%RSD n=6	% Recovery n=6
PCP-1	1.95	1.87	0.09	5.07	95.59
PCP-2	7.81	7.71	0.67	8.64	98.67
PCP-3	62.50	61.40	1.54	2.50	98.25
PCP-4	250.00	261.74	8.30	3.17	104.69
Mean			**2.6**	**4.8**	**99.3**

Criteria 4: The mean %RSD for both analysts should not be greater than 20%. The %recovery and the %RSD for each set are given in Table 3.13 for CBD and Table 3.14 for THC respectively.

Table 3.13. Precision summary from two analyst, different days for CBD

Precision Summary					
Label	Canabidiol (CBD) [Known] ng/mL	Mean n=9 (CBD) [Exp] ng/mL	s [Exp] n=6	%RSD n=6	% Recovery n=6
PHP-01	1.95	2.31	0.53	23.01	118.26
PHP-02	7.81	7.90	1.39	17.62	101.09
PHP-03	62.5	66.92	7.15	10.69	107.07
PHP-04	250.0	302.95	25.14	8.30	121.18
Mean			**8.6**	**14.9**	**111.9**

Table 3.14. Precision summary from two analyst, different days for THC

Precision Summary					
Label	Δ-9-THC [Known] ng/mL	Mean n=9 (Δ-9-THC) [Exp] ng/mL	s [Exp] n=6	%RSD n=6	% Recovery n=6
PHP-01	1.95	2.04	0.40	19.84	104.60
PHP-02	7.81	7.68	0.80	10.42	98.39
PHP-03	62.5	62.03	3.76	6.06	99.25
PHP-04	250.0	266.91	13.62	5.10	106.76
Mean			**4.6**	**10.4**	**102.3**

Results: The % recovery 111.9% ±14.9% for CBD and 102.3% ±10.4% for THC. The %RSD for a single analyst and two analyst should be < 20%.

3.2.7 Robustness

Robustness measures the ability of the method to remain unaffected by small, but deliberate variation in select parameters. This provides an indication of reliability during normal operation, and is used to set system suitability specifications[11][32][33][34][35][36][37][38][39].

For cannabinoids method, To check the extraction efficiency of the assay, the recovery of CBD and THC was determined by comparing the peak areas

from extracted samples at three concentrations without precipitation using organic solvent (acetonitrile) before the SPE procedure of equivalent concentrations. The % recovery of different pretreatment was calculated.

The calibration curves for CBD and THC had values for the $\mathbf{R^2}$ of **0.9933, and 0.9923 for CBD** and **THC** respetively, indicating that the method is linear through the range of interest (See Appendix A, Fig. A.26-A.29).

The % recovery for the samples for 1.95, 7.81, 62.50, and 250 ng/mL was within the range of 100 ± 25% . The %recovery and the %RSD for each set are given inTable 3.15 for CBD and Table 3.16 for THC.

Table 3.15.Robustness results for CBD in canine plasma

Label	Cannabidiol (CBD) [Known] ng/mL	Mean Calculated Cannabidiol (CBD) Conc. (ng/mL)	s [Exp] n=6	%RSD n=6	% Recovery n=6
PCP-1	1.95	2.19	0.96	43.86	112.09
PCP-2	7.81	7.45	1.64	22.04	95.38
PCP-3	62.50	51.60	9.40	18.22	82.56
PCP-4	250.00	321.15	36.93	11.50	128.46
Mean			**12.2**	**23.9**	**104.6**

Table 3.16. Robustness results for THC in canine plasma

Label	Theoretical Δ-9-THC Conc. (ng/mL)	Mean Calculated Δ-9-THC Conc. (ng/mL)	s [Exp] n=6	%RSD n=6	% Recovery n=6
PCP-1	1.95	2.23	0.55	24.53	114.31
PCP-2	7.81	6.96	0.61	8.82	89.09
PCP-3	62.50	51.66	1.51	2.92	82.66
PCP-4	250.00	244.97	5.34	2.18	97.99
Mean			**2.0**	**9.6**	**96.0**

Results:

The % recovery was 104.6% ±23.9% for CBD and 96.0% ±9.6% for THC.

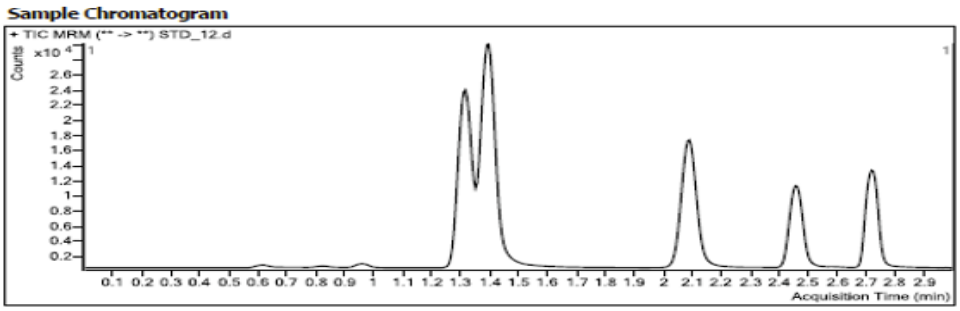
The results shows that we can analize the samples without the precipitation step but the %RSD (23.9%) was higher compared with using precipitation previously to the SPE (%RSD 15%). This confirmed that it is highly recommended the precipitation step previously to the SPE for the LC/MS/MS because it will help to remove efficiently the matrix from canine plasma.

3.2.8 System Suitability

Capacity Factor (k')

This value gives an indication of how long each component is retained on the column. The time elapsed between injection of sample components in the column and their detection is known as retention time (t_R). A non-retained substance passes through the column at a time t_M, called the void time. Figure 3.6 was used for the system suitability calculus.

Criteria 1: Value of k' must be ≥ 2 for optimum resolution. Results are in Table 3.17.



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBG		1.318	57901			972.5868	97.26
CBD		1.399	23990			959.4709	95.95
CBN		2.094	49708			990.4544	99.05
THC		2.459	8085			1005.3223	100.53
CBC		2.723	7077			1042.9633	104.30

Figure 3.6 . Chromatogram and mass spectra for the cannabinoids in methanol (1000 ng/mL)

Applicable equation

$$k' = \frac{t_R - t_M}{t_M}$$

Where:

t_R = retention time

t_M = void time

k'= capacity factor

Table 3.17.Capacity factor results for the cannabinoids mixture

Cannabinoids	*t*R	*t*M	k'
CBG	1.318	0.61	1.16
CBD	1.399	0.61	1.29
CBN	2.094	0.61	2.43
THC	2.459	0.61	3.03
CBC	2.723	0.61	3.46

A good retention on the column for the peaks of interest should have a k' ± 2 for optimum resolution. Here CBD and CBG values were below 2, the reason could be related to their molecular weight, the physical and chemical characteristics were so close each other that makes difficult to find the optimal column for all of them. For our purpose these values are acceptable.

Resolution Factor (RS)

The resolution factor measures the extent of separation between two adjacent peaks and accounts for the difference between retention times of the two peaks relative to their width.

Criteria 1: Value of R_S must be ≥ 2. Results are in Table 3.18.**Error! Reference source not found.**

Applicable equation (values taken in seconds).

$$R_S = \frac{t_{R(B)} - t_{R(A)}}{0.5(W_A + W_B)}$$

$t_{R(A)}$ = Retention time of peak 1

$t_{R(B)}$ = Retention time of peak 2

W_A = Width of peak 1

W_B = Width of peak 2

Table 3.18. Resolution factor results for the cannabinoids

Cannabinoids	*t*R(B)	*t*R(A)	*W*A	*W*B	*R*S
CBG	1.318	0.61	0.10	0.1	5.260
CBD	1.399	1.318	0.20	0.1	0.540
CBN	2.094	1.318	0.25	0.1	4.434
THC	2.459	1.318	0.15	0.1	9.128
CBC	2.723	1.318	0.20	0.1	9.367

Tailing Factor W0.5 (USP Method)

The tailing factor is measured at 5% of total peak height and is a measure of peak tailing. Peak tailing results from secondary retention effects that further retard the elution of a component from the column.

Criteria 1: Value of T must be ≤ 2. Results are in Table 3.19.

Applicable equation:

$$T = \frac{a + b}{2a}$$

Where:

T = tailing factor (measured at 5% of peak height)

b = distance from the point at peak midpoint to the trailing edge

a = distance from the leading edge of the peak to the midpoint

The method meets the criteria T ≤ 2

Table 3.19.Tailing factor results for the cannabinoids

Cannabinoids	a	b	Height (mm)	5%	T
CBG	3	3	51	2.55	1
CBD	4	4	65	3.25	1
CBN	4	4	37	1.85	1
THC	3	3	24	1.2	1
CBC	3	3	29	1.5	1

Theoretical plates (N) (USP Method)

Theoretical plates measure the sharpness of the peak and therefore the efficiency of the column.

Criteria 1: The value of N should be ≥ 2000. Results are in Table 3.20.

Table 3.20.Theoretical plates (N) results for the cannabinoids

Cannabinoids	Ve (mm)	*W*b (mm)	N
CBG	81	7	34278
CBD	87	8	30276
CBN	130	7	88294
THC	152	6	164295
CBC	168	6	200704

Applicable equation:

$$N = 16\left(\frac{V_e}{W_b}\right)^2$$

Where:

N = Number of theoretical plates

V_e = elution volume, retention time or retention distance (mL, sec, or cm)

h = peak height

w_b = width of the peak at the base line (mL, sec, or cm)

- Therefore the higher the plates number the more efficient the column.
- The plate number depends on column length - ie the longer the column the larger the plate number.

  Criteria: In general N > 2000

The C18 column used for this analytical method demonstrated to have a high efficiency.

- **Stability**

The chemical stability of an analyte in a given matrix under specific conditions for given time intervals is assessed in several ways. Drug stability in a biological fluid is a function of the storage conditions, the physicochemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems.

- **Freeze and thaw stability**

During freeze/thaw stability evaluations, the freezing and thawing of stability samples should mimic the intended sample handling conditions to be used during sample analysis. Stability should be assessed for a minimum of three freeze-thaw cycles. Three cannabinoid canine samples low, medium and high were analyzed fresh prepared, after freezing at 4 °C and thawing (in s cycle of 2 days and after a week).

- **Processed sample stability**

The stability of processed samples in one step of the method should be determined. For cannabinoids, one calibration curve was analyzed immediately after the sample preparation was performed. For a second calibration curve precipitation, SPE steps were performed, then after the eluted was dried, the residue was stored at 4 °C in the refrigerator. The residue was reconstituted the next day. The criteria was to observe significant differences in the cannabinoids concentration between the two tests.

3.2.9Application of the validated method to canine clinical samples

Upon successful validation of an analytical assay for the quantitation of THC and CBD in canine serum, the assay was then applied to clinical patients receiving commercial cannabinoids therapeutically. Samples were obtained from the Clinical Pharmacology Laboratory at The College of Veterinary Medicine at Auburn University. Samples were obtained through the Therapeutic Drug Monitoring (TDM) service. Plasma samples are received daily from various veterinarian clinics in USA. Samples were solicited through the TDM service at its web site, which provides accession forms as well as surveys the submitting veterinarians. Plasma samples flagged for cannabinoid analysis are transferred from their tube into 2 ml cryovials

from ThermoFisher™ (Waltham, MA, USA) for storage in the -80 °C (to maintain stability) were they remain until analysis.

Canine clinical samples (n=67) were identified for testing as described for the UPLC-MS validated analytical method. No attempt was made to exclude samples based on what product was being used or why (that is the disease being treated). The only criteria for inclusion was that the patient was receiving a commercial cannabinoid.

Figure 3.7 demonstrates the concentrations quantitated for each of the 67 samples. Concentrations of CBD and THC markedly varied among animals. Much of this variability likely reflects dosing but also is likely to reflect product differences as well as differences in the disposition of cannabinoids in individual dogs. Representative chromatograms and the mass spectra for randomly selected representative canine samples are demonstrated in Figures 3.8 to 3.10. These results demonstrate the applicability of this method to quantitation of CBD and THC in canine plasma, and thus its utility in pharmacokinetic and clinical trial studies.

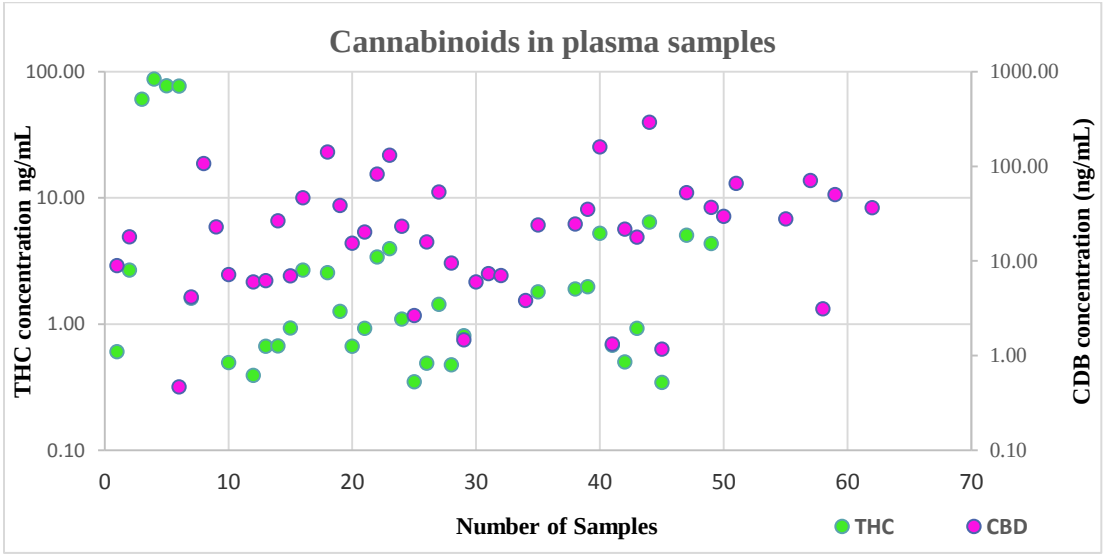


Figure 3.7. Plasma cannabinoids (ng/mL) concentrations for 62 TDM canine samples. Right scale is for CBD whereas the left scale is for THC demonstating CBD is the predominate cannabinoid in these products.

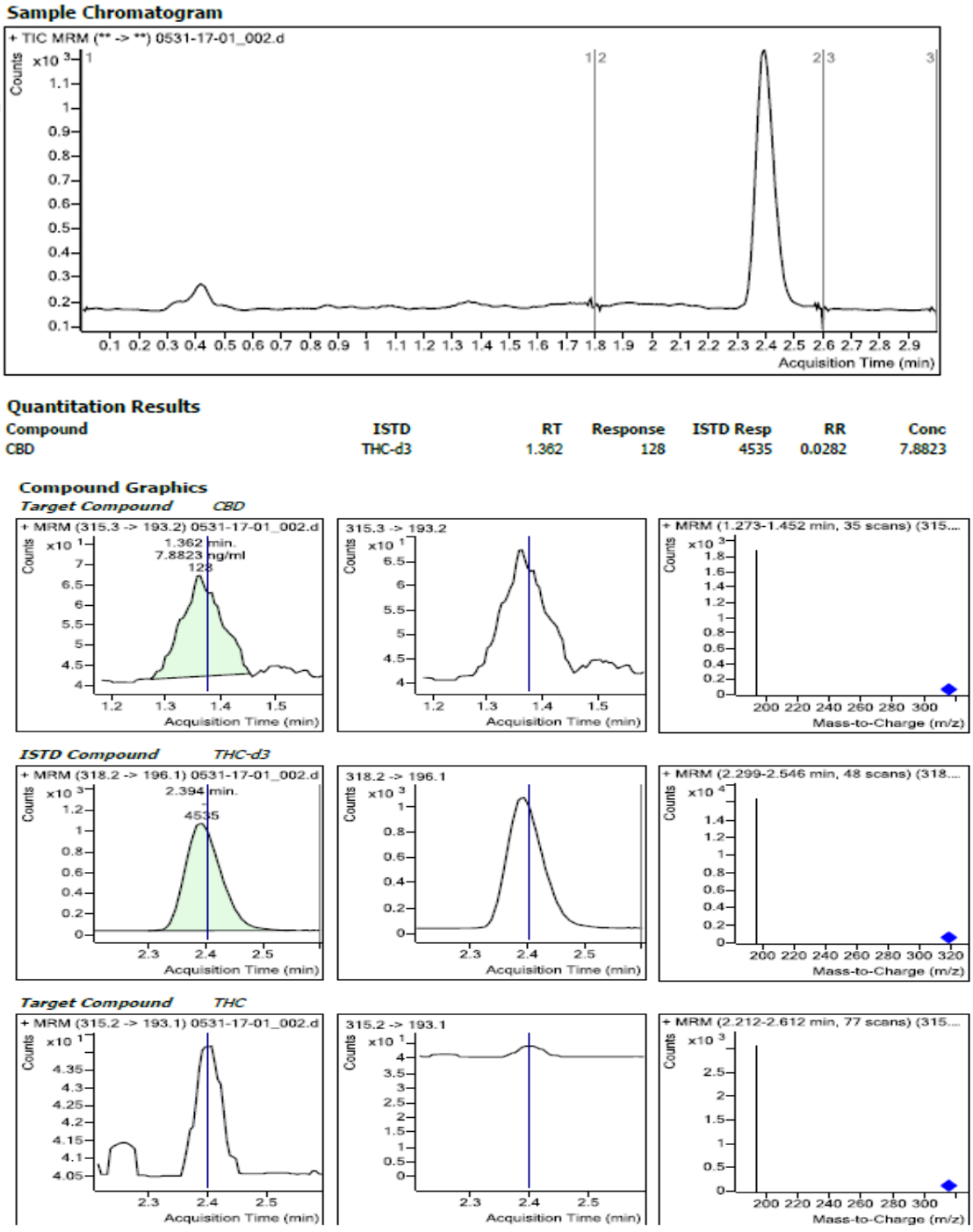


Figure 3.8 . Representative chromatographs and mass spectra of detected CBD or THA in canine TDM samples (TDM accession number 0531-17-01)

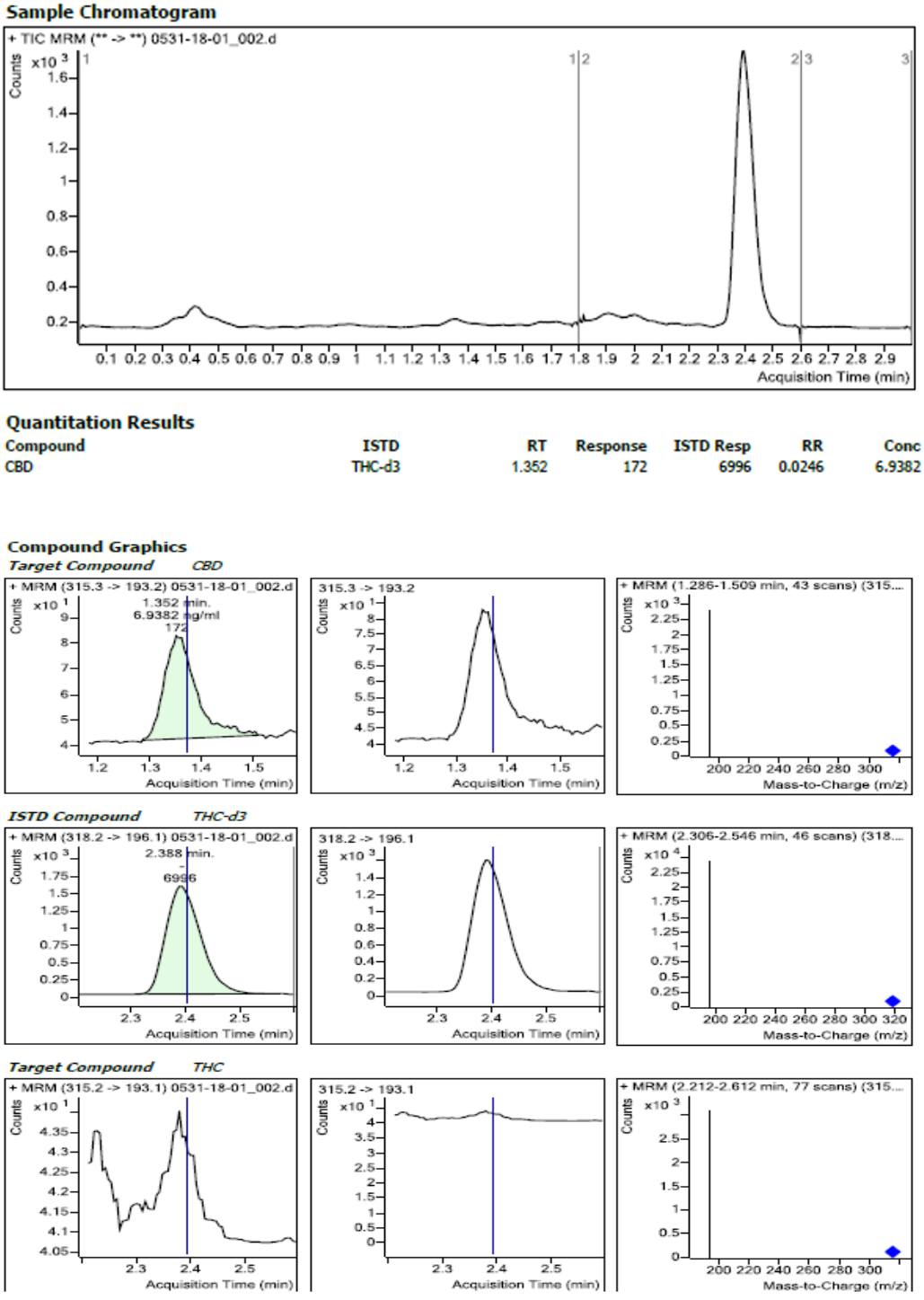


Figure 3.9. Representative chromatographs and mass spectra of detected CBD or THA in canine TDM samples (TDM accession number 0531-18-01)

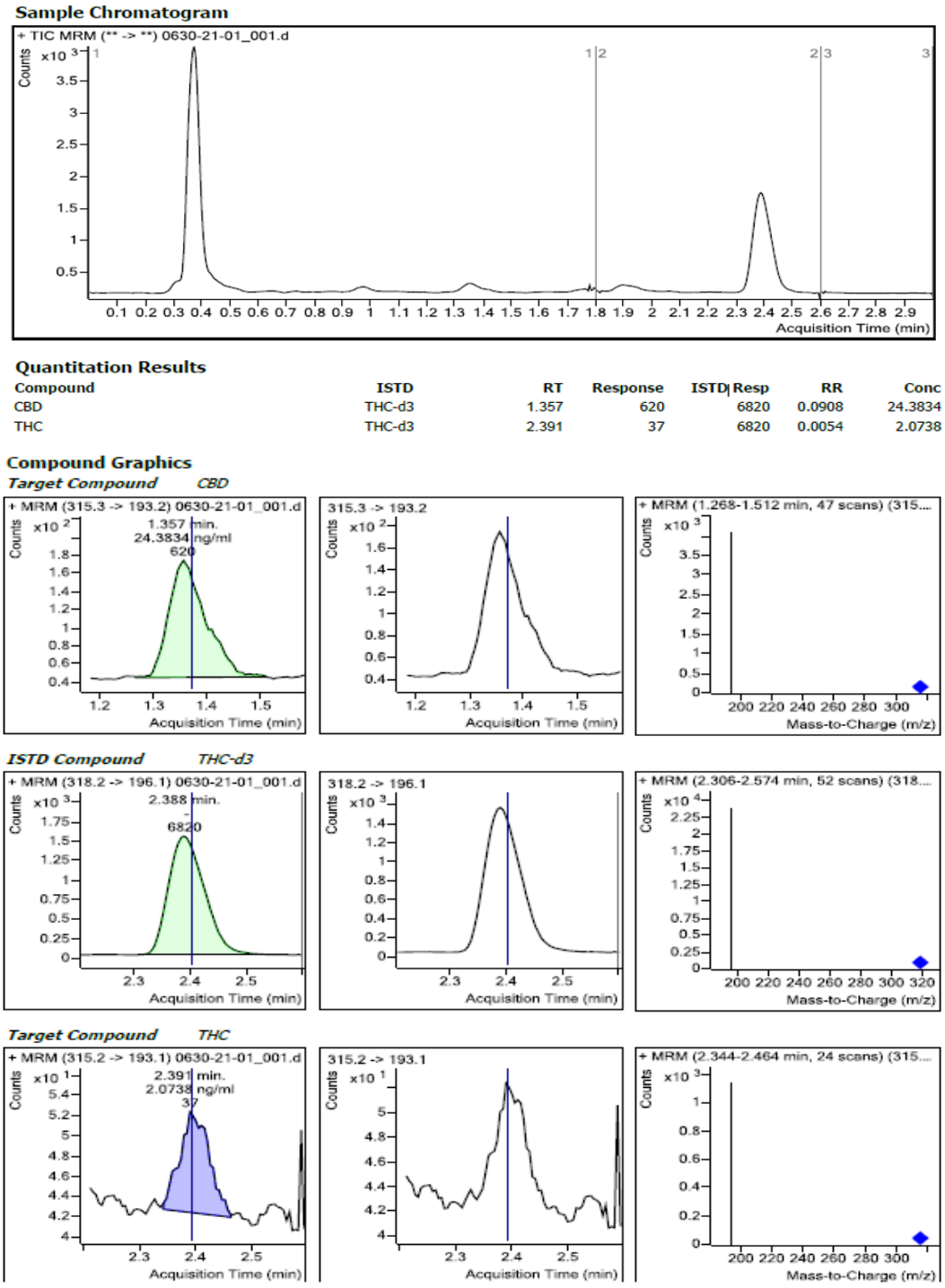


Figure 3.10. Representative chromatographs and mass spectra of detected CBD or THA in canine TDM samples (TDM accession number 0630-21-01

Chapter 4 Commercial cannabis products

In 2015 Vandrey R. and colleges worked in a research study for which the purpose was to review the accuracy in edible medical cannabis products. Of 75 products purchased (47 brands), 17% were accurately labeled, 23% were mis-labeled (thus, "misbranded") by overstating the cannabinoid content, and 60% were misbranded because of underestimating the THC content. Additionally, 44 products (59%) contained detectable levels of CBD, whereas only 13 had CBD content labeled. Four products were under-labeled and 9 were over-labeled for CBD (Table 4.1) [40][41][42].

CBD and THC variability for the canine plasma concentrations (Figure 3.7) reflects dosing as well as differences in the disposition of cannabinoids in individual dogs. However, a major contributing factor like is differences in product cannabinoid concentrations as is demonstrated by these studies. Based on these reports, and the marked variability in cannabinoid content of canine plasma samples, the decision was to test the concentration of cannabinoids in commercial products. As with humans, pets are consuming commercial products that undergo no federally mandated quality control assessment.

The difficulty in developing a single method that will quantify cannabinoids in both plasma and commercial compounds is the variability in the matrices and the lack of availability of control material for these different commercial matrices.

Further, the might higher concentration allowed use of methods other than UPLC-MS. As such, the method validated for canine plasma was not used for commercial products leading to different sample preparation techniques as well as different separation (chromatographic conditions) and detection methods.

Table 4.1 Example literature describing methods for the detection and quantitation of cannabinoids in commercial products.

Article Name	Authors	Date	Product	Standards	Detector	% difference from target	Accurate labeled	Underlabeled labeled	Overlabeled labeled	CBD detectable	CBD less than labeled	Mobile Phase	Sample Prep	LOD	LOQ
Pharmaceutical and biomedi	Citti C., et al.	2018	Whole blood, pl	N/A	GC-MS, HPLC-UV, L	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Deproteination (aceto	THC: MS:	THC: MS-0.25, 0.58, 0.8, 1, 3, 5, 7.5 UV-16 CBD: MS-0.44, 0.5, 0.8, 2
Pharmaceutical and biomedical analysis of cannabinoids: A critical review	Citti C., et al.	2018	Cannabis plant, dried hemp flowers , Fiber-type plant, Industrial-grade hemp, Medicinal cannabis.	N/A	GC-MS, HPLC-UV, HPLC-DAD, UPLC-MS (ESI), HPLC-QTOF,	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Extraction with organic solvents (Ethanol, MeOH, chloroform, EtOAc, Hexane)	0.05, 0.3 µg/mL MS:3 ng/mL	THC: DAD-0.05, 0.1, 0.125 µg/mL MS: 1 µg/mL , CBD: DAD-0.05, 0.1, 0.188 µg/mL, 0.041 MS: 1 µg/mL
Cannabinoid Dose and Label Accuracy in Edible Medical Cannabis Products	Vandrey R., et al.	2015	Study of accuracy of legal USA cannabis products	N/A	No reported	47 brands, 75 products	THC: 17% CBD: 13%	THC: 23% CBD: 4 products	THC: 60% CBD: 9 products	0.44	>50%	N/A	Entire package contents were homogenized (crushed or mixed)	N/A	N/A
Identification and quantification of cannabinoids in Cannabis sativa L. plants by high performance liquid chromatography-mass spectrometry	Aizpurua-Olaizola O., et al.	2014	Plants	CBD, THC from Cerilliant, THCA, CBG, and THCV from Echo Pharmaceuticals BV. THC-D3 (IS)	HPLC-triple Quad, APCI, postitive mode, MRM. Corona discharge current 5 uA, capillary voltage 3500 V, heated vaporizer at 280°C, nitrogen flow of 7 L/min, source temp 210°C, and nebulizer pressure 32 psi.	N/A	N/A	N/A	N/A		Kinetix C18 column (150 x 3 mm, 2.6 um).	0.1% formic acid in water : 0.1% formic acid in methanol. Gradient: 50:50, 20:80, 5:95, 50:50. Flow 0.25 ml/min.	Plants pulverized with neem oil, cryo-milled under liquid nitrogen, SFC, extraction with EtOG at 35°C, collection in EtOH.	0.2 ng/ml (CBD), 0.05 ng/ml (THC)	No reported

Sample preparation and chromatographic conditions:

The cannabinoids calibration curves and controls were prepared in methanol. The chromatographic conditions and the extraction procedure were developed based on a validated method for analysis in cannabis plant [39][11] with minor modifications in the column length and the composition of the mobile phase. Two different chromatographic columns (C8 and C18, 250 mm and 150 mm x 4.6 mm, 5µm), using temperature (35 °C and 40 °C). Two different mobile phases (acetonitrile : water and ammonium formate buffer pH 3.7: acetonitrile) were tested at different flow rates (1.0 and 1.5 mL/min). Different wavelengths (240 nm, 212 nm, and 272 nm) were tested. The HPLC System used was an Alliance system with a 2487 UV detector from Waters (Milford, MA, USA). Empower software (From Waters) was used for system control, data acquisition, and quantification. The external standard method was used for the quantification of the cannabinoids (cannabinoids standards in methanol with a high purity (98%) were used to prepare the calibration curve).

Results:

The optimal identification and separation were performed on a chromatographic column C18 Sunfire 250 mm x 4.6 mm, 5µm, using temperature (40 °C). The mobile phase was a mixture of 80% acetonitrile:20% water at a flow rate of 1.5 mL/min. The optimal wavelength for detection was 212 nm. The retention times are showed on Figures 4.2, 4.3 an 4.4.

Briefly, the cannabinoids were extracted from the commercial preparations with methanol and sonication for 30 min. The extract was filtered, diluted and tested with the optimal conditions for the HPLC-UV analytical method.

The calibration range was linear from 2 mg/ml to 20 µ/ml for CBD, and from 25 to 500 ng/mL for the CBG, CBN, CBC and THC in methanol. The R^2 was 0.9996, 0.998, 0.998, 0.997 and 0.999 for CBD, CBG, CBN, THC and CBC respectively. The LOD was 25 ng/mL and the LOQ was 50 ng/mL for CBG, CBN, THC and CBC. For CBD the concentrations were in µg/mL. The results were as follow,

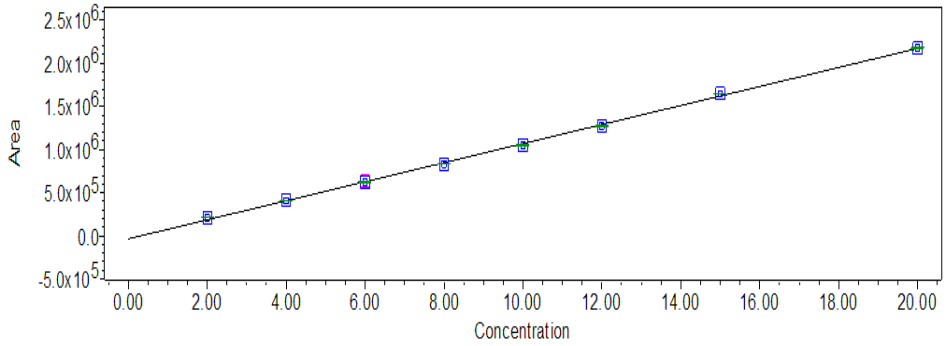


Figure 4.1CBD calibration curve in methanol

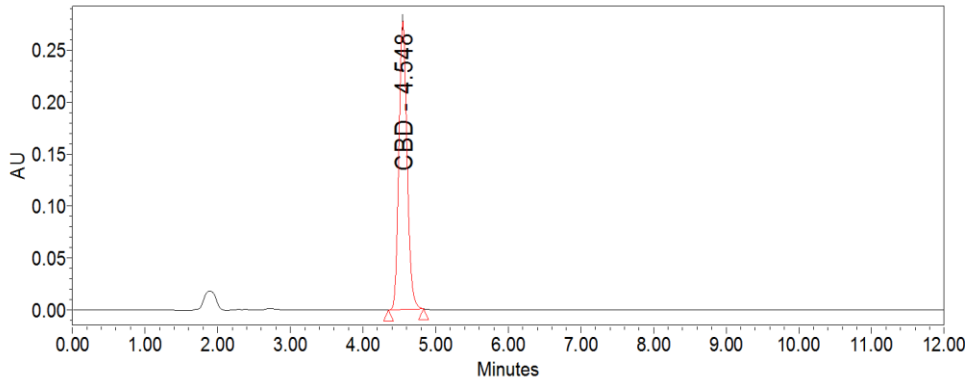


Figure 4.2 CBD chromatogram in methanol, 20 µg/mL

Name	Retention Time	Area	% Area	Height	Amount	Units
CBD	4.548	209276	100	25719	50	ug/mL

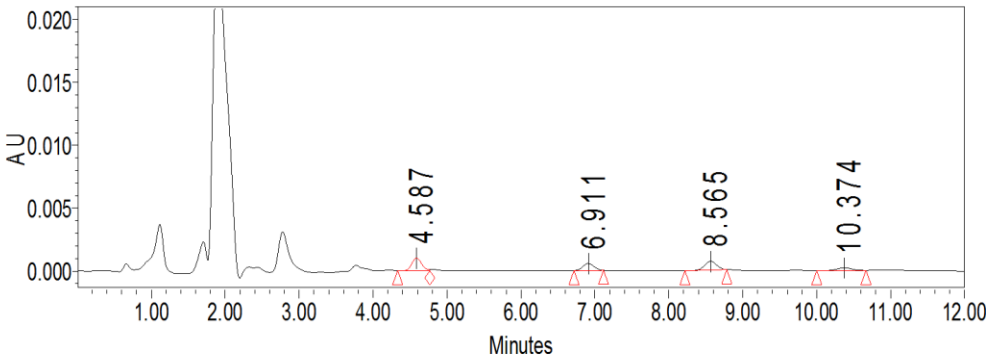


Figure 4.3. CBG, CBN, CBC, and THC standards (mixture) in methanol (25 ng/mL)

Name	Retention Time	Area	% Area	Height	Concentration	Units
CBG	4.587	9452	36.47	1025	25	ng/mL
CBN	6.911	5716	22.05	549	25	ng/mL
THC	8.565	7771	29.98	692	25	ng/mL
CBC	10.374	2981	11.5	215	25	ng/mL

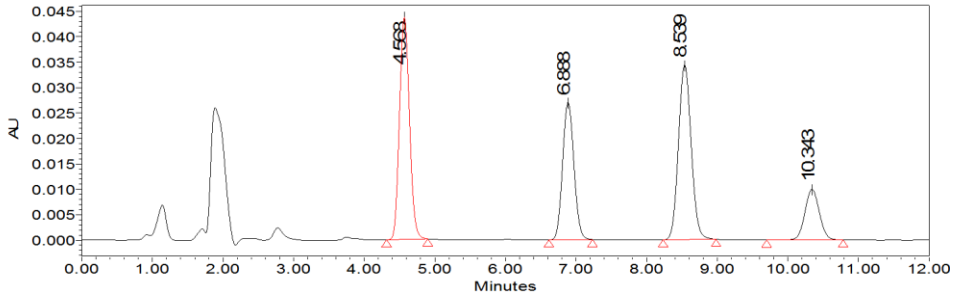


Figure 4.4. CBG, CBN, CBC, and THC Standards (mixture) in methanol, 1000 ng/mL

Name	Retention Time	Area	% Area	Height	Concentration	
CBG	4.568	429441	33.01	43742	1000	ng/mL
CBN	6.888	300380	23.09	27007	1000	ng/mL
THC	8.539	430045	33.05	34340	1000	ng/mL
CBC	10.343	141153	10.85	9980	1000	ng/mL

Upon successful identification and separation of an analytical assay for the cannabinoids in methanol, the assay was then applied to commercial samples that were obtained from the Clinical Pharmacology Laboratory at The College of Veterinary Medicine at Auburn University. Samples were obtained through the Therapeutic Drug Monitoring (TDM) service. Commercial samples were received from same clinics that sent the canine samples, we solicited if possible to submit the commercial sample preparation that the patient was receiving. Samples were solicited through the TDM service at its web site, which provides accession forms as well as surveys the submitting veterinarians. Product samples do not require -80 storage, and so they were stored at room temperature in a secure location in the laboratory were they remain until analysis. Figure 4.5 to 4.8 are the chromatograms that shows some of the oil products that veterinarian patients were taking.

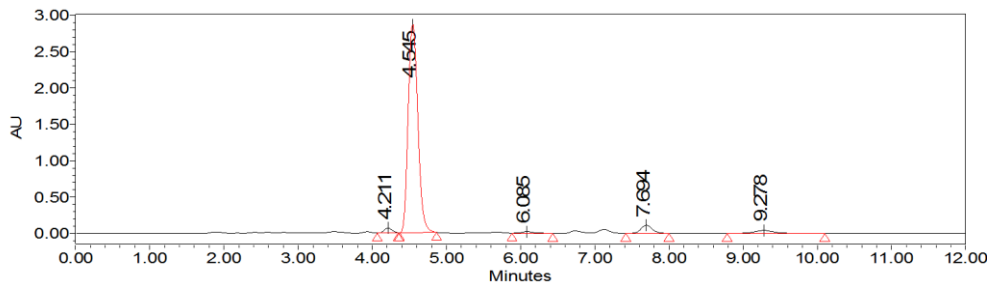


Figure 4.5. Hemp Rx oil sample

	Name	Retention Time	Area	% Area	Height
1		4.211	499279	1.74	68331
2	CBD	4.545	26013188	90.4	2867477
3	CBN	6.085	270348	0.94	22906
4	THC	7.694	1175657	4.09	112604
5	CBC	9.278	817613	2.84	44029

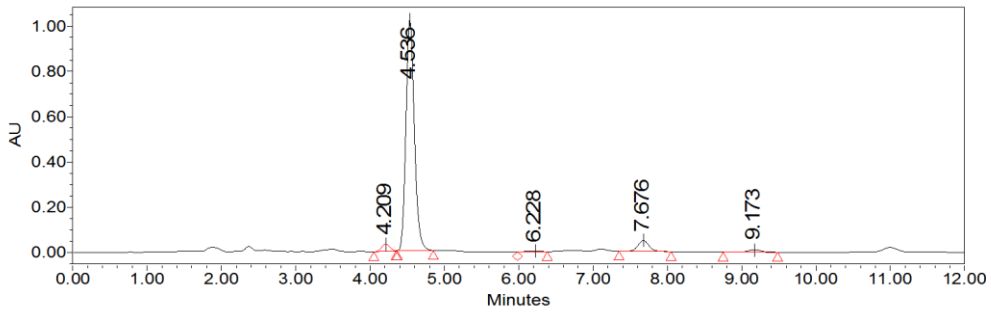


Figure 4.6. Sweet Jane oil sample

	Name	Retention Time	Area	% Area	Height
1		4.209	244440	2.73	31709
2	CBD	4.536	8045499	89.72	1024008
3	CBN	6.228	32552	0.36	2843
4	THC	7.676	503981	5.62	48503
5	CBC	9.173	140938	1.57	11522

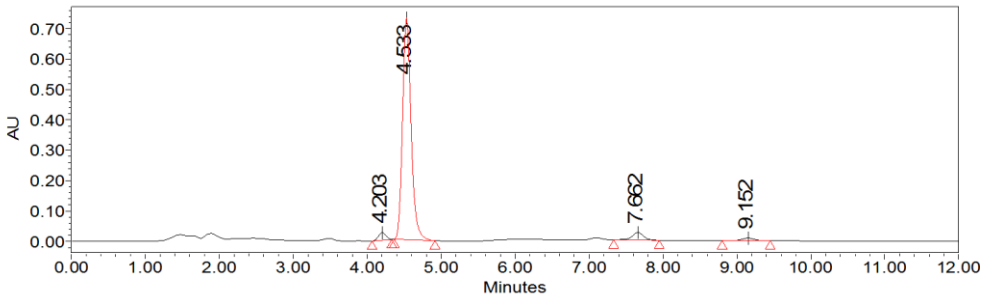


Figure 4.7. TDM oil sample, accession 0531-17

	Name	Retention Time	Area	% Area	Height
1		4.203	178333	2.78	24895
2	CBD	4.533	5863783	91.38	727529
3	THC	7.662	271536	4.23	25188
4	CBC	9.152	103478	1.61	8587

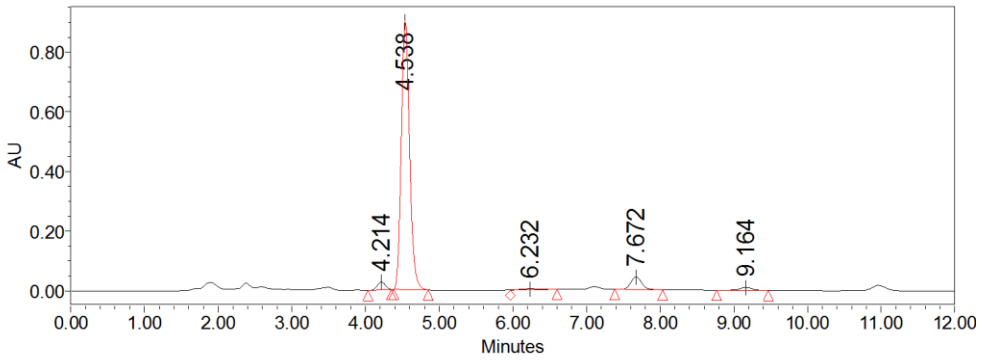


Figure 4.8. TDM oil sample, accession number 0531-18

	Name	Retention Time	Area	% Area	Height
1		4.214	208979	2.65	27006
2	CBD	4.538	7062101	89.44	903987
3	CBN	6.232	69463	0.88	4290
4	THC	7.672	437317	5.54	42740
5	CBC	9.164	117900	1.49	9864

Summary of cannabinoids concentration in commercial cannabinoid products:

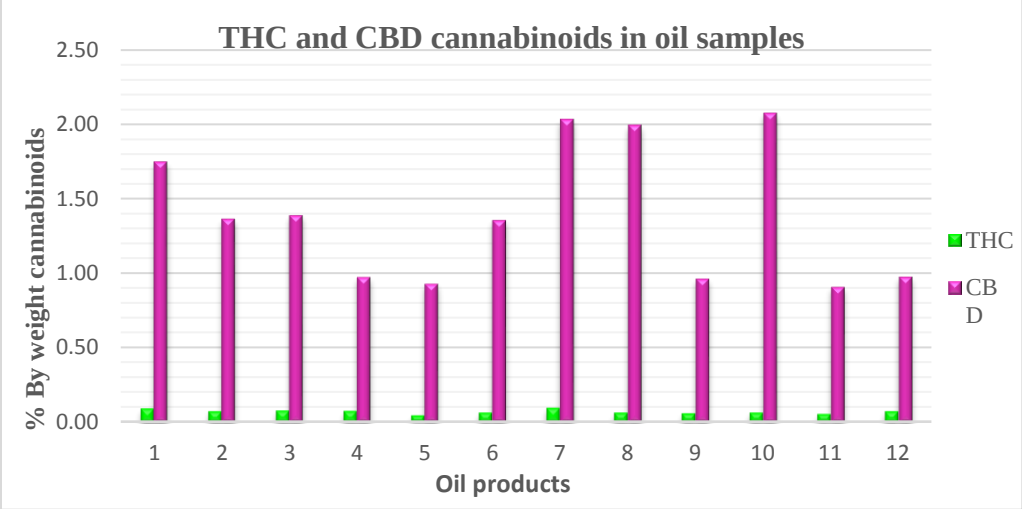


Figure 4.9. Results for CBD in oil samples

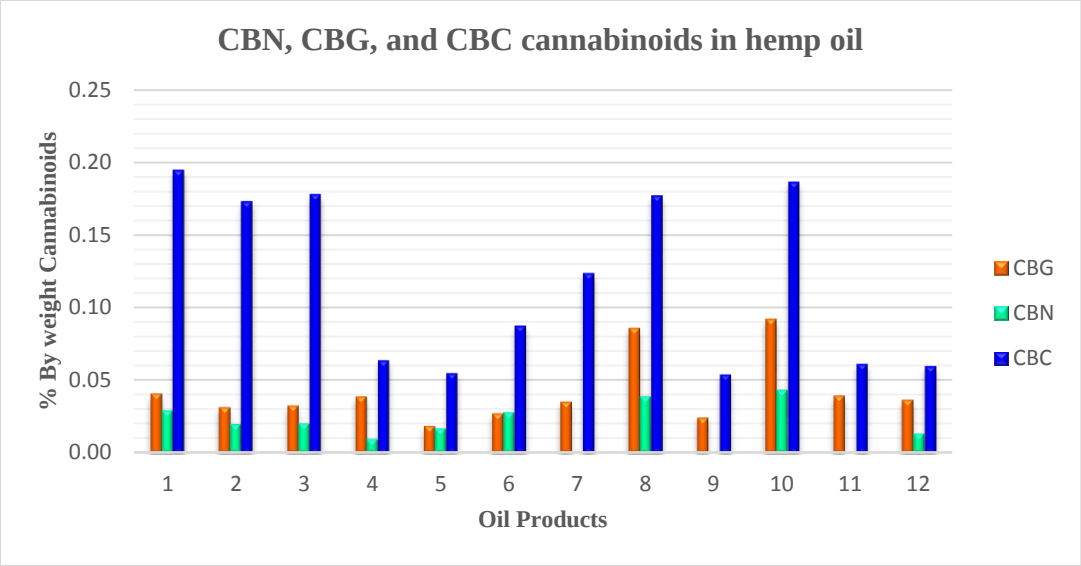


Figure 4.10. Results for CBG, CBN, THC and CBC in TDM oil samples

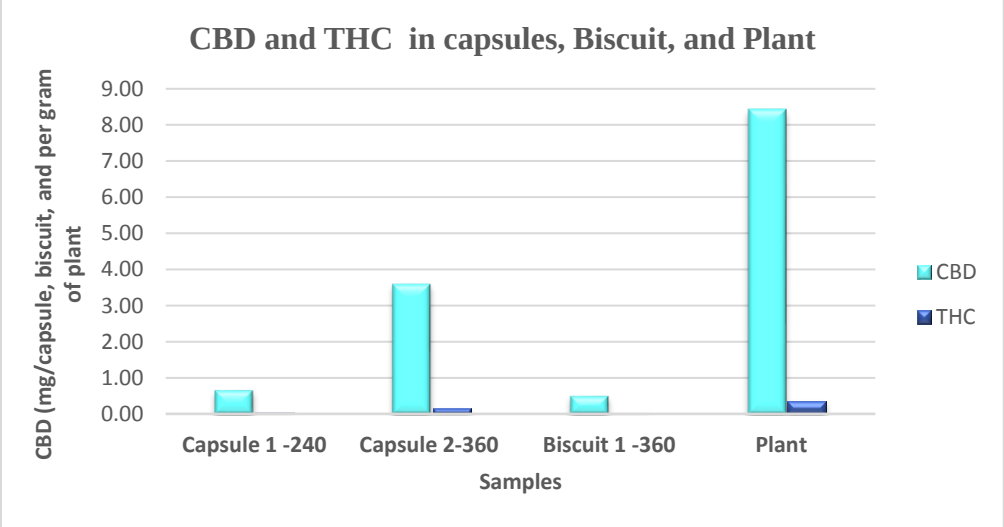


Figure 4.11. Cannabinoids results in capsules, biscuit and plant

A full validation is still needed to test the commercial products with accuracy and precision. These parts of the validation will be in progress as a second part of this work.

The objective to described the HPLC-UV method and his aplications to commercial samples in this section (even if it is not totally validated) was to demostrate some of the

differences between HPLC-UV and LC-MS/MS as lenght of the column (250 mm vs 50 mm), particle size (5 µg vs 1.7 µg), packing material (C8 vs C18) and sample preparation technique (Precipitation combined with SPE vs extraction in methanol). Consider the nature of the sample (type: plasma, capsules, oil etc.) is very important because based on this information the chromatographic condictions and type of detection can be selected.

Chapter 5 Discussion and future research

Cannabinoids are the important chemicals in cannabis plant with medicinal [55] value and cannabinoid medications are utilized for an increasing number of indications. However, effective and safe use is best based on studies that describe their behavior in the plasma of the species being treated. This requires a sensitive and robust method for accurate and precise quantification of these closely chemically related. Several LC-MS [44] [53]and GC-MS [52] methods have been described in the literature that quantify cannabinoids in human plasma [43] [50], rat urine, waste water, surface water, cannabis plant, and cannabis oil [54]. But the quantification of cannabinoids in canine plasma has not being described and this can yield the novel insight into cannabinoids pharmacokinetic and toxicology studies in the veterinary field. Also, this can help for a future therapeutic drug monitoring in pets.

This LC-MS/MS analytical method specifically quantifies 2 of the main cannabinoids in canine plasma CBD and THC, which are the typical cannabinoids of interest in the samples, also include a detection of the minor cannabinoids CBG, CBN and CBC. Thus, this analytical method for simultaneous analysis of CBD and THC in the plasma sample is a significant advancement in the detection and quantification of this important class of compounds for veterinary practice.

5.1 Validation method

The method was validated according to the criteria described in Chapter 3: Description of development and validation procedures. LOD, LLOQ and linearity results for each cannabinoid can be seen in Appendix A, Fig. A.1 to A.4. LLOQ were determined through analysis of low concentrations of drug-fortified canine plasma and were 3.91 ng/mL for CBD and THC, with a 0.5 mL canine plasma.

The LOQ could be lower than 3.91 ng/mL, but either the canine plasma sample size has to be larger than 0.5 mL (at least 1.0 mL), or the SPE cleaning procedure need more optimization.

For linearity R^2 values were acceptable ($R^2 > 0.980$) for all the cannabinoids. Linear ranges for CBD and THC were 3.91 to 1000 ng/mL, and 3.95 ng/mL to 250 ng/mL for CBG, CBN and CBC. These ranges should be useful for pharmacokinetic, toxicological studies, and for clinical therapeutic monitoring.

Quality controls (QCs) in canine plasma for CBD predicted the theoretical concentrations within ±20%, and for the LLOD (1.95 ng/mL) ± 25%. For THC the QCs predicted the theoretical concentrations within ±15%, and for the LLOD (1.95 ng/mL) ± 25% when quantified against the calibration curve. Clinical studies will help to establish a better range of concentrations.

There were not many deuterium-labeled analogues commercially available by the time this validations was prepared, but based in similarities in extraction efficiency/matrix effects the best option was to use THC-D3 as internal standard. In future research another deuterated internal standards can be used, and this will allow more stringent criteria (±15%) to be applied to all cannabinoids at concentrations below the LOQ.

The matrix of the internal standard (THC-D3) was methanol and this did not interfere with the canine plasma matrix. Furthermore, we investigated matrix effect in 10 different commercial canine plasma batches demonstrating that low QC quantification remained within ± 20%. Anyway differential matrix effect cannot be excluded totally, and cannabinoid quantification could still be affected at the low concentrations.

The precision was evaluated at four concentrations across the linear range for CBD and THC including the LLOD. Inter-assay precision (% RSD) was less than 20% for CBD except for the LLOD (1.95 ng/mL, 25%). For THC the inter-assay precision (% RSD) was less than 15% except for the LLOD (1.95 ng/mL, 25%). Intermediate precision (% RSD) was less than 20% for CBD except for the LLOD (1.95 ng/mL, 25%). For THC the inter-assay precision (% RSD) was less than 15% except for the LLOD (1.95 ng/mL, 20%). The precision was calculated as the percent of target concentrations at different concentrations (including low, mid and high). The Precision (%RSD) for CBD at the LLOQ (3.91 ng/mL) was 20 % and for THC was 11%

These differences in the quantification range (3.91 – 250 ng/mL) for CBD and THC were less than 20% and this is considered acceptable for pharmacokinetic studies and therapeutic drug monitoring in veterinary.

Development of an effective solid phase extraction sample cleanup that removed matrix interferences while maintaining high extraction efficiency proved to be the greatest challenge during method development. The extraction procedure (Oasis HLB reversed-phase polymeric SPE cartridge) with a gentle wash step (water) and polar elution solvent (methanol) yielded high concentrations of cannabinoids in extracts No major interferences from the blank canine plasma were observed.

Stability at 4 °C for the samples was tested: A calibration curve (3.91 to 250 ng/mL) was prepared for CBD and THC, and after the precipitation/SPE procedure, up to the dry step under nitrogen, the sample was stored at the refrigerator at 4 °C for 2 days and then, the residue was diluted with methanol and analyzed through the LC-MS/MS. The data was compared with a calibration curve that was prepared up to the final step and analyzed immediately. Cannabinoids at all concentrations were stable under these conditions, with mean concentrations differing from samples injected immediately by less than 10%. However, calibration curves were prepared (spiked) fresh every time, the variation for the stability test was on the SPE method.

5.2. Application of Method

As it was mentioned before some canine clinical samples from a therapeutic drug monitoring (TDM) were tested using the LC-MS/MS validated analytical method. These TDM samples were received at the clinical pharmacology laboratory for another TDM test, but the canine patients also were using some of the cannabis commercial products, and the decision was to test these samples with the validated method for a research purpose. The cannabinoids concentrations for the TDM samples were between 3 to 160 ng/mL with a mean of 39 ng/mL for CBD, this means that they were between the range concentrations of the validated method for CBD (Figure 3.7) For THC the concentration in the canine samples were between 0.39 to 87 ng/mL, with a mean value of 10 ng/mL, but most of the samples had less than 3 ng/mL and bellow of the LOD demonstrating the necessity for a lower LOQ than this method can achieved. Cannabinoids concentrations below the LLOQ should be reported as “0” or less than the LLOQ.

Most of the concentrations for the other minor cannabinoids, such as CBD and CBC were also below the LOQ, except for CBN (1.5 to 24 ng/mL). A further research will be focus on quantification of traces of these cannabinoids.

5.3 Conclusions

A new robust, sensitive and specific cannabinoid LC-MS/MS method for a simultaneous quantitation of CBD and THC in canine plasma samples was developed and validated. The method consists on SPE extraction and MS detection with electrospray ionization. The efficient cannabinoids extraction sample procedure using SPE allows to have a low LOD (1.95 ng/mL) and a low LOQ (3.91 ng/mL), and a sample analysis of 3 min, this is beneficial; however, care should be taken to prevent and other matrix components, leading to increased LC backpressure and loss of resolution. This analytical method will support clinical trials and pharmacokinetic studies necessary to demonstrate safety and efficacy of these promising agents. This method can also be used for toxicology test for veterinary use. This new LC-MS/MS analytical method for cannabinoids in canine plasma offers advantages in sensitivity over HPLC-UV technique. For CBC, CBN and CBC detection in canine plasma can be performed with this method, but validation is still needed for them because the concentrations are below the LOQ.

The limit of detection (LOD) in the previous assays for humans in plasma and urine was from 1 ng/mL to 25 ng/mL. Also, the lower limit of quantification for CBD in dogs was 25 ng/mL for some of them, a better detection (1 ng/mL) was obtained with automatized SPE, which makes not accessible the assay to every laboratory. The % recovery on the previous assays for humans were low (43%-78%). Some of these methods have a low recovery (55%) and requires derivatization See Table 1.1

The advantages of this new method is that it has the capability to detect 1.95 ng/mL(LOD) and quantify 3.91 ng/mL (LLOQ) for CBD and THC Also, For CBD the % recovery was 100% ± 18% with a 16% precision and for THC the % recovery was 105% ± 5% with a 5% precision.

A final future applications of this method will be the development and validation of analytical methods to quantify cannabinoids in cats, horses and humans and endocannabinoids (Anandamide and 2-AG) [1] in plasma for dogs, horses, and cats.

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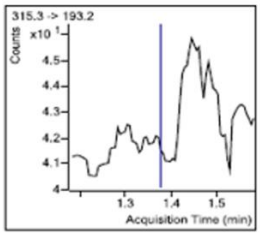
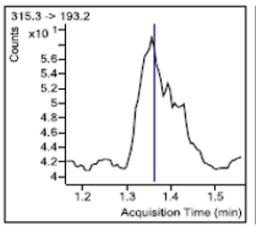
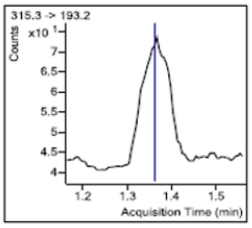
Appendix-A

LOD and LOQ

Fig. A.1







Canine blank plasma
Average response: 19.5

CBD Response
1.95 ng/mL: 60.21
3.91 ng/mL: 154.00
7.81 ng/mL; 209.33

THC Response
1.95 ng/mL: 78.62
3.91 ng/mL: 156.36
7.81 ng/mL: 228.50

For LOD

The LOD was determined as the lowest concentration of the cannabinoids with a signal to noise ratio of 3:1 Based on the response of the CBD and THC we can calculate how is the ratio between the the cannabinoid and the noise (from plasma).

For CBD LOD 1.95 ng/mL it has at least 3 to 1 relation = 60.21/19.5 = **3**

For THC LOD 1.95 ng/mL it has at least 3 to 1 relation = 78.62/19.5 = **4**

For LOQ

The LLOQ was determined by the lowest concentration of the cannabinoids that can be determined with acceptable precision and accuracy under the stated experimental conditions. Also this could correspond to a signal to noise ratio of at least 5:1 or 10 to 1.

Based on the response of the CBD and THC we could calculate how much is the ratio between the response of the cannabinoids and the noise (from the plasma). For CBD and THC the LOQ ratio was **8:1** with a 20% RSD, which mean is between the acceptable parameter (5-10).

The next concentration (7.81 ng/mL) also was tested, the results were **10:1** for CBD, and **11:1** for THC with an 11% RSD. For the purpose of the analytical method, 3.91 ng/mL was considered the LOQ because is still acceptable the 20% RSD, for small concentrations.

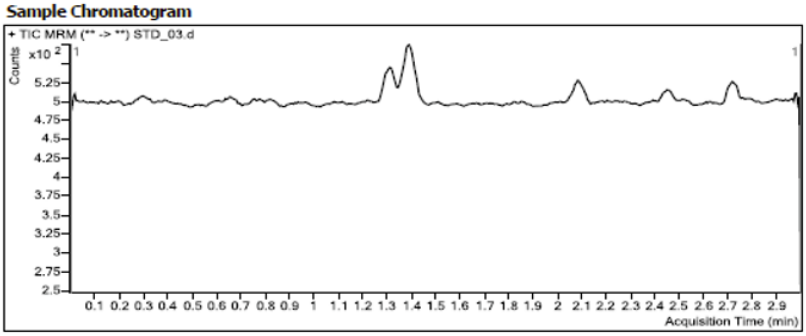
For CBD LOQ 3.91 ng/mL it has at least 5 to 1 relation = 154.00/19.5 = **8**

For THC LOQ 3.91 ng/mL it has at least 5 to 1 relation = 156.36/19.5 = **8**

For CBD LOQ 7.81 ng/mL it has at least 10 to 1 relation = 209.33/19.5 = **10**

For THC LOQ 7.81 ng/mL it has at least 10 to 1 relation = 228.5/19.5 = **11**

Fig. A.2 Chromatogram and mass spectra for cannabinoids in methanol (1.95 ng/mL) LOD



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBG		1.312	115			1.6755	85.79
CBN		2.089	103			1.8852	96.52
THC		2.459	11			1.7124	87.68

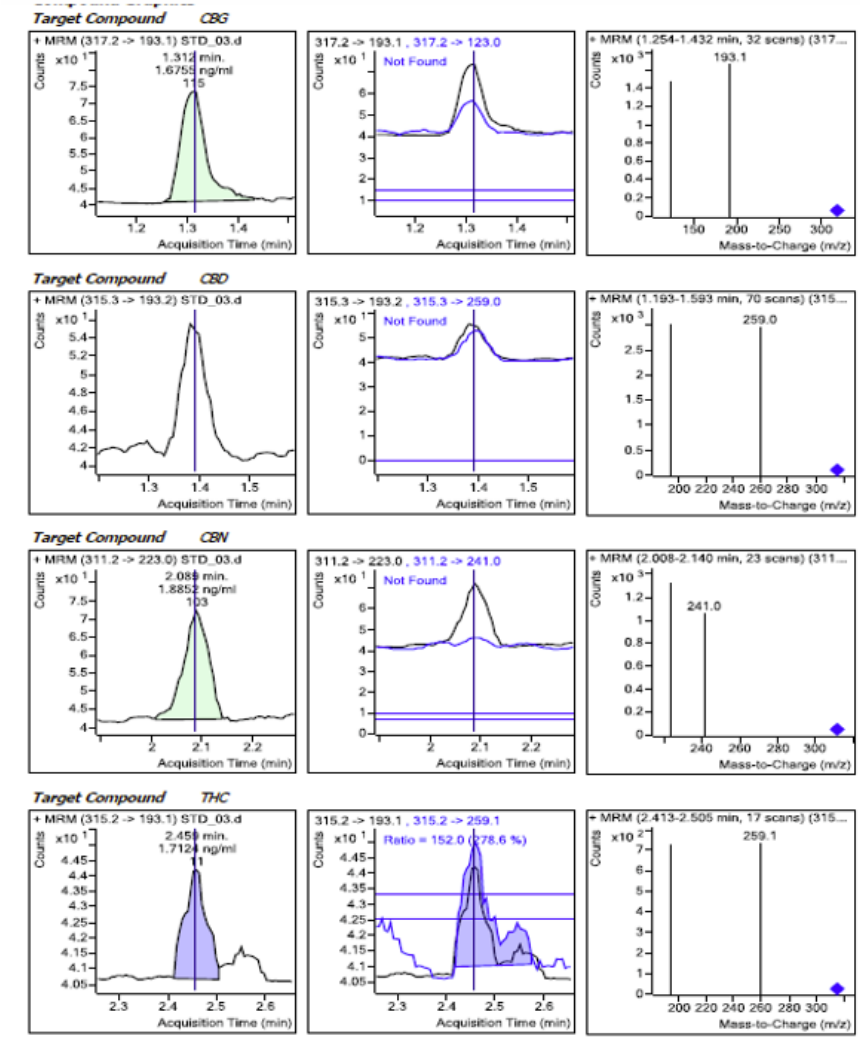
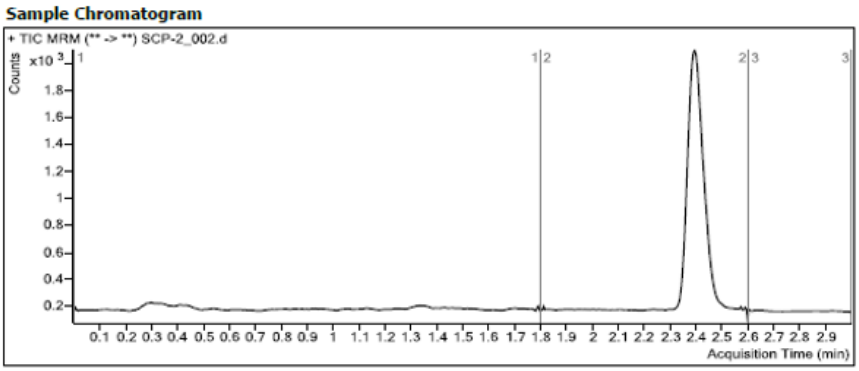


Fig. A.3 Chronmatogram and spectra for cannabinoids (CBD and THC) in canine plasma (1.95 ng/mL) LOD



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBD	THC-d3	1.357	87	8148	0.0107	2.3839	122.05
THC	THC-d3	2.396	36	8148	0.0044	2.0029	102.55

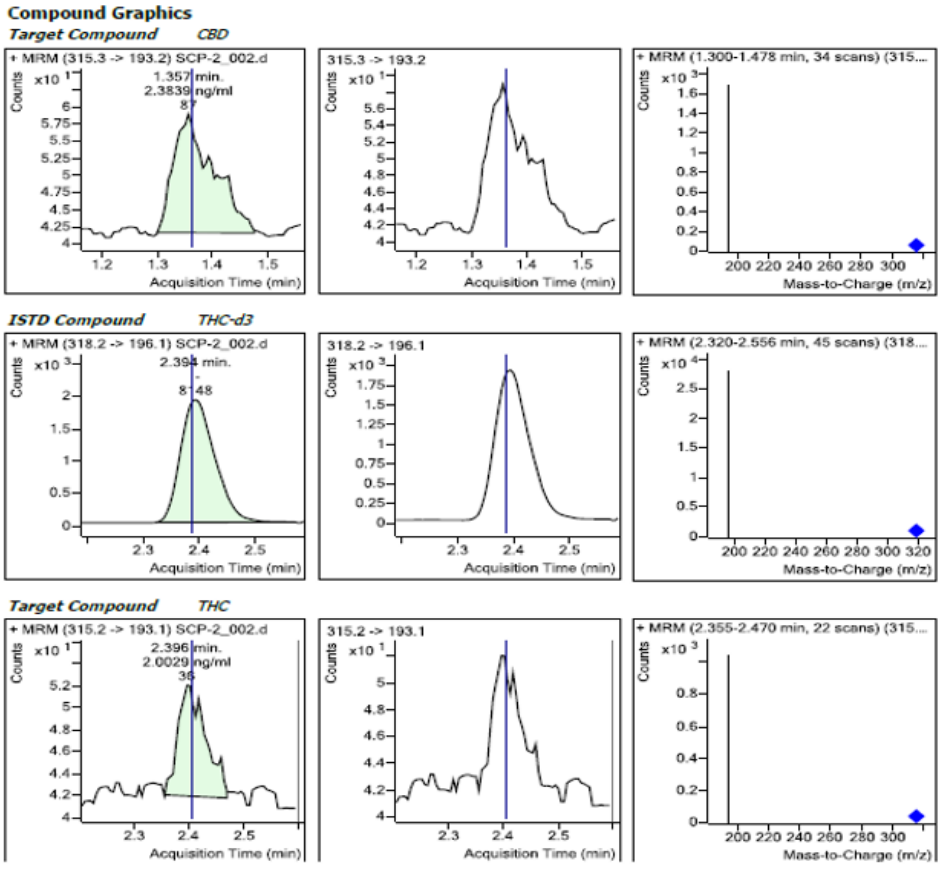
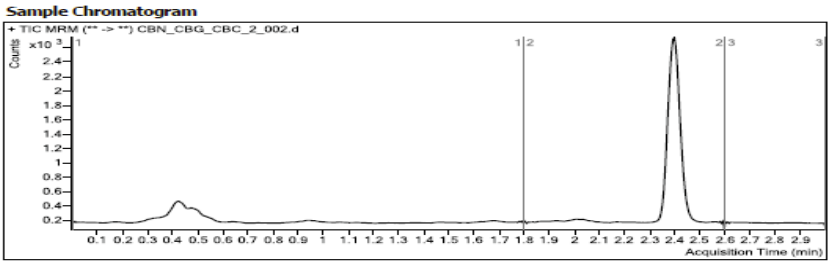


Fig. A.4 Chromatogram and mass spectra for cannabinoids (CBG, CBN, and CBC) in canine plasma (1.95 ng/mL) LOD.



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBG	THC-d3	1.481	7	8757	0.0008	0.7961	163.03
CBN	THC-d3	2.041	180	8757	0.0206	0.4937	101.11
CBC	THC-d3	2.677	16	8757	0.0018	1.2055	246.88

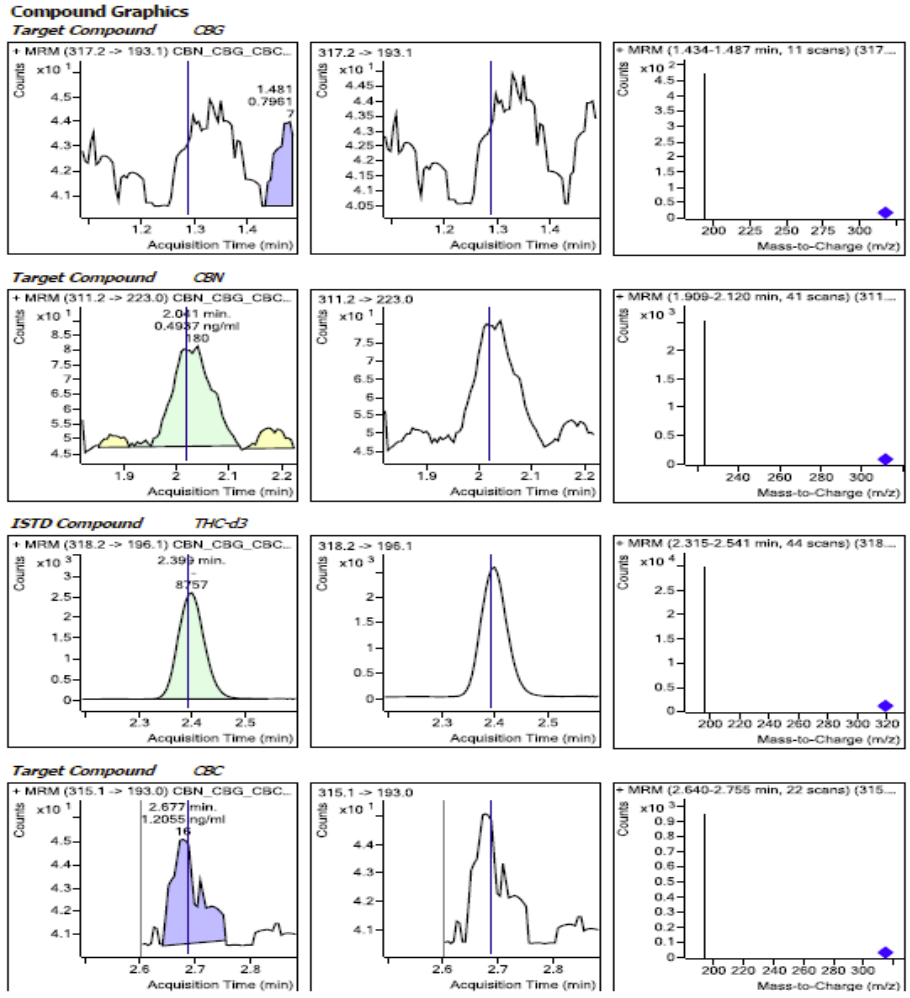
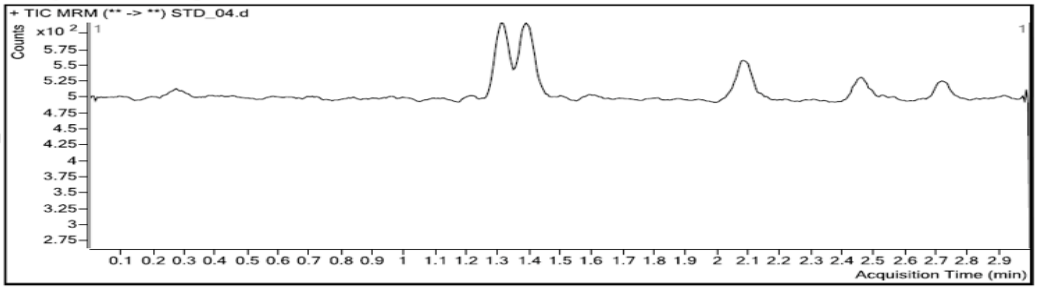


Fig. A.5 Chromatogram and mass spectra for CBD, CBG, CBN, CBC and THC in methanol (3.91 ng/mL) LLOQ

Sample Chromatogram

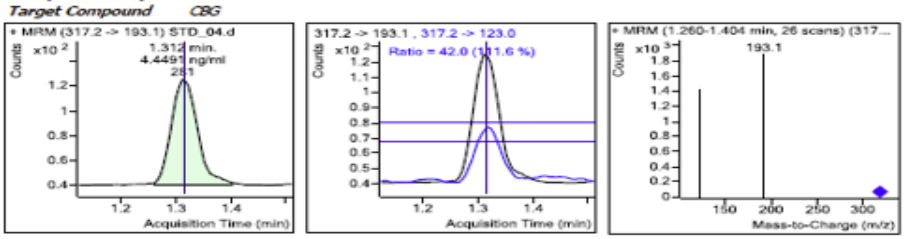


Quantitation Results

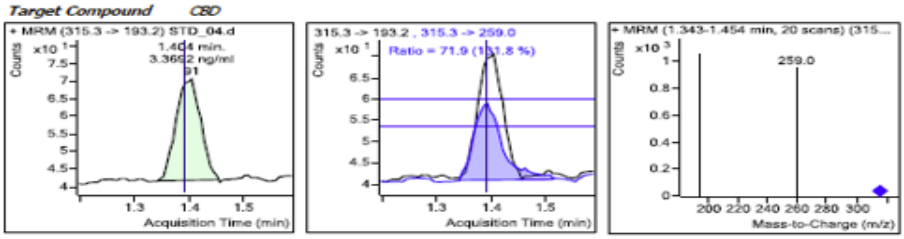
Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBG		1.312	281			4.4491	113.90
CBD		1.404	91			3.3692	86.25
CBN		2.089	193			3.6678	93.90
THC		2.464	31			4.1787	106.97

Compound Graphics

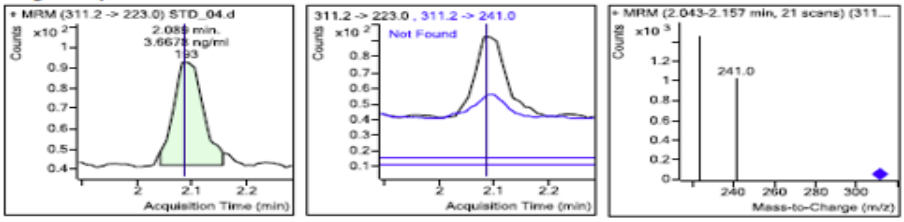
Target Compound CBG



Target Compound CBD



Target Compound CBN



Target Compound THC

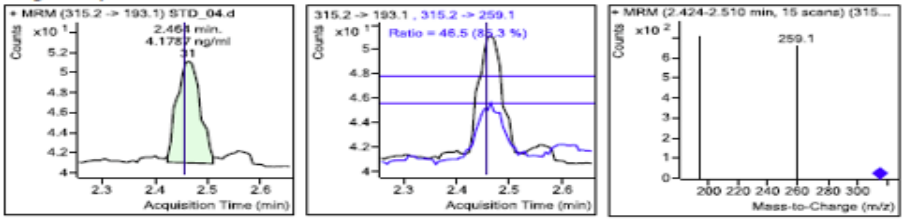
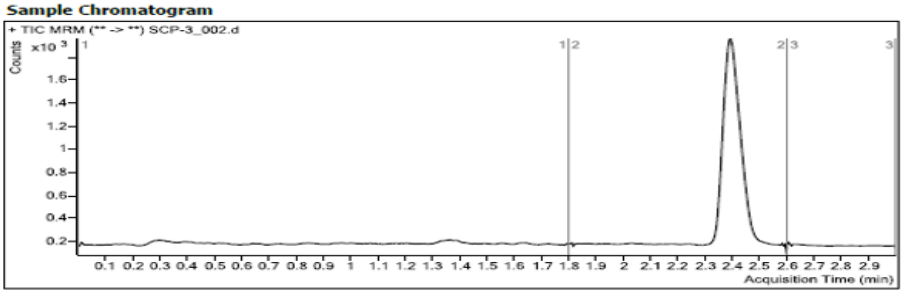


Fig. A.6 Chromatogram and mass spectra for cannabinoids (CBD and THC) in canine plasma (3.91 ng/mL) LLOQ



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBD	THC-d3	1.367	132	7842	0.0168	3.4511	88.35
THC	THC-d3	2.396	89	7842	0.0113	4.2380	108.49

Compound Graphics

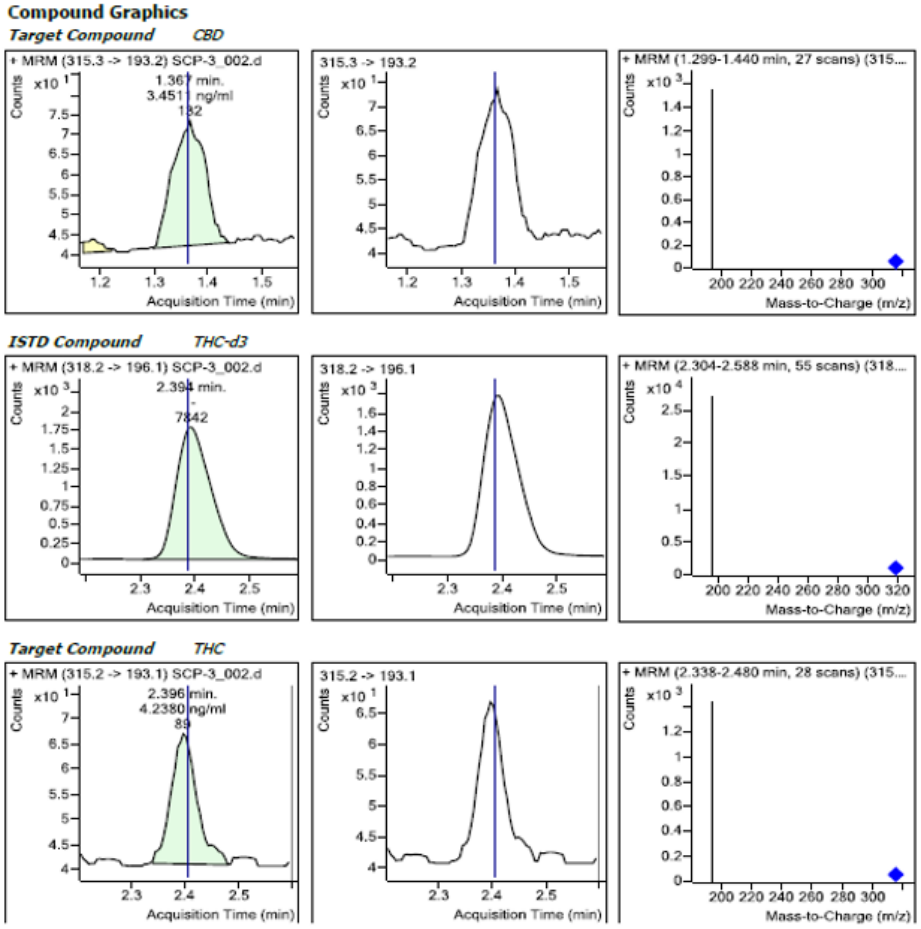
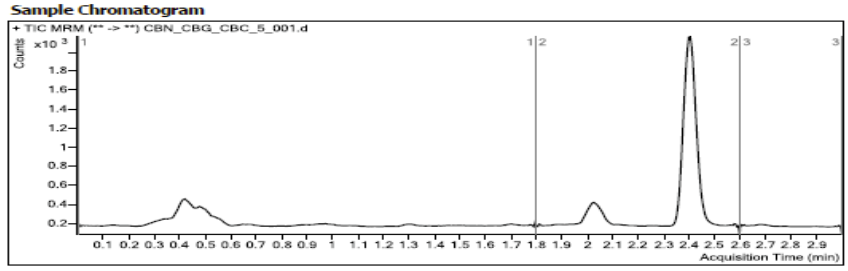


Fig. A.7 Chromatogram and mass spectra for cannabinoids (CBG, CBN, and CBC) in canine plasma (3.91 ng/mL) LLOQ



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBG	THC-d3	1.298	84	6922	0.0121	3.2345	82.80
CBN	THC-d3	2.030	1031	6922	0.1490	3.1600	80.90

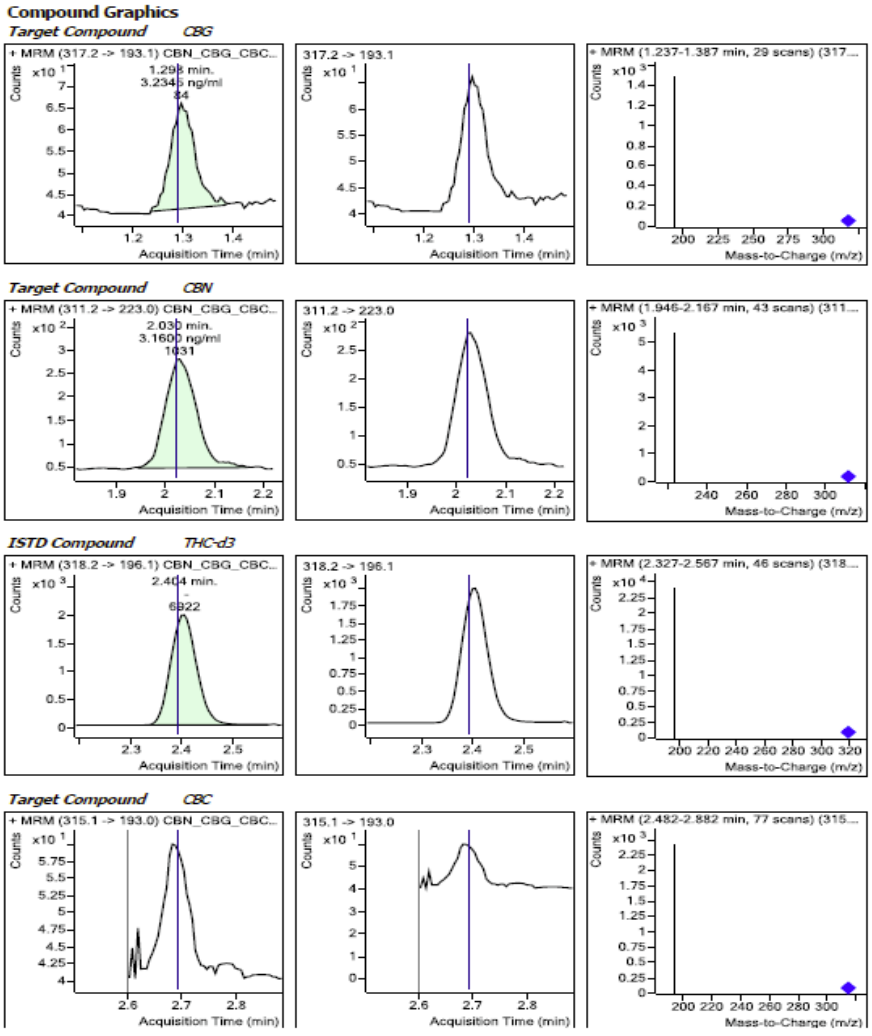
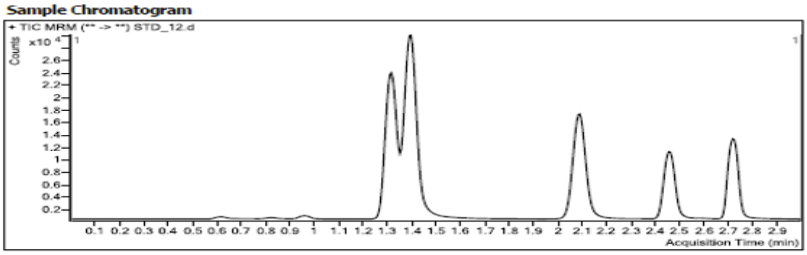


Fig. A.8 Chromatogram and mass spectra for cannabinoids in methanol (1000 ng/mL) ULOQ

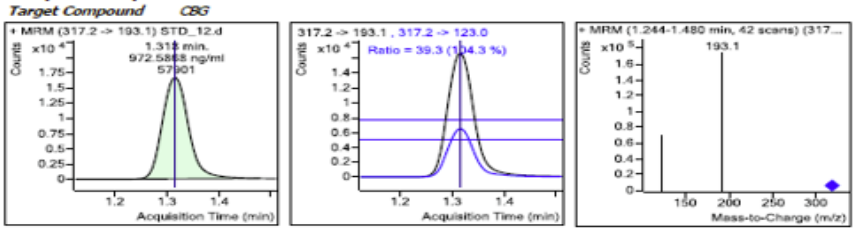


Quantitation Results

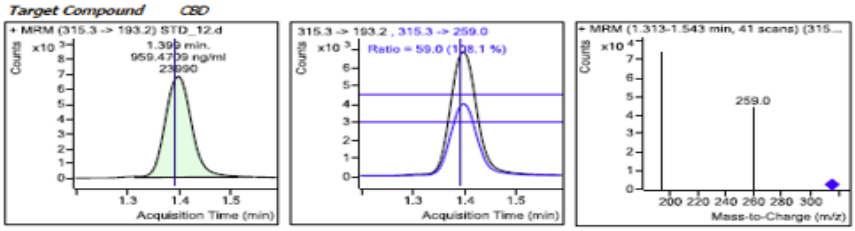
Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBG		1.318	57901			972.5868	97.26
CBD		1.399	23990			959.4709	95.95
CBN		2.094	49708			990.4544	99.05
THC		2.459	8085			1005.3223	100.53
CBC		2.723	7077			1042.9633	104.30

Compound Graphics

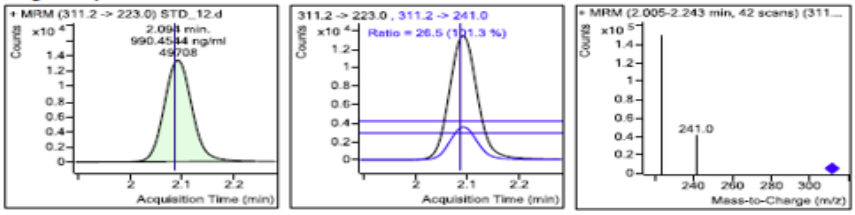
Target Compound *CBG*



Target Compound *CBD*



Target Compound *CBN*



Target Compound *THC*

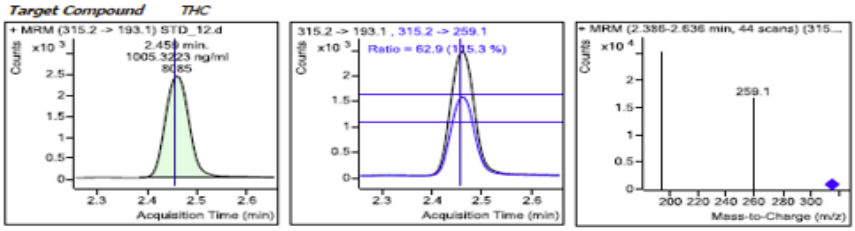
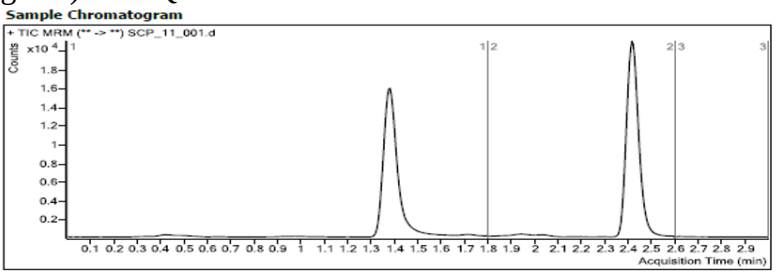


Fig. A.9 Chromatogram and mass spectra for cannabinoids (CBD and THC) in canine plasma (1000 ng/mL) ULOQ.



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc
CBD	THC-d3	1.383	66990	10959	6.1129	1085.4473
THC	THC-d3	2.417	65916	10959	6.0149	1012.7988

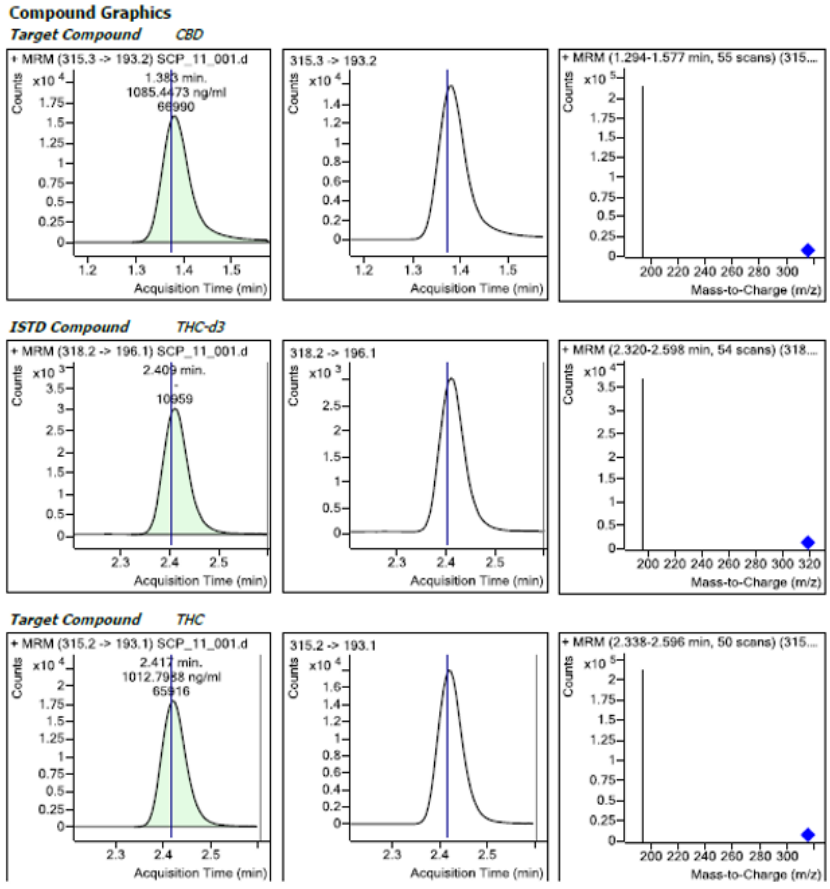
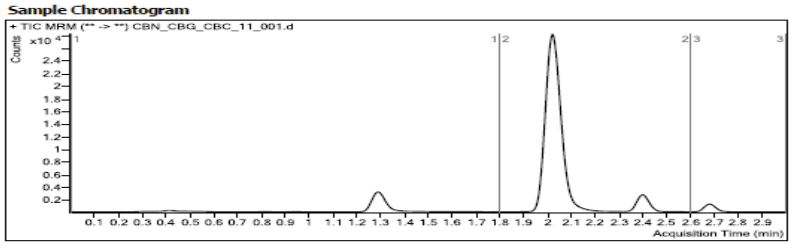
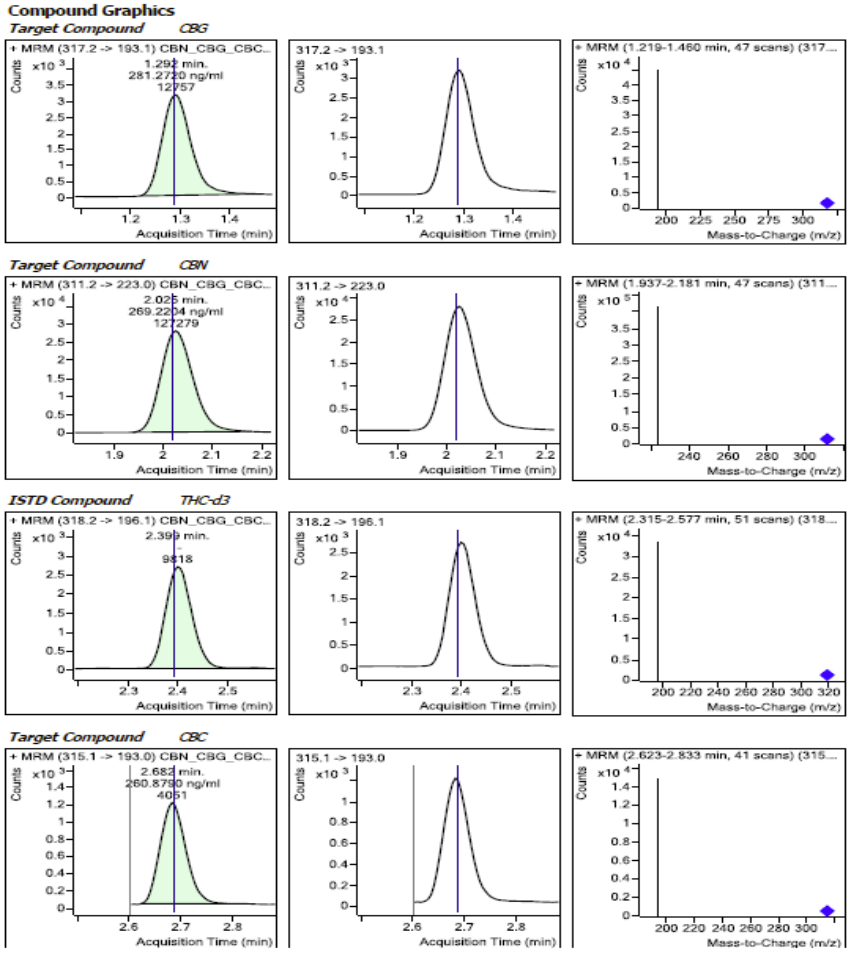


Fig. A.10 Chromatogram and mass spectra for cannabinoids (CBG, CBN, and CBC) in canine plasma (250 ng/mL) ULOQ.



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBG	THC-d3	1.292	12757	9818	1.2994	281.2720	112.51
CBN	THC-d3	2.025	127279	9818	12.9643	269.2204	107.69
CBC	THC-d3	2.682	4051	9818	0.4127	260.8790	104.35



Linearity

Fig. A.11 - Cannabinoids in methanol, no internal standard

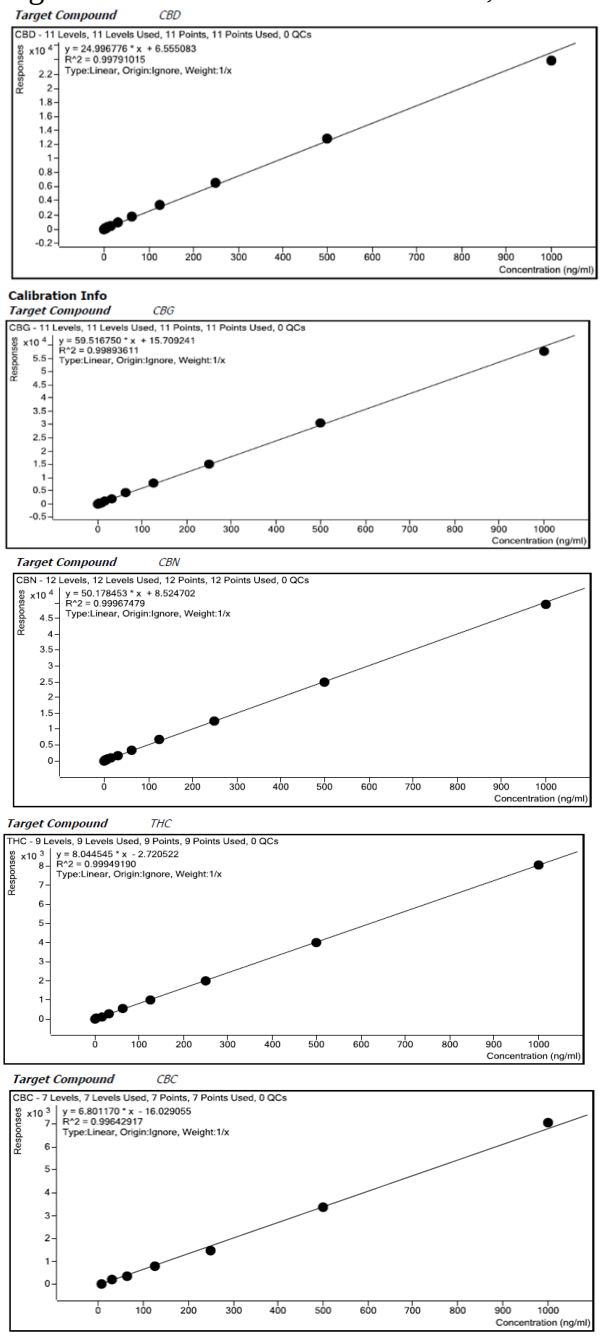
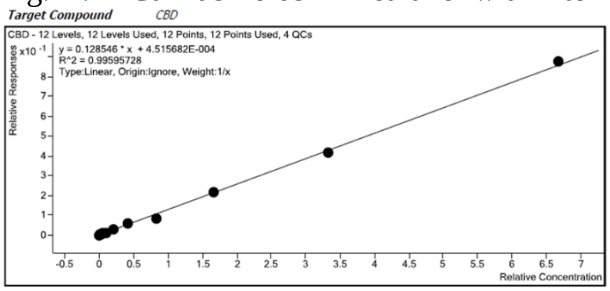
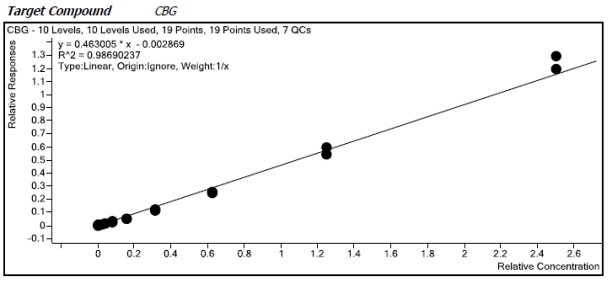
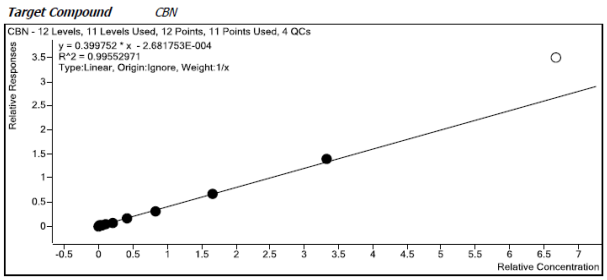
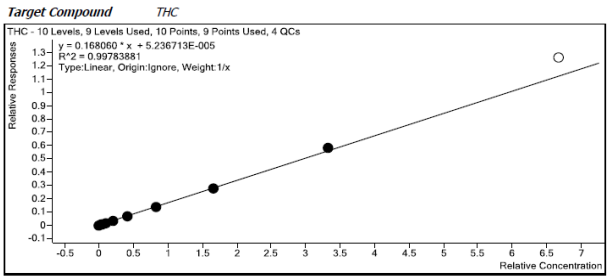


Fig. A.12 Cannabinoids in methanol with internal standard









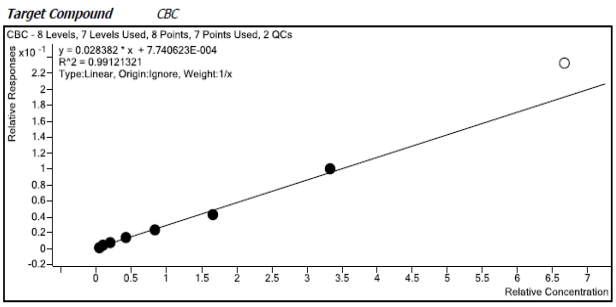
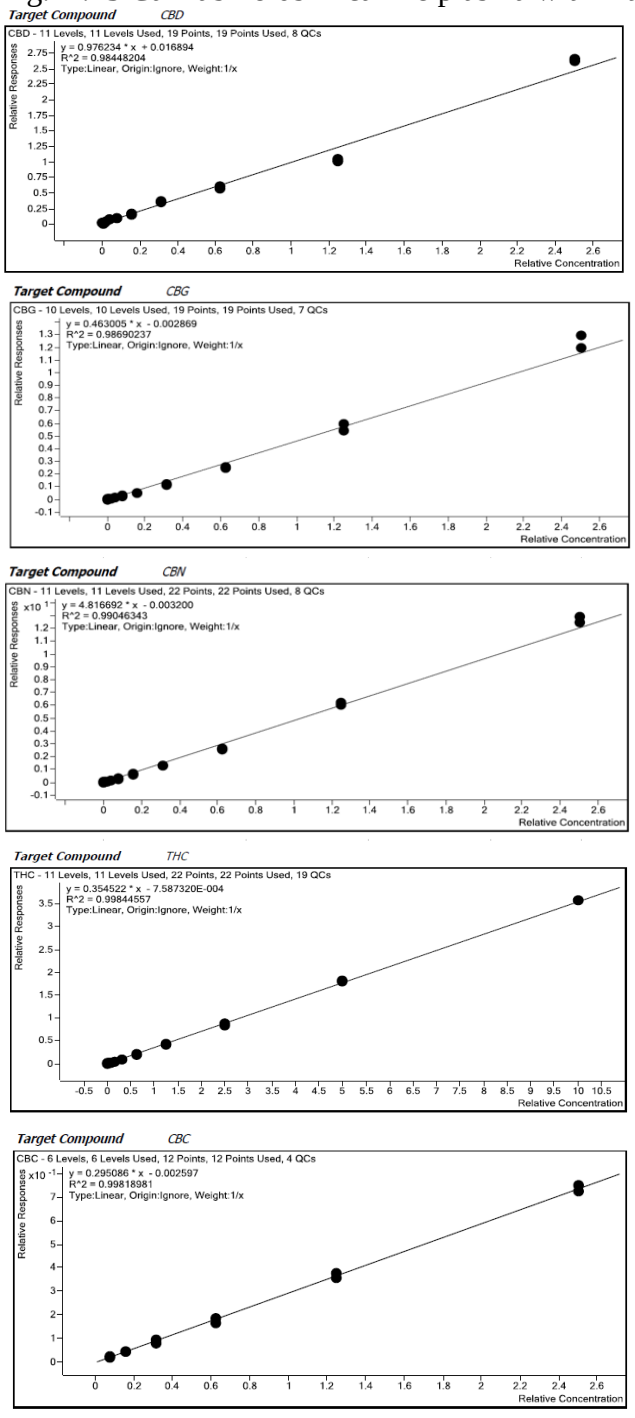


Fig. A.13 Cannabinoids in canine plasma with internal standard



Accuracy

Fig. A.14 Cannabinoids calibration curve in canine plasma used for accuracy (CBD and THC)

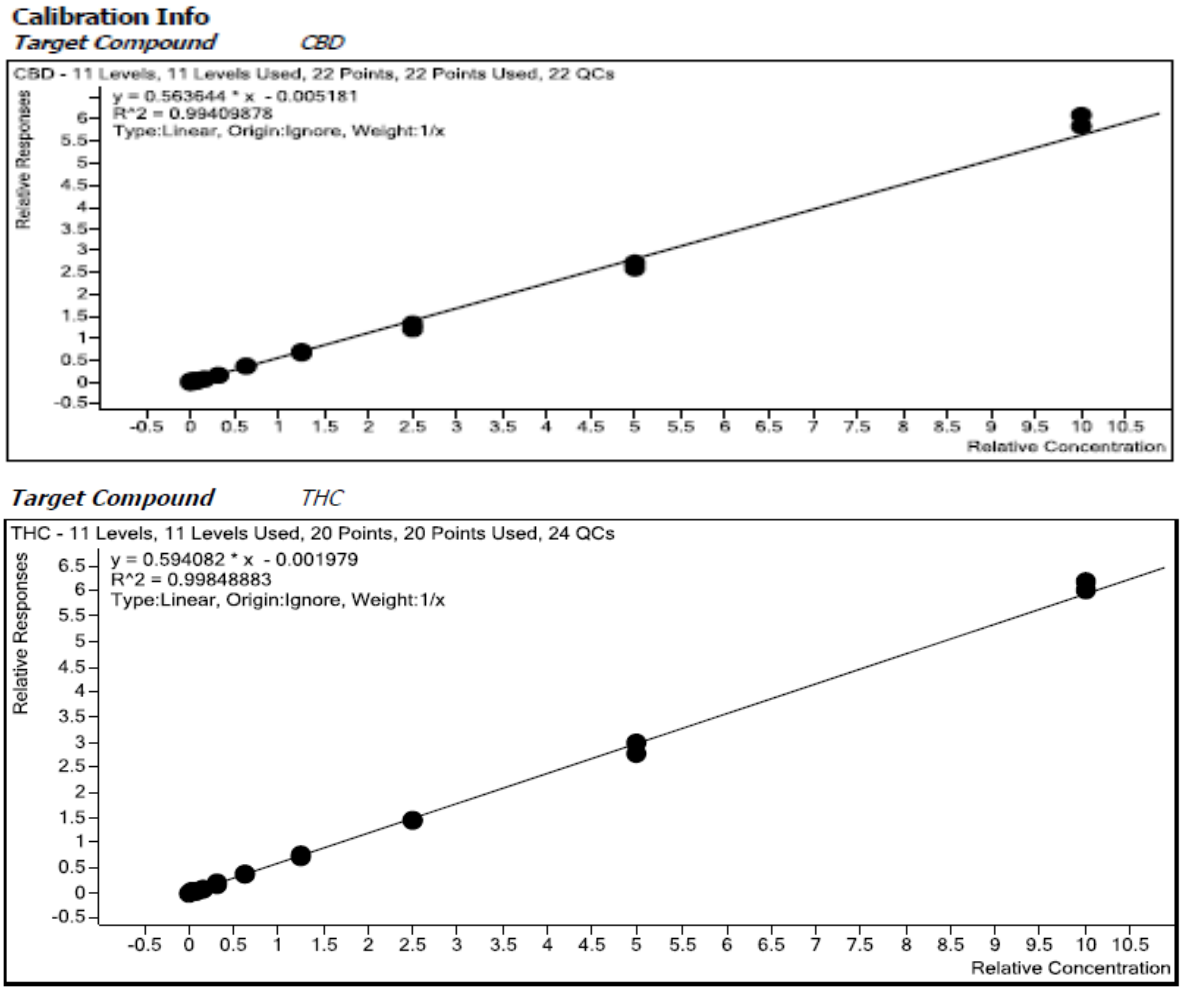


Fig. A.15 Chromatogram and mass spectra: Blank canine plasma with the internal standard (THC-D3)

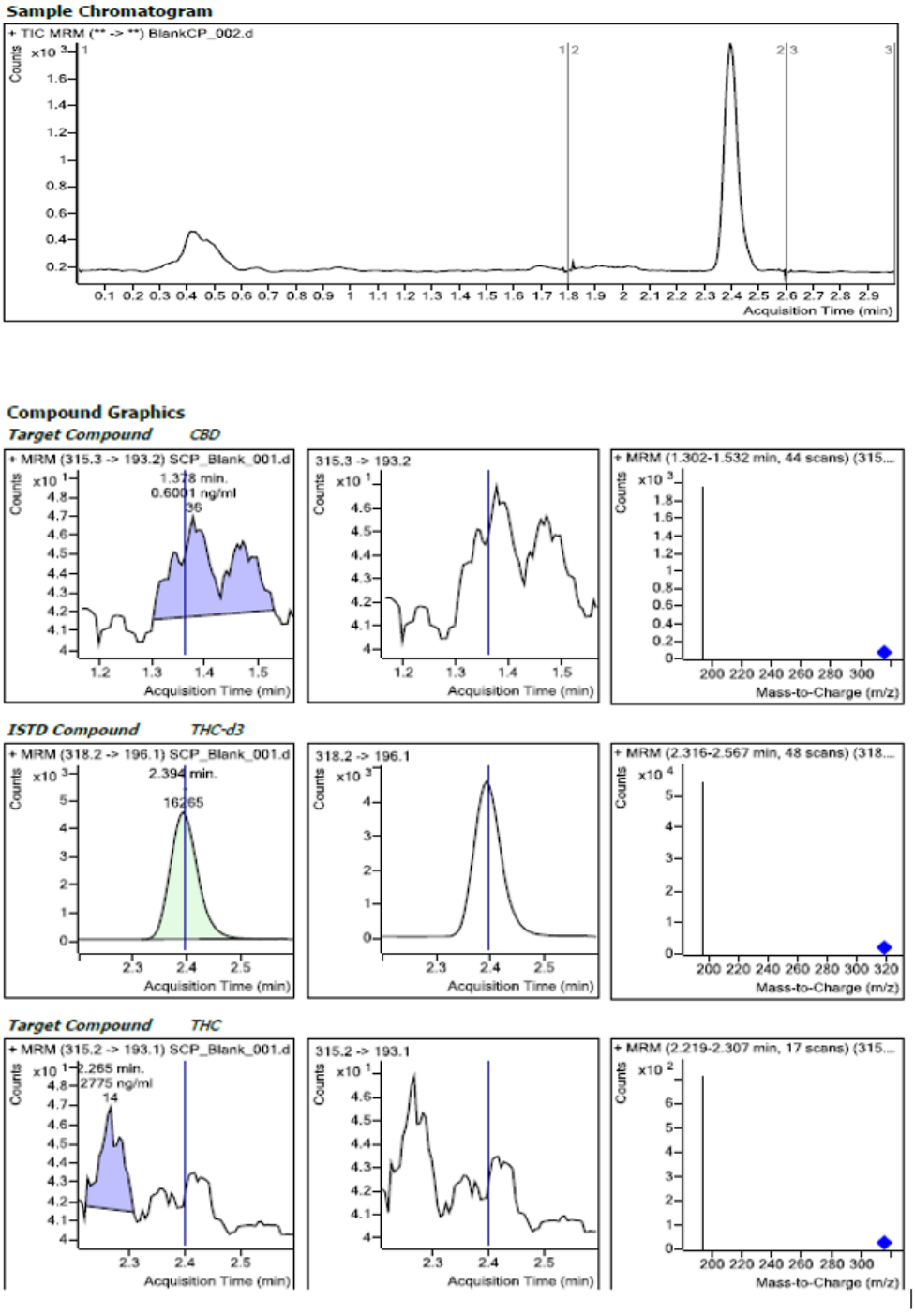
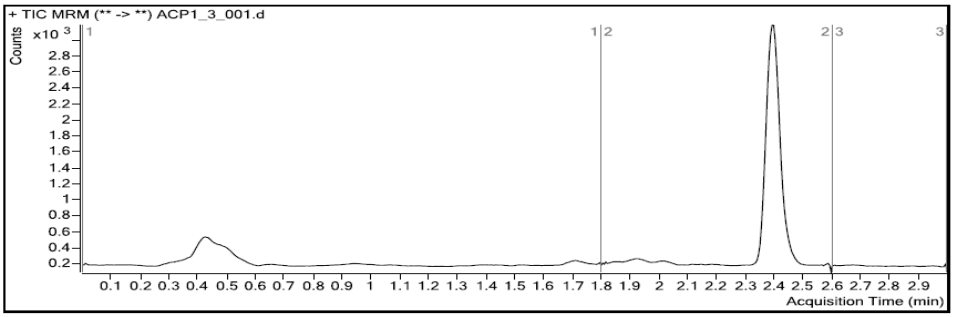


Fig. A.16 Chromatogram and MS spectra for the accuracy sample: 1.95 ng/mL (CBD and THC)

Analysis Info

Acq Time	2016-06-07 22:22	**Data File**	ACP1_3_001.d
Position	P1-E6	**Sample Name**	Sample39
Dilution	1	**Sample Info**	
Inj Vol	-1	**Acq Method File**	Cannabinoids.m
Sample Type	QC	**Comment**	

Sample Chromatogram

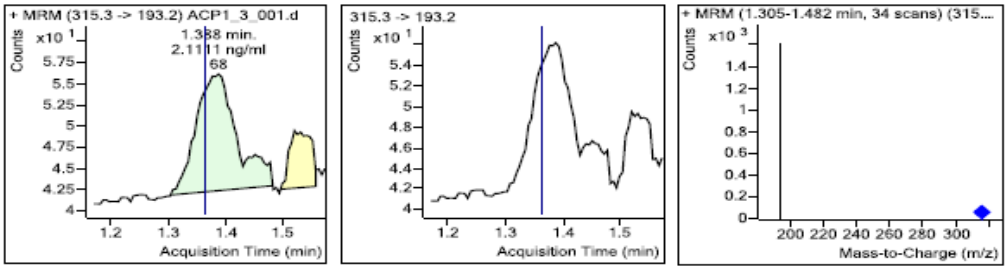


Quantitation Results

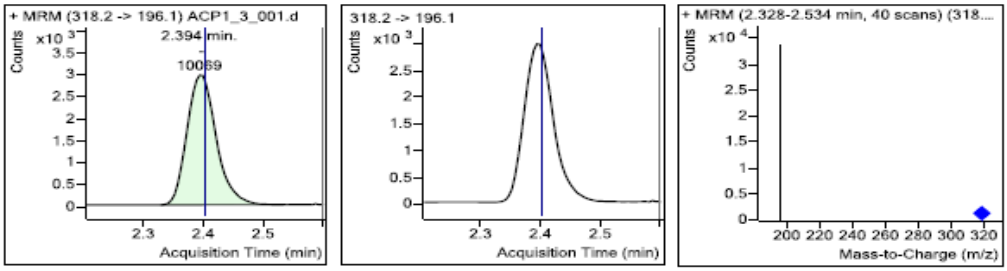
Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBD	THC-d3	1.388	68	10069	0.0067	2.1111	108.09
THC	THC-d3	2.407	120	10069	0.0119	2.3397	119.79

Compound Graphics

Target Compound ***CBD***



ISTD Compound ***THC-d3***



Target Compound ***THC***

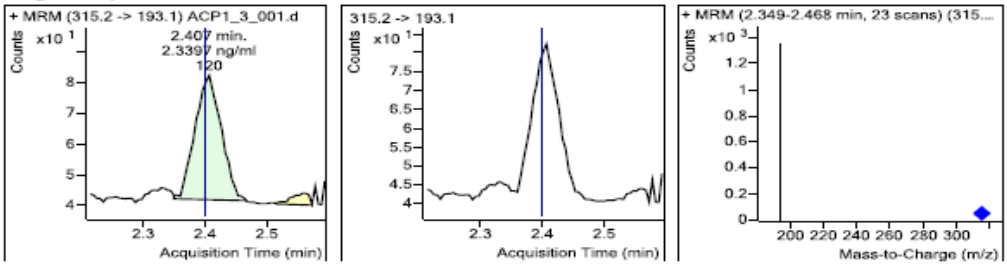
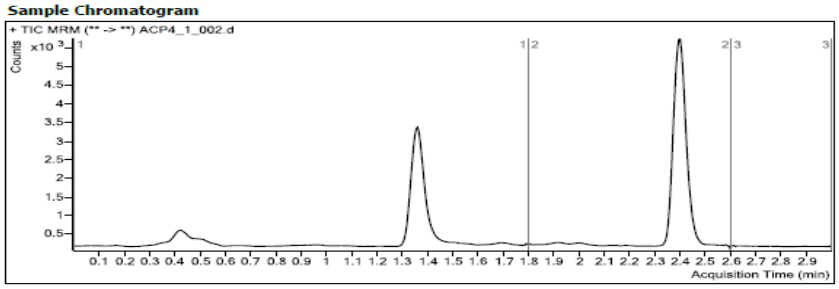


Fig. A.17 Chromatogram and MS spectra for the accuracy sample: 250 ng/mL (CBD and THC)

Analysis Info

Acq Time	2016-06-07 23:35	**Data File**	ACP4_1_002.d
Position	P1-F4	**Sample Name**	Sample46
Dilution	1	**Sample Info**	
Inj Vol	-1	**Acq Method File**	Cannabinoids.m
Sample Type	QC	**Comment**	

Sample Chromatogram

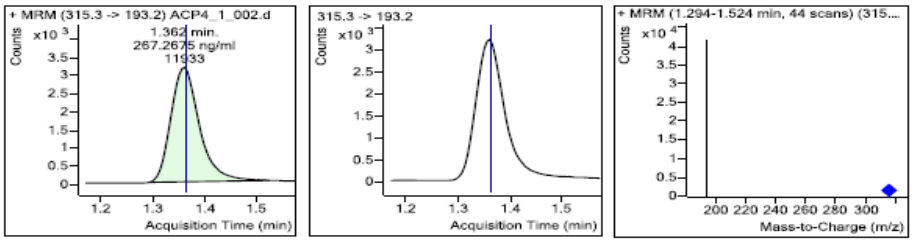


Quantitation Results

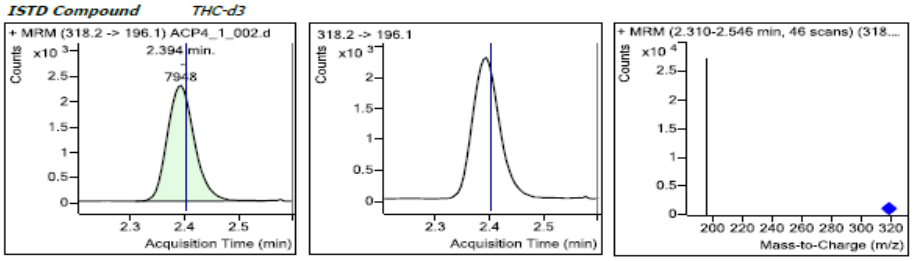
Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBD	THC-d3	1.362	11933	7948	1.5013	267.2675	106.91
THC	THC-d3	2.401	11954	7948	1.5040	253.4925	101.40

Compound Graphics

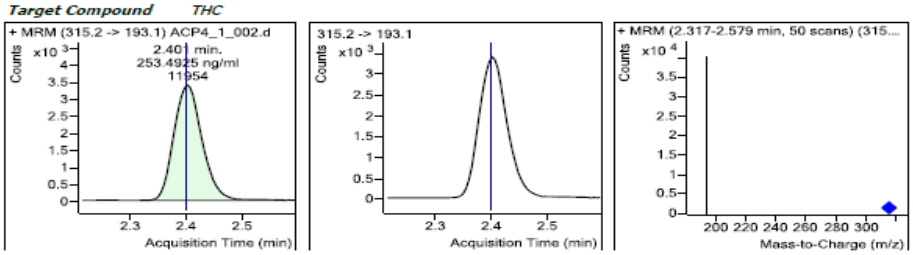
Target Compound ***CBD***



ISTD Compound ***THC-d3***



Target Compound ***THC***



Repeatability (Inter-assay precision)

Fig. A.18 Cannabinoids calibration curve in canine plasma used for repeatability (CBD and THC)

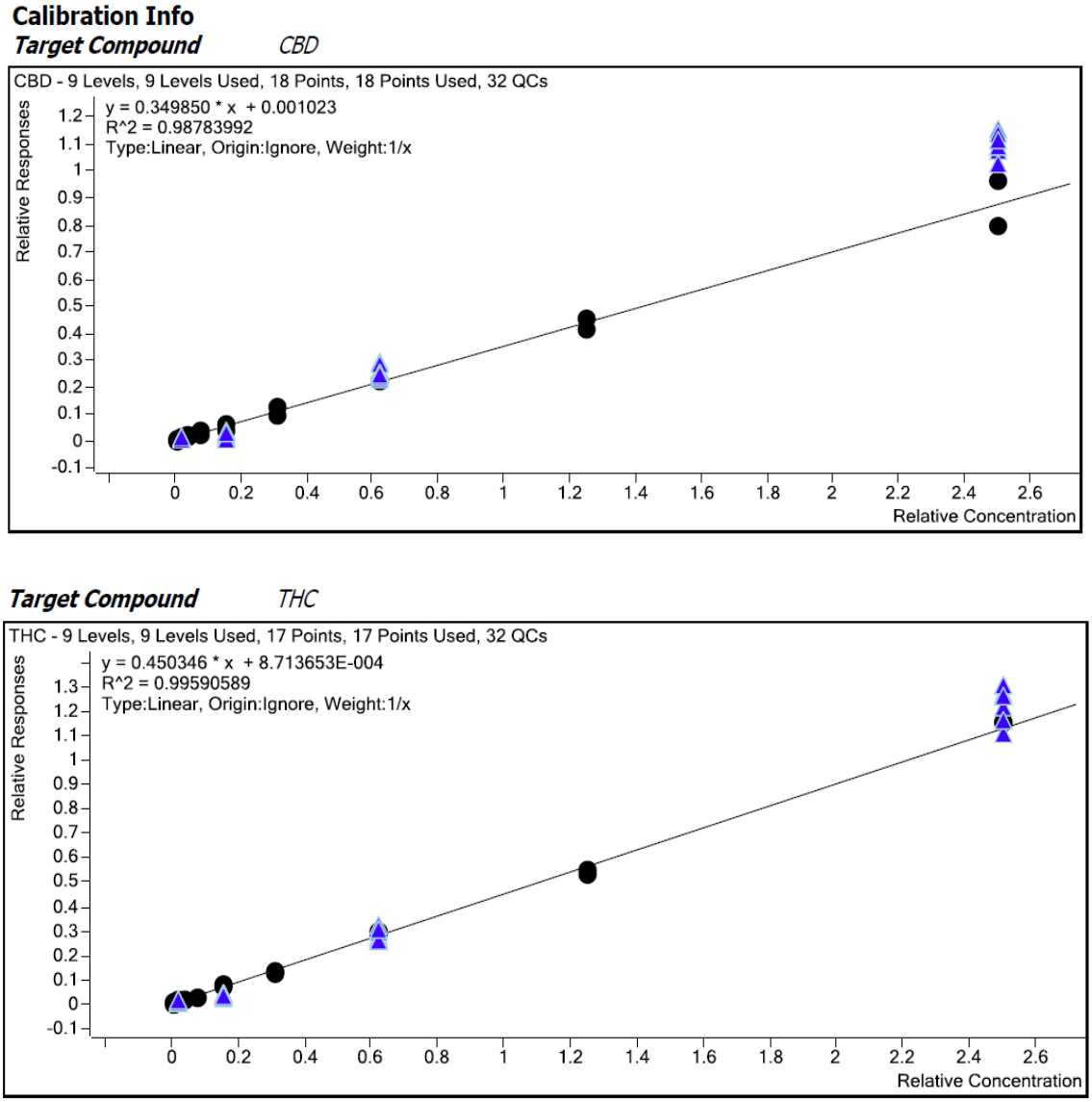


Fig. A.19 Chromatogram and mass spectra: Blank canine plasma with the internal standard (THC-D3)

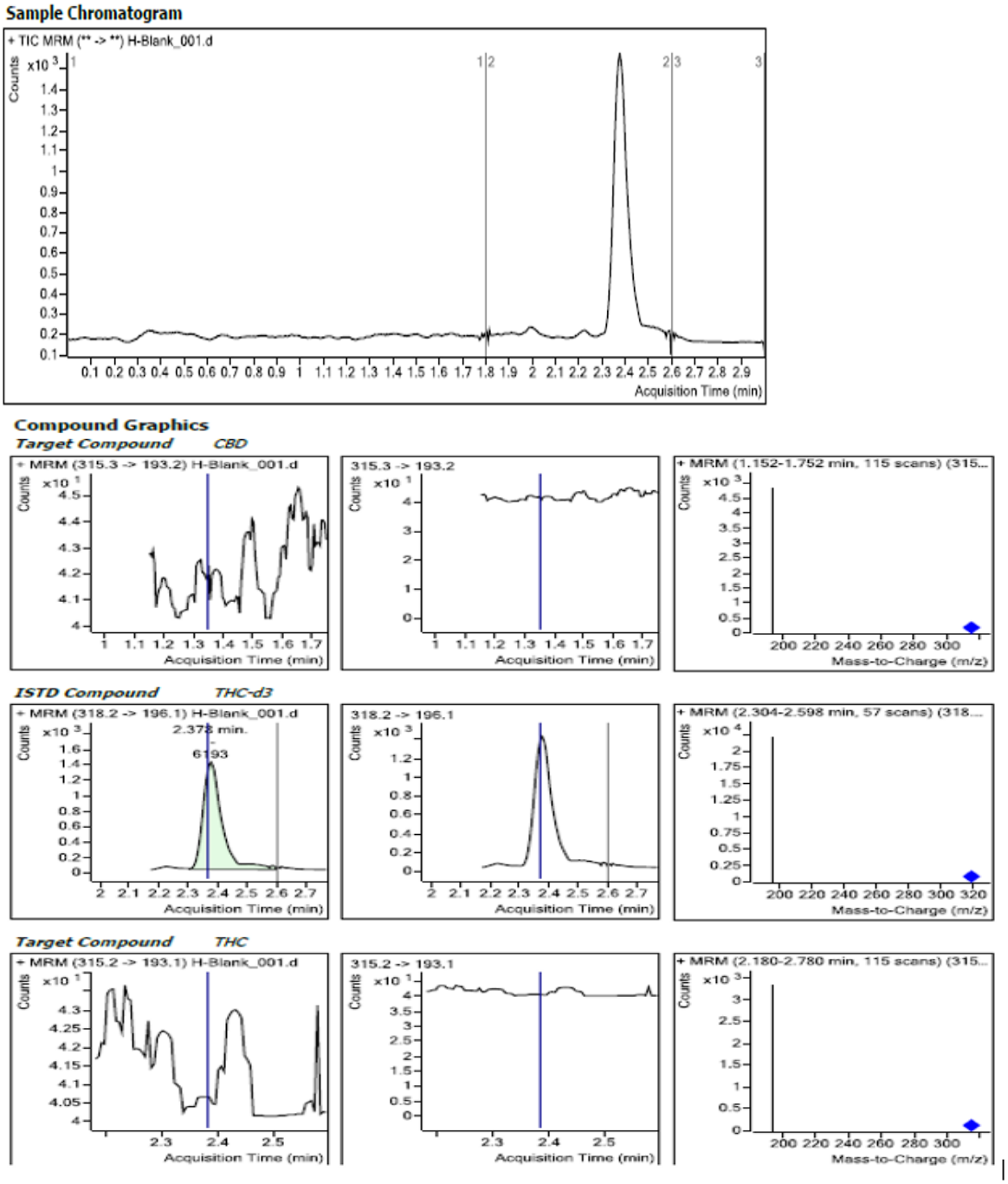


Fig. A.20 Chromatogram and MS spectra for the inter-assay precision sample: 1.95 ng/mL (CBD and THC)

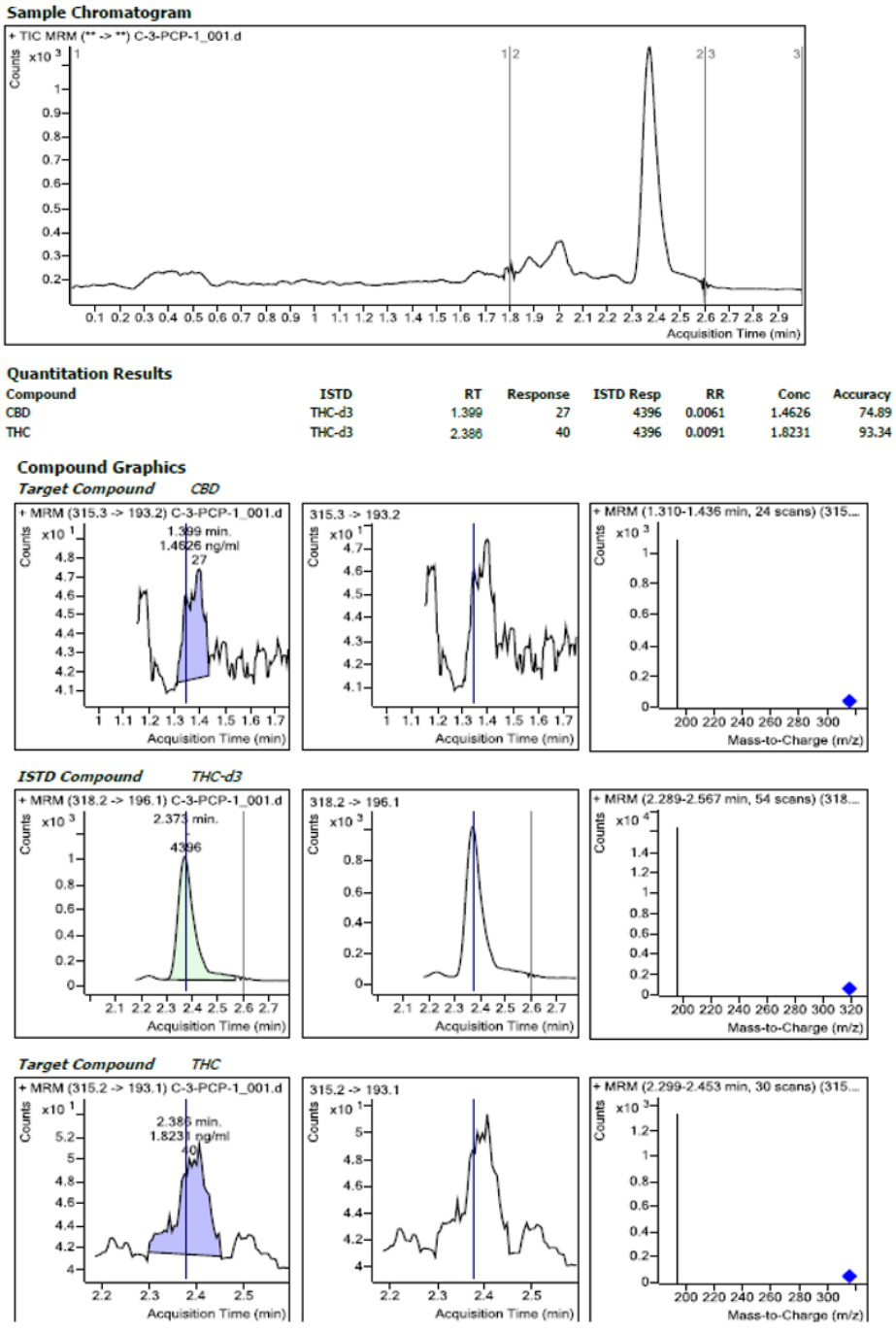
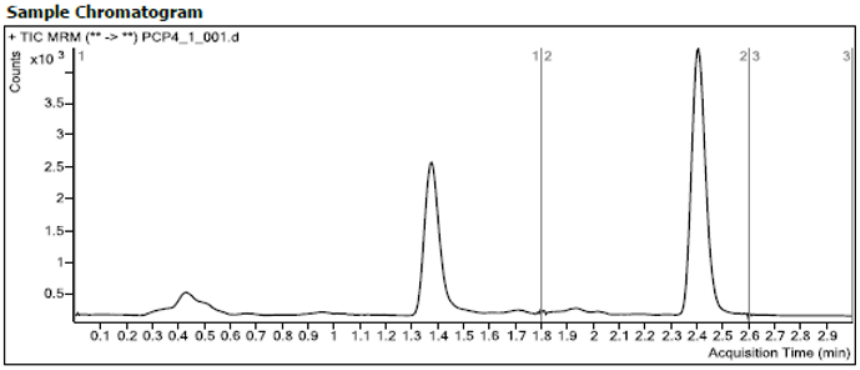


Fig. A.21 Chromatogram and MS spectra for the inter-assay precision sample: 250 ng/mL (CBD and THC)

Sample Chromatogram

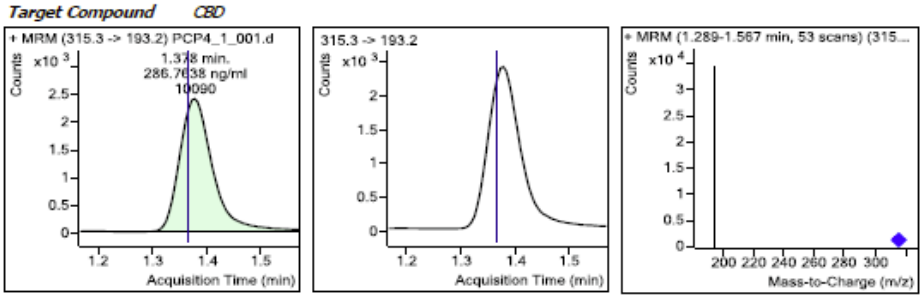


Quantitation Results

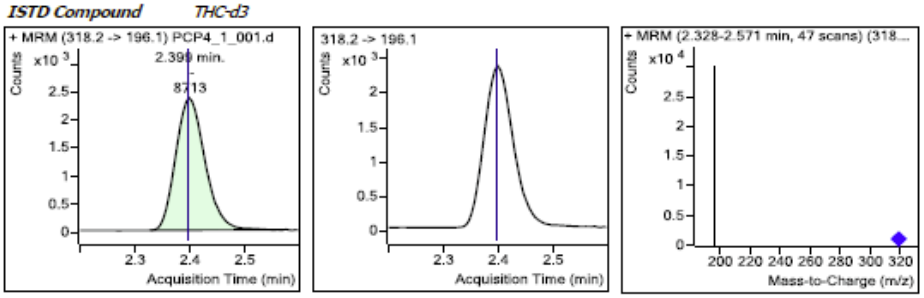
Compound	ISTD	RT	Response	ISTD Resp	RR	Conc
CBD	THC-d3	1.378	10090	8713	1.1580	286.7638
THC	THC-d3	2.412	7171	8713	0.8230	243.6254

Compound Graphics

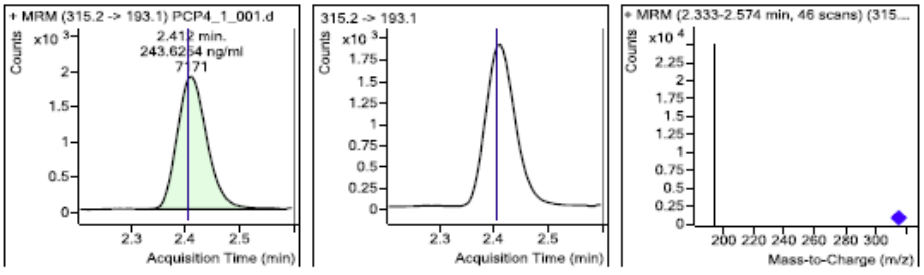
Target Compound *CBD*



ISTD Compound *THC-d3*



Target Compound *THC*



Intermediate Precision

Fig. A.22 (a) Cannabinoids calibration curve in canine plasma used for intermediate precision (CBD and THC)

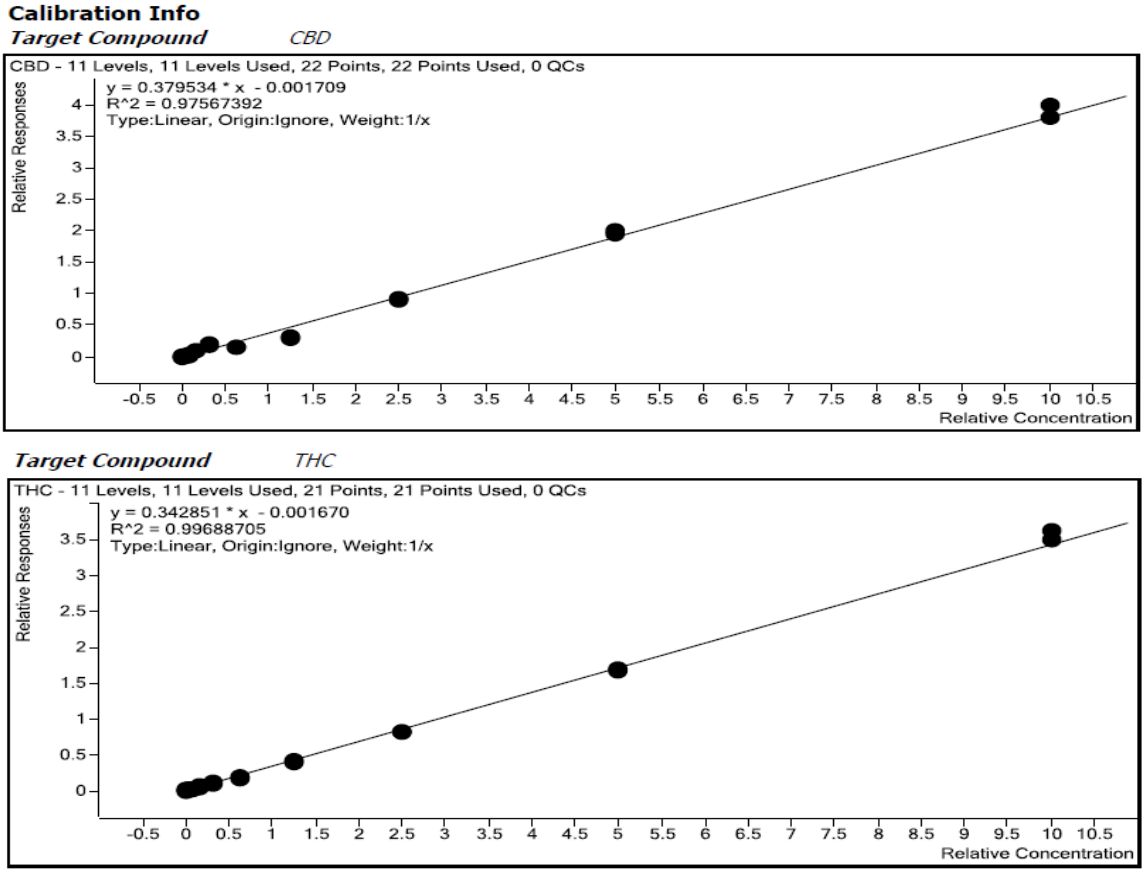


Fig. A.22 (b) Chromatogram and mass spectra: Blank canine plasma with the internal standard (THC-D3)

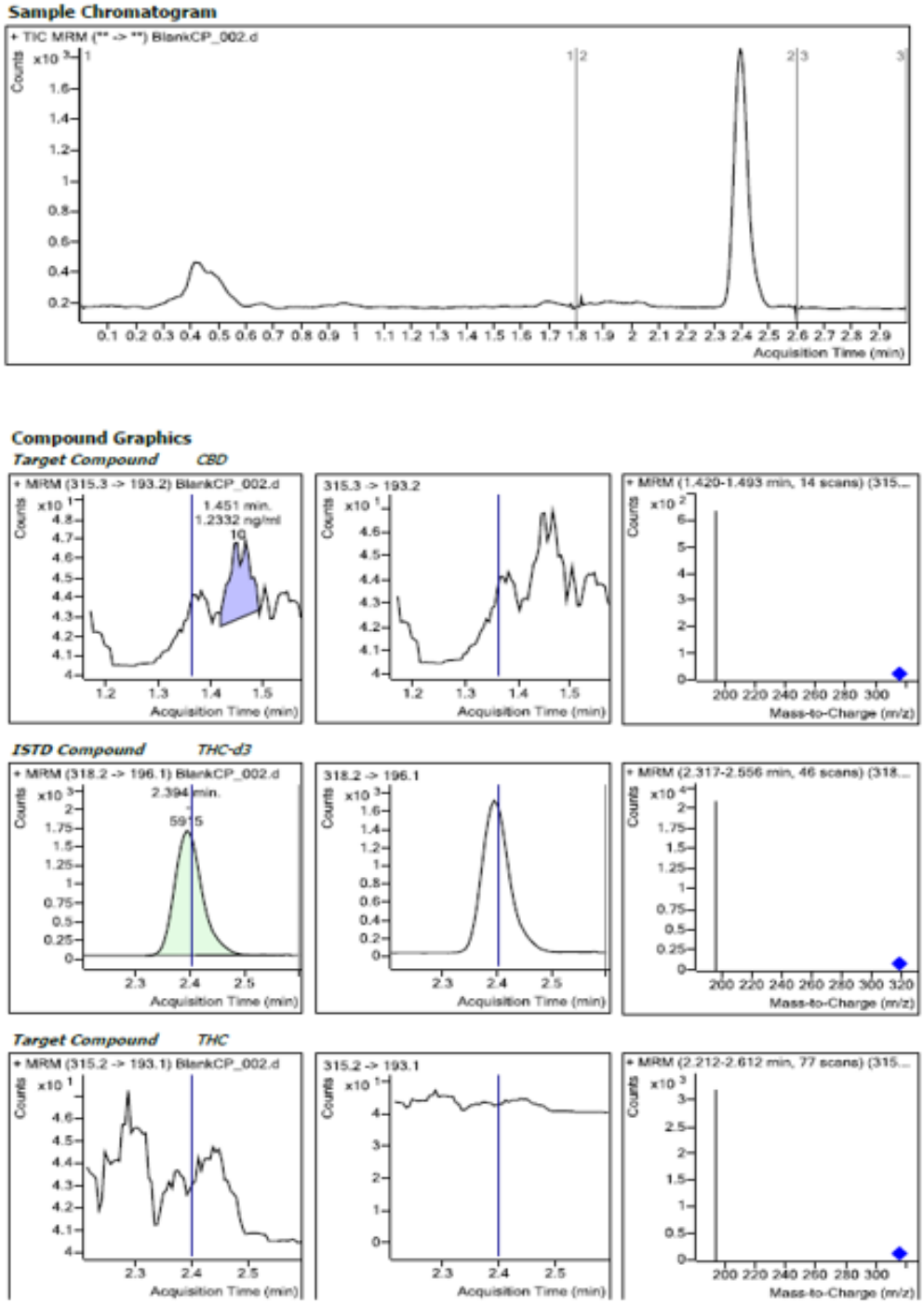
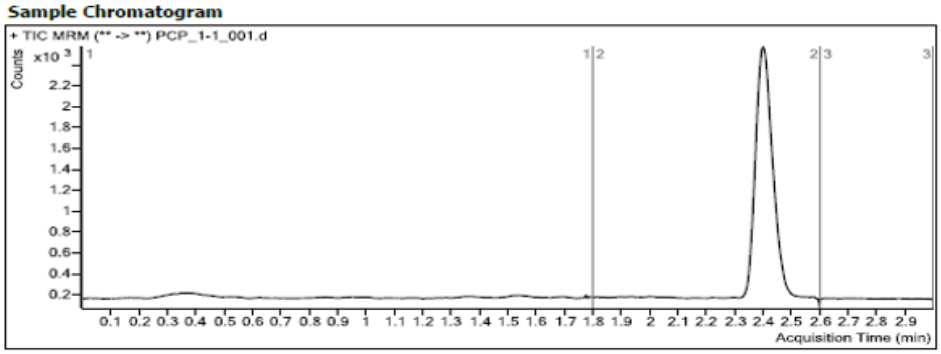


Fig. A.23 Chromatogram and MS spectra for the intermediate precision sample: 1.95 ng/mL (CBD and THC)

Sample Chromatogram



Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc
CBD	THC-d3	1.357	59	10157	0.0059	1.9927
THC	THC-d3	2.391	58	10157	0.0057	2.1402

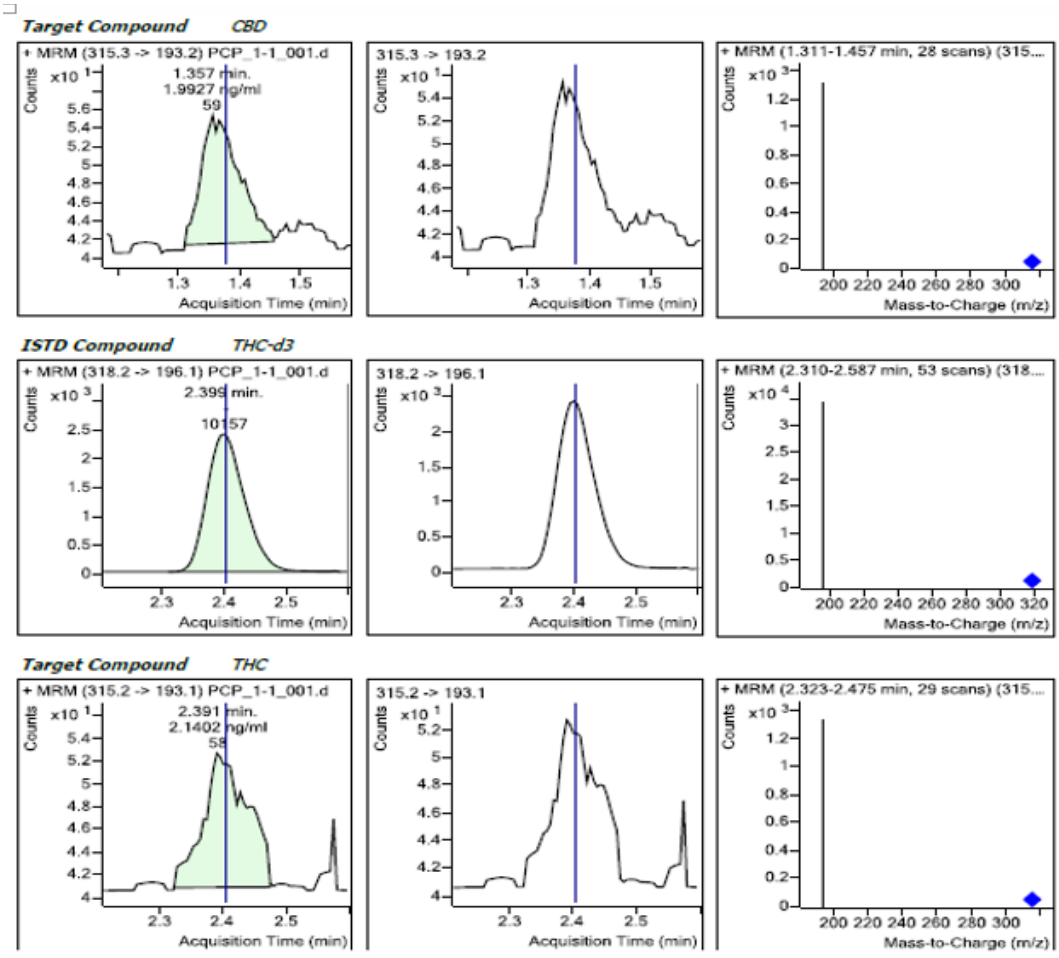
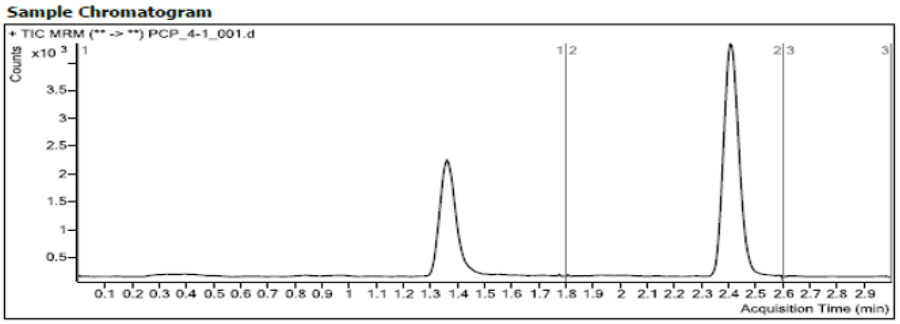
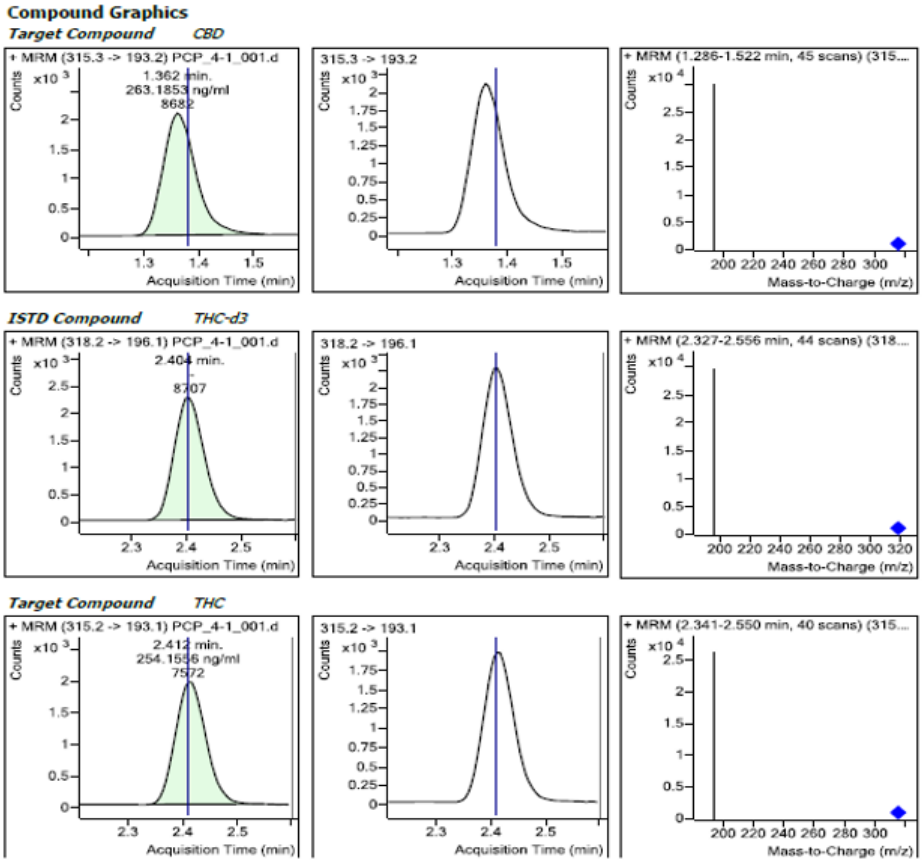


Fig. A.24 Chromatogram and MS spectra for the intermediate precision sample: 250 ng/mL (CBD and THC)



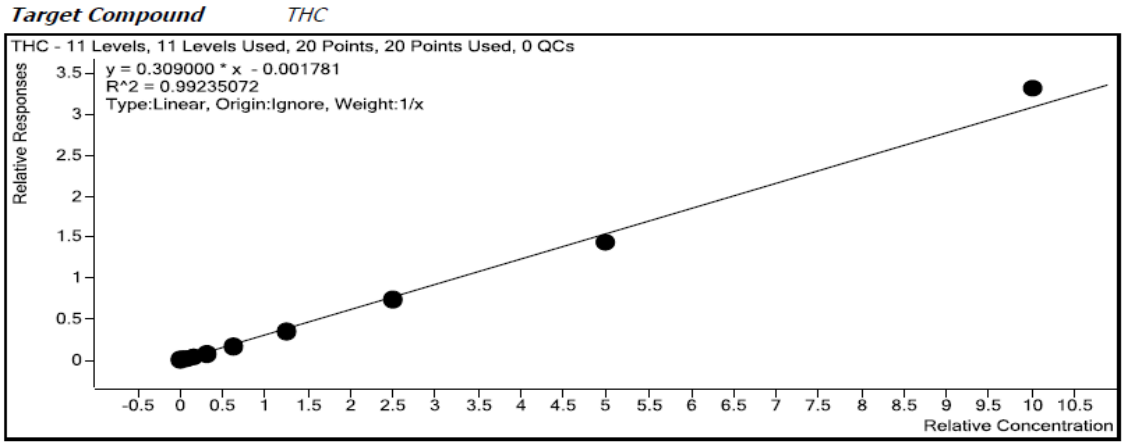
Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc
CBD	THC-d3	1.362	8682	8707	0.9972	263.1853
THC	THC-d3	2.412	7572	8707	0.8697	254.1556



Robustness

Fig. A.25 Cannabinoids calibration curve in canine plasma used for robustness (CBD and THC)



Calibration Info

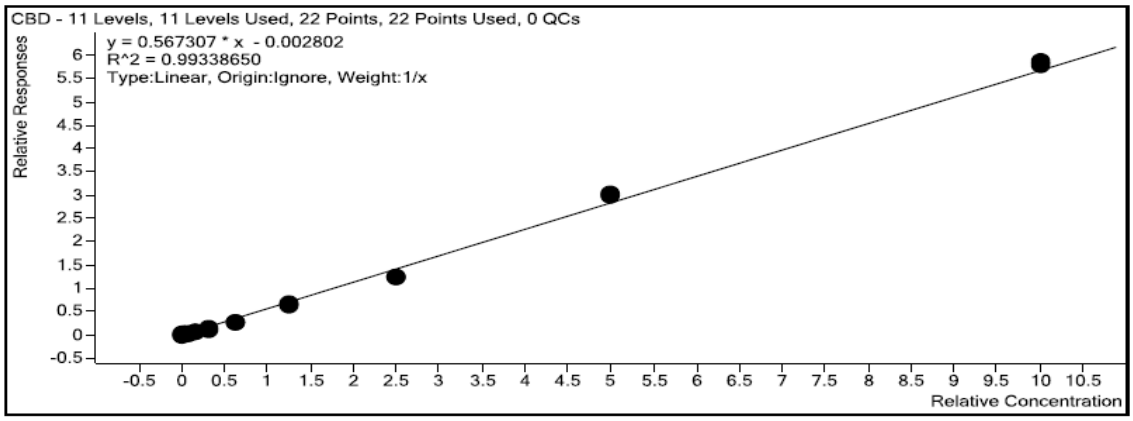


Fig. A.26 Chromatogram and mass spectra: Blank canine plasma with the internal standard (THC-D3)

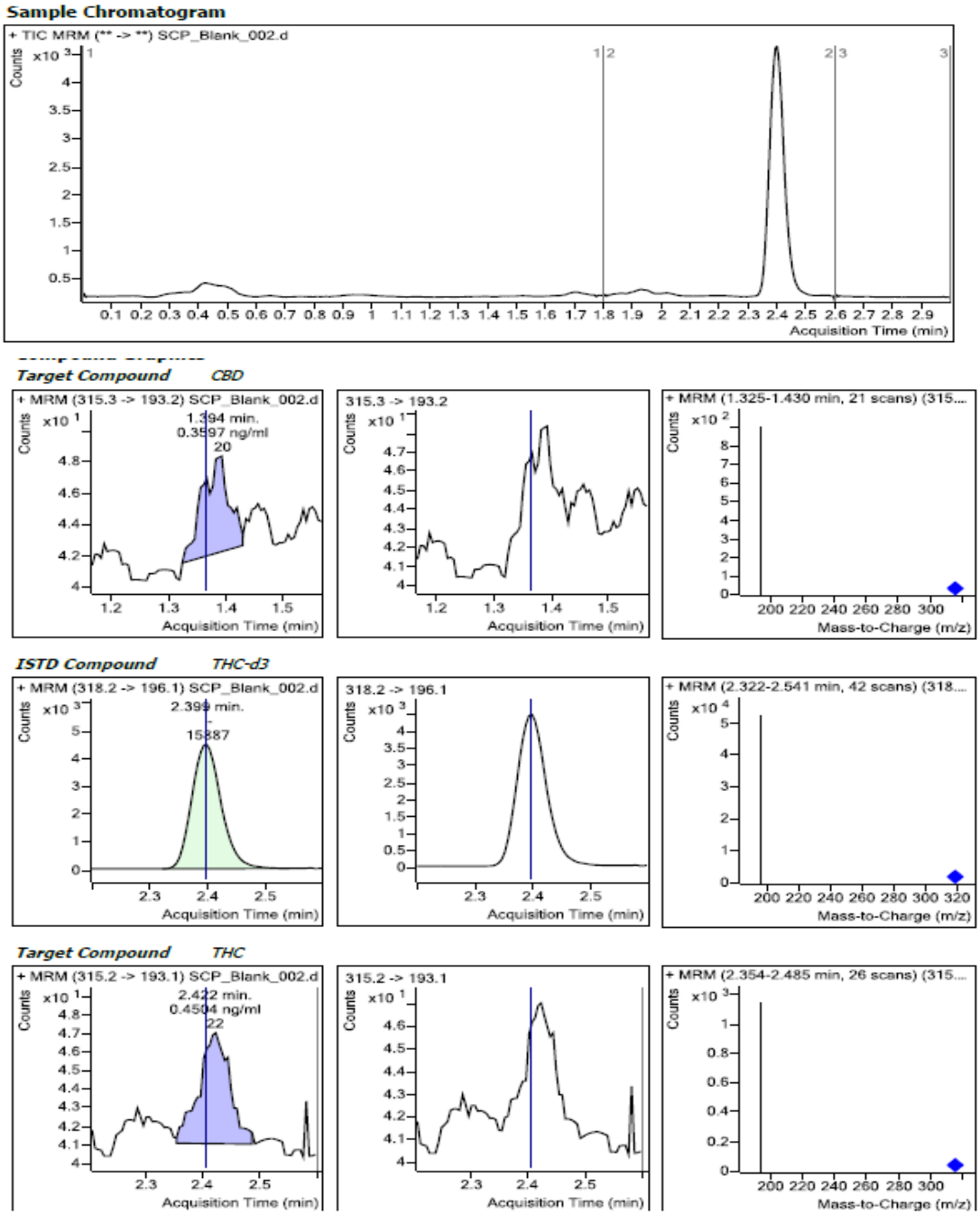
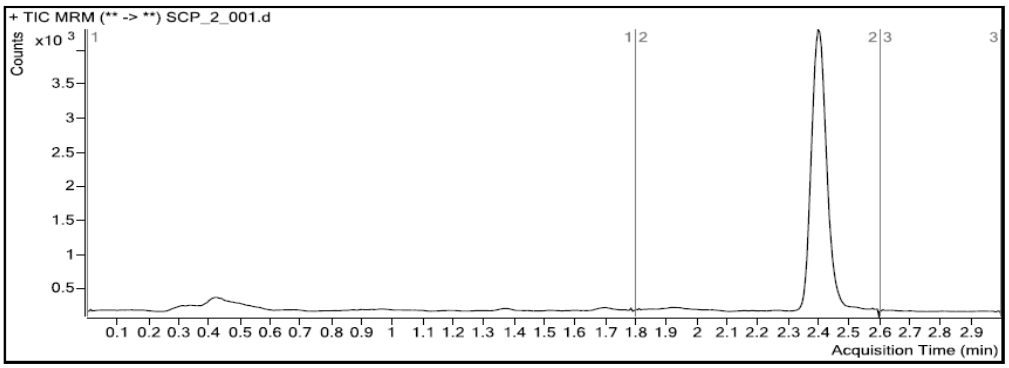


Fig. A.27 Chromatogram and MS spectra for the robustness sample: 1.95 ng/mL (CBD and THC)

Sample Chromatogram

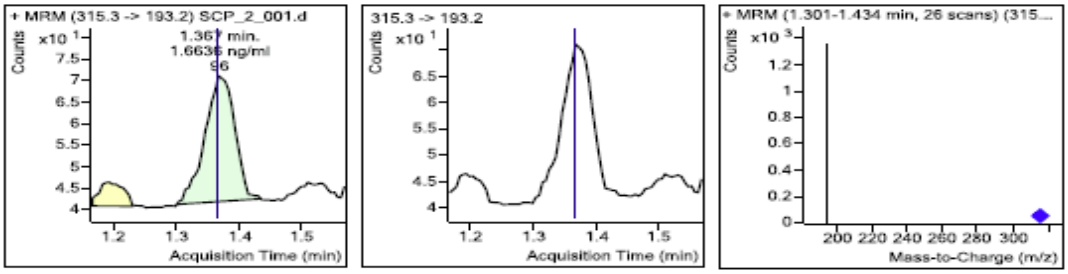


Quantitation Results

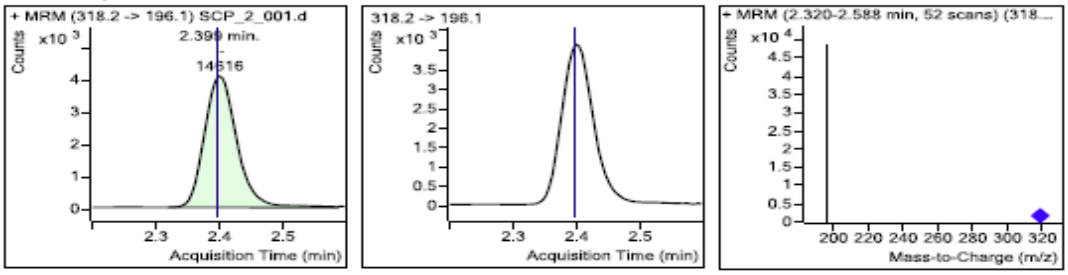
Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
CBD	THC-d3	1.367	96	14616	0.0065	1.6636	85.18
THC	THC-d3	2.401	90	14616	0.0061	1.8456	94.50

Compound Graphics

Target Compound *CBD*



ISTD Compound *THC-d3*



Target Compound *THC*

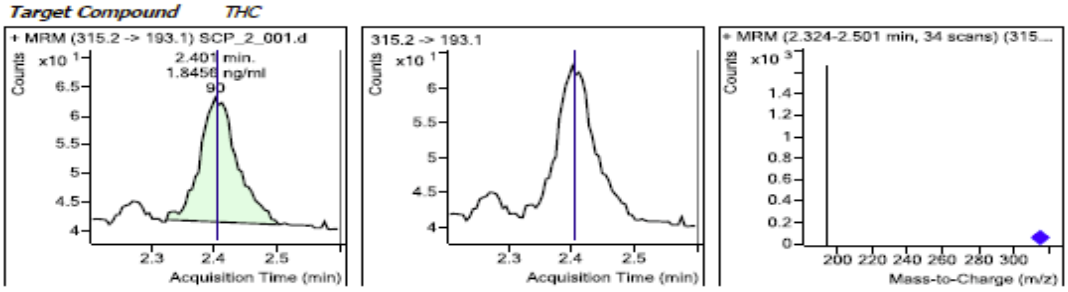
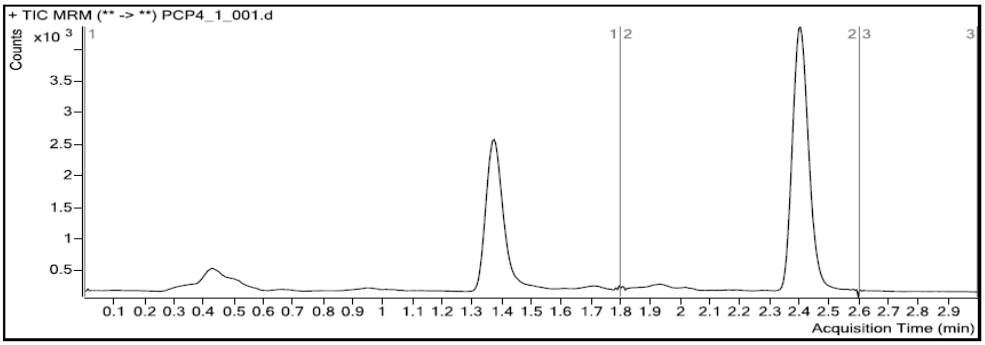


Fig. A.28 Chromatogram and MS spectra for the robustness sample: 250 ng/mL (CBD and THC)

Sample Chromatogram

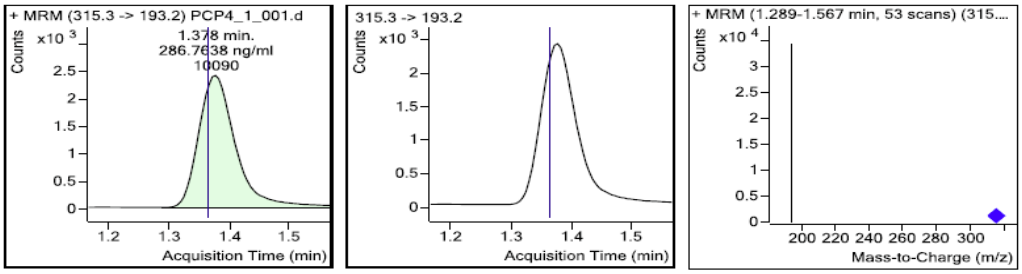


Quantitation Results

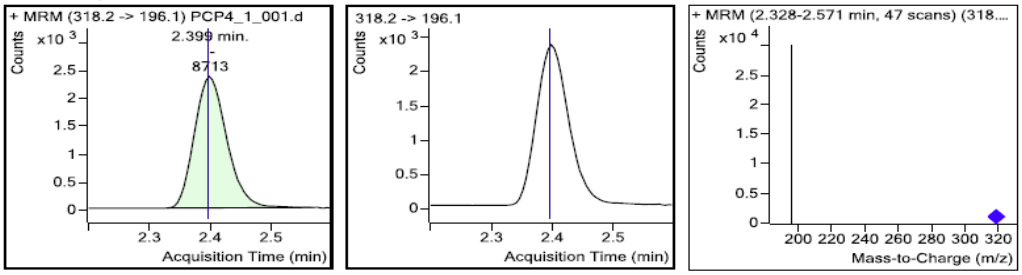
Compound	ISTD	RT	Response	ISTD Resp	RR	Conc
CBD	THC-d3	1.378	10090	8713	1.1580	286.7638
THC	THC-d3	2.412	7171	8713	0.8230	243.6254

Compound Graphics

Target Compound *CBD*



ISTD Compound *THC-d3*



Target Compound *THC*