Extraction and Determination of Tetrahydrocannabinol in Oil

Submitted in partial fulfilment of the requirements of the Degree of Master of Pharmacy

Miriana Cachia

Department of Pharmacy

2021

I would like to dedicate this dissertation to my parents, Alister and Josephine, my sister Lynn, my fiancé Ryan and my beloved grandparents, especially to my granddad, Carmel, the man with a strong mind and a soft heart who has always insisted on the importance of my education.

Without you I would not be the person I am today, and I am forever grateful to have you.

Abstract

Tetrahydrocannabinol (THC) is responsible for the psychoactive effects of cannabis. As interest in medicinal cannabis increased, extraction and determination of THC gained importance. [1]

The aim of this study was to develop an effective, efficient, and reproducible procedure for the extraction and determination of THC from oil. Reversed-Phase High Performance Liquid Chromatography (HPLC) was performed using an Agilent ® 1260 Infinity system with an ACE ® 5 C_{18} column (250 x 4.6mm id) using OpenLab ® software. Chemicals and reagents used included LGC ® standard solution of THC 0.1mg/ml in methanol, Sigma-Aldrich ® internal standard of ibuprofen, Natures aid ® MCT oil and Primadonna ® Extra Virgin Olive oil. Honeywell ® HPLC grade acetonitrile and methanol were used.

Methods of physical extraction were tested at different velocities and times using Vortex-Genie® 2, Langford Sonomatic® 1400 Ultrasonic Bath and Eppendorf® Minispin centrifuge. Method development was conducted at the department of Pharmacy, University of Malta.

[1] ElSohly AM, Murohy PT, Khan I, Walker WL and Gul W. Analysis of cannabidiol, Δ9-tetrahydrocannabinol, and their acids in CBD oil/hemp oil products. Medical Cannabis and Cannabinoids. 2020;3(1):1-13.

A rapid HPLC method for analysis and determination of THC, CBD and CBN in methanol was first developed. HPLC parameters that repeatedly resulted in good results were mobile phase: phosphate buffer (pH2.5) and acetonitrile (80:20, v/v) at flow rate of 2ml/min and UV detector wavelength of 220nm. Analyses were conducted in triplets to ensure reproducibility and precision of results. Resolution of chromatograph, peak shape and retention time of THC were observed while area under the peak of THC was measured.

Selected method for extraction of THC from oil involved: vortex mixing for 30 seconds, sonication for 15 minutes, followed by centrifugation for 15 minutes at 6000rpm. Two immiscible layers were yielded. The top layer, methanol and THC was extracted using a micropipette, refrigerated for 12 hours, and centrifuged for 15 minutes. Analyte was passed through a syringe filter prior to HPLC analysis.

Method development was initially conducted using MCT oil as a carrier, this method was not found efficient or reproducible due to blockage and damage of the stationary phase and long elution time of MCT oil. Method development was hence continued using Extra Virgin Olive oil as a carrier. HPLC parameters that repeatedly resulted in good THC peak shape and resolution following extraction of THC from Extra Virgin Olive oil were mobile phase: phosphate buffer (pH2.5) and acetonitrile (70:30, v/v) at flow rate of 2ml/min, UV detector wavelength of 220nm and column temperature of 40°C.

Acknowledgements

I would like to express my sincere gratitude to Ms Eva Tejada and Ms Pauline Falzon from laboratory 444 at the pharmacy department for their constant help and support during method development. My supervisor Profs Anthony Serracino Inglott, my co-supervisor Dr Janis Vella Szii, and Profs Everaldo Attard for their guidance and help throughout the past five years.

Last but not least, I would like to thank my parents, Alister and Josephine for making my happiness and success their priority. My fiancé, Ryan who has always believed in me, loved me and gave me all the support I required. My friends for all the beautiful memories, especially my classmate Michaela Vella for her honest friendship and for always being a shoulder to lean on all throughout my university experience.

Table of Contents

Chapter 1 Introduction.....11

1.1. Brief History of Cannabis.....12

1.2. The Cannabis Plant.....15

1.2.1. Botanical Description of Cannabis.....15

1.2.2. Constituents of *Cannabis*.....17

1.3. Medicinal Cannabis.....21

1.3.1. The Endocannabinoid System.....21

1.3.2. Medicinal Cannabis Products.....24

1.4. Cultivation of Medicinal Cannabis.....27

1.4.1. Methods of Cultivation.....27

1.4.2. Outdoor Cultivation.....28

1.4.3. Greenhouse Cultivation.....29

1.4.4. Indoor Cultivation.....30

1.5. Timeline of Laws and Regulations.....33

1.5.1. Local Regulations and Legislations.....36

1.6. Extraction and Analysis.....37

1.6.1. Extraction Techniques.....37

1.6.2. Sample Preparations.....38

1.6.3. Presumptive Tests.....40

1.6.4. Ion Mobility Spectrometry.....40

1.6.5. Thin-Layer Chromatography.....41

1.6.6. Gas Chromatography-Mass Spectrometry.....41

1.6.7. Gas Chromatography with Flame Ionization Detection.....41

1.6.8. High-Performance Liquid Chromatography.....42

1.7. Aims and Objectives 42

Chapter 2 Methodology 43

2.1. Project Overview 44

2.2. Instrumentation 44

2.3. Mobile Phase 44

2.4. Method Development for the Analysis of Cannabinoids 45

2.5. Method Development for the Extraction of THC in Oil 46

2.6. Method Development for the Determination of THC in Oil 47

Chapter 3 Results 49

3.1. Results Overview 50

3.2. Literature Review 50

3.3. Determination of THC, CBD and CBN in Methanol 57

3.4. Extraction and Determination of THC from Oil 58

Chapter 4 Discussion 61

4.1. Relevance of Developed Method 62

4.2. Main Outcomes 63

4.3. Conclusion 68

References 70

List of Publications and Abstracts 85

Appendices 93

List of Figures

Figure 1.1: Three main species of cannabis. 12

Figure 1.2: Resin glands found at the tip of the trichome. 16

Figure 1.3: Endocannabinoids anandamide and 2-arachidonoylglycerol. 23

Figure 3.1: Chromatograph produced using chosen parameters for the seperation and determination of THC, CBD and CBN in methanol. ... 57

Figure 3.2: Techniques and parameters used to extract THC from oil. 58

Figure 3.3: Chromatograph determining THC following extraction from MCT oil. (Average retention times; Ibuprofen: 3.27 minutes, THC: 10.90 minutes. Average area under the peaks; Ibuprofen: 736988 mAU and THC: 821169 mAU) 59

Figure 3.4: Chromatograph determining THC following extraction from Extra Virgin Olive oil. (Average retention times; Ibuprofen: 2.85 minutes, THC: 12.72 minutes. Average area under the peaks; Ibuprofen: 715800 mAU and THC: 835432 mAU) 60

Figure 4.1. Chromatograph of MCT oil. 66

Figure 4.2. Chromatograph of Extra Virgin Olive oil. 66

List of Tables

Table 1.1: Structures of Cannabinoids 20

Table 2.1: HPLC parameters tested during method development of analysis of THC, CBD and CBN in methanol.....46

Table 2.2: Parameters tested to determine best method of extraction of THC from oil. 47

Table 2.3: HPLC parameters tested during method development using MCT oil. 48

Table 2.4: HPLC parameters tested during method development using Extra Virgin Olive oil.....48

Table 3.1: HPLC parameters of different published methods. 51

Table 3.2: Chosen HPLC parameters for the analysis of THC, CBD and CBN in methanol. 57

Table 3.3: Chosen HPLC parameters for the analysis of THC following extraction from MCT oil. 58

List of Abbreviations

CBD – Cannabidiol

CBN – Cannabinol

CND – Commission on Narcotic Drugs

DAD – Diode Array Detector

ECS – Endocannabinoid System

GACP – Good Agricultural and Collection Practice

GC – Gas Chromatography

GMP – Good Manufacturing Practice

HID – High Intensity Discharge

HPLC – High Performance Liquid Chromatography

ICH – International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

IMS – Ion Mobility Spectrum

INCB – International Narcotics Control Board

MCT - Medium-Chain Triglyceride

MS – Mass Spectrometry

QC – Quality Control

QP – Qualified Person

SPH – Superintendent of Public Health

TCH –Tetrahydrocannabinol

TLC – Thin Layer Chromatography

UNODC – United Nations Office on Drugs and Crime

UV – Ultraviolet

Chapter 1

Introduction

1.1. Brief History of Cannabis

There are three main species of cannabis (Figure 1.1), *Cannabis sativa, Cannabis indica* and *Cannabis ruderalis*. There are more than 700 different Cannabis varieties. Cannabinoids, are the main biologically active constituents of the cannabis plant (Lehmann and Brenneisen, 1995; Stout et al, 2012).

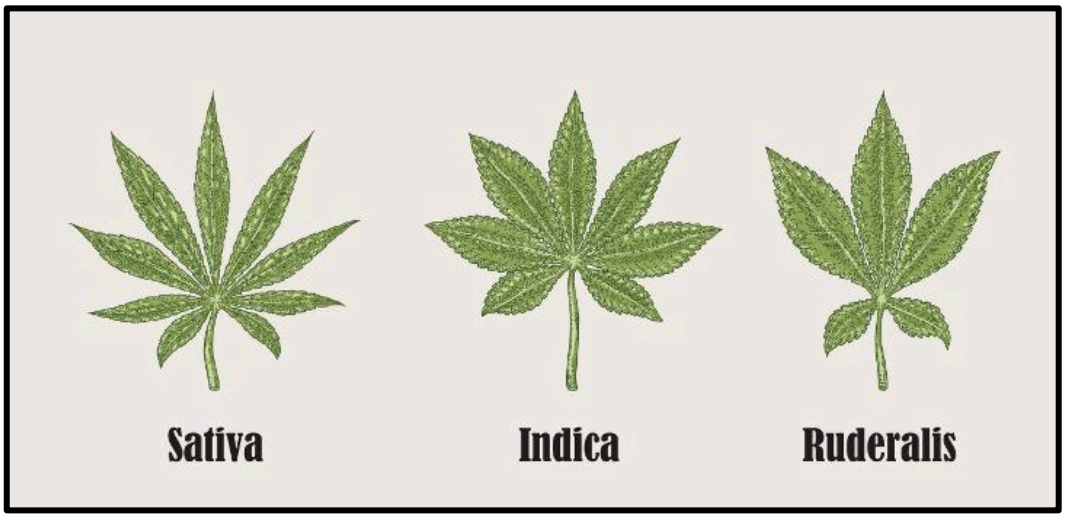


Figure 1.1: Three main species of cannabis.
Photo adopted from Shutterstock.com.

In previously published documents cannabis was referred to as 'Hemp'. Its main use was as a fibre and oil. The fibre produced from cannabis was superior to other fibres and was used in the production of sails and paper. Hempseed oil provided a beneficial nutritional value and had a major involvement in the development of a number of products, including food and cosmetics (Oomah et al. 2002).

Cannabis was first cultivated in Central Asia. Documents confirm that cannabis was excavated from tombs near the Flaming Mountains in Turpan. One tomb contained 789g of *Cannabis Sativa* and dated back to 750-550 BC. Collected evidence and botanical analysis suggests that cannabis was not only cultivated for food and fibre but also for "…pharmaceutical, psychoactive or divinatory purposes" (Russo et al. 2008).

Cannabis can be listed as one of the oldest known medicinal plants and its use is documented in various old literature and ancient handbooks, [1] including the following:

- The Ramesseum III Papyrus (1700 BC)
- The Eber's Papyrus (1600 BC)
- The Chester-Beatty VI (Medical) Papyrus (1300 BC)
- The Berlin Papyrus (1300 BC)
- The Hearst Papyrus (1,550 BC)
- The Vienna Papyrus 6257 (200 AD).

In ancient Egypt the plant was referred to "…the Arabic *ŝmŝm*" and was mainly consumed in the form of tea or tinctures (Dawson, 1932).

A study carried out in Egypt on natural and artificial mummies concluded that in both Peru and Egypt cannabis, previously referred to as 'Hashish', was already in use between 200-1500 AD (Parsche et al, 1993).

It was the Greeks and Romans who identified the therapeutic value of the plant and the term 'hempseed' and 'maijuana' were introduced. Bausanius and Glen document this discovery in Roman records in the 2nd century BC (Brunner, 1977).

[1] Antiquecannabisbook.com [Internet]. The Antique Cannabis Book. Chapter 2, The History of Medical Cannabis [cited 19 February 2019]. Available from: http://antiquecannabisbook.com/chap2B/Egypt/Egyptian.htm.

Dioscorides, a Greek physician had recommended cannabis seeds for the treatment of otalgia (pain in the ear) in his work 'De Materia Medica' that was written between 50-70 BC. 'Hashish' and its medical properties then started to become widely known by Arabic physicians (Gabriel and Nahas, 1982).

For a long period, the public was unaware of the psychoactive properties of the cannabis plant and therefore it was not abused of. In 1840 William O'Shaughnessy, a young Irish doctor, introduced cannabis as a medicine in Europe. In East India the medicinal use of cannabis had become widely common (Amar, 2006).

Issues related to cannabis being used as a medicinal product included:

- No equipment was available for the standardised cultivation of medicinal cannabis. This flaw in the cultivation process made it difficult to produce a reliable product of consistent composition. Patients were receiving the same quantity of the product but not of the same quality leading to different adverse effects (Aguilar et al. 2018).
- Difficulties in modes of administration of medicinal cannabis. Not being water-soluble the cannabis extract could not be injected, and oral administration was found to be unpredictable (Klumpers and Thacker 2018).
- Transportation and supply of cannabis overseas was problematic which resulted in an implementation of high tax on all cannabis products. By the beginning of the 20th century the medical use of cannabis started to decrease while cannabis abuse as a narcotic started to increase (Musto, 1972).

The activity of smoking cannabis for recreational purposes has become common in the Western world since the 1960s. Increase of cannabis use resulted in further interest in cultivation of cannabis, understanding of the psychoactive ingredients of the plant and in being able to quantify them. Development on the scientific knowledge of the active ingredients of the cannabis plant has led to controversies over both medicinal and recreational cannabis (Bewley-Taylor et al, 2014).

Cannabis has been cultivated and used for many years however studies on pharmacological properties and medicinal cannabis have only been carried out since the end of the nineteenth century and are still on-going. Laws and regulations on both cultivation and use of cannabis differ from one county to another (Amar, 2006).

1.2. The Cannabis Plant

Cannabis sativa L. has a worldwide distribution and is considered to be a cosmopolitan species. The plant is an annual herb, and it is naturally dioecious, meaning it has only female flowers (or only one male). The main source of the psychoactive drug delta-9-tetrahydrocannabinol (THC) is found in the female trichomes (Zuk-Golaszewska and Golaszewski, 2018).

1.2.1. Botanical Description of Cannabis

Cannabis sativa has an upright stem that is usually between 1 to 6m high depending on the phenotype and chemotype. The plant is covered in trichomes that are more concentrated at the female inflorescence. *Cannabis sativa* has a laterally branched root system that can reach up to 2.5m deep in loose soils. Leaves are arranged in an alternate manner, have a palmate shape, and are composed of petioles that vary in

length between 2 to 7cm. The leaves have serrate margins, which are a characteristic feature. At the tips of the slender trichome stalk there are resin glands (illustrated in figure 1.2 below). The yellowish-brown resin glands secrete an aromatic resin that contains terpenoids. With a very high content of cannabinoids and flavonoids, the resin glands play an important role in the biological activities of the cannabis plant. The resin secreted isolates itself from the secretory cells as it collects under a thin waxy membrane that protects the plant from enzymatic changes and oxidation. Inflorescences on the plant are male i.e. staminate or female i.e. pistillate (Farag, 2014; Farag and Kayser, 2017).

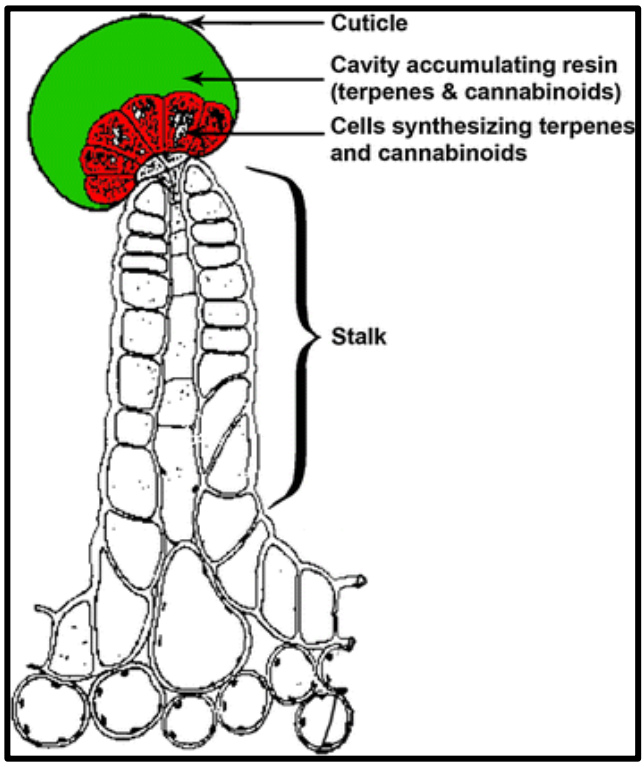


Figure 1.2: Resin glands found at the tip of the trichome.
Adapted from Pate D.W. Chemical ecology of Cannabis. International Hemp Association. 1994;2(29):32-37.

Cannabis trichomes and glands can be further classified at a microscopic level. Trichomes are epidermal in origin and can be subdivided into several morphotypes, all of which consist of a stalk and secretory cells (Szymanski et al, 2000).

Research indicates that the length of the stalk is directly related to the potency of THC. In 1992, Mahlberg and Kim have documented that *Cannabis sativa* with very small gland heads was reported to produce no cannabinoids while long stalked glands were found to have 20 times the cannabinoid content (Mahlberg and Kim, 1992; Small and Naraine, 2015).

The resin secreted offers the plant a defence mechanism against insects and fungal attack. The cannabis plant is still subject to other pests and contaminants including microbes, pesticides, and heavy metals (Small and Naraine, 2015).

1.2.2. Constituents of *Cannabis*

Mechoulam and Hanus carried out a historical overview of chemical research on cannabinoids in 2000. They documented the major events in the discovery of the main constituents of the cannabis plant as explained in section 1.2.2.

In 1899 Wood et al isolated the first constituent of cannabis by distillation which was named cannabinol (CBN). At the initial stages of the study, it was assumed that it was the main active compound of the plant (Wood et al, 1899). In the 1930s Cahn extracted pure cannabinol and it was recognised to be a weak active constituent, hence the search for the active constituents of cannabis continued (Cahn, 1933).

Cannabidiol (CBD) was isolated in the 1940's. Mechoulam and Shvo determined its structure and stereochemistry in 1963 (Mechoulam and Shvo, 1963). In the following years Gaoni and Mechoulam isolated the main active compound of cannabis that is delta-9-tetrahydrocannabinol (Δ -9-THC) (Gaoni and Mechoulam, 1967).

More than 100 terpenoids have been found and identified in cannabis by the 1980s and are classified into 5 classes (Turner et al, 1980).

Cannabinoids are odourless. It is the volatile compounds of monoterpenoids and sesquiterpenoids that give the cannabis plant a particular distinctive smell. The most unique terpenoids are caryophyllene oxide that is the compound sensed by search-dogs and m-mentha-1,8(9)-dien-5-ol (Russo, 2011).

Approximately 20 structurally diverse flavonoids have been isolated from the plant. Flavonoids can be classified into three different categories (Vanhoenacker et al., 2002).

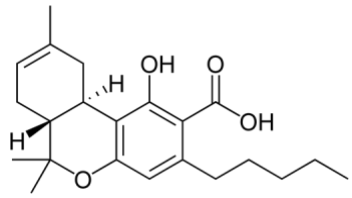
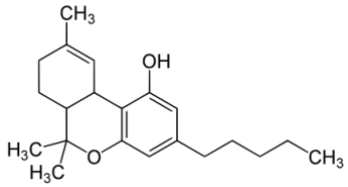
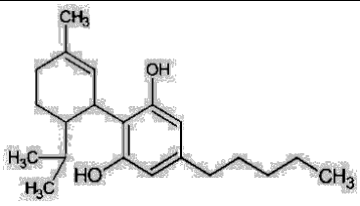
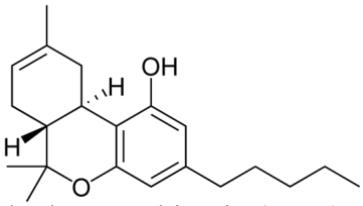
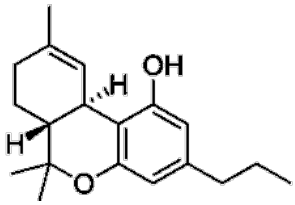
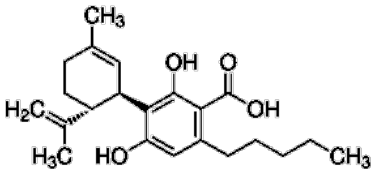
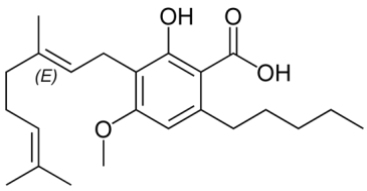
Alkaloids have also been identified as constituents of cannabis. Phenols, steroidal glycosides, esters, and other cannabis constituents have been reported (Turner et al, 1980).

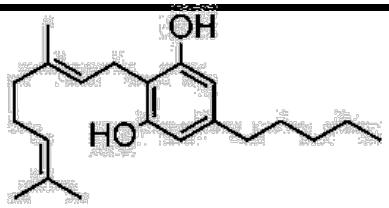
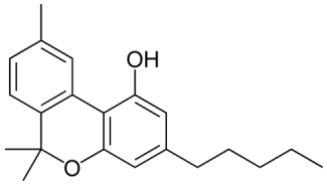
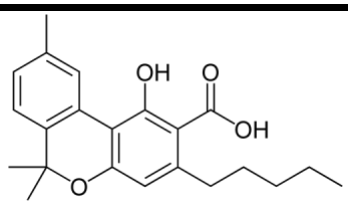
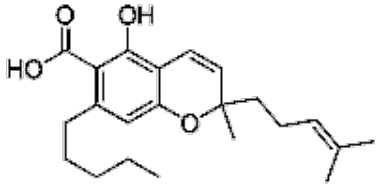
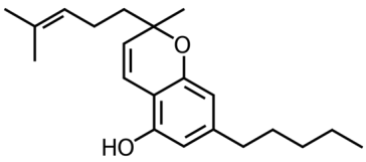
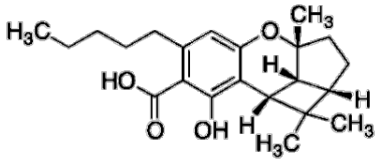
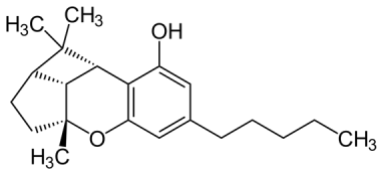
Cannabinoids, being part terpenes and part natural phenols are structurally classified as C_{21} terpenophenolic compounds. Cannabinoids are acyclic or cyclic monoterpene phenolic compounds unique to Cannabis. Cannabinoids are the main biological active constituents of the plant. Since the discovery of the chemical structure of tetrahydrocannabinol (THC), the compound responsible for the psychoactive effects of cannabis cannabinoids have been of great interest in the research field (Stout et al, 2012). Cannabinoids are classified according to their core structure. A total of 565 cannabinoids, have been reported so far (Radwan et al, 2017).

The most important cannabinoids are illustrated in Table 1.2. Cannabinoids are synthesised and stored in the glandular trichomes mentioned in section 1.2.1. Different quantities of cannabinoids are found in different parts of the plant. Cannabinoids are only not found in the seeds. (Ross et al, 2000).

The role of cannabinoids on the cannabis plant is that of a defence mechanism. Both cannabigerolic acid (CBGA) and tetrahydrocannabinolic acid (THCA) stimulate apoptosis, a natural process of cell death, in both plant and animal cells (Sirikantaramas et al, 2005).

Table 1.1: Structures of Cannabinoids

COMPOUND NAME AND STRUCTURE
 Tetrahydrocannabinolic acid (THCA)
 Tetrahydrocannabinol (THC)
 Delta-8-tetrahydrocannabinol (Δ-8-THC)
 Tetrahydrocannabivarin (THV)
 Cannabidiolic acid (CBDA)
 Cannabidiol (CBD)
 Cannabigerolic acid (CBDA)

c  Cannabigerol (CBG)
 Cannabinolic acid (CBNA)
 Cannabinol (CBN)
 Cannabichromenic acid (CBCA)
 Cannabichromene (CBC)
 Cannabicyclolic acid (CBLA)
 Cannabicyclol (CBL)

1.3. Medicinal Cannabis

1.3.1. The Endocannabinoid System

The Endocannabinoid system (ECS) is made up of cannabinoid receptors, their endogenous ligands, and other specific enzymes. ECS is a result of evolution and apart from humans it has also been found in birds, amphibians, and fish. The ECS involves physiological and pathological processes that have been studied in recent years (Sinclair, 2016).

Cannabinoids exert their antinociceptive effect, that is the action of blocking the detection of pain stimulus by activation of CB1 and CB2 receptors. In the 1980s the existence of these receptors was acknowledged and discovered by Devane et al. (1988). The discovery was carried out after it was established that cannabinoids are highly stereoselective and they can inhibit the adenylate cyclase second messenger pathway in both the brain tissue as well as neuroblastoma cell lines (Amar, 2006).

Matsuda et al carried out further research in 1990 leading to the discovery and cloning of a stereospecific G-protein-coupled cannabinoid receptor, called CB1 (Matsuda et al, 1990). CB1 receptors are present throughout the pain pathway in the central nervous system and present in the certain peripheral organs such as the heart and liver and adipose tissue (Maccarrone et al, 2001).

Analgesic activity of CB1 receptors has been localised in different regions from supraspinal areas, the spinal cord, and peripheral terminals. CB1 receptors are able to inhibit adenylate cyclase activity and open N-type calcium channels (Mackie and Hille, 1992).

Munro et al discovered the second type of cannabinoid receptors. This receptor was named CB2. CB2 receptors are primarily and highly expressed by the immune system in a wide range of leukocytes mainly in tissues like the tonsils and spleen (Munro et al, 1993). CB2 receptors can also be found in smaller quantities in cells of the central nervous system, by microglia, astrocytes, and some neurons (Cabral and Griffin, 2009). CB2 receptors are distributed in other tissues such as pulmonary endothelial cells, in osteocytes and in the gastrointestinal system (Atwood and Mackie, 2010).

CB2 receptors are mainly found in immune cells during inflammation, indicating their involvement in neuroinflammation and neuropathic pain. In 2011 Onaivi has provided evidence that CB2 receptors also have a role in schizophrenia, depression and in the effects of substance abuse (Onaivi, 2011).

Other studies also provided evidence that CB2 receptors are highly important in neuroinflammation present in multiple sclerosis, HIV-induced encephalitis, traumatic brain injury, Parkinson's disease, Alzheimer's disease, and Huntington's disease (Pazos et al, 2004; Benito et al, 2008).

Endocannabinoids are endogenous lipid agonists of cannabinoid receptors CB1 and CB2. All natural cannabinoids are highly lipid soluble, and their isolation was challenging. Devane et al. carried out the first compound isolation in 1992 from pig brain tissue. The compound isolated had a higher affinity to CB1 receptors and was named anandamide (arachidonic acid ethanolamine, AEA) (figure 1.3).

In 1995, Mechoylam et al. isolated 2-arachidonoylglycerol (2-AG) (figure 1.3) from a dog's gut. 2-AG has high affinity for the CB1 and CB2 receptors. In recent years more endocannabinoid compounds have been isolated however these 2 compounds are the most widely investigated, both of which are synthesised postsynaptically (Sinclair, 2016)

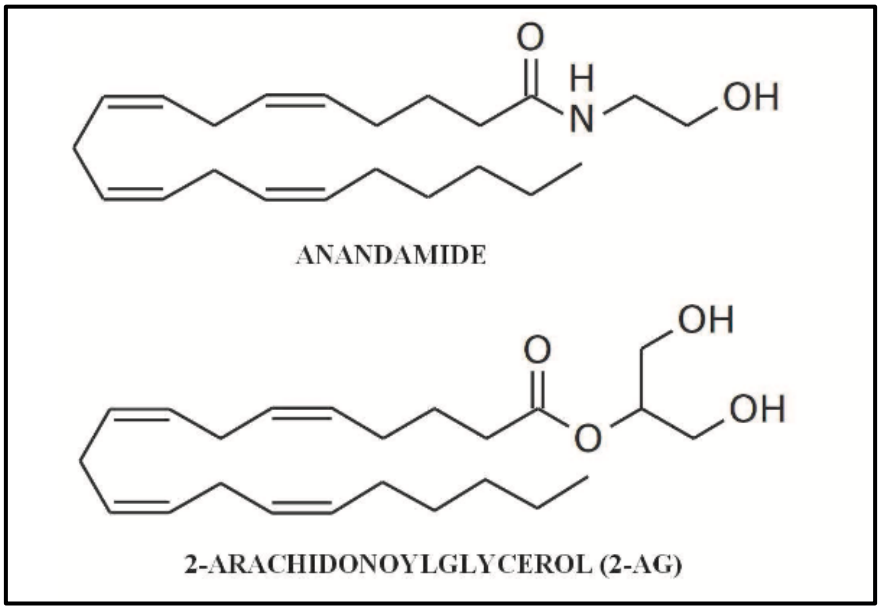


Figure 1.3: Endocannabinoids anandamide and 2-arachidonoylglycerol. Adopted from Dinu et al, 2011.

1.3.2. Medicinal Cannabis Products

The goal of pharmaceutical companies is to be able to cultivate and produce a constant and standardised product up to Good Manufacturing Practice (GMP) and Quality Control (QC) standards.

Studies have been carried out regarding the therapeutic indications of THC for conditions such as pain in multiple sclerosis (Markova et al, 2018), insomnia (Vigil et al, 2018), epilepsy (Thiele et al, 2019), asthma (Tashkin et al, 1974) and glaucoma (Merritt, 1982), and on how cannabinoids can act as anti-emetics (Jones et al, 1982), appetite stimulators (Haney et al, 2007) and analgesics (Van De Donk et al, 2018).

The main advantage of using cannabinoids clinically is their lack of toxicity. No deaths have been reported following use of cannabinoids and on comparison with other drugs their benefit to risk ratio is considered to be higher. In clinical trials the most common adverse effects were as follows: sedation, psychological effects such as euphoria, anxiety and depersonalization and physical effects such as dry mouth and ataxia (Ashton, 1999).

In Canada, in 2005 the first pharmaceutical laboratories received approval of authorisation for production of medicinal cannabis. Nabiximols an extract of cannabis under the trade name of Sativex ®, was one of the first cannabis extract product to be approved in Canada, United Kingdom, Germany, and Spain. The main active compounds of the product are delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) at a ratio of 1:1. Sativex ® is an oral spray used to treat neuropathic pain such

as muscle pain and stiffness in multiple sclerosis. Sativex ® provides symptomatic relief. [3]

Cannador ®, an approved medicinal product by the society for clinical research in Germany was reported to aid in the reduction of tremors related to multiple sclerosis. Cannador ® is administered orally as a capsule and contains both THC and CBD at a ratio of 2:1. [4]

Bedrocan ® was introduced in 2003. The company currently produce 5 different products that are Bedrocan ® that contains 22% THC and 1% CBD, Bedrobinol ® that contains 13.5% THC and 1% CBD, Bediol ® that contains 6.3% THC and 8% CBD, Bedica ® that contains 14% THC and 1% CBD and Bedrolite ® that contains 1% THC and 9% CBD.[5]

---

[3] Government of Canada. Bayer Inc. Approval Of Sativex® With Conditions Fact Sheet [Internet]. Canada: Government of Canada; 2005 [cited 26 July 2021]. Available from: http://medicalcannabis.it/documenti/Sativex_factsheet_en.pdf.

[4] International Association for Cannabis as Medicine. Cannador [Internet]. Cannabis-med.org; 2019 [cited 5 March 2019]. Available from: https://www.cannabis-med.org/index.php?tpl=def&id=241&lng=en&red=deflist.

[5] Bedrocan ®. Products and Services [Internet]. Netherlands; 2017 [cited 26 July 2021]. Available from: https://bedrocan.com/products-services/.

Aurora ® was first stationed in Canada in 2013, the company has now grown and emerged globally and consists of both medical and consumer brands. In terms of medicinal cannabis two main products are Pedanios ® 20/1 that contains 20% THC and 1% CBD and Pedanios ® 22/1 that contains 22% THC and 1% CBD. [6]

So far in Malta only 4 medicinal cannabis products are available on the market. These are: Bedrocan ® 22/1 (sativa), Bediol ® 6/8 (sativa) Pedanios ® 20/1 (indicta) and Pedanios ® 22/1 (sativa). All of which are in the form of cannabis dried flowers (floss or granular) to be administered by vaporisation.

[6] Canada Newswire. Aurora Cannabis and Grow Group PLC Renew Market Access Services Agreement for Medical Cannabis in the United Kingdom. [Internet]. Canada; 2021 [cited 26 July 2021]. Available from: https://www.newswire.ca/news-releases/aurora-cannabis-and-grow-group-plc-renew-market-access-services-agreement-for-medical-cannabis-in-the-united-kingdom-863683498.html.

1.4. Cultivation of Medicinal Cannabis

1.4.1. Methods of Cultivation

Cannabis is an annual plant. In outdoor conditions, in Europe the plant is sown according to temperature and climate between the beginning of March and late April. The difference between cultivating the plant for fibre and for therapeutic use is the period of harvesting. When cultivating for fibre harvesting takes place in the early stages of the flowering period while harvesting to obtain the drug is carries out later. The method used traditionally to separate the fibre from the rest of the plant was a natural process of decomposition by bacteria and fungi (Merfield, 1999).

Along the years the cultivation for fibre started to decrease while that to obtain the psychoactive form of the drug increased. Cultivators started to prioritise the potency of cannabinoids present in their crop and to appreciate the importance of obtaining a standardised and constant product. Different conditions and methods used to cultivate the Cannabis plant for medicinal use have evolved and improved throughout the years. The main aims during cultivation were to be able to produce a consistent product of high quality with no contaminations, produced under a GMP and QC. GW Pharmaceutics has been conducting indoor cultivation of cannabis since 1999 (Chandra et al, 2017).

Methods for cultivation of cannabis can be classified and divided into three, outdoor cultivation, greenhouse cultivation and indoor cultivation.

1.4.2. Outdoor Cultivation

According to the World Drug Report carried out by United Nations Office on Drugs and Crime (UNODC) in 2009 [7] it is estimated that the cannabis plant is suitable for cultivation on approximate one third of the earths land mass. It was estimated that in 2008 between 2000 and 6418 km^2 have been used for outdoor cultivation.

Products cultivated outdoors are more likely to contain heavy metals, pesticides, moulds, bacteria, and other contaminants. The growth rate of cannabis in outdoor conditions is slower and the CBD yield is significantly lower per unit area when compared to product cultivated indoors. UNODC, 2006 world drug report suggested that only 4% of herbal cannabis cultivated globally is cultivated outdoors in Europe. [8] As a result, outdoor cultivation of pharmaceutical cannabis is not the optimal method for cultivation. (Chandra et al, 2010).

Large volumes of outdoor, low-potency Cannabis have been cultivated in Albania for exporting purposes and in Morocco as mixed-sex plant for resin. Being grown in open fields when the cultivation was carried out in larger quantities for commercial use the crops were either stolen or seized (Carpentier et al, 2012). Outdoor cultivation in Europe is rarely reported except for small plants intended for personal use (Jansen, 2002).

[7] United Nations Office on Drugs and Crime (UNODC). World drug report 2009, United Nations Publications, New York; 2009, [cited 25 April 2020]. Available from: https://www.unodc.org/documents/wdr/WDR_2009/WDR2009_eng_web.pdf.

[8] United Nations Office on Drugs and Crime (UNODC). World drug report 2006, United Nations Publications, New York; 2006, [cited 25 April 2020]. Available from: https://www.unodc.org/pdf/WDR_2006/wdr2006_volume1.pdf.

Outdoor cultivation is problematic due to the humid environments in autumn. During this season the plant would be in its flowering period and is highly prone to disease and can be easily and quickly damaged by fungi. In a research carried out in the United Kingdom these environmental factors have been reportedly overcome. In 2009 Potter documented that for 10 consecutive years outdoor cultivators have achieved the production of high quality crops with similar yields and THC potency to crops grown in indoor conditions (Carpentier et al, 2012).

Outdoor cultivation is significantly cheaper than indoor cultivation. Limiting factors include that day length and light intensity cannot be altered. As a result, it is a nature dependent process, producing only one type of crop at a time and harvesting time cannot be scheduled (Potter et al, 2008).

1.4.3. Greenhouse Cultivation

Greenhouse cultivation requires controlled conditions, which are less exact than those used in indoor cultivation. GW Pharmaceuticals have concluded that greenhouse cultivation of cultivation is feasible however some environmental conditions must be modified including the addition of artificial lighting in the vegetative period, the installation of blinds on the roof to be able to decrease day length in the flowering period and the addition of gas heaters during harvesting period for the drying of the plant to take place.

A limiting factor of greenhouse cultivation is that the flowering period can only be supported from spring to end of summer when the sufficient natural light intensity is reached. Good quality material can be produced in greenhouse conditions. Limitation

of greenhouse cultivation is that yields are significantly lower when compared to indoor cultivation (Chandra et al, 2010).

1.4.4. Indoor Cultivation

The aim of indoor cultivation is to be able to produce a uniform and constant product. This is possible due to the ability to control parameters such as temperature, light intensity, hours of light and irradiance levels. Controlling these parameters will favour and facilitate yield improvements, regulate the life cycle and harvest timing, avoid contamination and pest control, prevent cross or self-pollination.

To be able to perform indoor cultivation of cannabis for medicinal and research purposes a pharmaceutical company requires a special license and to be GMP certified (Chandra et al, 2017).

A technique used to cultivate cannabis indoors is hydroponics growing. This method involves the cultivation of the plant in a nutrient solution instead of soil. Hydroponic technique aids in obtaining consistency. The nutrient solution is composed of potassium, calcium, magnesium, nitrate, dihydrogen phosphate and sulphate. These ions need to be in the right ratio for optimal growth and can be monitored using a pH meter (Steiner, 1961).

An important factor for growth is light, for photosynthesis to take place. In indoor cultivation artificial light is used to facilitate both photosynthesis and photoperiodic processes. Since the rate of photosynthesis also depends on carbon dioxide concentration delivery of carbon dioxide is essential to increase the rate of growth of

the plant. Throughout the process of cultivation documentation is highly important for traceability and maximal care is taken from the selection of the seed to the end and final standardised packed product (Chandra et al, 2017).

Germination initiates within three days of planting, of which only the well-developed seedlings will be selected and transferred to another location into a peat growth medium for optimal growth. After the pots develop roots and sufficient body mass. Germination usually takes 2 weeks of humid conditions and constant light. Seedlings are then transferred to a multi-flow hydroponic system. Hydroponic system controls the flow of the solution that is high in nutrients (Chandra et al, 2017).

The selected genotypes need to be kept in continuous light and supply of nutrition. Altering the day length to an 18-hour day instead of 24 hours is recommended, which results in compromised slower plant growth along with reduced electrical consumption. With the use of clones, the same genotype of the mother plant can be retained throughout subsequent generations (Chandra et al, 2017).

Branches are then removed, and rooted cuttings (clones) are produced. Clones are then relocated into a seeding tray holding a small amount of culture medium. Nutrition is still provided via a hydroponic system. Hence, all clones are exposed to constant conditions for 1 month. After an average of 14 days as the roots reach an adequate size, the clones are then potted up in growth medium into a larger hydroponic system promoting maximum and optimal growth (Chandra et al, 2017).

The cannabis plant then reaches the vegetative growth period that is approximately 3 weeks long. All clones are maintained in the same light intensity, temperature, relative humidity, and carbon dioxide concentration in a cultivation room. In this period the plant reaches the height of approximately 50 cm and develops a root system. When the required growth of the plant is reached the next period would be flower formation and maturation (Chandra et al, 2017).

Altering light duration and introducing a 12-hour light/12-hour dark routine will help achieve the flowering and maturation period. This will cause the cannabinoid content and inflorescences to increase gradually. Flowers develop within 8-10 weeks after which the stigmas and trichomes change colour becoming orange/brown and the secretion of cannabinoids decreases (Chandra et al, 2017).

The plant is then harvested and dried. The plant is suspended to dry in a dehumidified environment for 1 week and then the botanic raw material is stripped from the stem. The material is then inspected visually and tested in the laboratories to ensure standardisation and no contamination (Chandra et al, 2017).

1.5. Timeline of Laws and Regulations

Regulation of cannabis for medicinal use has been an on-going worldwide issue. Consensus among the countries has not been reached and regulations and policies range significantly globally (Aguilar et al. 2018).

In 1961, The International Drug Control Conventions listed cannabis as a prohibited psychotropic substance. “Cannabis and cannabis resin and extracts and tinctures” are listed in schedule I of the 1961 single convention on Narcotic Drugs. As well as “cannabis and cannabis resin” being listed in schedule IV. [9]

In The International Drug Control Convention, drugs are classified according to their therapeutic value, risk of abuse and health hazard. Since currently cannabis is listed in both schedule I and IV this implies that the drug has a much greater risk to benefit ratio and a narrow medical or therapeutic value. [10]

In 1961, countries including India, Mexico, Pakistan, and Myanmar, which used cannabis in their traditional and religious rituals objected with the 1961 single convention on Narcotic Drugs and attempted suspending the inclusion of cannabis in both schedule I and IV. This was done by comparing the potential drug dependence of cannabis and how it differed from other substances listed in the same schedule and by presenting medical benefits of cannabis. The Indian society argued on how their

[9] United Nations, Commission on Narcotic Drugs (CND). The International Drug Control Conventions. Schedules of the Single Convention on Narcotic Drugs of 1961 as amended by the 1972 Protocol, as at 16 May 2018 [Internet]. New York; 2018 [cited 18 March 2019]. Available from: http://undocs.org/ST/CND/1/Add.1/Rev.4.

[10] European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Overview of legal topics: Classification of controlled drugs [Internet]. Lisbon; 2012 [cited 25 April 2021]. Available from: https://www.emcdda.europa.eu/publications/topic-overviews/classification-of-controlled-drugs_es.

traditional drink (bhang) was “far less harmful than alcohol”. The attempt was unsuccessful. After the Convention was put forward India was the country that kept the cannabis plant from being demolished as prohibition of cannabis was waived in some traditional regions and continued practicing traditional and religious celebrations using bhang. (Bewley-Taylor et al, 2014). Bhang is a traditional Hindu drink composed of cannabis flowers and leaves, milk, water, spices, and yoghurt. [11]

In 1971, the Convention on Psychotropic Substances was issued. This included Tetrahydrocannabinol (THC), its isomers and their stereochemical variants in schedule I. This act classified THC, the main active ingredient, as a psychotropic drug. [12]

It was in the late 19th and early 20th centuries that the cannabis regulations started to be developed, as the pharmaceutical industry became more interested in the medicinal potential of cannabis (Bewley et al, 2014). According to the International Narcotic Control Board report published in 2017 the legal use of cannabis has increased significantly since the year 2000. In the following years multiple countries have initiated the use of cannabis for medical purposes and more scientific research have been conducted. As a result, total production of cannabis has increased from 1.3 tons per year to 100.2 tons by 2015 and doubled to 211.3 tons by 2016. [13]

[11] What Is Bhang, Where Did It Come From & How Is It Made? [Internet]. Royalqueenseeds.com; 2017 [cited 19 February 2019]. Available from: https://www.royalqueenseeds.com/blog-what-is-bhang-and-how-is-it-made-n683.

[12] United Nations. Schedules of the Convention on Psychotropic Substances of 1971, as at 19 November 2019 [Internet]. New York; 2018. [cited 25 April 2020]. Available from: https://undocs.org/ST/CND/1/Add.2/Rev.5.

[13] International Narcotic Control Board. Report of the International Narcotics Control Board for 2017 [Internet]. New York; 2017 [cited 25 April 2020]. Available from:

In 2020, the United Nations have accepted recommendations made by the World Health Organisation to remove cannabis and cannabis resin from schedule IV of the 1961 single convention on Narcotic Drugs list. This reclassification will aid in removing barriers with regards to both research and development and accessibility of patients to medicinal cannabis. [14]

Differentiation should also be made between regulations of different cannabinoids. This is because different cannabinoids example CBD and THC have different properties, pharmacological effects and potential for dependence or abuse. As a result, the World Health Organisation also suggested that standardised preparations of cannabis containing mainly CBD (example CBD oil preparations) and a percentage of less than 0.2% of THC are not classified as a narcotic drug. [15] No country authorises the smoking of cannabis for medicinal purposes. [16]

---

https://www.incb.org/documents/Publications/AnnualReports/AR2017/Annual_Report/E_2017_AR_ebook.pdf.

[14] UN Commission on Narcotic Drugs reclassifies cannabis to recognize its therapeutic uses [Internet]. Who.int. 2021 [cited 26 July 2021]. Available from: https://www.who.int/news/item/04-12-2020-un-commission-on-narcotic-drugs-reclassifies-cannabis-to-recognize-its-therapeutic-uses.

[15] Annex 1- Extract from the Report of the 41st Expert Committee on Drug Dependence: Cannabis and cannabis-related substances [Internet]. Who.int. 2021 [cited 26 July 2021]. Available from: https://www.who.int/medicines/access/controlled-substances/Annex_1_41_ECDD_recommendations_cannabis_22Jan19.pdf?ua=1.

[16] European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), Cannabis legislation in Europe: an overview [Internet]. Publications Office of the European Union, Luxembourg; 2018 [cited 25 April 2020]. Available from: https://www.emcdda.europa.eu/system/files/publications/4135/TD0217210ENN.pdf.

1.5.1. Local Regulations and Legislations

In Malta the legislation regarding medicinal cannabis has been approved in March 2018. [17] The law currently states that a licenced medical practitioner can prescribe medicinal preparations of cannabis products that are licenced under the 'Medicines Act or manufactured under Good Manufacturing Practice'. Symptoms and conditions that can be treated with medicinal cannabis are not listed.

Regulations and General Guidelines on the Production of cannabis for medical and research purposes have been implemented in Malta in December 2018. [18] With regards to cannabis activities carried out for research and development purposes a licence is also required. The site in which the activities are carried out needs to be approved by the regulatory authorities while a detailed proposal describing the activities, aims and objectives need to be submitted. Cultivation of the cannabis plant should be carried out with reference to the 'European Medicines Agency Guideline on Good Agricultural and Collection Practice (GACP) for Starting Materials of Herbal Origin'. [19]

[17] Drug Dependence (Treatment not Imprisonment) ACT 2015 (CAP. 537) [Internet]. Malta; 2018[cited 2020 January 20]. Available from: http://justiceservices.gov.mt/DownloadDocument.aspx?app=lom&itemid=12289&l=1.

[18] Malta Medicines Authority. General Guidelines on the Production of cannabis for medical and research purposes [Internet]. Malta; 2018 [cited 1 April 2019]. Available from: http://www.medicinesauthority.gov.mt/file.aspx?f=4226.

[19] European Medicines Agency (EMEA). Committee On Herbal Medicinal Products (HMPC). Guideline On Good Agricultural And Collection Practice (GACP) For Starting Materials Of Herbal Origin [Internet]. European Medicines Agency; 2006 [cited 25 April 2020]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-good-agricultural-collection-practice-gacp-starting-materials-herbal-origin_en.pdf.

1.6. Extraction and Analysis

1.6.1. Extraction Techniques

Extraction can be divided into two; physical and chemical. Physical extraction makes use of mainly the centrifugal force, the force of gravity and filtration. On comparison physical extraction is more user friendly, cheaper and makes use of instrumentation that is readily available in most laboratories, however in terms of larger industrial scales such methods might not be as efficient hence why chemical extraction is used (Wise, 2019).

Throughout the process of extraction chemical composition, chemical properties and quantity of cannabinoids being extracted from the sample may be altered. Therefore, during method development the main concern would be efficiency, reproducibility, availability of equipment, convenience, environmental impact, and the volume of analyte being yielded. A Soxhlet extractor is the apparatus of choice used in the industry for extraction of cannabinoids from oil. Using a Soxhlet extractor is favoured as it is easy to use, efficient and economical. Supercritical fluid extraction is another method of choice using carbon dioxide as a solvent. Ultrasonication can also be used, using both physical extraction and chemical (Devi and Khanam, 2019).

Equipment used in small scale laboratories for physical extraction are explained as follows:

Vortex mixing

Vortex mixers are found in most of the laboratories. It is fast and easy to use instrument used to mix small samples of liquid in a rapid manner. Speed and duration

of mixing are two parameters that can be controlled. The rapid mixing in circular motion creates a spiral flow within the sample. [20]

Sonication

Sonication is the process of using ultrasonic waves to aid in extraction. This method has been used for cannabinoid extraction mainly since it does not use any chemicals and due to its efficiency, making the method more economical as well as environmentally friendly. [21]

Centrifugation

Centrifugation is a separation technique in which the centrifugal force is used to separate component in a heterogeneous mixture. The component liquids will separate and sediment according to their density. This method of extraction coupled up with sonication was also the method of choice for sample preparation by Zivovinovic et al.

1.6.2. Sample Preparations

Sample preparation is an important step prior to analysis. This varies according to the dosage formulation of cannabis as different forms of standardised preparations are available. These include tinctures, raw herbal cannabis in the form of flower buds, powder capsules and the most common form; oils (ElSohly et al, 2020).

[20] Vortex Mixers [Internet]. Conduct Science. 2021 [cited 26 July 2021]. Available from: https://conductscience.com/vortex-mixers/.

[21] A Leap Forward in Cannabinoid Extraction with Ultrasonic Techniques [Internet]. Cannabistech.com. 2017 [cited 26 July 2021]. Available from: https://www.cannabistech.com/articles/a-leap-forward-in-cannabinoid-extraction-with-ultrasonic-techniques/.

The ‘Recommended methods for the Identification and Analysis of Synthetic Cannabinoid Receptor Agonists in Seized Materials’ published by the United Nations Office on Drugs and Crime in 2020 [22] suggests two different methods for extraction and sample preparation for qualitative or quantitative analysis. Solvents recommended to extract the samples are non-polar or polar example methanol and acetonitrile. Sonication, centrifugation, and filtration are recommended physical extraction methods. Soxhlet extraction is also recommended when quantitative analysis is carried out.

With regards to extraction of cannabinoids from oil performing further extractions might be required. Cannabis oil products can be found in various forms and are composed of different carrier oils example olive oil, medium chain triglyceride (MCT) oil and hemp seed oil. Different carrier oils require different extraction methods, sample preparation and parameters for analysis. (Urasaki et al, 2020).

[22] United Nations Office on Drugs and Crime (UNODC), Recommended Methods for the Identification and Analysis of Synthetic Cannabinoid Receptor Agonists in Seized Materials (Revised and updated) [Internet]. New York; 2020. [cited 3 August 2021]. Available from: https://www.unodc.org/documents/scientific/STNAR48_Rev.1_ebook.pdf.

1.6.3. Presumptive Tests

Colour tests are used for screening for the presence of cannabis and not definitive identification. Colour tests are used in combination with other analytical tests to ensure identification. When performing such tests, it is recommended to use a cannabis control sample as a reference material. Immunoassays can be performed for presumptive identification of cannabis. On comparison with other tests immunoassays are expensive.[23]

1.6.4. Ion Mobility Spectrometry

Performing Ion Mobility Spectrometry (IMS) test can screen THC. In 2001 it was documented that this method could be problematic and has limited selectivity when distinguishing from heroin signals and hence it was not the ideal test to be performed.[23]

In 2018 a paper was published by Hadener et al. in which the "suitability of direct analysis of cannabis extracts by ion mobility spectrometry coupled to mass spectrometry" was investigated. An IMS-MS method was developed and validated.

[23] United Nations Office on Drugs and Crime (UNODC), Recommended methods for the identification and analysis of cannabis and cannabis products [Internet]. New York; 2009. [cited 1 April 2019]. Available from: https://www.unodc.org/documents/scientific/ST-NAR-40-Ebook_1.pdf.

[23] United Nations Office on Drugs and Crime (UNODC), Recommended methods for the identification and analysis of cannabis and cannabis products [Internet]. New York; 2009. [cited 1 April 2019]. Available from: https://www.unodc.org/documents/scientific/ST-NAR-40-Ebook_1.pdf.

1.6.5. Thin-Layer Chromatography

Thin-Layer Chromatography (TLC) methods have been developed and validated all of which have a variety of different mobile and stationary phases which are accompanied by different sample preparation and techniques. The distance travelled by a given component divided by the distance travelled by the solvent that is the Retention Factor (Rf) is subject to laboratory conditions as well as sample age and quality. It is recommended to use cannabinoid standards on the same plate while performing TLC testing. [7]

1.6.6. Gas Chromatography-Mass Spectrometry

Gas Chromatography –Mass Spectrometry (GC-MS) method has been used for the analysis of the cannabinoids. To avoid decarboxylation of the acidic cannabinoids in the heated injected port of gas chromatography cannabinoids must be derived prior to analysis. A broad range of sample types can be analysed by this method such as food, beverages, oral supplements, and plant extracts detecting both acidic and neutral cannabinoids (Ciolino et al, 2018a).

1.6.7. Gas Chromatography with Flame Ionization Detection

This method is used to identify qualitatively and quantitatively cannabinoids that are synthetically produced. This method is therefore mainly used in the forensic area to confirm identity of drug and not for pharmaceutical or medicinal products of cannabis. [22]

[7] United Nations Office on Drugs and Crime (UNODC). World drug report 2009, United Nations Publications, New York; 2009, [cited 25 April 2020]. Available from: https://www.unodc.org/documents/wdr/WDR_2009/WDR2009_eng_web.pdf.

1.6.8. High-Performance Liquid Chromatography

A method of choice for the analysis and determination of cannabinoids is high-performance liquid chromatography (HPLC). Separation and determination of cannabinoids can be performed rapidly, and quantification can be subsequently carried out (Mandrioli et al, 2019; Zivovinovic et al, 2018)

For optimum analysis it is fundamental to select the best mobile phase and buffer for the sample. It is at lower wavelengths that the UV cut-offs become significant. Analytes have individual UV absorbance maxima hence the UV detector can be set at a specific wavelength in which the analyte gives optimal selectivity (Swartz, 2010).

1.7. Aims and Objectives

To develop an effective, efficient, and reproducible method for the extraction and determination of THC from oil, making use of instrumentation and equipment that is readily available in most laboratories.

Chapter 2

Methodology

2.1. Project Overview

Phase 1 of this project consisted of a detailed literature review using HyDi, PubMed and Google Scholar focusing on cultivation and analysis of cannabis. Followed by the development of a method for the analysis of THC, CBD and CBN in methanol.

In phase 2 an effective, efficient, and reproducible method for the extraction and determination of THC from oil was developed.

Project was conducted at the Department of Pharmacy, University of Malta at the Pharmaceutical Synthesis and Technology Laboratory.

2.2. Instrumentation

Reversed-Phase High Performance Liquid Chromatography (HPLC) was performed using an Agilent ® 1260 Infinity system with an ACE ® 5 C_{18} column (250 x 4.6mm id) using OpenLab ® software.

2.3. Mobile Phase

Isocratic elution was performed using mobile phase of:

(A) Acetonitrile (Honeywell Chemical)

(B) 2.84 g/L phosphate buffer (Carlo Erba Reagents), with phosphoric acid (Fisher Chemical) for pH adjustment.

Buffer solution (B) was prepared as follows:

1. 2.84g of disodium hydrogen phosphate anhydrous was weighed.
2. Added to 1L of HPLC grade water in a beaker.
3. Solution was sonicated for 5 minutes.

4. Phosphoric acid was added until the required pH value was obtained. pH was measured using a Hanna® pH meter.
5. Buffer solution was filtrated using a funnel and filter paper.
6. Buffer was stored in a labelled bottle.

2.4. Method Development for the Analysis of Cannabinoids

A literature review for the analysis of THC, CBD and CBN was first conducted using HiDy, Google Scholar and PubMed using keywords 'HPLC' and 'Cannabinoids' in title (2009-2019). Parameters used in different published HPLC methods were compared in the form of a table (Table 3.1).

HPLC method development was conducted using LGC ® standard solution of THC 0.1mg/mL, CBD 1.0mg/mL and CBN 1.0 mg/mL in methanol. Standard solutions of CBD and CBN were diluted with HPLC-grade methanol (Honeywell Chemical) to match the concentration of THC (0.1mg/mL). The analyte used consisted of equal amounts of standard solutions, of concentration 0.1 mg/mL of THC, CBD and CBN in methanol. HPLC parameters were tested as illustrated in table 2.1. including ratio of mobile phase, Acetonitrile (ACN) is to Buffer, pH, and flow rate of mobile phase. The selected UV detection wavelength was 220nm and temperature of stationary phase was 25ºC. UV detection wavelengths of 228nm and 270nm were used.

Table 2.1: HPLC parameters tested during method development of analysis of THC, CBD and CBN in methanol.

Mobile Phase	ACN:Buffer 90:10	ACN:Buffer 80:20	ACN:Buffer 70:30
pH	2.5	3.0	4.0	6.0
Flow Rate (ml/min)	0.5	1.0	1.5	2.0

Cannabinoid standards (THC, CBD, CBN) were run separately to determine their elution times and order of elution.

Parameters that repeatedly resulted in good chromatogram resolution, area under the peaks, baseline and retention time at an efficient rate were determined.

2.5. Method Development for the Extraction of THC in Oil

Chemicals and reagents used included LGC ® standard solution of THC 0.1mg/ml in methanol, Sigma-Aldrich ® internal standard of ibuprofen, Natures aid ® MCT oil and Primadonna ® Extra Virgin Olive oil. Sample was prepared using a stock solution of 5 μg/ml of THC in MCT oil. 0.3ml of stock solution was hence added to 0.6ml of methanol into a 1ml microcentrifuge tube. Sample (total volume of 0.9ml) was then ready for extraction and analysis.

Extraction was performed using Vortex-Genie ® 2, Langford Sonomatic ® 1400 Ultrasonic Bath and Eppendorf ® Minispin centrifuge. Parameters tested included different velocities and times as listed in table 2.2. Conditions for extraction of THC from oil were selected by comparing areas under the peak of THC using constant

HPLC parameters of the previously developed analytical method. HPLC parameters used were mobile phase: phosphate buffer (pH2.5) and acetonitrile (80:20, v/v) at flow rate of 2ml/min, UV detector wavelength of 220nm and column temperature of 25°C.

Table 2.2: Parameters tested to determine best method of extraction of THC from oil.

Technique	**Parameter/s**	**Values**			
Vortex Mixing	Time (minutes)	0.5	1	3	5
Sonication	Time (minutes)	15		20	
Centrifugation	Time (minutes)	15	20	25	
	Velocity (rpm)	3500	4500	6000	10000

2.6. Method Development for the Determination of THC in Oil

Following the development of an efficient and effective method for the extraction of THC from oil, HPLC parameters of previously developed method had to be optimised. Parameters tested included ratio of buffer to acetonitrile in the mobile phase, pH of buffer, injection volume of analyte, flow rate of mobile phase, detection wavelength and temperature of stationary phase.

Analyses were conducted in triplicate to ensure reproducibility and precision of results. In between each HPLC run methanol was injected on its own to clean the stationary phase from oil residue and any other contaminants. Resolution of chromatograph, peak shape and retention time of THC were observed while area under the peak of THC were measured.

Method development was initially conducted using MCT oil (table 2.3 illustrates tested parameters) however this method was not found to be reproducible due to blockage and damage of stationary phase. Method development was continued using Extra Virgin Olive oil (table 2.4 illustrates tested parameters).

Table 2.3: HPLC parameters tested during method development using MCT oil.

Mobile Phase	% Volume Acetonitrile	% Volume Phosphate Buffer	Flow Rate (ml/min)	Injection Volume (µl)	pH	UV Detection Wavelength (nm)	
1	80	20	1.5	20	2.5	220	228
2	70	30	1.5	20	2.5	220	228
3	70	30	2	20	2.5	220	228
4	65	35	2	20	2.5	220	228
5	80	20	0.5	20	2.5	220	228
6	80	20	1.5	20	2.5	220	278
7	80	20	1.5	20	2.5	220	275
8	80	20	1.5	20	2.5	220	280
9	80	20	2	20	2.5	220	278
10	80	20	2	50	2.5	220	278
11	80	20	2	100	2.5	220	278
12	80	20	1.5	100	2.5	275	278
13	80	20	1.5	50	2.5	275	278
14	80	20	1.5	20	6	220	228
15	90	10	1	20	6	220	228
16	90	10	1	20	6	275	278
17	85	15	1	20	6	275	278
18	75	25	1	20	6	220	228
19	70	30	1.5	20	6	220	278
20	70	30	1.5	50	6	220	278
21	70	30	1.5	100	6	220	278

Table 2.4: HPLC parameters tested during method development using Extra Virgin Olive oil.

Mobile Phase	% Volume Acetonitrile	% Volume Phosphate Buffer	Flow Rate (ml/min)	Temperature of Column (°C)
1	90	10	1	25
2	90	10	1	30
3	90	10	1	40
4	80	20	1.5	25
5	80	20	0.5	40
6	85	15	1	40
7	85	15	1.5	40

Chapter 3

Results

3.1. Results Overview

Results obtained can be summarised as follows:

1. A table outlining the main parameters, conditions, and instrumentation of different HPLC methods described in literature for the analysis of cannabinoids. These results helped in understanding better different HPLC methods used for the analysis of cannabinoids. Knowledge acquired was an important part in method development.
2. A method for the analysis of THC, CBD and CBN standard solutions in methanol was developed. Optimal chromatogram peak shape, size and retention time were achieved.
3. A method for the extraction of THC from MCT oil was developed followed by determination of THC. This method was not found efficient or reproducible due to column blockage and long elution times of MCT oil.
4. Method development was then continued using Extra Virgin Olive Oil as a carrier oil. The developed method was found to be effective, efficient, and reproducible.

3.2. Literature Review

Table 3.1. illustrates results of literature review for the analysis of THC, CBD and CBN. This was conducted utilising HiDy, Google Scholar and PubMed while using keywords 'HPLC' and 'Cannabinoids' in title (2009-2019). Table 3.1. outlines the main parameters, conditions, and instrumentation of different HPLC methods for the analysis of cannabinoids published in literature.

Table 3.1: HPLC parameters of different published methods.

Author-Date	HPLC Unit	Stationary Phase	Mobile Phase	Injection Volume (µL)	Detection Wavelength (nm)	Retention Time (Min)	Flow Rate (ml/min)	Run time (min)	Comments
Aizpurua et al, 2016	HPLC-MS/MS method. (Infinity liquid 1260 chromatographic system)	Kinetex C18 100A column Dimensions: 150mm x 3mm, i.d. 2.6µm	(a) Milli-Q ® water with 0.1% formic acid (b) Methanol with 0.1% formic acid	10	/	THC=15.5 CBD=12.8 CBN=14.9	0.3	/	Two step gradient elution was used
Brighenti et al, 2017	HPLC-UV/DAD method. (Agilent Technologies modular model 1100 system)	Ascentis Express C18 column Dimensions: 150mm × 3.0mm, i.d. 2.7µm	(a) 0.1% Formic acid in water (b)Acetonitrile in water	3	190–600 nm. However chromatograms were acquired at 210nm for decarboxylated cannabinoids and at 220nm for cannabinoic acids.	/	0.4	15	Two step gradient elution was used

Author-Date	HPLC Unit	Stationary Phase	Mobile Phase	Injection Volume (µL)	Detection Wavelength (nm)	Retention Time (Min)	Flow Rate (ml/min)	Run time (min)	Comments
Chang et al, 2016	HPLC-MS/MS method. (Agilent 1200 series HPLC system)	Supelco Ascentis C18 column Dimensions: 100mm × 2.1mm, i.d. 3µm	(a)Pure water with 0.1% formic acid (b)Acetonitrile with 0.1% formic acid	20	/	THC=13.17 CBD=11.16 CBN=12.40	0.3	20	Two step gradient elution was used.
Ciolino, Ranieri and Taylor, 2018	HPLC-DAD method. (Agilent 1100, 1200, or 1260 HPLC-DAD systems)	MacMod ACE 5 C18-AR analytical columns Dimensions: 250mm x 4.6mm, i.d. 5µm	66:34 Acetonitrile 0.5% is to Acetic Acid No pH adjustment, nominal pH 2.9	25	220 – 307 Overall optimal wavelengths: 220nm and 240nm (240nm preferred due to better resolution) Exception CBN: 285nm	THC=29 CBD=15 CBN=25	1.0	50	/
Cirimele et al, 2014	HPLC-MS/MS method. (Finnigan Surveyor HPLC system coupled with a triple-quadrupole ABSciex 5500 QTRAP instrument)	KinetexTM XB-C18 column. Dimensions: 2.1mm x 100mm, i.d. 2.6µm.	/	/	/	/	/	/	/

Author-Date	HPLC Unit	Stationary Phase	Mobile Phase	Injection Volume (µL)	Detection Wavelength (nm)	Retention Time (Min)	Flow Rate (ml/min)	Run time (min)	Comments
De Backer et al, 2009	HPLC/DAD method. (Hewlett Packard HPLC System)	MS C18 analytical column Dimensions: 2.1mm x 250mm, i.d. 5µm.	(a)Methanol containing 50 mM of ammonium formate (b)Water containing 50 mM of ammonium formate pH adjusted to 5.19	30	220-380	THC=23.5 CBD=16.5 CBN=21.6	0.3	36	Two step gradient elution was used.
Jin et al, 2017	HPLC-DAD method followed by GS-MS. (Agilent 1100 Series)	Agilent Z0RBAX RX-C18 Column. Dimensions: 4.6mm × 150mm, i.d. 3.5µm.	1:1 (v/v), water and acetonitrile.	/	/	/	/	/	/

Author-Date	HPLC Unit	Stationary Phase	Mobile Phase	Injection Volume (µL)	Detection Wavelength (nm)	Retention Time (Min)	Flow Rate (ml/min)	Run time (min)	Comments
Krizman, 2019	HPLC-DAD method. (Finnigan Surveyor HPLC system equipped with a photodiode-array UV–Vis detector)	Luna C18 (2) Dimensions: 150mm × 3mm, i.d. 3µm.	9:31 (*v*/*v*) water and acetonitrile with 0.1% formic acid (v/v) and 10 mM ammonium formate No pH adjustment.	5	275	THC= 6.1 CBD=3.3 CBN=4.8	0.5	8.5	Isocratic mobile phase.
Labutin and Temerdashev, 2015	HPLC-MS/MS method.	Poroshell 120 C18 column Dimensions: 2.1mm × 75mm, i.d. 1.8µm	(a) 0.1% formic acid in water (b)Acetonitrile and 85% formic acid	5	/	/	0.3	/	Two step gradient elution was used
Layton and Reuter, 2015	HPLC using PDA detector. (PerkinElmer AltusTM HPLC system)	PerkinElmer Brownlee Analytical C18 column. Dimensions: 4.6mm x 150mm, i.d. 3.0µm	(a) 0.1% formic acid in water Solvent (b) 0.1% formic acid in acetonitrile	25	210	THC= 6.7 CBD=5.2 CBN=6.0	1.0	8	Two step gradient elution was used.

Author-Date	HPLC Unit	Stationary Phase	Mobile Phase	Injection Volume (µL)	Detection Wavelength (nm)	Retention Time (Min)	Flow Rate (ml/min)	Run time (min)	Comments
Mandrioli et al, 2019	RP-HPLC-UV Method.	Reverse phase C18 column, Nex-Leaf CBX Dimensions: 150mm × 4.6mm, i.d. 2.7µm	(a)Water and 0.085% Phosphoric Acid (b)Acetonitrile and 0.085% Phosphoric Acid	5	220	THC=6.5 CBD=4.1 CBN=5.7	1.6	8	Two step gradient elution was used.
Mwanza et al, 2016	HPLC-MS/MS method. (Agilent 1200 HPLC system)	Reverse phase C18 column, Dikma technologies Dimensions: 50mm × 4.6mm, i.d. 5µm	Acidic mixture of methanol and deionized water. pH 4.5	20	/	/	0.5	/	Gradient elution was used.
Ofitserova and Nerkara, 2017	HPLC method with post column derivatization.	C18 reversed phase column. Dimensions: 150mm x 4.6mm	70:30 Acetonitrile is to sodium phosphate buffer 6mM pH 3.5	/	475	/	1.0	/	/

Author-Date	HPLC Unit	Stationary Phase	Mobile Phase	Injection Volume (µL)	Detection Wavelength (nm)	Retention Time (Min)	Flow Rate (ml/min)	Run time (min)	Comments
Patel, Wene and Fan, 2017	HPLC-DAD system.	Agilent Poroshell 120 SB-C18 column. Dimensions: 3.0mm x 75 mm, i.d. 2.7µm	Ammonium acetate 25mM. pH adjusted to 4.75 with acetic acid	10	235	THC=8.32 CBD=5.55 CBN=7.54	/	10	Method to analyse a large number of samples in a short time.
Protti et al, 2019	HPLC−DAD−MS/MS Method. (Waters Alliance e2695 chromatographic pump system)	Reversed phase Waters Cortecs C18+ column. Dimensions: 100mm × 2.1mm, i.d. 2.7µm	(a) 0.1% FA in Acetonitrile (b) 0.1% formic acid in water	10	/	/	0.3	13	Two step gradient elution was used.

3.3. Determination of THC, CBD and CBN in Methanol

Figure 3.1. illustrates HPLC parameters that resulted in the optimal chromatograph during method development for the analysis of THC, CBD and CBN in methanol in terms of peak shape, size, retention time and reproducibility were as listed in table 3.2.

Table 3.2: Chosen HPLC parameters for the analysis of THC, CBD and CBN in methanol.

Mobile Phase:	Acetonitrile : Buffer 80:20 (v/v)
pH of Buffer:	2.5
Injection Volume of Analyte:	20 µL
Flow Rate of Mobile Phase:	2 ml/min
Detection Wavelength:	220 nm
Temperature of Column:	25 °C
Retention Time of CBD:	3.6 Minutes
Retention Time of CBN:	5.5 Minutes
Retention Time of THC:	6.9 Minutes

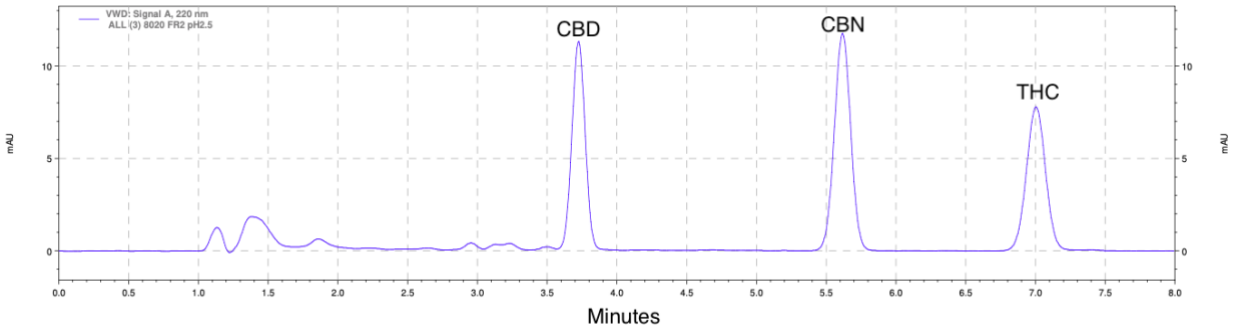


Figure 3.1: Chromatograph produced using chosen parameters for the seperation and determination of THC, CBD and CBN in methanol.

3.4. Extraction and Determination of THC from Oil

Extraction of THC from oil was carried out using parameters described in figure 3.2. These parameters resulted in the extraction of the optimal analyte for analysis. Two immiscible layers were yielded. The supernatant (i.e. the top layer), consisted of THC in methanol, and was separated using a micropipette, refrigerated for 12 hours and centrifuged for 15 minutes at a velocity of 6000 rpm. Analyte was filtered using a 0.45 mm syringe filter and ready for HPLC analysis.

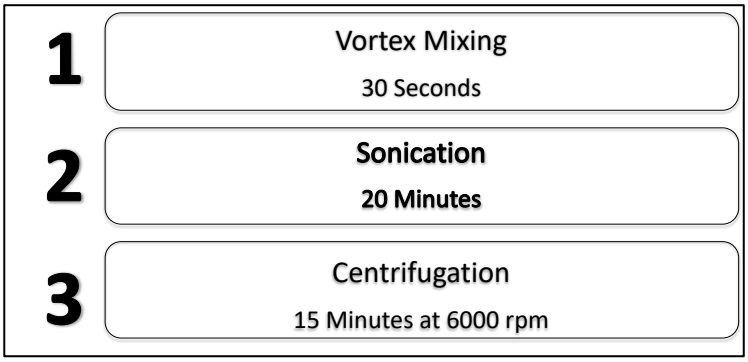


Figure 3.2: Techniques and parameters used to extract THC from oil.

Selected HPLC parameters for determination of THC in MCT oil were as listed in table 3.3.

Table 3.3: Chosen HPLC parameters for the analysis of THC following extraction from MCT oil.

Mobile Phase:	Acetonitrile : Buffer 80:20 (v/v)
pH of Buffer:	2.5
Injection Volume of Analyte:	20 µL
Flow Rate of Mobile Phase:	1.5 ml/min
Detection Wavelength:	220 nm
Temperature of Column:	25 °C
Retention Time of THC:	10.9 Minutes

Figure 3.3 illustrates corresponding chromatograph. Resolution and peak shapes observed were sufficiently good. Average retention times and area under the peaks of Ibuprofen and THC were 3.27 minutes and 10.90 minutes, 736988 mAU and 821169 mAU respectively. Method was not found efficient or reproducible due to column blockage and long elution times of MCT oil.

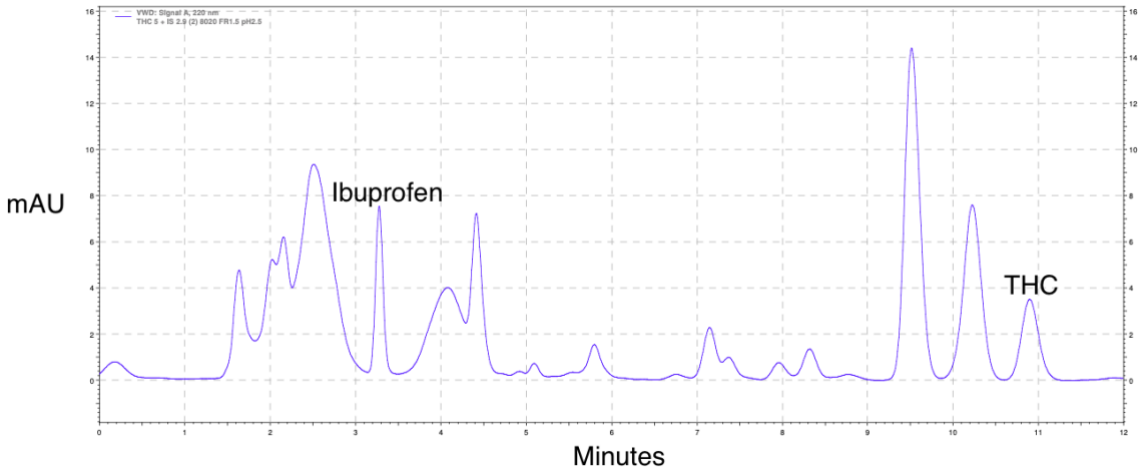


Figure 3.3: Chromatograph determining THC following extraction from MCT oil. (Average retention times; Ibuprofen: 3.27 minutes, THC: 10.90 minutes. Average area under the peaks; Ibuprofen: 736988 mAU and THC: 821169 mAU)

Method development was continued using Extra Virgin Olive oil. HPLC parameters that repeatedly resulted in good THC peak shape and resolution are listed in table 3.4.

Table 3.4: Chosen HPLC parameters for the analysis of THC following extraction from Extra Virgin Olive oil.

Mobile Phase:	Acetonitrile : Buffer 70:30 (v/v)
pH of Buffer:	2.5
Injection Volume of Analyte:	20 µL
Flow Rate of Mobile Phase:	2 ml/min
Detection Wavelength:	220 nm
Temperature of Column:	40 °C
Retention Time of THC:	12.72 Minutes

Figure 3.4 illustrates corresponding chromatograph. Resolution and peak shapes observed were better when compared to results obtained with MCT oil. Average retention times and area under the peaks of Ibuprofen and THC were 2.85 minutes and 12.72 minutes, 715800 mAU and 835432 mAU respectively.

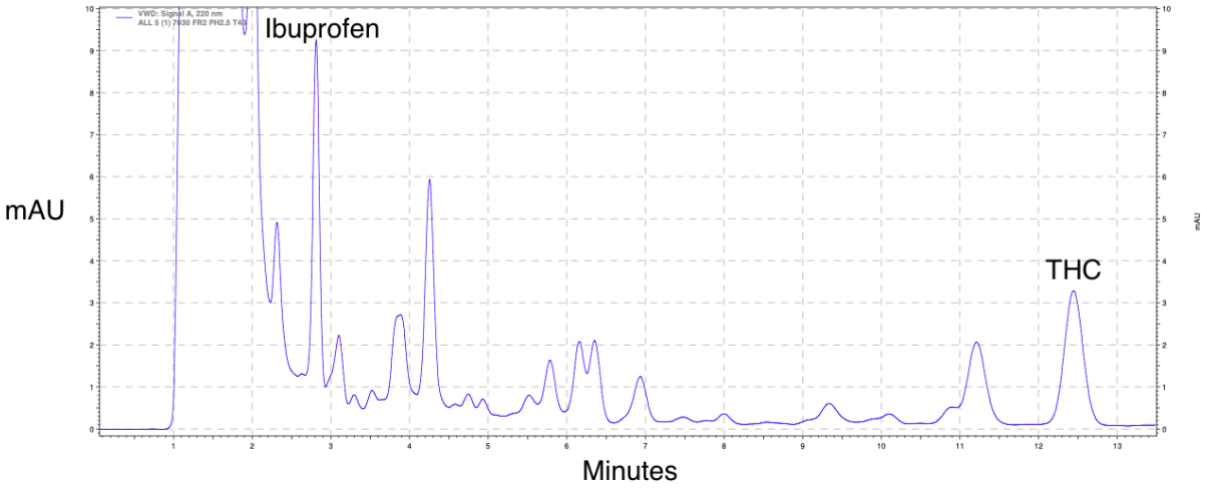


Figure 3.4: Chromatograph determining THC following extraction from Extra Virgin Olive oil. (Average retention times; Ibuprofen: 2.85 minutes, THC: 12.72 minutes. Average area under the peaks; Ibuprofen: 715800 mAU and THC: 835432 mAU)

Chapter 4

Discussion

4.1. Relevance of Developed Method

According to the World Health Organisation cannabis preparations containing CBD with less than 0.2% of THC should be removed from the 1961 single convention on Narcotic Drugs list. It was stated that on comparison with other drugs making part of this list CBD does not have significant psychoactive effects, abusive and dependence properties. In 2020, the European Court of Justice used this recommendation in court for case C-663/18 in which it was concluded that CBD is not classified as a narcotic drug. [26]

Standardised preparations of cannabis which contain mainly CBD are commonly produced in the form of oils. Example of carrier oils include sunflower oil, MCT oil and olive oil. Analysis from an oil as a carrier is challenging due to complexity of mixtures and presence of fatty acids. The challenge can be divided into 1) extraction and separation of the analyte from the oil matrix to prepare sample for analysis 2) choosing the right parameters to be able to produce reproducible results with good resolution and resolved peaks. [27]

The relevance of developing this method of extraction and determination of THC in oil was to overcome these challenges and be able to effectively apply it for the determination of THC from CBD containing oils for medicinal and commercial use.

[26] Cannabidiol (CBD) is not considered a 'narcotic drug' under European law [Internet]. Emcdda.europa.eu. 2021 [cited 26 July 2021]. Available from: https://www.emcdda.europa.eu/news/2020/cannabidiol-cbd-is-not-considered-a-narcotic-drug-under-european-law_en.

[27] Analysis of Vegetable Oils by High Performance Liquid Chromatography Using Evaporative Light Scattering Detection and Normal Phase Eluents [Internet]. www.waters.com. 2008 [cited 26 July 2021]. Available from: https://www.waters.com/waters/library.htm?locale=172&cid=514428&lid=10095599.

4.2. Main Outcomes

In this project a method was first developed using THC, CBD and CBN standard solutions in methanol. Developing this method helped in understanding better method of analysis and how to apply in practice all the principles of HPLC analysis. During this project HPLC parameters published in literature were first observed in the form of a systematic literature review followed by method development. The following was concluded:

HPLC Detector

In table 3.1. one can observe that various HPLC detectors are used to analyse cannabinoids. Detectors are very specific due to their sensitivity. If this specificity is met both a qualitative and a quantitative measure can be obtained (Swartz, 2010). A Diode Array Detector (DAD) was frequently used (De Backer et al, 2009; Layton and Reuter, 2015; Brighenti et al, 2017; Jin et al, 2017; Patel, Wene and Fan, 2017; Ciolino, Ranieri and Taylor, 2018; Krizman, 2019; Protti et al, 2019). Diode Array detectors can scan a range of wavelengths, differently from UV detectors that can only detect a single wavelength at a time. Using a DAD result in a more efficient method in which samples containing different cannabinoids (with different UV absorbance) can be detected at once (Swartz, 2010).

HPLC coupled up with Mass Spectrometry (MS) was another method frequently used in methods described in literature (Cirimele et al, 2014; Labutin and Temerdashev, 2015; Aizpurua et al, 2016; Chang et al, 2016; Mwanza et al, 2016; Protti et al, 2019).

Methods using UV detectors were observed the least. UV detectors are categorised as analyte specific property detectors with the specificity being the precise wavelength at

which a particular analyte absorbs the UV light (Swartz, 2010). A UV detector was used for this project.

A detection wavelength commonly used in methods described in literature was 220nm (De Backer et al, 2009; Brighenti et al, 2017; Ciolino, Ranieri and Taylor, 2018; Mandrioli et al, 2019). During method development the optimal range of UV absorbance was also at 220nm. Superior results and larger area under the peaks of THC, CBD and CBN were observed. It could also be observed that even though the same concentration of THC, CBD and CBN were used in equal amounts area under the peak of THC was relatively smaller. This is because cannabinoids have different UV absorptivity at 220nm (Hazekamp et al, 2005).

pH of Buffer

During method development, on increasing the pH of the buffer, the baseline of the chromatographs became less regular. This did not affect the peak shape or area under the peak of the cannabinoids. This is because the pH was still lower than their relative pKa. Hazekamp et al has also described this trend in pH alteration.

Elution time and order of the compounds reflect their pKa values. pKa of THC, CBD and CBN is greater than 9, therefore at pH lower then 9 they are stable and fully protonated compounds (Vacek et al, 2021).

Mobile Phase

Percentage of acetonitrile used and retention time of cannabinoids was inversely proportionate. The higher percentage of acetonitrile used in the mobile phase the faster the compounds were eluted. Higher percentages of acetonitrile also affected the

resolution of the chromatograph as well as peak sizes. This could be due to the compounds losing their buffering capacity when larger volumes of acetonitrile are used (Vella et al, 2014).

Flow Rate of Mobile Phase

Flow rate mainly affected time of elution and total run time of the method. In the method for determination of THC, CBD and CBN in methanol as the flow rate was doubled from 1 ml/min to 2ml/min total run time decreased by half to a total run time of 7 minutes. This parameter made the method more efficient.

MCT Oil and Extra Virgin Olive Oil

MCT oil and Extra Virgin Olive oil are composed of different fatty acids which have different carbon chain lengths and bond types, hence have different properties. Viscosity, lipophilicity, and stability, as well as elution times of fatty acids present in the carrier oils vary. This was a challenge that was encountered during method development (Gharby et al, 2014).

On running both carrier oils on their own, different peaks of compounds found in each oil were observed. In figure 4.1. it can be observed that MCT oil on its own produces a chromatograph with several peaks which might compromise resolution of analyte peaks. Elution of all fatty acids and compounds found in MCT oil also require a long period of time. These properties made both reproducibility and resolution of chromatograph problematic.

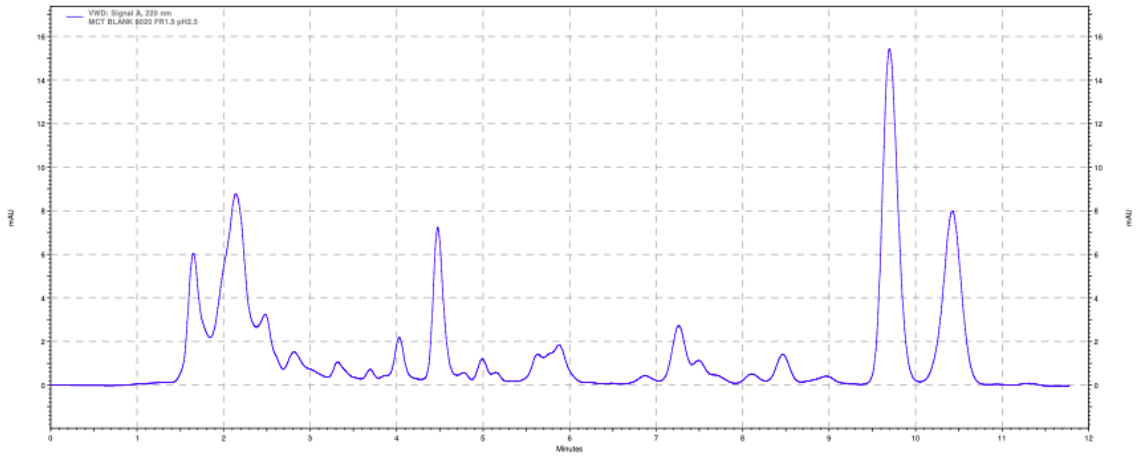


Figure 4.1. Chromatograph of MCT oil.

Figure 4.2. illustrates chromatograph of extra virgin olive oil, one can observe that extra virgin olive oil contains fewer fatty acids on comparison with MCT oil resulting in a cleaner chromatograph and better results in terms of baseline, peak shape, and resolution.

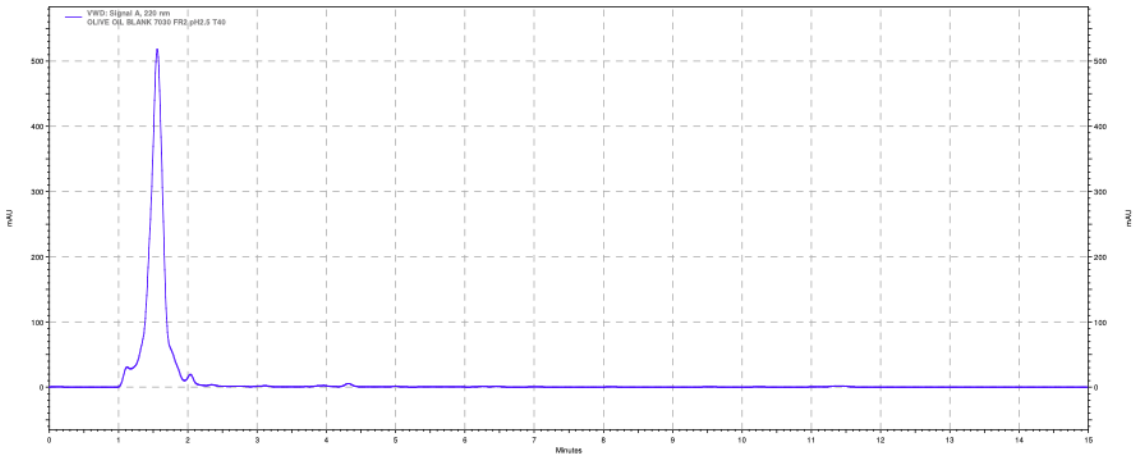


Figure 4.2. Chromatograph of Extra Virgin Olive oil.

Blank injections of the carrier oils were run to ensure selectivity and specificality in identifying analyte of interest (THC). Peaks of the fatty acids found in both carrier oils did not interfere or overlap with the retention time of THC.

On comparing the two developed methods the following was concluded:

1. Extraction and determination of THC from MCT oil was possible but not reproducible mainly due to contamination of the stationary phase of the HPLC system.
2. Area under the peak of THC was greater when Extra Virgin Olive oil was used. This illustrates that greater volumes of THC can be extracted and detected using the developed method and Extra Virgin Olive oil as a carrier.

In general, peaks of fatty acids present in the carrier oil (both MCT and Extra Virgin Olive oil) did not interfere with the peaks of the Ibuprofen standard and the THC, however overall resolution of chromatographs were better when Extra Virgin Olive oil was used as a carrier. This observation however does not imply that a method for the extraction and determination of THC from MCT oil cannot be successfuly developed.

Method and instrumentation of choice for quantitative analysis of cannabinoids is Gas Chromatography (GC). In GC high column temperatures are used, therefore prior to analysis cannabinoids are first decarboxylated. In general GC results in better detection and determination of cannabinoids (Pourseyed et al, 2020).

On comparing to other developed methods described in literature GC as well as HPLC-MS/MS and HPLC-DAD have superior sensitivity and specificity (Pourseyed et al, 2020). Differently, HPLC-UV costs less, is easier to operate and is readily available in most laboratories (Leghissa et al. 2018).

4.3. Conclusion

A method for the extraction and determination of THC from Extra Virgin Olive oil was successfully developed. HPLC analysis coupled to UV detection was used. Parameters that repeatedly resulted in good THC peak shape and resolution were mobile phase: phosphate buffer (pH2.5) and acetonitrile (70:30, v/v) at flow rate of 2ml/min, UV detector wavelength of 220nm and column temperature of 40°C.

The developed method is effective. THC, being the analyte of interest could be successfully identified and determined both qualitatively and quantitatively. During method validation Tejada Rodríguez has also concluded that the level of accuracy and limit of quantification (0.039 μg/mL) has been the lowest published in literature.

Average retention time of THC was 12.72 minutes, as a result total run time of the analysis was less than a total of 13 minutes. Preparing sample for analysis was the most time consuming. In total the extraction process required more than an hour of using different instrumentation and extraction methods (excluding 12-hour refrigerated period). Overall, given the obtained results, the developed method was time efficient.

Reproducibility and repeatability of the developed method was a requirement. All runs were conducted in triplicates to ensure intra-assay precision and regularity of results. During method development Tejada Rodríguez published Relative Standard Deviation results of both intra-day precision (less than 4.5%) and inter-day precision

(less than 6.9%). These results concluded that the developed method has acceptable precision according to the ICH guidelines. [28]

Limitations

The developed method was only applied to extract and determine THC from the standard solution of THC and Extra Virgin Olive oil that was prepared in the laboratory. Other THC containing oils that have Extra Virgin Olive oil as a carrier or other carrier oils were not tested. Therefore, one cannot state that the developed method can be effectively applied for determination of THC from CBD containing oils found in the market for medicinal and commercial use.

Another limitation was that LGC ® standard solution of THC in methanol of concentration 0.1 mg/mL was diluted in the carrier oil to produce a stock solution of concentration 5 μg/ml. Hence, only concentrations of THC below this concentration were tested in the developed method.

Suggestions for Future Work

Developed method can be improved if the above limitations are challenged. Other carrier oils can be used while different parameters for the extraction and determination of THC from MCT oil can be further tested. More cannabinoids can be introduced which makes the method more applicable for the analysis of standardised CBD oils found in the market for medicinal and commercial use that contain a more complex composition. Higher concentrations of THC can also be used to ensure that the method is still effective when higher concentrations are used.

[28] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Validation of analytical procedures: text and methodology Q2 (R1) [Internet]. 2005. [cited 17 August 2021]. Available from: https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf.

References

Aguilar S, Gutiérrez V, Sánchez L, Nougier M. Medicinal cannabis policies and practices around the world [Internet]. International Drug Policy Consortium: London; 2018 [cited 25 April 2020]. Available from: http://fileserver.idpc.net/library/Medicinal%20cannabis%20briefing_ENG_FINAL.PDF.

Aizpurua O, Zarandona I, Ortiz L, Navarro P, Etxebarria N and Usobiaga A. Simultaneous quantification of major cannabinoids and metabolites in human urine and plasma by HPLC-MS/MS and enzyme-alkaline hydrolysis. Drug Testing and Analysis. 2016;9(4):626-633.

Amar BM. Cannabinoids in medicine: A review of their therapeutic potential. Journal of Ethnopharmacology. 2006;105(1-2):1-25.

Ashton C. Biomedical benefits of cannabinoids. Addiction Biology. 1999;4(2):111-126.

Atwood B, Mackie K. CB2: a cannabinoid receptor with an identity crisis. British Journal of Pharmacology. 2010;160(3):467-479.

Benito C, Tolon R, Pazos M, Nunez E, Castillo A, Romero J. Cannabinoid CB2 receptors in human brain inflammation. British Journal of Pharmacology. 2008;153(2):277-285.

Bewley TD, Blickman T, Jelsma M. The rise and decline of cannabis prohibition the history of cannabis in the UN drug control system and options for reform [Internet]. Chapter 1: Amsterdam: Transnational Institute; 2014 [cited 19 February 2019]. Available from: https://www.tni.org/en/publication/the-rise-and-decline-of-cannabis-prohibition.

Brighenti V, Pellati F, Steinbach M, Maran D and Benvenuti S. Development of a new extraction technique and HPLC method for the analysis of non-psychoactive cannabinoids in fibre-type Cannabis sativa L. (hemp). Journal of Pharmaceutical and Biomedical Analysis. 2017;143:228–236.

Brunner T. Marijuana in ancient Greece and Rome? The literary evidence. Journal of Psychedelic Drugs. 1977;9(3):221-225.

Cabral G, Griffin TL. Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation. Expert Reviews in Molecular Medicine. 2009;11(3):1-25.

Cahn R. Cannabis indica resin, part IV. The synthesis of some 2:2-dimethyldibenzopyrans, and confirmation of the structure of cannabinol. Journal of the Chemical Society (Resumed). 1933;1(4):1400-1405.

Carpentier C, Mulligan K, Laniel L, Potter D, Hughes B, Vandam L et al. Cannabis production and markets in Europe [Internet]. Luxembourg: Publication Office of the European Union; 2012 [cited 25 April 2020]. Available from: http://www.emcdda.europa.eu/attachements.cfm/att_166248_EN_web_INSIGHTS_CANNABIS.pdf.

Chandra S, Lata H, ElSohly M, Walker L, Potter D. Cannabis cultivation: Methodological issues for obtaining medical-grade product. Epilepsy & Behavior. 2017;70:302-312.

Chandra S, Lata I, Khan A, ElSohly M. Propagation of elite Cannabis sativa for the production of Δ9-Tetrahydrocannabinol (THC) using 104 biotechnological tools. In: Rajesh A, editor. Medicinal Plant Biotechnology, 1st ed. United Kingdom: The Centre for Agriculture and Bioscience International (CABI); 2010:98-114.

Chang C, Tung C, Tsai C, Wu Y and Hsu M. Determination of cannabinoids in hemp nut products in Taiwan by HPLC-MS/MS coupled with chemometric analysis: quality evaluation and a pilot human study. Drug Testing and Analysis. 2016;9(6):888-897.

Ciolino L, Ranieri T, Taylor A. Commercial cannabis consumer products part 1: GC–MS qualitative analysis of cannabis cannabinoids. Forensic Science International. 2018;289:429-437.

Ciolino L, Ranieri T, Taylor A. Commercial cannabis consumer products part 2: HPLC-DAD quantitative analysis of cannabis cannabinoids. Forensic Science International. 2018;289:438-447.

Cirimele V, Klinger N, Etter M, Duez M, Humbert L, Gaulier J and Allorge, D. O21: Testing for 18 synthetic cannabinoids in hair using HPLC-MS/MS: Method development and validation, its application to authentic samples and preliminary results. Toxicologie Analytique et Clinique. 2014;26(2):13.

Dawson R. Studies in the Egyptian Medical Texts. The Journal of Egyptian Archaeology. 1932;18(3):150-154.

De Backer B, Debrus B, Lebrun P, Theunis L, Dubois N, Decock L, Verstraete A, Hubert P and Charlier C. Innovative development and validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in cannabis plant material. Journal of Chromatography B. 2009; 877(32): 4115-4124.

Devane W, Dysark F, Johnson M, Melvin S, Howlett C. Determination and characterization of a cannabinoid receptor in rat brain. Molecular Pharmacology. 1988;34:605–613.

Devane W, Hanus L, Breuer A, Pertwee R, Stevenson L, Griffin G et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science. 1992;258(5090):1946-1949.

Devi V, Khanam S. Comparative study of different extraction processes for hemp (Cannabis sativa) seed oil considering physical, chemical and industrial-scale economic aspects. Journal of Cleaner Production. 2019;207:645-657.

Dinu R, Popa S, Mot M. The Role of the Endocannabinoid System in the Pathogeny of Type 2 Diabetes. In: Role of the Adipocyte in Development of Type 2 Diabetes. London: United Kingdom; 2011. p. 289-308.

ElSohly AM, Murohy PT, Khan I, Walker WL and Gul W. Analysis of Cannabidiol, Δ9-Tetrahydrocannabinol, and Their Acids in CBD Oil/Hemp Oil Products. Medical Cannabis and Cannabinoids. 2020;3(1):1-13.

Farag S. Cannabinoids production in Cannabis sativa L.: An in vitro approach [dissertation]. Technical University of Dortmund; 2014.

Farag S, Kayser O. The Cannabis Plant: Botanical Aspects. Handbook of Cannabis and Related Pathologies. 2017:4-5.

Gabriel G, Nahas M. Hashish in Islam 9th to 18th century. Bulletin of the New York Academy of Medicine. 1982;58(9);814-831.

Gharby S, Harhar H, Boulbaroud S, Bouzoubaa Z, El Madani N, Chafchaouni I et al. The stability of vegetable oils (sunflower, rapeseed and palm) sold on the Moroccan market at high temperature. International Journal of Chemical and Biochemical Sciences. 2014;5:47-54.

Hadener M, Kamrath M, Weinmann W, Groessl M. High-resolution ion mobility spectrometry for rapid cannabis potency testing. Analytical Chemistry. 2018;90(15):8764-8768.

Haney M, Gunderson E, Rabkin J, Hart C, Vosburg S, Comer S et al. Dronabinol and marijuana in HIV-positive marijuana smokers. Journal of Acquired Immune Deficiency Syndromes. 2007;45(5):545-554.

Hazekamp A, Peltenburg A, Verpoorte R, Giroud C. Chromatographic and spectroscopic data of cannabinoids from cannabis sativa L. Journal of Liquid Chromatography & Related Technologies. 2005;28(15):2361-2382.

Jansen A. The economics of cannabis-cultivation in Europe [Internet]. Cedro-uva.org. 2002 [cited 5 March 2019]. Available from: http://www.cedro-uva.org/lib/jansen.economics.html.

Jin D, Jin S, Yu Y, Lee C and Chen J. Classification of cannabis cultivars marketed in canada for medical purposes by quantification of cannabinoids and terpenes using HPLC-DAD and GC-MS. Journal of Analytical and Bioanalytical Techniques. 2017;08(01).

Jones S, Durant J, Greco AF, Robertone A. A multi-institutional Phase III study of nabilone vs. placebo in chemotherapy-induced nausea and vomiting. Cancer Treatment Reviews. 1982;9:45-48.

Klumpers L, Thacker D. A brief background on cannabis: From plant to medical indications. Journal of AOAC International. 2018;102(2):412-420.

Krizman M. A simplified approach for isocratic HPLC analysis of cannabinoids by fine tuning chromatographic selectivity [Internet]. European Food Research and Technology. 2019 [cited 5 March 2021]. Available from: https://link.springer.com/article/10.1007%2Fs00217-019-03344-7. Subscription required.

Labutin A and Temerdashev A. Nontarget screening of the markers of synthetic cannabinoids in urine using HPLC–MS/MS. Journal of Analytical Chemistry. 2015;70(14):1620-1628.

Layton C and Reuter M. Analysis of Cannabinoids in Hemp Seed Oils by HPLC Using PDA Detection [Internet]. PerkinElmer, Inc. 2015 [cited 5 March 2021]. Available from: https://resources.perkinelmer.com/lab-solutions/resources/docs/APP_Analysis-of-Cannabinoids-in-Hemp-Seed-Oils-by-HPLC-012317_01.pdf.

Leghissa A, Hildenbrand Z, Schug K. A review of methods for the chemical characterization of cannabis natural products. Journal of Separation Science. 2018;41(1):398-415.

Lehmann T, Brenneisen R. High performance liquid chromatographic profiling of cannabis products. Journal of Liquid Chromatography. 1995;18(4):689-700.

Maccarrone M, Bari M, Battista N, Di Rienzo M, Finazzi AA. Endogenous cannabinoids in neuronal and immune cells: toxic effects levels and degradation. Functional Neurology 2001;16(4):53–60.

Mackie K, Hille B. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proceedings of the National Academy of Sciences. 1992;89(9):3825-3829.

Mahlberg P, Kim E. Secretory vesicle formation in glandular trichomes of cannabis sativa (cannabaceae). American Journal of Botany. 1992;79(2):166.

Mandrioli M, Tura M, Scotti S and Gallina TT. Fast Detection of 10 cannabinoids by RP-HPLC-UV method in cannabis sativa L. Molecules. 2019;24(11):2113.

Markova J, Essner U, Akmaz B, Marinelli M, Trompke C, Lentschat A et al. Sativex® as add-on therapy vs. further optimized first-line Antispastics (SAVANT) in resistant multiple sclerosis spasticity: a double-blind, placebo-controlled randomised clinical trial. International Journal of Neuroscience. 2018;129(2):119-128.

Matsuda L, Lolait S, Brownstein M, Young A, Bonner T. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature. 1990;346,561–564.

Mechoulam R, Gaoni Y. The absolute configuration of Δ1-tetrahydrocannabinol, the major active constituent of hashish. Tetrahedron. 1967;12:1109–1111.

Mechoulam R, Hanus L. A historical overview of chemical research on cannabinoids. Chemistry and Physics of Lipids. 2000;108(1-2):1-13.

Mechoulam R, Shvo Y. Hashish. I. The Structure of Cannabidiol. Tetrahedron. 1963;19:2073-2078.

Merfield C. Industrial hemp and its potential for New Zealand [Internet]. European Food Research and Technology. 1999 [cited 5 March 2021]. Available from: https://www.researchgate.net/publication/277111429_Industrial_hemp_and_its_potential_for_New_Zealand. Subscription required.

Merritt J. Glaucoma, hypertantion and marijuana. Journal of the National Medical Association. 1982;74(8):715-716.

Munro S, Thomas K, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature. 1993;365(6441):61-65.

Musto, D.F. The history of the marihuana tax act of 1937. Archives of General Psychiatry. 1972;26:101-108.

Ofitserova M and Nerkar S. Analysis of cannabinoids using HPLC with post-column derivatization. 2017 [cited 5 March 2021]. Available from: https://www.pickeringlabs.com/wp-content/uploads/2021/05/Cannabinoids-in-plant-and-consumer-products-MA243.pdf.

Onaivi E. Commentary: Functional neuronal CB2 cannabinoid receptors in the CNS. Current Neuropharmacology. 2011;9(1):205-208.

Oomah B, Busson M, Godfrey D, Drover J. Characteristics of hemp (Cannabis sativa L.) seed oil. Food Chemistry. 2002;76(1):33-43.

Parsche F, Balabanova S, Pirsig W. Drugs in ancient populations. The Lancet. 1993;341(8843):503.

Pate DW. Chemical ecology of Cannabis. International Hemp Association. 1994;2(29):32-37.

Patel B, Wene D and Fan Z. Qualitative and quantitative measurement of cannabinoids in cannabis using modified HPLC/DAD method. Journal of Pharmaceutical and Biomedical Analysis, 2017;146:15–23.

Pazos M, Nunez E, Benito C, Tolon R, Romero J. Role of the endocannabinoid system in Alzheimer's disease: New perspectives. Life Sciences. 2004;75(16):1907-1915.

Potter D, Clark P, Brown M. Potency of Δ9–THC and other cannabinoids in cannabis in England in 2005: Implications for psychoactivity and pharmacology. Journal of Forensic Sciences. 2008;53(1):90-94.

Pourseyed LM, Torres S, Hooker T, Fowlie C, Young O, Seyfoddin A. Methods for quantification of cannabinoids: a narrative review. Journal of Cannabis Research. 2020;2(1).

Protti M, Brighenti V, Battaglia M, Anceschi L, Pellati F and Mercolini L. Cannabinoids from cannabis sativa L.: A new tool based on HPLC–DAD–MS/MS for a rational use in medicinal chemistry. ACS Medicinal Chemistry Letters. 2019;10(4):539-544.

Radwan M, ElSohly M, Slade D, Ahmed S, Wilson L, El-Alfy A et al. Non-cannabinoid constituents from a high potency Cannabis sativa variety. Phytochemistry. 2008;69(14):2627-2633.

Radwan M, Wanas A, Chandra S, ElSohly M. Natural cannabinoids of cannabis and methods of analysis. In: Chandra S, Lata H, ElSoholy M, editors. Cannabis sativa L - Botany and Biotechnology. New York; 2017. p. 161-182.

Russo E, Jiang H, Li X, Sutton A, Carboni A, del Bianco F et al. Phytochemical and genetic analyses of ancient cannabis from central Asia. Journal of Experimental Botany. 2008;59(15):4171-4182.

Russo E. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. British Journal of Pharmacology. 2011;163(7):1344-1364.

Sinclair J. An introduction to cannabis and the endocannabinoid system. Australian Journal of Herbal Medicine. 2016;28(4):1-12.

Small E, Naraine S. Size matters: evolution of large drug-secreting resin glands in elite pharmaceutical strains of Cannabis sativa (marijuana). Genetic Resources and Crop Evolution. 2015;63(2):349-359.

Steiner A. A universal method for preparing nutrient solutions of a certain desired composition. Plant and Soil. 1961;15(2):134-154.

Stout J, Boubakir Z, Ambrose S, Purves R, Page J. The hexanoyl-CoA precursor for cannabinoid biosynthesis is formed by an acyl-activating enzyme in Cannabis sativa trichomes. The Plant Journal. 2012;71:353-385.

Swartz M. HPLC detectors: A brief review. Journal of Liquid Chromatography & Related Technologies. 2010;33(9-12):1130-1150.

Sytsma K, Morawetz J, Pires J, Nepokroeff M, Conti E, Zjhra M et al. Urticalean rosids: circumscription, rosid ancestry, and phylogenetics based on rbcL, trnL-F, and ndhF sequences. American Journal of Botany. 2002;89(9):1531-1546.

Szymanski D, Lloyd A, Marks M. Progress in the molecular genetic analysis of trichome initiation and morphogenesis in Arabidopsis. Trends in Plant Science. 2000;5(5):214-219.

Tashkin D, Shapiro B, Frank I. Acute effects of smoked marijuana and oral delta-9-tetrahydrocannabinol on specific airway conductance in asthmatic subjects. The American review of respiratory disease. 1974;109(4):420-428.

Tejada Rodríguez E. Aspects of Cannabis in the Laboratory [dissertation]. Degree of Doctorate in Pharmacy: University of Malta; 2021.

Thiele E, Marsh E, Mazurkiewicz BM, Halford J, Gunning B, Devinsky O et al. Cannabidiol in patients with Lennox-Gastaut syndrome: Interim analysis of an open-label extension study. Epilepsia. 2019;60(3):419-428.

Turner C, Elsohly M, Boeren E. Constituents of Cannabis sativa L. XVII. A Review of the Natural Constituents. Journal of Natural Products. 1980;43(2):169-234.

Urasaki Y, Beaumont C, Workman M, Talbot J, Hill D, Le T. Potency assessment of CBD oils by their effects on cell signalling pathways. Nutrients. 2020;12(2):357.

Vacek J, Vostalova J, Papouskova B, Skarupova D, Kos M, Kabelac M et al. Antioxidant function of phytocannabinoids: Molecular basis of their stability and cytoprotective properties under UV-irradiation. Free Radical Biology and Medicine. 2021;164:258-270.

Van De Donk T, Niesters M, Kowal M, Olofsen E, Dahan A, Van Velzen M. An experimental randomized study on the analgesic effects of pharmaceutical-grade cannabis in chronic pain patients with fibromyalgia. PAIN. 2019;160(4):860-869.

Vella J, Mifsud M, Bartolo NS, Ferrito V, Serracino-Inglott A, Azzopardi LM et al. The combined effects of pH and acetonitrile composition on the separation of two lincosamide antibiotics. Asian Journal of Pharmaceutical and Clinical Research. 2014;7;96-100.

Vigil J, Stith S, Diviant J, Brockelman F, Keeling K, Hall B. Effectiveness of Raw, Natural Medical Cannabis Flower for Treating Insomnia under Naturalistic Conditions. Medicines. 2018;5(3):75.

Wise A. An overview of extraction terminology, specific types of extraction methodologies, and clarification on some common misconceptions. Cannabis Science and Technology. 2019; 2(1):20-26.

Wood T, Spivey W, Easterfield T. Cannabinol. Part I. Journal of the Chemical Society, Transections. 1899;75:20-36.

Zivovinovic S, Alder R, Allenspach M and 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. 2018;9(1):1-10.

Zuk-Golaszewska K, Golaszewski J. Cannabis sativa L. - Cultivation and quality of raw material. Journal of Elementology. 2018;23(3):971-984.

List of Publications and Abstracts

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 14, Issue 4, 2021

Online - 2455-3891
Print - 0974-2441

Research Article

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

EVA TEJADA, JANIS VELLA SZIJJ*, MIRIANA CACHIA, PAULINE FALZON, LILLIAN MAZZOPARDI, ANTHONY SERRACINO INGLOTT

Department of Pharmacy, Faculty of Medicine and Surgery, University of Malta, Msida, Malta MSD 2080, Malta.
Email: janis.vella@um.edu.mt

Received: 20 January 2021, Revised and Accepted: 01 March 2021

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.

 DOI: http://dx.doi.org/10.22159/ajpcr.2021v14i4.40844. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

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 r etention 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

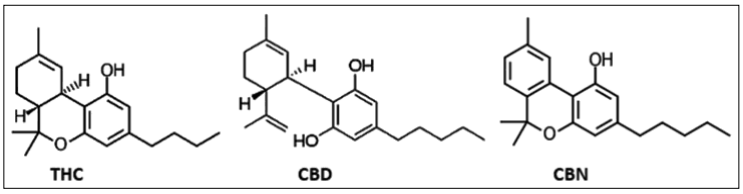


Fig. 1: Molecular structure of tetrahydrocannabinol, cannabidiol, and cannabinol

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 ㎎/mL of THC, CBD, and CBN were prepared in HPLC-grade methanol (Fisher Chemical, Leicestershire, UK). Equal volumes of the 5 ㎎/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 ㎜ 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 ㎖ 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 ㎎/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

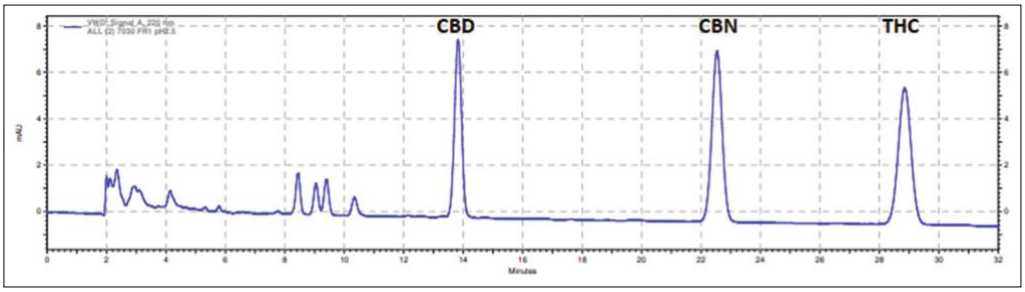


Fig. 2: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (30:70 v/v); detection wavelength 220 nm; flow rate 1 mL/min

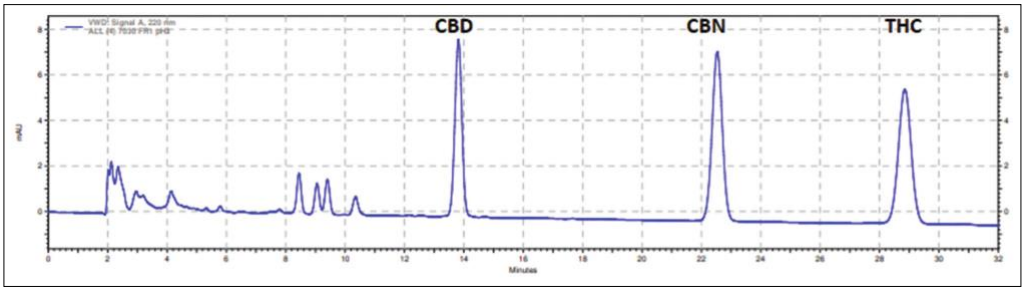


Fig. 3: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (30:70 v/v); detection wavelength 220nm; flow rate 1 mL/min

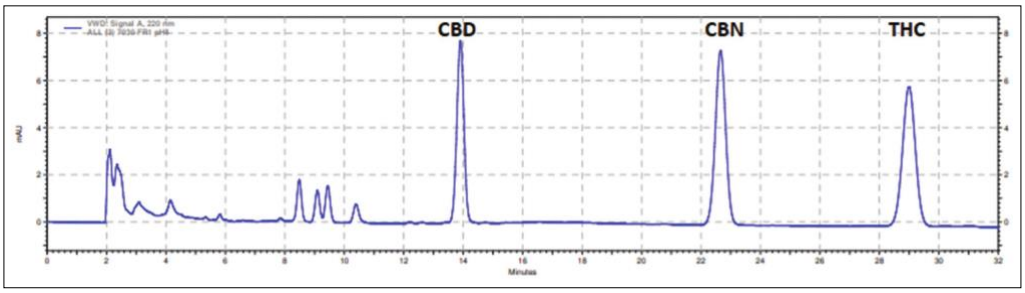


Fig. 4: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (30:70 v/v); detection wavelength 220 nm; flow rate 1 mL/min

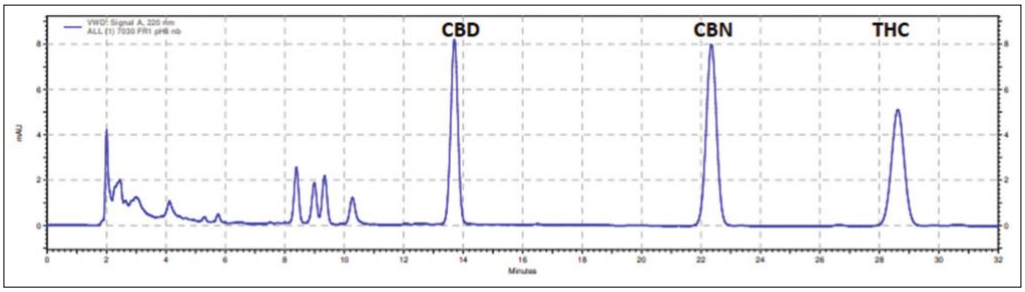


Fig. 5: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (30:70 v/v); detection wavelength 220 nm; flow rate 1 mL/min

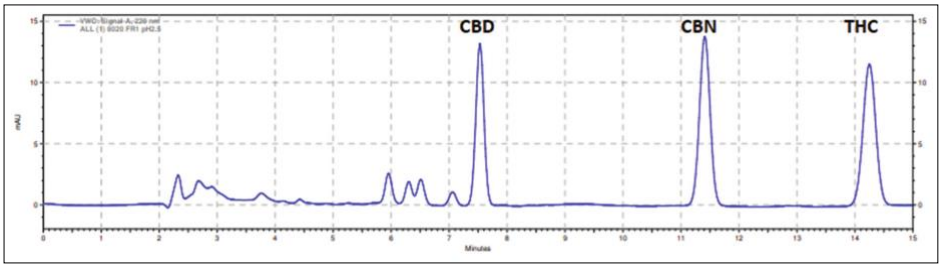


Fig. 6: Chromatogram produced using phosphate buffer (pH2.5) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1mL/min

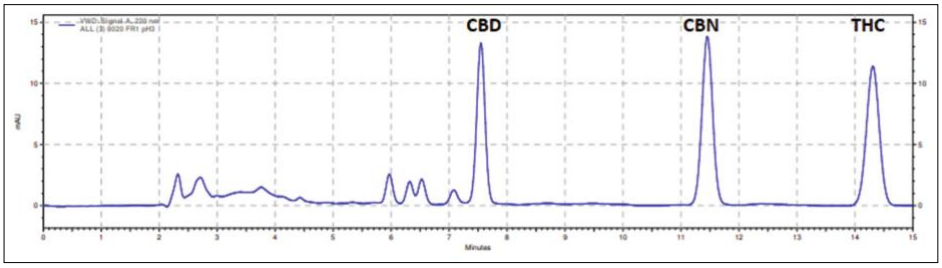


Fig. 7: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (20:80 v/v); detection wavelength 220nm; flow rate 1 mL/min

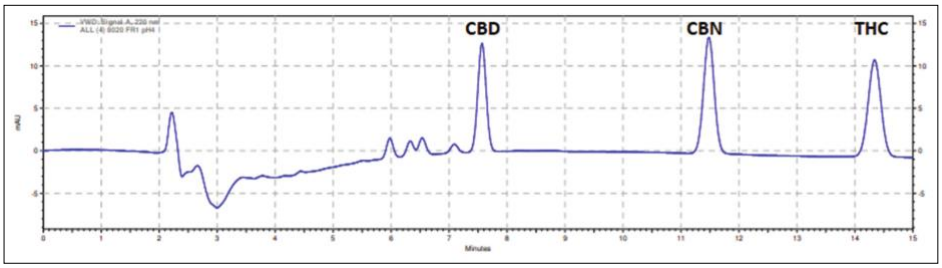


Fig. 8: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (20:80 v/v); detection wavelength 220 nm; flow rate 1 mL/min

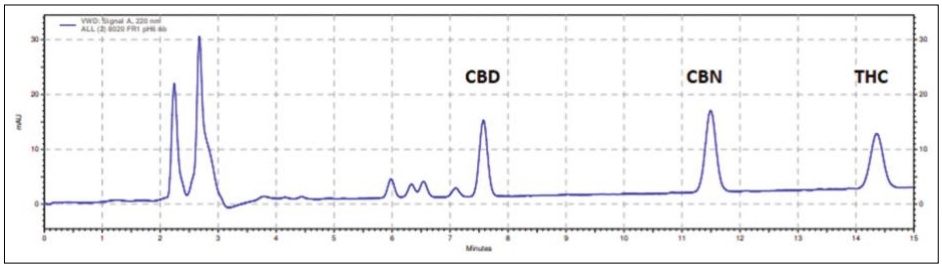


Fig. 9: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (20:80 v/v); detection wavelength 220 nm; flow rate 1 mL/min

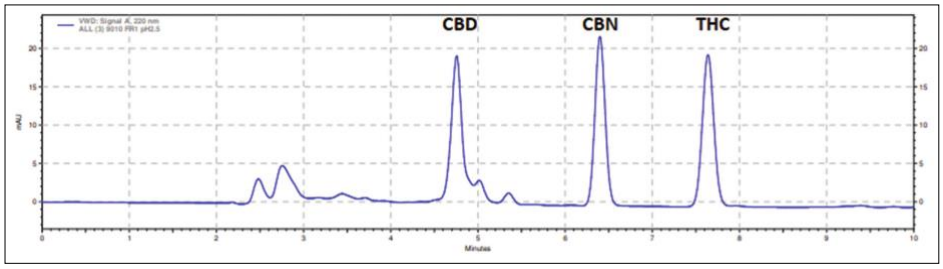


Fig. 10: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (10:90 v/v); detection wavelength 220 nm; flow rate 1 mL/min

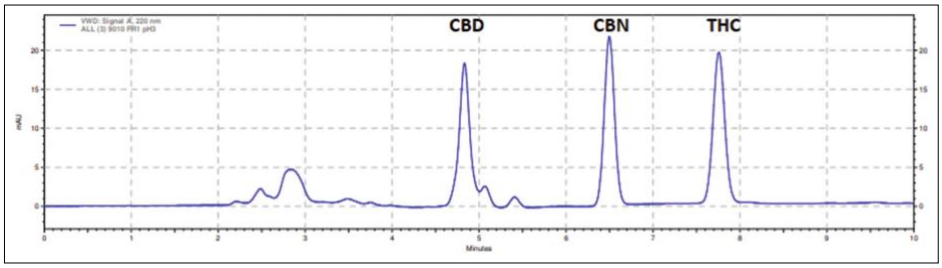


Fig. 11: Chromatogram produced using phosphate buffer (pH 3) and acetonitrile (10:90 v/v); detection wavelength 220 nm; flow rate 1 mL/min

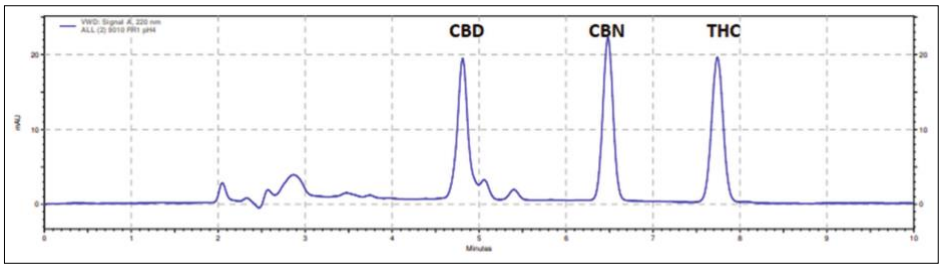


Fig. 12: Chromatogram produced using phosphate buffer (pH 4) and acetonitrile (10:90 v/v); detection wavelength 220 nm; flow rate 1 mL/min

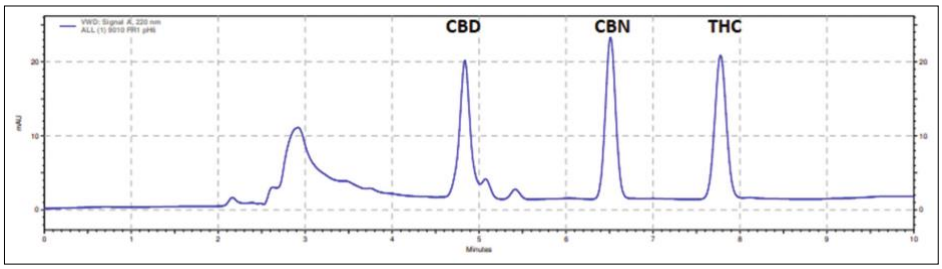


Fig. 13: Chromatogram produced using phosphate buffer (pH 6) and acetonitrile (10:90 v/v); detection wavelength 220 nm; flow rate 1 mL/min

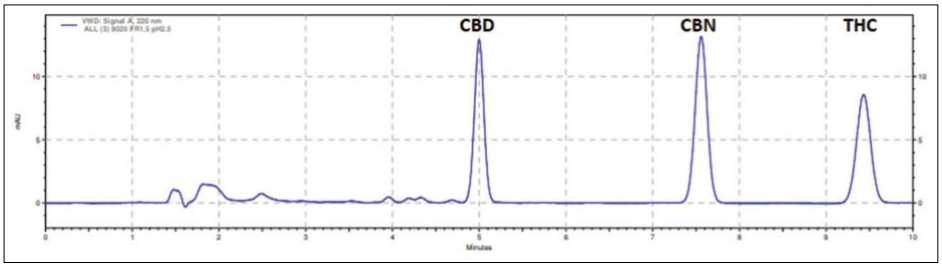


Fig. 14: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220 nm; flow rate 1.5 mL/min

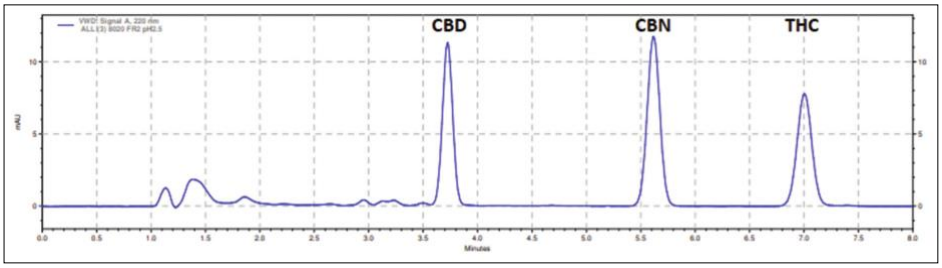


Fig. 15: Chromatogram produced using phosphate buffer (pH 2.5) and acetonitrile (20:80 v/v); detection wavelength 220 nm; flow rate 2 mL/min

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.

REFERENCES

1. Nahar L, Onder A, Sarker SD. A review on the recent advances in HPLC, UHPLC and UPLC analyses of naturally occurring cannabinoids (2010-2019). Phytochem Anal 2020;31:413-57.

2. Nikolin B, Imamović B, Medanhodzić S, Sober M. High performance liquid chromatography in pharmaceutical analyses. Bosn J Basic Med Sci 2004;4:5-9.
3. Rafferty JL, Siepmann JI, Schure MR. Mobile phase effects in reversed-phase liquid chromatography: A comparison of acetonitrile/water and methanol/water solvents as studied by molecular stimulation. J Chromatogr A 2011;1218:2203-13.
4. Wilson RE, Groskreutz SR, Weber SG. Improving the sensitivity, resolution and peak capacity of gradient elution in capillary liquid chromatography with large-volume injections by using temperature-assisted on-column solute focusing. Anal Chem 2016;88:5112-21.
5. Locatelli M, Melucci MD, Carlucci G, Locatelli C. Recent HPLC strategies to improve sensitivity and selectivity for the analysis of complex matrices. Instrum Sci Technol 2012;40:112-37.
6. Vella J, Busuttil F, Bartolo NS, Sammut C, Ferrito V, Serracino-Inglott A, *et al*. A simple HPLC-UV method for the determination of ciprofloxacin in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2015;989:80-5.
7. Venn RF. Principles and Practice of Bio-Analysis. 2nd ed. London: Taylor and Francis; 2005.
8. Muchohi SM, Thuo N, Karisa J, Muturi A, Kokwaro GO, Maitland K. Determination of ciprofloxacin in human plasma using high-performance liquid chromatography coupled with fluorescence detection: Application to a population pharmacokinetics study in children with severe malnutrition. J Chromatogr B 2011;879:146-52.
9. Yang Y. High-Temperature Liquid Chromatography, LcGC North America; 2008. Available from: https://www.researchgate.net/publication/269874595_high-temperature_liquid_chromatography. [Last accessed on 2020 Oct 05].
10. Wenclawiak BW, Sascha G, Tuetenberg T. High-temperature liquid chromatography. Anal Lett 2008;41:1097-105.
11. Hendriks G. Theoretical models in LC based bioanalytical method development. J Pharm Biomed Anal 2009;49:1-10.
12. Marques RM, Schoenmakers PJ. Modelling retention in reversed-phase liquid chromatography as a function of pH and solvent composition. J Chromatogr A 1992;592:157-82.
13. Galaon T, David V. The influence of mobile phase pH on the retention and selectivity of related basic compounds in reversed-phase liquid chromatography. Rev Roum Chim 2012;57:131-40.
14. Mifsud-Buhagiar L, Scorpiniti M, Bartolo NS, Szijj JV, Ferrito V, Serracino-Inglott A, *et al*. Implications of mobile phase composition and pH of the chromatographic separation of amitriptyline and its metabolite nortriptyline. Int J Pharm Pharm Sci 2018;10:132-8.
15. Vella J, Mifsud M, Bartolo NS, Ferrito V, Serracino-Inglott A, Azzopardi LM, *et al*. The combined effects of pH and acetonitrile composition on the separation of two lincosamide antibiotics. Asian J Pharm Clin Res 2014;7:96-100.
16. Ahuja A, Rasmussen H. HPLC Method Development for Pharmaceuticals. Amsterdam: Elsevier, Academic Press; 2007.
17. Sanli S, Sanli N, Alsancak G. Spectrophotometric determination of acidity constants of some macrolides in acetonitrile-water binary mixtures. Acta Chim Slov 2010;57:980-7.
18. Deidda R, Avohou TH, Orlandin S, Baronti R, Pasquini B, Furlanetto S, *et al*. Analytical quality by design: Development and control strategy for a RP-HPLC method implemented in routine to evaluate the cannabinoids content in *Cannabis* olive oil extracts. J Pharm Biomed Anal 2019;166:326-35.
19. de Petrocellis L, Ligresti A, Moriello AS, Allarà M, Bisogno T, Petrosino S, *et al*. Effects of cannabinoids and cannabinoid-enriched *Cannabis* extracts on TRP channels and endocannabinoid metabolic enzymes. Br J Pharmacol 2011;163:1479-94.
20. Baron EP. Comprehensive review of medicinal marijuana, cannabinoids, and therapeutic implications in medicine and headache: What a long strange trip it's been. Headache 2015;55:885-916.
21. Pellati F, Borgonetti V, Bighenti V, Biagi M, Benvenuti S, Corsi S. *Cannabis sativa* L. and nonpsychoactive cannabinoids: Their chemistry and role against oxidative stress, inflammation, and cancer. Biomed Res Int 2018;2018:1691428.
22. Zuardi AW. History of *Cannabis* as a medicine: A review. Braz J Psychiatry 2006;28:153-7.
23. Eliott J, Deirdre D, Clifford T, Coyle D, Potter B, Skidmore B, *et al*. *Cannabis* for pediatric epilepsy: Protocol for a living systematic review. Syst Rev 2018;7:95.
24. Klumpers LE, Thacker DL. A brief background on *Cannabis*: From plant to medical indications. J AOAC Int 2019;12:412-20.
25. Lafaye G, Karila L, Blecha L, Benyamina A. *Cannabis*, cannabinoids and health. Dialogues Clin Neurosci 2017;19:309-16.
26. Zgair A, Wong JC, Sabri A, Fischer PM, Barrett DA, Constantinescu CS, *et al*. Development of a simple and sensitive HPLC-UV method for the simultaneous determination of cannabidiol and Δ9-tetrahydrocannabinol in rat plasma. J Pharm Biomed Anal 2015;114:145-51.
27. Gallo-Molina AC, Castro-Vargas HI, Garzón-Méndez WF, Ramirez JA, Monro ZJ, King JW, *et al*. Extraction, isolation and purification of tetrahydrocannabinol from the *Cannabis sativa* L. plant using supercritical fluid extraction and solid phase extraction. J Supercrit Fluids 2019;146:208-16.
28. Jamwal R, Topletz AR, Ramratnam B, Akhlaghi F. Ultra-high performance liquid chromatography tandem mass-spectrometry for simple and simultaneous quantification of cannabinoids. J Chromatogr B Analyt Technol Biomed Life Sci 2017;1048:10-8.
29. Mazina J, Spiljova A, Vaher M, Kaljurand M, Kulp M. A rapid capillary electrophoresis method with LED-native fluorescence detection for analysis of cannabinoids in oral fluid. Anal Methods 2015;7:7741.
30. ChEMBL; 2018. Available from: https://www.ebi.ac.uk/chembl/compound_report_card/chembl74415. [Last accessed on 2020 Oct 06].
31. Cas MD, Casagni E, Saccardo A, Arnoldi S, Young C, Scotti S, *et al*. The Italian panorama of *Cannabis* light preparation: Determination of cannabinoids by LC-UV. Forensic Sci Int 2020;307:110-3.
32. Elkins AC, Deseo MA, Rochfort S, Ezenieks V, Spangenberg G. Development of a validated method for the qualitative and quantitative analysis of cannabinoids in plant biomass and medicinal *Cannabis* resin extracts obtained by super-critical fluid extraction. J Chromatogr B Analyt Technol Biomed Life Sci 2019;1109:76-83.
33. Burnier C, Esseiva P, Rouseel C. Quantification of THC in *Cannabis* plants by fast-HPLC-DAD: A promising method for routine analyses. Talanta 2019;192:135-41.
34. Mandrioli M, Tura M, Scotti S, Gallina T, Toschi T. Fast detection of 10 cannabinoids by RP-HPLC-UV method in *Cannabis sativa* L. Molecules 2019;24:2113.
35. Bettiol A, Lombardi N, Crescioli G, Maggioni V, Gallo E, Mugelli A, *et al*. Galenic preparations of therapeutic *Cannabis sativa* differ in cannabinoids concentration: A quantitative analysis of variability and possible clinical implications. Front Pharmacol 2018;9:1543.
36. Citti C, Linciano P, Forni F, Vandelli MA, Gigli G, Laganà A, *et al*. Analysis of impurities of cannabidiol from hemp. Isolation, characterization and synthesis of cannabidibutol, the novel cannabidiol butyl analog. J Pharm Biomed Anal 2019;175:112752.
37. Križman M. A simplified approach for isocratic HPLC analysis of cannabinoids by fine tuning chromatographic selectivity. Eur Food Res Technol 2020;2:315-22.
38. Namdar D, Mazuz M, Ion A, Koltai H. Variation in the compositions of cannabinoid and terpenoids in *Cannabis sativa* derived from inflorescence position along the stem and extraction methods. Ind Crops Prod 2018;113:376-82.
39. Hazekamp A, Peltenburg A, Verpoorte R, Giroud C. Chromatographic and spectroscopic data of cannabinoids from *Cannabis sativa* L. J Liq Chromatogr Relat Technol 2005;28:2361-82.
40. United Nations Office on Drugs and Crime. WHO Scheduling Recommendations on *Cannabis* and *Cannabis*-Related Substances; 2020. Available from: https://www.unodc.org/unodc/en/commissions/cnd/mandate_functions/current-scheduling-recommendations.html. [Last accessed on 2020 Oct 26].

Appendix I Ethics Form

UNIQUE FORM ID: 1044:05032019-Miriana Cachia

Ticked one or more self-assessment issues. Submitting to FREC for review.

L-Università ta' Malta

ETHICS & DATA PROTECTION

PART 1: APPLICANT AND PROJECT DETAILS

1. Name and surname: Miriana Cachia
2. Applicant status: UM student
3. Faculty: Medicine and Surgery
4. Department: Pharmacy

If applicable

5. Principal supervisor's name: Prof Anthony Serracino Inglott
6. Co-supervisor's name: Dr Janis Vella
7. Study-unit code: B.Sc Pharmaceutical science. PHR 2019, 3116, 4120, 5123
8. Student number: 1

9. Title of research project: Cultivation of Cannabis
10. Research question/statement & method: Conditions required for indoor cultivation of medicinal cannabis (MC) to obtain a standardised product under Good Manufacturing Practice will be studied. A review of validated methods for the analysis of MC will be carried out. A high-performance liquid chromatography method for determination of active ingredients in medicinal cannabis will be developed and validated.
11. Collection of primary data from human participants?

No (PROCEED TO PART 2. SELF-ASSESSMENT)

12. If applicable, explain:

PART 2: SELF-ASSESSMENT

Human Participants

1. Risk of harm to participants:
2. Physical intervention:
3. Vulnerable participants:
4. Identifiable participants:
5. Sensitive personal data:
6. Human tissue/samples:
7. Withheld info assent/consent:
8. Opt-out at consent/assent:
9. Deception in data generation:
10. Incidental findings:

Unpublished secondary data

11. Was the data collected from human participants?

UNIQUE FORM ID: 1044:05032019-Miriana Cachia
Ticked one or more self-assessment issues. Submitting to FREC for review.

12. Was the data collected from animals?
13. Is written permission from the data controller still to be obtained?

Animals

14. Live animals out of habitat:
15. Live animals, risk of harm:
16. Dead animals, illegal:

General considerations

17. Cooperating institution: Yes or Unsure
18. Risk to researcher/s:
19. Risk to environment:
20. Commercial sensitivity
21. Other potential risks:

Self-assessment outcome: Ticked one or more self-assessment issues. Submitting to FREC for review.

PART 3: DETAILED ASSESSMENT

1. Risk of harm to participants:
2. Physical intervention on participants:
3. Vulnerable participants:
4. Identifiable participants:
5. Sensitive personal data:
6. Collection of human tissue/samples:
7. Withholding information at consent/assent:
8. Opt-out at consent/asset:
9. Deception in data generation:
10. Incidental findings:
11. Unpublished secondary data - human participants :
12. Unpublished secondary data - animals:
13. Unpublished secondary data - no written permission from data controller:
14. Lasting harm to animals out of natural habitat:
15. Risk of harm to live animals :
16. Use of non legal animals/tissue:
17. Permission from cooperating institution: i. Cooperation from the Department of Pharmacy has already been approved.
This includes: 1. The use of the department's lab resources (example: HPLC apparatus). 2. Help and guidance from the staff expertise both from my supervisor and sub supervisor and also from other professors and lecturers within the department.

ii. No approval of another Research Ethics Committee or Data Protection Office is required

UNIQUE FORM ID: 1044:05032019-Miriana Cachia

Ticked one or more self-assessment issues. Submitting to FREC for review.

18. Risk to researcher/team:
19. Risk of harm to environment:
20. Commercial sensitivity:
21. Other issues
    - 21a. Dual use and/or misuse:
    - 21b. Conflict of Interest:
    - 21c. Dual role:
    - 21d. Use research tools:
    - 21e. Collaboration/data/material collection in low/lower-middle income country:
    - 21f. Import/export of records/data/materials/specimens:
    - 21g. Harvest of data from social media:
    - 21h. Other considerations:

PART 4: SUBMISSION

1. Which FREC are you submitting to? : Medicine and Surgery
2. Attachments:
3. Cover note for FREC :
4. Declarations: I hereby confirm having read the University of Malta Research Code of Practice and the University of Malta Research Ethics Review Procedures., I hereby confirm that the answers to the questions above reflect the contents of the research proposal and that the information provided above is truthful., I hereby give consent to the University Research Ethics Committee to process my personal data for the purpose of evaluating my request, audit and other matters related to this application. I understand that I have a right of access to my personal data and to obtain the rectification, erasure or restriction of processing in accordance with data protection law and in particular the General Data Protection Regulation (EU 2016/679, repealing Directive 95/46/EC) and national legislation that implements and further specifies the relevant provisions of said Regulation.
5. Applicant Signature: Miriana Cachia
6. Date of submission: 05032019
7. If applicable data collection start date:
8. E-mail address (Applicant): miriana.cachia.16@um.edu.mt
9. E-mail address (Principal supervisor):
10. Conclude: Proceed to Submission