# ASPECTS OF CANNABIS IN THE LABORATORY

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**Abstract**

There is a need for accredited methods for determination of the amount of $\Delta^9$-tetrahydrocannabinol (THC) in cannabidiol (CBD) products as a result of a consideration being discussed at the United Nation Narcotics Board in respect to the reclassification of CBD as a narcotic limiting the amount of THC present in CBD products to less than 0.2%-0.3% (w/w).

The objective of this study was the development and validation of a simple, rapid and effective High-Performance Liquid Chromatography (HPLC) method for the determination and quantification of THC in CBD oil.

The method was divided in two parts: [1] Determination of conditions for extraction of THC from CBD oil and [2] Development and validation of method of analysis to determine THC in CBD oil. The best conditions for extraction of THC from oil were determined by changing three different ratios of solvent to THC in oil (0.3mL MeOH and 0.3mL 5 µg/mL THC in oil, 0.3mL MeOH and 0.6mL 5 µg/mL THC in oil and 0.6mL MeOH and 0.3 mL 5 µg/mL THC in oil), sonication times (15 and 20 minutes), centrifugation force (4500, 6000 and 10000 rpm), time (15 and 25 minutes) and temperature (4ºC and -20ºC). Analysis was conducted using an Agilent 1260 Infinity Series® II HPLC unit with ultra-violet (UV) detection at 220 nm. Separation was carried out on an ACE 5µm $C_{18}$ Column (250 x 4.6 mm) using a mobile phase composed of acetonitrile and pH 2.5 phosphate buffer (70:30 v/v) at a flow rate of 2 mL/min at temperature of 40ºC using ibuprofen as the internal standard. Areas under the peak (AUP) and retention times (RT) were compared for each scenario. All the runs were carried out in triplicates.

The results obtained indicated that the best conditions for extraction of THC from CBD oil were 0.6mL MeOH and 0.3 mL 2.5 µg/mL THC in oil, 20 minutes of sonication, 15

minutes at 6000rpm of centrifugation and two hours at -20ºC. Retention time of Ibuprofen was 2.85 minutes and retention time of THC was 12.72 minutes.

The developed method is innovative, quick, and easy to use and can determine THC in oil with good peak shape and resolution. Application of the analytical method will help in the determination of THC in CBD oil for medicinal use.

**Keywords:** cannabis, cannabinoids, high performance liquid chromatography, cannabidiol, $\Delta^9$-tetrahydrocannabinol.

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## List of Abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| AUP | Area Under the Peak |
| CBC | Cannabichromenic Acid |
| CBCAS | Cannabichromenic Acid Synthase |
| CBD | Cannabidiol |
| CBDA | Cannabidiolic Acid |
| CBDAS | Cannabidiolic Acid Synthase |
| CBGA | Cannabigerolic Acid |
| CBN | Cannabinol |
| DAD | Diode-Array |
| DM | Dynamic Maceration |
| DRE | Drug-Resistant Epilepsy |
| ECS | Endocannabinoid system |
| ESI | Electrospray ionization |
| EtOH | Ethanol |
| EVOO | Extra Virgen Olive Oil |
| FDA | Food and Drug Administration |
| FL | Fluorescence |
| FR | Flow Rate |
| GC | Gas Chromatography |
| GPP | Geranyl-Pyrophosphate |
| HPLC | High-Performance Liquid Chromatography |
| HPTLC | High Performance Thin Layer Chromatography |
| IBD | Inflammatory Bowel Disease |

| | |
|---|---|
| ICH | International Council for Harmonisation |
| IV | Injection Volume |
| LLE | Liquid Liquid Extraction |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| MAE | Microwave-Assisted Extraction |
| MCT | Medium Chain Triglycerides |
| MeOH | Methanol |
| MS | Mass Spectrometry |
| NMR | Nuclear Magnetic Resonance |
| OLA | Olivetolic Acid |
| PP | Protein precipitation |
| RP | Reverse Phase |
| RSD | Relative Standard Deviation |
| SALLE | Salting-out Assisted Liquid-Liquid Extraction |
| Sc $CO_2$ | Supercritical Carbon Dioxide |
| SFE | Supercritical fluid extraction |
| SPE | Solid Phase Extraction |
| THC | $\Delta^9$-tetrahydrocannabinol |
| THCA | $\Delta^9$-tetrahydrocannabinolic Acid |
| THCAS | $\Delta^9$-tetrahydrocannabinolic Acid Synthase |
| THF | Tetrahydrofuran |
| TWIM | Travelling Wave Ion Mobility |
| UAE | Ultrasound-Assisted Extraction |
| UHPLC | Ultra High-Performance Liquid Chromatography |

| | |
|---|---|
| UV | Ultraviolet |
| WHO | World Health Organization |

# Chapter 1 Introduction

## 1.1 Background

Cannabis belongs to the plant family Cannabacea (Baron, 2015) and is recommended in the management of epilepsy, cancer and pain (Elkins, et al 2019). Cannabis has medicinal properties which are attributed to a group of compounds known as cannabinoids (Jamwal, 2017). The main cannabinoids having medicinal properties are $\Delta^9$-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) (Zgair et al, 2015).

Analytical techniques used for the determination and separation of cannabinoids include gas chromatography (GC) and high-performance liquid chromatography (HPLC) (Wang et al, 2017). Analysis of cannabinoids using GC can be difficult due to need of chemical derivatization.[1] Analysis of cannabinoids using HPLC allows for determination of both neutral and acidic forms of cannabinoids without the need for derivatization (Mandrioli et al, 2019). Different kind of detectors can be coupled to HPLC: ultraviolet (UV), fluorescence (FL) or mass spectrometry (MS) detector (Vella et al, 2015). Although coupling the HPLC system with MS detectors can offer excellent selectivity and sensitivity of the analysis (Elkins et al, 2019), it requires expensive instrumentation and skilled expertise (Vella et al, 2015). FL detector coupled to an HPLC system cannot be used to analyse CBN because CBN does not possess a fluorophore (Citti et al, 2018). HPLC-UV is a common method chosen for analysis of cannabinoids as it is more convenient and economic than other methods of analysis.[2]

[1] Zivovinovic S, Alder R, Allenspach MD, Steuer C. Determination of cannabinoids in Cannabis sativa L. samples for recreational, medical, and forensic purposes by reversed-phase liquid chromatography-ultraviolet detection. Journal of Analytical Science and Technology [Internet]. 2018 [cited 2010 Jan 19]. Available from: https://jast-journal.springeropen.com/articles/10.1186/s40543-018-0159-8

[2] He Q, Li M, Wang X, Xia Z, Du Y, Li Y, et al. A simple, efficient and rapid HPLC–UV method for the detection of 5-HT in RIN-14B cell extract and cell culture medium. BMC Chemistry [Internet]. 2019[cited 2020 May 18]. Available from: https://link.springer.com/article/10.1186/s13065-019-0591-

## 1.2 Cannabis

*Cannabis sativa* is a dioecious annual flowering plant (Farag and Kayser, 2017) that was mainly cultivated in China and India (Russo et al, 2008). The medicinal use of *Cannabis sativa* dates around twenty-eighth century B.C., when Shen Nung who was considered the "Father of Chinese Medicine" drew up the *Pen Ts'ao*, the first Chinese pharmacopeia (Abel, 1980). *Cannabis sativa* was prescribed for menstrual fatigue, gout, rheumatism, malaria, beriberi, constipation and absentmindedness (Abel, 1980). Chinese physicians recommended hempseeds, rich in polyunsaturated fatty acids, of *Cannabis sativa* for treatment of atopic dermatitis, inflammation and rheumatoid arthritis (Jeong et al, 2014). Dioscorides, a Greek physician, wrote *De Materia Medica* recommending the use of *Cannabis sativa* juice for earache, around the fifth century B.C. (Carod-Artal, 2013; Greydanus, 2014). In the last century B.C, *Cannabis sativa* was used by women in Egypt to improve their mood and mitigate pain (Bonini et al, 2018). In the first century A.C. the Roman historian Pliny the Elder recommended the decoction of roots of *Cannabis sativa* for reducing stiffness in joints, gout, and pain (Ryz et al, 2017). *Cannabis sativa* was used in different countries to treat convulsions, to alleviate toothache and to treat fevers (Benet, 1975). In the mid-eighteenth century, Carl Linnaeus, a Swedish botanist, drew up the Species Plantarum naming *Cannabis sativa* for the first time and categorized hemp as *Cannabis sativa* (Benet, 1975; Bonini et al 2018). In 1785, Jean Lamarck wrote the *Encyclopédique de Botanique I*, where he differentiated between *Cannabis sativa* for the plant in Europe and *Cannabis indica* for the plant in India (Small and Cronquist, 1976). In the nineteenth century, cannabis was reported to have anti-convulsant, anti-inflammatory and anti-emetic properties (O'Shaughnessy 1843). In Portugal, effects of cannabis such as stimulation of appetite, hallucinations, euphoria, aphrodisiac effects and sedation were reported (Lee, 2012). In the first decades of the twentieth century, the three

main medicinal uses of *Cannabis sativa*, were sedative or hypnotic, analgesic and for stimulation of appetite (Zuardi, 2006). In the mid-twentieth century, the use of Cannabis was prohibited in USA and Europe due to its psychoactive effects (Miller, 2017). The World Health Organization (WHO) affirmed that Cannabis causes dependence (Lister, 1969). In 1961 the United Nations Single Convention on Narcotic Drugs, and in 1971 the Convention of Psychotropic Substances classified cannabis as a Schedule I drug and its use was restricted for medical and research purposes (Nutt, 2015). The cannabinoid receptors were discovered in 1988 and the endogenous cannabinoid anandamide was discovered in 1992 (Russo, 2007). In 1996, California was the first state which legalized the medical use of cannabis. In the 21st century, the medicinal use of cannabis remains controversial (Greydanus et al, 2015). In June of 2018 the WHO's Expert Committee on Drug Dependence met for reviewing Cannabis for medicinal use (Welling et al, 2019). During these past two decades countries approved the use of Cannabis for medicinal use. In 2018, Malta authorized the *Production of Cannabis for Medicinal and Research Purposes Act* which sets out legislative measures to allow production of cannabis for medicinal and research purposes. This law acts in accordance with the amendment of the *Drug Dependence Act* regarding the prescribing of medicinal cannabis' preparations.[3]

### 1.3 Cannabinoids

Cannabis has a number of medicinally important compounds, such as cannabinoids, terpenoids, flavonoids and alkaloids (Andre et al, 2016). Cannabinoids are the most studied compounds of the Cannabis plant, and have a wide range of therapeutic effects (Andre et al, 2016). Phytocannabinoids can be classified in two groups based on the presence or absence of a carboxyl group; neutral cannabinoids (without carboxyl group)

[3]Legislation Malta. Production of cannabis for medicinal and research purposes act [Internet] Malta: Valleta;2018[cited 2020 Jun 10]. Available from: https://legislation.mt/eli/cap/578/eng/pdf

and acidic cannabinoids (with carboxyl group) (Hanus et al, 2006). In the Cannabis plant, cannabinoids are biosynthesized and accumulated as their acid form and non-enzymatically decarboxylated as neutral cannabinoids (Sirikantaramas and Taura, 2017). The alkylation of olivetolic acid (OLA) with geranyl-pyrophosphate (GPP) by a synthase enzyme known as olivatolate geranyltransferase, produces cannabigerolic acid (CBGA) the main precursor of different cannabinoids in a carboxylated form (Andre et al, 2016; Bonini et al, 2018; Elkins et al, 2019). CBGA is catalyzed by three oxidocyclases; $\Delta^9$-tetrahydrocannabinolic acid synthase (THCAS) to form $\Delta^9$-tetrahydrocannabinolic acid (THCA); cannabidiolic acid synthase (CBDAS) to form cannabidiolic acid (CBDA) and cannabichromenic acid synthase (CBCAS) to form cannabichromenic acid (CBCA) (Andre et al, 2016; Bonini et al, 2018; Elkins et al, 2019). THCA and CBDA are descarboxylated at high temperatures and in UV light (Zivovinovic et al, 2018). The descarboxylation of THCA produces THC, and the descarboxylation of CBDA produces CBD (Bonini et al, 2018; Zivovinovic et al, 2018). CBN is produced from the oxidative aromatization of THC (Bonini et al, 2018; Zivovinovic et al, 2018) (Figure 1.1).

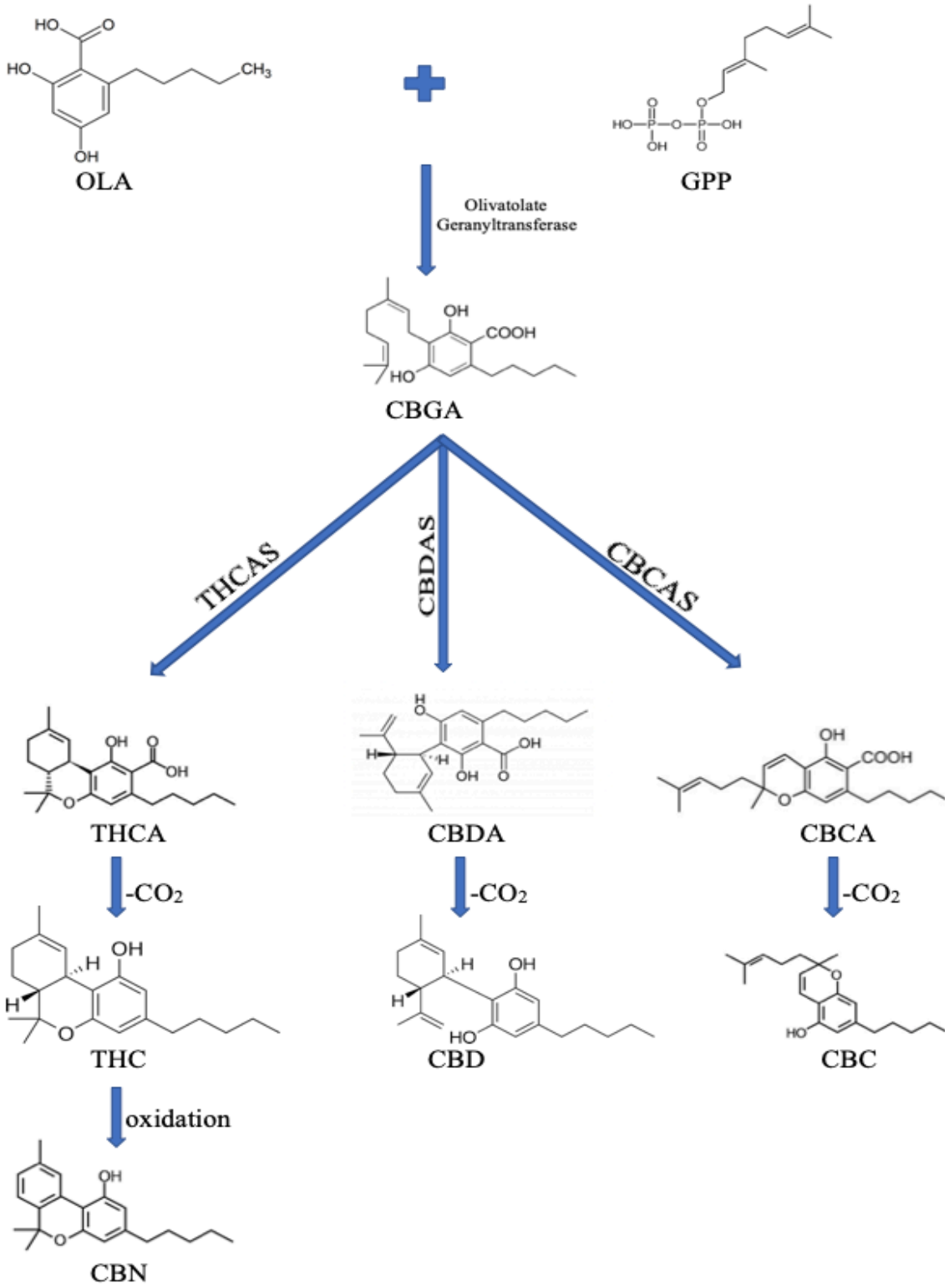


**Figure 1.1: Synthesis of the main cannabinoids**

adopted from Bonini SA, Premoli M, Tambaro S, Kumar A, Maccarinelli G, Memo M, et al. Cannabis sativa: A comprehensive ethnopharmacological review of a medicinal plant with a long history. J.Ethnopharmacol. 2018; 227: 300-15.

At the end of the 19th century, CBN was the first cannabinoid isolated (Mechoulam and Hanus, 2000; Pertwee, 2006). CBN was isolated from a red oil extract of cannabinoids (Pertwee, 2006) and by that time, CBN was considered to be the main cannabinoid having the greatest psychoactive effects (Mechoulam and Hanus, 2000). In 1940, the second cannabinoid, CBD was isolated by chemist Roger Adam (Mechoulam and Shvo, 1963; Pertwee, 2006; Atakan, 2012). In 1963, Raphael Mechoulam and Youval Shvo elucidated CBD in Raphael Mechoulam´s laboratory (Pertwee, 2006; Atakan, 2012). The following year, 1964, R. Mechoulam and Yechiel Gaoni isolated and synthetized THC, (Mechoulam and Gaoni, 1965), the main psychoactive cannabinoid (Bonini et al, 2018; Citti et al, 2018).

Dronabinol (Marinol®) was the first FDA-approved cannabinoid-based preparation of synthetic THC in sesame seed oil (Greydanus et al, 2015; Zgair et al, 2015; Welling et al, 2019). In 1985, Nabilone (Cesamet ®), another synthetic cannabinoid, was approved by the FDA (Greydanus et al, 2015). Nabilone is a synthetic molecule analogous to THC (Greydanus et al, 2015). Dronabinol and Nabilone are approved in the management of chemotherapy- induced nausea and vomiting and for appetite stimulation in patients with Acquired Immunodeficiency Syndrome (Zgair et al, 2015; Greydanus et al, 2015). Dronabinol was also indicated to treat anorexia in Human immunodeficiency virus/ Acquired Immunodeficiency Syndrome patients (Greydanus et al, 2015; Badowski, 2017). Some of the difference between Nabilone and Dronabinol lies on bioavailability and side effects, Nabilone has an improved bioavailability and Dronabinol presents less incidence of dry mouth and muscle incoordination (Greydanus & Holt 2014; Greydanus et al, 2015). Another approved product containing cannabinoids was Nabiximols (Sativex®) (Zgair et al, 2015). Nabiximols is the first natural phytocannabinoid extract approved and it is an oral mucosal spray used for the treatment of cancer pain, and

neuropathic pain and spasticity associated with multiple sclerosis (Greydanus et al, 2015; Zgair et al, 2015). In 2018, the Food and Drug Administration (FDA) approved Epidiolex® that it is the first Cannabis-derived drug (Bonini et al, 2018; Welling et al, 2019). Epidiolex® is a CBD-based drug for the treatment of Lennox-Gastaut syndrome and Dravet syndrome (Bonini et al, 2018; Welling et al, 2019).

THC has been used in the management of chemotherapy- induced nausea and vomiting, for appetite stimulation in patients with Acquired Immunodeficiency Syndrome (Wang et al, 2017) and in the treatment of migraines (Baron, 2015).

CBD is the principal non-psychoactive cannabinoid (Zgair et al, 2015) and is known for having the largest number of medicinal properties (Elkins et al, 2019). CBD has been used in the management of drug-resistant epilepsy, principally in children (Leo et al, 2016). CBD has anti-inflammatory (Mudge et al, 2017), analgesic, antioxidant (Citti et al, 2018), anxiolytic, antidepressant, neuroprotective and antipsychotic activities (Fogaça et al, 2018). CBD can be used in different neuropsychiatric disorders such as autism spectrum disorder, anxiety and psychosis, and for neuropathic pain, cancer pain and multiple sclerosis.[4]

CBN has weak psychoactive properties[5] with potent sedative properties. CBN is an oxidation product of THC (Citti et al, 2018) and the amount of CBN found in the plant is limited (Perrotin-Brunel et al, 2010).

### 1.4 Endocannabinoid system

The Endocannabinoid System (ECS) consists of two main receptors CB1 (type-1) and CB2 (type-2) (Mechoulam et al, 1998) that are connected to G-proteins (Greydanus et al,

[4] Chayasirisobhon S. Cannabis and Neuropsychiatric Disorders: An Updated Review. Acta neurologica Taiwanica [Internet]. 2019 [cited 2020 June 20];28(2):27–39. Available from: http://www.ant-tnsjournal.com/Mag_Files/28-2/001new.pdf

[5] Morales P, Hurst DP, Reggio PH. Molecular Targets of the Phytocannabinoids: A Complex Picture. Progress in the Chemistry of Organic Natural Products [Internet]. 2017 [cited 2020 June 20];103–31. Available from: https://link.springer.com/chapter/10.1007%2F978-3-319-45541-9_4

2015), their endogenous ligands (endocannabinoids), and the enzymes which carry out the synthesis and degradation of the ligands (Naftali et al, 2014). CB1 receptors, are found in regions of the brain, such as the hippocampus, cortex, caudate-putamen, globus pallidus, substantia nigra, and cerebellum (Sim et al, 1996). The distribution of the receptors in these regions, which, involves mood, cognitive functions and motor control are in line with the observable effects that cannabinoids produce; memory interruption, reduced motor activity, catalepsy, antinociception, and hypothermia (Sim et al, 1996; Bonini et al, 2018). CB1 receptors are also found, in lower concentrations, in peripheral areas like the liver, teste and small intestine (Greydanus, 2015). CB2 receptors are found in immune cells and modulate immune cells, like T cell proliferation, B cell action and proinflammatory cytokine release (Greydanus, 2015). Both receptors are found in the human placenta and have been shown to participate in the regulation of serotonin transporter activity (Atakan, 2012). When neuronal excitations are present, cyclic adenosine monophosphate is converted to cyclic adenosine monophosphate due to receptor activation inhibiting adenylate cyclase, and also, release of multiple neurotransmitters is inhibited, including acetylcholine, dopamine, glutamate (Koppel et al, 2014). THC binds to the CB1 receptor, while CBD does not (McCoy et al, 2018). The human body synthetizes endogenous cannabinoids called arachidonoylethanolamine (anandamide) and 2-arachidonoyl glycerol (2-AG) which bind to both receptors (Greydanus, 2015). Endogenous cannabinoids present some similar effects to other cannabinoids. The presence of cannabinoid receptors and neurotransmitters (dopamine, glutamate, serotonin and gamma-aminobutyric acid) pursued researchers to carry out preclinical studies to seek the involvement of the ECS in health and disease (Pacher and Konus, 2013; Bonini et al, 2018).

## 1.5 Therapeutic uses of cannabinoids

### 1.5.1 Pain

The use of cannabis was not found to be beneficial for the treatment of acute pain. Cannabis and cannabis-based preparations appeared to have mild beneficial effects in the treatment for neuropathic pain, and in orthopedic musculoskeletal pain (Vulfsons et al, 2020). Whiting et al, carried out a metanalysis study on the use of cannabis to treat chronic pain: quality evidence for the management of chronic pain with cannabinoids is moderate (Whiting et al, 2004).

### 1.5.2 Colitis

Cannabis- based preparations have been used as an alternative to treat different gut diseases, like Crohn's diseases and ulcerative colitis (Bonini et al, 2018; Couch et al, 2018). Some studies have demonstrated that the isolation of cannabis constituents as CBD, CBC and CBG exerts positive effects in experimental models of Inflammatory Bowel Disease (IBD) (Jamontt et al, 2010; Pagano et al, 2016; Bonini et al, 2018). Pagano et al, demonstrated in a mouse model study, that CBD ameliorated colitis, and reduces the extent of injuries and offsets hypermotility of the intestine in experimental models of intestinal inflammation (Pagano et al, 2016). Couch et al, performed a systematic review and a metanalysis review on the use of cannabinoids for the treatment of colitis, demonstrating that cannabinoids reduce gut inflammation in rats and mice. In the light of this positive findings, further research in humans is required (Couch et al, 2018).

### 1.5.3 Spasticity

More than 85% of the people who suffer from Multiple Sclerosis present some spasticity (Bonini et al, 2018; Rice and Cameron, 2018). Currently available medication for management of MS-associated spasticity has limited effectiveness and is toxic (Flachenecker et al, 2014; Bonini et al, 2018; Nielsen et al, 2018). There is evidence that

cannabinoids reduce the symptoms of spasticity (Koppel et al, 2014; Allan et al, 2018; Nielsen et al, 2018; Rice and Cameron, 2018). Cannabinoids were well-tolerated in this group of patients (Rice and Cameron, 2018).

### 1.5.4 Nausea and vomiting

In 1975, the first placebo-controlled study showing the benefits of THC to treat chemotherapy- induced nausea and vomiting was published (Badowski, 2017). In 1985, nabilone and dronabinol were approved by the FDA in the management of chemotherapy-induced nausea and vomiting in patients who did not respond adequately to conventional antiemetic treatment (Sharkey et al, 2014; Greydanus et al, 2015). Nabilone was found to be more effective than Metoclopramide (D2 antagonist) in patients undergoing chemotherapy (Sharkey et al, 2014). Other studies demonstrated that either oral solution (dronabinol) or capsules (nabilone and dronabinol) were effective in the treatment of chemotherapy- induced nausea and vomiting (Badowski, 2017).

### 1.5.5 Anorexia

Cannabinoids and endocannabinoids are involved in the control of body weight by affecting central and peripheral regulatory circuits that coordinate energy homeostasis (Horn et al, 2018). The stimulation of CB1 signals can increase appetite. Andries et al, carried out a double-blind, randomized controlled crossover study that demonstrated that treatment of anorexia nervosa with dronabinol produces a small increase in weight, was well tolerated and did not produce severe adverse events during four weeks (Andries et al, 2014). Cannabinoids are also effective in the treatment of patients suffering from anorexia due to Acquired Immunodeficiency Syndrome (Gérard et al, 2011).

### 1.5.6 Sleep disorders

Short-term treatment with cannabinoids suggests to have a therapeutic benefit on sleep, particularly in decreasing sleep onset latency and slow wave sleep. Long-term chronic

treatment with cannabinoids is associated with a negative impact due to the habituation to the sleep-enhancing benefits and higher risk for dependence on cannabis (Babson et al, 2017).

### 1.5.7 Epilepsy

In recent years, cannabis has prompted a special interest in its role as an anticonvulsant in patients experiencing refractory epilepsy (Ben-Zeev, 2020). Pure CBD and CBD-enriched cannabis oil are effective in the control of seizures in pediatric and young adult patients, especially in Dravet syndrome and Lennox–Gastaut syndrome (Thomas and Cunningham, 2018; Ben-Zeev, 2020). It is important that cannabinoid formulations used to treat epilepsy present the lowest concentration of THC, since THC can aggravate seizures and can be associated with short-term and long-term side effects (Ben-Zeev, 2020). Elliot et al, carried out a systematic review which demonstrated that CBD is effective in the treatment of children with drug- resistant epilepsy (Elliot et al, 2020).

### 1.5.8 Alzheimer's disease

CBD is neuroprotective, avoids hippocampal and cortical neurodegeneration, presents anti-inflammatory and antioxidant properties, decreases tau hyperphosphorylation and balances microglial cell migration (Esposito et al, 2005; Esposito et al, 2006; Martín-Moreno et al, 2011; Watt and Karl, 2017). CBD can decrease reactive gliosis, neuroinflammatory response and promotes neurogenesis. In rodent models CBD reversed and prevented cognitive loss (Esposito et al, 2006; Watt and Karl, 2017). When comparing treatment with CBD alone with THC alone and with CBD and THC combined therapies, it was demonstrated that CBD can antagonize the psychoactive effects linked with THC and had better therapeutic benefits than THC or CBD alone (Aso et al, 2015; Watt and Karl, 2017).

## 1.6 Cannabis formulation for medicinal use

Cannabis-based preparations are an alternative medicinal strategy for different diseases, and in the last decade were approved in countries of the European Union (Carcieri et al, 2017).

### 1.6.1 Cannabis infusion

Cannabis can be infused in cold water and ingested as a cannabis infusion. The flowering tops of cannabis are used for this kind of preparation.[6]

### 1.6.2 Vaping

Vaping cannabis has become a very popular method of administration due to patient's perception as a safer and less harmful comparing to combustible smoking methods (Budney, et al 2015). Studies have demonstrated that the amount of inhaled toxic compounds are less in vaping, while the amount of cannabinoids produced in vaping is higher than smoking (Abrams et al, 2007; Pomahacova et al, 2009; Budney, et al 2015).

### 1.6.3 Cannabis oils

Cannabis oils are concentrated extracts with a sticky and viscous appearance which are obtained from the leaves or buds of the Cannabis plant by solvent extraction (Romano and Hazekamp, 2013). Cannabis oil can be applied topically and orally, inhaled or formulated into suppositories (Martini, 2016). Cannabis oils are used in the management of seizures in Drug-Resistant Epilepsy (DRE) (McCoy et al, 2018). Jemos et al carried out a study to monitor the benefits of cannabis oil in oncological patients and results have shown that cannabis oil was effective in some patients who did not respond to other treatments; but the majority did not present any benefit (Jemos et al,2018).

[6] Decreto 9 novembre 2015: Funzioni di Organismo statale per la cannabis previsto dagli articoli 23 e 28 della convenzione unica sugli stupefacenti del 1961, come modificata nel 1972. [Internet] 2016 [cited 26 Oct 2020]. Available from: http://www.gazzettaufficiale.it/eli/id/2015/11/30/15A08888/sg;jsessionid=p1rnwNujUKlqQ5azhA Q95A__.ntc-as3-guri2a.

## 1.7 Extraction techniques

To separate cannabinoids from oil, plant material and biological fluids, extraction must be performed using a procedure which is effective, selective and reproducible (Citti et al, 2018).

### 1.7.1 Liquid Liquid Extraction

Liquid Liquid Extraction (LLE) is a sample preparation technique where water-immiscible solvents are used to separate analytes from aqueous solutions. LLE presents some disadvantages such as it is time-consuming which limits the number of samples that can be handled at the same time (Battista et al, 2013). Before analysis, a pre-concentration step of the extract is usually required and large amount of toxic solvents can be used (Barrionuevo and Lanças, 2002).

### 1.7.2 Solid Phase Extraction

Solid Phase Extraction (SPE) is an extraction technique where analytes that are dissolved or suspended in a liquid matrix are separated according to their physical and chemical characteristics. SPE is faster, requires less quantity of solvent making it more environmentally friendly and the extraction procedure is more complete comparing to LLE (Barrionuevo and Lanças, 2002). SPE presents some disadvantages like adsorbents must be loaded into a SPE cartridge, which is not simple to operate (Shi et al, 2016) and cartridges are only used once and expensive.

### 1.7.3 Supercritical fluid extraction

Supercritical Fluid Extraction (SFE) is a technique used for the extraction and separation of cannabinoids from vegetable matrices (Rovetto and Aieta, 2017). Some of the advantages are that SFE uses solvents with low toxicity like Supercritical carbon dioxide (Sc $CO_2$) (Perrotin-Brunel et al, 2010; Gallo-Molina et al, 2019). Due to the low polarity of Sc $CO_2$ a small amount of a polar modifier (ethanol, water, acids) is required to

improve extraction range and selectivity (Rovetto and Aieta, 2017; Gallo-Molina et al, 2019).

## 1.8 Analytical techniques for the determination of cannabinoids

Different methods have been studied for the analysis of cannabinoids (Nahar et al, 2019). Some of the methods used are: High Performance Thin Layer Chromatography (HPTLC), Nuclear Magnetic Resonance (NMR) spectroscopy (Citti et al, 2018), GC with and without derivatization and HPLC (Wang et al, 2017).

### 1.8.1 High Performance Thin Layer Chromatography

HPTLC is an analytical technique derived from TLC which uses similar principles to TLC (Reich and Blatter, 2005). HPTLC is mainly used for preliminary semi-quantitively analysis of cannabinoids in plant material (Fischedick, et al 2009). HPTLC costs less and can be used for the analysis of various samples at the same time (Fischedick, et al 2009) HPTLC presents less specificity and sensitivity compared to HPLC (Citti et al, 2018).

### 1.8.2 Nuclear Magnetic Resonance spectroscopy

NMR is a tool for the analysis of cannabinoids which presents high accuracy and reproducibility in a short analysis time (Hazekamp, et al 2004; Citti et al, 2018). NMR presents lack of sensitivity to impurities containing in the sample like chlorophyll and lipids. This lack of sensitivity avoids the step of clean-up the plant material before the analysis like in techniques such as HPLC and GC (Hazekamp, et al 2004; Citti et al, 2018). NMR is not usually used due to the high instrumental costs and due to the requirements of highly technical skills required.

### 1.8.3 Gas chromatography

Gas chromatography (GC) was the main analytical technique used for the analysis of cannabinoids in both hemp products and biological matrices (Pellegrini et al, 2005; Citti

et al, 2018). GC can be carried out with or without derivatization (Hazekamp et al, 2007). In absence of derivatization, the sample requires to be heated at high temperature (about 280ºC) to be converted from its liquid phase to gaseous prior to analysis (Hazekamp et al, 2007; Citti et al, 2018). The high temperature, causes the decarboxylation of the cannabinoid acids to their corresponding neutral form (Hazekamp et al, 2007; Citti et al, 2018) resulting in the sum of both (Citti et al, 2018). To avoid decarboxylation, derivatization of the acidic form of cannabinoids is carried out (Hazekamp et al, 2007; Citti et al, 2018). During this procedure it is very difficult to obtain 100% derivatization yield for acidic cannabinoids (Dussy et al, 2005). Dussy et al demonstrated that quantities of $\Delta^9$ -THCA-A and $\Delta^9$ -THC calculated using HPLC analysis were higher than the amount of $\Delta^9$ -THC determined by GC (Dussy et al, 2005).

### 1.8.4 High Performance Liquid Chromatography

HPLC is an analytical technique which can be used for the identification, quantification and separation of analytes (Gupta et al 2012; Moreno et al, 2014). HPLC is based on the injection of a liquid sample into the column with a stationary phase and the mobile phase which is liquids pumped at high pressure through the column. The differences in migration rates across the column will determinate the separation of different compounds contained in a sample (Gupta et al, 2012). The migration rate depends on the interaction of the different compounds in the sample with the stationary phase, which affects their partition, and results in different elution times. A limiting factor in the analysis of compounds using HPLC is solubility, that is, the compounds have to be dissolved prior to analysis (Gupta et al, 2012).

In reversed phase chromatography, the packing material of the stationary phase is usually made of porous silica beads. These beads are coated with hydrophobic liquids which are usually made up of aliphatic chains: C4, C8, C18 (Kirkland 2004; Zhao 2017). C18

columns are the most commonly used columns in reversed phase chromatography. (Citti et al, 2018). The mobile phase used in reversed phase chromatography consists of water or an aqueous solution such as a buffer and an organic solvent, with methanol and acetonitrile being the most commonly used organic solvents. The presence of these two components in the mobile phase is important, since it controls how long the analyte will take to travel through the polar mobile phase and how long it will interact with the hydrophobic stationary phase (Van Gyseghem et al, 2005). When analysing cannabinoids, acetonitrile is the organic solvent preferred over methanol because of the large reduction of the total run time (Ricardo Deidda, 2019). There are two different types of HPLC methods, isocratic method: the mobile phase composition is kept constant during the entire elution process (Vibha Gupta et al, 2012) and gradient method: the mobile phase composition is programmed to change during the elution process and can employ two or more solvents systems, which differ significantly in polarity (Kirkland, 2004; Vibha Gupta et al. 2012).

After exiting the stationary phase, the separated compound travels through a detector and the chromatogram is obtained (Zhang et al, 2008). Different kinds of detectors can be coupled to HPLC: ultraviolet (UV), fluorescence (FL) or a mass spectrometry (MS) detector (Vella et al, 2015). Although coupling the HPLC system with MS detectors can offer excellent selectivity and sensitivity of the analysis (Elkins et al, 2019), it requires expensive instrumentation and skilled expertise (Vella et al, 2015). FL detectors coupled to an HPLC system cannot be used to analyse CBN because CBN does not possess a fluorophore (Citti et al, 2018). HPLC-UV is a common method chosen for analysis of cannabinoids for some reasons, it absorbs the chromophore of the substituted phenolic ring, which is a structural element present in the cannabinoids tested (Citti et al, 2018)

and it is more convenient and economic than other methods of analysis.[7]

### 1.9 HPLC method validation

The validation of an analytical method is a process aimed to prove and document that the performance characteristics of the procedure are suitable for its intended use (Vibha Gupta et al, 2012; Kishor et al, 2017). The validation of an analytical procedure can be done as per International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use guidelines[8] (Vibha Gupta et al, 2012). The different parameters to validate a method are:

- Linearity
- Specificity
- Accuracy
- Precision
- Limit of Detection
- Limit of Quantification

### 1.10 Aim and objectives

The aim of this study is to develop and validate an HPLC method to determine THC in CBD oil for medicinal use.

The objectives are:

1. To select appropriate HPLC parameters in terms of resolution and efficiency of analysis

[7] He Q, Li M, Wang X, Xia Z, Du Y, Li Y, et al. A simple, efficient and rapid HPLC–UV method for the detection of 5-HT in RIN-14B cell extract and cell culture medium. BMC Chemistry [Internet]. 2019[cited 2020 May 18]. Available from: https://link.springer.com/article/10.1186/s13065-019-0591-x

[8] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Validation of analytical procedures: text and methodology Q2(R1) . [Internet] 2017[cited 2021 Feb 08]. Available from https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf.

2. To develop an appropriate sample preparation technique for the extraction of THC from CBD oil.

3. To validate the developed HPLC method according to the ICH guidelines.[9]

[9] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Validation of analytical procedures: text and methodology Q2(R1) . [Internet] 2017[cited 2021 Feb 08]. Available from https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf.

# Chapter 2 Methodology

### 2.1 Methodology

The study was divided into four phases (i)Systematic literature review on analytical methods used for separation and determination of cannabinoids using HPLC (ii) Development and validation of HPLC method to determine CBD, CBN and THC in MeOH (iii) Development of procedure to extract THC from oil (iv) Development and validation of HPLC method to determine THC in oil.

### 2.2 Systematic Literature Review

A systematic literature review was carried out using the PRISMA method[10]. The systematic literature review included methods used for separation and determination of cannabinoids using LC. Sources included open access peer reviewed journal articles published in English between the years 2015 and 2020. Databases used for the literature search were Pubmed and Scopus. Keywords used in the search were: analysis, cannabinoids, cannabis, tetrahydrocannabinol, cannabidiol, cannabinol and LC. Data collected was presented in tables (Appendix I), according to the matrix in which the cannabinoids were presented. Data in each table compared the type of: type of matrix, type of cannabinoids analyzed, sample preparation method, stationary phase, mobile phase and detector.

### 2.3 Chemicals

- Cannabinoids standards were procured from LoGiCal® (LGC):
  - THC: 0.1 mg/mL in Methanol, Uncertainty (U)= 0.0006 mg/mL[3]
  - CBD: 1.0 mg/mL in Methanol, U = 0.0006 mg/mL[3]
  - CBN: 1.0 mg/mL in Methanol, U = 0.0006 mg/mL[3]
- HPLC-grade water

[10] prisma-statement.org [Internet] Prisma transparent reporting of systematic reviews and meta-analysis. [cited 2021 April 23]. Available from: http://prisma-statement.org/PRISMAStatement/FlowDiagram

- HPLC-grade MeOH (Methanol) (Honeywell Riedel-de Haën, France)
- HPLC-grade ACN (Acetonitrile) (Honeywell Riedel-de Haën, France)
- Anhydrous disodium hydrogen phosphate (Carlo Erba Reagents, Val-de-Reuil,France)
- HPLC-grade orthophosphoric acid (Fisher Chemical, Leicestershire, UK)
- Standard Hanna® calibrator buffer solutions (at pH values of 4.01, 7 and 10)
- Medium Chain Triglyceride (MCT) oil (Natures aid)
- Extra Virgin Olive Oil (EVOO) (Primadonna)
- Standard ibuprofen procured from Sigma-Aldrich (purity 99.7%)
- CBD oil 5%(500mg) 10mL (Formula Swiss)

**2.4 Consumables**

- ACE 5µm C18 LC Column 5µm (250 x 4.6 mm)
- Pursuit XRs 5 C18 MetaGuard (10 x 4.6 mm)
- Agilent Premium Syringe filter Regenerated Celulose 0.45 µm, 15mm
- Centrifuge tubes (1.5mL) (Isolab, Germany)
- Micropipettes labopipette® (Hirschmann ® laborgerate Germany)
- HPLC vials 1.5mL (Labbox)
- Pipettes 3.0mL
- Calibrator buffer solutions (at pH values of 4.01, 7 and 10).

**2.5 Instruments**

- Sartorius® analytical balance
- Hanna® Bench-top pH meter HI8521
- Agilent 1260 Infinity Series® II HPLC unit
- Langford sonomatic 1400 ultrasonic bath
- Vortex Genie 2
- Eppendorf minispin centifuge

## 2.6 Method development for the separation of CBD, CBN and THC

HPLC method for the detection and quantification of CBD, CBN and THC was developed and validated. The method was developed by selecting appropriate HPLC parameters in terms of resolution and efficiency of analysis by changing:

- pH of the buffer in the mobile phase
- amount of organic modifier in the mobile phase
- flow rate of the mobile phase
- column temperature

### 2.6.1 Materials

HPLC-grade water was used for preparation of buffer. The composition of mobile phase was acetonitrile and phosphate buffer, using an ultra-sonic cleaning bath prior to use. The preparation of the buffer solution was performed by dissolving 2.84 grams, anhydrous disodium hydrogen phosphate, in 1 Litre HPLC-grade water. The pH (2.5, 3, 4 and 6) was adjusted by adding HPLC-grade orthophosphoric acid drop by drop. Sample preparation for method development of CBD, CBN and THC.

Stock solutions of the cannabinoids were prepared in MeOH. To prepare the stock solutions, 1 mg/mL of CBD standard in MeOH was diluted to a concentration of 5 µg/ml, 1 mg/mL of CBN standard in MeOH was diluted to a concentration of 5 µg/ml and 0.1 mg/mL of THC standard in MeOH was diluted to a concentration of 5 µg/ml. The three cannabinoids were mixed together by transferring 0.5 ml of each solution into amber-colored vials. Samples containing the three cannabinoids were stored at -20ºC.

### 2.6.2 Chromatographic system

Chromatographic separation was achieved using an Agilent 1260 Infinity Series® II liquid chromatography system with UV detection. An ACE 5µm C18 LC Column 5µm (250 x 4.6 mm) at a temperature of 25 ˚C was used as stationary phase and acetonitrile and a

phosphate buffer as mobile phase. The injection volume was 20 µL. The UV detector was set at 220 and 228 nm. Separate runs for the individual cannabinoids were first carried out to help with peak identification.

### 2.6.3 Method development of the separation of CBD, CBN and THC

Different parameters such as mobile phase, pH, temperature, injection volume, flow rate, were tested to observe how the area under the peak and retention time, change for each chromatogram. Acetonitrile was selected as the organic. In the literature, methanol and acetonitrile are usually used as organic solvent, with acetonitrile being preferred in this scenario because it decreased the total run time with respect to methanol (Deidda et al, 2019). For the method, phosphate buffer was used, because it is effective over a range of pH values (Rao and Goyal, 2016). The pH and the percentage of acetonitrile in the mobile phase were changed as shown in Table 2.1 to determine which conditions result in adequate separation. The sample concentration and injection volume, for the HPLC runs were kept constant. The sample concentration was 5 ug/mL, and the injection volume was 20µL. The wavelength was set at 220nm and 228nm respectively for all runs. All the runs were carried out in triplicates. Once the mobile phase composition and buffer pH which give the best separation were chosen, different Flow Rates (FR) were tested (1, 1.5 and 2 mL/min). After selecting the best flow rate, different temperatures of stationary phase were tested (20, 25 and 30ºC) and the one which give the best chromatogram was selected.

Different amounts of ACN in the mobile phase, and different pH (from 2.5 to 6) by adding of HPLC-grade orthophosphoric acid drop by drop, were tested to evaluate the impact that they can cause on the chromatogram obtained.

**Table 2.1: HPLC method development for the analysis of CBD, CBN and THC**

| Mobile phase number | pH | Percentage of ACN used |
|---|---|---|
| 1 | 2.5 | 70 |
| 2 | 2.5 | 80 |
| 3 | 2.5 | 90 |
| 4 | 3 | 70 |
| 5 | 3 | 80 |
| 6 | 3 | 90 |
| 7 | 4 | 70 |
| 8 | 4 | 80 |
| 9 | 4 | 90 |
| 10 | 6 | 70 |
| 11 | 6 | 80 |
| 12 | 6 | 90 |

## 2.7 Method validation for the separation of the three cannabinoids

The HPLC method for the determination and separation of the three cannabinoids was validated according to the International Council on Harmonisation Q2 guideline (ICH Q2).[11] The objective of the validation was to determine linearity, range, accuracy, specificity/selectivity, precision, detection limit and quantitation limit.

### 2.7.1 Sample preparation for validation process

Different sample concentrations were prepared for the calibration curve. The preparation of the sample concentrations was as follows: 0.5 ml of the stock solution of each cannabinoid at a concentration of 5µg/ml was transferred into amber-colored vials. A total of seven concentrations at 5.00µg/ml, 2.50µg/ml, 1.25µg/ml, 0.63µg/ml, 0.16µg/ml, 0.04µg/ml and 0.02µg/ml were analysed.

### 2.7.2 Selectivity/Specificity

To demonstrate the selectivity/specificity a blank sample which consisted of methanol was ran in triplicates and compared with the analysis of the three cannabinoids, by observing both chromatograms it was confirmed that the peaks obtained were attributed to the three cannabinoids.

[11] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Validation of analytical procedures: text and methodology Q2(R1) . [Internet] 2017[cited 2021 Feb 08]. Available from https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf.

### 2.7.3 Linearity

To demonstrate the linearity of the three cannabinoids, the stock solution was diluted in a total of seven concentrations previously mentioned in section 2.6.1. The largest concentrations were analysed first, and the smallest concentrations were analysed later.

The evaluation was carried out by visual inspection of a plot of signals (AUP) as a function of the three cannabinoids' concentration. The regression line was calculated to observe if there was a linear relationship by the method of least squares. The correlation coefficient to measure the strength and the direction of the linear relationship between the concentration and the AUP was determined. The concentrations were analyzed in triplicates.

A line of best fit was plotted using the values attained (see section 3.3.2)

In the best-case scenario, a linear relationship- y=mx +b was attained.

Where y= detector response (mAU); x=concentration of cannabinoid (µg/ml); m= slope of straight line and b=intercept.

### 2.7.4 Stability

The stability of the three cannabinoids in MeOH was determined by storing samples of the three cannabinoids in MeOH at -20ºC. Three different concentrations of the three cannabinoids in MeOH were stored. These concentrations were: 0.04, 0.63 and 5.00 µg/ml. These concentrations were analysed after one and three weeks in triplicates.

### 2.7.5 Accuracy

To demonstrate the accuracy of the method, 3 concentrations of the 3 cannabinoids in MeOH: 0.04, 0.63 and 5.00 µg/ml, were analysed. The concentration of the three

cannabinoids detected in µg/ml was divided by the true concentration of the three cannabinoids that was injected in µg/ml. The results were multiplied by 100 to express it in percentage. This procedure was done in triplicates to ensure reproducibility

### 2.7.6 Precision

To demonstrate the precision of the method, the means of the percentage Relative Standard Deviation (RSD) were evaluated. The coefficient of variation was calculated by dividing the standard deviation with the mean and multiplying this by 100:

*Relative standard deviation (RSD)= Standard deviation / Mean · 100*

Precision inter-day was determined by injecting the sample at three different concentrations: 0.04, 0.63 and 5.00 µg/ml in triplicates, on three consecutive days.

Intra-day precision was assessed by the analysis of the sample at three different concentrations: 0.04, 0.63 and 5.00 µg/ml in triplicates, on the same day.

### 2.7.7 Limit of Detection

The limit of detection is the lowest concentration analysed that gave a detector signal. The concentration of the three cannabinoids in MeOH was decreased consecutively and injected. The lowest concentration which gave a signal on the detector without the necessity of being quantified and in triplicates was noted. This concentration was considered to be the limit of detection.

### 2.7.8 Limit of Quantification

The limit of quantification is the lowest concentration analysed that gave a detector signal and can be quantified. The concentration of the three cannabinoids in MeOH was decreased consecutively and injected. The lowest concentration which gave a signal on

the detector and was quantified and in triplicates was noted. This concentration was considered to be the limit of quantification.

## 2.8 Method development for extraction of THC from MCT oil

### 2.8.1 Preparation of THC in MCT oil

Stock solutions of THC was prepared in MCT oil. To prepare the stock solution, 0.1 mg/mL of THC standard in methanol was diluted in MCT oil to a concentration of 5 µg/ml. Samples were stored at 4ºC.

### 2.8.2 Solvent extraction

To develop a sample technique for the extraction of THC from MCT oil different conditions of ratio of solvent to THC in oil, vortex time, sonication time, centrifugation time and force were tested to observe how the peak of THC changed in terms of size and shape. The different ratios of solvent to THC in oil were as follows: 0.3mL MeOH and 0.3mL 2.5 µg/mL THC in oil, 0.3mL MeOH and 0.6mL 2.5 µg/mL THC in oil and 0.6mL MeOH and 0.3 mL 2.5 µg/mL THC in oil. Each centrifuge tube was vortex-mixed for 10 minute and centrifuged for 5 minutes at 3,000 revolution per minute (rpm). After chosen the best ratio of MeOH to THC in oil, other parameters were tested as shown in Table 2.2.

**Table 2.2: Parameters tested for the extraction of THC from MCT oil**

| Nº | Ratio | Vortex time | Sonicate time | Centrifuge time & force | Frezeer (-20ºC) | Centrifuge time |
|---|---|---|---|---|---|---|
| 1 | 0.6mL MeOH + 0.3mL THC in oil | 3 min | x | 25 min 3500rpm | x | x |
| 2 | 0.6mL MeOH + 0.3mL THC in oil | 5 min | x | 25 min 3500rpm | x | x |
| 3 | 0.6mL MeOH + 0.3mL THC in oil | 1 min | x | 25 min 3500rpm | x | x |
| 4 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | x | 20min 4500rpm | x | x |
| 5 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | x | 15 min 5000rpm | x | x |
| 6 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | 15 min | 15 min 4500rpm | x | 15 min 4500rpm |
| 7 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | 15 min | 15 min 4500rpm | 2h | 15 min 4500rpm |
| 8 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | 15 min | 15 min 10000rpm | x | 15 min 10000rpm |
| 9 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | 20 min | 15 min 6000rpm | 2h | 15 min 6000rpm |
| 10 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | 20 min | 15 min 10000rpm | 2h | 15 min 10000rpm |
| 11 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | 20 min | 25 min 6000rpm | 2h | 25 min 6000rpm |
| 12 | 0.6mL MeOH + 0.3mL THC in oil | 30 s | 20 min | 25 min 10000rpm | 2h | 25 min 10000rpm |

### 2.8.3 Method development for the analysis of THC in MCT oil

Different parameters such as mobile phase, pH, flow rate, injection volume, were tested to observe how the area under the peak, the size and the shape of the peak and retention time change for each chromatogram. For the method, phosphate buffer and ACN were selected as a mobile phase. In studies published in the literature, the % ACN in mobile phase ranged from 60% to 100%, and the flow rate used, ranged from 0.38 mL / min to 1 mL / min, it was decided to try 0.5, 1 and 1.5 due a shorter retention time. The pH, the percentage of acetonitrile in the mobile phase, the flow rate, the injection volume (IV) and the wavelength were changed as shown in Table 2.3:

**Table 2.3:Parameters tested for the method development of THC in oil**

| Mobile phase number | Percentage of ACN used | Flow Rate (mL/min) | WV (nm) | pH | IV(µL) |
|---|---|---|---|---|---|
| 1 | 80 | 1.5 | 220 & 228 | 2.5 | 20 |
| 2 | 70 | 1.5 | 220 & 228 | 2.5 | 20 |
| 3 | 70 | 2 | 220 & 228 | 2.5 | 20 |
| 4 | 65 | 2 | 220 & 228 | 2.5 | 20 |
| 5 | 80 | 0.5 | 220 & 228 | 2.5 | 20 |
| 6 | 80 | 1.5 | 220 & 278 | 2.5 | 20 |
| 7 | 80 | 1.5 | 220 & 275 | 2.5 | 20 |
| 8 | 80 | 1.5 | 220 & 280 | 2.5 | 20 |
| 9 | 80 | 2 | 220 & 278 | 2.5 | 20 |
| 10 | 80 | 2 | 220 & 278 | 2.5 | 50 |
| 11 | 80 | 2 | 220 & 278 | 2.5 | 100 |
| 12 | 80 | 1.5 | 275 & 278 | 2.5 | 100 |
| 13 | 80 | 1.5 | 275 & 278 | 2.5 | 50 |
| 14 | 80 | 1.5 | 220& 228 | 6 | 20 |
| 15 | 90 | 1 | 220& 228 | 6 | 20 |
| 16 | 90 | 1 | 275& 278 | 6 | 20 |
| 17 | 85 | 1 | 275& 278 | 6 | 20 |
| 18 | 75 | 1 | 220&228 | 6 | 20 |
| 19 | 70 | 1.5 | 220 & 278 | 6 | 20 |
| 20 | 70 | 1.5 | 220 & 278 | 6 | 50 |
| 21 | 70 | 1.5 | 220 & 278 | 6 | 100 |

Since THC was still not well separated from the fatty acids from MCT oil, it was decided to add one step more to the extraction procedure described in section 2.7.2. After the last centrifugation, the sample was passed through a RC syringe filter of 0.45 µm, 15mm.

### 2.8.4 Preparation of Internal Standard

Ibuprofen was chosen as the internal standard for THC (Figure 2.1). Ibuprofen was selected due to it showing the maximum amount of absorption at the same UV chosen in the method (Citti et al, 2019). Ibuprofen has been previously used as an internal standard in the analysis of THC (Giese et al, 2015; Citti et al, 2019). The internal standard ibuprofen was prepared as follows, 0.002g of Ibuprofen were weighed and dissolved in 20mL of MeOH, to prepare a stock solution of 100µg/mL.

## 2.9 Method development for extraction of THC from EVOO

The validation of the method in MCT oil was not carried out due to reproducibility problems. It was decided to change the carrier oil to EVOO. HPLC method for the detection and quantification of THC in EVOO was developed and validated. The main steps for the method development for the determination and quantification of THC in EVOO included:

**Step 1:** Preparation of solutions for chromatographic analysis as described in Section 2.8.1

**Step 2:** Developed of an appropriate extraction preparation technique as described in Table 2.2.

**Step 3:** Selection of the most favorable chromatographic parameters in terms of resolution and efficiency of analysis as described in Section 2.8.3

**Step 4:** Preparation of Internal Standard as described in Section 2.8.4

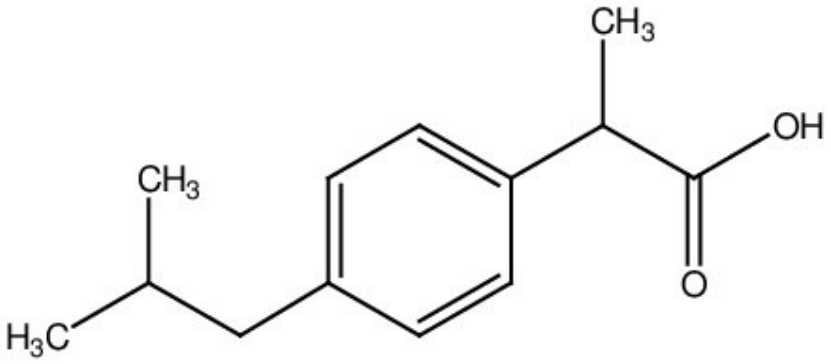


**Figure 2.1: Chemical structure of Ibuprofen**

### 2.9.1 Preparation of THC in EVOO

Stock solutions of THC was prepared in EVOO. To prepare the stock solution, 0.1 mg/mL of THC standard in methanol was diluted in EVOO to a concentration of 5 μg/ml. Samples were stored at 4ºC.

### 2.9.2 Solvent Extraction

The developed sample technique for the extraction of THC from MCT oil was used for the extraction of THC from EVOO. Described in Section 2.7.2.

## 2.10 Method development for the analysis of THC in EVOO

Different parameters such as mobile phase, flow rate and temperature, were tested to observe how the area under the peak, the size and the shape of the peak and retention time change for each chromatogram. For the method, phosphate buffer and ACN were selected as a mobile phase. In studies published in the literature, the % ACN in mobile phase ranged from 60% to 100%, and the flow rate used, ranged from 0.38 mL / min to 1 mL / min, it was decided to try 0.5,1,1.5 and 2 due a shorter retention time. The percentage of acetonitrile in the mobile phase, the flow rate and the temperature were changed as shown in Table 2.4.

**Table 2.4: Parameters tested for the method development of THC in EVOO**

| Mobile phase number | Percentage of ACN used | Flow Rate (mL/min) | Temperature (Cº) |
|---|---|---|---|
| 1 | 90 | 1 | 25 |
| 2 | 90 | 1 | 30 |
| 3 | 90 | 1 | 40 |
| 4 | 80 | 1.5 | 25 |
| 5 | 80 | 0.5 | 40 |
| 6 | 85 | 1 | 40 |
| 7 | 85 | 1.5 | 40 |
| 8 | 85 | 1.5 | 25 |
| 9 | 70 | 1.5 | 40 |
| 10 | 70 | 2 | 40 |

### 2.10.1 Preparation of Internal Standard

Six mL of the IS stock solution of 100µg/mL were placed in a volumetric flask and filled in with MeOH to a concentration of 30 µg/mL.

## 2.11 Method validation for the analysis of THC in EVOO

The HPLC method for the determination and separation of THC from EVOO was validated according to the International Council on Harmonisation Q2 guideline (ICH Q2).[12] The objective of the validation was to determine linearity, range, accuracy, specificity/selectivity, precision, detection limit and quantitation limit.

### 2.11.1 Sample preparation for validation process of THC in EVOO

Different sample concentrations were prepared for the calibration curve. The stock solution of THC in EVOO store in the fridge was placed outside for 2 minutes to assure complete defrost of the oil. The preparation of the sample concentrations was as follows: 0.5 ml of the stock solution of THC at a concentration of 5µg/ml in EVOO was transferring into a centrifuge tube where 0.5 mL of EVOO was placed to proceed with the dilutions. The final concentrations were 5.00 µg/mL, 2.50 µg/mL 1.25 µg/mL, 0.63 µg/mL, 0.16 µg/mL, 0.04 µg/mL Each centrifuge tube was added a couple of drops of IS at concentration of 30µg/ml.

[12] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Validation of analytical procedures: text and methodology Q2(R1) . [Internet] 2017[cited 2021 Feb 08]. Available from https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf.

### 2.11.2 Selectivity/Specificity

To demonstrate the selectivity/specificity a blank sample which consisted of EVOO was ran in triplicate and compared with the analysis of THC in EVOO, by observing both chromatograms it was confirmed that the peak obtained was attributed to THC.

### 2.11.3 Linearity

To demonstrate the linearity of THC in EVOO, the stock solution was diluted in a total of six concentrations previously mentioned in section 2.9.1. To each of the six concentrations a couple of drops of 30 µg/ml of ibuprofen in MeOH were added and the extraction solvent procedure as described in Section 2.7.2 was carried out. The largest concentrations were analysed first, and the smallest concentrations were analysed later. Following every injection of THC in EVOO, an injection of MeOH alone was made onto the column to avoid any carry over of THC.

A calibration curve of the ratio of the AUP of THC to that of ibuprofen (both in mAU.min) against concentration of THC (in µg/ml) was plotted. The correlation coefficient to measure the strength and the direction of the linear relationship between the concentration and the AUP was determined. The concentrations were analyzed in triplicates.

A line of best fit was plotted using the values attained (see section 3.9.2)

In the best-case scenario, a linear relationship- y=mx +b was attained.

Where y= detector response (mAU); x=concentration of THC (µg/ml); m= slope of straight line and b=intercept.

### 2.11.4 Accuracy

To demonstrate the accuracy of the method, three concentrations of THC in EVOO: 0.04, 0.63 and 5.00 µg/ml, were analysed. The concentration of THC in EVOO detected in µg/ml was divided by the true concentration of THC in EVOO that was injected in µg/ml. The results were multiplied by 100 to express it in percentage. This procedure was done in triplicates to ensure reproducibility.

### 2.11.5 Precision

To demonstrate the precision of the method, the means of the percentage Relative Standard Deviation (RSD) were evaluated. The coefficient of variation was calculated by dividing the standard deviation with the mean and multiplying this by 100:

*Relative standard deviation (RSD)= Standard deviation / Mean · 100*

Precision inter-day was determined by injecting the sample at six different concentrations: 0.04, 0.16, 0.63, 1.25, 2.5 and 5.00 µg/ml in triplicates, on three consecutive days.

Intra-day precision was assessed by the analysis of the sample at six different concentrations: 0.04, 0.16, 0.63, 1.25, 2.5 and 5.00 µg/ml in triplicates, on the same day.

### 2.11.6 Stability

The stability of THC in EVOO was determined by storing samples of THC in EVOO at 4ºC. Three different concentrations of THC in EVOO in MeOH were stored. These concentrations were: 0.04, 0.63 and 5.00 µg/ml. These concentrations were analysed after one and three weeks in triplicates.

### 2.11.7 Limit of detection

The Limit Of Detection (LOD) is the lowest concentration analysed that gave a detector signal. The concentration of THC in EVOO was decreased consecutively and injected. The lowest concentration which gave a signal on the detector without the necessity of being quantified and in triplicates was noted. This concentration was considered to be the limit of detection.

### 2.11.8 Limit of quantification

The Limit Of Quantification (LOQ) is the lowest concentration analysed that gave a detector signal and can be quantified. The concentration of THC in EVOO was decreased consecutively and injected. The lowest concentration which gave a signal on the detector and was quantified and in triplicates was noted. This concentration was considered to be the limit of quantification.

## 2.12 Application of the method

Developed and validated method was applied to determine concentration of THC in commercially available CBD in Olive Oil. Extraction and analysis were performed in triplicates and AUP were determined for each chromatogram.

## 2.13 Publications

The following articles were published:

I. Effects of pH and amount of acetonitrile on the separation of cannabinoids, published in the Asian Journal of Pharmaceutical and Clinical Research in 2021.

II. Analytical Techniques Used for Analysis of Cannabinoids published in Cannabis science and technology in 2021.

The articles are available in appendix IV.

# Chapter 3 Results

## 3.1 Results

This section is divided in nine sections : 1)Systematic review 2)Method development for the separation of the three cannabinoids 3)Method validation for the separation of the three cannabinoids 4)method development for the extraction of THC from MCT oil 5)Method development for the analysis of THC in MCT oil 6)Method validation for THC in MCT oil 7)Method development for the extraction of THC from Extra Virgin Olive Oil(EVOO) 8)Method development for the analysis of THC in EVOO 9)Method validation for THC in EVOO

## 3.2 Systematic Review

One hundred and three articles were identified from the literature of which 41 were included in the comparative analysis.

Data of the systematic review is presented in four tables (Appendix I) according to the matrix from which cannabinoids were extracted from:

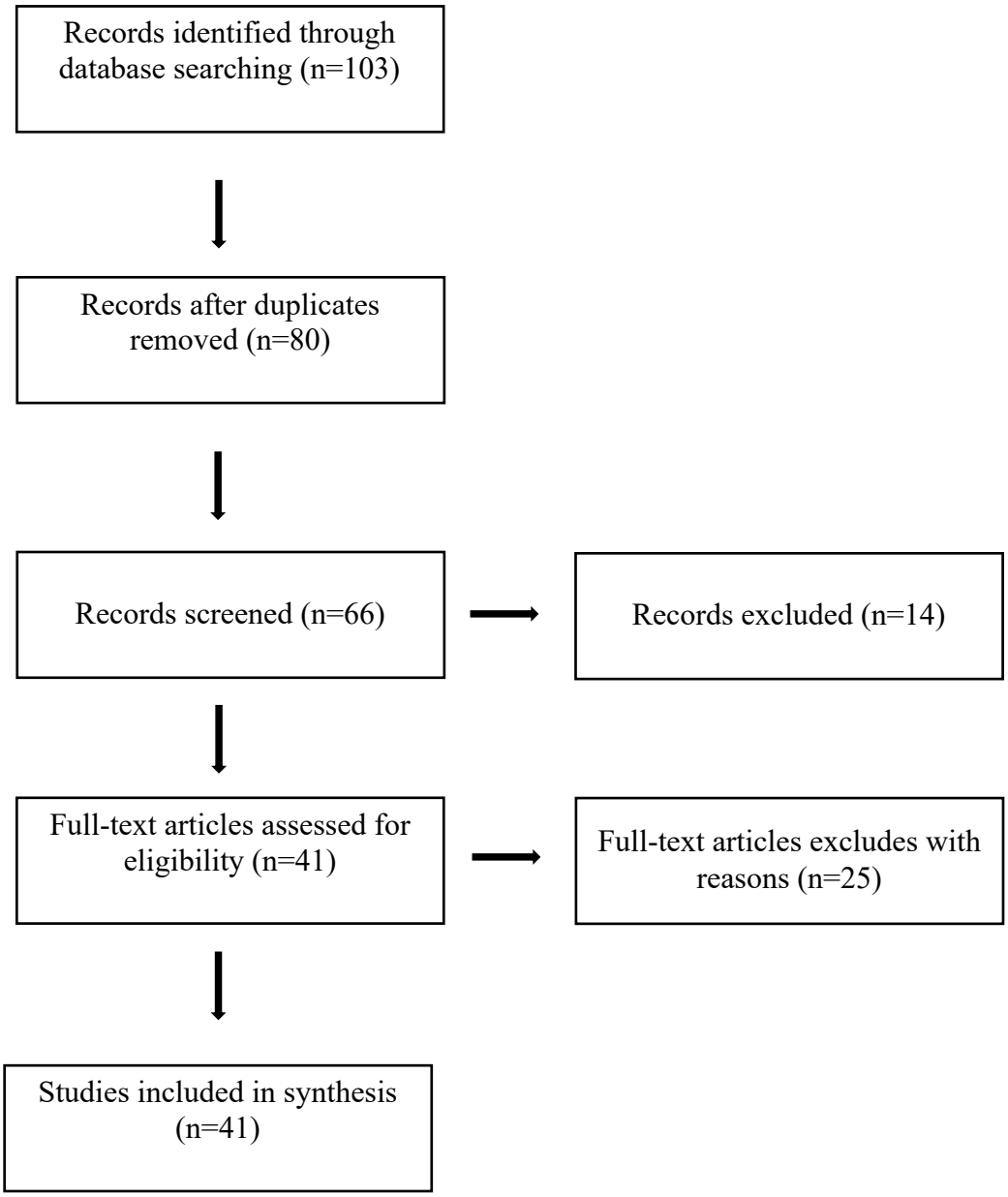


**Figure 3.1: Systematic literature review using PRISMA method**

adapted from Gurusamy K, Toon C, Virendrakumar B, Morris S, Davidson B. Feasibility of comparing the results of pancreatic resections between surgeons: a systematic review and meta-analysis of pancreatic resections. HPB Surgery. 2015; 2015: 1-14

### 3.2.1 Liquid Chromatographic Methods of Analysis of Cannabis from plant material

HPLC is the most commonly used (n=12) Liquid Chromatography (LC)-based method to analyse cannabinoids in plant material. Other methods used were Ultra High-Performance Liquid Chromatography (UHPLC) (n=7), LC(n=2) and a Fast-HPLC(n=1) (Tables 3.1-3.4). Different detectors can be coupled to LC-based methods, for the analysis of cannabinoids in plant material with the most popular are UV and/or Diode-Array (DAD)(n=16). Combined detectors as UV-DAD, Electrospray ionization (ESI)-MS and MS/MS are used in 4 studies (Table 3.3). Other methods used for the analysis of cannabinoids were, UPLC- Travelling Wave Ion Mobility (TWIM)-MS and HPLC-Q-Exactive-Orbitrap-MS (Table 3.4). Solvent extraction is the most commonly used (n=22) analytical technique for sample preparation to extract cannabinoids from plants. Solvents used for extraction of cannabinoids from plants are MeOH and ethanol, used in 7 out of 22 and 7 out 22 studies, respectively. Other extraction methods with solvents, used ACN or mix of solvents. Deville et al, performed the extraction with a mix of methanol/ chloroform (90/10: v/v). Solvent extraction is usually performed with Dynamic Maceration (DM). Brighenti et al, performed four different extraction techniques DM, Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Supercritical fluid extraction (SFE) and compared the results. Ribeiro Grijó et al, carried out the extraction process using SPE with supercritical carbon dioxide (scCO2).

**Table 3.1: Analysis of cannabinoids from plant using HPLC coupled to UV and/or DAD**

| Method | Cannabinoids | Author and date |
|---|---|---|
| HPLC-UV/DAD | CBD, CBDA, CBG & CBGA | Brighenti et al, 2019 |
| HPLC-DAD | THC, THCA, CBD, CBDA & CBN | Ciolino et al, 2018 |
| HPLC-UV | CBD, CBDV & CBDB | Citti et al, 2019 |
| HPLC-DAD | THC, THCA, CBDA, CBD, CBG, CBC, Δ-8 THC & CBN | Giese et al, 2015 |
| HPLC-UV | THC, CBD, CBN, CBDA, CBGA, THCA, THCV, CBG & d8-THC | Križman, 2019 |
| HPLC-UV | Δ9-THC, THCA, Δ8-THC, CBD, CBDA, CBG, CBN, CBC & THCV | Mudge et al,2017 |
| HPLC-DAD | THC, CBD & CBN | Ribeiro Grijó et al, 2019 |

Table 3.1 shows the analysis of different cannabinoids using an HPLC method coupled to UV or DAD detector.

**Table 3.2 : Analysis of cannabinoids from plant using UHPLC**

| Method | Cannabinoids | Author and date |
|---|---|---|
| UHPLC-MS | THC, CBD & THCA | Bala et al, 2019 |
| UHPLC-DAD | THC, CBDA, CBG, CBGA, THCA, CBD & CBN | Deville et al, 2020 |
| UHPLC-DAD | THC, CBD, CBC, CBN, CBG, THCA & CBDA | Elkins et al, 2019 |
| UHPLC-DAD | THC, CBC, CBD, Δ8-THC, THCA, CBDA, THCV & CBDV | Fekete et al,2018 |
| UHPLC-UV | THC, CBD, CBN, THCA, CBDA, CBG, CBDVA, CBL, CBGA, CBDV, CBC, THCV & Δ8-THC | Mudge et al, 2018 |
| UHPLC-UV<br>UHPLC-MS/MS | THC, CBD, CBN, CBDA, CBGA, CBDV, THCA, CBG & Δ8-THC | Nemeškalová et al, 2020 |

Table 3.2 shows the analysis of different cannabinoids using an UHPLC.

**Table 3.3 : Analysis of cannabinoids from plant using HPLC coupled to combined detectors**

| Method | Cannabinoids | Author and date |
|---|---|---|
| HPLC-UV/DAD<br>HPLC-ESI-MS | CBD, CBDA, CBG & CBGA | Brighenti et al,2017 |
| HPLC-MS/MS | THC, CBD, CBC, CBG, CBN, CBDV, THCA CBGA & CBDA | Palmieri et al, 2019 |
| HPLC-ESI-MS<br>HPLC-MS/MS | CBDA, CBGA, CBG & CBD | Pellati et al,2018 |
| HPLC-MS/MS | THC, CBD, CBN, CBG, CBDA & THCA | Zweigenbaum, 2020 |

Table 3.3 shows the analysis of different cannabinoids using an HPLC coupled to combined detectors.

**Table 3.4 : Analysis of cannabinoids from plant using different U/HPLC methods**

| Method | Cannabinoids | Author and date |
|---|---|---|
| Fast-HPLC-DAD | THC, CBN, CBD & THCA | Burnier et al,2019 |
| UPLC-MS<br>UPLC-TWIM-MS | Δ9-THC, CBD, CBC, CBN, CBG, Δ9-THCA A & CBDA | Dossantos et al, 2018 |
| HPLC-Q-Exactive-Orbitrap-MS | THC, CBD, CBN, CBG, CBC, CBDV, THCV, CBDA, THCA, CBNA, CBCA, CBGA, CBDVA & THCVA | Pavlovic et al, 2019 |

Table 3.4 shows the analysis of different cannabinoids using different U/HPLC methods.

**Table 3.5 : Analysis of cannabinoids from plant using LC**

| Method | Cannabinoids | Author and date |
|---|---|---|
| LC-MS | THC, CBD, CBC, THCA, CBDA, THCV, CBDV, THCVA, CBDVA, CBCA & CBL | Dong et al, 2019 |
| LC-MS/MS | CBN | Hidayati et al, 2020 |

The most commonly used HPLC unit was Agilent system (n=11), different modular model systems were used. Among those studies using Agilent system, the modular model 1100 and 1290 were the most popular and were used in three studies, two studies used modular model 1200 system, one study used modular model 1260 system and other study used modular model 1220 system. Waters HPLC unit was used in 4 studies out of 22. Other HPLC systems used were Thermo LTQ XL by Dong et al, Finnigan Surveyor by Križman, and Nexera LC20AD XR system by Palmieri et al.

The majority of the studies published used a C18 column with Poroshell® (n=4), Kinetex® (n=3) and Ascentis®(n=3) being the brands of the stationary phases most commonly used. Among the different mobile phases used in the literature, gradient mobile phase is the most commonly used and 4 studies out 22 used an Isocratic mobile phase. The majority of the mobile phases were composed of water and an organic solvent (n=16), usually MeOH and ACN being the last one preferred. The flow rate of the mobile phases ranged from 0.3mL/min to 3mL/min, with 0.4mL/min(n=5) and 0.3mL/min (n=5) being the most commonly used.

### 3.2.2 Liquid Chromatography Methods of Analysis of Cannabis from human fluids and hair

UHPLC is the most popular method of analysis of cannabinoids from biological fluids and hair. Six studies were carried out using UHPLC (Table 3.6), three used HPLC (Table 3.7) and Toennes et al, used Agilent 1290 Infinity LC system (Table 3.8). The samples included urine (n=4), hair (n=1), human plasma (n=3), human serum (n=2), blood (n=1) and sweat (n=1). The detector most widely used in the analysis of cannabinoids from biological fluids is MS/MS (n=9).

**Table 3.6 : Analysis of cannabinoids from biological fluids using UHPLC**

| Method | Cannabinoids | Author and date |
|---|---|---|
| UHPLC-MS/MS | THC-COOH | Cho et al,2018. |
| UHPLC-MS | CBD | Dybowski et al,2020 |
| UHPLC-MS/MS | THC, CBD & CBN | Moorthy et al,2019 |
| UHPLC-MS/MS | THC, CBD, THCA-A, CBDA, THC-COOH, THC-COOH-gluc,11-OH-THC & THC-gluc | Pichini et al, 2019 |
| UHPLC-MS/MS | THC, CBD, CBN, 11-OH-THC & THC-COOH | Pires da Silva 2020 |
| UHPLC–MS/MS | THC, COOH–THC, OH–THC, CBD & CBN | Wei et al, 2015 |

**Table 3.7 : Analysis of cannabinoids from biological fluids using HPLC**

| Method | Cannabinoids | Author and date |
|---|---|---|
| HPLC-MS/MS | THC, CBD, CBN and THC-COOH | Chang et al, 2016 |
| HPLC-MS/MS | THC, 11-OH-THC & THC-COOH | Dziadosz et al, 2016 |
| HPLC-MS/MS | THC, 11-OH-THC, THC-COOH, THC-C-gluc, CBD, CBN, CBG, CBDV, THCV & THCV-COOH | Klawitter et al, 2017 |

Table 3.7 shows the three studies which used an HPLC method for the analysis of cannabinoids in biological fluids and hair.

**Table 3.8 : Analysis of cannabinoids from biological fluids using LC**

| Method | Cannabinoids | Author and date |
|---|---|---|
| LC-MS/MS | THC, THCOH & THCCOOH | Toennes et al, 2014 |

Table 3.8 shows the only study which used a LC method for the analysis of cannabinoids in biological fluids and hair.

Different techniques were performed to extract cannabinoids from human fluids. Protein precipitation (PP) is a popular technique used for the sample preparation in blood (Zhang et al, 2011). Dybowski et al, Dziadosz et al, and Klawitter et al, carried out protein precipitation. Klawitter et al, performed the same sample preparation for both matrices, plasma and urine. Moorthy et al, 2009 used VAMS$^{TM}$ devices and used SPE, Toennes et al, and Weit et al, used SPE. Pires de Silva et al, used Salting-out Assisted Liquid-Liquid Extraction (SALLE). Chang et al, performed hydrolysis of the urine specimen before the extraction method. Pichini et al, carried out the study in oral fluid, serum, urine and sweat patch samples, the sample preparation for the three first matrices were the same with further alkaline hydrolysis for urine samples for the quantification of CBD as it appears as glucuronide in urine. The extraction of cannabinoids from sweat patch samples was performed with MeOH as extraction solvent. Cho et al, carried out the study in hair and the sample preparation consisting of washing the hair twice with MeOH to eliminate any external contaminants and performed the digestion with 1 M NaOH to free the cannabinoids from the matrix.

The HPLC unit commonly used was Agilent system, Cho et al, performed the analysis in a system consisted of a binary pump, Agilent 1290 UHPLC pump (pump 1), and the additional Agilent 1260 pump (pump 2). Klawitter et al, Chang et al, and Toennes et al, also performed the analysis with an Agilent HPLC unit.

The analyses were performed using C18 columns, 3 out 10 studies used Acquity ®, and 2 out of 10 Kinetex®. The majority (n=9) of the studies used gradient mobile phase, Dybowski et al, performed an Isocratic method. The mobile phases were composed of ammonium formate or water and an organic solvent. ACN and MeOH are the organic solvents more commonly used for the mobile phase, ACN was used in seven studies. The

flow rate of the mobile phases ranged from 0.15 mL/min to 1mL/min, FR 0.4mL/min was the most commonly used (n=3).

### 3.2.3 Liquid Chromatographic Methods of Analysis of Cannabis from oil

HPLC is the method of analysis preferred (n=5) for the determination and quantification of cannabinoids in oil (Table 3.9). Nemeškalová et al, and Pichini et al carried-out analysis using UHPLC (Table 3.10). The detectors most commonly used are UV and PDA(n=5). Araneda et al, performed analysis of cannabinoids using benchtop NMR instruments to compare the results with the ones obtained in HPLC-UV.

Solvent extraction is the method chosen for extracting cannabinoids from oil. Bettiol et al, and Deidda et al, used the same method to extract different cannabinoids from oil, but Bettiol et al used ACN as an organic solvent and Deidda et al, MeOH. Mudge et al, performed solvent extraction with MeOH while Nemeškalová et al, used isopropanol: ethyl acetate (1:1, v/v). Ciolino et al, carried out the method using Ethanol (EtOH) or isopropyl alcohol.

Three different brands of HPLC units were used among the articles published in the literature for the extraction of cannabinoids from oil with the HPLC unit most commonly used being Agilent(n=4), other brand used was Thermo- Fisher Surveyor(n=2) and Pichini et al, used a Waters® Xevo® TQ-S. Bettiol et al, and Deidda et al, used a Thermo-Fisher Surveyor PlusTM HPLC system. The majority of studies were performed using an Agilent Poroshell® as a stationary phase (n=4), Ciolino et al, carried out analysis using an ACE column and Mudge et al, used a Kinetex® column. The seven studies used C18 column. The methods used for the mobile phase were gradient for four studies and isocratic for the other three. The majority of the methods(n=5) used ACN as organic solvent and it ranged from 60 to 100 %. The FR ranged from 0.38 mL/min to 1mL/min

**Table 3.9 : Analysis of cannabinoids from oil using HPLC**

| Method | Cannabinoids | Author and date |
|---|---|---|
| HPLC-DAD | THC & CBD | Araneda et al, 2020 |
| HPLC-DAD | THC, CBD, CBN & THCA | Bettiol et al, 2019 |
| HPLC-DAD | THC, THCA, CBD, CBDA & CBN | Ciolino et al, 2018 |
| HPLC-UV<br>HPLC-MS | THC, CBD, THCA, CBDA, CBDV, CBG & CBN | Citti et al, 2018 |
| RP-HPLC/UV | THC & CBD | Deidda et al, 2019 |
| HPLC-UV | Δ9-THC, THCA, Δ8-THC, CBD, CBDA, CBG, CBN, CBC & THCV | Mudge et al,2017 |

Table 3.9 shows the studies which used an HPLC method for the analysis of cannabinoids in oil.

**Table 3.10 : Analysis of cannabinoids from oil using UHPLC**

| Method | Cannabinoids | Author and date |
|---|---|---|
| UHPLC-UV-MS/MS | THC, CBD, CBN, CBDA, CBGA, CBDV, THCA, CBG & Δ8-THC | Nemeškalová et al, 2020 |
| UHPLC-MS/MS | THC, CBD, THCA-A, CBDA, THC-COOH, THC-COOH-gluc,11-OH-THC & THC-gluc | Pichini et al, 2019 |

Table 3.10 shows the studies which used an UHPLC method for the analysis of cannabinoids in oil.

### 3.2.4 Liquid Chromatography Methods of Analysis of Cannabis from miscellaneous matrices

Some studies were carried in different matrices such as cannabis concentrates, honey, hemp nut, resin, vaporized fluid, milk, liver, capsules, wastewater, cotton cloths, gummies. The majority of the studies(n=6) used an Agilent HPLC unit. Brighenti et al, and Chang et al, carried out their studies using an Agilent 1200 HPLC system. Ciolino et al, also used an Agilent 1200 HPLC system, as well as an Agilent 1100 and 1260 HPLC system. HPLC 1260 system was also used by Escrivá et al, and by Jornet-Martínez et al, but Escrivá et al,used an UHPLC and Jornet-Martínez et al, using an Infinity nanoLC chromatograph instead of HPLC. Elkins et al, and Nemeškalová et al, carried out their studies in an Agilent 1290. Among the others HPLC units used, Duffy et al, and Jacox et al, performed their studies using HPLC from Shimadzu but Jacox et al, used UHPLC instead of HPLC. The stationary phase used were three out of 11 studies used a Kinetex® column, other brands used were Poroshell ®, Phenomenex ®, ACE®, Supelco Ascentis ®, Zorbax ® and Water Acquity. All the studies used a C18 column. The majority of the studies used gradient mobile phase (n=8) with ACN (n=5) as the most common organic solvent ranging from 5% to 100%. The flow rate ranged from 0.18mL/min to 1.5 mL/min.

### 3.3 Method development for the separation of the three cannabinoids

During method development, different parameters were tested and area under the peak, retention time, peak resolution, peak tailing and shape of peak were compared.

The UV detector was set at 220 and 228 nm. The areas under the peak attained for CBD, CBN and THC at 220nm were larger when compared to 228nm. The area under the peak for THC was smaller than the peak for CBD and CBN for equal concentrations (5µg/mL) of the three cannabinoids (Figure 3.2 and 3.7)

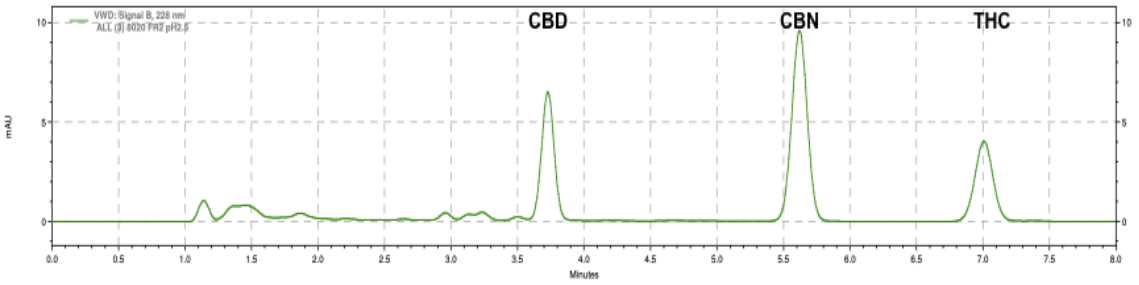


**Figure 3.2: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 228nm; flow rate 2 mL/min**

To investigate the effect of the pH in the analysis, different pHs were tested, when observing the chromatograms of pH 2.5 and pH 3 (Appendix II), almost no difference can be noted in terms of peak shape or area under the peak. When the buffer pH was raised to 4 and 6 there were some irregularities in the baseline but this did not have any effect in the shape and area under the peak of the three cannabinoids (Appendix II)

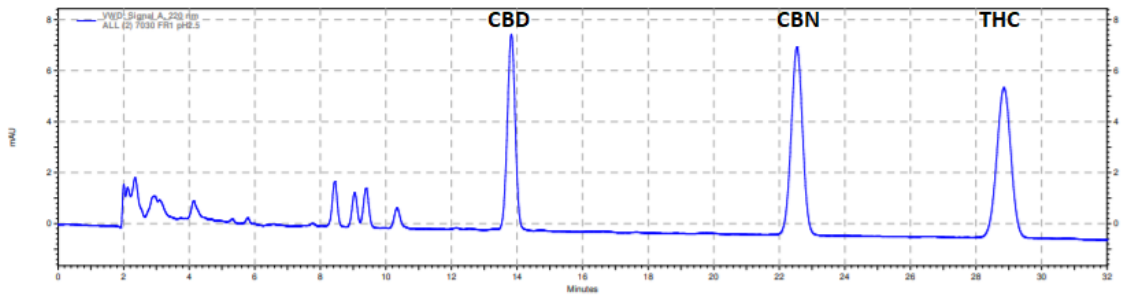


**Figure 3.3: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 1mL/min**

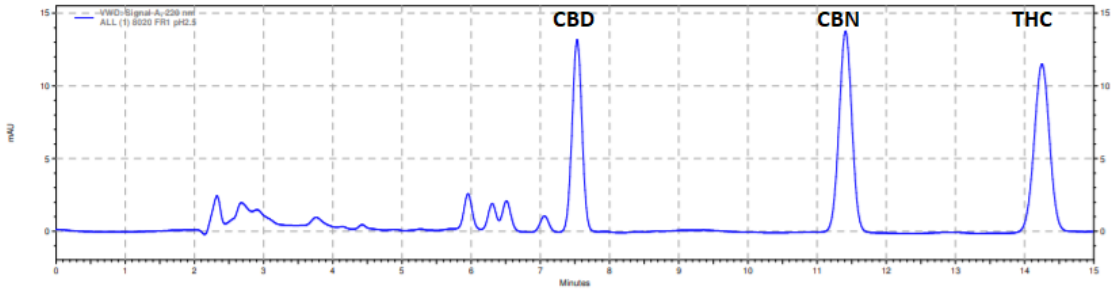


**Figure 3.4: Chromatogram produced using phosphate buffer (pH2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1mL/min**

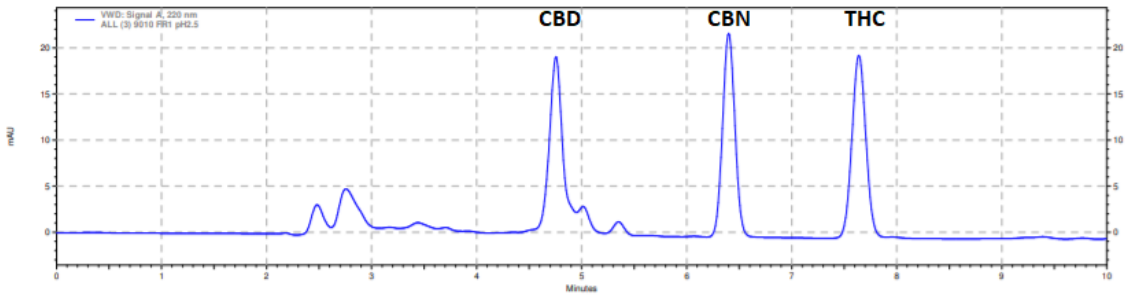


**Figure 3.5: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (10:90 v/v); detection wavelength 220nm; flow rate 1mL/min**

When observing the chromatograms generated, as the percentage of acetonitrile in the mobile phase was raised from 70% to 80% the retention time of the three cannabinoids decreased (Figures 3.3 and 3.4). The retention time kept decreasing when the percentage of acetonitrile was raised to 90% but the peak shape of CBD was compromised and unsymmetrical (Figure 3.5). The decrease in retention time with a loss of symmetry of peak occurred at all pH values 2.5, 3, 4 and 6 (Appendix II).

The mobile phase consisting in buffer (pH2.5): ACN 20:80 (v/v) was chosen as it produced great results in terms of area under the peak, shape of the peak and retention time. When the flow rate of the mobile phase raised from 1 to 1.5 to 2mL/minute, the total run time for the analysis of the three cannabinoids decreased from 14.3 to 9.4 to 7.0 minutes respectively (Figure 3.4, 3.6 and 3.7).

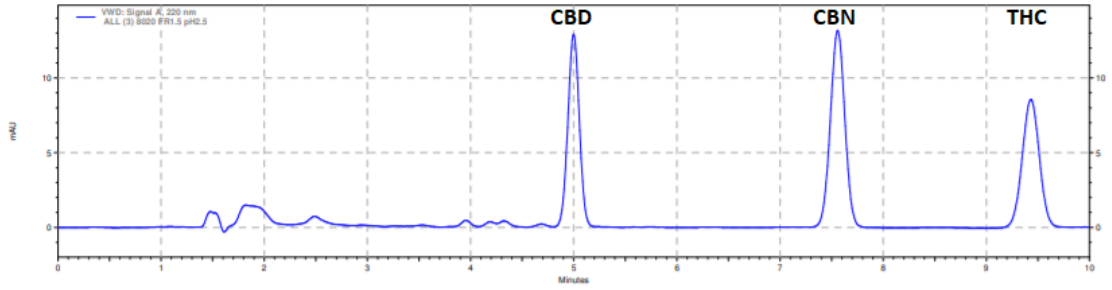


**Figure 3.6: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1.5 mL/min**

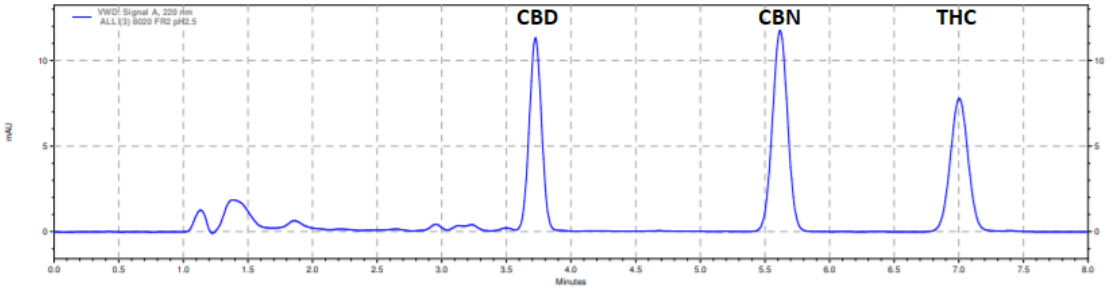


**Figure 3.7: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 2 mL/min**

The last parameter tested was the temperature of the stationary phase, but no difference in the areas under the peak of CBD, CBN and THC were found when the column temperature was changed from 25°C to 20°C and 30°C.

The parameters which gave the peaks of CBD, CBN and THC with good areas under the peak and good shapes were as shown in Table 3.11.

**Table 3.11 : Selected chromatographic parameters for the determination of CBD, CBN and THC in MeOH**

| | |
|---|---|
| Instrumentation | Agilent 1260 Infinity Series® II HPLC unit |
| Stationary phase | RP ACE® C18 column (250 x4.6mm; 5µm particle size) at 25°C |
| Mobile phase | 0.02M phosphate buffer (pH 2.5) and ACN 20:80 v/v, Flow rate 2mL/min |
| UV detection | 220 nm |

### 3.4 Method validation for the separation of the three cannabinoids

The following results were obtained when CBD, CBN and THC were analysed in MeOH during the validation of the method developed. The method was found to have linearity, selectivity to CBD, CBN and THC, precision, with a relatively low quantification and detection limit. CBD, CBN and THC were stable when stored in MeOH at -20ºC.

#### 3.4.1 Selectivity/Specificity

When a blank of MeOH was injected, the chromatogram produced was compared with the chromatogram produced for the analysis of the three cannabinoids. As there were no peaks or signals produced at the retention time of CBD, CBN and THC, method is specific to the analytes of interest

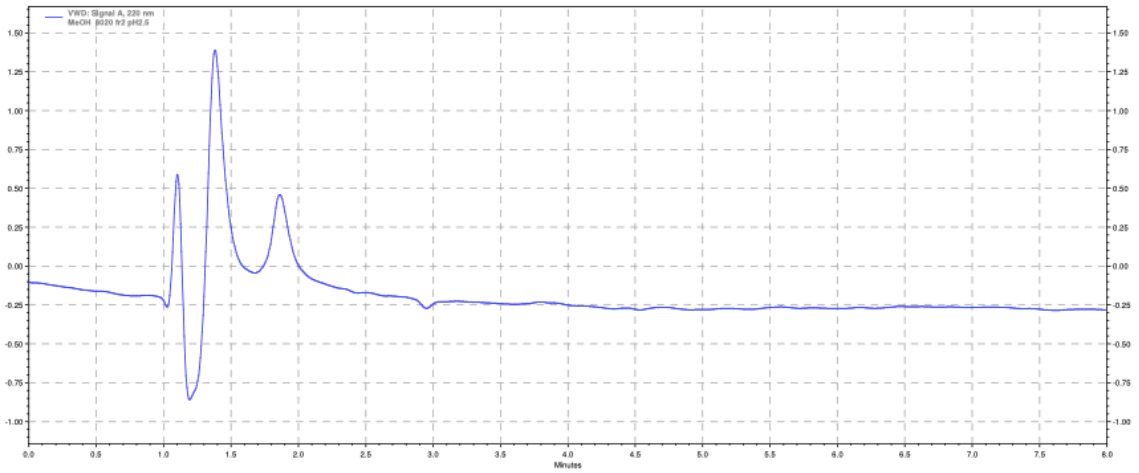


**Figure 3.8: Chromatogram of a blank MeOH produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 2 mL/min:**

When blank of MeOH was analysed using the developed method there were no peaks eluting at the retention time when CBD (around 3.9 minutes), CBN (around 6 minutes) and THC (around 7.5 minutes). This shows that the method is selective for CBD, CBN and THC.

### 3.4.2 Linearity

The calibration curve of CBD, CBN and THC was set up by injecting 7 different concentration levels, ranging from 0.04 to 5 µg/ml. Linearity was determined by obtaining a linear relationship between the AUP of CBD, CBN and THC against 7 different sample concentrations of CBD, CBN and THC.

Figures 3.9-3.12 show the calibration curve obtained when solutions of:

- 5 µg/ml
- 2.5 µg/ml
- 1.25 µg/ml
- 0.63 µg/ml
- 0.31 µg/ml
- 0.16 µg/ml
- 0.04 µg/ml

of CBD, CBN and THC were analysed in MeOH.

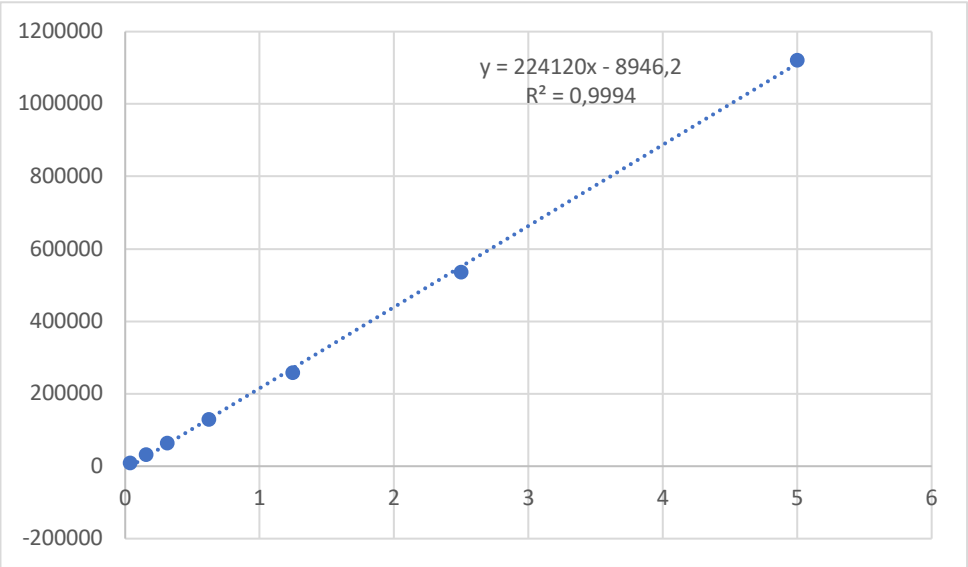


**Figure 3.9: Calibration curve for CBD in MeOH:**

Plot of AUP vs concentration in µg/ml. When the seven concentrations of CBD in MeOH were analysed, an $r^2$ of 0.9994 was obtained, indicating that the detector signal and AUP obtained is linearly proportional to the concentration of CBD being analysed in MeOH

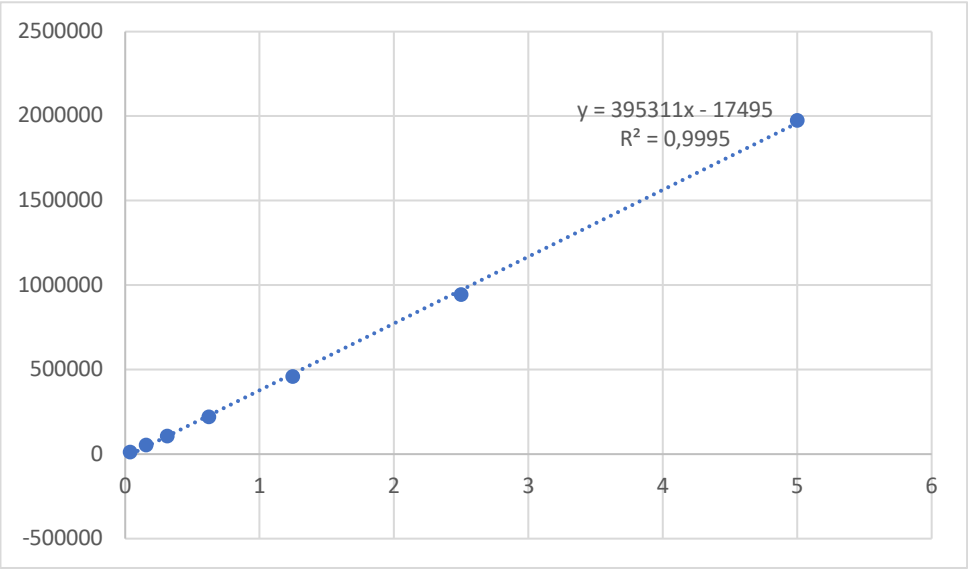


**Figure 3.10: Calibration curve for CBN in MeOH:**

Plot of AUP vs concentration in µg/ml. When the seven concentrations of CBN in MeOH were analysed, an $r^2$ of 0.9995 was obtained, indicating that the detector signal and AUP obtained is linearly proportional to the concentration of CBN being analysed in MeOH

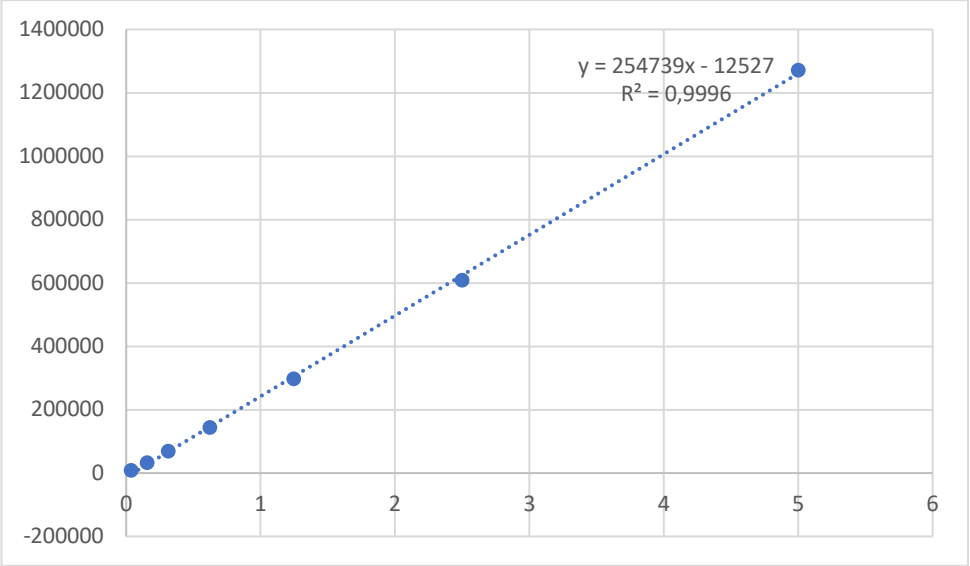


**Figure 3.11: Calibration curve for THC in MeOH:**

Plot of AUP vs concentration in µg/ml. When the seven concentrations of THC in MeOH were analysed, an $r^2$ of 0.9996 was obtained, indicating that the detector signal and AUP obtained is linearly proportional to the concentration of THC being analysed in MeOH

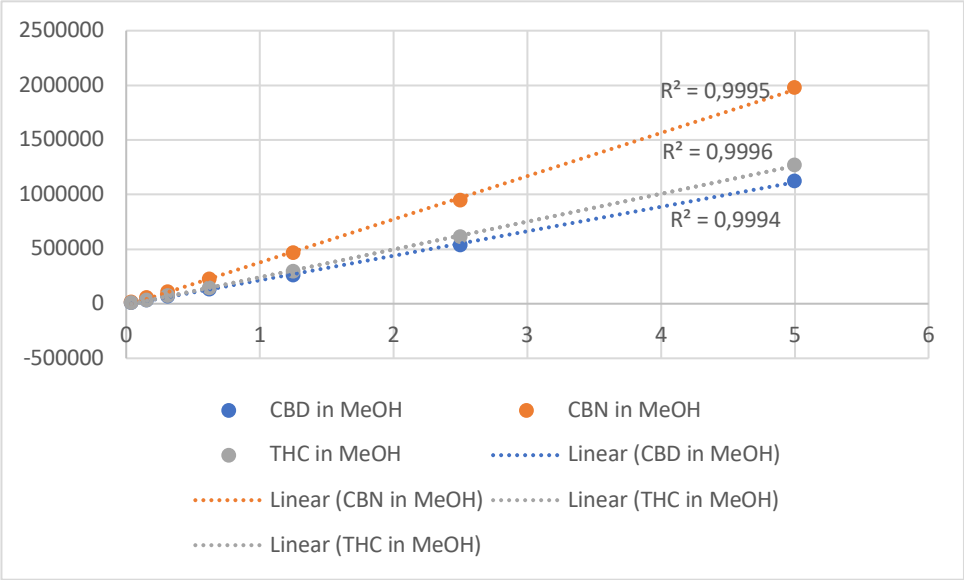


**Figure 3.12: Calibration curve for the three cannabinoids in MeOH:**

Plot of AUP vs concentration in µg/ml.

### 3.4.3 Accuracy

Results of accuracy and recovery from the three cannabinoids were acceptable (Tables 3.12-3.14). All percentage recoveries calculated were between 96.648% and 104.302%.

**Table 3.12 : Accuracy for CBD analysis**

| Accuracy CBD | | |
|---|---|---|
| Standard concentration of CBD (µg/ml) | Concentration of CBD which was quantified in MeOH (µg/ml) | Percentage recovery |
| 0.039 | 0.041 | 104.302% |
| 0.039 | 0.039 | 99.050% |
| 0.039 | 0.038 | 96.648% |
| 0.625 | 0.615 | 98.382% |
| 0.625 | 0.615 | 98.378% |
| 0.625 | 0.646 | 103.412% |
| 5.000 | 4.941 | 98.823% |
| 5.000 | 5.020 | 100.399% |
| 5.000 | 5.039 | 100.777% |

The developed method is accurate since all percentage recoveries calculated were above 96.645%

**Table 3.13 : Accuracy for CBN analysis**

| Accuracy CBN | | |
|---|---|---|
| Standard concentration of CBN (µg/ml) | Concentration of CBN which was quantified in MeOH (µg/ml) | Percentage recovery |
| 0.039 | 0.039 | 100.692% |
| 0.039 | 0.038 | 98.008% |
| 0.039 | 0.040 | 101.300% |
| 0.625 | 0.627 | 100.299% |
| 0.625 | 0.628 | 100.537% |
| 0.625 | 0.620 | 99.164% |
| 5.000 | 4.981 | 99.629% |
| 5.000 | 5.015 | 100.292% |
| 5.000 | 5.004 | 100.079% |

The developed method is accurate since all percentage recoveries calculated were above 98.005%

**Table 3.14: Accuracy for THC analysis**

| Accuracy THC | | |
|---|---|---|
| Standard concentration of THC (µg/ml) | Concentration of THC which was quantified in MeOH (µg/ml) | Percentage recovery |
| 0.039 | 0.038 | 96.930% |
| 0.039 | 0.038 | 97.932% |
| 0.039 | 0.041 | 105.140% |
| 0.625 | 0.630 | 100.634% |
| 0.625 | 0.617 | 98.707% |
| 0.625 | 0.630 | 100.656% |
| 5.000 | 4.953 | 99.686% |
| 5.000 | 5.063 | 101.251% |
| 5.000 | 4.984 | 99.681% |

The developed method is accurate since all percentage recoveries calculated were above 96.925%

### 3.4.4 Precision

The method was found to have an acceptable intra-day and inter-day precision

#### 3.4.4.1 Intra-day precision

The results obtained when the seven concentrations of CBD, CBN and THC were analysed in triplicates to calculate intra-day precision of the method are shown in tables 3.15-3.17.

The RSD results attained were below 7.800% showing acceptable intra-day precision.

#### 3.4.4.2 Inter-day precision

The RSD results obtained when each of the seven concentrations of CBD, CBN and THC were analysed once every day on three different days are shown in tables 3.18-3.20. RSD were all below 10.015% indicating acceptable inter-day precision.

**Table 3.15 : Intra-day precision of CBD in MeOH**

| Intra-day precision | | | | | | | |
|---|---|---|---|---|---|---|---|
| Replicate number | Concentration CBD in MeOH (µg/ml) | | | | | | |
| | 5.000 | 2.500 | 1.250 | 0.625 | 0.313 | 0.156 | 0.039 |
| 1 | 4.941 | 2.494 | 1.25 | 0.61 | 0.30 | 0.14 | 0.041 |
| 2 | 5.020 | 2.491 | 1.25 | 0.61 | 0.31 | 0.16 | 0.039 |
| 3 | 5.039 | 2.515 | 1.26 | 0.64 | 0.33 | 0.17 | 0.038 |
| Mean | 5.000 | 2.500 | 1.25 | 0.63 | 0.31 | 0.16 | 0.039 |
| SD | 0.052 | 0.013 | 0.007 | 0.018 | 0.012 | 0.012 | 0.002 |
| RSD(%) | 1.037 | 0.520 | 0.577 | 2.904 | 3.719 | 7.755 | 3.915 |

The RSD results attained were below 7.800% showing acceptable intra-day precision for CBD.

**Table 3.16 : Intra-day precision CBN in MeOH**

| Intra-day precision | | | | | | | |
|---|---|---|---|---|---|---|---|
| Replicate number | Concentration CBN in MeOH (µg/ml) | | | | | | |
| | 5.000 | 2.500 | 1.250 | 0.625 | 0.313 | 0.156 | 0.039 |
| 1 | 4.981 | 2.489 | 1.246 | 0.627 | 0.315 | 0.158 | 0.039 |
| 2 | 5.015 | 2.509 | 1.254 | 0.628 | 0.309 | 0.158 | 0.038 |
| 3 | 5.004 | 2.502 | 1.247 | 0.620 | 0.313 | 0.152 | 0.040 |
| Mean | 5.000 | 2.500 | 1.249 | 0.625 | 0.313 | 0.156 | 0.039 |
| SD | 0.017 | 0.010 | 0.004 | 0.005 | 0.003 | 0.003 | 0.001 |
| RSD(%) | 0.338 | 0.396 | 0.338 | 0.734 | 1.060 | 2.156 | 1.752 |

The RSD results attained were below 2.200% showing acceptable intra-day precision for CBN.

**Table 3.17: Intra-day precision THC in MeOH**

| Intra-day precision | | | | | | | |
|---|---|---|---|---|---|---|---|
| Replicate number | Concentration CBD in MeOH (µg/ml) | | | | | | |
| | 5.000 | 2.500 | 1.250 | 0.625 | 0.313 | 0.156 | 0.039 |
| 1 | 4.953 | 2.495 | 1.241 | 0.629 | 0.313 | 0.154 | 0.038 |
| 2 | 5.063 | 2.509 | 1.259 | 0.617 | 0.298 | 0.155 | 0.038 |
| 3 | 4.984 | 2.496 | 1.243 | 0.629 | 0.326 | 0.159 | 0.041 |
| Mean | 5.000 | 2.500 | 1.248 | 0.625 | 0.313 | 0.156 | 0.039 |
| SD | 0.056 | 0.008 | 0.010 | 0.007 | 0.014 | 0.002 | 0.002 |
| RSD(%) | 1.126 | 0.313 | 0.771 | 1.120 | 4.463 | 1.534 | 4.480 |

The RSD results attained were below 4.500% showing acceptable intra-day precision for THC.

**Table 3.18 : Inter-day precision of CBD in MeOH**

| Inter-day precision | | | | | | | |
|---|---|---|---|---|---|---|---|
| DAY | Concentration CBD in MeOH (µg/ml) | | | | | | |
| | 5.000 | 2.500 | 1.250 | 0.625 | 0.313 | 0.156 | 0.039 |
| 1 | 4.941 | 2.494 | 1.248 | 0.615 | 0.303 | 0.144 | 0.041 |
| 2 | 5.151 | 2.571 | 1.330 | 0.676 | 0.329 | 0.161 | 0.039 |
| 3 | 4.712 | 2.420 | 1.144 | 0.567 | 0.282 | 0.135 | 0.039 |
| Mean | 4.935 | 2.495 | 1.241 | 0.619 | 0.305 | 0.147 | 0.040 |
| SD | 0.219 | 0.076 | 0.093 | 0.055 | 0.023 | 0.013 | 0.001 |
| RSD(%) | 4.445 | 3.030 | 7.511 | 8.861 | 7.700 | 9.135 | 2.525 |

The RSD results attained were below 9.150% showing acceptable inter-day precision for CBD.

**Table 3.19 : Inter-day precision CBN in MeOH**

| Inter-day precision | | | | | | | |
|---|---|---|---|---|---|---|---|
| DAY | Concentration CBN in MeOH (µg/ml) | | | | | | |
| | 5.000 | 2.500 | 1.250 | 0.625 | 0.313 | 0.156 | 0.039 |
| 1 | 4.981 | 2.489 | 1.246 | 0.627 | 0.315 | 0.158 | 0.039 |
| 2 | 5.070 | 2.518 | 1.252 | 0.628 | 0.348 | 0.170 | 0.040 |
| 3 | 5.292 | 2.695 | 1.261 | 0.645 | 0.306 | 0.160 | 0.042 |
| Mean | 5.114 | 2.567 | 1.253 | 0.633 | 0.323 | 0.163 | 0.040 |
| SD | 0.160 | 0.112 | 0.007 | 0.010 | 0.022 | 0.007 | 0.002 |
| RSD(%) | 3.123 | 4.345 | 0.586 | 1.585 | 6.816 | 4.049 | 4.380 |

The RSD results attained were below 6.820% showing acceptable inter-day precision for CBN.

**Table 3.20: Inter-day precision THC in MeOH**

| Inter-day precision | | | | | | | |
|---|---|---|---|---|---|---|---|
| DAY | Concentration THC in MeOH (µg/ml) | | | | | | |
| | 5.000 | 2.500 | 1.250 | 0.625 | 0.313 | 0.156 | 0.039 |
| 1 | 4.953 | 2.500 | 1.241 | 0.630 | 0.313 | 0.154 | 0.038 |
| 2 | 5.046 | 2.510 | 1.252 | 0.632 | 0.345 | 0.174 | 0.040 |
| 3 | 4.964 | 2.527 | 1.166 | 0.615 | 0.282 | 0.168 | 0.040 |
| Mean | 4.988 | 2.511 | 1.220 | 0.625 | 0.314 | 0.165 | 0.039 |
| SD | 0.051 | 0.016 | 0.047 | 0.009 | 0.031 | 0.010 | 0.001 |
| RSD(%) | 1.021 | 0.633 | 3.827 | 1.508 | 10.011 | 6.182 | 2.594 |

The RSD results attained were below 10.015% showing acceptable inter-day precision for THC.

### 3.4.5 Stability

The three chosen concentrations of CBD, CBN and THC in MeOH (5.00, 0.63 and 0.04 μg/mL) were stored at -20ºC and analysed after one and three weeks, the quantities detected in chromatograms produced following analysis after one week and three weeks were not significantly different from the quantities analysis immediately after the sample was prepared.

Results for stability after one and three weeks using this method can be seen in Tables 3.21-3.26.

**Table 3.21 : Stability of CBD after one week**

| Stability of CBD after one week | | | |
|---|---|---|---|
| Replicate number | Concentration CBD in MeOH (µg/ml) | | |
| | 5.000 | 0.625 | 0.039 |
| 1 | 4.868 | 0.599 | 0.043 |
| 2 | 4.912 | 0.658 | 0.039 |
| 3 | 4.909 | 0.656 | 0.042 |
| Mean | 4.896 | 0.638 | 0.041 |
| SD | 0.024 | 0.033 | 0.002 |
| RSD | 0.493 | 5.244 | 5.096 |

When concentrations of CBD were analysed after 1 week of stored at -20ºC, RSD results attained were below 5.245% showing CBD is stable when stored in that conditions.

**Table 3.22: Stability of CBN after one week**

| Stability of CBN after one week | | | |
|---|---|---|---|
| Replicate number | Concentration CBN in MeOH (µg/ml) | | |
| | 5.000 | 0.625 | 0.039 |
| 1 | 5.426 | 0.647 | 0.042 |
| 2 | 5.455 | 0.663 | 0.040 |
| 3 | 5.463 | 0.660 | 0.039 |
| Mean | 5.448 | 0.656 | 0.040 |
| SD | 0.019 | 0.009 | 0.002 |
| RSD | 0.355 | 1.320 | 4.802 |

When concentrations of CBN were analysed after 1 week of stored at -20ºC, RSD results attained were below 4.810% showing CBN is stable when stored in that conditions.

**Table 3.23: Stability of THC after one week**

| Stability of THC after one week | | | |
|---|---|---|---|
| Replicate number | Concentration THC in MeOH (µg/ml) | | |
| | 5.000 | 0.625 | 0.039 |
| 1 | 5.078 | 0.608 | 0.041 |
| 2 | 5.043 | 0.624 | 0.040 |
| 3 | 5.107 | 0.623 | 0.038 |
| Mean | 5.076 | 0.618 | 0.040 |
| SD | 0.032 | 0.009 | 0.002 |
| RSD(%) | 0.630 | 1.425 | 4.503 |

When concentrations of THC were analysed after 1 week of stored at -20ºC, RSD results attained were below 4.510% showing THC is stable when stored in that conditions.

**Table 3.24 : Stability of CBD after three weeks**

| Stability of CBD after three weeks | | | |
|---|---|---|---|
| Replicate number | Concentration CBD in MeOH (µg/ml) | | |
| | 5.000 | 0.625 | 0.039 |
| 1 | 4.911 | 0.640 | 0.038 |
| 2 | 4.958 | 0.687 | 0.035 |
| 3 | 4.929 | 0.676 | 0.032 |
| Mean | 4.933 | 0.668 | 0.035 |
| SD | 0.024 | 0.025 | 0.003 |
| RSD(%) | 0.480 | 3.667 | 9.687 |

When concentrations of CBD were analysed after 3 weeks of stored at -20ºC, RSD results attained were below 9.700% showing CBD is stable when stored in that conditions.

**Table 3.25 :Stability of CBN after three weeks**

| Stability of CBN after three weeks | | | |
|---|---|---|---|
| Replicate number | Concentration CBN in MeOH (µg/ml) | | |
| | 5.000 | 0.625 | 0.039 |
| 1 | 5.140 | 0.666 | 0.031 |
| 2 | 5.151 | 0.657 | 0.033 |
| 3 | 5.136 | 0.670 | 0.034 |
| Mean | 5.143 | 0.665 | 0.033 |
| SD | 0.008 | 0.007 | 0.002 |
| RSD(%) | 0.154 | 1.035 | 5.524 |

When concentrations of CBN were analysed after 1 week of stored at -20ºC, RSD results attained were below 5.530% showing CBN is stable when stored in that conditions.

**Table 3.26 : Stability of THC after three weeks**

| Stability of THC after three weeks | | | |
|---|---|---|---|
| Replicate number | Concentration THC in MeOH (µg/ml) | | |
| | 5.000 | 0.625 | 0.039 |
| 1 | 5.115 | 0.659 | 0.041 |
| 2 | 5.043 | 0.685 | 0.043 |
| 3 | 5.087 | 0.663 | 0.041 |
| Mean | 5.082 | 0.669 | 0.041 |
| SD | 0.036 | 0.014 | 0.001 |
| RSD(%) | 0.713 | 2.076 | 2.245 |

When concentrations of THC were analysed after 1 week of stored at -20ºC, RSD results attained were below 2.250% showing THC is stable when stored in that conditions.

### 3.4.6 Limit of detection

The lowest concentration of CBD, CBN and THC in MeOH that gave a signal but could not be quantified was 0.019 µg/ml (Figure 3.13).

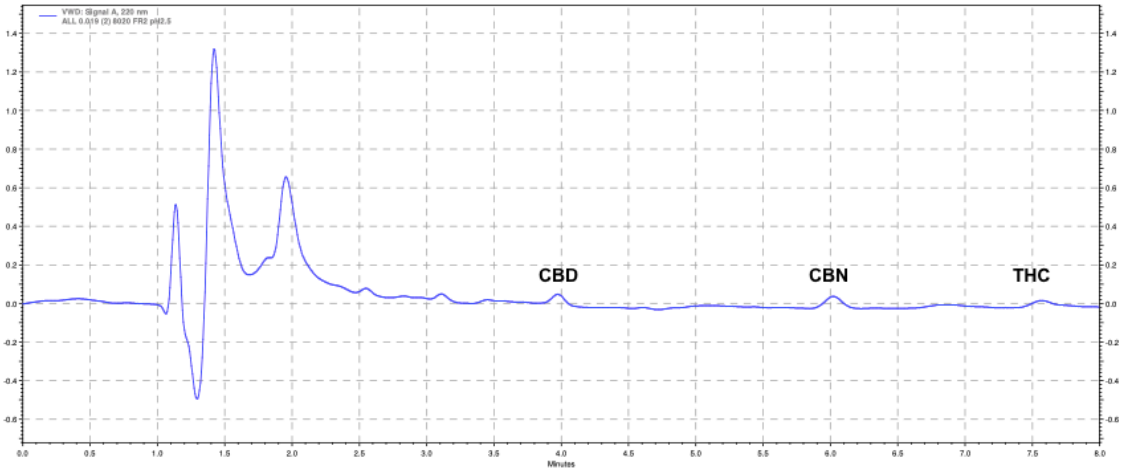


**Figure 3.13: Chromatogram produced when running CBD, CBN and THC at a concentration of 0.019 µg/ml**

### 3.4.7 Limit of quantification

The lowest concentration of CBD, CBN and THC in MeOH that gave a signal and could be quantified with acceptable precision and accuracy was 0.039 µg/ml . (Figure 3.14).

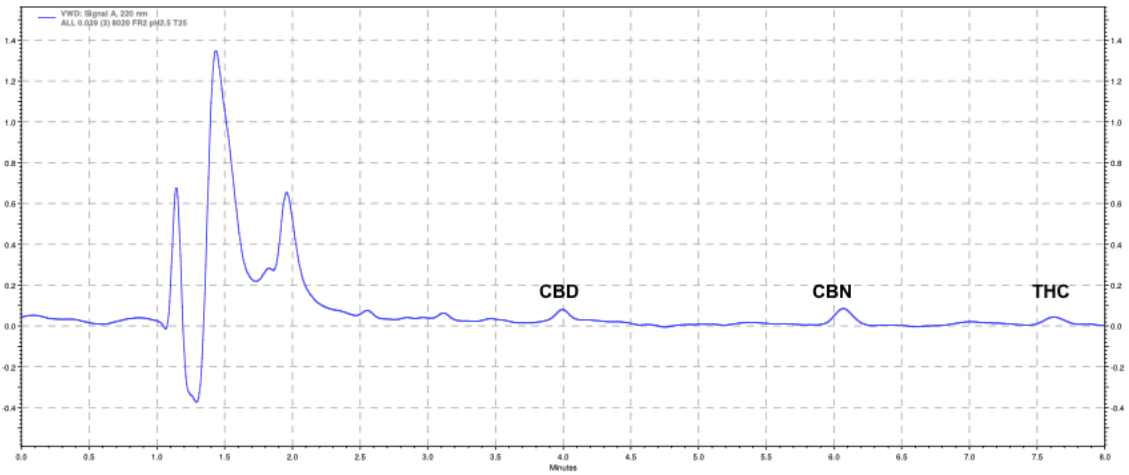


**Figure 3.14: Chromatogram produced when running CBD, CBN and THC at a concentration of 0.039 µg/ml**

### 3.5 Method development for extraction of THC from MCT oil

The best conditions for the extraction of THC from MCT oil, using solvent extraction is illustrated in Figure 3.15.

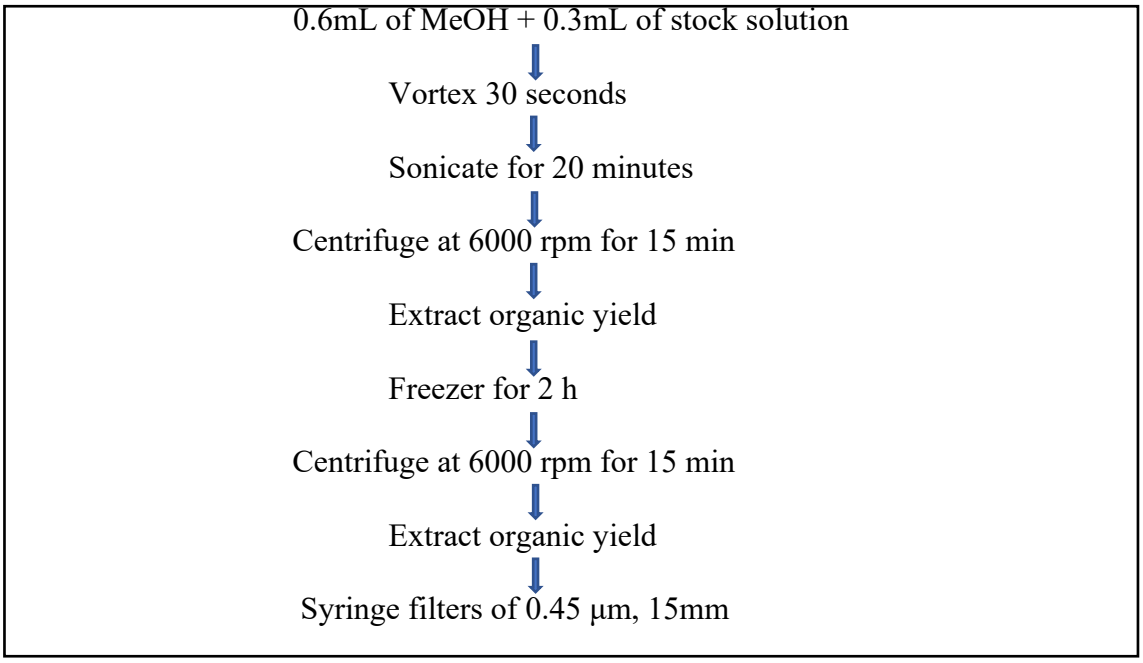


**Figure 3.15: Methodology used for the extraction of THC from MCT oil using MeOH as a solvent**

### 3.6 Method development for the analysis of THC in MCT oil

Different conditions were tested during method development.

The UV detector was set at 220 and 228 nm. Larger areas under the peak were obtained for THC at 220nm when compared to 228nm (Figures 3.16 and 3.22)

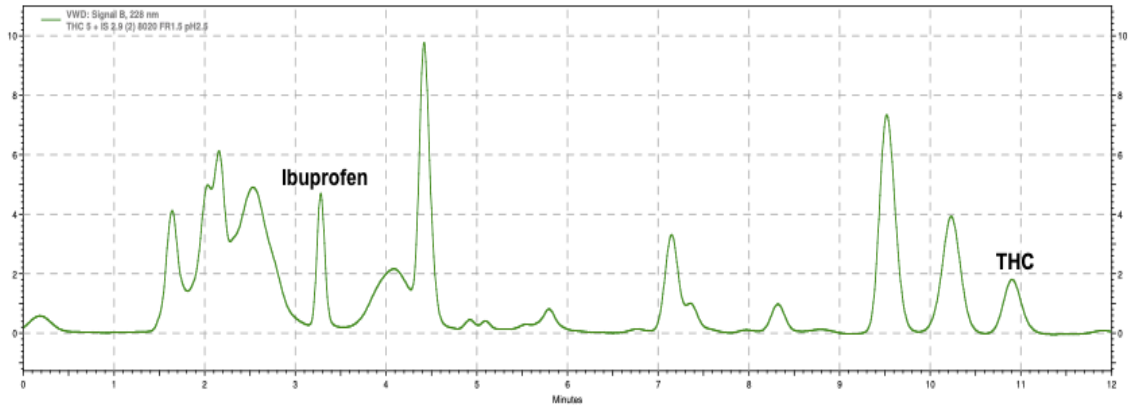


**Figure 3.16: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 228nm; flow rate 1.5 mL/min**

To investigate the effect of the % of ACN in the mobile phase in the analysis, different mobile phases were used. When observing the chromatograms generated, as the amount of acetonitrile in the mobile phase was decreased from 80% to 75% to 70% the retention time of THC increased (Figures 3.17-3.19).

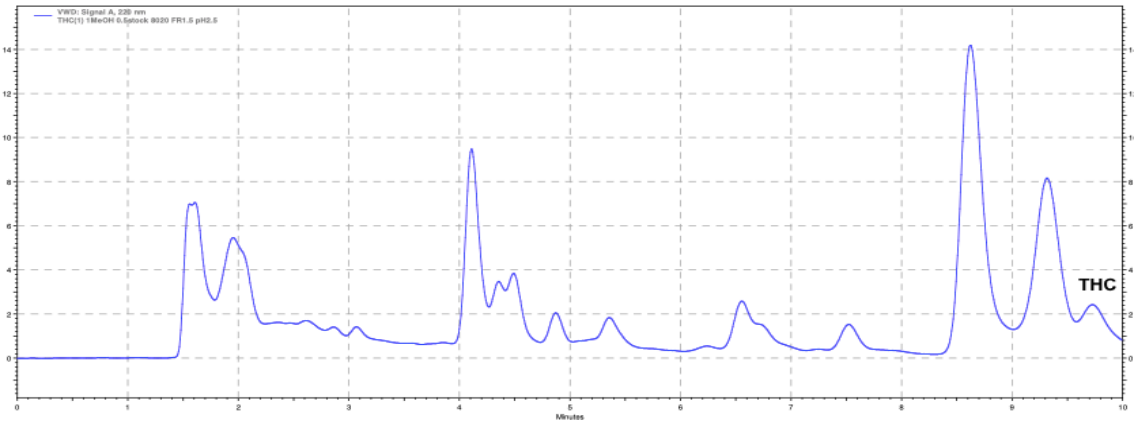


**Figure 3.17: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1.5 mL/min**

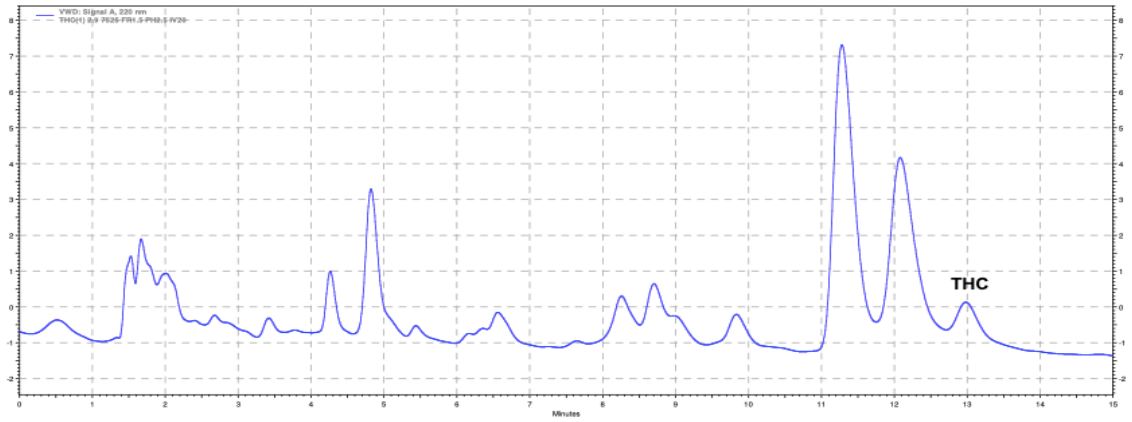


**Figure 3.18: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (25:75 v/v); detection wavelength 220nm; flow rate 1.5 mL/min**

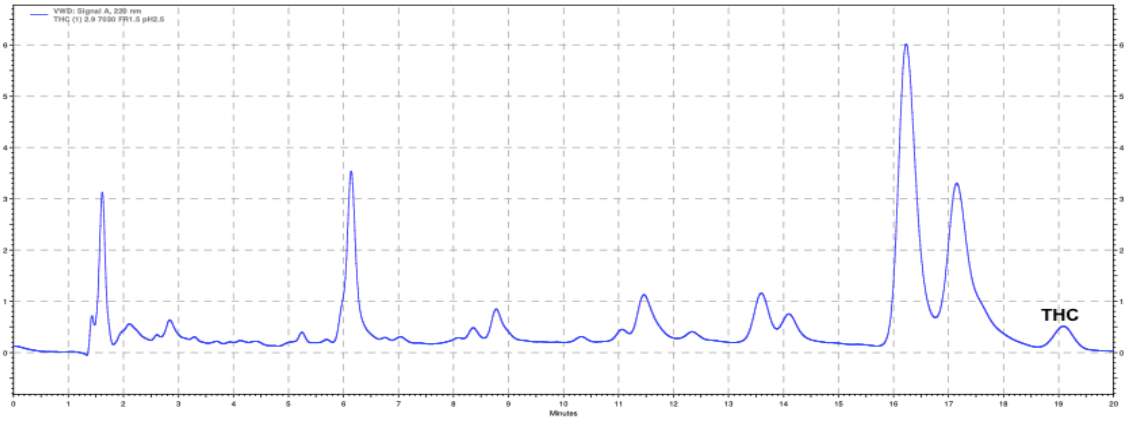


**Figure 3.19: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 1.5 mL/min**

Since the chromatogram using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v) had a long retention time, it was decided to increase the FR to 2 (Appendix III). The peaks were not well separated and the % of ACN was decreased to 65%. As observed in appendix III the peaks were separated, but the peak of THC was smaller and with a longer retention time.

The mobile phase using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v) was chosen. As the flow rate of the mobile phase was increased from 0.5 to 1.5 to 2mL/minute, the total run time for the analysis of THC decreased (Figures 3.20-3.22)

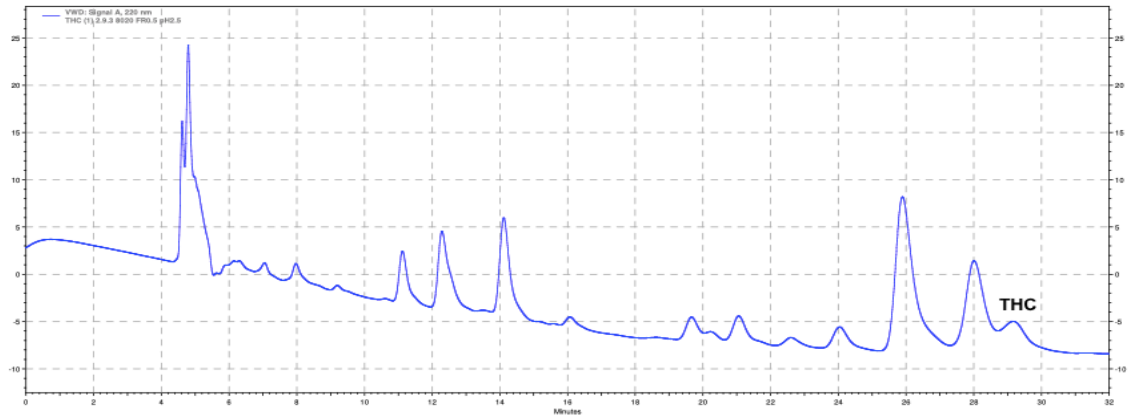


**Figure 3.20: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 0.5 mL/min**

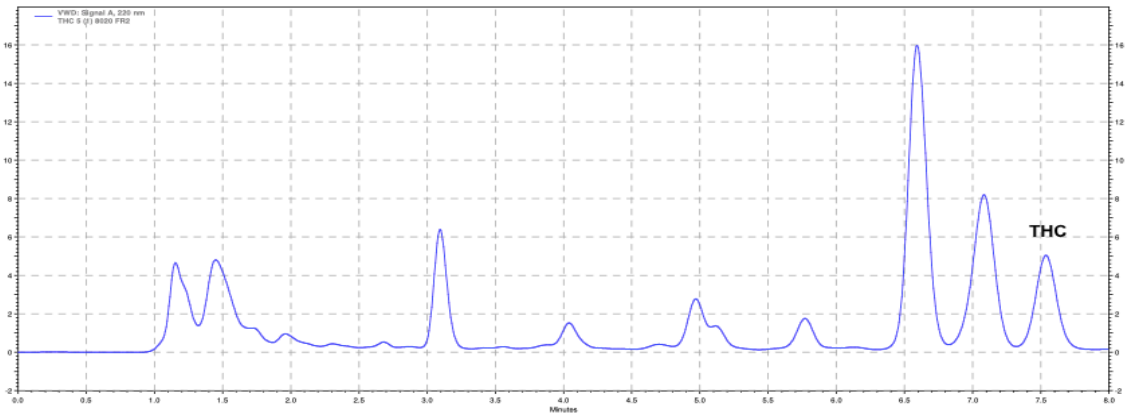


**Figure 3.21: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 2 mL/min**

Flow rates of 1.5 and 2mL/minute gave good chromatograms (Figures 3.21 and 3.22). The next parameter changed was the wavelength but it did not improve the separation of the peaks. The last parameter changed was pH to 6 (Appendix III).

When the % of ACN increased the retention time of THC decreased. When the mobile phase used was phosphate buffer and acetonitrile (10:90 v/v), the peak of THC overlapped with one of the peaks of the fatty acids from MCT (Appendix III).

As observed in Appendix III, pH 6 and mobile phase using phosphate buffer and acetonitrile (20:80 v/v) produced some irregularities in the baseline.

The mobile phase used was phosphate buffer and acetonitrile (30:70 v/v) produced good separation of the peaks but a long retention time (Appendix III).

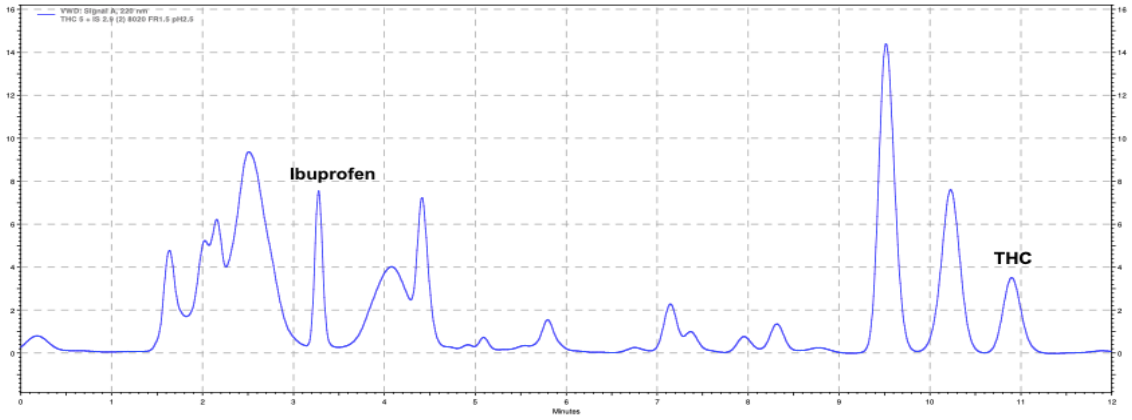


**Figure 3.22: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1.5 mL/min**

Better separation was achieved after the use of syringe filter of RC of 0.45 µm, 15mm (Figure 3.22).

The parameters which gave a peak of THC with good area under the peak, good shape, and was well resolved for neighboring peaks for fatty acids presented in MCT oil were as shown in Table 3.27.

**Table 3.27 : Selected chromatographic parameters for the determination of THC in MCT oil**

| | |
|---|---|
| Instrumentation | Agilent 1260 Infinity Series® II HPLC unit |
| Stationary phase | RP ACE® C18 column (250 x4.6mm; 5µm particle size) at 25°C |
| Mobile phase | 0.02M phosphate buffer (pH 2.5) and ACN 20:80 v/v, Flow rate 1.5mL/min |
| UV detection | 220 nm |

### 3.7 Method validation for THC in MCT oil

The validation of the method for the analysis of THC in MCT oil, was not carried out because the used of MCT as a carrier oil results in many problems of reproducibility. Due to this, it was decided to change the carrier oil to Extra Virgin Olive Oil (EVOO)

### 3.8 Method development for extraction of THC from EVOO

The best conditions for the extraction of THC from EVOO oil, using solvent extraction were the same as the extraction of THC from MCT oil illustrated in Figure 3.15.

### 3.9 Method development for the analysis of THC in EVOO

Different conditions were tested during method development. The UV detector was set at 220 and 228 nm. Larger areas under the peak were obtained for THC at 220nm when compared to 228nm (Figures 3.23 and 3.27)

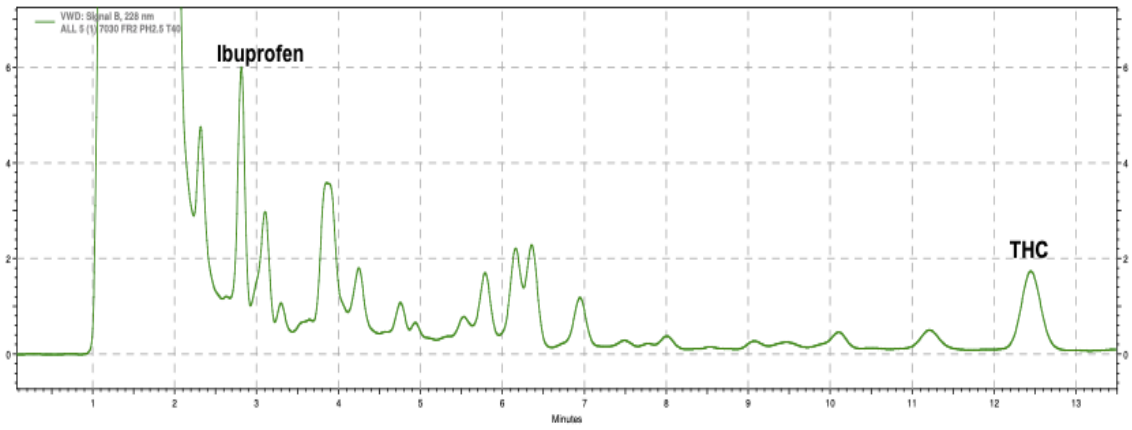


**Figure 3.23: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 228nm; flow rate 2 mL/min; T°40°C**

Different mobile phases were tested, when observing the chromatograms generated, as the amount of acetonitrile in the mobile phase was decreased from 90% to 85% to 80% the retention time of THC increased (Figures 3.24-3.27)

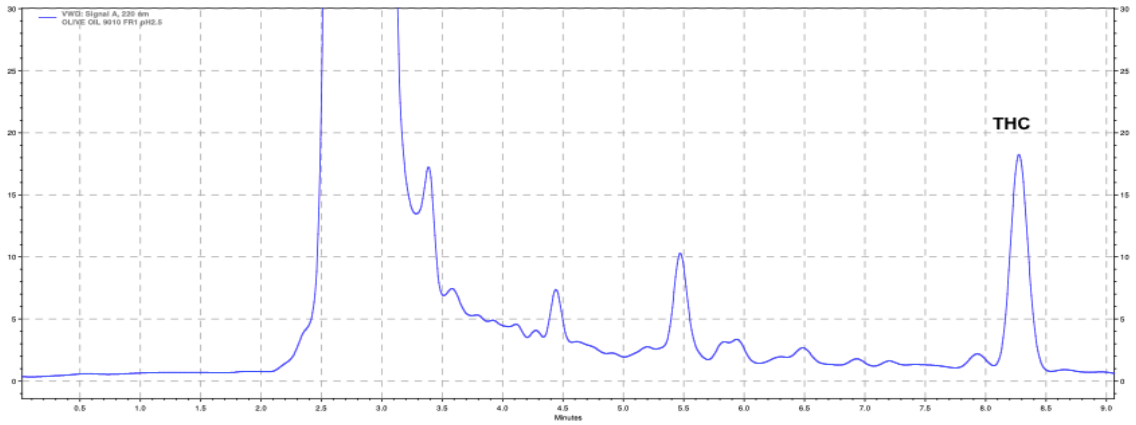


**Figure 3.24: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (10:90 v/v); detection wavelength 220nm; flow rate 1mL/min; Tª 25ºC**

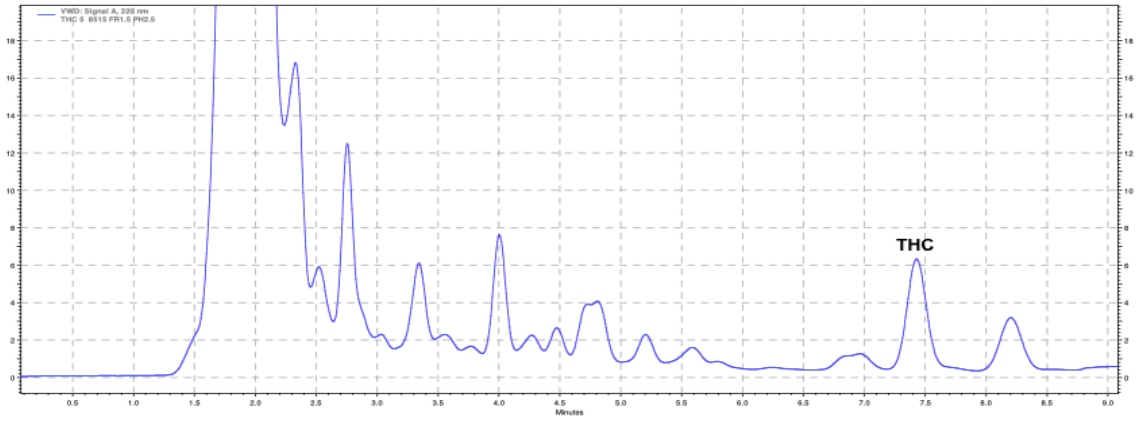


**Figure 3.25: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (15:85 v/v); detection wavelength 220nm; flow rate 1.5mL/min; Tª 25ºC**

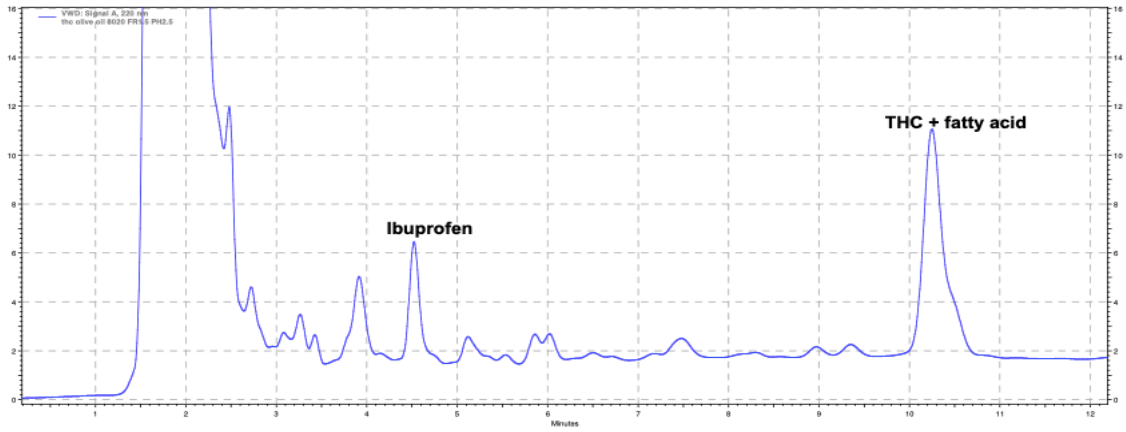


**Figure 3.26: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1.5mL/min; Tª 25ºC**

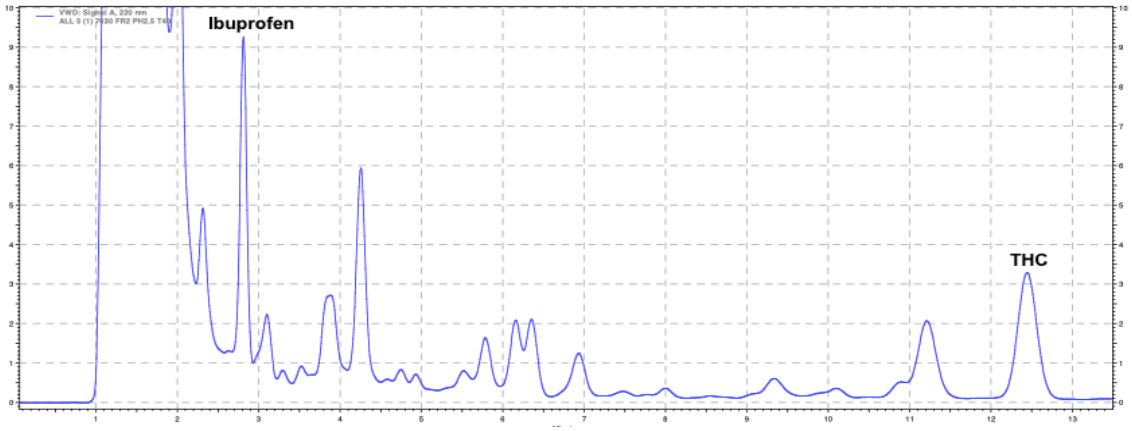


**Figure 3.27: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 2mL/min; Tª 40ºC**

When using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1.5 mL/min, a peak from EVOO overlapped with THC (Figure 3.26).

The parameters which gave a peak of THC with good area under the peak, good shape, and was well resolved for neighboring peaks for fatty acids presented in EVOO were as shown in Table 3.28.

**Table 3.28 : Selected chromatographic parameters for the determination of THC in EVOO**

| | |
|---|---|
| Instrumentation | Agilent 1260 Infinity Series® II HPLC unit |
| Stationary phase | RP ACE® C18 column (250 x4.6mm; 5µm particle size) at 25°C |
| Mobile phase | 0.02M phosphate buffer (pH 2.5) and ACN 30:70 v/v, Flow rate 2mL/min |
| UV detection | 220 nm |

## 3.10 Method validation for THC in EVOO

### 3.10.1 Selectivity/Specificity

When a blank of EVOO was injected, the chromatogram produced was compared with the chromatogram produced for the analysis of THC. As there we no peak or signal produced in the retention time of THC, it can be concluded that the method is specific to the analyte of interest (Figure 3.28).

### 3.10.2 Linearity

The calibration curve of THC in EVOO was set up by injecting 6 different concentrations levels, ranging from 0.039 to 5.000 µg/ml. Linearity was determined by obtaining a linear relationship between the AUP of the ratio THC/ibuprofen against 6 different sample concentrations of THC in EVOO.

Figure 3.29 show the calibration curve obtained when solutions of:

- 5.000 µg/ml
- 2.500 µg/ml
- 1.250 µg/ml
- 0.625 µg/ml
- 0.156 µg/ml
- 0.039 µg/ml

of THC were analysed in EVOO.

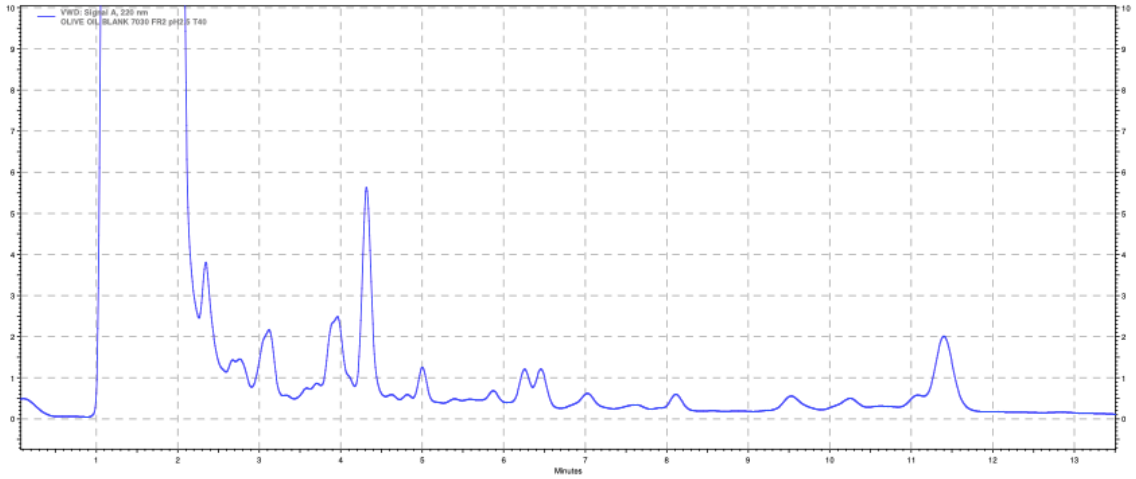


**Figure 3.28: Chromatogram of blank EVOO produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 2 mL/min and temperature 40ºC:**

When blank of EVOO was analysed using the developed method there were no peaks eluting at the retention time when THC (around 12.5 minutes). This shows that the method is selective for THC

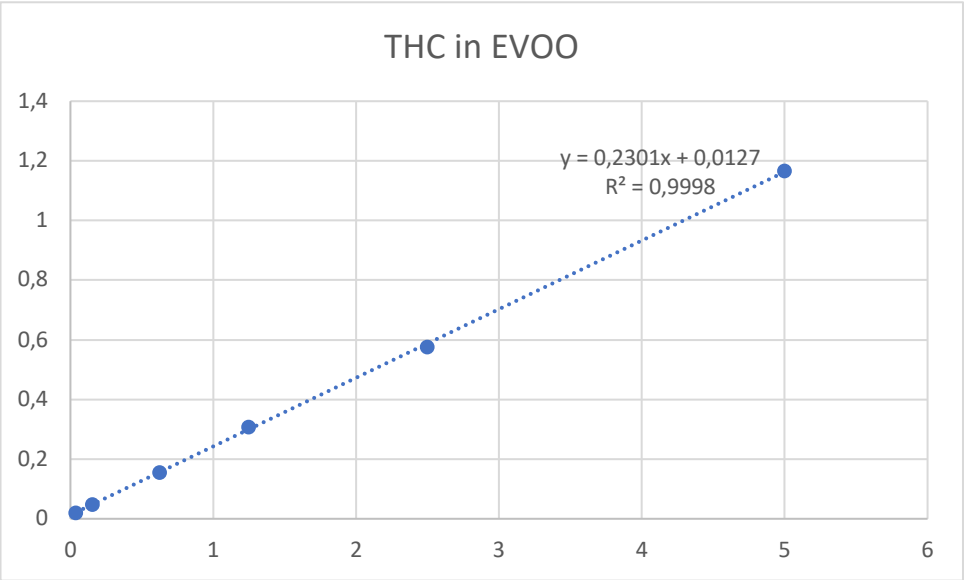


**Figure 3.29: Calibration curve for THC in EVOO:**

Plot of AUP vs concentration in µg/ml. When the six concentrations of THC in EVOO were analysed, an r2 of 0.9998 was obtained, indicating that the detector signal and AUP obtained is linearly proportional to the concentration of THC being analysed in EVOO

### 3.10.3 Accuracy

Results of accuracy and recovery from THC in EVOO were acceptable (Table 3.29). All percentage recoveries calculated were between 93.408% and 99.959%.

**Table 3.29 : Accuracy of THC in EVOO**

| Standard concentration of THC (µg/mL) | Concentration of THC which was quantified in EVOO (µg/mL) | Percentage recovery |
|---|---|---|
| 0.039 | 0.039 | 99.753% |
| 0.039 | 0.036 | 93.408% |
| 0.039 | 0.037 | 95.544% |
| 0.625 | 0.585 | 93.636% |
| 0.625 | 0.625 | 99.959% |
| 0.625 | 0.591 | 94.578% |
| 5.000 | 4.950 | 98.994% |
| 5.000 | 4.979 | 99.588% |
| 5.000 | 4.981 | 99.611% |

The developed method is accurate since all percentage recoveries calculated were above 93.400%

### 3.10.4 Precision

#### 3.10.4.1 Intra-day precision

The results obtained when the six concentrations of THC in EVOO were running in triplicates to calculate intra-day precision of the method are shown in Table 3.3'

The RSD results attained were below 4.520% showing acceptable intra-day precision.

#### 3.10.4.2 Inter-day precision

The RSD results obtained when each of the six concentrations of THC in EVOO were analysed once every day in three different days are shown in Table 3.31. RSD were all below 6.900% indicating acceptable inter-day precision.

**Table 3.30: Intra-day precision of THC in EVOO**

| Intra-day precision | | | | | | |
|---|---|---|---|---|---|---|
| Replicate number | Concentration THC in EVOO (µg/ml) | | | | | |
| | 5.000 | 2.500 | 1.250 | 0.625 | 0.156 | 0.039 |
| 1 | 5.003 | 2.552 | 1.217 | 0.657 | 0.149 | 0.040 |
| 2 | 5.027 | 2.482 | 1.285 | 0.605 | 0.162 | 0.039 |
| 3 | 4.970 | 2.466 | 1.248 | 0.613 | 0.157 | 0.039 |
| Mean | 5.000 | 2.500 | 1.250 | 0.625 | 0.156 | 0.039 |
| SD | 0.029 | 0.045 | 0.034 | 0.028 | 0.007 | 0.001 |
| RSD(%) | 0.576 | 1.809 | 2.750 | 4.516 | 4.340 | 0.722 |

RSD results attained for intra-day of THC in EVOO was below 4.520%, showing acceptable precision of the method.

**Table 3.31 : Inter-day precision of THC in EVOO**

| Inter-day precision | | | | | | |
|---|---|---|---|---|---|---|
| DAY | Concentration THC in EVOO (µg/ml) | | | | | |
| | 5.000 | 2.500 | 1.250 | 0.625 | 0.156 | 0.039 |
| 1 | 5.003 | 2.552 | 1.217 | 0.657 | 0.149 | 0.040 |
| 2 | 4.950 | 2.485 | 1.252 | 0.585 | 0.152 | 0.036 |
| 3 | 4.908 | 2.437 | 1.268 | 0.584 | 0.141 | 0.039 |
| Mean | 4.954 | 2.491 | 1.245 | 0.609 | 0.147 | 0.038 |
| SD | 0.047 | 0.058 | 0.026 | 0.042 | 0.005 | 0.002 |
| RSD(%) | 0.953 | 2.310 | 2.111 | 6.896 | 3.594 | 4.555 |

RSD results attained for inter-day precision of THC in EVOO was below 6.900%, showing acceptable precision of the method.

### 3.10.5 Stability

The three chosen concentration of THC in EVOO (5.000, 0.625 and 0.039 μg/mL) were stored at -20ºC and analysed after one and three weeks, the quantities detected in chromatograms produced following analysis after one week and three weeks were not significantly different from the quantities detected after the sample was prepared. Results for stability after one week and three weeks using this method can be seen in Table 3.32 and Table 3.33, respectively.

**Table 3.32 : Stability of THC in EVOO after one week**

| Stability of THC after one week | | | |
|---|---|---|---|
| Replicate number | Concentration THC in EVOO (µg/mL) | | |
| | 5.000 | 0.625 | 0.039 |
| 1 | 4.967 | 0.563 | 0.035 |
| 2 | 4.966 | 0.574 | 0.037 |
| 3 | 4.973 | 0.576 | 0.037 |
| Mean | 4.969 | 0.571 | 0.036 |
| SD | 0.004 | 0.007 | 0.001 |
| RSD | 0.078 | 1.301 | 2.933 |

All RSD values were below 2.935%. This indicates that THC is stable in EVOO after one week when stored in such conditions.

**Table 3.33 : Stability of THC in EVOO after three weeks**

| Stability of THC after three weeks | | | |
|---|---|---|---|
| Replicate number | Concentration THC in EVOO (µg/ml) | | |
| | 5.000 | 0.625 | 0.039 |
| 1 | 4.958 | 0.575 | 0.036 |
| 2 | 5.004 | 0.597 | 0.031 |
| 3 | 4.973 | 0.576 | 0.033 |
| Mean | 4.978 | 0.583 | 0.033 |
| SD | 0.023 | 0.012 | 0.002 |
| RSD | 0.464 | 2.140 | 6.976 |

All RSD values were below 6.980%. This indicates that THC is stable in EVOO after three weeks when stored in such conditions.

### 3.10.6 Limit of detection

The lowest concentration of THC in EVOO that gave a signal but could not be quantified was 0.019 µg/ml (Figure 3.30).

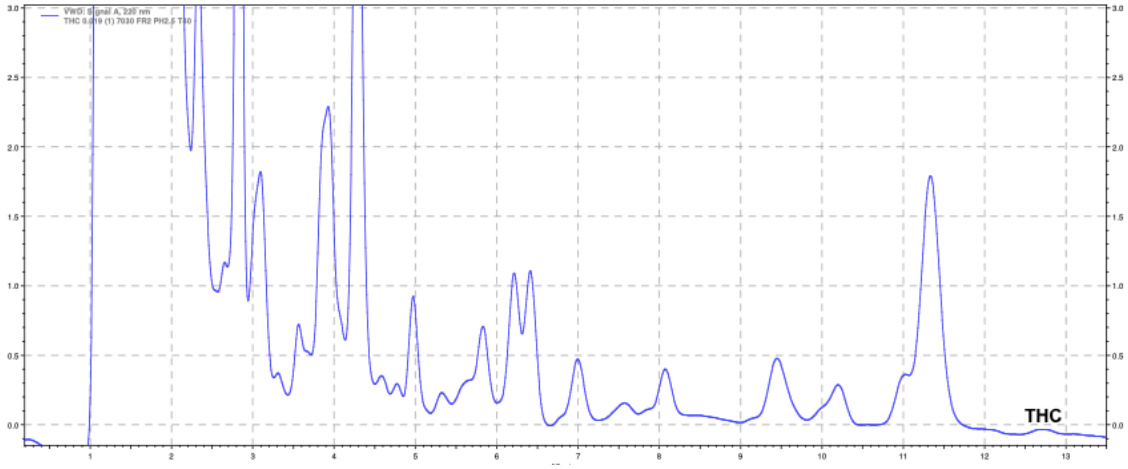


**Figure 3.30: Limit of detection of THC in EVOO**

### 3.10.7 Limit of quantification

The lowest concentration of THC in EVOO that gave a signal and could be quantified was 0.039 µg/ml (Figure 3.31).

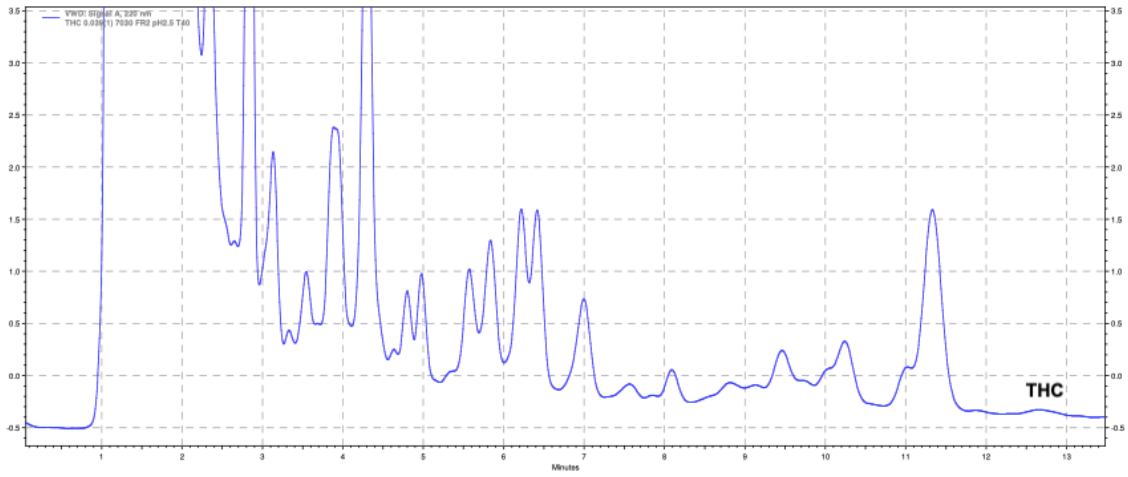


**Figure 3.31: Limit of quantification of THC in EVOO**

### 3.11 Application of the method

When analyzing the commercial CBD oil using the same method of extraction and method developed, the peak of THC appeared, was well defined and with a good AUP and shape but the peak of ibuprofen was compromised (Figure 3.32). Figure 3.33 shows the chromatogram of commercial CBD oil without adding ibuprofen, a peak can be observed at the same retention time as ibuprofen.

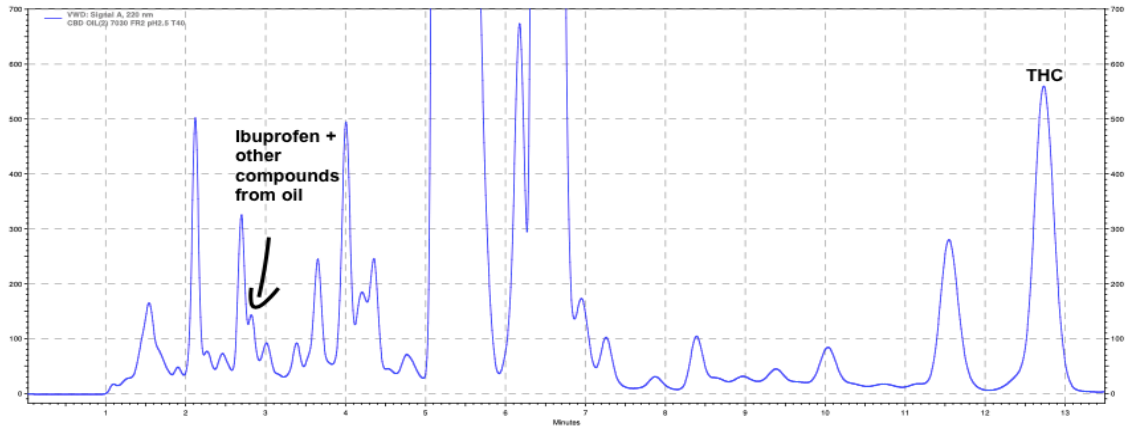


**Figure 3.32: Chromatogram of CBD oil and Ibuprofen produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 2 mL/min and temperature 40ºC**

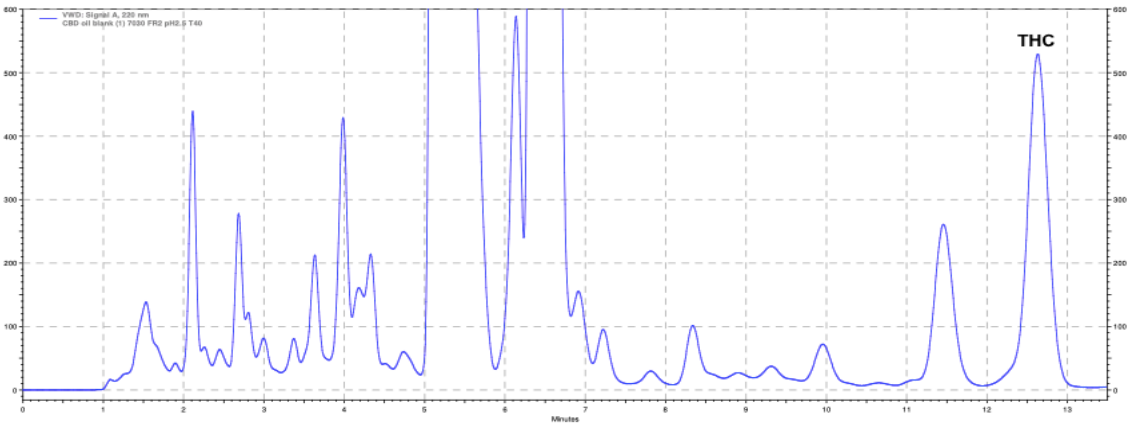


**Figure 3.33: Chromatogram of CBD oil produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 2 mL/min and temperature 40ºC**

# Chapter 4 Discussion

## 4.1 Systematic Review

The most commonly used LC-based system for the analysis of cannabinoids is HPLC. UHPLC is becoming popular due to faster analysis and less amount of solvent is needed. The detector used for the analysis of cannabinoids depends on the complexity of the matrices from which cannabinoids are extracted from. In general, MS/MS detector is used for more complex matrices as blood and urine and for matrices which present a small quantity of cannabinoids while DAD and UV are used in plant material where the quantity of cannabinoids is higher. The most popular mobile phase is gradient mode using water with 0.1% HCOOH and ACN with 0.1% HCOOH. C18 columns are the most commonly used. Identification and comparison of analytical methods for determination of cannabinoids plays a key role in the development of efficient and effective methods of analysis which are useful for high throughput analysis. Determination of concentrations of this class of compounds accurately and precisely can help in the better understanding of physiological effects and medicinal properties of cannabis.

### 4.1.1 Liquid Chromatography Methods of Analysis of Cannabis from plant material

The availability of analysis of Cannabis plants and plant-based products has become important due to the increased interest in growing different varieties such as *Cannabis sativa* and *Cannabis indica* (Krizman, 2019). CBD and THC, are among the most important cannabinoids due to their presence in higher concentrations in the Cannabis plant and their different pharmacological effects (Bala et al, 2019). HPLC is a commonly used analytical technique for determination of cannabinoids in plants (Tejada Rodríguez et al, 2021). The use of UHPLC is increasing due to the need of less amount of solvent and shorter run times (Nováková et al, 2006). Results from the review identified UHPLC coupled to DAD as the most commonly analytical technique for the analysis of

cannabinoids from plants (Fekete et al, 2018; Elkins et al,2019; Deville et al,2020). The use of DAD as a detector for HPLC and UHPLC is quite common but combined detectors as UV-DAD, ESI-MS and MS/MS have increased in their popularity (Tejada Rodríguez et al, 2021). The use of ESI-MS and MS/MS present some advantages such as the possibility of performing analysis in negative and positive ion mode. The uses of both ion modes produce better analysis of neutral and acidic cannabinoids since in general neutral cannabinoids gives a better signal in the positive ion-mode and acidic cannabinoids in negative ion-mode (Bala et al, 2019). The higher sensitivity of HPLC and UHPLC replaced the use of LC. LC is still used in some studies due to its simple equipment of analysis and decreased costs (Fekete et al, 2018). Analysis using a LC system requires that the analytes of interest are soluble, and have to be dissolved prior to analysis (Tejada Rodríguez et al, 2021). For that a sample preparation technique must be carried out, including an extraction method. The selected extraction method influences qualitative and quantitative presence of cannabinoids (Citti et al, 2018). Most of the studies, carried out solvent extraction to extract cannabinoids from plant material. EtOH and MeOH are the most popular organic solvents used for the extraction of cannabinoids from plants (Tejada Rodríguez et al, 2021). The use of ethanol as an organic solvent is more eco-friendly (Zweigenbauma and Pierrib, 2020), but ethanol presents higher viscosity than MeOH (Dong et al, 2019). The use of MeOH as an organic solvent is due to its great efficacy as an extraction solvent (Bala et al, 2019). Other solvent extraction techniques are carried out with a mix of solvents as methanol/ chloroform (90/10: v/v), but the use of chloroform for long periods of time might produce liver and kidney injury to the operator using them and have a negative impact in the environment (Križman, 2020). The use of chloroform as an organic solvent produces CBD instability (Ciolino et al, 2018). Extraction techniques which include the use of solvent extraction are DM, UAE, MAE

and SFE. DM is carried out using a solvent and vortex or stirring at room temperature. UAE and MAE consist in the extraction of cannabinoids from plant material using ultra-sound waves and microwave energy for a greater extraction in a shorter period of time. SFE is an eco-friendlier extraction technique than solvents extraction (Tejada et al, 2021). Brighenti et al, compare the four different extraction techniques, drawing the conclusion that the extraction of acidic cannabinoids like CBDA is better with DM and the extraction of CBD, with MAE (Brighenti et al, 2019). Ribeiro Grijó et al, performed other extraction techniques consisting of the use of Solid Phase extraction (SPE) with scCO2 which avoided that the sample preparation contains trace of organic solvents (Ribeiro Grijó et al, 2019).

### 4.1.2 Liquid Chromatography Methods of Analysis of Cannabis from biological fluids and hair

Cannabis can be used for medicinal and recreational uses (Nováková et al, 2006). The analysis of drugs in biological fluids is important for the determination of the physiological characteristics of the drug. Analysis of drugs in biological fluids provides critical information about their pharmacokinetics and pharmacodynamic properties (Mullet, 2007). Blood, plasma or serum are popular biological samples used in forensic analysis for the determination of cannabinoids (Nahar et al, 2019). Analysis of cannabinoids in blood is important to stablish the correct dosage of cannabinoids and to understand their pharmacology in humans (Dybowski et al,2020).

Urine is used for the analysis of THC and its metabolites since THCA, the major metabolite of THC, is secreted in the urine as its glucuronide conjugate (Klawitter et al, 2017; Nahar et al, 2019). Hair is used as a matrix because traces of some compounds can collect in hair. THC and THCA-A can be detected in hair after a substantial amount of time following consumption. The presence of traces of THC and THCA-A in the hair can

be due to its absorption of passive smoke or other external contamination (Cho et al, 2018).

LC, HPLC and UHPLC methods are used for the analysis of cannabinoids from different biological fluids (Tejada Rodríguez et al, 2021). Due to the complexity of the matrices, MS/MS is the detector most widely used. The uses of both ion mode in MS/MS allow the detection of minor and major cannabinoids due to its high selectivity and sensitivity (Tejada Rodríguez et al, 2021).

The nature and complexity of the matrices make the sample preparation a key step because it needs to eliminate the greatest amount of interference as possible and be reproducible, efficacious and selective (Citti et al, 2018). One of the most popular techniques used for the extraction of cannabinoids from blood is PP due to its higher capacity to eliminate protein when adding an appropriate reagent (Toennes et al,2015). Other techniques used are Volumetric Absorptive Microsampling (VAMSTM), which is used to obtain dried biological matrices to improve the accuracy in the sample volume. Salting-out assisted liquid-liquid extraction (SALLE), carried out by Pires de Silva et al, consists in the use of an extraction solvent that is water miscible organic solvent. This technique is simpler to use and costs less than SPE (Tejada Rodríguez et al, 2021).

Sample preparation in urine requires hydrolysis to improve sample accuracy and to quantify CBD due its presence as glucuronide in urine. Sample preparation in hair requires extra steps, such as washing and digestion (Tejada Rodríguez et al, 2021).

### 4.1.3 Liquid Chromatography Methods of Analysis of Cannabis from oil

In recent years, CBD oil has become very popular due to its use for a variety of conditions (Pavlovic et al, 2018). There is a lack of standardized regulation related to extraction from cannabis oil (Alshishani et al, 2017; Citti et al, 2018). CBD oil is present on the market

in different carrier oils such as olive oil, MCT, hemp seed oil and avocado oil (Tejada Rodríguez et al, 2021).

The most commonly used method for the determination and quantification of cannabinoids in oil is HPLC coupled to UV detectors. Sample preparation is important in the analysis of cannabinoids from oil due to the fact that oil cannot be injected directly because it presents a high viscosity (Tejada Rodríguez et al, 2021). Different organic solvents were used for the extraction of cannabinoids from oil. Bettiol et al, and Deidda et al, extracted different cannabinoids using the same method which consisted in the use of tetrahydrofuran (TFH) as extraction solvent followed by dilution in ACN in the study of Bettiol et al, and in MeOH in the study of Deidda et al, before injecting the sample in the HPLC (Bettio et al, 2019; Deidda et al, 2019). Mudge et al, used MeOH as extraction solvent while Nemeškalová et al, used isopropanol: ethyl acetate (1:1, v/v) and Ciolino et al used EtOH or isopropyl alcohol (Tejada Rodríguez et al, 2021).

### 4.1.4 Liquid Chromatography Methods of Analysis of Cannabis from miscellaneous matrices

There is a need of quantitative analysis to determine cannabinoids like CBD and THC in commercial products such as honey, capsules, serum skin, etc., to calculate the amount of each cannabinoids to evaluate the dosage and the exposure of the patient when the product is consumed (Ciolino et al, 2018).

The determination of cannabinoids in wastewater gives information about the community used of cannabis in a determinate area. The extraction and separation of cannabinoids from wastewater is a difficult process because these compounds are hydrophobic (Pandapolus et al, 2020).

### 4.2 Method development

This study proposed an innovative, quick, and easy to use method for the determination and quantification of CBD, CBN and THC in MeOH and for the determination and quantification of THC in Oil. The method for quantification of THC in oil, was first developed in MCT oil, but due to reproducibility problems it was decided to change the carrier oil to EVOO.

Different parameters were changed to obtain the best conditions for efficient analysis that can be carried out in in most laboratories and can be validated and applied to separate and determine cannabinoids such as THC in CBD preparations in a relatively short period of time.

The proposed method can be applied to different CBD oils using EVOO as a carrier oil to verify that the product is in line with the law of many countries which sustain that products containing less than 0.2% (w/w) of THC are not controlled under drugs laws pertaining to narcotics[13].

Systematic method development is key to successfully implement a robust method that can be applied on a daily bases and is effective. Analysis should be developed step by step to attain the best conditions.

### 4.3 Method development of the separation of the three cannabinoids

The method development of the three cannabinoids in MeOH started with the selection of an appropriate pH and mobile phase due to the importance of these two parameters in terms of resolution, retention time, area under the peak and shape. The selection of the pH value of the phosphate buffer in the mobile phase was done according with the pKa of the analytes of interest. The pKa values of CBD, CBN and THC are 9.5, 9.32 and 10.5

[13] European Monitoring Centre for Drugs and Drug Addiction. Low-THC cannabis products being sold in the EU – key legal issues.[Internet] Lisbon 2018. [cited 2021 May 03] Available from: https://www.emcdda.europa.eu/news/2018/low-thc-cannabis-products-being-sold-in-the-EU–key-legal-issues_en

respectively (Mazina et al, 2015; Tejada et al, 2021). CBD, CBN and THC are weak acid substances that at pH lower than 9.32 are present in their protonated form (Tejada et al, 2021). The use of a pH higher than 10.5 will cause the conversion of CBD, CBN and THC to their unprotonated form increasing their polarity, however the usual pH of the buffer recommended for Reverse Phase (RP)-HPLC ranged from 2 to 8 (Tejada et al, 2021). pH values higher than 7 increase the solubilization of silica and reduce the lifetime of the column (Gupta et al, 2012; Vella et al, 2014; Tejada et al, 2021). Due to this, pH values used were: 2.5, 3, 4 and 6. The increased of pH did not affect the area under the peak, peak shape or retention time, even if some irregularities were observed in the baseline, this finding is in line with the study carried out by Hazekamp et al,( Hazekamp et al, 2005).

Mobile phase plays an important role due to its effects in terms of resolution, selectivity and efficacy. In RP-HPLC, the mobile phases consisted in an aqueous solution, usually buffer, and an organic solvent with non-UV activity (Gupta et al, 2012). Phosphate buffers are commonly used in HPLC due to their relatively low cost and different buffer capacity ranges: pH 1.1-3.1, pH 6.2-8.2 and pH 11.3-13-3 (Tejada et al, 2021). ACN was chosen over MeOH as an organic solvent because ACN produces shorter elution times and less of a raise in pressure (Sanli et al,2010; Deidda et al,2019). When the percentage of ACN was higher, the cannabinoids eluted faster, but peak of CBD was compromised when the mobile phase consisted of 10:90 phosphate buffer:ACN (v/v). The increase of percentage volume of organic solvent produces that the three cannabinoids interacted less with the stationary phase and eluted earlier. The use of larger amount of ACN is favorable because it might produce a conversion in the partition coefficient. The percentage of organic solvent should not be more than 50% due to loss of buffer capacity but the use of the buffer with a high concentration might correct this (Tejada et al, 2021).

The total retention time of the three cannabinoids decreased when the flow rate was increased without a compromise in resolution. The development of quick HPLC methods are useful to meet the requirements of high throughput analyses (Tejada et al,2021).

The change of temperature did not result in any change in terms of area under the peak or shape of the peak. The use of higher temperatures was not attempted to keep the analytical method relatively energy efficient (Tejada et al,2021).

Published UV spectra of cannabinoids show absorption in the region between 190–350 nm. Analyses with HPLC-UV has been conducted using double wavelengths, like 220 nm or 228 nm (Clifford et al, 2020). The selected wavelength was 220nm due to higher areas under the peak for the three cannabinoids when compared to 228nm. At the selected wavelength THC presented lower absorptivity than CBD and CBN. This might be due to a higher degree of conjugation within the molecule (Tejada et al, 2021).

**4.4 Method validation for the separation of the three cannabinoids**

The developed method for the determination of CBD, CBN and THC was validated. The method was selective for CBD, CBN and THC (as can be in section 3.3.1). The linearity of the method was acceptable for CBD, CBN and THC with and $R^2$ of 0.9994, 0.9995 and 0.9996, respectively. The accuracy and recovery of the method resulted also acceptable with the lowest % of recovery 96.648% corresponding to the lowest concentration of THC in MeOH. The precision of a method is acceptable if all RSD results are below of 15% according to ICH guidelines[14].

[14] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Validation of analytical procedures: text and methodology Q2(R1).[Internet] 2017[cited 2021 May 03]. Available from https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf.

### 4.5 Method development for the analysis of THC in MCT oil

The method development for the analysis of THC in MCT oil was started with the preparation of the stock solution of THC in MCT oil at a concentration of 5 µg/ml. The next step in the method development was the selection of an efficient extraction technique. An appropriate extraction method is required for the analysis of THC in MCT oil, because the injection of a sample in oil directly in the HPLC columns produces reproducibility problems due to high viscosity of the oil (Tejada Rodríguez et al, 2021). Solvent extraction is a popular technique used to extract cannabinoids from different matrices (Tejada Rodríguez et al, 2021). MeOH is commonly used due to its higher efficacy in extraction procedures (Tejada Rodríguez et al, 2021). Another popular organic solvent used is EtOH as its use is more environmentally friendly, but its use as an extraction solvent with ultrasonication produces the presence of numerous interfering analytes (Brighenti et al, 2019). Due to the low miscibility of oil and MeOH at room temperatures, vortex mixing and sonication were required followed by centrifugation. The sample was left in the freezer (-20ºC) for 2 hours to help complete separation of MeOH from MCT oil due to the different freezing points of MeOH (-98 °C)[15] and MCT oil (-5ºC)[16]. The sample preparation improved when a second centrifugation step was carried out before injecting the sample into the HPLC. A crucial step in the development of the extraction procedure was the introduction of RC syringe filters of 0.45 µm pore size. The second centrifugation and the use of syringe filters were carried out to decrease the possibilities of blocking the column due to the presence of impurities and oil in the solution containing THC.

[15] HONEYWELL. Methanol [Internet] 2021 [cited 2021 May 03] Available from: https://lab.honeywell.com/shop/methanol-m1775

[16] Kraft Chemical. Safety data sheet MCT oil. [Internet] Chicago 2015[ cited 2021 May 03] Available from: https://greenfield.com/wp-content/uploads/2018/11/MCT-Oil-SDS.pdf

The following step in the method development of THC in MCT oil was the selection of the internal standard. Previous studies have reported the use of ibuprofen when analysing cannabinoids (Giese et al, 2015; Citti et al, 2019). Ibuprofen is also a weak acid with a pKa 4.4 (Moghadamnia et al, 2019) with a wavelength max (nm) at 220 and 273 (Citti et al, 2019). Ibuprofen like THC presents substantial degree of conjugation and has maximum UV absorption at the wavelength used for the method developed. Due to these chemical similarities Ibuprofen was selected as the internal standard. Ibuprofen eluted earlier than THC, keeping the same total chromatographic run time.

The next step was the selection of an adequate mobile phase in terms of percentage of ACN using a set pH of 2.5. When the amount of ACN was higher, THC eluted faster, but the separation of THC from the fatty acids of MCT oil was compromised. Since the retention time was too long with lower amount of ACN, the flow rate was increased but no improvements were observed. Since retention time was appropriate using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v), but peaks were not well separated, it was decided to decrease flow rate but peaks remained similar.

The fatty acids presented in MCT oil are caprylic acid, capric acid and lauric acid with a pKa of 4.89 ,4.9 and 5.3, respectively. The pKa for the three fatty acids is lower than for THC, and in the previous method developed, changing of pH did not affect the area under the peak, peak shape or retention time of THC. Increasing the pH to 6, which is a value above the one of the three fatty acids' pKa, will cause the conversion of caprylic acid, capric acid and lauric acid to their unprotonated form. The unprotonated form of analytes are more polar and analytes elute earlier (Tejada, et al 2021).

When the amount of ACN was 90%, THC eluted as the same time as one of the fatty acids. Decreasing the amount of ACN and comparing the chromatograms with those produces at pH 2.5, results were not improved. This could be due that the use of more

than 50% of organic solvent might decrease buffering capacity (Tejada et al,2021). Separation of THC from fatty acids was achieved after the introduction of syringe filter of RC of 0.45 μm pore size as last step in the sample preparation.

The developed method for the determination of THC in MCT oil was not validated because the use of MCT as a carrier oil results in problems of reproducibility. Due to this, it was decided to change the carrier oil to EVOO.

**4.6 Method development for the analysis of THC in EVOO**

The method development for the analysis of THC in EVOO was started with the preparation of the stock solution of THC in EVOO at a concentration of 5 μg/ml. The next step in the method development was the selection of an efficient extraction technique. The developed solvent extraction technique used for the extraction of THC from MCT oil was used for the extraction of THC from EVOO.

The amount of ACN was decreased in the method using EVOO to improve peak separation. Studies published in literature using EVOO as a carrier oil used pH 3.45 for mobile phase buffer (Bettiol et al, 2019; Deidda et al, 2019). Other methods published in literature using hemp seed oil as carrier oil used pH 2.9 and 4.2 (Ciolino et al, 2018). Other methods published in literature but, which do not specify the carrier oil used, used pH 3.6, 3 and 4.45 (Mudge et al,2017; Pichini et al,2019; Nemeškalová et al, 2020). The pH selected for this study was 2.5 since it was stablished in the method of CBD, CBN and THC in MeOH and it gave good chromatograms. When the amount of ACN was higher, THC eluted faster, but the peak of ibuprofen was compromised. Decreasing the amount of ACN to 70%, produced a great peak of ibuprofen and THC. Among the studies published in the literature, only three carried out analysis using an Isocratic mode mobile phase and ACN as an organic solvent, the percentages of ACN used were 66% (Ciolino et al,2018) and 75% (Bettiol et al, 2019; Deidda et al, 2019). The total retention time

decreased when the flow rate increased. The flow rate used in the studies published in literature ranged from 0.38mL/min to 1mL/min, however the use of a flow rate 2mL/min decreased the retention time of the analysis which is very important for high throughput analyses. Even if the use of high flow rate as 2mL/min is unusual in analytical HPLC methods (Nahar et al, 2019), the developed method worked well for the separation and quantification of THC in EVOO.

When comparing the new developed method in this study to other methods published in the literature, this study presents a relatively short retention time for THC. Four studies presented shorter retention time for the analysis of THC. Two of the studies involving the analysis of THC with shorter retention time (7.8 min and 10 min), used an UHPLC (Pichini et al, 2019; Nemeškalová et al, 2020). UHPLC is an advanced analytical technique with significantly shorter analysis time than HPLC (Nahar et al, 2019). However, HPLC is more robust and presents lower costs[17]. Other study involving the analysis of THC with shorter retention time (8 min) used a HPLC-DAD (Bettiol et al,2019) which can be more expensive than UV[18].The last study with shorter retention time for the analysis of THC (5 min) was the one carried out by Deidda et al. Pichini et al, Bettiol et al, and Deidda et al , who used higher temperatures (50ºC, 53ºC ,53ºC, respectively) than the one used in this study, causing the THC peak to elute earlier (Bettiol et al,2019; Deidda et al, 2019;Pichini et al,2019). In this study the temperature was not increased more than 40ºC to keep the analytical method as much energy efficient as possible. The four studies having a shorter retention time for THC performed analysis

[17] SHIMADZU Excellence in Science. HPLC vs UHPLC-How to Choose? [Internet] 2021 [cited 2021 May 03] Available from: https://www.ssi.shimadzu.com/products/liquid-chromatography/knowledge-base/hplc-vs-uhplc.html

[18] He Q, Li M, Wang X, Xia Z, Du Y, Li Y, et al. A simple, efficient and rapid HPLC–UV method for the detection of 5-HT in RIN-14B cell extract and cell culture medium. BMC Chemistry [Internet]. 2019[cited 2021 May 10]. Available from: https://link.springer.com/article/10.1186/s13065-019-0591-x

using a shorter analytical column than the one used in this study. The use of shorter columns decrease the retention time of the analyte of interest but also decrease the resolution of the analysis (Fekete et al, 2018). Ciolino et al, performed the analysis using the same analytical column used in this study with a total run time of 50 minutes (Ciolino et al, 2018).

### 4.7 Method validation for the analysis of THC in EVOO

The developed method for the determination of THC in EVOO was validated. The developed method was validated and all validation parameters met the requirements described in the ICH guidelines[19]. The developed method achieved relatively low limits of quantification and detection. Pichini et al, reported the lowest limit of detection (0.012 µg/mL) than the one reported in this study (0.019 µg/mL). Pichini et al, performed the analysis using an MS/MS as a detector. HPLC coupled to MS increased sensitivity of the analysis (Elkins et al, 2019). The use of MS/MS increased even more the sensitivity of the analysis due to decrease of the noise, but it requires skilled expertise and expensive instrumentation (Vella et al, 2015). The limit of quantification (LOQ) achieved in this study (0.039 µg/mL) is the lowest compared to ones in the published literature.

### 4.8 Application of the method

When validated method for analysis of THC in EVOO was applied to determine THC in commercially available CBD oil well defined peak was produced for THC but not for ibuprofen. The reason for this might be because the commercially available oil also contained other cannabinoids, terpenoids, flavonoids, antioxidant and nutrients. Peaks produced by other cannabinoids than CBD and THC, terpenoids, flavonoids, antioxidant

[19] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Validation of analytical procedures: text and methodology Q2(R1).[Internet] 2017[cited 2021 May 03]. Available from https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf.

and nutrients might have overlapped with peak produced by ibuprofen making accurate quantification of THC in the commercially available product problematic. Further work would be recommended to apply validated method in commercially available product containing only CBD and THC.

### 4.9 Limitations

1. Concentrations of THC in oil higher than 5 μg/mL were not analysed making method not appropriate to determine higher concentrations of THC.
2. THC was not extracted and analysed from other available carrier oils such hemp seed oil, avocado oil and sunflower oil.
3. Developed and validated method for determination of THC was not applied on other commercially available EVOO products.

### 4.10 Recommendations

1. Higher concentrations of THC could be analysed to determine whether method can be used on oil preparations containing more THC.
2. Study can be performed to extract and determine THC in different carrier oils such as hemp seed oil, avocado oil and sunflower oil to help determination of THC in other commercially available products.
3. Study can be performed on a larger sample of commercially available CBD oils to ensure amount of THC present is as indicated.
4. Study can be performed for determination of other cannabinoids which might be present in commercially available CBD oils.
5. Analysis can be conducted using more sensitive detectors such MS detector to determine smaller concentrations of cannabinoids.

## 4.11 Conclusion

The innovative method for the determination of THC in EVOO is simple, reproducible and relatively quick to perform. The developed and validated method has acceptable retention time, accuracy and precision with the lowest LOQ and temperature of analysis compared to other literature describing determination of THC in EVOO. Developed and validated method for extraction and determination of THC from oil can be effectively applied and useful to meet the recommendations of the United Nations Commission on Narcotic drugs for rescheduling of cannabis.[20]

[20] United Nations Office on Drugs and Crime. WHO Scheduling Recommendations on Cannabis and Cannabis-Related Substances. [Internet]2020. [cited 2021 May 12] Available from: https://www.unodc.org/unodc/en/commissions/CND/Mandate_Functions/current-scheduling-recommendations.html

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# Appendices

# Appendix I Tables of Systematic Review

## Table I.I: Liquid Chromatographic Method of analysis of Cannabis from plant material.

| Author and date | Cannabinoids | Method | Sample preparation | HPLC unit used | Stationary phase | Mobile phase |
|---|---|---|---|---|---|---|
| Bala et al, 2019 | THC, CBD & THCA | UHPLC-MS | Extracted sequentially with n-hexane, DCM, and MeOH by 1 hour of maceration, followed by filtration and evaporation in a rotary vacuum evaporator. | Waters UPLC system with a Waters Synapt G2 QTOF | Acquity UPLC BEH C18 1.7dμm (2.1 × 100 mm column) | Gradient: A) water + 0.1% HCOOH & B) ACN (3-100%) + 0.1% HCOOH FR0.4mL/min |
| Brighenti et al, 2019 | CBD, CBDA, CBG & CBGA | HPLC-UV/DAD | 0.25g of sample were extracted for 15 min under magnetic stirring with 10mL EtOH. The solution was filtered with a paper filter and the residue was extracted twice more with the same procedure with 10 and 5 mL of solvent. The three extractions were then combined and brought to 25 mL with the solvent in a volumetric flaskand pass through a 0.45 μm PTFE filter | Agilent Technologies modular model 1100 system | Ascentis Express C18 column (150 mm × 3.0 mm I.D., 2.7μm) | Gradient: A) water + 0.1% HCOOH & B) ACN (60-90%) + 0.1% HCOOH FR0.4mL/min |
| Brighenti et al,2017 | CBD, CBDA, CBG & CBGA | 1.HPLC-UV/DAD 2.HPLC-ESI-MS | 0.25g of sample were extracted for 15 min under magnetic stirring with 10mL EtOH. The solution was filtered with a paper filter and the residue was extracted twice more with the same procedure with 10 and 5 mL of solvent. The three extractions were then combined and brought to 25 mL with the solvent in a volumetric flaskand pass through a 0.45 μm PTFE filter | Agilent Technologies modular model 1100 system | Ascentis Express C18 column(150 mm × 3.0 mm I.D., 2.7μm) | Gradient: A) water + 0.1% HCOOH & B) ACN (60-90%) + 0.1% HCOOH FR0.4mL/min |
| Burnier et al,2019 | THC, CBN, CBD & THCA | Fast-HPLC-DAD | 100 mg of sample were extracted with ethanol containing tribenzylamine at a concentration of 0.5 mg/mL, tubes were vortexed for 1 min, sonicated for 15 min and finally centrifuged for 60 min at 1509g (3000 rpm, r = 15cm). The resulting samples were filtered on a 0.45 μm PTFE | Waters 1515® HPLC-DAD system | Nucleodur® C18 Gravity column (250 mm x 4.6 mm, particles of 5 μm) | Isocratic: phosphoric acid aqueous solution at 50 mM & ACN (15/85, v/v) FR 3 mL/min |
| Ciolino et al, 2018 | THC, THCA, CBD, CBDA & CBN | HPLC-DAD | Samples were extracted with EtOH, then capped, then vortexed thoroughly to dissolve or disperse the matrix.Samples were filtered 0.45 mm nylon membrane filter. | Agilent 1100, 1200, or 1260 systems. | ACE 5 C18-AR analytical columns (5 μm, 4.6 mm ID x 250 mm length) | 1)Isocratic : 66:34 ACN & 0.5% acetic acid 2)Isocratic: 83:17 MeOH & 50mM citrate FR 1mL/min |
| Citti et al, 2019 | CBD, CBDV & CBDB | HPLC-UV | 10 mg of solid crystals were dissolved in 1 mL of IS working solution. A 100 μL aliquot of the solution was diluted with 900 μL of IS working solution | Agilent 1220 Infinity LC System | C18 column (Poroshell 120 SB-C18, 3.0 × 150 mm, 2.7μm) | Isocratic: A) water + 0.1% HCOOH & B) ACN + 0.1% HCOOH (30:70, v/v) FR 0.5mL/min |
| Deville et al, 2020 | THC, CBDA, CBG, CBGA, THCA, CBD & CBN | UHPLC-DAD | Samples were dried for 24h at 40ºC in a ventilated oven and after grinded. Samples were extracted with a mix of methanol/ chloroform (90/10: v/v) and then filtered on cellulose filter paper. 100 μL of the filtrates were diluted in 3100 μL methanol.25 μL of the IS solution was added to 100 μL of this diluted sample before evaporation under a gentle nitrogen stream. Dried extract was redissolved in 100 μL of a mixture of methanol/water (50/50: v/v) | Acquity UPLC system from Waters | C18 SB 1.8 μm column (2.1 x 100 mm) | Gradient: A) MeOH & B) water + 0.1% HCOOH (50/50) FR0.35 mL/min |
| Dong et al, 2019 | THC, CBD, CBC, THCA, CBDA, THCV, CBDV, THCVA, CBDVA, CBCA & CBL | LC-MS | 50mg samples were extracted with 50mL MeOH. Samples were vortexed at 2000 rpm for 1 min and sonicated for 60 min. An aliquot of 1 mL extract was filtered by 0.22-μm nylon syringe filter. After 100 μL 1% formic acid was added to the vial | Thermo LTQ XL mass spectrometer | Waters Acquity UPLC BEH shield RP18 column (50 × 2.1 mm i.d., 1.7 μm, 130 Å) | Gradient: A) 0.2% HCOOH in water, v/v & B) ACN (50%-85%) FR 0.3 mL/min. |
| Dossantos et al, 2018 | Δ9-THC, CBD, CBC, CBN, CBG, Δ9-THCA A & CBDA | UPLC-MS and UPLC-TWIM-MS | 10mg samples were solubilized in MeOH | Waters Acquity UPLC I-Class | | Gradient:A) 0.1% v/v water/HCOOH & B) 0.1% v/v methanol/HCOOH (60-95%) FR0.50 μL/min |
| Elkins et al, 2019 | THC, CBD, CBC, CBN, CBG, THCA & CBDA | UHPLC-DAD | Samples were cured at 120 °C for 2 h then ground to a fine powder with liquid nitrogen for 1 min at 1500 rpm. After, 10 mg of each sample was weighed into a 2.0 mL microcentrifuge tube . Sample was extracted with 1 mL MeOH, vortexed for 30 s, sonicated for 5 min and centrifuged at 13,000 rpm for 5 min. supernatant to a 2 mL d diluted 1:3 for analysis. Resin was extracted using a super-critical fluid liquid CO2 extractor transferred to a pre-weighed vial and diluted 1:125 with MeOH. Samples were sonicated for 5 min | Agilent 1290 UHPLC system | Phenomenex Luna Omega C18 (150 × 2.1 mm × 1.6 μm) | Gradient: A) water+ 0.1% HCOOH & B, ACN (40-100%) + 0.1% HCOOH FR0.3 mL/min |

## Table I.I: (Continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| Fekete et al,2018 | THC, CBC, CBD, Δ8-THC, THCA, CBDA, THCV & CBDV | UHPLC-DAD | Sample was grinded and homogenised using grinder for 20 min at 6000 rpm. 100mg sample was extracted with 1mL MeOH for 15 min in an ultrasonic bath. Sample was then vortex. Sample was centrifuged 1 min at 14500rpm. Supernatant filtered with 0.2 µm filter | Waters Acquity UPLC H-class system | BEH Shield RP18 column (100 × 2.1 mm, 1.7 µm) ) | Gradient: A) 20 mM ammonium acetate & B) ACN (45-100%). FR0.57mL/min |
| Giese et al, 2015 | THC, THCA, CBDA, CBD, CBG, CBC, Δ-8 THC & CBN | HPLC-DAD | Pregrinding samples in a stainless steel coffee grinder. 1000 mg of sample was placed with 1 mL 2.0 mm zirconia beads and added 15.0 mL EtOH, via a solvent dispenser gravimetrically calibrated to and homogenized at 1500 rpm for 6 min. An aliquot was placed in a 2.0 mL centrifuge tube, and centrifuged at 10000 rpm for 5 min. An aliquot was placed in an HPLC vial and diluted 6-fold with diluent for quantification of the minor cannabinoids, while another was placed in a separate HPLC vial and diluted 96-fold with diluent for quantification of the major cannabinoids | Agilent 1290 HPLC system | Poroshell 120 EC-C18 column (2.7 µm, 150 × 2.1 mm) | Gradient: A) 0.1% HCOOH in water + B,)0.1% HCOOH in ACN(66-95%) FR0.5mL/min |
| Hidayati et al, 2020 | CBN | LC-MS/MS | 1g of sample was extracted with 30mL MeOH, then sonicated and macerated under mechanical shaker at 150 rpm for 5 h | Waters Acquity | Select HSS T3 Waters (1.8 µm, 1 mm × 100 mm) | Gradient: A, Water + 0.1 % HCOOH & B, ACN (20-85%) + 0.1 % HCOOH |
| Nemeškalová et al, 2020 | THC, CBD, CBN, CBDA, CBGA, CBDV, THCA, CBG & Δ8-THC | UHPLC-UV-MS/MS | Samples were extracted with 0.025 µg/mL IS in isopropanol: ethyl acetate (1:1, v/v) and then vortex for 4h. 50 µL aliquot of the solution o was diluted with 950 µL of 60% aqueous ACN with IS at 0.025 µg/mL. Solution was vortexed for 1 min and passed through 0.2 µm PVDF centrifuge filters | Agilent 1290 Infinity liquid chromatography system | Poroshell 120 EC-C18 (100 mm ×2.1mm, 2.7µm) | Gradient: A) 0.1% acetic acid + 10 mmol/L ammonium acetate in 5% aqueous MeOH & B)5% MeOH in ACN(60-100%). FR 0.4mL/min |
| Palmieri et al, 2019 | THC, CBD, CBC, CBG, CBN, CBDV, THCA CBGA & CBDA | HPLC-MS/MS | Dried samples were tritured and crushed and extracted with 1 mL of ethanol in an ultrasonic water bath for 30 min, followed by centrifugation at room temperature at 10,000 rpm for 15 min. The supernatant was passed through a 0.2 µm PTFE filter, diluted 2000 times and collected in an HPLC vial. | Nexera LC20AD XR system | Kinetex C18-XB column (100 × 2.1 mm,2.6µm ) | Gradient: A)water + 5 mM HCOOH & B) ACN(70-99%) + 5 mM HCOOH FR 0.3mL/min |
| Križman, 2019 | THC, CBD, CBN, CBD, CBG, THCA, THCV, CBGA, CBDA & d8-THC | HPLC-UV | Pulverized hemp plant were extracted by sonication for 30 min in EtOH containing IS (20 µg/mL) at a rate of 0.5 g/50 mL. Extracts were further diluted 10- to 20-fold with ethanolic IS solution (20 µg/mL). Hemp resin extracts were dissolved in 2-propanol at 0.5 g/10 mL and then diluted with IS solution 500- to 1000-fold. Samples were then centrifuged at 16.000g for 10 min and the supernatant transferred into HPLC vials. | Finnigan Surveyor HPLC system | Luna C18 (octadecyl silica)(150 mm × 3 mm i.d., 3 µm) | Isocratic: A) water/ACN 9:31 (v/v), + 0.1% HCOOH (v/v) & B) 10 mM ammonium formate. FR 0.8mL/min |
| Mudge et al, 2018 | THC, CBD, CBN, THCA, CBDA, CBG, CBDVA, CBL, CBGA, CBDV, CBC, THCV & Δ8-THC | UHPLC-UV | Samples were extracted with 25 mL of 80% methanol in a 50 mL amber centrifuge tube for 15 minutes by sonication at room temperature with vortexing every 5 minutes, followed by centrifugation at 4500 g for 5 minutes and filtration with a 0.22 µm PTFE | Agilent 1200 UHPLC | Kinetex C18 column (100 mm × 3.0 mm, 1.8 µm ) | Gradient: A) 10 mM ammonium formate & B) ACN |
| Mudge et al,2017 | Δ9-THC, THCA, Δ8-THC, CBD, CBDA, CBG, CBN, CBC & THCV | HPLC-UV | Samples were extracted with 25 mL of 80% methanol in a 50 mL amber centrifuge tube for 15 minutes by sonication at room temperature with vortexing every 5 minutes, followed by centrifugation at 4500 g for 5 minutes and filtration with a 0.22 µm PTFE | Agilent 1200 RRLC system | Kinetex® C18 column (1.7 µm, 100 × 3.0 mm) | Gradient:A) 10 mM ammonium formate & B) ACN(66-80%). FR0.6 mL/min |
| Pavlovic et al, 2019 | THC, CBD, CBN, CBG, CBC, CBDV, THCV, CBDA, THCA, CBNA, CBCA, CBGA, CBDVA & THCVA | HPLC-Q-Exactive-Orbitrap-MS | 100 mg of sample was homogenized with an equal weight of diatomaceous earth and transferred into the cell. 100 µl of extraction solution containing the IS (diazepam 1 mg ml−1) was added. The remaining empty part of the cell was filled-up with diatomaceous earth. temperature of 25°C, pressure of 1500 psi), 2 static cycles, 5 min each, 60 s purging with nitrogen and 90% rinse volume were used for the study. Organic extracts (25 ml) were obtained using pure MeOH and were dried under vacuum in a centrifugal evaporator. The residue was dissolved in 1 ml of ACN, and after proper dilution (1:10) in starting mobile phase, 2 µl were submitted to analysis | HPLC system Thermo Fisher Scientific | RP HPLC column (150 × 2 mm, 4 µm) | Gradient: A) water + 0.1% HCOOH & B) ACN + 0.1% HCOOH FR 0.3mL/min |

## Table I.I: (Continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| Pellati et al,2018 | CBDA, CBGA, CBG & CBD | HPLC-ESI-MS & HPLC-MS/MS | 0.25g of sample were extracted for 15 min under magnetic stirring with 10mL EtOH. The solution was filtered with a paper filter and the residue was extracted twice more with the same procedure with 10 and 5 mL of solvent. The three extractions were then combined and brought to 25 mL with the solvent in a volumetric flaskand pass through a 0.45 μm PTFE filter | Agilent Technologies modular model 1100 system | Ascentis Express C18 column (150 mm × 3.0 mm I.D., 2.7 μm) | Gradient: A)0.1% HCOOH + H2O & B) 0.1% HCOOH + ACN(60-90%). FR 0.4mL/min |
| Ribeiro Grijó et al, 2019 | THC, CBD & CBN | HPLC-DAD | 1.Samples were decarboxylation (heating) followed by extraction with pure scCO2 in a single step. 2 Samples were extracted with sequential extraction steps under different conditions (temperature and pressure) and a change of solute/solvent polarity (previous decarboxylation , and 5% ethanol was used as co-solvent to obtain a fraction with higher cannabinoid content. | HPLC Chromatograph (Shimadizu, 20A) | RP-8 column (SUPELCOSIL (TM): 250 × 4.5 mm, 5 μm) | Isocratic: ACN & water (8:2 v/v) FR1.0mL/min |
| Zweigenbaum, 2020 | THC, CBD, CBN, CBG, CBDA & THCA | HPLC-MS/MS | 1g of homogenized sample was extracted with15 mL ACN. and shaken for 120s. Solution is introducedin an unconditioned SPE cartridge, allowed to elute by gravity and collected in another container. The Tube containing 1g aliquot of sample is washed with 5mL of ACN and this is decanted into the SPE cartridge and collected. This is repeated one more time with 5 mL ACN. The total volume is brought to 25mL. Finally, 100μL of this extract is pipetted into a 2mL autosampler vial and diluted with 900μL of ACN. | Agilent 1260 Infinity II | Poroshell 120 EC-C18, 2.1 x 50mm, 1.9μm | Gradient: A) 0.1% acetic acid + 5mM ammonium acetate in ultra-high purity water & B)ACN(5-100%). FR 0.65mL/min |

## Table I.II: Liquid Chromatographic Method of analysis of Cannabis from human fluids and hair.

| Author and date | Cannabinoids | Method | Sample preparation | HPLC unit used | Stationary phase | Mobile phase |
|---|---|---|---|---|---|---|
| Chang et al, 2016 | THC, CBD, CBN and THC-COOH | HPLC-MS/MS | 30 µL of IS (1 µg/mL) were added to 2 mL of urine specimen. 60 µL of 11.8 M sodium hydroxide was added and mixed at 60°C for 20 min for hydrolysis. 350 µL of 0.1 M acetic acid and 6 mL of an isopropanol:dichlo- romethane mixture (1:9 v/v) were added and mixed. The sample was centrifugated at 2500 rpm for 5 min and then dried under nitrogen at 60°C. The residue was added to 300 µL of 0.02% formic acid in a water:methanol mixture (4:6 v/v) | Agilent 1200 series HPLC system | Supelco Discovery HS C18 column (50 × 2.1 mm i.d.; 3 µm) | Gradient: A) pure water + 0.02% HCOOH & B) MeOH (40-90%). FR0.3mL/min |
| Cho et al,2018. | THC-COOH | UHPLC-MS/MS | Hair was washed twice each with 2 mL of MeOH and distilled water. Hair was digested with 1 M NaOH, extracted twice with mixed n-hexane:ethyl acetate | Agilent 1290 UHPLC (pump 1), Agilent 1260 (pump 2) | Halo C18 column (2.1 mm × 75 mm, 2.7 µm) | Gradient (1290)A) 2mM ammonium formate/0.2% HCOOH in DW & B) 2mM ammonium formate/0.2% HCOOH in ACN(41-95%). FR0.5-0.8mL/min Gradient (1260)A) 0.5mM ammonium formate in DW & B) 0.5mM ammonium formate in ACN(5-90%). FR0.3mL/min |
| Dybowski et al,2020 | CBD | UHPLC-MS | Precipitation agent was added to 500µL of human plasma or pig plasma or HSA solution or pig plasma for tified in protein by HSA addition or egg white protein solution, containing CBD. The samples were vortex mixed, incubated and centrifuged for 5 min at 18600 xg | UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) | Gemini C18 column (4.6 x 100 mm, 3 µm) | Isocratic:A) 25 mM HCOOH + water & B) 25 mM HCOOH + ACN.60:40 v/v FR0.5mL/min |
| Dziadosz et al, 2016 | THC, 11-OH-THC & THC-COOH | HPLC-MS/MS | 15 µL of human serum spiked with THC and metabolites and 150 µL of 70 % mobile phase B were added to 10-µL of IS. Protein precipitation was performed by vigorous shaking for 10 min and centrifugation | HPLC system Shimadzu | Luna 5µm C18 (2) (100 A, 150 x 2mm ) | Gradient: A) H2O/MeOH 95:5, v/v +10 mM ammonium acetate + 0.1 % acetic acid & B) H2O/MeOH, 3:97, v/v + 10 mM ammonium acetate + 0.1 % acetic acid (70-100%) FR 0.4-0.5mL/min |
| Klawitter et al, 2017 | THC, 11-OH-THC, THC-COOH, THC-C-gluc, CBD, CBN, CBG, CBDV, THCV & THCV-COOH | HPLC-MS/MS | 10µL of 20-fold aliquots of 200 µL of the calibrator, quality control, or blank sample (urine or plasma) were added to 800 µL of 0.2 mol/L ZnSO4 30% water/70% methanol (v/v) and IS (5 ng/mL) . Samples were vortexed for 10 minutes and then centrifuged at 27,500·g, 4 °C for 10 minutes | Agilent 1200 series LC system | Poroshell Eclipse C18 analytical column (4.6 x 50 mm, 2.7µm) | Gradient: A) water + 0.1%HCOOH & B) 20% isopropanol + 20% MeOH + 60% ACN (60-100%) FR0.75-1 mL/min |
| Moorthy et al,2019 | THC, CBD & CBN | UHPLC-MS/MS | VAMSTM devices speak with blood were dried at room t[a] for 60 h . After , each device was placed into a 96-well plate and added 0.1 M zinc sulfate-0.1 M ammonium acetate (50:50, v/v) (40 µL), a 5.00 ng/mL IS working solution (20 µL), and a precipitation solution containing 0.1% HCOOH in ACN (250 µL). Samples were vortexed for 15 min at 800 rpm, sonicated for 15 min, and centrifuged for 15 min at 4000 rpm at 4 °C. Supernatants (250 µL) were transferred to a new 96-well plate and each sample was diluted (1:1, v/v) with water then mixed. Each sample is washed with a 25% methanol solution (2x 250 µL) and eluted (2x 25 µL) with a solution of MeOH-ACN (10:90, v/v). Samples were diluted with 50 µL of water through the SPE columns and vortexed for 3 min at 1100 rpm | AB Sciex Exion UHPLC system | Acquity UPLC HSS C18 column (1.8 µm, 2.1 mm × 100 mm) | Gradient: A) 5 mM ammonium formate in water +0.05% HCOOH & B) ACN (50-96%) FR0.4mL/min |

## Table I.II: (Continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| Pichini et al, 2019 | THC, CBD, THCA-A, CBDA, THC-COOH,THC-COOH-gluc,11-OH-THC & THC-gluc | UHPLC-MS/MS | 100 µL of biological sample were added to 100 µL M3® reagent, 200 µL ISs solution ( 25 ng/mL) and 200 µL acetone:ACN (8:2, v/v) in polypropylene microcentrifuge tubes . Tubes were capped, vortexed for 10 s and centrifuged at 5,000 g for 5 min. Urine samples were further analyzed following alkaline hydrolysis to quantify total CBD. The sweat patches: sample extracted with 2 mL MeOH spiked with deuterated ISs (2.5 ng/mL). Tubes were capped, vortexed for 10 s and centrifuged at 5,000 g for 5 min. | Waters® Xevo® TQ-S | ACQUITY UPLC® BEH C18 column from Waters® (50 mm x 2.1 mm, 1.7 µm) | Gradient: A) ammonium formate 50 mM & B) MeOH(40-100%), FR 0.4mL/min |
| Pires da Silva 2020 | THC, CBD, CBN, 11-OH-THC & THC-COOH | UHPLC-MS/MS | 100µl of plasma were added to 200 µl IS. Sample vortex-mixed for 60 s and then 50 mg of a salt mixture composed of magnesium sulfate, sodium chloride and sodium citrate dihydrate (4:1:1, w/w/w) was added. A 5 mm diameter stainless steel ball was added to avoid compaction of the salt mixture. The sample was again vortex-mixing step for 60 s. After,the mixture was centrifuged at 10,000g for 10 min | Acquity UPLC® chromatog- raphy system | Acquity® BEH C18 (100 × 2.1 mm, 1.7 µm) | Gradient: A)water+ 0.1% HCOOH & B) ACN + 0.1% HCOOH (5-95%) FR0.15mL/min |
| Toennes et al, 2014 | THC, THCOH & THCCOOH | LC-MS/MS | 0.2 ml serum were added to 4.8 ml of deionized water and 50 µl IS mixed. The samples were slowly loaded onto the extraction cartridges preconditioned with MeOH (3 ml) and water (3 ml). After washing with water, 0.25 M acetic acid, and 70 % ACN (1 ml each), the extraction cartridges were dried with air for 15 min. Samples were added to 2 ml of acetone which were evaporated with air to dryness at 25 °C. The dry residue was reconstituted in 100 µl of ACN/ MeOH/water (6:6:4, v/v/v) | Agilent 1290 Infinity LC | KinetexTM XB-C18, 100 Å, (100 × 2.1 mm) | Gradient: A) 0.01 % HCOOH + 5 mM ammonium formate & B) ACN + 0.1 % HCOOH (50-100%) FR0.5mL/min |
| Wei et al, 2015 | THC, COOH–THC, OH–THC, CBD & CBN | UHPLC–MS/MS | 500 µL of each urine sample were added to 50 µL of IS and 50 µL of enzyme solution. After mixing, 96-well plate was incubated at 37 °C for 2 h. Then, 50 µL of 10 N NaOH was added and incubated at 70 °C for 20 min. After, plate to room temperature, 400 µL of HCOOH, water, and MeOH (v/v/v 12.5:12.5:75) was added . Enzyme and NaOH solutions were replaced with 450 µL of formic acid, water, and methanol (v/v/v 5.5:27.8:66.7). After mixing for 5 min, the plate was centrifuged for 30 min at −5 °C, and then 0.96 mL mixtures from each well was transferred onto the 96-well SPE plate, pre-equilibrated with 1.0 mL of MeOH and 1.0 mL of buffer (5 mM ammonium formate, 0.05% HCOOH). After soaking for 10 min, the mixtures were pushed through the SPE under approximately 1.0 psi positive pressure. Samples were washed with 1.0 mL of water and 1.0 mL of MeOH and water (v/v 60:40) | Shimadzu UHPLC system | Kinetex C18 column (100 mm × 2.1 mm, 2.6 µm) | Gradient: A) 5.0 mM of ammonium formate + 0.05% HCOOH & B) ACN FR0.4mL/min |

## Table I.III: Liquid Chromatographic Method of analysis of Cannabis from oil.

| Author and date | Cannabinoids | Method | Sample preparation | HPLC unit used | Stationary phase | Mobile phase |
|---|---|---|---|---|---|---|
| Araneda et al, 2020 | THC & CBD | HPLC-DAD | Samples were weighted diluted with MeOH. | Thermo Dionex Ultimate 3000 system | Phenomenex Aqua (150 mm x 4.6 mm; 5 µm particles) | Gradient :A) water & B) ACN |
| Bettiol et al, 2019 | THC, CBD, CBN & THCA | HPLC-DAD | 40 µl of the sample were added to 960 µl tetrahydrofuran (THF) and vortex. Then 50 µl of such solution were added to 950 µl ACN and vortex. | Thermo- Fisher Surveyor Plus | Agilent PoroshellR 120 SB-C18 column, (2.1 mm × 150 mm; 2.7 µm) | Isocratic:ACN/5mM phosphate buffer rate 75/25 v/v FR0.38mL/min |
| Ciolino et al, 2018 | THC, THCA, CBD, CBDA & CBN | HPLC-DAD | Ground material were extracted with 30ml EtOH or isopropyl alcohol. Sample were filtered (0.45µm nylon membrane filter), solvent was evaporated heated water bath (45–50 ° C) under a nitrogen purge | Agilent 1100, 1200, or 1260 HPLC-DAD systems | ACE 5 C18-AR analytical columns (5 mm, 4.6 mm ID x 250 mm length) | 1)Isocratic : 66:34 ACN & 0.5% acetic acid 2)Isocratic: 83:17 MeOH & 50mM citrate FR 1mL/min |
| Citti et al, 2018 | THC, CBD, THCA, CBDA, CBDV, CBG & CBN | HPLC-UV & HPLC-MS | 100 µL of hemp seed oil were diluted with 395 µL of 2-propanol and 5 µL of IS solution. The sample was vortex mixed for 1 min | Agilent Technologies modular model 1200 system, | A Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 µm) | Gradient A) water + 0.1% HCOOH and B)ACN (70-95%) + 0.1% HCOOH FR0.4mL/min |
| Deidda et al, 2019 | THC & CBD | RP-HPLC/UV | 40µL solution A (FM2® olive oil extracts samples) were added to 960 µL of THF(solution B"). 50µL of solution B were added to 950µL MeOH | Thermo- Fisher Surveyor PlusTM HPLC system | Agilent Poroshell®120 SB-C18 analytical column (2.7 µm, 150 mm × 2.1 mm) | Isocratic:ACN/5mM phosphate buffer rate 75/25 v/v FR0.38mL/min |
| Mudge et al,2017 | Δ9-THC, THCA, Δ8-THC, CBD, CBDA, CBG, CBN, CBC & THCV | HPLC-UV | 50.0 mg of oil were added to 10.00 mL of MeOH and vortexed for 30 s. Extracts were sonicated for 15 min with vortexing every 5 min. Samples were filtered with 0.22-µm Teflon filters | Agilent 1200 RRLC system | Kinetex® C18 column (1.7 µm, 100 × 3.0 mm) | Gradient:A) 10 mM ammonium formate & B) ACN(66-80%) FR0.6mL/min |
| Nemeškalová et al, 2020 | THC, CBD, CBN, CBDA, CBGA, CBDV, THCA, CBG & Δ8-THC | UHPLC-UV-MS/MS | 250mg of oil were added to 0.025 µg/mL IS in isopropanol: ethyl acetate (1:1, v/v) and then mixed using a rotating mixer for 15 min at room temperature.50 µL of the sample was diluted with 950 µL of 60% aqueous ACN with IS at 0.025 µg/mL. The sample solution was vortexed for 1 min and passed through 0.2 µm MicroSpin PVDF centrifuge filters | Agilent 1290 Infinity liquid chromatography system | Poroshell 120 EC-C18 (100 mm ×2.1mm, 2.7µm) | Gradient: A) 0.1% acetic acid + 10 mmol/L ammonium acetate in 5% aqueous MeOH & B)5% MeOH in ACN(60-100%). FR 0.4mL/min |
| Pichini et al, 2019 | THC, CBD, THCA-A, CBDA, THC-COOH,THC-COOH-gluc,11-OH-THC & THC-gluc | UHPLC-MS/MS | 50 µL of Oil spiked with 100 µL IS (100 µ g/mL) was added to 5 ml hexane/ethyl acetate 9:1 (v/v). 1 µL of this solution was diluted in 1 ml of MeOH. 50 µL of this solution was added to 950 µL of mobile phase | Waters® Xevo® TQ-S | ACQUITY UPLC® BEH C18 column from Waters® (50 mm x 2.1 mm, 1.7 µm) | Gradient: A) ammonium formate 50 mM & B) MeOH(40-100%), FR 0.4mL/min |

## Table I.IV: Liquid Chromatographic Method of analysis of Cannabis from miscellaneous matrices.

| Author and date | Cannabinoids | Method | Sample preparation | HPLC unit used | Stationary phase | Mobile phase |
|---|---|---|---|---|---|---|
| Brighenti et al, 2019 | THC, CBD, CBG, THCA, CBDA & CBGA | HPLC-ESI-MS/MS | 2g of honey were added to 8mL of H2O under continuous agitation. 10 mL of ACN and 50 µL of IS were added too. The roQ QuEChERS kit was added too and sample was shaken immediately for 1 min. Sample was centrifuged at 4000 rpm for 5 min and the upper organic layer was brought to dryness under vacuum. The residue was dissolved into 150 µL of a 2.0 mM aqueous CH3 COONH4 -ACN (50:50, v/v). | Agilent 1200 HPLC system | Kinetex EVO C18 column (100 × 2.1 mm, 5 µm particle size) | Gradient: A)2.0 mM aqueous CH3COONH4 and B) ACN(60-90%) FR 0.35mL/min |
| Chang et al, 2016 | THC, CBD, CBN and 11-nor-9-carboxy-THC | HPLC-MS/MS | 1g hemp nut was added to 1 mL of IS (1 µg/mL) and to 19 mL of solvent 60% isopropanol. Mixture underwent ultrasound-assisted extraction for 15 min. Extract was filtered using filter paper, and solvent 60% isopropanol was added to make a total volume of 20 mL. The final extract was then filtered using a syringe filter PTFE membrane, 0.2 µm | Agilent 1200 series HPLC system | Supelco Ascentis C18 column (100 × 2.1 mm i.d.; 3 µm) | Gradient: A) pure water + 0.1% HCOOH & B) ACN(40-90%) + 0.1%HCOOH FR0.3mL/min |
| Ciolino et al, 2018 | D9-THC, THCA, CBD, CBDA & CBN | HPLC-DAD | Samples were extracted with EtOH, then capped, then vortexed thoroughly to dissolve or disperse the matrix.Samples were filtered 0.45 mm nylon membrane filter. | Agilent 1100, 1200, or 1260 HPLC-DAD systems | ACE 5 C18-AR analytical columns (5 µm, 4.6 mm ID x 250 mm length) | 1)Isocratic : 66:34 ACN & 0.5% acetic acid 2)Isocratic: 83:17 MeOH & 50mM citrate FR 1mL/min |
| Duffy et al, 2020 | THC, CBD, CBN, CBG, THCA, THCV, CBC, CBDV, CBDA, CBGA & Δ8-THC | 1)LC-HRMS/MS 2)HPLC-PDA | 10 mg of residual fluid, was diluted with ACN (1.00 mL). When less than 10 mg of fluid was available, the amount of ACN added was adjusted accordingly to maintain a similar concentration. | Shimadzu HPLC | 2)A Poroshell EC-C18 HPLC column (Agilent, 2.1 × 100 mm; 2.7-µm particle size) 3)Agilent Poroshell 120 column (3.0 × 150 mm with 2.7-µm) | 2)Gradient A) 0.1% v/v HCOOH + water & B,) 5 mM ammonium formate +MeOH(20-80% FR1.5mL/min 3)Isocratic 73% v/v ACN + water with 0.1% v/v HCOOH FR0.25mL/min |
| Escrivá et al, 2017 | THC, THC-COOH & THC-OH | LC-MS/MS | 3 g of samples (milk, liver or hemp seeds) were spiked with 100 ng of each IS adding 10 lL of a mixture at a concentration of 10 µg/mL-1. In the case of liver, 5 mL of 0.1 M potassium phosphate buffer (pH 6.8), and 200 lL b-glucuronidase solution (50,000 U/mL 0.1 M phosphate buffer) were added and incubated the mixture at 37 ºC for 16 h. 10 mL of MeOH were added to the sample, shaken 1 min, sonicated at 40 ºC for 5 min and centrifuged at 4000 rpm for 5min. Supernatant was transferred into a 100 mL volumetric flask and diluted with distilled water and SPE was carried out. Extracts were evaporated to dryness under a gentle stream of N2 at 30ºC, dissolved in 0.2 mL of MeOH with 0.1% HCOOC by an ultrasonic bath for 5 min | Agilent 1260 UHPLC series | Kinetex C18 (1.7 µm, 100 A, 50 x 2.10 mm) | Gradient: A) water + 0.1% HCOOH & B) MeOH (70-95%) + 0.1% HCOOH FR0.2mL/min |
| Heo et al, 2016 | THC | 1)UHPLC-UV 2)UHPLC-MS/MS | 1 g of the homogenized samples was added to 100% MeOH and vortexed briefly and sonicated for 30 min. Additional MeOH was added to a 20-mL volumetric flask after cooling. The stock solution was filtered through a 0.22-µm PVDA filter | Acquity UPLCTM system | 1)Waters Acquity UPLC HSS C18 (2.1 mm × 150 mm, 1.8 µm) 2)Waters Acquity UPLC BEH C18 column (2.0 mm × 100 mm,1.7 µm) | 1)Gradient:A) 25 mM sodium phosphate + 0.01% sodium hexane sulfonate in deionized water with phosphoric acid & B) ACN (60-100%) FR0.18mL/min 2)Gradient: A) 0.1% HCOOH + distilled water & B) 0.1% HCOOH + ACN(5-80%) FR0.25mL/min |
| Jacox et al, 2017 | THC, THCCOOH & THCCOOH-glucuronide | UHPLC-MS/MS | 100 mL of each wastewater sample was spiked with 50 mL of IS mixture (0.1 mg/mL), and filtered through a glass microfiber filter. Then, 0.5 mL of HCl was added immediately to acidify the solution to maximize retention onto mixedmode cartridges, SPE was carried out | UHPLC from Shimadzu | Kinetex C18 (2.1 mm x 100 mm, 1.7 µm) | Gradient: A) 0.1% HCOOH + water and B) 0.1% HCOOH + ACN(40-95%) FR 0.5mL/min |
| Jornet-Martínez et al, 2018 | THC, CBD & CBN | IT-SPME-nanoLC-UV | Cotton tips of the swabs were introduced into 2-mL glass vials, and added 1 mL of MeOH. Vials were introduced an ultrasonic bath for 15 min. Liquid phase was removed and filtered , IT-SMPE was carried out. | Agilent 1260 Infinity nanoLC chromatograph | Zorbax 300SB C18 (50 × 0.075 mm id, 3.5 µm) | Gradient: water + ACN(55-75%) FR0.5mL/min |
| Nemeškalová et al, 2020 | THC, CBD, CBN, CBDA, CBGA, CBDV, THCA, CBG & Δ8-THC | UHPLC-UV-MS/MS | For every gram of gelatinous gummies sample, 5 mL of 0.025 µg/mL IS in ultrapure water was added, and the tubes were vortexed until the content dissolved. An aliquot of 500–2000 µL was placed in a separate tube and mixed with the same amount of acetonitrile (with IS at 0.025 µg/mL) to precipitate proteins. The mixture was vortexed for 1 min and allowed to settle for 5 min. Ointments were weighed into test tubes, 5 mL of 0.025 µg/mL IS in isopropanol: ethyl acetate (1:1, v/v) was added and the mixture was vortexed for 15 min. Liquids were weighed into 5 mL volumetric flasks and an appropriate solvent (0.025 µg/mL IS in in isopropanol,) was added. The flasks were sealed and then mixed using a rotating mixer for 15 min at laboratory temperature. Regardless of the matrix type, a 50 µL aliquot of the obtained solution or supernatant was diluted with 950 µL of 60% aqueous acetonitrile with IS at 0.025 µg/mL. The resulting solution was vortexed for 1 min, passed through 0.2 µm MicroSpin PVDF centrifuge filters | Agilent 1290 Infinity liquid chromatography system | Poroshell 120 EC-C18 (100 mm ×2.1mm, 2.7µm) | Gradient: A) 0.1% acetic acid + 10 mmol/L ammonium acetate in 5% aqueous MeOH & B)5% MeOH in ACN(60-100%). FR 0.4mL/min |
| Pichini et al, 2019 | THC, CBD, THCA-A, CBDA, THC-COOH,THC-COOH-gluc,11-OH-THC & THC-gluc | UHPLC-MS/MS | 100 µL of sample was added to 300 µL M3® reagent spiked with 10 µL deuterated ISs (100 µg/mL). Sample was vortexed for 10 s and centrifuged at 5,000 g for 5 min. | Waters® Xevo® TQ-S | ACQUITY UPLC® BEH C18 column from Waters® (50 mm x 2.1 mm, 1.7 µm) | Gradient: A) ammonium formate 50 mM & B) MeOH(40-100%), FR 0.4mL/min |

## Appendix II Chromatograms for method development for the separation of the three cannabinoids

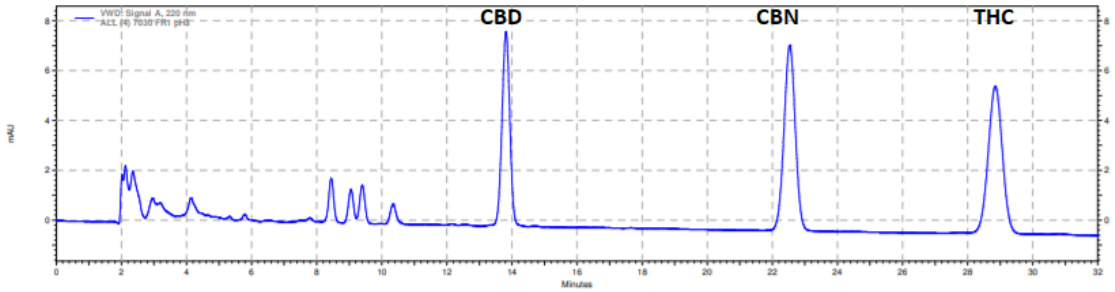


**Figure II.I: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 1mL/min**

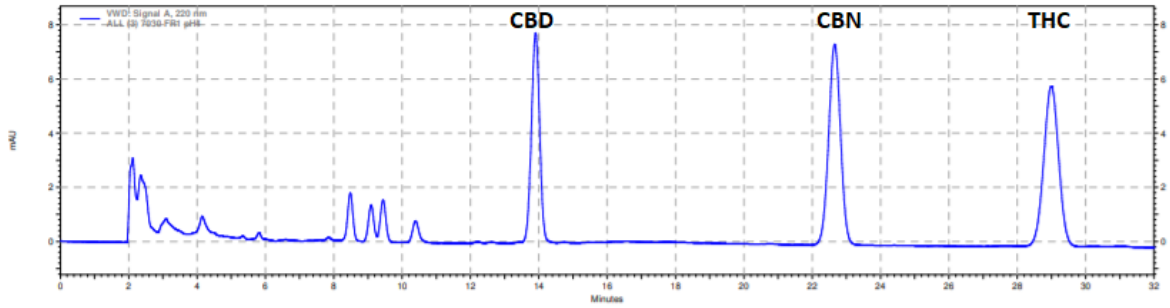


**Figure II.II: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 1mL/min**

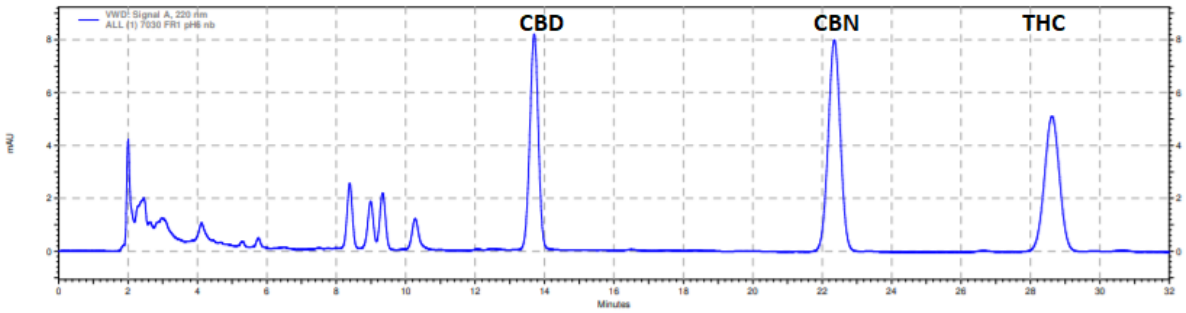


**Figure II.III: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 1mL/min**

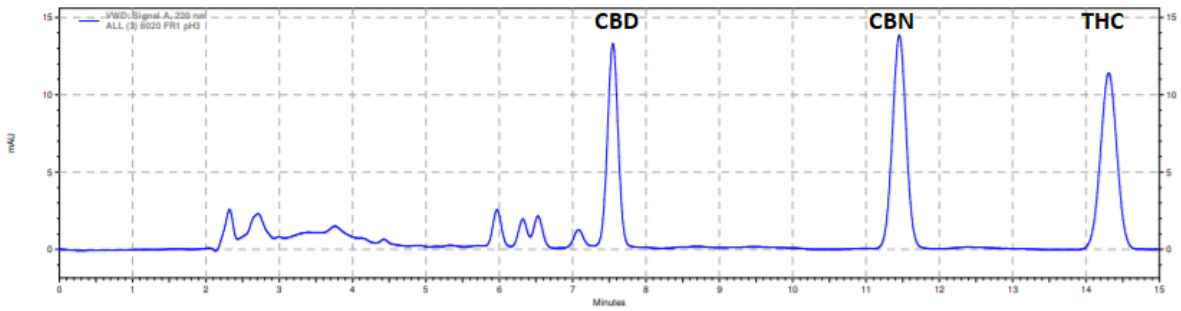


**Figure II.IV: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1mL/min**

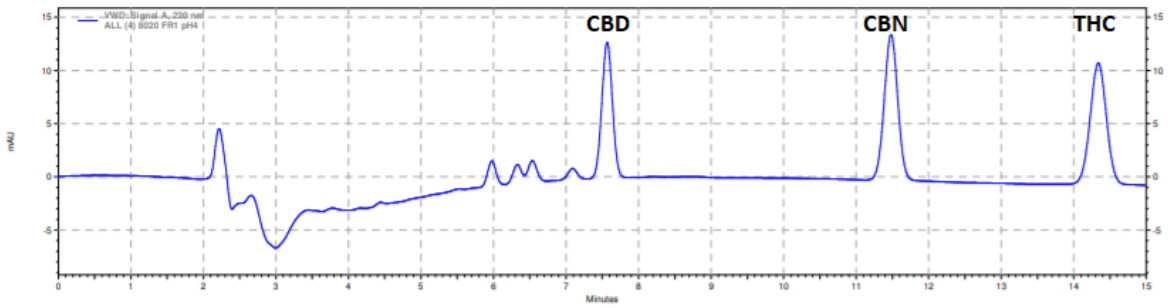


**Figure II.V: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1mL/min**

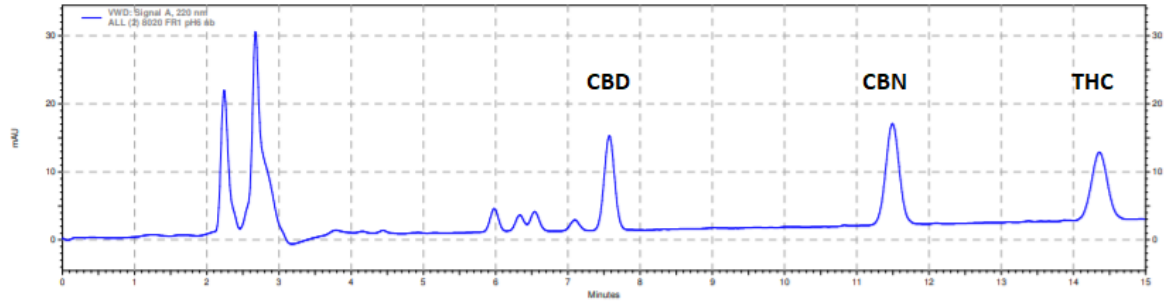


**Figure II.VI: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1mL/min**

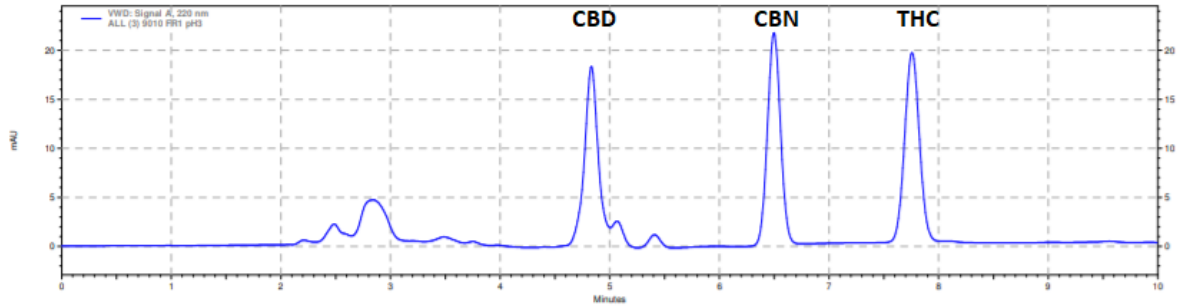


**Figure II.VII: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (10:90 v/v); detection wavelength 220nm; flow rate 1mL/min**

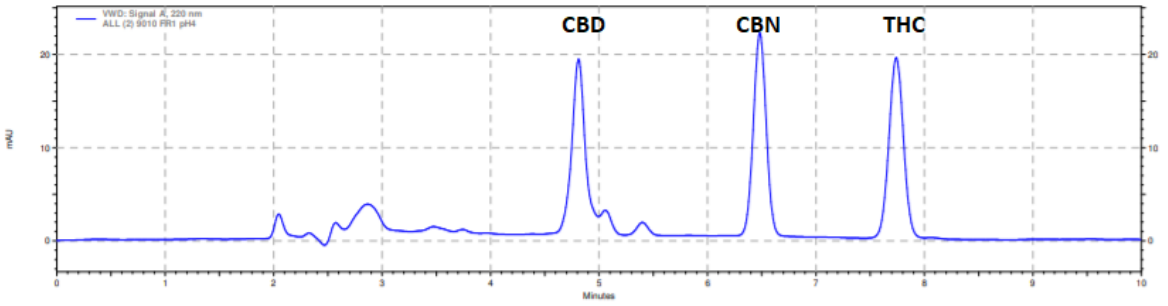


**Figure II.VIII: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (10:90 v/v); detection wavelength 220nm; flow rate 1mL/min**

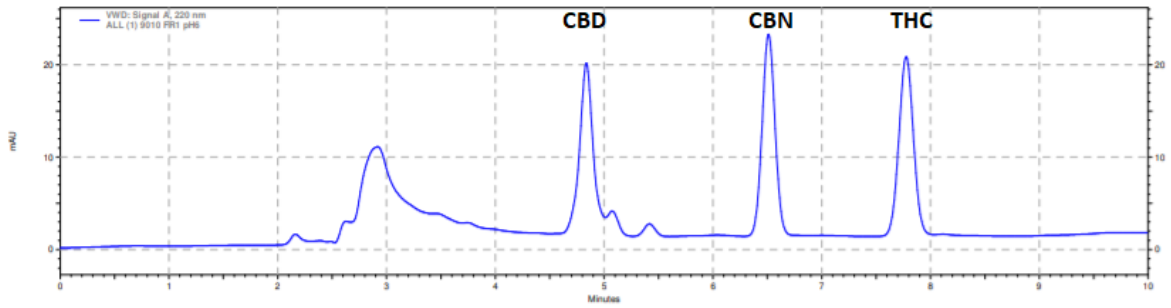


**Figure II-IX: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (10:90 v/v); detection wavelength 220nm; flow rate 1mL/min**

## Appendix III Chromatograms for the analysis of THC in MCT oil

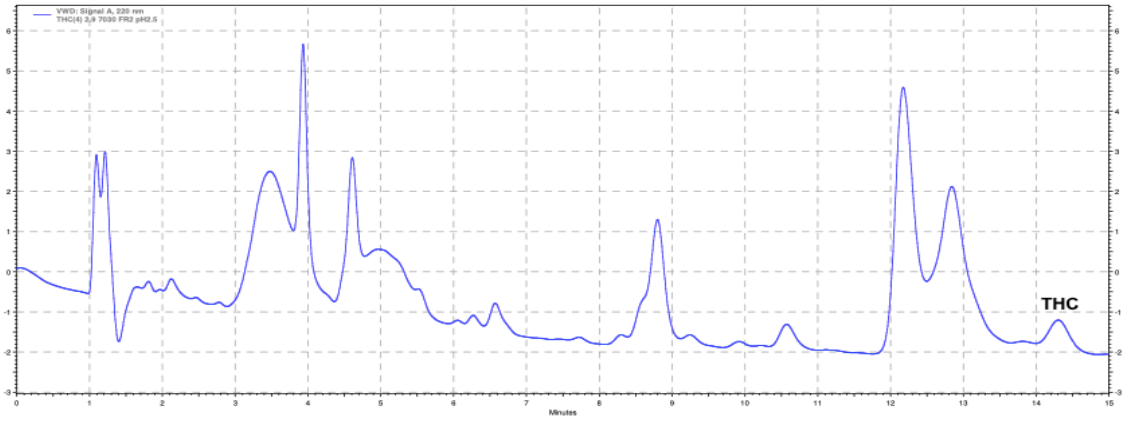


**Figure III.I: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 2 mL/min**

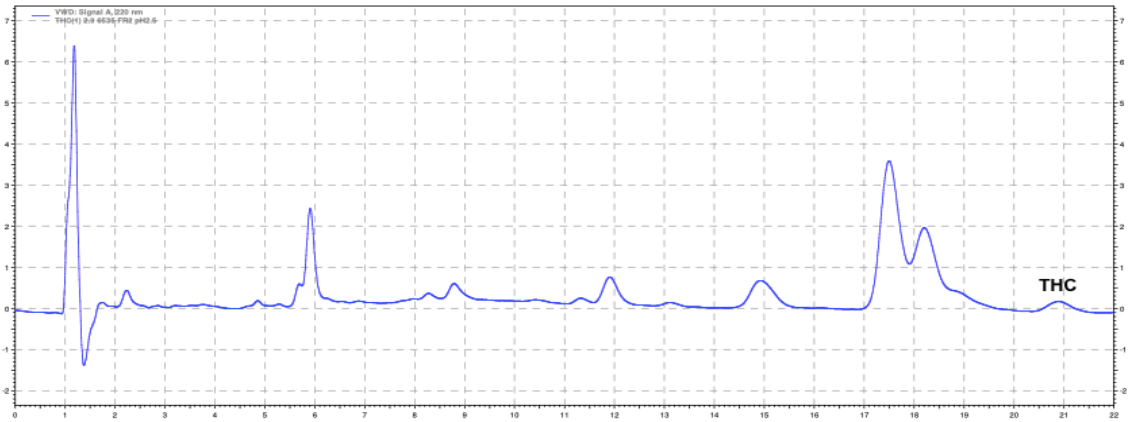


**Figure III.II: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (35:65 v/v); detection wavelength 220nm; flow rate 2 mL/min**

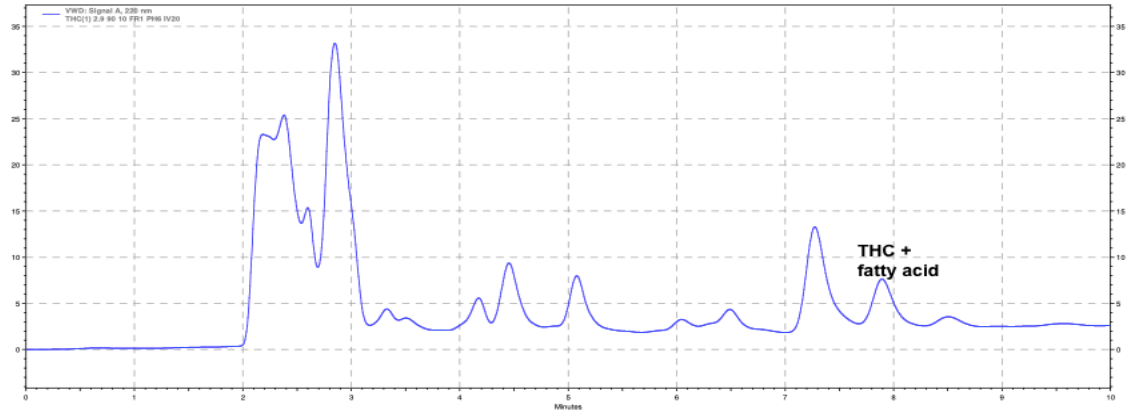


**Figure III.III: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (10:90 v/v); detection wavelength 220nm; flow rate 1 mL/min**

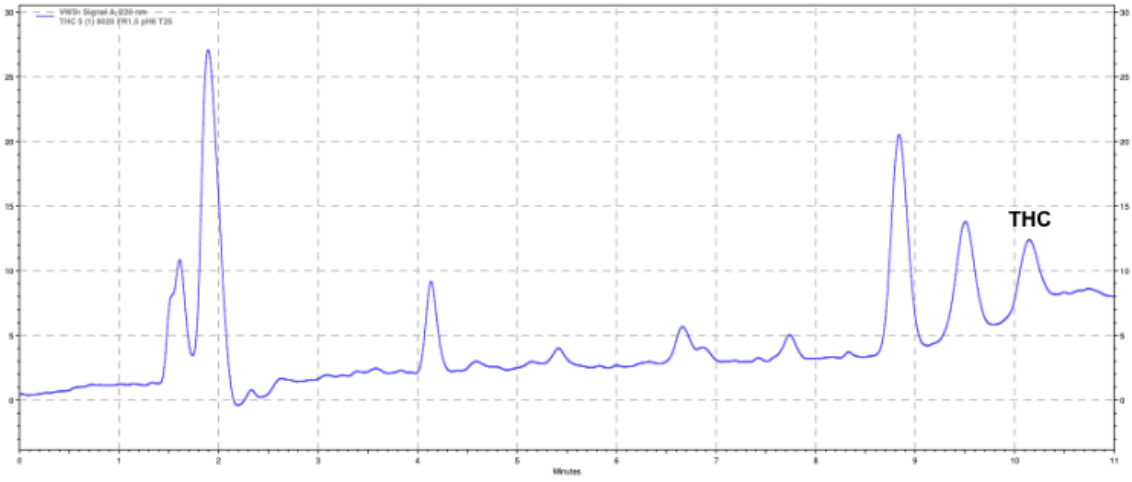


**Figure III.IV: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1 mL/min**

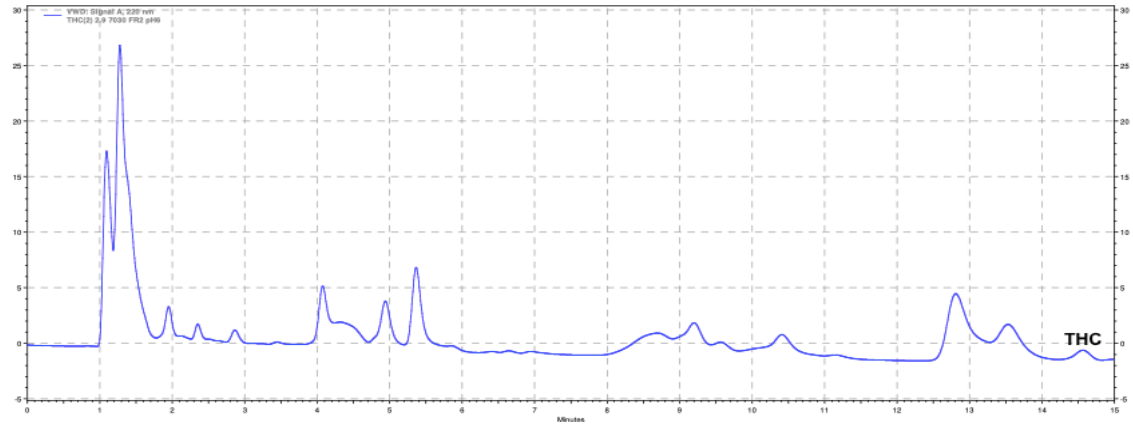


**Figure III.V: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 2 mL/min**

**Appendix IV Articles published**

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Research Article

# EFFECTS OF PH AND AMOUNT OF ACETONITRILE ON THE SEPARATION OF CANNABINOIDS

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**ABSTRACT**

**Objective:** During reversed-phase high-performance liquid chromatography (HPLC) analyses, optimization of separation can be achieved by selecting appropriate chromatographic conditions. The retention time, peak shape, and peak size of chromatographic peaks are dependent on amount of organic modifier in the mobile phase and buffer pH. The aim of this study was to investigate the effects of varying pH, acetonitrile composition and flow rate of the mobile phase, and temperature of the stationary phase and wavelength in the development of a method to separate Δ9 tetrahydrocannabinol, cannabidiol, and cannabinol.

**Methods:** Mobile phases with different buffer pHs and acetonitrile composition were used with ultraviolet (UV) detection wavelength of 220 nm and 228 nm. The AUPs and retention times were observed using different mobile phase flow rates and stationary phase temperatures.

**Results:** The best results were obtained when using a mobile phase composition of 20% phosphate buffer pH 2.5 or pH 3 and 80% acetonitrile v/v at a flow rate of 2 mL/min at 220 nm.

**Conclusion:** This rapid and easy-to-use HPLC method describes the effect of changing important chromatographic parameters on separation and retention time of cannabinoids and can be effectively applied for high throughput analysis.

**Keywords:** Reversed-phase high-performance liquid chromatography, pH, Acetonitrile, Δ9 tetrahydrocannabinol, Cannabidiol, Cannabinol.

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## INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) separates analytes based on differences in polarity [1] and is the most commonly used type of liquid chromatographic analytical technique, preferred by the pharmaceutical industry [2,3]. When developing analytical HPLC methods, various parameters have to be considered to achieve favorable resolution, specificity, peak shape, retention time, and total run time [4,5]. Different detectors such as ultraviolet (UV), fluorescence, and mass spectrometry (MS) can be coupled to HPLC. MS detectors have very good sensitivity and selectivity but require skilled expertise to operate and are relatively expensive. UV detectors are often preferred as they are easier to operate, cheaper, and more readily available [6-8].

Optimization of separation of analytes can be achieved by selecting appropriate stationary phase and mobile phase characteristics and appropriate UV detection wavelength. Shorter retention times may be achieved with an increase in temperature of the stationary phase although stationary phase stability can be compromised if temperatures are too high [9,10]. The type and amount of organic modifier, pH of buffer, and flow rate of the mobile phase have an effect on peak shape, retention time, and resolution [11-14]. Chromatographic run times should ideally not be too long for more efficient analyses but not too short so as to compromise resolution and selectivity [15].

The majority of reversed-phase chromatographic analyses operate at pH values in the range of 2–8 [16]. Control of pH of the buffer used in the mobile phase is important when separating analytes can be ionized. pH control can affect symmetry and peak shape which is enhanced when the analytes are present in either an ionized or unionized form. Ionization also affects relative distribution of analytes between mobile and stationary phase, influencing retention time in the process. Phosphate buffers are widely used in HPLC analyses as they are inexpensive, produce good chromatograms, and can be used for a range of pH values since phosphoric acid has three different buffering ranges: pH 1.1–3.1, pH 6.2–8.2, and pH 11.3–13.3 [15]. Methanol and acetonitrile are the most commonly used buffers in isocratic reversed-phase HPLC. Acetonitrile produces less of an increase in pressure and shorter run times when compared to methanol [17,18].

The versatility of HPLC allows for the identification and quantification of a variety of compounds, both of natural and synthetic origin. HPLC can be effectively used to analyze compounds which present in the cannabis plant. *Cannabis sativa* is a dioecious plant belonging to the *Cannabaceae* family and has been used for millennia for recreational purposes, as a folk medicine and as a source of textile fiber [19-21]. Following a renewed interest in the properties of the cannabis plant after the description of cannabinoid receptors and the endogenous endocannabinoid system [22], efforts are now being put in trials and research on cannabis for medicinal purposes such as management of epilepsy, pain, and chemotherapy-induced nausea and vomiting [23-25]. The three most commonly studied cannabinoids known to exert physiological effects are Δ9 tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN) [26-28] (Fig. 1).

THC, CBD, and CBN are weakly acidic compounds having pKa values of 10.5, 9.5, and 9.32, respectively [29,30]. At pH values lower than 9.32, THC, CBD, and CBN exist in their protonated form.

A number of chromatographic techniques describing the separation and determination of cannabinoids have been described with reversed-phase HPLC being commonly used for analysis [27,31-33]. Analysis of cannabinoids using HPLC allows for the determination of both neutral and acidic forms of cannabinoids without the need for derivatization [34]. HPLC methods using gradient and elution modes

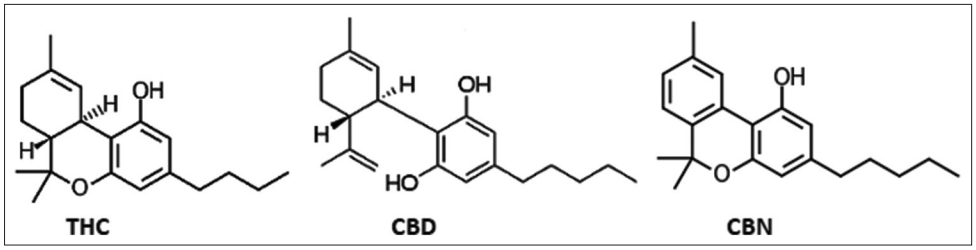


**Fig. 1: Molecular structure of tetrahydrocannabinol, cannabidiol, and cannabinol**

**Text**

for the separation of cannabinoids have been described with isocratic elution being favored due to lower cost, ease of use, and no need of column re-equilibration between runs [35-38].

The aim of the study was to investigate the effects of varying pH, acetonitrile composition and flow rate of the mobile phase, and temperature of the stationary phase and wavelength in the development of a comparatively simple and rapid method to separate THC, CBD, and CBN.

## METHODS

### Mobile phases

Mobile phases were prepared using HPLC-grade acetonitrile (Fisher Chemical, Leicestershire, UK) and phosphate buffer. The buffer was prepared by dissolving anhydrous extra pure disodium hydrogen phosphate (Scharlau, Sentmenat, Spain) in HPLC-grade water (Fisher Chemical, Leicestershire, UK) to make up a solution of 0.02M, and pH was then adjusted by the dropwise addition of HPLC-grade orthophosphoric acid (Fisher Chemical, Leicestershire, UK). pH readings were taken using a Mettler Toledo FiveGo® pH meter which was calibrated before every reading using standard Hanna® calibrator buffer solutions at pH values of 4.01 and 7.01. Twelve different mobile phases were used (Table 1).

### Sample preparation

Standards of (-)-delta 9-THC 0.1 mg/mL in methanol, (-)-CBD 1.0mg/mL, and CBN 1.0 mg/mL were purchased from LGC Standards GmBH (Wesel, Germany). Stock solutions of 5 µg/mL of THC, CBD, and CBN were prepared in HPLC-grade methanol (Fisher Chemical, Leicestershire, UK). Equal volumes of the 5 µg/mL stock solutions of THC, CBD, and CBN were mixed in amber-colored flasks.

### Instrumentation

An Agilent 1260 Infinity Series® liquid chromatographic system having a quaternary pump and multiwavelength detector was used. The stationary phase used was an ACE® RP $C_{18}$ column (250 mm×4.6 mm; 5 µm particle size). The temperature of the stationary phase was first set at 25°C. The UV/visible detector was set at 220 nm and 228 nm. Sample volumes of 20 µL containing THC, CBD, and CBN were injected. Before analysis, solutions containing only THC, CBD, and CBN, respectively, were injected to assist with peak identification. Three replicate runs using each type of mobile phase prepared were carried out to ensure precision. Column equilibration was carried out before changing the mobile phase. The flow rate of the mobile phase was set at 1 mL/min. The best mobile phase characteristics according to amount of acetonitrile and pH were identified and then tested at two other different flow rates - of 1.5 and 2 mL/min. Following the choice of the flow rate which gave the best results in terms of resolution and speed of analysis, the temperature of the stationary phase was tested at two other different temperatures, namely 20°C and 30°C, and the temperature giving the best results in terms of resolution and speed of analysis was chosen.

The run times were adjusted for each chromatogram according to the mobile phase used. The average values for the areas under the peak, area percentages, and retention times were calculated for each run.

**Table 1: Properties of the mobile phases used for high-performance liquid chromatography separation of cannabinoids**

| Mobile phase number | pH of buffer | Percentage of acetonitrile in mobile phase |
|---|---|---|
| 1 | 2.5 | 70 |
| 2 | 2.5 | 80 |
| 3 | 2.5 | 90 |
| 4 | 3.0 | 70 |
| 5 | 3.0 | 80 |
| 6 | 3.0 | 90 |
| 7 | 4.0 | 70 |
| 8 | 4.0 | 80 |
| 9 | 4.0 | 90 |
| 10 | 6.0 | 70 |
| 11 | 6.0 | 80 |
| 12 | 6.0 | 90 |

## RESULTS

### Wavelength of analysis

Larger areas under the peak were obtained for CBD, CBN, and THC at 220 nm when compared to 228 nm. The areas under the peak for CBD and CBN were larger than the peak for THC for equal concentrations (5 µg/mL) of the three cannabinoids.

### Buffer pH

There was no difference in peak shape or area under the peak when pH 2.5 buffer and pH 3 buffer were used. As the buffer pH was increased to 4 and 6, there were some irregularities in the baseline although this did not affect the shape and area under the peak of the three cannabinoids (Figs. 2-9).

### Percentage of acetonitrile in the mobile phase

As the amount of acetonitrile in the mobile phase was increased from 70% to 80%, the retention time of the three cannabinoids decreased (Figs. 5 and 6). The retention time continued to decrease as the amount of acetonitrile was increased to 90%, but the peak shape of CBD was compromised and unsymmetrical. The decrease in retention time with a loss of symmetry of peak occurred at all pH values - 2.5, 3, 4, and 6 (Figs. 10-13).

### Flow rate of mobile phase

The mobile phase having a buffer pH of 2.5 and 80% acetonitrile was chosen as it gave favorable results in terms of peak shape, size, and retention time. As the flow rate of the mobile phase was increased from 1 to 1.5 to 2 mL/min, the total run time for the analysis of the three cannabinoids decreased from 14.3 to 9.4 to 7.0 min, respectively (Figs. 6, 14 and 15).

### Column temperature

There was no difference in the areas under the peak or retention time of CBD, CBN, and THC when the column temperature was changed from 25°C to 20°C and 30°C.

## DISCUSSION

Published UV spectra of cannabinoids have shown maximum UV absorption for THC, CBD, and CBN to lie in the region of around

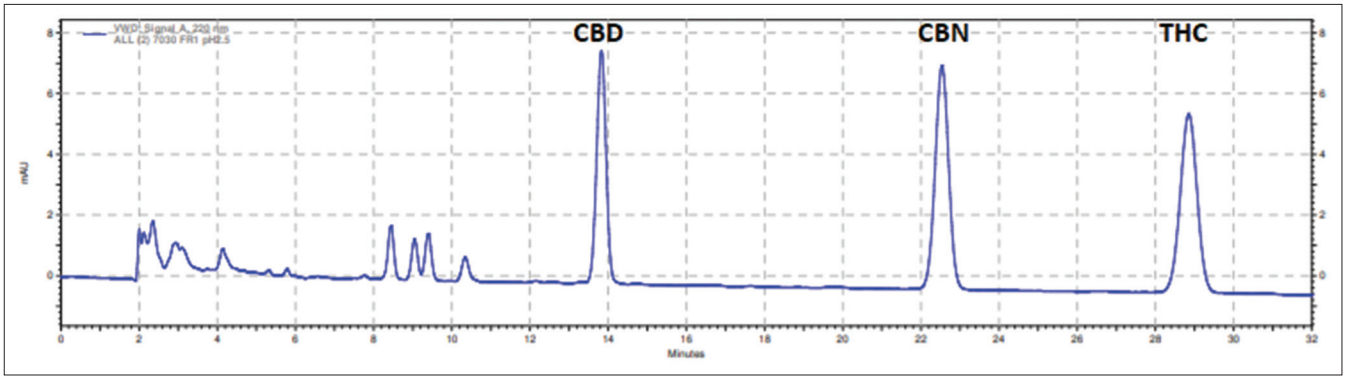


**Fig. 2: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

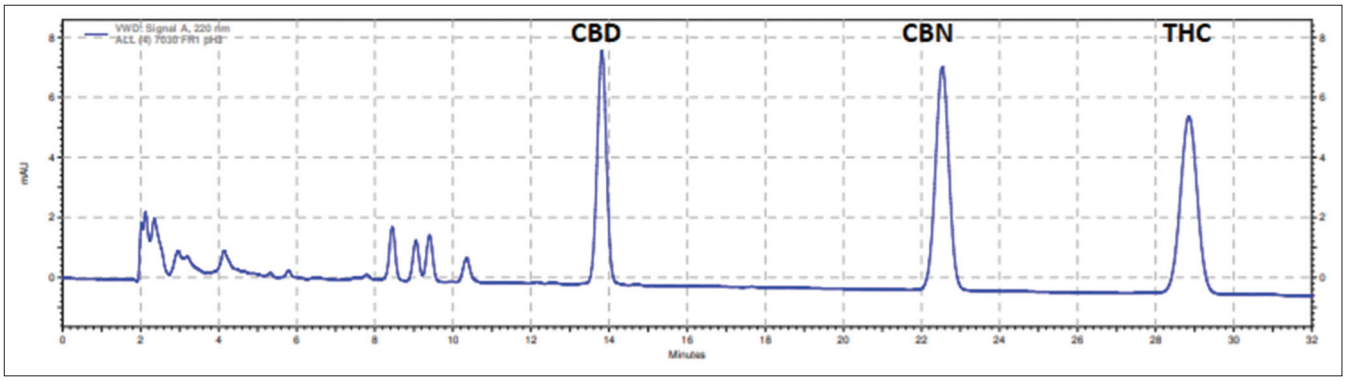


**Fig. 3: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 1 mL/min**

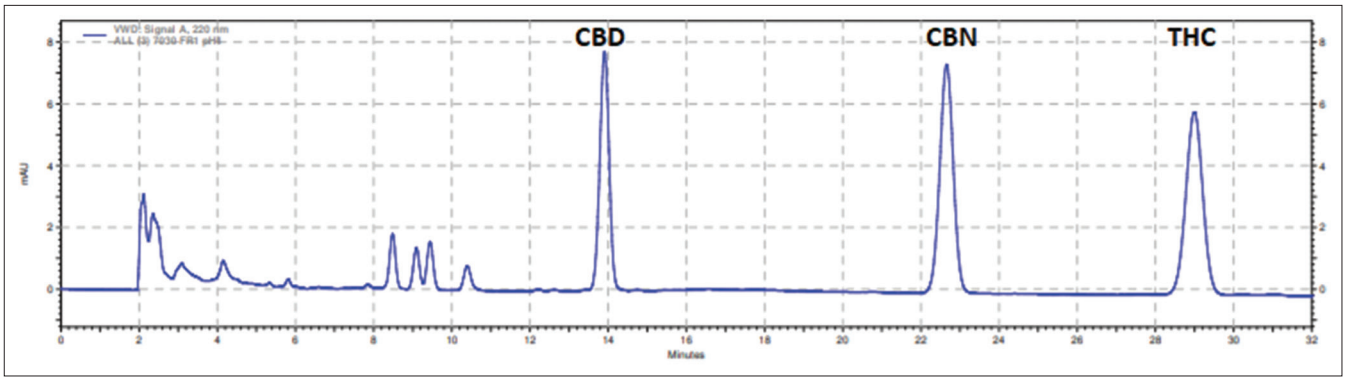


**Fig. 4: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (30:70 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

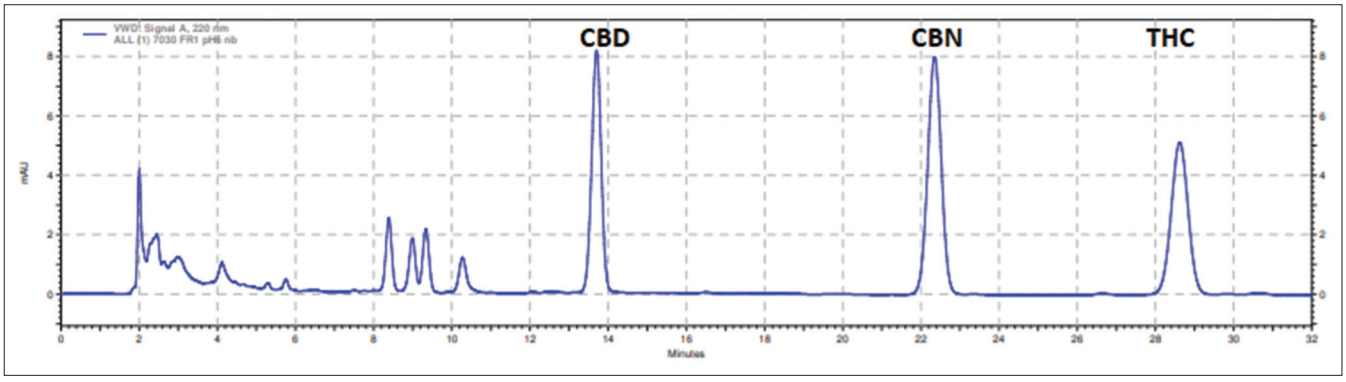


**Fig. 5: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (30:70 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

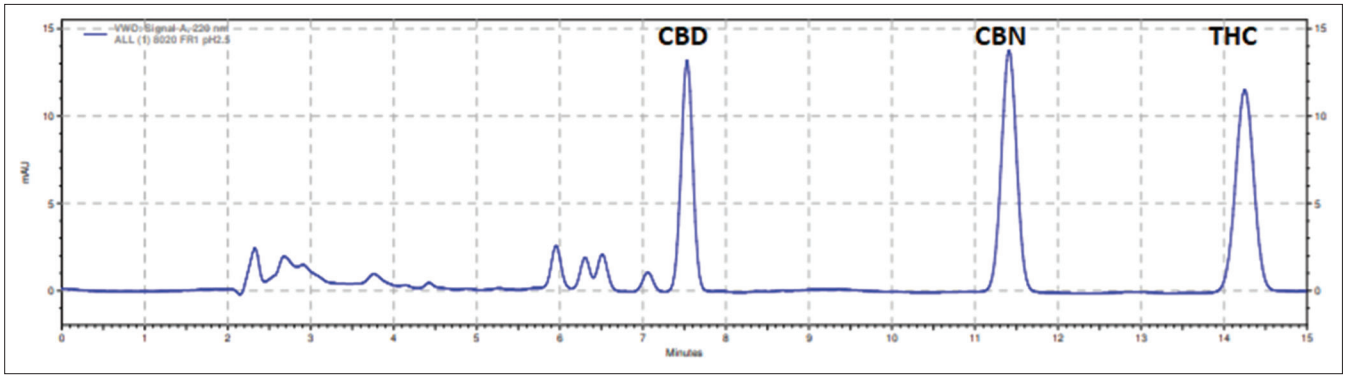


**Fig. 6: Chromatogram produced using phosphate buffer (pH2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1mL/min**

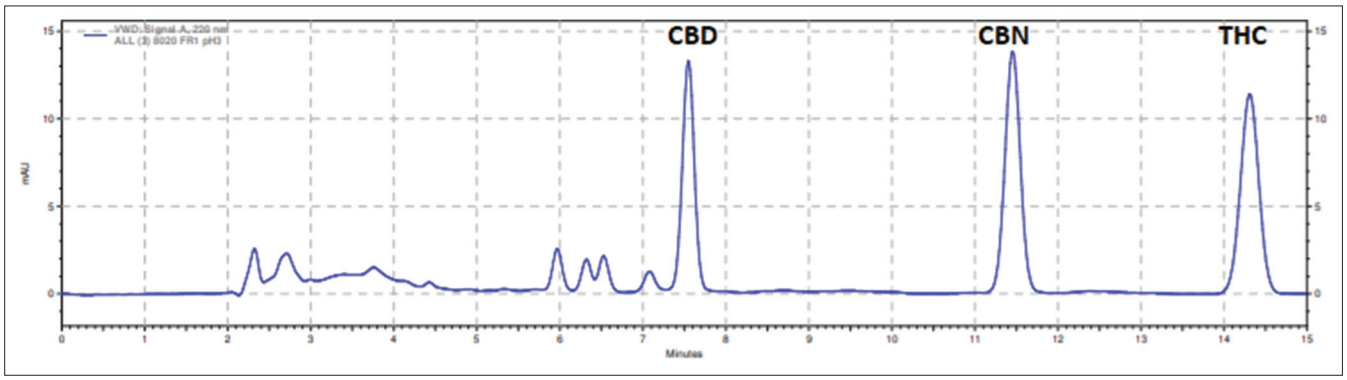


**Fig. 7: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1 mL/min**

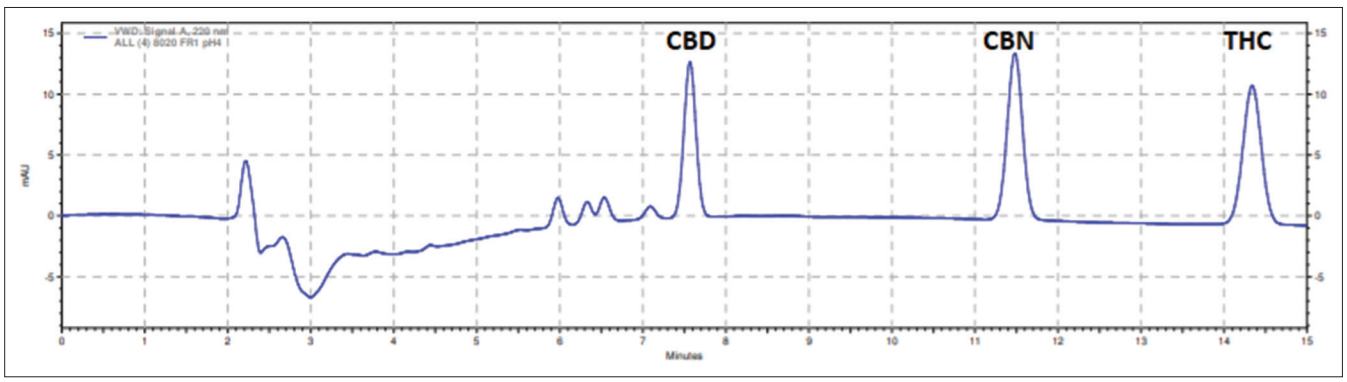


**Fig. 8: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (20:80 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

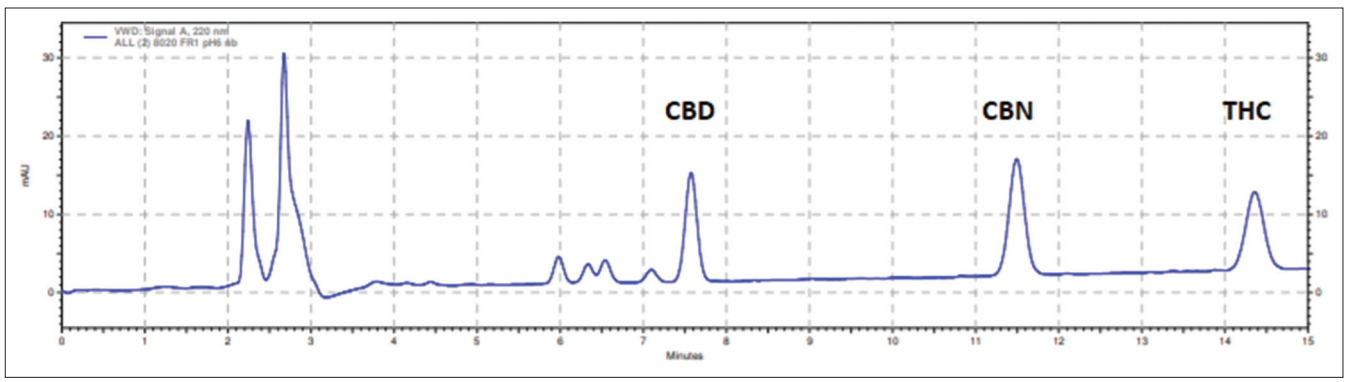


**Fig. 9: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (20:80 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

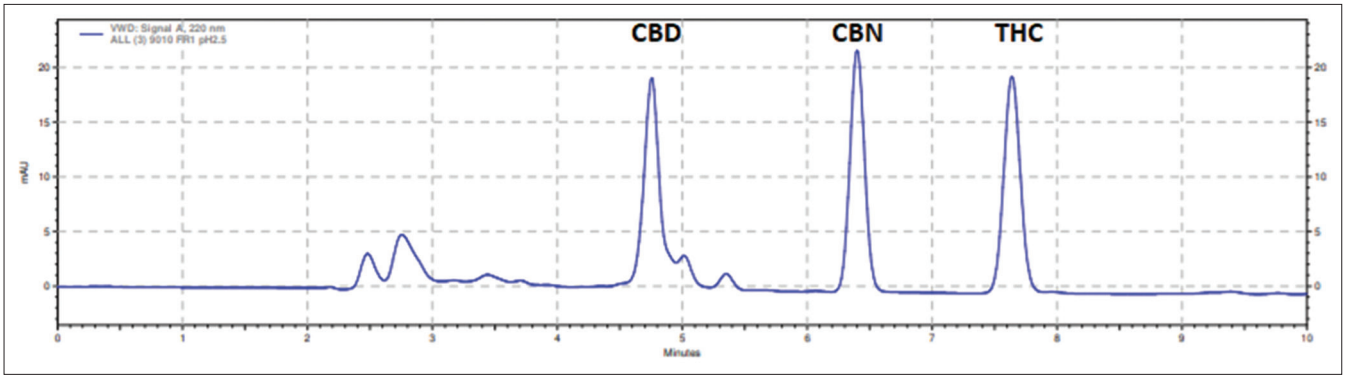


**Fig. 10: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (10:90 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

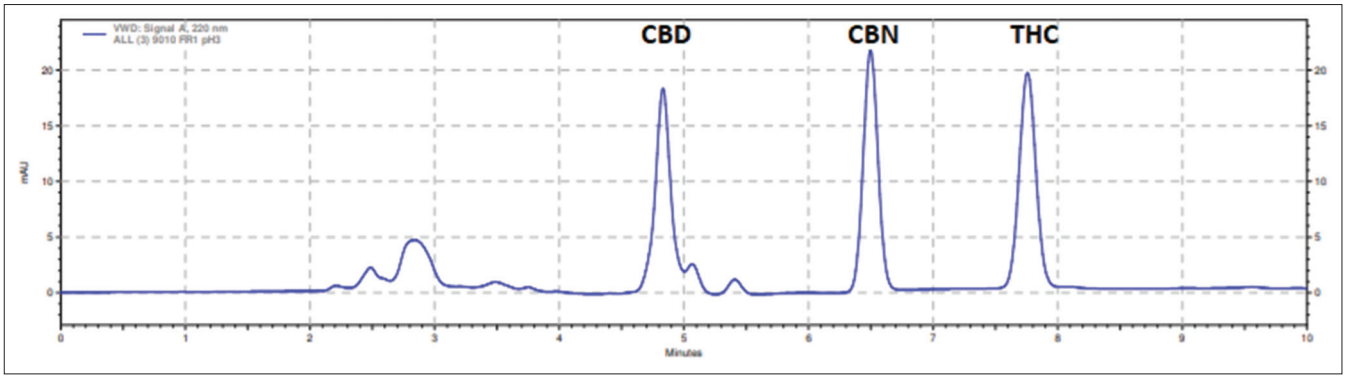


**Fig. 11: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (10:90 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

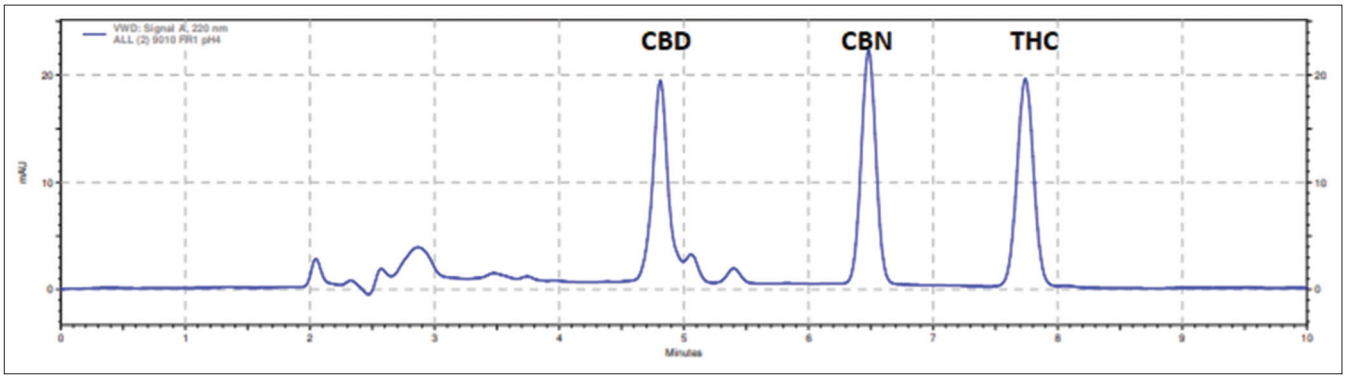


**Fig. 12: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (10:90 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

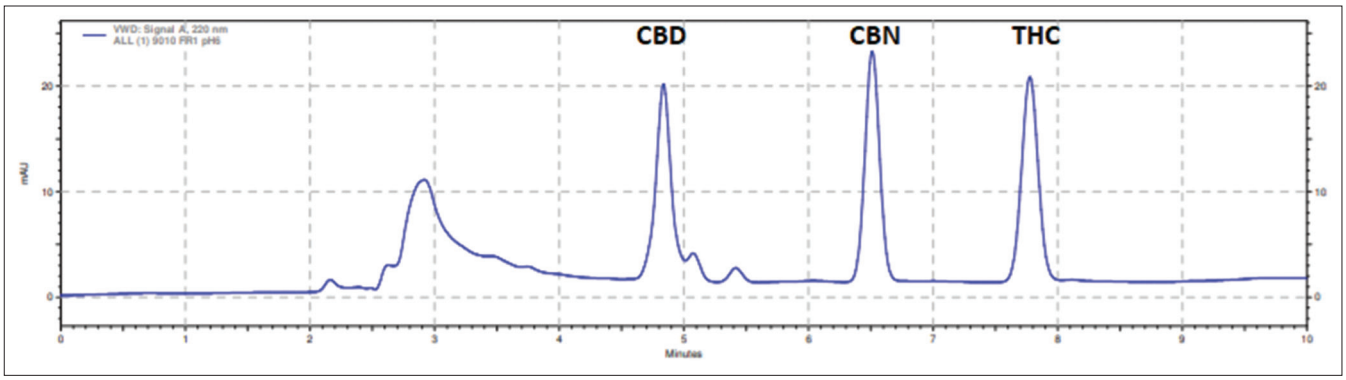


**Fig. 13: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (10:90 v/v); detection wavelength 220 nm; flow rate 1 mL/min**

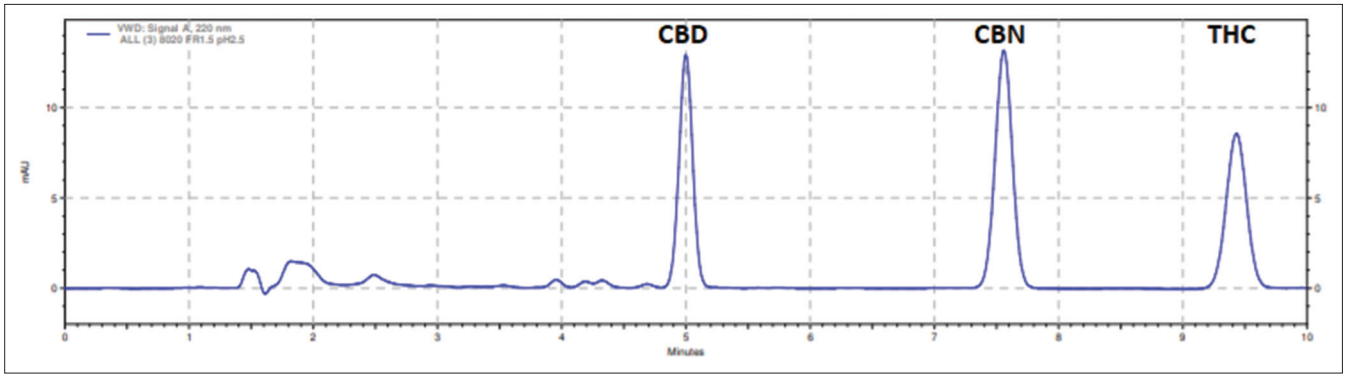


**Fig. 14: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220 nm; flow rate 1.5 mL/min**

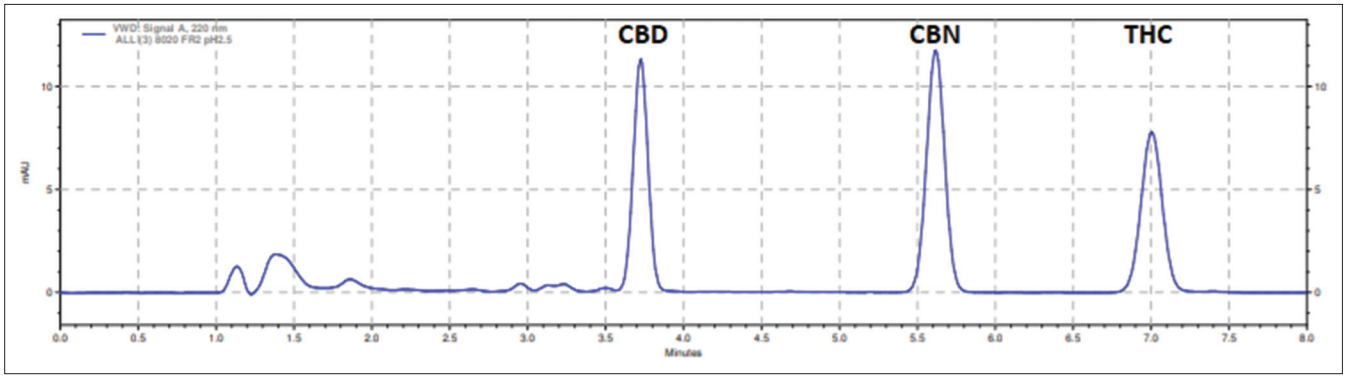


**Fig. 15: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220 nm; flow rate 2 mL/min**

220 nm [39]. CBD and CBN have higher absorptivity than THC at the selected wavelength for detection which could be due to a greater degree of conjugation within the molecule.

Although affecting the baseline, increasing the pH of the buffer did not affect peak size, shape, or order of elution of the cannabinoids, and this finding is in agreement with the study conducted by Hazekamp *et al*. [39]. At a pH lower than their pKa, the three cannabinoids were present in their protonated form and would have been present in their unprotonated form at pHs higher than their pKa. Conversion of analytes from a protonated to an unprotonated form will probably cause shifts in chromatograms due to changes in the amounts of the two forms. The use of a buffer with a higher pH is not usually recommended in RP-HPLC analyses as this can result in solubilization of the silica support present in the column [15].

When larger volumes of acetonitrile were used, the retention time decreased but peak shape was compromised when mobile phases containing 90% acetonitrile were used. As larger volumes of organic modifier were used, there is less interaction of the three cannabinoids with the stationary phase resulting in quick elution, probably due to a shift in the partition coefficient which favors the mobile phase having larger amounts of acetonitrile. Buffering capacity could have been lost at higher percentages of acetonitrile in the mobile phase and having a buffer with a stronger concentration might counteract this [15].

Increasing the flow rate resulted in a decrease in total retention time for the three cannabinoids without a compromise in resolution. Having chromatographic methods which are quick are useful when conducting high throughput analyses [6]. Although changing the temperature did not result in any change in peak size, shape, and retention time, higher temperatures were not used so as to maintain the analytical procedure relatively energy efficient.

## CONCLUSION

A rapid and easy to follow HPLC technique using readily-available instrumentation to separate and determine concentrations of THC, CBD, and CBN in a mixture of cannabinoids in methanol are described. Different amounts of acetonitrile result in differences in retention time, peak size, and shape for THC, CBD, and CBN. The best chromatograms in terms of peak shape, peak size, baseline characteristics, and retention time are given when using 80% acetonitrile with a buffer pH of 2.5 and pH 3 at a flow rate of 2 mL/min, detected at a wavelength of 220 nm.

The Expert Committee on Drug Dependence proposed that pure CBD preparations should not be scheduled within international drug control conventions [40]. This method would be useful to meet the recommendations of the United Nations Commission on narcotic drugs for rescheduling of cannabis. A simple and rapid technique using instrumentation which is available in most laboratories, such as the one proposed, can be validated and applied to separate and determine cannabinoids such as THC in CBD preparations.

## AUTHOR CONTRIBUTIONS

- Ms. Eva Tejada- Main researcher
- Dr. Janis Vella Szijj- corresponding author, reviewer, and project supervisor
- Ms. Miriana Cachia- contributed in laboratory work and review
- Ms. Pauline Falzon- contributed in laboratory work and review
- Prof. Lilian.M.Azzopardi- head of department and reviewer
- Prof. Anthony Serracino Inglott-project supervisor.

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# Analytical Techniques Used for Analysis of Cannabinoids

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*Cannabinoids can be analyzed using different techniques. The aim of this review was to identify and compare analytical methods used for the determination of cannabinoids in different matrices using liquid chromatography (LC)-based systems. A systematic literature review was carried out using the preferred reporting items for systematic reviews and meta-analyses (PRISMA) method. In the results, 41 relevant articles were identified. The most commonly used methods for the analysis of cannabinoids were high performance liquid chromatography-photodiode array (HPLC-DAD) (n= 8), ultrahigh-pressure liquid chromatography–mass spectrometry (UHPLC–MS) (n= 8), and HPLC–tandem mass spectrometry (HPLC–MS/MS) (n= 8). Matrices from which cannabinoids were extracted included plants, oil, hair, human biological fluids, resin, honey, wastewater, and commercial products (n=41). The most commonly used stationary phases were C18 Poroshell (n= 9) and C18 Kinetex (n=8). The identification and comparison of methods used for the determination of cannabinoids can help in the development of more efficient and effective methods of analysis.*

**CANNABIS IS PART** of the plant family *Cannabacea* (1). Cannabis sativa is an annual dioecious flowering plant (2) known for its medicinal and textile uses since ancient times (1,3). Cannabis sativa contains chemically active compounds called cannabinoids, which have a wide range of therapeutic effects in humans (3). Medicinal uses of cannabinoids include management of spasticity related to multiple sclerosis (MS), chronic neuropathic and cancer pain, nausea and vomiting, sleep disorders, anxiety, epilepsy, and Tourette syndrome (4).

The principal cannabinoids known to have medicinal properties are $\Delta^9$-tetrahydrocannabinol (THC) and cannabidiol (CBD)(5). THC and CBD are synthesized and accumulate in their acidic form in Cannabis sativa (6). The alkylation of olivetolic acid (OLA) with geranyl-pyrophosphate (GPP) by olivatolate geranyltransferase produces cannabigerolic acid (CBGA) (3,4,7). The catalysis of CBGA by three oxidocyclases—$\Delta^9$-tetrahydrocannabinolic acid synthase (THCAS), cannabidiolic acid synthase (CBDAS), and cannabichromenic acid synthase (CBCAS)—produces $\Delta^9$-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA), respectively (3,4,7). The decarboxylation of THCA, CBDA, and CBCA due to high temperatures (8) produces THC, CBD, and cannabichromene (CBC), respectively (4,8). Cannabinol (CBN) is produced as a result of oxidation of THC (4,8) and is a sign of deterioration of the plant (9).

THC is the main psychoactive component in cannabis and has been used in the management of chemotherapy-induced nausea and vomiting, for appetite stimulation in patients with acquired immunodeficiency syndrome (AIDS) (10), for suppressing spasticity related to multiple sclerosis (MS) (6), and in the treatment of migraines (1).

CBD is known to have the largest number of therapeutic properties (7) and is the main nonpsychoactive component in cannabis (11). CBD presents potent antioxidant and anti-inflammatory properties (6). CBD has anticonvulsive, neuroprotective, anxiolytic, antipsychotic, and antidepressant properties (12). CBD is used principally in children in the treatment of drug-resistant epilepsy, Dravet and Lennox-Gastaut syndromes (13,14).

The endocannabinoid system has two principal receptors CB1 (type-1) and CB2 (type-2) (15) connected to G-proteins, endogenous cannabinoids called arachidonoylethanolamine (anandamide) and 2-arachidonoyl glycerol (2-AG) and enzymes that are involved in synthesis and degradation of endocannabinoids (9,16). CB1 receptors are present in different regions of the human brain (17). Distribution of these receptors is in areas involved in cognitive function and mood (4,17). CB1 receptors can be also found in the liver, testes, and small intestine (16).

There are different analytical techniques for the determination and quantification of cannabinoids (10,18). Gas chromatography (GC) has been the method of choice for analysis of cannabinoids (10), but chemical derivarization is required to avoid decarboxylation of acid cannabinoids (18). Liquid chromatography (LC) allows determination of cannabinoids in neutral and acidic forms without the need for derivarization. LC has become more popular with the introduction of high performance liquid chromatography (HPLC) and ultrahigh-pressure liquid chromatography (UHPLC) (7,18,19). LC, HPLC, and UHPLC can be coupled to different detectors: fluorescence, diode-array detection (DAD), mass spectrometry (MS), or ultraviolet (UV) detector (20). The use of MS coupled to HPLC and UHPLC increases the selectivity and the sensitivity of analysis (7), but the cost is higher and requires higher skilled expertise to operate (20). CBN does not have a fluorophore and therefore use of a fluorescence detector is unfavourable (2). HPLC and UHPLC coupled to a UV-visible detector is a method commonly used because it can be economic and more convenient than other methods of analysis. DAD offers a range of detection wavelengths but can be more expensive than UV (21). The aim of this study was to conduct a systematic literature search to compare and identify analytical methods and parameters used in the determination of naturally occurring cannabinoids in different matrices.

## Experimental

A systematic literature review was carried out using the preferred reporting items for systematic reviews and meta-analyses (PRISMA) method. (Note: PRISMA. Transparent reporting of systematic review and meta-analysis. [Internet] PRISMA, 2021 [cited 2021, 23 April] Available from: http://prisma-statement.org/PRISMAStatement/FlowDiagram.) The systematic literature review included methods used for separation and determination of cannabinoids using LC. Sources included open access peer-reviewed journal articles published in English between the years 2015 and 2020. Databases used for the literature search were Pubmed and Scopus. Keywords used in the search were: analysis, cannabinoids, cannabis, tetrahydrocannabinol, cannabidiol, cannabinol, and LC. Data collected was presented in tables, according to the matrix in which the cannabinoids were presented. Data in each table compared the type of matrix, cannabinoids analyzed, sample preparation method, stationary phase, mobile phase, and detector.

## Results and Discussion

In the study, 41 articles were identified. The articles were classified depending on the matrices used for the analysis: 18 articles analyzed cannabinoids from plant material and four articles in plant material and other matrices. Ten articles analyzed cannabinoids from biological fluids and hair, and one article from biological fluids and hair and other matrices. Four articles

**Table I:** Analysis of cannabinoids from plants using HPLC coupled to UV or DAD

| Method | Cannabinoids | Author and Date |
|---|---|---|
| HPLC-UV/DAD | CBD, CBDA, CBG, and CBGA | Brighenti et al, 2019 |
| HPLC-DAD | THC, THCA, CBD, CBDA, and CBN | Ciolino et al, 2018 |
| HPLC-UV | CBD, CBDV, and CBDB | Citti et al, 2019 |
| HPLC-DAD | THC, THCA, CBDA, CBD, CBG, CBC, $\Delta^8$-THC, and CBN | Giese et al, 2015 |
| HPLC-UV | THC, CBD, CBN, CBDA, CBGA, THCA, THCV, CBG, and $\Delta^8$-THC | Križman, 2019 |
| HPLC-UV | $\Delta^9$-THC, THCA, Δ8-THC, CBD, CBDA, CBG, CBN, CBC, and THCV | Mudge et al, 2017 |
| HPLC-DAD | THC, CBD, and CBN | Ribeiro Grijó et al, 2019 |

**Table II:** Analysis of cannabinoids from plants using UHPLC

| Method | Cannabinoids | Author and Date |
|---|---|---|
| UHPLC-MS | THC, CBD, and THCA | Bala et al, 2019 |
| UHPLC-DAD | THC, CBDA, CBG, CBGA, THCA, CBD, and CBN | Deville et al, 2020 |
| UHPLC-DAD | THC, CBD, CBC, CBN, CBG, THCA, and CBDA | Elkins et al, 2019 |
| UHPLC-DAD | THC, CBC, CBD, $\Delta^8$-THC, THCA, CBDA, THCV, and CBDV | Fekete et al, 2018 |
| UHPLC-UV | THC, CBD, CBN, THCA, CBDA, CBG, CBDVA, CBL, CBGA, CBDV, CBC, THCV, and $\Delta^8$-THC | Mudge et al, 2018 |
| UHPLC-UV UHPLC-MS/MS | THC, CBD, CBN, CBDA, CBGA, CBDV, THCA, CBG, and $\Delta^8$-THC | Nemeškalová et al, 2020 |

analyzed cannabinoids in oil, and four articles, in oil and other matrices. Ten articles analyzed cannabinoids from miscellaneous matrices.

## *Methods of Analysis of Cannabinoids from Plant Material*

LC, HPLC, and UHPLC have been performed for the separation, determination and quantification of different cannabinoids in Cannabis sativa. The samples included aerial parts of the plant (n=1), male and female inflorescences (n=5), leaves (n=2), roots (n=1), colas (n=1), resins (n=1), buds (n=2), and flowers (n=8) (7,22–42). HPLC is the most popular analytical technique used for the analysis of cannabinoids in plants (n=13) (22–27,32,34,36,38–42). HPLC can be coupled to UV, DAD, MS, or fluoresence detectors (20).

The detector most commonly used for the analysis of cannabinoids in plant material is UV or DAD (n=13) (23–27,32,34,36,40,41). For example, Križman completed a study using HPLC-UV (34). Križman carried out a simple isocratic HPLC method for the analysis of THC, CBD, CBN, cannabigerol (CBG), THCA, tetrahydrocannabivarin (THCV), CBGA, CBDA, and $d^8$-THC. The mobile phase consisted of water and actonitrile (ACN) in the ratio of 9:31 (v/v), with 0.1% formic acid (v/v) and 10 mM ammonium formate, using a Luna C18 (150 mm × 3 mm i.d., 3 µm) column and UV at 275 nm (34).

In recent years, UHPLC has become more popular (n=7) because of the small quantity of solvent needed in the mobile phase and a shorter anaylsis time (43). Bala and colleagues carried out a study using UHPLC coupled to MS to detect THC, CBD, and THCA which are present in large amounts in the cannabis plant and have therapeutic properties (22). The most commonly used detector to analyse cannabinoids from plants with UHPLC is DAD (n=3) (7,28,31). Elkins and colleagues analyzed THC, CBD, CBN, CBDA, CBC, and THCA using a simple method consisting of a mobile phase based in water containing 0.1% formic acid (HCOOH) and ACN containing 0.1% HCOOH (ranging between 40–100%) with gradient elution mode using a Phenomenex Luna Omega C18 (150 × 2.1 mm × 1.6 µm) column and DAD detection monitored at 280 nm (7).

While the use of DAD as a detector for HPLC and UHPLC is quite common, combined detectors as UV-DAD, ESI-MS, and MS/MS have also increased in their popularity. One of the advantages of using ESI-MS or MS/MS is that the analysis can be performed in negative and positive ion mode. Neutral cannabinoids give a better signal in the positive ion-mode while acidic cannabinoids give better signal in the negative ion-mode (24). Brighenti and colleagues developed a method for the analysis of nonpsychoactive cannabinoids using the three combined detectors (24).

Other methods used for the analysis of cannabinoids were a fast-HPLC-DAD (25), UHPLC-travelling wave ion

mobility (TWIM)-MS (30), and HPLC-Q-Exactive-Orbitrap-MS (39). Burnier and colleagues analysed THC, CBD, CBN, and THC-A in a total run time less than 5 min using a fast-HPLC-DAD method that could be an alternative to UHPLC but with a lower cost (25).

LC presents less sensitivity than HPLC and UHPLC. The use of LC requires less expensive and simpler equipment (33). Dong and colleagues developed a thermal desorption direct analysis in real time mass spectrometry method and compared the results with those obtained using a simple LC–MS (29).

One of the limiting factors in the analysis of compounds using a LC system is the solubility of cannabinoids prior to analysis. The analysis of cannabinoids from plant material using LC, HPLC, or UHPLC requires an extraction method to determine the presence of cannabinoids qualitatively and quantitatively (2).

Solvent extraction is the most commonly used analytical extraction method to extract cannabinoids from plant material. In our findings, 20 studies used solvent extraction as a part of the sample preparation method for the analysis of cannabinoids. The most commonly used solvents for the extraction of cannabinoids from plants are ethanol (EtOH) and methanol (MeOH), used in 7 out of 22 and 7 out of 22 studies, respectively. Ethanol is an organic solvent commonly used because of its higher eco-friendly behavior, even if it is more viscous than MeOH (31) and due to its high extraction efficacy because of its high affinity for the molecular structure of cannabinoids (44). MeOH is also commonly used because it presents a high extraction efficiency (24). Other extraction methods with solvents make use of ACN or a mix of solvents. Deville and colleagues performed the extraction technique with a mix of methanol/chloroform (90:10 v/v). The long-term use of chloroform by the analyst can cause liver and kidney injury to the operator of the method of analysis. Reducing the use of chloroform will increase safety in the laboratory and decrease costs of reagent disposal while improving the impact in the environment (36).

Sample preparation is usually accompanied by dynamic maceration (DM), which consists of extraction of analytes of interest from plant material using a solvent and vortex or stirring at ambient temperature (24). Brighenti and colleagues compared four different extraction techniques DM, ultrasound-assisted extraction (UAE), microwave-assisted

**Table III:** Analysis of cannabinoids from plants using HPLC coupled to combined detectors

| Method | Cannabinoids | Author and Date |
|---|---|---|
| HPLC-UV/DAD<br>HPLC-ESI-MS | CBD, CBDA, CBG, and CBGA | Brighenti et al, 2017 |
| HPLC-MS/MS | THC, CBD, CBC, CBG, CBN, CBDV, THCA CBGA, and CBDA | Palmieri et al, 2019 |
| HPLC-ESI-MS<br>HPLC-MS/MS | CBDA, CBGA, CBG, and CBD | Pellati et al, 2018 |
| HPLC-MS/MS | THC, CBD, CBN, CBG, CBDA, and THCA | Zweigenbaum, 2020 |

**Table IV:** Analysis of cannabinoids from plants using different HPLC methods

| Method | Cannabinoids | Author and Date |
|---|---|---|
| Fast-HPLC-DAD | THC, CBN, CBD, and THCA | Burnier et al,2019 |
| UPLC-MS<br>UPLC-TWIM-MS | $\Delta^9$-THC, CBD, CBC, CBN, CBG, $\Delta^9$-THCA, and CBDA | Dossantos et al, 2018 |
| HPLC-Q-Exactive-Orbitrap-MS | THC, CBD, CBN, CBG, CBC, CBDV, THCV, CBDA, THCA, CBNA, CBCA, CBGA, CBDVA, and THCVA | Pavlovic et al, 2019 |

**Table V:** Analysis of cannabinoids from plants using LC

| Method | Cannabinoids | Author and Date |
|---|---|---|
| LC–MS | THC, CBD, CBC, THCA, CBDA, THCV, CBDV, THCVA, CBDVA, CBCA, and CBL | Dong et al, 2019 |
| LC–MS/MS | CBN | Hidayati et al, 2020 |

extraction (MAE), and supercritical fluid extraction (SFE). UAE and MAE are extraction techniques that use ultrasound waves and microwave energy for a faster and higher extraction of the secondary metabolites of cannabinoids (24). SFE is a more environmentally friendly technique than the extraction techniques of cannabinoids from plant material that use organic solvents (24), Elkins and colleagues extracted the resin from cannabis using a biobotanical SFE liquid $CO_2$ extractor (7). DM is the best method to extract acidic cannabinoids such as CBDA and MAE for CBD (24). Ribeiro Grijó and colleagues carried out the extraction process using solid phase extraction (SPE) with supercritical carbon dioxide ($scCO_2$) avoiding trace of organic solvents in the sample prepared (41).

The majority of the analysis of cannabinoids in plant material were carried out using an Agilent system (n=11) with different modular model systems (7,23,24,26,27,32,35–37,40,42). Among those studies using Agilent systems, the modular model 1100 and 1290 were the most popular and were used in three studies: (23–25,40,7,32,37) two studies used modular model 1200 system (35,36), one study used modular model 1260 system (42), and another study used modular model 1220 system (27). Ciolino and colleagues conducted the analysis using Agilent 1100, 1200, or 1260 HPLC-DAD systems (26). Another HPLC unit used was the Waters system, this unit was used in four studies (22,25,28,30). Other HPLC systems used were Thermo LTQ XL by Dong and colleagues, Finnigan Surveyor by Križman, and Nexera LC20AD XR system by Palmieri and colleagues (29,34,38).

The majority of the studies used C18 Poroshell (n=4), Kinetex (n=3), and Ascentis (n=3) columns. Gradient mode elution of the mobile phase was the most common method chosen for the analysis of cannabinoids from plants and only four studies out of 22 used an isocratic mode of elution. The majority of the mobile phases are composed of water and an organic solvent (n=16), usually MeOH and ACN. ACN was preferred because it decreases the total run time with respect to MeOH. The flow rate of the mobile phase ranged from 0.3 mL/min to 3 mL/min, with 0.4 mL/min (n=5) and 0.3 mL/min (n=5) being the most commonly used.

## *Methods of Analysis of Cannabinoids from Biological Fluids and Hair*

Cannabis can be determined in biological fluids and hair (45). The analysis of cannabinoids in human fluids is important to understand their pharmacology in humans and to be able to establish the correct dosage (46). The availability of analytical techniques to detect and quantify THC in blood, saliva, hair, and urine is necessary to demonstrate consumption of illicit preparations (47).

The concentration of THC and its metabolites from blood and urine depends on the amount and route of administration and the time of analysis following consumption (48)

LC, HPLC, and UHPLC methods are used for the analysis of cannabinoids from different biological fluids

**Table VI:** Analysis of cannabinoids from biological fluids using UHPLC

| Method | Cannabinoids | Author and Date |
|---|---|---|
| UHPLC–MS/MS | THC-COOH | Cho et al, 2018. |
| UHPLC–MS | CBD | Dybowski et al, 2020 |
| UHPLC–MS/MS | THC, CBD, and CBN | Moorthy et al, 2019 |
| UHPLC–MS/MS | THC, CBD, THCA-A, CBDA, THC-COOH, THC-COOH-gluc, 11-OH-THC, and THC-gluc | Pichini et al, 2019 |
| UHPLC–MS/MS | THC, CBD, CBN, 11-OH-THC, and THC-COOH | Pires da Silva 2020 |
| UHPLC–MS/MS | THC, COOH–THC, OH–THC, CBD, and CBN | Wei et al, 2015 |

**Table VII:** Analysis of cannabinoids from biological fluids using HPLC

| Method | Cannabinoids | Author and Date |
|---|---|---|
| HPLC–MS/MS | THC, CBD, CBN, and THC-COOH | Chang et al, 2016 |
| HPLC–MS/MS | THC, 11-OH-THC, and THC-COOH | Dziadosz et al, 2016 |
| HPLC–MS/MS | THC, 11-OH-THC, THC-COOH, THC-C-gluc, CBD, CBN, CBG, CBDV, THCV, and THCV-COOH | Klawitter et al, 2017 |

**Table VIII:** Analysis of cannabinoids from biological fluids using LC

| Method | Cannabinoids | Author and Date |
|---|---|---|
| LC-MS/MS | THC, THCOH, and THCCOOH | Toennes et al, 2014 |

(46,48–56). Six studies were carried out using UHPLC, three used HPLC and one study by Toennes and colleagues used an Agilent 1290 Infinity LC system. Analysis was performed using a Kinetex XB-C18, 100 Å, (100 × 2.1 mm) column with a gradient mode mobile phase composed of 0.01% formic acid with 5 mM ammonium formate and ACN with 0.1 % formic acid ranging between 50–100%, the flow rate was 0.5 mL/min (53). The samples included urine (n=4), hair (n=1), human plasma (n=3), human serum (n=2), blood (n=1), and sweat (n=1) (46,48–55).

The detector most commonly used in the analysis of cannabinoids from biological fluids is MS/MS (n=9) because it presents higher selectivity and sensitivity allowing for the detection of major and minor cannabinoids in small quantities (2,7). THC and its metabolites (THC-OH and THC-COOH) are, in general, the cannabinoids analyzed in blood and urine because of the psychoactive effects of THC (2). Analysis of other cannabinoids such as CBD, CBN, CBG, CBDV, and CBDA were also identified in the literature (46,50–54,56).

Sample preparation is an important step in the analysis of compounds from biological fluids and has an effect on reproducibility, efficiency, and selectivity and eliminates interferences (2). Different techniques were performed to extract cannabinoids from biological fluids and hair. Protein precipitation (PP) is a popular technique used for the sample preparation in blood and can eliminate up to 98% of the protein (57). Dybowski and colleagues, Dziadosz and colleagues, and Klawitter and colleagues, carried out protein precipitation studies (46,48,51). Dybowski and colleagues analyzed CBD using an UHPLC–MS/MS system with a Gemini C18 column (4.6 x 100 mm, 3 µm) and an isocratic mode mobile phase consisting of 60% 25 mM formic acid with water and 40% 25 mM formic acid with ACN with a flow rate of 0.5 mL/min (46). Dziadosz and colleagues used an HPLC–MS/MS system with a gradient mode mobile phase and MeOH as organic solvent for the analysis of THC, 11-OH-THC, and THC-COOH (48). Klawitter and colleagues performed protein precipitation for plasma and urine and carried out analysis from both matrices using an HPLC–MS/MS (51). Moorthy and colleagues used volumetric absorptive microsampling (VAMS) devices in the sample preparation technique. VAMS is a relatively new microsampling tool used for obtaining dried biological matrices, which improves the accuracy of the sample volume (52,58). Pires de Silva and colleagues used salting-out assisted liquid-liquid extraction (SALLE), another recent extraction technique where the extraction solvent is a water miscible organic solvent (54,59). SALLE is cheaper and easier to use than SPE (59). Toennes and colleagues, and Weit and colleagues, performed sample preparation using SPE (55,56).

Chang and colleagues performed hydrolysis of the urine specimen before the extraction method to improve sample accuracy (50). Pichini and colleagues carried out the study in oral fluid, serum, urine, and sweat patch samples. Sample preparation from oral fluid, serum, and urine were the same

with further alkaline hydrolysis for urine samples for the quantification of CBD as it appears as glucuronide in urine. The extraction of cannabinoids from sweat patch samples was performed with MeOH as the extraction solvent (53).

Hair is also used as a matrix because traces of some compounds can be present in hair (49). Hair is a complex matrix that requires longer sample preparation times because washing and digestion steps are required (2,49). Cho and colleagues carried out the sample preparation washing the hair twice with MeOH to eliminate any external contaminants and performed the digestion with 1 M NaOH to free the cannabinoids from the matrix. Analysis was carried out using a system consisting of a binary pump, Agilent 1290 UHPLC pump (pump 1), and an additional Agilent 1260 pump (pump 2) (49).

Klawitter and colleagues, Chang and colleagues, and Toennes and colleaguesl also performed analysis with an Agilent HPLC unit. Ten studies used C18 columns. Three out of ten studies used Acquity and two out of ten used Kinetex. The majority of the studies used gradient mobile phase (n=9). Dybowski and colleagues performed an isocratic method of elution. The mobile phases were composed of ammonium formate or water and an organic solvent. ACN and MeOH are the organic solvents more commonly used for the mobile phase, with ACN being preferred (n=7) because of shorter elution times for cannabinoids (60). The flow rate ranged from 0.15 mL/min to 1 mL/min. A flow rate of 0.4 mL/min was the most commonly used (n=3).

### *Methods of Analysis of Cannabinoids from Oil*

In recent years, CBD oil has become popular for use in different conditions (61). There is a lack of standardized extraction regulation (2,61). Different carrier oils on the market are olive oil, medium chain triglyceride (MCT), hemp seed oil, and black cumin seed oil.

HPLC is the method of analysis most commonly used (n=5) for the determination and quantification of cannabinoids in olive oil (n=2) and hemp seed oil (n=2) (26,27,36,60,66). The detectors most commonly used are UV (27,36,60) and DAD (26,64). Two studies carried out the analysis using UHPLC (37,53).

**Table IX:** Analysis of cannabinoids from oil using HPLC

| Method | Cannabinoids | Author and Date |
|---|---|---|
| HPLC-DAD | THC and CBD | Araneda et al, 2020 |
| HPLC-DAD | THC, CBD, CBN, and THCA | Bettiol et al, 2019 |
| HPLC-DAD | THC, THCA, CBD, CBDA, and CBN | Ciolino et al, 2018 |
| HPLC-UV<br>HPLC-MS | THC, CBD, THCA, CBDA, CBDV, CBG, and CBN | Citti et al, 2018 |
| RP-HPLC/UV | THC and CBD | Deidda et al, 2019 |
| HPLC-UV | $\Delta^9$-THC, THCA, $\Delta^8$-THC, CBD, CBDA, CBG, CBN, CBC, and THCV | Mudge et al,2017 |

**Table X:** Analysis of cannabinoids from oil using UHPLC

| Method | Cannabinoids | Author and Date |
|---|---|---|
| UHPLC-UV-MS/MS | THC, CBD, CBN, CBDA, CBGA, CBDV, THCA, CBG, and $\Delta^8$-THC | Nemeškalová et al, 2020 |
| UHPLC-MS/MS | THC, CBD, THCA-A, CBDA, THC-COOH, THC-COOH-gluc, 11-OH-THC, and THC-gluc | Pichini et al, 2019 |

Nemeškalová and colleagues carried out analysis in a wide variety of oils—paraffin oil, sunflower oil, castor oil, jojoba oil, shea oil, argan oil, almond oil, coconut oil, and aviril baby massage oil—using an UHPLC-UV-MS/MS method. UV-visible detection was used for the analysis of cannabis with a high amount of cannabinoids, while MS/MS was used for low quantities of major cannabinoids such as THC and CBD and for minor concentrations of cannabinoids (37).

Efficient extraction procedures are required for the analysis of cannabinoids in oil because oil cannot be injected directly in the HPLC due to its high viscosity (62). Bettiol and colleauges and Deidda and colleagues performed the same method to extract different cannabinoids from olive oil, consisting of 40 µL of sample in olive oil added to 960 µL of tetrahydrofuran (TFH) and vortex-mixed. Next, 50 µL of this solution were added to 950] µL of ACN in the study of Bettiol and colleagues, and in MeOH in the study by Deidda and colleauges (60,63). Mudge and colleagues carried out a solvent extraction with MeOH while Nemeškalová and colleagues used isopropanol/ethyl acetate (1:1, v/v). Ciolino and colleagues used EtOH or isopropyl alcohol (26,36,37). Araneda and colleagues performed analysis of cannabinoids using benchtop nuclear magnetic resonance (NMR) instruments to compare the results with the ones obtained using HPLC-UV. Analysis was carried out for five different concentrates of cannabinoids. The relative standard deviation for the samples analyzed with benchtop NMR was higher than that with the HPLC-UV. In the analyses performed with benchtop NMR, the amount of CBD in sample 1 and THC in sample 2 could not be quantified while in the analysis with HPLC both samples were quantified (64).

Three different brands of HPLC units were used among the articles published in the literature for the extraction of cannabinoids from oil. The HPLC unit most commonly used was Agilent (26,27,36,37).The second brand used was Thermo Fisher Surveyor and Pichini and colleagues used a Waters Xevo TQ-S. Bettiol and colleagues and Deidda and colleagues used a Thermo-Fisher Surveyor Plus HPLC system using an Agilent PoroshellR 120 SB-C18 column, (2.1 mm × 150 mm; 2.7 µm)as a stationary phase and an isocratic mode mobile phase composed of ACN/5 mM phosphate buffer rate 75/25 v/v with a flow rate of 0.38 mL (60,63). Ciolino and colleagues also performed analysis using the isocratic mode for the mobile phase, but used two types of mobile phases 66:34 ACN: 0.5% acetic acid and 83:17 MeOH:50 mM citrate both using a flow rate of 1 mL/min. Analysis was carried out using an ACE column (26).

Seven studies used a C18 column as a stationary phase. The majority of the studies were performed using the brand Agilent Poroshell (n=4). The methods used for the mobile phase were gradient in four studies and isocratic in the other three.

The majority of the methods used ACN (n=5) as organic solvent and the amount ranged from 60–100% in mobile phase composition. The flow rate ranged from 0.38 mL/min to 1 mL/min.

### *HPLC Methods of Analysis of Cannabinoids from Miscellaneous Matrices*

There is a need for quantitative analyses to determine cannabinoids such as CBD and THC in commercial products such as honey, capsules, and serum to calculate the amount of each cannabinoid and evaluate the dosage and the exposure of the patient when the product is consumed (26). Studies were carried out in different matrices such as cannabis concentrates, honey (n=1), hemp nut (n=1), vaporized fluid (n=1), milk (n=1), liver (n=1), capsules (n=2), wastewater (n=1), cotton cloths (n=1), and gummies (n=1).

Ciolino and colleagues carried out

analysis of cannabinoids in different commercial products (26). Methods of sample preparation were the same for all the matrices: the sample was weighed and MeOH (95% or 100%) was added as an extraction solvent. The sample was then vortex-mixed and filtered with nylon membrane filter of 0.45 µm. Depending on the quantity of cannabinoids, the sample was further diluted or directly injected in an Agilent 1100, 1200, or 1260 HPLC-DAD system with an ACE 5 C18-AR analytical column (5 µm, 4.6 mm i.d. x 250 mm) (26).

Jornet-Martínez and colleagues detected traces of cannabinoids in different matrices such as plastic bags, cotton tip, aluminium foil, office paper, piece of cotton cloth, and skin. Due to the complex nature of the matrices and the small quantity of cannabinoids, Jornet-Martínez and colleagues performed analysis using an in-tube solid-phase microextraction (IT-SPME) coupled on-line to nanoliquid chromatography (nanoLC), which improved the selectivity of the analysis. The study was carried out using a Zorbax 300SB C18 (50 × 0.075 mm i.d., 3.5 µm) column with a simple gradient mode mobile phase consisting of water and ACN ranging between 55–75%. Jornet-Martínez and colleagues performed an ultrasound assisted extraction for the preparation of the sample using just 1 mL of MeOH per sample making the sample preparation an eco-friendly technique (63).

Nemeškalová and colleagues performed analysis of cannabinoids in oils and plant materials as well as in cosmetics and gelatinous gummies. The large amount of therapeutic benefits of CBD has led to a varied market of CBD based-products such as as candies and cosmetics, which contain smalls amounts of THC that need to be quantified due to its psychoactive effects. The method proposed by Nemeškalová and colleagues demonstrated its feasibility on 13 CBD-based products using an UHPLC-UV-MS/MS with a Poroshell 120 EC-C18 (100 mm × 2.1 mm, 2.7 µm) column. The sample preparation was different for hydrophilic liquids, gummies, and hydrophobic cosmetics but it consisted of dissolution and dilution (37).

Heo and colleagues performed analysis of different synthetic cannabinoids and THC in tablets, capsules, powders, liquids, cookies, and candy using an UHPLC-UV and UHPLC–MS/MS. Analysis

with the UHPLC system was carried out using a Waters Acquity UPLC HSS C18 (2.1 mm × 150 mm, 1.8 µm) column with a gradient mode mobile phase. The column used for the analysis with UHPLC–MS/MS was a smaller one: Waters Acquity UPLC BEH C18 column (2.0 mm × 100 mm,1.7 µm). Both methods can be used for adulterant inspection and sample analysis in food and dietary supplements (66). Analysis from wastewater was carried out to study the exposure of individuals living in a community to cannabinoids (67). Determination of cannabinoids in wastewater can give information about the use of cannabis in a determinate area. The extraction and separation of cannabinoids from wastewater is a difficult process because these compounds are hydrophobic in nature (67–71).

Jacox and colleagues developed a method for the analysis of THC and its metabolites THCCOOH and THCOOH-glucuronide and other licit and illicit drugs, using an UHPLC–MS/MS with a Kinetex C18 (2.1 mm x 100 mm, 1.7 µm) column and a gradient mode mobile phase consisting of 0.1% formic acid with water and 0.1% formic acid with ACN ranging between 40–95% at a flow rate of 0.5 mL/min (67).

Brighenti and colleagues carried out analysis in honey since apiary products are extensively consumed. Extraction of cannabinoids from honey was performed comparing two methods 1) ultrasonication in a water bath, and liquid–liquid (L/L) purification step and 2) SPE with QuEchERS. The use of L/L extraction can be time consuming and large amounts of solvent are required (71). Brighenti and colleagues reported reproducibility problems that occurred because of the emulsion formation. QuEchERS extraction has become more popular in the last year because is easier and quicker to use, and smaller amounts of solvent and samples are required (23, 72). QuEchERS consists of two steps: extraction and partition of the homogenised sample with an organic solvent and salt solution; and the use of the dispersive solid-phase extraction (dSPE) technique to extract and clean the supernatant (23,72). Brighenti and colleagues used the first step of this procedure for the extraction of cannabinoids from honey and analyzed them using an Agilent 1200 HPLC–MS/MS system with a Kinetex EVO C18 column (100 × 2.1 mm, 5 µm particle size). The mobile phase used consisted of 2.0 mM aqueous $CH_3COONH_4$ and ACN at a flow rate 0.35 mL/min (21).

## Conclusion

HPLC is the most commonly used LC-based system for the analysis of cannabinoids. UHPLC is becoming popular because of its shorter analysis time and use of less solvent. The detector used can depend on the matrix from which cannabinoids are extracted from. MS/MS is used for matrices such as blood and urine, which are more complex and contain less quantities of cannabinoids while DAD and UV are used in plant material where the quantity of cannabinoids is higher. The most popular mobile phase is water and ACN in both with 0.1% HCOOH in gradient mode. C18 columns are the most commonly used. Identification and comparison of analytical methods for determination of cannabinoids in different matrices can help in the development of efficient and effective methods of analysis, which are useful for high throughput screening. Accurate and precise determination of concentrations of cannabinoids can help in better understanding the physiological effects and therapeutic properties of this class of compounds.

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