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Understanding cannabinoid profile variation: from cannabis extraction to final medicinal product.

Ana Rita de Jesus Ribeiro Fortes Morais

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Capita, A. M., Morais, A. F., Dinis-Oliveira, R. J., Ribeiro, C. J. A., & Silva, E. M. P. Analysis of terpenes in cannabis by GC-MS: method development and its application to Cannabis sativa L extracts. IV 1H-TOXRUN International Congress - No Boundaries for Toxicology: One Health, One Society, One Planet. Hotel Cristal, Porto, Portugal (8 and 9 May 2025)

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Resumo

As plantas da espécie *Cannabis sp.* contêm centenas de compostos químicos, incluindo fitocanabinoides, terpenoides e flavonoides. Os canabinoides de maior interesse medicinal são o Δ^9-tetrahidrocanabinol (Δ^9-THC) e o canabidiol (CBD), os quais estão presentes na planta nas suas formas ácidas. A caracterização do perfil de canabinoides em cultivares, amostras biológicas e produtos à base de canabis, através da utilização de metodologias analíticas precisas e rápidas, é fulcral não apenas na indústria farmacêutica, como também no domínio forense, especialmente devido às limitações legais relativas aos canabinoides. No âmbito da toxicologia forense, que auxilia nas questões judiciárias e judiciais através da deteção do uso de substâncias ilícitas, o canabinoide Δ^9-THC é uma das substâncias psicoativas mais controladas a nível mundial.

Esta dissertação pretendeu compreender o processamento da canábis, começando com a pulverização das flores secas e culminando na preparação de extratos finais adequados para integração em produtos de canábis medicinal. O foco principal foi a investigação do protocolo utilizado pela empresa farmacêutica Avextra para cultivares de canábis com predominância de Δ^9-THC, em pequena escala. Especificamente, o trabalho teve como objetivo identificar e avaliar os fatores que influenciam a pulverização, extração, descarboxilação e o refinamento do extrato de canábis através da winterização e da descoloração utilizando carvão ativado.

Para este propósito, foram otimizados e validados dois métodos analíticos de cromatografia líquida de alta eficiência (HPLC) acoplada à deteção por matriz de díodos (DAD). Para caracterizar o perfil de canabinoides na flor, foi otimizada uma corrida de HPLC de 30 minutos para quantificar 14 canabinoides. Um segundo método, mais rápido (12 minutos), foi desenvolvido para avaliar apenas três variações de canabinoides ao longo do processamento da amostra: ácido Δ^9-tetrahidrocanabinólico (THCA), Δ^9-THC e canabinol (CBN). Adicionalmente, foi também desenvolvido um método de extração rápido que combina pulverização (3 minutos) e extração (10 minutos) num único recipiente e equipamento (moinho de bolas) para facilitar as medições analíticas.

As condições de descarboxilação finais estabelecidas foram 120 °C durante 60 minutos, resultando numa formação mínima de CBN (0.13 ± 0.01%) para cultivares com 16% (m/m) de THCA. A extração final por Soxhlet foi realizada a 125 °C durante 2 horas a partir de 1.0 g de flor pulverizada. O processo de refinamento, que incluiu a winterização (-80 °C durante 24

horas) e o tratamento com carvão ativado (50% à temperatura ambiente durante 1 hora), permitiu remover com sucesso compostos lipossolúveis e pigmentos indesejáveis do extrato. Futuramente, a aplicabilidade das variáveis estudadas que afetam os processos de extração e o refinamento de canabis deve ser avaliada em larga escala, com o objetivo de aperfeiçoar o processo industrial. Adicionalmente, o método rápido de preparação de amostras e a metodologia analítica de HPLC-UV desenvolvida para a quantificação de canabinoides podem ser úteis para uma rápida análise de amostras ilícitas em laboratórios forenses.

Palavras-chave: canábis; Δ^9-THC; HPLC-DAD; pulverização; descarboxilação; técnicas de extração; Soxhlet; técnicas de refinamento.

Abstract

Cannabis sp. plants contain hundreds of chemical compounds, including phytocannabinoids, terpenoids, and flavonoids. The cannabinoids of greatest medicinal interest are Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), which are present in the plant in their acid forms. Profiling cannabinoids in plant material, biological samples, and cannabis-based products using accurate and rapid analytical methods is essential not only in the pharmaceutical industry but also in the forensic field, particularly due to legal limits on cannabinoids. In forensic toxicology, which supports judicial contexts by detecting illicit substance use, Δ^9-THC is among the most regulated psychoactive substances globally.

This dissertation tracked the processing of cannabis, beginning with the pulverisation of dry flowers and culminating in the preparation of final extracts suitable for integration into medicinal cannabis products. The primary focus was on investigating the protocol used by the pharmaceutical company Avextra for THC-dominant cannabis cultivars on a small scale. Specifically, it aimed to identify and assess the factors influencing cannabis pulverisation, extraction, decarboxylation, and extract refinement through winterization and decolourisation using activated carbon.

For this purpose, two analytical high-performance liquid chromatography (HPLC) methods coupled with diode array detection (DAD) were optimised and validated. To characterise the cannabinoid profile of the flower, a 30-minute HPLC run was optimised to quantify 14 potential cannabinoids. A second, faster method (12 minutes) was developed to assess only three targeted cannabinoid variations throughout sample processing: Δ^9-tetrahydrocannabinolic acid (THCA), Δ^9-THC, and cannabinol (CBN). Additionally, a rapid extraction method that combines both pulverisation (3 minutes) and extraction (10 minutes) in a single vessel and piece of equipment (ball mill) was also developed to facilitate analytical measurements.

The final decarboxylation conditions established were 120 °C for 60 minutes, resulting in minimal CBN formation (0.13 ± 0.01%) for cultivars with 16% (w/w) THCA. Final Soxhlet extraction was performed at 125 °C for 2 hours on 1.0 g of ground plant material. The refinement process, including winterization (-80 °C for 24 hours) and activated charcoal treatment (50% at room temperature for 1 hour), successfully removed undesirable waxes and pigments from the extract.

In the future, this study on the variables affecting the various processes of cannabis extraction and refinement of the extract should be evaluated for application in scaled-up environments to

potentially enhance the industrial process. Furthermore, the rapid cannabis sample preparation and the HPLC-UV method developed for quantifying cannabinoids can be helpful for screening illicit flower samples in forensic laboratories.

Keywords: cannabis; Δ^9-THC; HPLC-DAD; pulverisation; decarboxylation; extraction techniques; Soxhlet extraction; refinement techniques.

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List of Abbreviations and Acronyms

The following abbreviations and acronyms are used in this thesis:

2-AG	2-arachidonoylglycerol
AA	Arachidonic acid
AC	Activated Charcoal
ACE	Acetone
ACN	Acetonitrile
AEA	Anandamide
AOAC	Association of Official Analytical Collaboration
APT	Olivetolic acid geranyltransferase
Approx	Approximately
BUT	Butane
C. *sativa*	*Cannabis sativa*
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBCA	Cannabichromenic acid
CBC	Cannabichromene
CBCO	Cannabichromeorcin
CBCV	Cannabichromevarin
CBCVA	Cannabichromevarinic acid
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDA-ME	Cannabidiolic acid methyl ester
CBDP	Cannabidiphorol
CBDV	Cannabidivarin
CBDVA	Cannabidivarinic acid
CBE	Cannabielsoin
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBGV	Cannabigerovarin
CBGVA	Cannabigerovarinic acid
CBL	Cannabicyclol

CBLA	Cannabichromenic acid
CBN	Cannabinol
CBNA	Cannabinolic acid
CBT	Cannabitriol
CH2Cl2	Dichloromethane
CH3Cl	Chloromethane
CH5H12	Pentane
CHCl3	Chloroform
CINV	Chemotherapy-induced nausea and vomiting
CNS	Central Nervous System
CO_2	Carbon dioxide
DAD	Diode-Array Detector
DES	Deep Eutectic Solvents
DL	Detection Limit
DM-NS	Dynamic Maceration - non-specified
DM-Shaking	Dynamic Maceration by Shaking
DM-Stirring	Dynamic Maceration by Stirring
DM-Vortexing	Dynamic Maceration by Vortexing
DMAPP	Dimethylallyl pyrophosphate
DME	Dimethyl Ether
DS	Dravet syndrome
ECS	Endocannabinoid System
Eq	Equation
ES	Eutectic Solvent
EtOAc	Ethyl Acetate
EtOH	Ethanol
F	Frequency
FA	Formic acid
FID	Flame Ionisation Detection
GC	Gas Chromatography
(G)	Gradient method
GPP	Geranyl pyrophosphate
H_2O	Water
HBA	Hydrogen Bond Acceptor

HPLC-DAD	High-Performance Liquid Chromatography-Diode Array
HPLC-UV	High-Performance Liquid Chromatography-Ultraviolet
IC	Ice Bath
ICH	International Council for Harmonisation
(I)	Isocratic method
IPP	Isopentenyl pyrophosphate
LC	Liquid Chromatography
LGS	Lennox–Gastaut syndrome
MAE	Microwave-Assisted Extraction
ME	Matrix Effect
MeOH	Methanol
MS	Mass Spectrometry
NR	Not Reported
NSAIDs	Non-steroidal anti-inflammatory drugs
OLA	Olivetolic acid
Ph. Eur.	European Pharmacopoeia
QC	Quality Control
QL	Quantification limit
R&D	Research and Development
R^2	Correlation coefficient
RP-HPLC	Reverse-Phase High-Performance Liquid Chromatography
Rs	Resolution
Rt	Retention time
RSD	Relative standard deviation
RT	Room Temperature
S/N	Signal-to-noise
SD	Standard Deviation
SE-NS	Solvent Extraction - non specified
SFE	Supercritical Fluid Extraction
T	Temperature
TFA	Trifluoracetic acid
THCA-C4	Δ^9-Tetrahydrocannabinolic acid-C4
THCB	Δ^9-Tetrahydrocannabutol
THCH	Δ^9-Tetrahydrocannabihexol

THCP	Δ^9-Tetrahydrocannabiphorol
THCV	Δ^9-Tetrahydrocannabivarin
THCVA	Δ^9-Tetrahydrocannabivarinic acid
TRP	Transient Receptor Potential
TRPA1	Transient Receptor Potential Ankyrin 1
TSC	Tuberous sclerosis complex
UAE	Ultrasound-Assisted Extraction
UC	Unknown Compound
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
Δ^8-THC	Δ^8-Tetrahydrocannabinol
Δ^9-THC/THC	Δ^9-Tetrahydrocannabinol
Δ^9-THCA/THCA	Δ^9-Tetrahydrocannabinolic acid
11-OH-THC	11-hydroxy-Δ^9-tetrahydrocannabinol

1. Introduction

1.1 Historical origins and the present of *Cannabis sativa*

Human civilisation has long sought plants for their unique medicinal potential, with evidence dating back 60,000 years [1]. Cannabis sp**.** plant is a dioecious, annual plant member of the genus Cannabis and the family Cannabaceae, with origins in East Asia, where it was initially used for recreational, religious, and spiritual purposes [2]. The plant's ability to adapt to diverse environmental conditions has contributed to its wide distribution across different geographic areas. Key environmental parameters, including climate and altitude, can significantly affect not only plant growth but also the nutritional composition and organoleptic characteristics of the seeds produced [3].

Before the Common Era, cannabis played a significant role in ancient China, where it was cultivated for both nutritional and textile applications. In India, where the plant held deep religious and spiritual significance, cannabis was widely employed in traditional medicine, particularly for the treatment of infectious diseases. The Indian Hemp Drugs Commission, in 1894, notably referred to it as the "penicillin of Ayurvedic medicine", highlighting its important therapeutic properties [4]. This plant accompanied the development of early human societies. However, in the early 20th century, the isolation and identification of the cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) by Gaoni and Mechoulam, marked a significant milestone in cannabis history [5]. This discovery contributed to cannabis becoming one of the most widely used controlled substances worldwide, raising concerns about the potential side effects of its abuse. In 1971, the Parliament of the United Kingdom classified it as a banned substance for therapeutic use in England [6].

Today, the plant is still cultivated for multiple reasons, including production of cannabis oil for medical treatments and the extraction of fibres for industrial and textile applications [7]. Nevertheless, the legal status of *Cannabis sativa* remains a highly debated topic in science and politics. Advocates for its legalisation — both for medical and recreational use — argue that such measures represent a significant advance in civil rights and a necessary improvement of the justice system. Conversely, detractors view the normalisation of *C. sativa* consumption as a serious sociocultural challenge, prompting substantial discourse about its public health implications [8].

The 2025 European Drug Report, published by the European Monitoring Centre for Drugs and Drug Addiction [9], identifies cannabis as the most widely used illicit substance in Europe, with an estimated 8.4% of European adults being consumers. Current estimates suggest that around 1.5% of adults in the European Union consume cannabis daily or nearly daily, up from 1.3% in 2024. While synthetic and semi-synthetic cannabinoids are on the rise, traditional forms such as herbal cannabis (marijuana) and cannabis resin (hashish) remain the preferred choices among consumers.

1.2 Cannabinoids

1.2.1 Phytocannabinoids

The *Cannabis* plant contains hundreds of different classes of compounds, including cannabinoids, terpenoids, flavonoids, alkaloids, and others. Terpenes constitute the largest group of phytochemicals found in the plant and are responsible for the characteristic aromas and flavours associated with various *Cannabis* strains. Both terpenes and cannabinoids are important chemotaxonomic markers for the genus and are recognised as key physiologically active secondary metabolites [10]. Phytocannabinoids comprise a diverse group of closely related chemical compounds, each characterised by a distinct 21-carbon carbocyclic structure and by specific pharmacological properties [11].

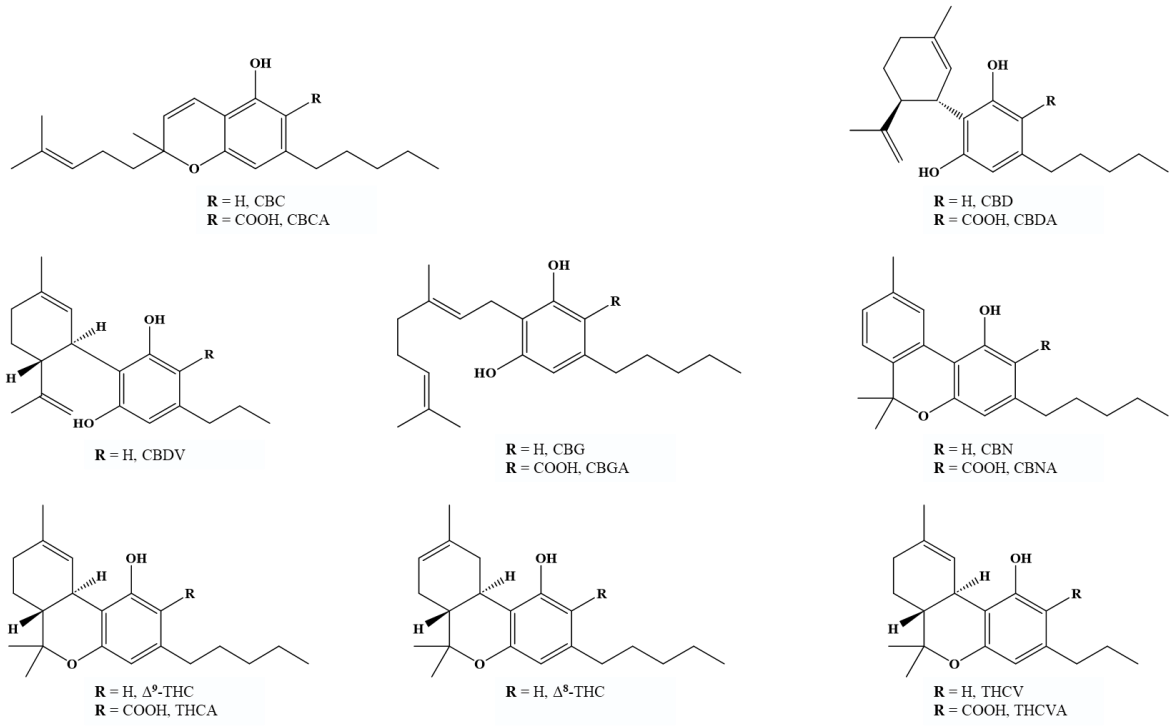


Figure 1. Chemical structures of acid and neutral cannabinoids.

These compounds are primarily synthesised in the secretory cavity of glandular trichomes, epidermal glandular protuberances covering the leaves, bracts, stems and particularly the inflorescences of female plants. These metabolites are involved in defence and in interaction with herbivores [12]. No glandular trichomes are found on the root surfaces; therefore, root tissue therefore does not accumulate phytocannabinoids [13]. The cannabinoid profile and concentration in *Cannabis* plants can differ significantly between varieties and even in the same plant. This variability is influenced by several factors, including cultivation methods, environmental conditions, the season in which cultivation occurs, and mineral nutrients [14,15]. The *Cannabis* plant comprises two primary subspecies: *Cannabis indica* Lam. and *Cannabis sativa* L. They can be differentiated by chemical composition: the indica subspecies typically contains a higher concentration of cannabidiol (CBD), whereas sativa plants are generally characterised by THC dominance. Because of THC's psychoactive effects, consumers often prefer *Cannabis sativa* [16]. The biosynthetic pathway of cannabinoids begins with olivetolic acid (OLA), a benzene ring compound with a carboxyl group, which is synthesised from hexanoyl-CoA (Fig. 2). OLA is converted into cannabigerolic acid (CBGA), reacting with geranyl pyrophosphate (GPP), through the action of olivetolic acid geranyltransferase (APT). GPP is synthesised by the condensation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), catalysed by geranyl pyrophosphate synthase. Once synthesised, CBGA is enzymatically converted into Δ^9-tetrahydrocannabinolic acid (THCA/Δ^9-THCA) and cannabidiolic acid (CBDA) by THCA synthase and CBDA synthase, respectively [17]. Δ^9-THC and CBD, neutral compounds formed by non-enzymatic and organic reaction named decarboxylation, are the cannabinoids of greatest regulatory concern worldwide [18]. Decarboxylation converts acidic cannabinoids into their neutral forms, with the replacement of an aromatic carboxylic acid group with a hydrogen atom. There are several circumstances where acidic cannabinoids might undergo decarboxylation, discussing them in the logical time progression of *Cannabis* plant growth, harvesting/processing, storage, and human consumption [19]. Figure 2 illustrates only two pairs of acidic and neutral cannabinoids, but several other compounds have also been isolated from *Cannabis* (e.g., Fig. 1).

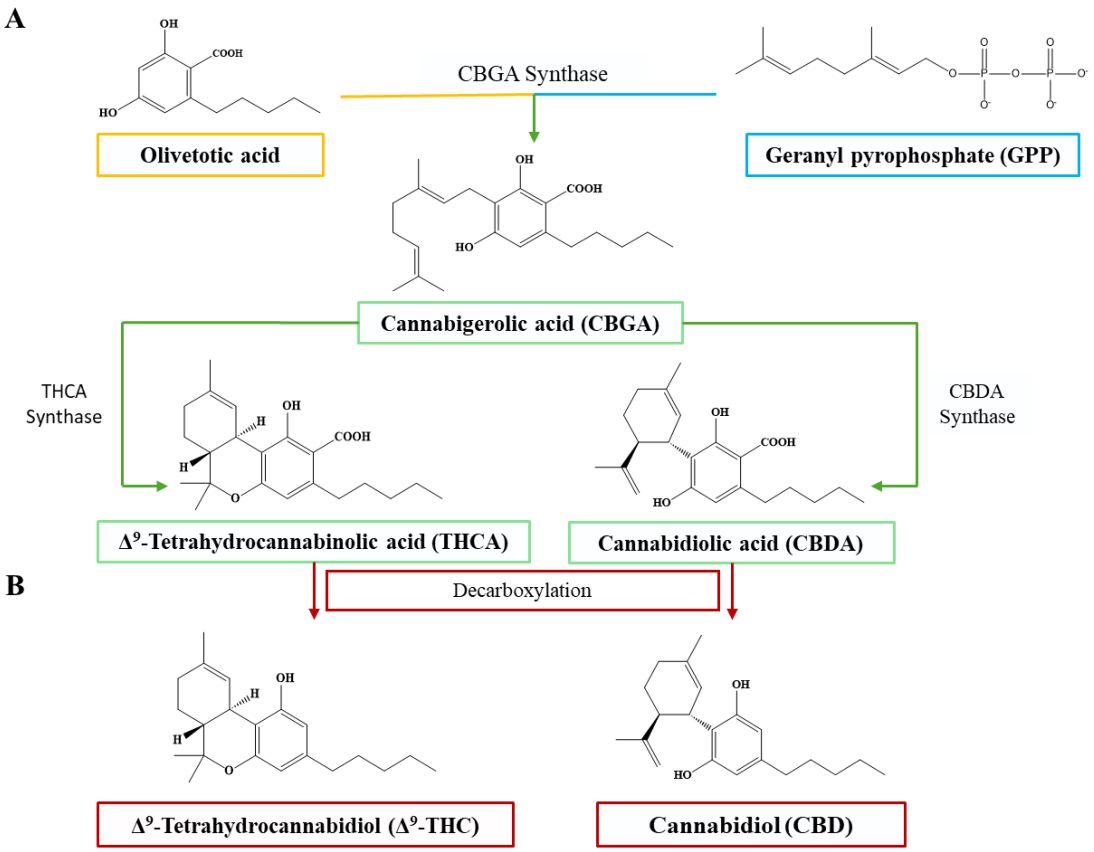


Figure 2. (A) The biosynthetic pathways of cannabinoids, specifically Δ^9-tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), (B) Highlighted in red is the decarboxylation process that yield Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD).

The ECS comprises a network of receptors, enzymes, and ligands that are essential in regulating various physiological processes, including pain perception, appetite, mood, memory, motility, and immune function [20]. In the human body, cannabinoids interact with the endocannabinoid system (ECS). These compounds exhibit marked affinity for the endocannabinoid receptors CB1 and CB2, which belong to the larger family of G protein-coupled receptors. CB1 receptor, discovered in 1990, is predominantly expressed in the central nervous system (CNS), and is mainly located at the terminals of central and peripheral neurons. CB2 receptor is primarily found in immune cells and is much less abundant in the brain than the CB1 receptor. Mainly expressed in the spleen, tonsils, and thymus tissues, CB2 plays important roles in the production and regulation of immune cells [21]. The interaction of cannabinoids with these receptors influences multiple physiological functions, including motor skills, cognition, memory, and analgesic responses [22]. In contrast to Δ^9-THC, CBD exhibits a markedly lower binding affinity for both CB1 and CB2 receptors [23]. Co-administration of CBD and Δ^9-THC has been shown to significantly attenuate various effects induced by Δ^9-THC, including anxiety and tachycardia. This attenuation appear to stem from CBD's antagonic action at CB1, which

contributes to the modulation of THC's psychoactive effects [24]. Non-psychoactive cannabinoids such as cannabigerol (CBG) and Δ^9-tetrahydrocannabivarin (THCV) exhibit low to negligible affinities for CB1 and CB2 receptors, with cannabichromene (CBC) as an agonist of CB2 receptor [25]. Despite this, these compounds play essential roles in modulating biological processes. CBG exhibits significant activity as both an agonist and inhibitor of various transient receptor potential (TRP) channels within the TRP superfamily [26]. CBC has been reported to inhibit endocannabinoids inactivation and activate TRPA1 [27,28], and THCV can act as a partial agonist at the CB1 receptor [29].

In the early decades of *Cannabis* research for therapeutic purposes, the term "cannabinoids" was largely tied to “phytocannabinoids” [30]. Today, the term cannabinoids refers to a diverse array of compounds that interact with cannabinoid receptors, including endogenous ligands synthesised within the body and numerous synthetic analogues [31].

1.2.2 Endocannabinoids and Synthetic cannabinoids

Endocannabinoids are endogenous lipid molecules that selectively binding to and activating different cannabinoid receptors [32]. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are two well-known endogenous ligands derived from arachidonic acid (AA) [33]. These endocannabinoids are agonists of CB_1 and CB_2 receptors [34]. 2-AG is mainly involved in broad physiological functions like synaptic plasticity and neuroprotection, supporting homeostasis, development, and adaptive behaviour. AEA is important in the modulation of functions such as learning and memory. [35].

Ongoing research into the endocannabinoid system has inspired medicinal chemists to investigate structural modifications of Δ^9-THC to deepen the understanding of its pharmacological properties and improve its therapeutic effectiveness [36]. This line of inquiry has yielded significant advancements in cannabinoid synthesis, exemplified by the development of Dronabinol (Marinol®), a synthetic form of Δ^9-THC, and Nabilone (Cesamet®), a synthetic analogue of Δ^9-THC [37,38]. However, the significant potency exhibited by certain synthetic cannabinoids, such as HU-21 and CP47,497-C8, makes them particularly dangerous for consumers [39].

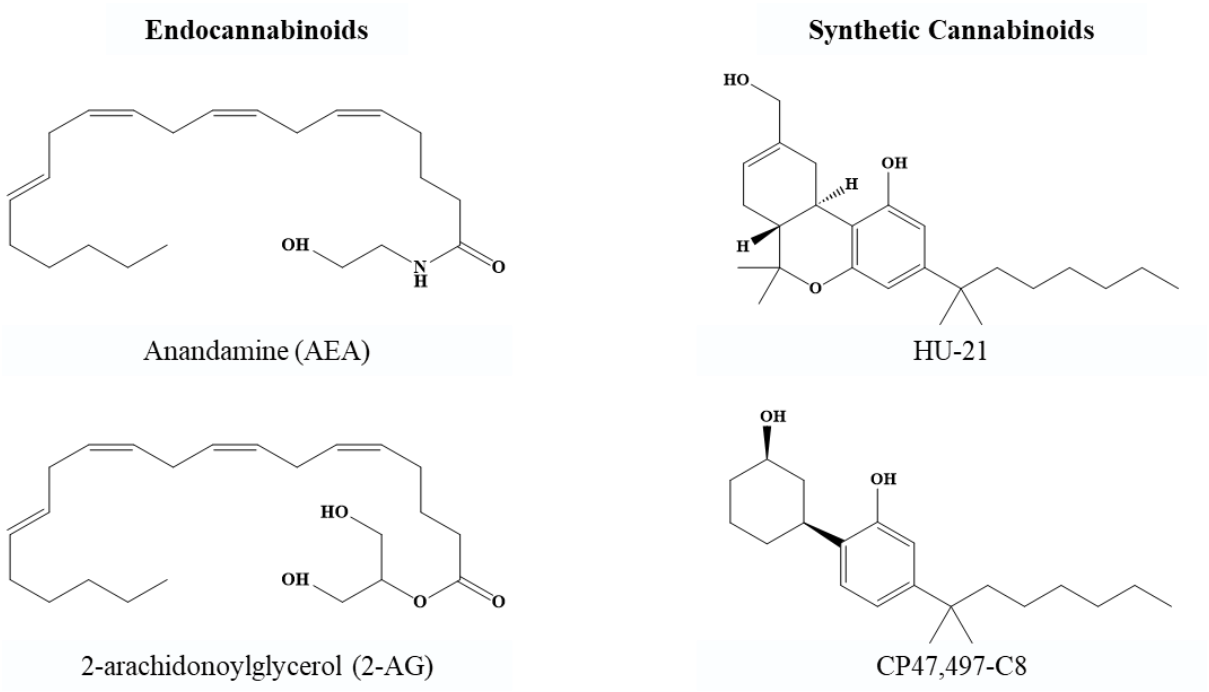


Figure 3. Chemical structures of endocannabinoids – Anandamine (AEA) and 2-arachidonoylglycerol (2-AG) – and synthetic cannabinoids – HU-21 and CP47,497-C8.

1.3 Medicinal benefits and drawbacks of *Cannabis* consumption

The medicinal use of *Cannabis sativa* can be traced back around 5,000 years to the era of Emperor Chen Nung, with the compilation of the first Chinese pharmacopoeia, documenting various herbal medicines for fatigue, rheumatism, and malaria conditions [40]. In recent decades, building on this accumulated knowledge, there has been a strong interest within the scientific community in investigating the pharmacology of these compounds and researching their safety and effectiveness [41]. This interest can be attributed to the therapeutic properties of cannabinoids, which have shown potential in the treatment of multiple diseases [42,43]. For example, the non-psychoactive cannabinoid CBD has emerged as a significant pharmacological agent in the treatment of neurodegenerative disorders, including multiple sclerosis, Parkinson's disease, and Alzheimer's disease [44].

Due to extensive scientific research, cannabis-based medicines are nowadays approved and available in several European countries. Epidiolex® serves as an excellent therapeutic option based on CBD, designed to reduce the effects of numerous conditions such as Lennox–Gastaut syndrome (LGS), Dravet syndrome (DS), and tuberous sclerosis complex (TSC) [45,46]. Neuropathic pain associated with multiple sclerosis, a chronic autoimmune condition impacting the CNS, can be effectively managed with Sativex®, an oromucosal spray featuring a ratio 1:1 formulation of THC and CBD in an ethanolic solution [47]. Despite the generally low oral

bioavailability of both cannabinoids, Sativex® benefits from enhanced absorption characteristics due to its sublingual and oromucosal administration routes. This delivery method optimises the pharmacokinetic profile, making Sativex® a viable adjunctive therapy for patients experiencing moderate to severe spasticity inadequately addressed by conventional treatments [48]. To alleviate the adverse side effects of anti-cancer treatments - such as pain, anxiety, nausea, loss of appetite, and insomnia - cannabinoids, particularly CBD, have been prescribed to cancer patients [49]. Examples of FDA-approved formulations include Dronabinol (Marinol®) and Nabilone (Cesamet®), which are used to treat chemotherapy-induced nausea and vomiting (CINV) in patients who have not found relief with standard antiemetic therapies [50]. Additionally, the Cannabis plant contains a variety of minor non-psychoactive compounds that may have therapeutic value [51]. Cannabidivarian (CBDV), a homologue compound of CBD, was shown by Hill A.J. et al. [52] to have anticonvulsant potential without impairing normal motor function, in mice and rats. Preclinical studies indicated that CBC possesses significant anti-inflammatory properties, effectively reducing pain and inflammation associated with osteoarthritis in rodent models, while exhibiting a more favourable safety profile compared to traditional non-steroidal anti-inflammatory drugs (NSAIDs). Interestingly, the combination of CBC with Δ^9-THC has been shown to enhance the anti-inflammatory efficacy of both cannabinoids, producing greater effects than when each compound is administered alone [53]. CBG, according to the preclinical trial conducted by Brierley D. et al. [54], in rats, also exhibits significant anti-inflammatory activity and shows potential for neuroprotective applications.

Despite all the benefits, the consumption of *Cannabis* has the potential to stimulate the brain's reward system, which introduces a risk of developing addictive behaviours. [55].

Acute intoxication and chronic exposure to *Cannabis* can lead to notable long-term consequences, while occasional users tend to experience slighter effects. Although acute intoxication typically persists for several hours, studies indicate that Δ^9-THC, being a lipophilic compound, can be retained in adipose tissue. This allows for the gradual release of Δ^9-THC and hydroxy metabolites (e.g.,11-OH-THC) into the bloodstream over an extended period, potentially lasting for months [56]. For chronic users, the duration of exposure is a more critical factor determinant of long-term outcomes than dose or frequency of use. Cognitive functions, particularly memory processes such as verbal learning and recall, are among the most consistently affected areas [57]. Chronic cannabis use has been associated with a range of adverse outcomes, including reduced educational attainment and increased risks to physical health, manifesting as alterations in brain morphology and respiratory and cardiovascular

complications. A decreased motivation to engage in everyday activities is also reported among these consumers and may be related to reduced reward sensitivity [58].

1.4 Extraction and chromatographic approaches for the determination of cannabinoids

1.4.1 Extraction Techniques

The political discussions and legalisation of *Cannabis* consumption have increased interest in medicinal cannabis, creating an urgent demand for accurate analytical methods to identify and quantify cannabinoids in various matrices such as flowers, leaves, and resin. The method should be chosen according to the scope of the investigation [59].

For example, the CBN and Δ^9-THC ratio is a marker for sample stability in order to control the quality of the product [60], and the CBD and Δ^9-THC ratio is of primary importance to understand the origin of the sample [61]. In most countries within the European Union, including Portugal, the threshold value for industrial hemp is ≤0.3% to prevent the cultivation of illicit drug-type *Cannabis* in hemp fields. The maximum level refers to the combined total of Δ^9-THC and THCA, expressed as Δ^9-THC. Regarding the hemp seed oil, for medicinal purposes, the permissible percentage limit is higher, set at 7.5% [62]. On the other hand, due to social problems related to recreational cannabis abuse, different analytical techniques are required to identify cannabinoids or their metabolites in biological matrices, such as urine [63] and hair [64], to assess evidence of drug abuse [65].

Over the years, extraction techniques have advanced significantly, moving from traditional methods like maceration, digestion, and decoction to modern approaches that prioritise automation and green chemistry – reducing the need for solvents and minimising energy consumption [66]. With this evolution, a classification emerged for extraction techniques that allowed them to be subdivided into two designations: “conventional” and “unconventional” [67]. Soxhlet extraction is a classic example of a conventional extraction technique, while modern "green techniques" encompass supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) [68]. When selecting the most suitable extraction technique for cannabinoids, several important factors have to be considered, including the ultimate goal, required sample volume, need for purification processes, stability and physicochemical properties of the compounds, extraction efficiency, and the ecological and economic impacts [69]. For the first time, a monograph on *Cannabis* flower was adopted by the European Pharmacopoeia (Ph. Eur.) Commission which became official in 1 of July 2024 [70].

In the last three years, extraction techniques applied to cannabinoids from plant matrix have been explored [71]. A comprehensive summary of the operational extraction conditions used in studies on Cannabis flower is available in Appendix 1A. This appendix highlights the diverse experimental methodologies employed.

The choice of extraction method is crucial for the efficiency of cannabinoid recovery. In the reviewed literature, ultrasound-assisted extraction (UAE) and dynamic maceration by shaking (DM-Shaking) emerged as the most prevalent techniques. Other methods, including simple maceration, reflux, and hydrodistillation, were also documented (Fig. 4A). UAE was particularly common, appearing in 34 of the 67 assessed articles (entries 34 to 67). The effectiveness of UAE is heavily influenced by operational parameters like frequency and temperature, which are critical for controlling cavitation [72]. Among the studies, the highest reported frequency was 40 kHz (e.g., entries 37, 44, 50, and 54). Conditions characterised by elevated frequencies and temperatures typically resulted in the shortest extraction times (e.g., entries 45, 54, 55, and 63). Finding the right balance between extraction time, operational parameters, and potential degradation of target compounds is essential to maximise extraction efficiency and yield [73]. Most studies identified optimal extraction times within a 10 to 30-minute range (e.g., entries 36, 40 and 41), which markedly contrasts with maceration techniques that required significantly longer extraction durations – entry 1 reports a total extraction time of 4320 minutes (3 days) to achieve effective results.

In the context of dynamic maceration, the second most frequently used extraction method, studies summarised in Appendix 1A (e.g., entries 2, 6, 7, 20, 22, and 23) highlight the necessity of extending extraction duration or implementing multiple extraction cycles to enhance efficiency. Extraction times for these studies varied widely, ranging from 5 to 120 minutes. There is a clear tendency to use one to three extraction cycles, as also shown in the overall analysis of all studies (Fig. 4B), with no single approach being predominant. For instance, Wilson et al. (entry 7) performed dynamic maceration in two 30-minute cycles. While this traditional approach was effective for extracting 11 cannabinoids, the authors noted that the lengthy 70-minute total extraction time was impractical for the application in forensic laboratories. In addition to extended process times, some articles reported using a vortex or other secondary techniques after the main extraction to improve compound recovery. Fernandez et al. (entry 3) combined a 30-minute dynamic maceration with a subsequent 15-minute sonication. This coupled strategy successfully characterised cannabinoids across different chemovars, revealing cannabinol (CBN) concentrations below the limit of quantification and demonstrating the method's effectiveness in minimising sample degradation.

Sample preparation is also a fundamental step in cannabinoid extraction [74]. Most reported studies utilised powdered or ground samples with particle sizes ranging from <0.2 mm to 2.0 mm (Fig. 4C), an approach that enhances extraction efficiency. The importance of pre-extraction drying was also highlighted by Birenboim et al. (entry 14). The authors examined the effects of various drying conditions on the yield of cannabinoids and terpenes from cannabis inflorescences across different cultivars. Their findings showed that distinct chemovars, with varying secondary metabolite profiles and genetic traits, respond differently to drying methods, underscoring the need for specific optimisation of drying conditions for each cultivar. Moreover, the effectiveness of any extraction method is highly dependent on the plant-to-solvent ratio. Studies employing ultrasound-assisted extraction (UAE) (e.g., entries 40, 43, and 48) commonly used high ratios (1:100 to 1:200).

Commonly utilised solvents included ethanol (EtOH) and methanol (MeOH), due to their ability to extract a broad spectrum of compounds. Some studies have also explored binary organic solvent systems (Fig. 4D). Organic solvents are particularly versatile, capable of extracting both hydrophilic and hydrophobic compounds [75].

Regarding the use of innovative techniques, Skala et al. (entry 31) assessed the antimicrobial and antifungal properties of two medicinal cannabis strains using maceration with ethanol and a Dexso extractor with dimethyl ether and butane. The results indicated that the Dexso extraction with dimethyl ether was more efficient at extracting cannabinoids than the traditional ethanol maceration. Furthermore, the ethanol extracts contained higher levels of chlorophyll, suggesting that additional purification steps may be required for maceration-based methods. Despite the high extraction efficiencies of organic solvents, they have significant drawbacks, including toxicity, flammability, and environmental concerns [76]. In this context, Green Chemistry provides a viable framework by promoting the use of safer, more environmentally friendly solvents [77]. Deep eutectic solvents (DES) present a promising alternative. Mastellone G. et al. (entry 54) developed a simple ultrasound-assisted dispersive solid-liquid microextraction technique for determining phytochemicals in hemp. The study compared two types of DES: one based on [Ch+] [Br-]-modified salts (N16) and another on natural compounds (ML). The N16 solvent yielded superior results for extracting cannabidiol (CBD) and cannabidiolic acid (CBDA) from inflorescences.

These efforts, alongside the preference for solvents such as EtOH and MeOH, and the optimisation of plant-to-solvent ratios and extraction time, reflect a broader goal in the analysed articles. The use of green techniques like UAE collectively underscores the scientific

community's focus on achieving complete, reproducible, and rapid extractions of phytocannabinoids.

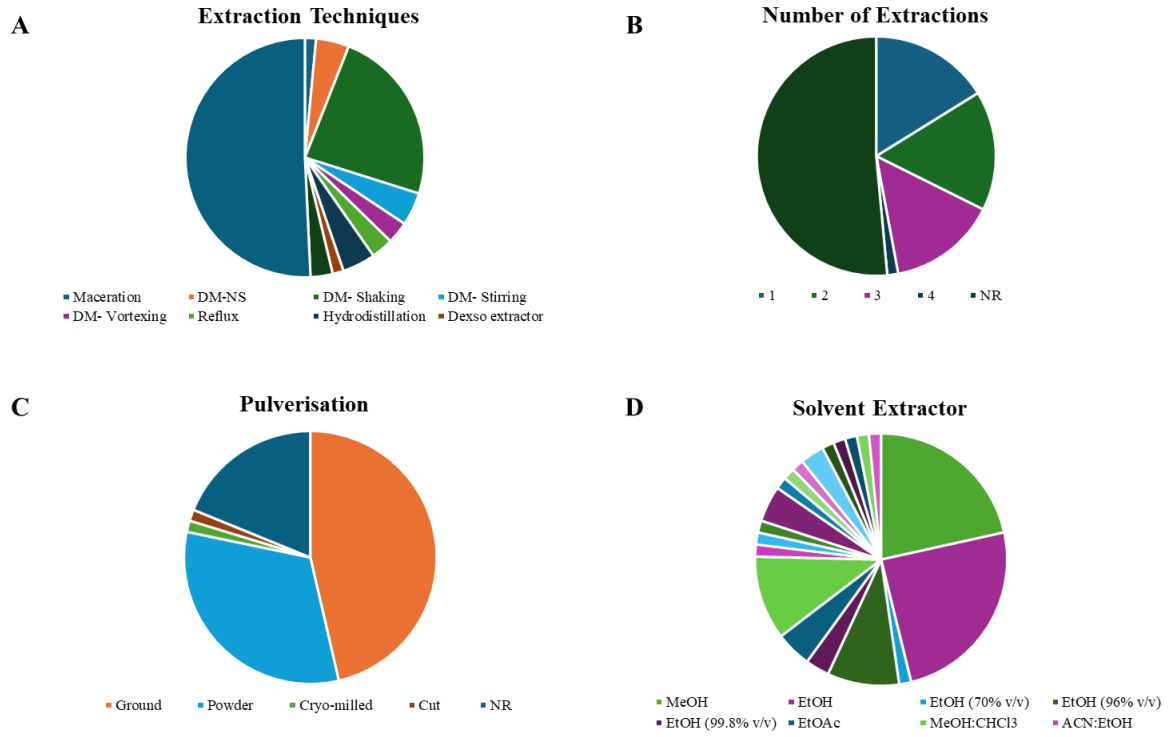


Figure 4. Representation of the conditions used by the reviewed articles (Appendix 1A), relating to (A) extraction techniques, (B) number of extractions, (C) pulverisation and (D) solvent extractor. DM-NS: dynamic maceration-non specified; SE-NS: solvent extraction-non specified; UAE: ultrasound-assisted extraction; T: temperature: F: frequency; NR: not reported; RT: room temperature; IC: ice bath; EtOAc: ethyl acetate; MeOH: methanol; EtOH: ethanol; CHCl3: chloroform. Regarding pulverisation, samples categorised as "ground" had a particle size > 1 mm, while those described as "powder" had a particle size of ≤1 mm.

1.4.2 Detection Techniques

Phytocannabinoids offer a unique chemical fingerprint in *Cannabis*, which can be clearly identified by various analytical methods [78]. The continuous discovery of new phytocannabinoids in *Cannabis sativa* has been gradually increasing in recent years, highlighting an urgent need for the development of new separation methods for their detection and quantification. Gas Chromatography (GC) and Liquid Chromatography (LC) are the most widely used equipment for quantitative analysis. The main objective is to optimise and validate methods that are highly reproducible and easily standardised, allowing for the separation of an extensive number of cannabinoids in a short timeframe. This can be challenging, as many cannabinoids share similar chemical profiles and thus tend to elute within a narrow retention time on the chromatogram [79].

Gas chromatography, generally coupled with mass spectrometry (MS) or flame ionisation detection (FID), allows for the detection of cannabinoids in both plant materials and biological matrices [80]. However, the main drawback associated with this technique is the need for a derivatisation step in the sample preparation process. This step is critical to prevent the decarboxylation of acidic compounds, ensuring that their neutral forms do not interfere with the analysis [81]. In comparison, the HPLC (High-Performance Liquid Chromatography) and UPLC (Ultra-Performance Liquid Chromatography) system allows for the determination of the cannabinoid composition, both neutral and acid forms, without the necessity of the derivatisation step. HPLC is one of the most popular and powerful chromatographic separation techniques that has been routinely used to separate, identify and quantify components from complex matrices, as an example of cannabinoids from *Cannabis* samples. This versatile technique can be coupled with different detection technologies, including mass spectrometry (MS), ultraviolet (UV) and diode-array (DAD) [82]. The efficiency of HPLC separation is associated with numerous parameters regarding the stationary phase, mobile phase, including polarity and flow rate, as well as the inherent characteristics of the sample matrix [83].

The analysis of cannabinoids has seen a significant increase in recent years, with numerous authors highlighting the versatility, sensitivity, and importance of HPLC for this purpose. A summary of the chromatographic conditions for the analysis of 14 or more cannabinoids by LC, published over the last three years, is presented in Table 1.

The type of the stationary phase is one of the main factors in optimising separation outcomes [84,85]. Its particle size directly influences peak width and, consequently, the resolution between compounds. Reverse-phase high-performance liquid chromatography (RP-HPLC), utilising a non-polar stationary phase, is the most common technique for separating a wide range of compounds, including cannabinoids [86]. Different types of HPLC columns are available, but reversed-phase C18 packed columns are the most frequently used for cannabinoid analysis, according to Table 1. In the context of an HPLC-DAD method detailed in entry 10, the detection of seventeen cannabinoids was achieved utilising a diode array detector monitoring at the absorbance maxima of the analysed compounds (190 and 410 nm). This separation was achieved on three different C18 columns – a Shim-Pak and two Phenomenex Synergy – with varying dimensions (75 × 3.0 mm with 2.2 µm and 100 × 3.0 mm with 2.5 µm, respectively), using the same mobile phase. The data in Table 1 indicate a trend toward the utilisation of columns featuring smaller particle diameters (for instance, 1.6 µm, 1.9 µm, and 2.7 µm) along with shorter lengths (such as 100 and 150 mm).

The resolution of cannabinoids by LC can be challenging, but significant improvements can be achieved by modifying the mobile phase. This is based on the principle that chromatographic selectivity is a result of the interaction between the stationary phase and the mobile phase [87]. In RP-HPLC, the mobile phase is typically a mixture of an organic solvent (the organic phase) and water (the aqueous phase). Small amounts of buffers and modifier agents are often added to enhance the separation of compounds, particularly between acidic and neutral forms [88].

Acetonitrile (ACN), methanol (MeOH), and water, typically incorporating a small percentage – usually 0.1% – of formic acid (FA) or trifluoracetic acid (TFA), along with formate buffers, predominantly constitute the mobile phase, as detailed in Table 1. The use of ACN as the organic phase is prevalent across all articles (Fig. 5A), with MeOH serving as a less common alternative (e.g., entry 7). The aqueous phase is consistently acidified with a modifier. FA is the most frequently employed additive (e.g., entries 2, 5 and 8); however, trifluoracetic acid (TFA) and phosphoric acid (PA) were also utilised (Fig. 5B). An HPLC-DAD method described in entry 6 exemplifies an effective approach, developed for the identification and quantification of 26 cannabinoids across six different matrices in a rapid 19-minute analysis. This was accomplished using a gradient mobile phase with an aqueous phase (A) of 0.1% PA in water and an organic phase (B) of 0.1% PA in ACN.

55% of the reviewed articles, according to Table 1, employed gradient elution (G), where the percentage of the organic phase increases during the run. Gradient elution offers superior flexibility and control over selectivity compared to isocratic elution, which is limited to a fixed eluent strength. Specifically, factors such as gradient steepness and initial solvent composition can be precisely controlled to enhance separation outcomes, making it a preferred choice for complex samples [89]

Column temperature and flow rate are also pivotal factors in optimising compound separation in HPLC. A key advantage of elevated temperatures in rapid HPLC is the reduced viscosity of the eluent, which minimizes pressure drop and enhances analyte diffusion [90] Increasing the column temperature is arguably the most effective strategy to diminish peak tailing; however, it is important to note that this approach may shorten the useful lifespan of the column, which presents a significant concern [91]. Furthermore, optimal flow rate is defined as the solvent flow rate that maximises the separation of a specific peak pair or enhances the separation capacity of the overall compounds analysis. Empirical findings indicate that the optimal flow rate determined under isocratic conditions may differ substantially from that which is actually optimal for gradient analysis [92].

Across the studies that reported temperature, values ranged from 25 °C (entry 1) to 54 °C (entry 19) (Fig. 5D). Regarding flow rate, this parameter ranged from 0.3 to 2 mL/min (Fig. 5C). For example, a study (entry 8) successfully optimised the separation of 18 cannabinoids using a multi-step isocratic mobile phase. This method involved a mobile phase composed of three solvents: (A) 0.015% FA in water and (B) a 75/25 (v/v) mixture of MeOH and ACN. Ultraviolet (UV) detection was set at 230 nm for all neutral cannabinoids, with specific exceptions for CBCO (229 and 278 nm), CBN (220 and 284 nm), and CBC (279 nm). The separation protocol utilised a four-step isocratic approach. It started with an initial flow rate of 0.3 mL/min for 17.5 minutes, followed by a gradual increase in the organic phase and flow rate to 0.5 mL/min over 6.5 minutes to effectively isolate the target compounds. This unique four-step optimisation significantly improved the resolution of late-eluting phytocannabinoids, such as CBC, CBNA, CBT, THCA, CBLA, and CBCA.

In absorbance studies, UV and DAD detectors can help to improve specificity in cannabinoid analysis, as acidic and neutral cannabinoids have distinct absorption spectra [93]. This difference is based on the absorption of the chromophore in the phenolic ring, as a common structural element among the cannabinoids (see Figure 1). The length of the alkyl sidechain does not affect the absorbance. Changes in the non-phenolic part of the cannabinoids only has influence on the absorbance, when it implies the formation of another aromatic ring (CBN and CBNA) or a conjugated double bond (CBC and CBCA) [94].

The utilisation of ultraviolet (UV) detection for cannabinoid analysis is prevalent; however, its efficacy and specificity are contingent upon the optimisation of absorption wavelengths, which differ among various classes of cannabinoids. A recent review by Silva E. et al. [84], encompassing publications from 2022 to 2024 regarding HPLC in the detection of cannabinoids, highlights the criticality of the optimisation of absorption wavelengths, which differ among various classes of cannabinoids, thereby enhancing both the specificity and sensitivity of the analytical method. In the reported data, neutral cannabinoids (e.g., CBD, CBG, and Δ^9-THC) typically show a single absorption peak around 210 nm. In contrast, acidic cannabinoids (e.g., CBDA and THCA) exhibit a predominant peak near 220 nm, with two smaller peaks at 260–270 nm and 300 nm. Notable exceptions, such as CBN and CBC, have unique absorption characteristics that allow for analysis at higher wavelengths (280–285 nm), along with their acidic counterparts (254–262 nm). In instances where a single wavelength was employed, the wavelengths most frequently used were 220 nm and 228 nm, as many cannabinoids absorb significantly within this range. Nevertheless, the review also identifies

scenarios where elevated wavelengths – such as 269 nm, 275 nm, or 280 nm – were adopted to reduce interference from co-eluting compounds like terpenes or to enhance detection sensitivity.

The wavelength spectra outlined in Table 1 align closely with these findings. Most studies utilized wavelengths between 220 nm and 285 nm, a range where the majority of cannabinoids, including acidic and neutral forms, exhibit significant absorbance.

The observed variability in the data of Table 1 for optimisation of chromatographic conditions – such as the incorporation of small-particle columns, selection of suitable mobile phase, absorbance spectra and the regulation of flow rates and temperatures – underscores the necessity for a case-by-case approach, according to the analysed matrix and analytical objective.

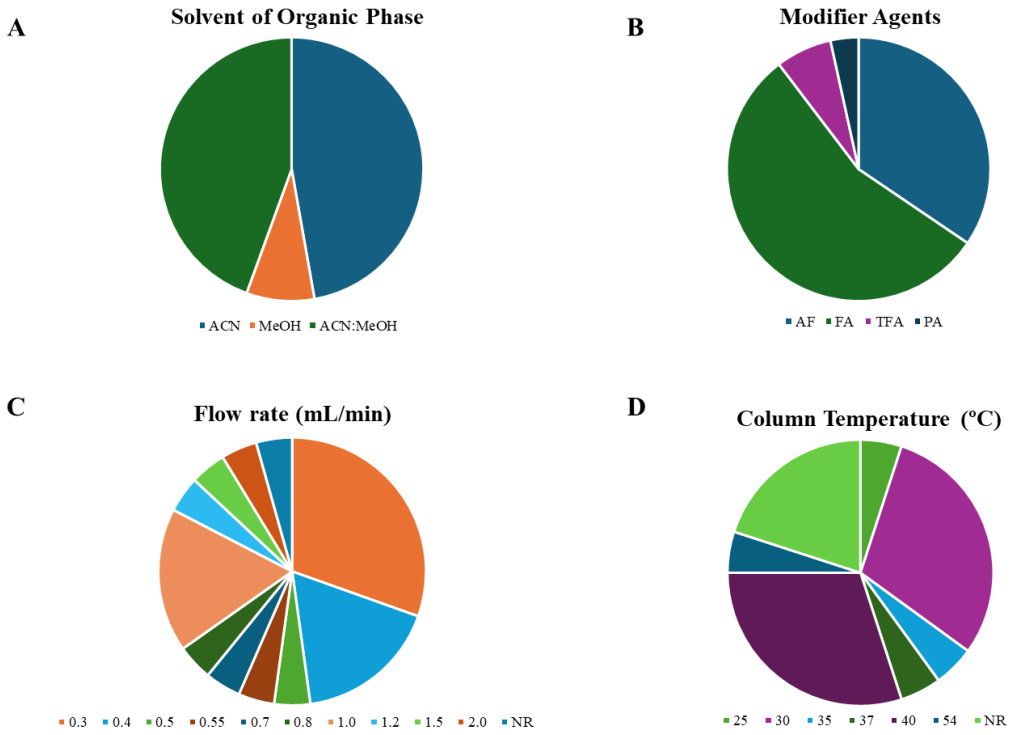


Figure 5. Representation of the HPLC conditions used in the reviewed articles (Table 1), relating to the (A) solvent of the organic phase, (B) modifier agents, (C) flow rate (mL/min) and (D) column temperature (ºC). NR: not reported; MeOH: methanol; ACN: acetonitrile.

Table 1. Summary of HPLC conditions used for chromatographic separation.

Entry	Method	Mobile phase			Stationary phase (column)	Run time (min)	Flow rate (mL/min)	Temperature (°C)	Injection Volume (µL)	UV detection (nm)	Cannabinoids Analysed	Ref
		Aqueous phase (A)	Organic phase (B)	*I* [a] (A:B ratio) / *G* [b] (%B)								
1	HPLC-DAD	AF 1.0mM (pH: 3.53)	ACN	Mer70-99% ***(G)***	Hypersil C18 (150 × 4.6 mm, 3µm)	18	1.0	25	10	NR	14: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBN, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[95]
2	UHPLC-DAD	AF 5mM-0.1%FA	ACN-0.1%FA	25:75 ***(I)***	Raptor ARC-18 (100 × 3.0 mm, 1.8 µm)	6	1.0	40	2	228	15: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBN, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[96]
3	HPLC-VWD	AF 0.5mM -0.1%FA	ACN-AF 0.5mM -0.1%FA	25:75 ***(I)***	Restek Raptor ARC18 (150 × 4.6 mm, 2.7µm)	9	1.5	NR	NR	220	16: CBC, CBCA, CBD, CBDA, CBDV CBDVA, CBG, CBGA, CBL CBN, CBNA, Δ^8-THC, Δ^9-THC THCA, THCV, THCVA	[97]
4	HPLC-DAD	AF 20mM-FA(Ph: 2.9)	ACN-0.1%FA	30:70 ***(I)***	Phenomenex Kinetex XB-C18 (150 × 2.1 mm, 1.7 µm)	16	0.3	NR	2	228	16: CBC, CBCA, CBD, CBDA, CBDVA, CBG, CBGA, CBL, CBN, CBNA, Δ^8-THC, Δ^9-THC, THCA, THCA-C4, THCV, THCVA	[100]
5	HPLC-DAD	AF 0.1 M-0.1%FA	ACN-AF 0.1 M-0.1%FA	22.5:77.5 ***(I)***	Phenomenex Luna C18 (2) (250 × 3 mm, 3 µm)	21	0.55	37	8	275	17: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBLA, CBN, CBNA, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[98]
6	HPLC-DAD	0.1%PA	ACN-0.1%PA	60-100% ***(G)***	Infinity Lab Poroshell 120 EC-C18 (50 x 3.0 mm, 2.7 µm)	19	NR	NR	1	208, 220, 230, 240	26: CBC, CBCO, CBCV, CBCVA, CBD, CBDA, CBDA-ME, CBDP, CBDV, CBE, CBG, CBGA, CBGQA, CBGV, CBGVA, CBL, CBN, CBT, Δ^8-THC, Δ^9-THC, THCA, THCB, THCH, THCP, THCV, THCVA	[99]

Table 1. *Cont.*

Entry	Method	Mobile phase			Stationary phase (column)	Run time (min)	Flow rate (mL/min)	Temperature (ºC)	Injection Volume (µL)	UV detection (nm)	Cannabinoids Analysed	Ref
		Aqueous phase (A)	Organic phase (B)	*I* [a] (A:B ratio) / *G* [b] (%B)								
7	HPLC-DAD	0.1 %TFA	MeOH	15:85 ***(I)***	Raptor ARC-18 (150 × 2.1 mm, 2.7 µm)	10	0.3	30	3	230, 269	18: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBLA, CBN, CBNA, CBT, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[100]
8	HPLC-DAD	0.015%FA	MeOH: ACN (75:25, v/v)	74.5-80.5% ***(G)***	Restek Raptor ARC-18 (150 × 2.1 mm, 2.7 µm)	32	0.3, 0.5	30	3	230, 262, 271, 284	18: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBLA, CBN, CBNA, CBT, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[101]
9	UHPLC-DAD	0.028%FA (pH:3.6)	ACN	23:73 ***(I)***	Luna Omega Polar C18 (150 × 2.1 mm, 1.6 µm)	15	0.3	30	3	223, 230, 251, 269, 285	15: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBLA, CBN, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[102]
10	HPLC-DAD	0.1%FA	(B) ACN - 0.1%FA (C)MeOH	55%(B),0%(C)-0%(B),100%(C) ***(G)***	Shim-Pak C18 (75 x 3 mm, 2.2 µm); 2 Phenomenex Synergy C18 (100 x 3 mm, 2.5 µm)	88	0.3-0.41	40	5-30	190-410	17: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBLA, CBN, CBNA, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[103]
11	HPLC-DAD	AF 0.5 mM - 0.02%FA (pH: 3.0)	ACN	25:75 ***(I)***	Raptor ARC-18 (150 2.1 mm, 2.7 mm)	20	0.4	30	4	223, 230, 251, 261, 269, 285	20: CBC, CBCA, CBCV, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBLA, CBN, CBNA, CBT, Δ^8-THC, Δ^9-THC, Δ^8-THCA, THCA, THCV, THCVA	[104]
12	HPLC-UV/Vis	0.1%FA	ACN-0.1%FA	25-100% ***(G)***	Bondapak C18 (300 × 3.9 mm)	45	2.0	NR	10	225	14: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBN, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[105]
13	HPLC-DAD	0.1%FA	ACN-0.1%FA	25:75 ***(I)***	Phenomenex Luna C18(2) (250 x 4.6 mm, 3 µm)	30	1.0,1.2	40	10	220	15: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBN, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[106]

Table 1. *Cont.*

Entry	Method	Mobile phase			Stationary phase (column)	Run time (min)	Flow rate (mL/min)	Temperature (ºC)	Injection Volume (µL)	UV detection (nm)	Cannabinoids Analysed	Ref
		Aqueous phase (A)	Organic phase (B)	*I* [a] (A:B ratio) / *G* [b] (%B)								
14	UHPLC-UV	AF 5 mM-0.1%FA	ACN-0.1%FA	70-98% ***(G)***	Ascenti Express C18 (150× 2.1 mm, 2 µm)	8	0.4	30	25	228	17: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBLA, CBN, CBNA, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[107]
15	UHPLC-UV	0.05% FA	ACN-0.05% FA	70–100% ***(G)***	Waters Cortecs UPLC C18 (100 × 2.1 mm, 1.6 µm)	12.5	0.3	35	10	228	17: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBN, CBNA, CBL, CBLA, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[108]
16	HPLC-DAD	0.1% FA	ACN-0.1% FA	70–100% ***(G)***	Phenomenex Luna Omega C18 (150 × 2.1 mm × 1.6 µm)	8	0.4	40	5	214, 280	16: CBC, CBCA, CBD, CBDA, CBDV CBDVA, CBG, CBGA, CBL, CBN, CBNA, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[109]
17	UHPLC-DAD	AF 5 mM–0.1% FA	ACN-0.1% FA	67–95% ***(G)***	Ascentis Express C18 (150 × 3.0 mm, 2.7 µm)	8	1.0	40	5	228	16: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBN, CBNA, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[110]
18	HPLC-DAD	AF 10 mM (pH 3.6)	ACN-0.1% FA	70–100% ***(G)***	InfinityLab Poroshell 120 EC-C18, (100 × 2.1 mm, 1.9 µm)	11	0.8	40	1	NR	16: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBL, CBLA, CBN, CBNA, Δ^9-THC, THCA, THCV, THCVA	[111]
19	HPLC-DAD	0.1%TFA	MeOH-0.1% TFA	79–100% ***(G)***	Waters Cortecs C18 (150 × 4.6 mm, 2.7 µm)	21	0.7	54	NR	226	14: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBN, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[112]
20	HPLC-DAD	AF 2 Mm-0.011% FA (pH 3.6)	ACN	27:73 ***(I)***	Luna Omega Polar C18 (150 × 2.1 mm, 1.6 µm)	18	0.3	30	3	223, 230, 251, 269, 285	15: CBC, CBCA, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBLA, CBN, Δ^8-THC, Δ^9-THC, THCA, THCV, THCVA	[113]

[a] Isocratic method; [b] Gradient method.

2. Aim of the study

This work requires collaboration across various disciplines, including botany, chemistry, pharmaceutical technology, toxicology, and forensic sciences. Improving the efficiency and sustainability of extraction and analytical procedures can lead to increased availability of medicinal cannabis products within the pharmaceutical sector and reduce the time required in the laboratory for the analysis of forensic samples.

This work primarily focuses on analysing extraction protocols, including the classical industrial method—Soxhlet—and the purification process employed by Avextra pharmaceutical company to produce medicinal products containing cannabis extracts. It examines how certain variables affect the extraction and refinement processes, from flower pulverisation to the final cannabis industrial product, particularly their impact on cannabinoid content at each unit operation (e.g., pulverisation, extraction, and decarboxylation). Additionally, this study is expected to provide insights that may lead to protocol recommendations for adoption by the pharmaceutical industry and forensic science laboratories, fostering innovation in extraction and refinement methods while potentially creating more efficient, safer, and environmentally friendly processes.

Chapter 3 describes the optimisation and validation of two analytical HPLC-DAD methods. The CannProVar method A, capable of identifying and quantifying 14 cannabinoids, is used for the characterisation of the flower cannabinoid profile and for comparison between extraction techniques. A second procedure – CannProVar method B – was developed for a faster evaluation of the three key cannabinoids monitored throughout sample processing: THCA, Δ^9-THC, and CBN. Various factors that can impact the procedure are evaluated, including mobile phase solvents and modifiers, gradient, flow rate, column length and temperature, run time, and UV wavelength.

Chapter 4 and 5, describes the optimisation of the decarboxylation and extraction processes, respectively. Since THC-dominant cultivars (Z-face strain) are used in this project, the focus of this decarboxylation study is on the conversion of THCA (the acid form) into the psychoactive Δ^9-THC (the neutral form), as well as on CBN, the byproduct of Δ^9-THC oxidation. Experiments following various temperatures and exposure time in an oven are studied.

Three extractions procedures are studied: (i) Dynamic maceration described in the European Pharmacopoeia cannabis monograph; (ii) Avextra's extraction method – Soxhlet extraction;

and (iii) a ball mill extraction, developed to respond quickly to incoming cannabis samples that require cannabinoid analytical analysis.

Chapter 6 outlines the assessment of two purification steps: winterization, which removes lipids and waxes, and decolourisation using activated charcoal to enhance the extract organoleptic profile by eliminating chlorophyll and other pigments. Studies assessing different percentages of activated charcoal, along with various exposure durations and temperatures, are conducted. The goal of this chapter is to find the best purification conditions that minimise Δ^9-THC loss.

3. Development and validation of an HPLC-DAD method for cannabinoids

3.1 Optimisation of the analytical method – CannProVar method A

Due to the complexity of cannabis extracts, optimisation of chromatographic conditions is essential for accurate detection and quantification of the target compounds. Proper method development improves the separation of cannabinoids and reveals potential interferences, such as co-eluting matrix components, that could compromise analytical results. To establish a method with good sensitivity, selectivity and resolution for 14 cannabinoids (CBC, CBCA, CBD, CBDA, CBDV, CBG, CBGA, CBN, CBNA, THCV, THCVA, Δ^8-THC, Δ^9-THC, THCA), numerous experiments were conducted to optimise critical parameters, including the mobile phase composition, gradient, pH, and flow rate, as well as the length of the chromatographic column, and oven temperature. The research was conducted using an Agilent 1260 Infinity II HPLC-DAD system with Open-Lab software for sample management, data acquisition and data analysis.

3.1.1 Optimising studies using InfinityLab Poroshell 120 EC-C18, 3.0×50 mm, 2.7 µm column

An analytical method for the identification and quantification of 11 commonly targeted cannabinoids found in *Cannabis sativa*, namely CBC, CBD, CBDA, CBDV, CBG, CBGA, CBN, THCV, Δ^8-THC, Δ^9-THC and Δ^9-THCA, using a Poroshell 120 EC-C18, 3.0×50 mm, 2.7 µm column was developed by Agilent [114]. This method, presented in Appendix 2A, served as the basis for optimisation of the analytical procedure in this study, using the same column and equipment. Briefly, the analytical run lasted 9.5 minutes and used a gradient elution between two mobile phases: methanol with 0.05% formic acid (organic phase; 60 to 95%) and water with 0.1% formic acid (aqueous phase). The flow rate was 1 mL/min, and the oven temperature was 50 °C. This analytical method successfully separated 11 cannabinoid standards with good peak resolution. However, since cannabinoids primarily exist in their acidic forms within the flower [6], the present study included three additional cannabinoids (CBCA, CBNA, and THCVA), which revealed insufficient separation when using this Agilent method (Fig. 6A). Preliminary tests were conducted on cannabis cultivar extracts, and the Agilent method also proved insufficient for accurately characterise Avextra's cultivar cannabinoid profile (Fig. 6B). Several issues were immediately highlighted: (i) Δ^9-THC and THCVA, both expected to

be present in Avextra's cultivars, co-eluted; (ii) CBC, CBNA, THCA, and CBCA co-eluted with other matrix compounds; (iii) THCA and CBCA eluted close to the end of the runtime. To address these issues, the first set of modifications adapted the Agilent method to allow for longer runtimes and to explore mobile phases at different pH values.

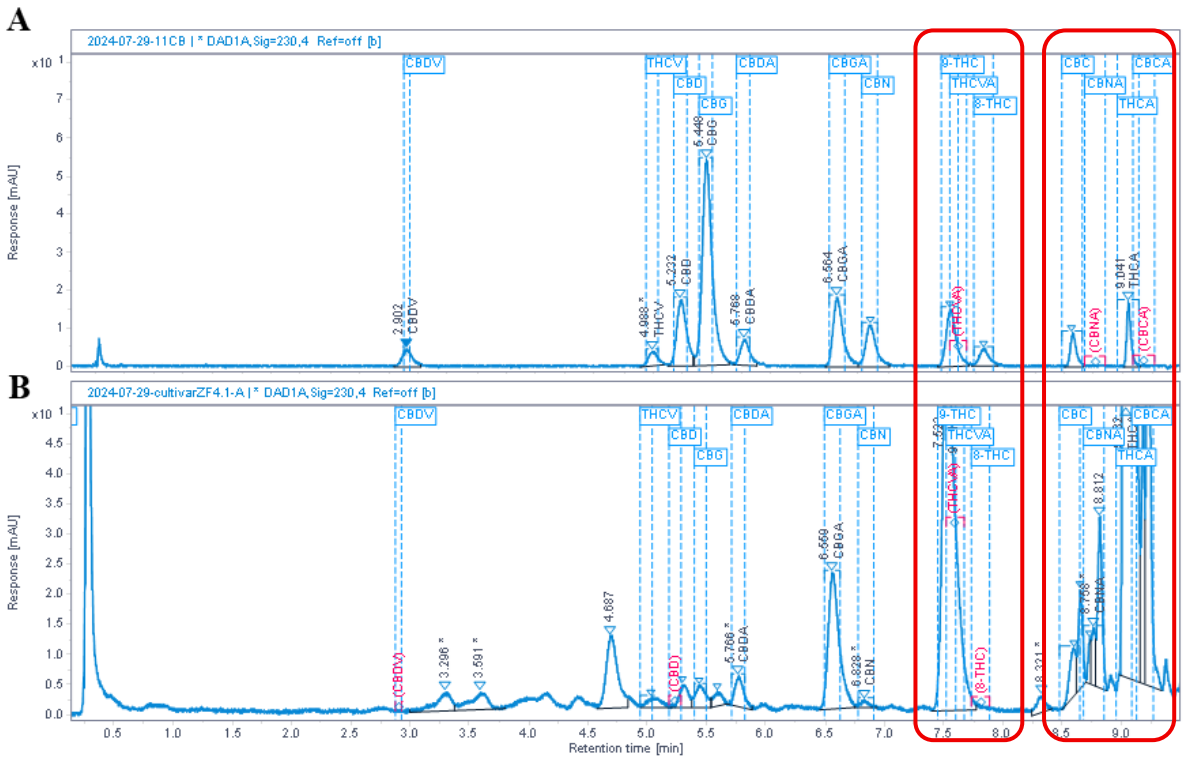


Figure 6. Representative chromatograms, using Agilent method (Appendix 2A), of the (A) 11 standard cannabinoids mixture and (B) Avextra's cultivar.

3.1.1.1 Increasing run time

General strategies to diminish matrix interference recommend adjusting the retention times of the analytes of interest to avoid the front solvent and the end of the chromatographic gradient [115,116]. Initially, with all other parameters kept constant, the runtime was extended to allow for a slower increase in the percentage of the organic mobile phase (e.g., Method 1, Appendix 2B). Since the goal was to work with THC-dominant cultivars, high levels of THCA were expected; therefore, this change should also allow the main cannabinoid to elute well before the end of the run.

This strategy greatly improved separation among cannabinoids and from matrix interferents. Nevertheless, at least CBNA continued to co-elute with other matrix compounds, and, most importantly, Δ^9-THC and THCVA remained unresolved (Fig. 7).

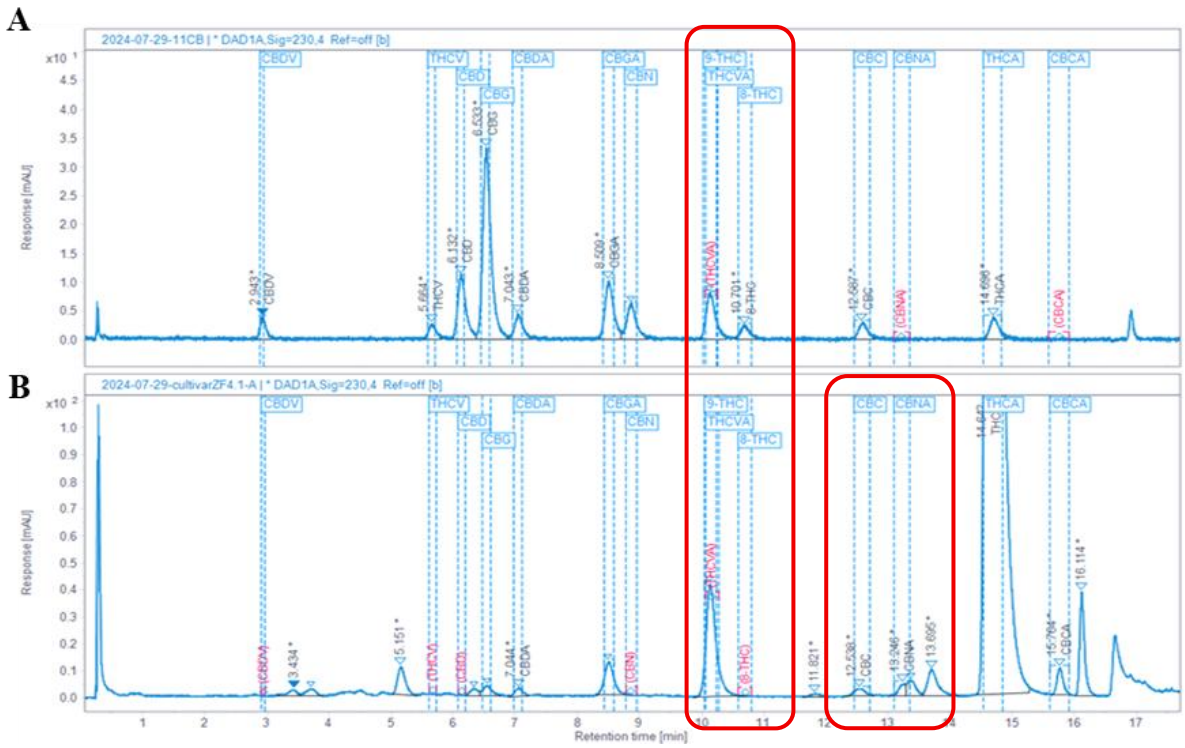


Figure 7. Representative chromatograms, using Method 1 (Appendix 2B), of the (A) 11 standard cannabinoids mixture and (B) Avextra's cultivar.

3.1.1.2 Altering mobile phase pH

Incorporating pH-modifying agents into the mobile phase can affect the selectivity factor by enhancing ionic strength and changing the pH [117]. For instance, the inclusion of formic acid (FA) at different percentages in the mobile phase can improve the peak shape and resolution of the compounds [81]. Acidic cannabinoids will be more influenced, exhibiting greater retention at lower pH values, while neutral cannabinoids are significantly less affected by pH variations. Since Δ^9-THC and THCVA were co-eluted, different combinations of aqueous phases (2.5–10 mM ammonium formate with 0.05–0.1% FA or trifluoracetic acid (TFA), with pH values ranging from 1.87 to 3.19, and MeOH (containing 0.05–0.1% FA) were tested. Increasing the pH through the addition of ammonium formate enabled the separation between Δ^9-THC and THCVA; consequently, FA also revealed greater resolution results compared to TFA.

The best condition was obtained when 10 mM of ammonium formate with 0.05% of FA was used as aqueous phase (Fig. 8A, Method 2, Appendix 2C). However, matrix interference with cannabinoid signals was still present in the extract (Fig. 8B), and they exhibited increased peak widths, which potentially affect resolution, detection and quantification limits.

To enhance the interaction between extract components and the stationary phase for better resolution, several strategies were implemented: (i) increasing the length of the column; (ii) reducing the column temperature; (iii) adjusting mobile phase pH; (iv) decreasing the flow rate; and (v) slowing the gradient rate by extending the run time.

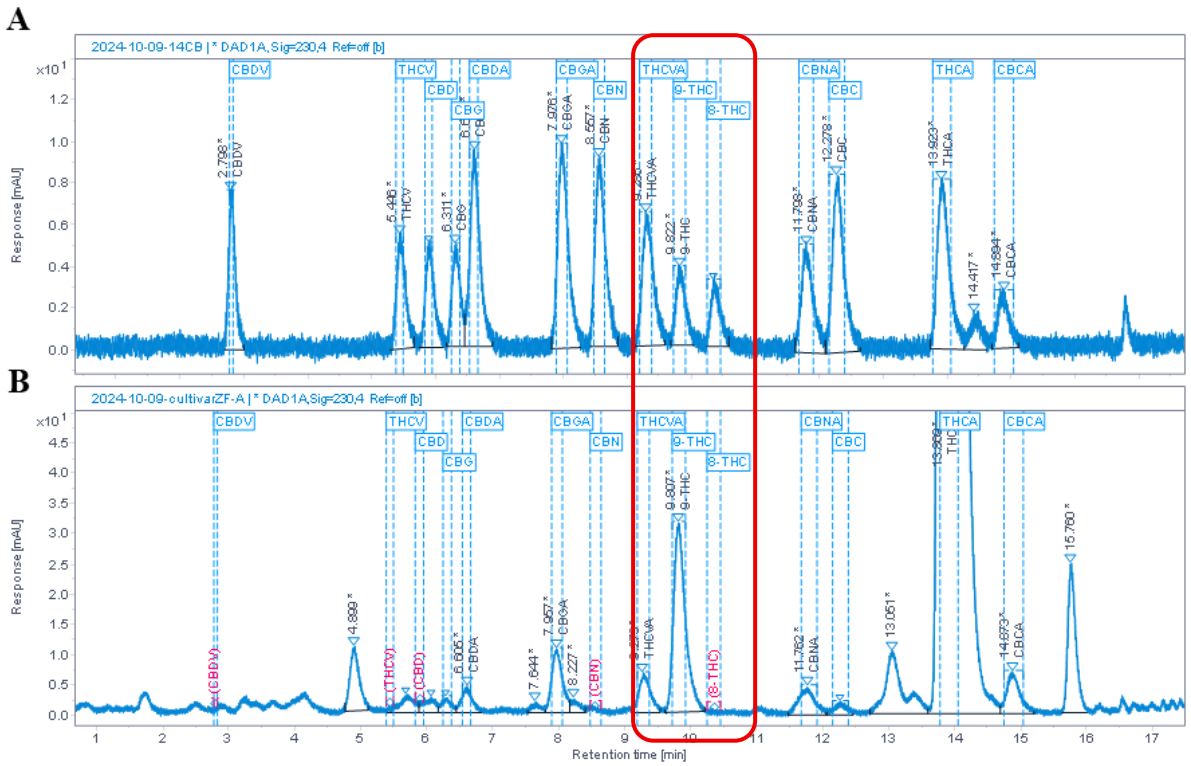


Figure 8. Representative chromatograms, using Method 2 (Appendix 2C), of the (A) 14 standard cannabinoids mixture (2.5 µg/mL) and (B) Avextra's cultivar.

3.1.2 Increasing column length: Optimisation studies with InfinityLab Poroshell 120 EC-C18, 3.0×150 mm, 2.7 µm column

By extending the length of a chromatography column, the separation of analytes can be improved by allowing more interaction time between the sample and the stationary phase [118]. Increasing the interaction time can be particularly beneficial for compounds with similar structures and physicochemical properties like cannabinoids. However, increasing the column length is accompanied by greater backpressure and may require longer analysis times. For this reason, initial changes to the chromatographic runs were implemented. Specifically, while maintaining the same mobile phases, the flow rate was lowered from 1 to 0.7 mL/min, and the post-run time was extended from 1.5 to 3 minutes to ensure proper column stabilisation.

As a consequence, adjustments to the gradient were needed to enable all cannabinoids to elute, including increasing the run time and raising the initial percentage of organic phase (between 65% and 70%) while maintaining a final percentage of 95%.

This experimental series (e.g., Method 3, Appendix 2D) revealed new resolution issues for CBD, CBG, and THCV (Fig. 9A), while matrix interference persisted (Fig. 9B). Moreover, a modification in the elution order of THCVA (retention time (t_R) = 11.543) was observed, now appearing between Δ^9-THC (t_R = 11.388) and Δ^8-THC (t_R = 11.732). Given the improved peak sharpness with the longer column, optimisation was continued on the 150 mm column.

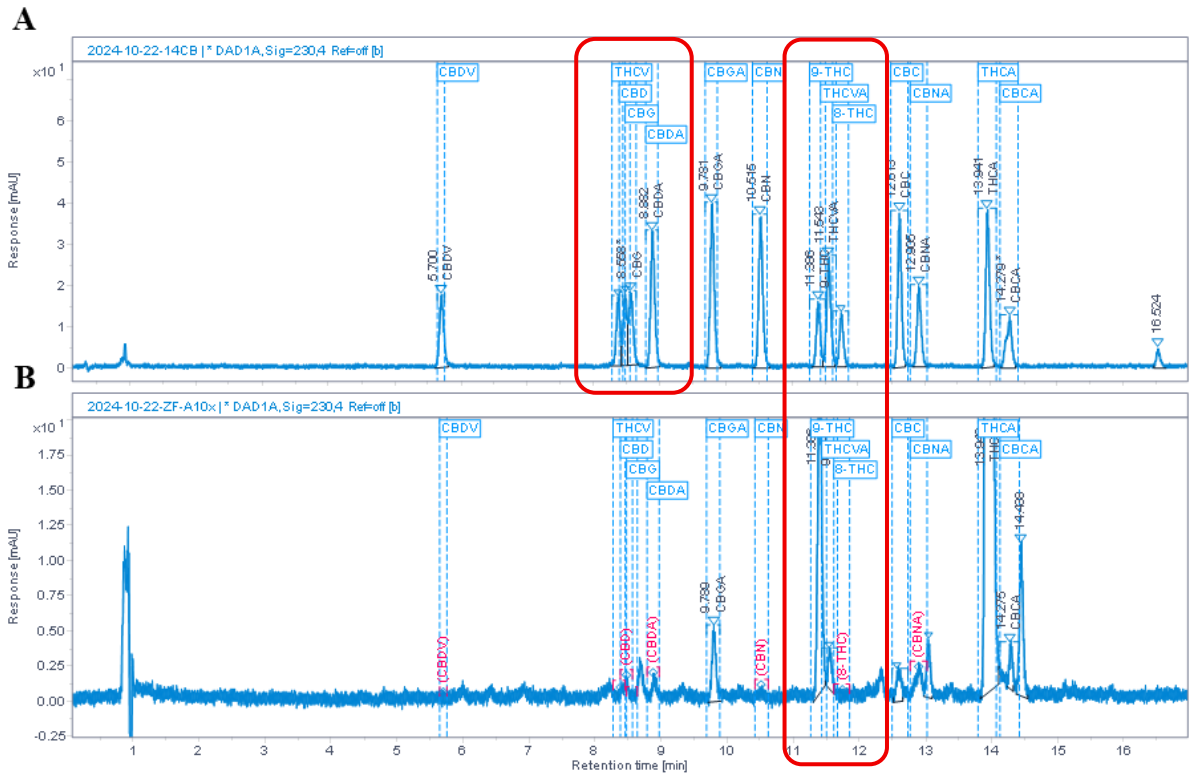


Figure 9. Representative chromatograms, using Method 3 (Appendix 2D), of the (A) 14 standard cannabinoids mixture (2.5 µg/mL) and (B) Avextra's cultivar.

3.1.2.1 Changing Column Temperature, Mobile Phase pH and Flow rate

Reducing the temperature slows the movement of molecules within the column, enhancing sample interaction with the stationary phase and potentially improving the separation of compounds that elute closely together [119]. Using the previous gradient condition (Method 3, Appendix 2D), the column temperature was lowered, and the flow rate was reduced as needed (Method 4, Appendix 2E) to prevent a rise in backpressure.

Although changing the column temperature was primarily intended to improve the separation between the earliest-eluted cannabinoids (CBD, CBG, and THCV), all compounds were affected. Δ^8-THC and THCVA co-eluting (Table 2), and some cannabinoids (THCA and CBCA) did not elute from the column at all during the current runtime.

Interestingly, the elution order of three neutral cannabinoids shifted from THCV–CBD–CBG to CBD–CBG–THCV. Spiking experiments with selected cannabinoids were carried out in the extract throughout the optimisation to better understand the elution orders and matrix interference.

Table 2. Resolution (*Rs*) of target compounds using different conditions of temperature and ammonium formate (AF) buffer concentrations.

AF Conc.	**10 mM**				**7.5 mM**	**5 mM**	**2.5 mM**	**0 mM**
pH aqueous phase	3.19				3.02	2.96	2.81	2.57
Temperature	**50º C**	**40º C**	**35 ºC**	**30 ºC**	**30 ºC**	**30 ºC**	**30 ºC**	**30 ºC**
Flow rate (mL/min)	0.7	0.7	0.5	0.5	0.5	0.5	0.5	0.5
***Rs* (CBD–CBG)**	0.02	1.76	2.03	2.12	2.26	2.29	2.20	2.35
***Rs* (CBG–THCV)**	0.04	3.47	6.41	5.28	6.04	6.04	6.03	5.92
***Rs* (THCVA–Δ^8-THC**	3.23	1.67	0.0	0.0	0.0	0.0	1.84	2.27

Baseline resolution between CBD, CBG, and THCV was achieved with decreasing temperature; however, resolution was lost between Δ^8-THC and THCVA at 30 ºC and 35 ºC. Since THCVA is an acidic cannabinoid, changes in the pH were pursued again, using Method 4 as a reference (Appendix 2E). Different conditions were tested using decreasing concentrations of ammonium formate (10 mM, 7.5 mM, 5 mM, and without the buffer) along with 0.1% FA, leading to a progressive decrease in pH. As expected, the retention time of THCVA increased with decreasing pH, while Δ^8-THC remained largely unaffected, which allowed for good resolution under conditions of 2.5 mM and without buffer (Method 5, Appendix 2F), with the elution order of Δ^9-THC–Δ^8-THC–THCVA. Interestingly, the resolution between CBD and CBG also slightly improved in the absence of ammonium formate; therefore, this condition was selected for further optimisation (Appendix 2G).

Maintaining the same mobile phases, several new conditions were tested to ensure that all 14 cannabinoids eluted without significantly extending the run time, while also attempting to improve resolution against other matrix interferences. Several strategies were implemented: (i)

extending the run time, (ii) increasing the initial percentage of the organic phase, and (iii) reducing the final gradient slope to enhance the separation of the latest eluted compounds; leading to Method 6 (Fig. 10, Appendix 2H). The purpose of slightly increasing the initial percentage of the organic phase (65%–70%) was to enable all cannabinoids to elute earlier without significantly prolonging the run time (17.7min–21min) or causing substantial interference in resolution. Furthermore, by lowering the final gradient slope, the rate of increase in the organic phase is slowed, allowing for better resolution of the final eluted compounds.

Method 6 promoted sufficient resolution between all 14 cannabinoids in the standard mixture (Fig. 10A). However, some matrix components continued to potentially interfere with the readings, and in certain instances, minor shifts in retention times were observed in specific signals. This may suggest the presence of co-eluting matrix components or indicate that they do not correspond to the assigned cannabinoid (e.g., CBD, Fig. 10B vs. 10A). For this reason, the next set of experiments considered the UV spectrum of each cannabinoid to evaluate the specificity of the method, in addition to its selectivity.

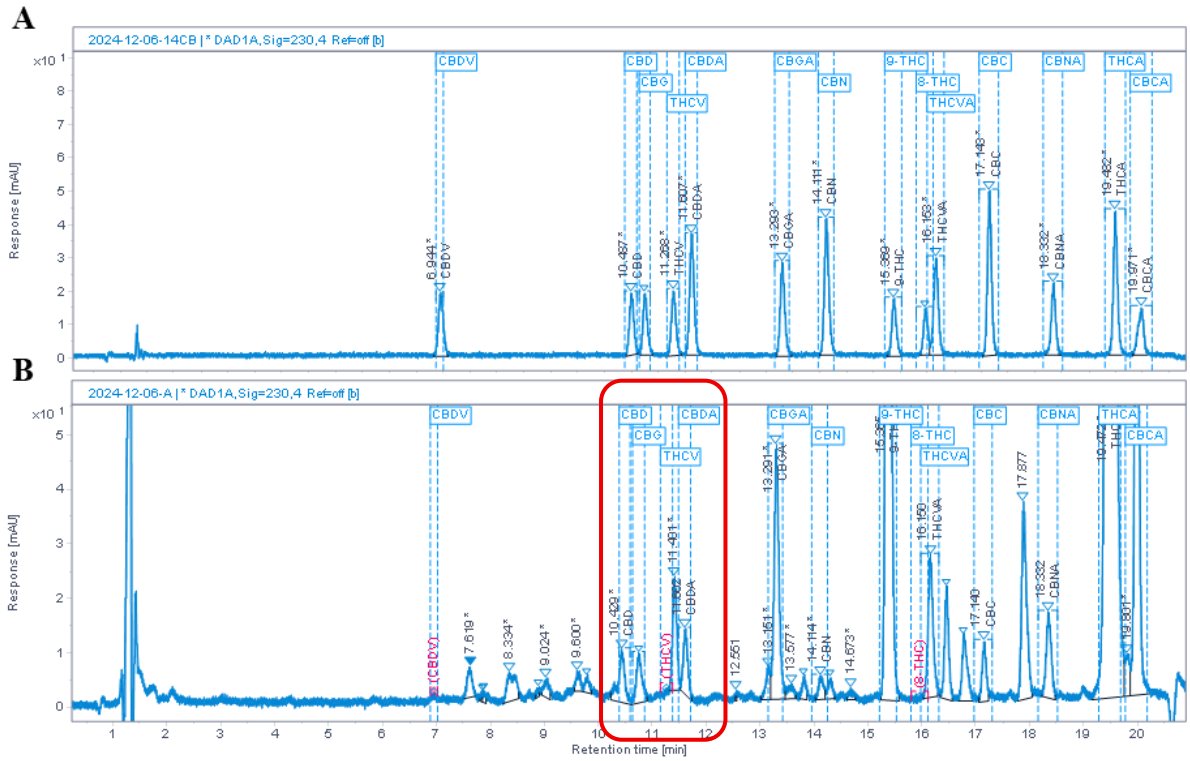


Figure 10. Representative chromatograms, using method 6 (Appendix 2H), of the (A) 14 standard cannabinoids mixture (2.5 µg/mL) and (B) Avextra's cultivar.

3.1.2.2 Absorbance profile studies

Detection relies on the analyte's UV absorption properties. For cannabinoids, the primary chromophore is a substituted phenolic ring (see Fig. 1, section 1.1.2). Additional contributions to the UV spectrum arise when the non-phenolic portion cyclizes to form a conjugated double bond (CBC and CBCA) or when a second phenyl ring is produced (CBN and CBNA) [120]. As a result, many cannabinoids exhibit similar UV spectra, which can reduce specificity. Diode array detection (DAD) helps to address this limitation by enabling peak characterisation through spectral comparison, particularly between neutral (Appendix 2I) and acidic (Appendix 2J) cannabinoids. Moreover, exploiting these UV spectral differences can enhance sensitivity by allowing the measurement of different cannabinoids at optimised wavelengths [3].

At this stage of the optimisation, all chromatograms were recorded only at 230 nm. As mentioned previously, to determine whether each signal assigned to a cannabinoid in the extract was truly that compound or a matrix interference with the same retention time, absorbance spectra were recorded and analysed from 190 to 400 nm. Based on the absorption maxima for the different cannabinoids, nine wavelengths were selected: 224, 230, 254, 264, 268, 270, 272, 280, and 284 nm (see Appendix 2K). Analysing this table revealed that only the extract signal designated as CBCA exhibited a significantly different absorbance profile compared to the corresponding standard. This discrepancy suggested two possibilities: either CBCA was absent in the analysed cannabis cultivar, and the observed signal was attributable to another compound with a similar retention time, or CBCA was present but co-eluted with a compound whose maximum absorbance was at 230 nm instead of 254 nm. To clarify, two long run methods were applied: an isocratic method of 60 minutes (Method 7, Appendix 2L), and a slow gradient over 49 minutes (Method 8, Appendix 2M, Fig. 11). The results indicated that this signal attributed to CBCA corresponded to two signals with different maximum absorptions (Fig. 11B). Additionally, these longer methods allowed for the effective separation of the target cannabinoids, with the main exception of THCVA and Δ^8-THC (Fig. 11A).

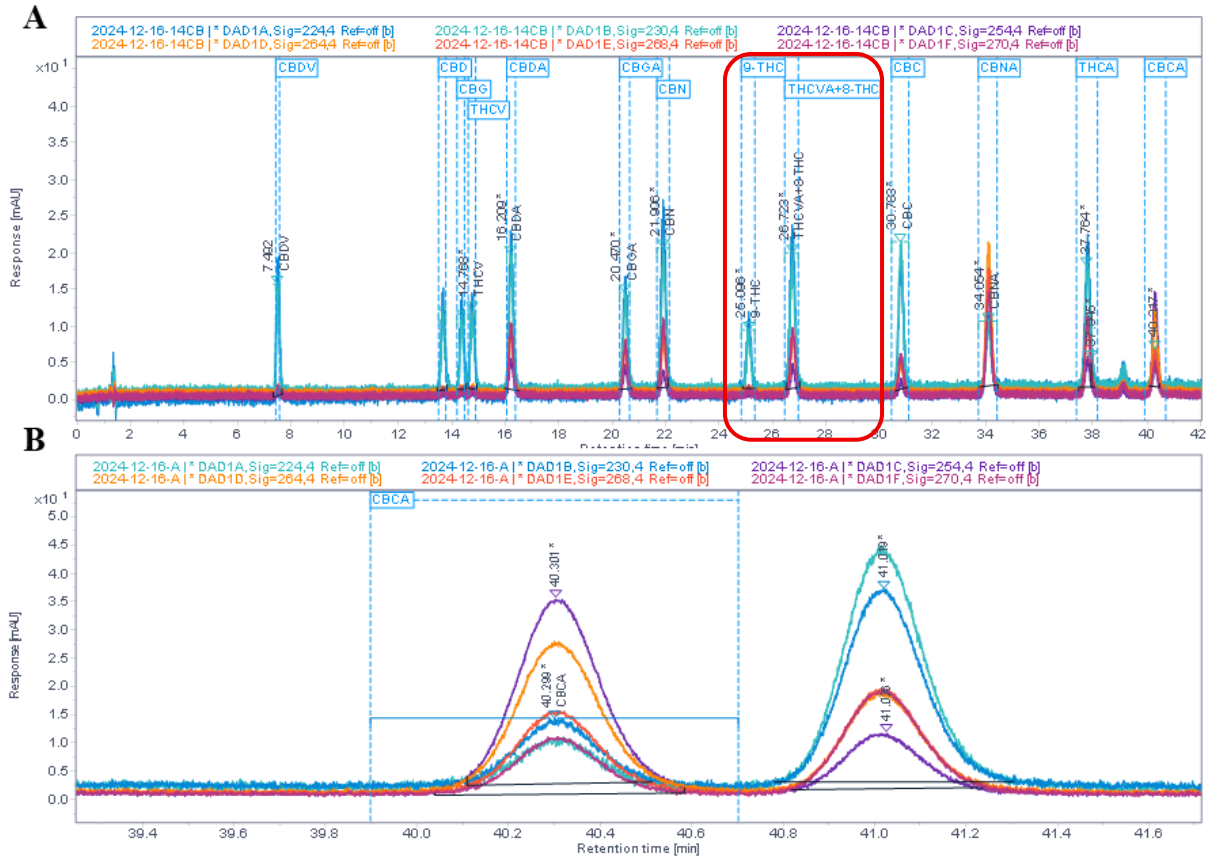


Figure 11. Representative chromatograms, using Method 8 (Appendix 2M), of the (A) 14 standard cannabinoids mixture (2.5 µg/mL) and (B) Avextra's cultivar showing run times between 39 and 42 min, with selected wavelengths: 224, 230, 254, 264, 268, and 270 nm.

The final optimised method was based mainly on four observations gathered during the entire optimisation process: (i) Lowering the initial organic content or slowing its gradient rise improves separation for all analytes, except for Δ^8-THC and THCVA, (ii) an initial isocratic hold enhances resolution between the early-eluting compounds (CBD, CBG and THCV) from matrix interferences, (iii) a rapid increase in polarity after those elutions improves the separation of Δ^8-THC-THCVA, and (iv) a longer run time is necessary to separate CBCA from the adjacent matrix peak discussed earlier.

Maintaining the same mobile phases, numerous approaches were evaluated using different mobile-phase mixtures, including multilevel isocratic and isocratic-gradient-isocratic strategies. These adjustments led to the development of gradient Method 9 (Fig. 12, Appendix 2N), which achieved a resolution greater than 1.5 for most cannabinoids and matrix compounds at 230 nm (discussed in validation section 3.3.1). For further studies, 230 nm was chosen instead of 224 nm because methanol and FA absorbance at this wavelength caused a noisier baseline and reduced sensitivity. Additionally, with the exception of five neutral cannabinoids (CBDV,

CBD, CBG, THCV, Δ^9-THC, and Δ^8-THC) that do not present additional absorbance maxima, a secondary wavelength was also used for all other analytes to confirm specificity throughout this work. This was also implemented as a backup plan in case new cultivars with a different matrix and cannabinoid profile were tested: CBDA, CBGA, THCVA, and THCA at 272 nm; CBN and CBC at 280 nm; and CBNA and CBCA at 260 nm (Fig. 12).

The pair with lower resolution was Δ^8-THC and THCVA. Although THCVA could be measured at 272 nm, as mentioned above, Avextra's cultivar does not contain Δ^8-THC; therefore, it was measured using 230 nm, like the other cannabinoids. Moreover, in addition to Δ^8-THC, neither CBDV, CBD nor THCV was detected in Avextra's cultivar (Fig. 13).

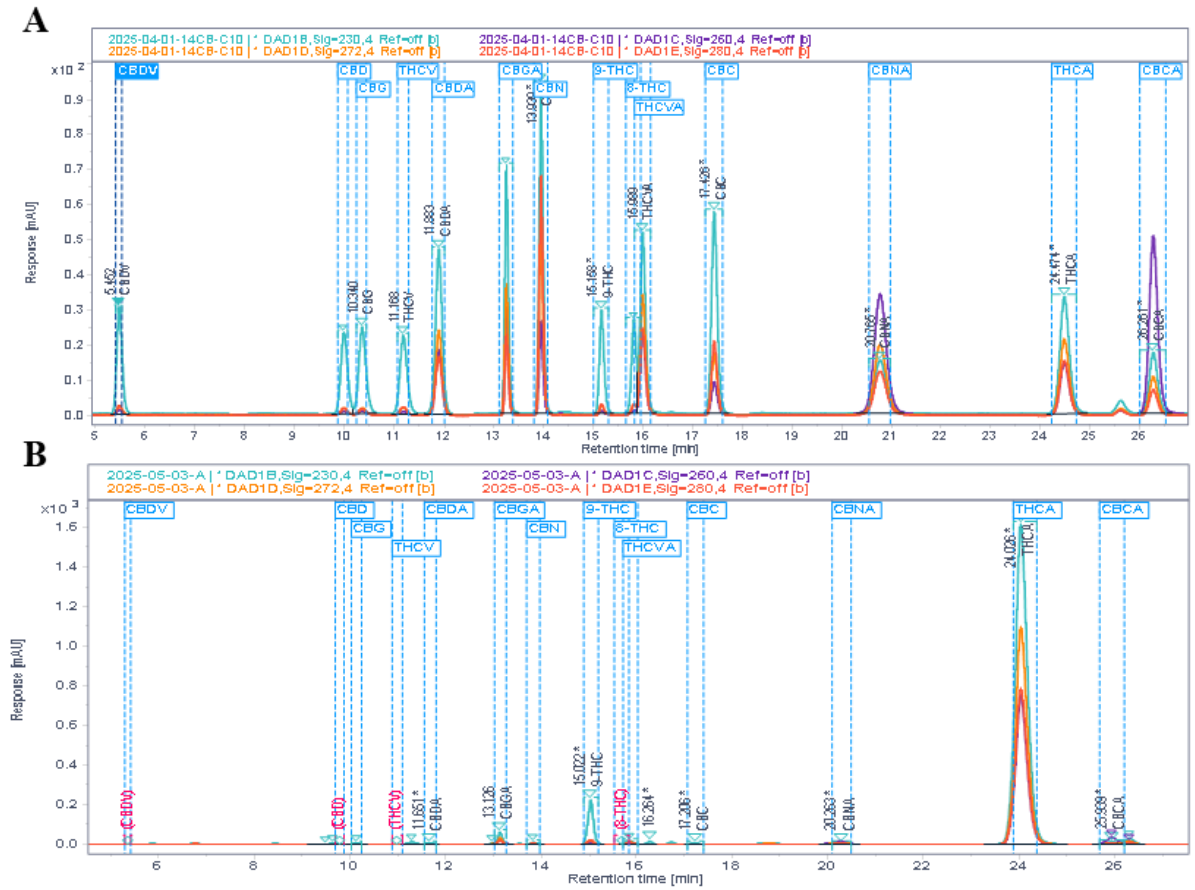


Figure 12. Representative chromatograms, using the final method (CannProVar method A, Appendix 2N), of the (A) 14 standard cannabinoids mixture (2.5 µg/mL) and (B) Avextra's cultivar.

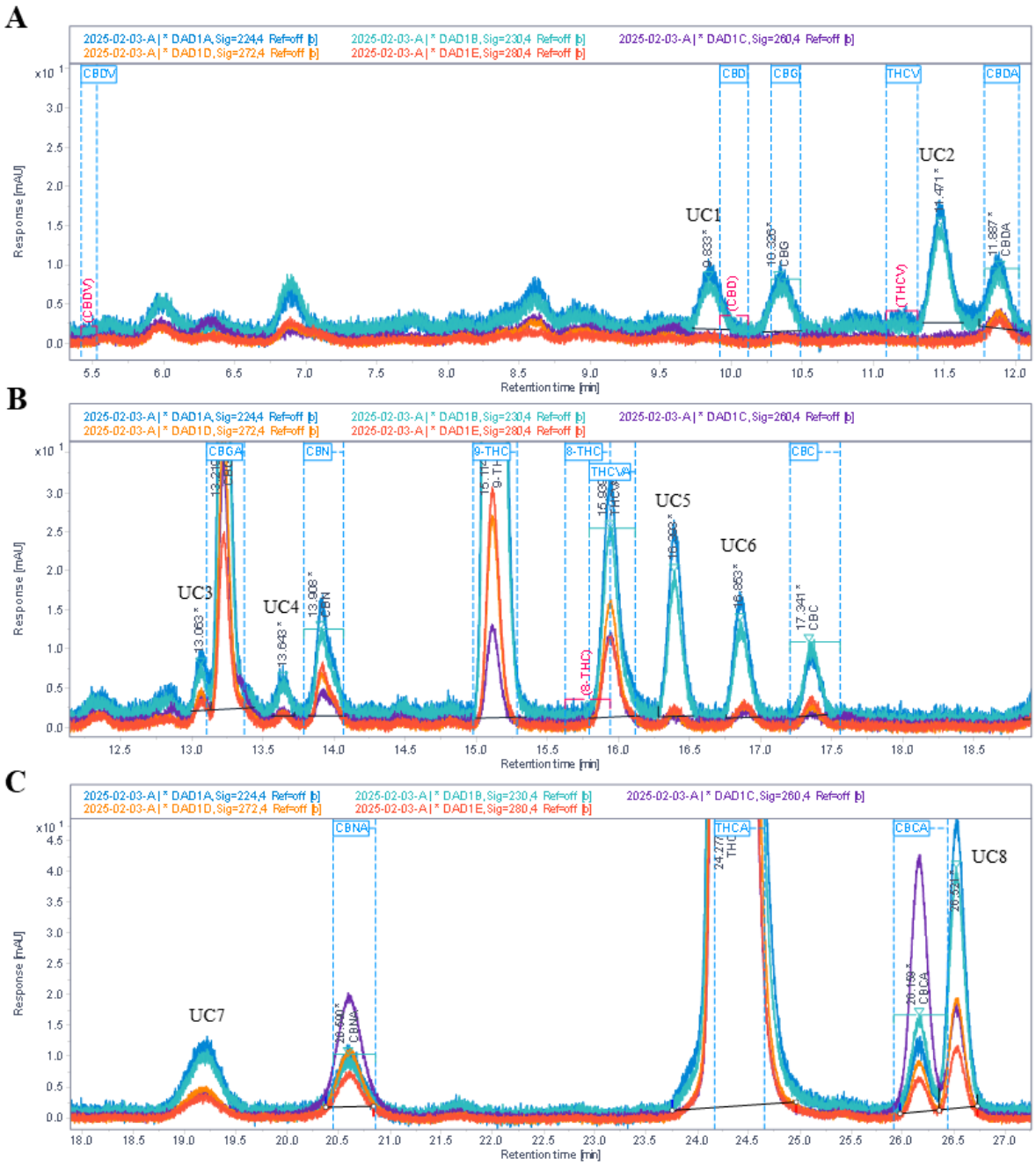


Figure 13. Representative chromatograms, using the final method (CannProVar method A, Appendix 2M), of the Avextra's cultivar showing run times between (A) 5.5 and 12 min, (B) 12 and 19 min, (C) 18 and 27.5 min, with selected wavelengths: 224, 230, 260, 272, and 280 nm. UC: unknown compounds.

3.2 Optimisation of the analytical method – CannProVar method B

The previously developed method enables comprehensive characterisation of the flower's cannabinoid profile, providing effective separation of the 14 cannabinoids examined and preventing their co-elution with other matrix compounds. However, because the goal is also to monitor variations of two main cannabinoids in this cultivar - Δ^9-THC and THCA - as well as

the Δ^9-THC oxidative by-product CBN throughout sample processing (decarboxylation, extraction, winterization and purification by activated charcoal), a faster 12-minute HPLC–UV method was developed (CannProVar method B, Fig. 14, Appendix 2O).

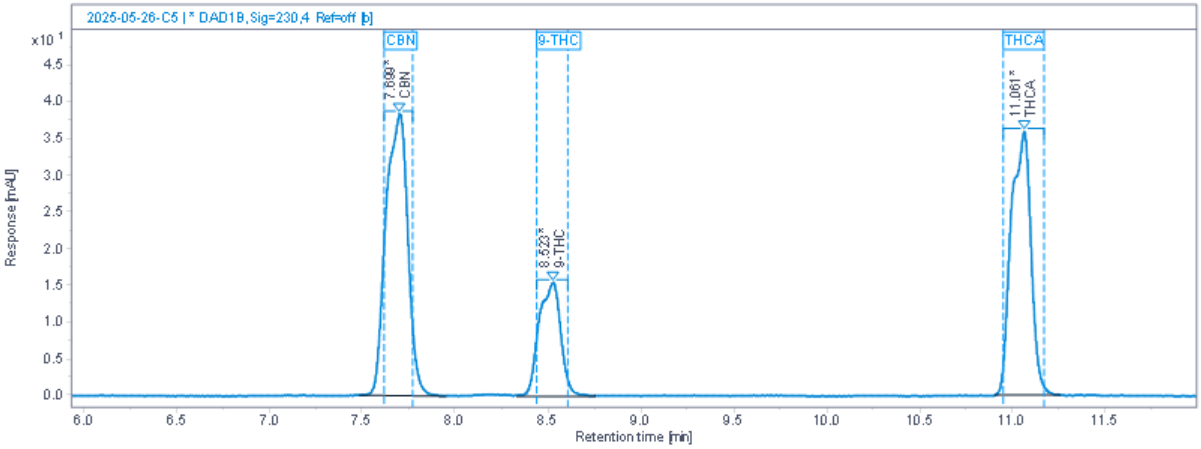


Figure 14. Representative chromatogram, using CannProVar method B (Appendix 2O), of CBN, Δ^9-THC and THCA, from the standards injections in methanol (5 µg/mL).

3.3 Validation of the analytical method – CannProVar method A

This section aims to validate the analytical method previously developed for quantifying 14 cannabinoids. The objective of method validation is to demonstrate that the analytical procedure fulfils its intended purpose for the analysis. A method can be validated by applying an appropriate set of acceptance criteria defined initially according to the method's intended use. The tests conducted to verify each validation parameter, including accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantification limit, linearity and range, were carried out following primarily the regulatory guidelines provided by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use - ICH Q2-R2 document [121].

3.3.1 Specificity/Selectivity

Specificity describes an analytical method's ability to distinctly identify the target analytes in the potential presence of other compounds in the matrix [121]. The optimisation of the analytical method resulted in the separation of all 14 analysed cannabinoids, with no compounds sharing the same retention time. The selectivity of the analytical method can be assessed by calculating the resolution between the peaks of adjacent compounds. In

chromatography, a resolution (R_s) of at least 1.5 is generally considered indicative of good separation, especially when baseline separation is desired [122].

In this study, resolutions were determined using Equation 1 [123], where t_{R1} and t_{R2} are the retention times of compounds 1 and 2 ($t_{R2} > t_{R1}$), and W_{h1} and W_{h2} are the peak widths at half-heights.

$$Rs = \frac{1.18(tR2 - tR1)}{Wh2 + Wh1} \quad \text{(Eq. 1)}$$

Baseline resolution greater than 1.5 was achieved for most tested cannabinoids (Table 3). Only two pairs fell below this threshold: CBD–CBG (Rs = 1.4) and Δ^8-THC–THCVA (Rs = 0.9). However, because Avextra's cultivar lacks CBD and Δ^8-THC (both below the detection limit), the CBG and THCVA signals can be integrated without resolution issues. As described in the UV-optimization section, even if a cultivar contains Δ^8-THC, the absorbance spectrum of THCVA still permits its analysis at an alternative wavelength (272 nm), ensuring baseline resolution. Resolution between cannabinoids and unidentified compounds in Avextra's cultivar was also evaluated (Table 4). The results showed that the method achieved a resolution of at least 1 between all matrix compounds and analytes of interest, except for UC4–CBN pair (Rs = 0.9). Nevertheless, smaller resolution values were obtained for impurities with a very small signal area compared to the analysed cannabinoid (UC4–CBN and UC3–CBGA, Fig.13), reducing their effect on cannabinoid quantification.

Table 3. Retention times and resolution values for a 14 cannabinoids solution (2.5 µg/mL) in methanol, detected at 230 nm.

Cannabinoids *(in order of elution)*	***Retention time*** *(min)*	***Resolution*** ***(R_s)*** [a]		***Cannabinoids*** *(in order of elution)*	***Retention time*** *(min)*	***Resolution*** ***(R_s)*** [a]
CBDV	5.45	20.14		Δ^9-THC	15.16	3.66
CBD	9.98	1.43		Δ^8-THC	15.81	0.93
CBG	10.34	3.10		THCVA	15.99	6.95
THCV	11.17	2.60		CBC	17.43	9.95
CBDA	11.83	5.15		CBNA	20.77	9.23
CBGA	13.24	2.19		THCA	24.47	5.43
CBN	13.94	4.91		CBCA	26.26	

[a] The resolution presented is between two consecutive cannabinoids (e.g., R_s = 20.14 between CBDV and CBD).

Table 4. Retention times and resolution values for the cannabinoids and unknown compounds in Avextra's cultivar, detected at 230nm.

Compounds *(in order of elution)*	***Retention time*** *(min)*	***Resolution*** ***(R_s)***[a]		***Compounds*** *(in order of elution)*	***Retention time*** *(min)*	***Resolution*** ***(R_s)***[a]
UC1	9.83	1.61		THCVA	15.91	2.29
CBG	10.33	3.33		UC5	16.36	2.36
UC2	11.43	1.38		UC6	16.85	2.12
CBDA	11.85	4.74		CBC	17.34	4.15
UC3	13.02	1.18		UC7	19.21	2.24
CBGA	13.20	2.24		CBNA	20.59	7.69
UC4	13.64	0.94		THCA	24.28	5.21
CBN	13.91	5.15		CBCA	26.13	1.22
Δ^9-THC	15.11	4.31		UC8	26.50	

[a] The resolution presented is between two consecutive cannabinoids (e.g., R_s = 1.61 between UC1 and CBG). UC: Unknown Compounds.

3.3.2 Linearity and Range

The linearity of an analytical method indicates its ability to yield results that are directly proportional to analyte concentration within a defined range. Linearity is assessed by the correlation coefficient (R^2), which quantifies the strength of the association between the independent variable (concentration, X) and the dependent variable (response area, Y). An R^2 value of 0.99 or greater is generally regarded as evidence of a strong linear relationship. According to ICH guidelines, to assess linearity, a minimum of five concentrations adequately distributed over the range is recommended [121]. The range of an analytical method is the interval between the lowest and the highest concentration of the analyte in the sample for which it is possible to verify that an acceptable level of precision, accuracy and linearity is achieved. From 1 mg/mL standard solutions, ten concentrations (0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 200 µg/mL) were prepared by serial dilution in methanol to assess linearity. At least three independent experiments for each cannabinoid were analysed to ensure linearity and robustness of each calibration curve. All calibration curves were performed at a wavelength of 230 nm. All R^2 values obtained ranged from 0.998 to 1.000 (Appendix 2P).

3.3.3 Detection Limit (DL) and Quantification Limit (QL)

The detection limit is the lowest possible concentration at which a given analyte can be detected, but not necessarily quantified. This level signifies the lowest concentration where the compound can still be differentiated from the baseline noise [121].

The Detection Limit (DL) can be expressed through Equation 2, where σ (Sigma) represents the standard deviation of the response and *S* is the slope of the calibration curve, estimated from the regression line of the analyte. DL can also be assessed based on signal-to-noise ratio ≥ 3 (Eq. 3) and through visual evaluation. The Quantification Limit (QL) is the lowest concentration at which a given analyte can be accurately and precisely quantified. This parameter can be evaluated using approaches similar to those used for DL, including visual evaluation, a signal-to-noise ratio of ≥ 10, the standard deviation of a linear response and a slope (Eq. 4), as well as accuracy and precision at lower range limits [121].

$$\mathrm{DL} = \frac{3.3\sigma}{\mathrm{S}} \quad \text{(Eq. 2)} \qquad \mathrm{S/N} = \frac{2\mathrm{H}}{\mathrm{h}} \quad \text{(Eq. 3)} \qquad \mathrm{QL} = \frac{10\sigma}{\mathrm{S}} \quad \text{(Eq. 4)}$$

In a preliminary assessment, the DL and QL were determined using Equation 2 and Equation 4, respectively, with the standard deviation of the y-intercepts of the regression lines used as σ (Sigma). Using these values as a reference, several concentrations near the DL and QL were analysed. The DL was defined as the lowest tested concentration, ranging from 0.1 to 0.2 µg/mL (Appendix 2P), with a signal-to-noise ratio of ≥ 3. The QL was defined as the lowest tested concentration, varying from 1.0 to 3.0 µg/mL with a signal-to-noise ratio of ≥ 10, along with adequate accuracy (bias ± 15%) and precision (RSD ≤10%), as discussed in the "Accuracy and Precision" section (3.3.4).

For the most concentrated flower sample extract tested, the measurements obtained for the CannProVar method A, the DL ranged from 0.004 to 0.008% (w/w) for the target cannabinoids, while the QL varied between 0.04 and 0.12% (Appendix 2P).

3.3.4 Accuracy and Precision

Accuracy is the measure of how close the experimental results are to the accepted reference value. According to the ICH guidelines, the validation of this parameter is demonstrated through the comparison of the measured results with the expected values [121].

In this study, accuracy was assessed using the percentage of bias, as outlined in the AOAC guidelines, and was calculated using Equation 5 [124].

$$\%\mathrm{Bias} = \frac{\text{Determined concentration} - \text{Known concentration}}{\text{Known concentration}} \times 100 \quad \text{(Eq. 5)}$$

Precision is defined as the extent of variation observed in repeated measurements conducted under controlled conditions. In analytical methodologies, precision is determined by the consistency of results obtained from multiple injections of a sample while employing specified chromatographic conditions. Repeatability and intermediate precision were evaluated at three levels. Repeatability reflects the consistency of measurements conducted under identical conditions within a short time interval. It is also referred to as intra-assay precision, but it is often represented as intra-day precision. Intermediate precision accounts for intra-laboratory variations and considers various sources of variability, including, for example, different days, environmental conditions, and equipment [121]. In this study, only inter-day assessments were performed. Precision is expressed as the relative standard deviation (RSD) of several results obtained (Eq. 6).

$$\%\text{RSD} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100 \quad \text{(Eq. 6)}$$

Precision and accuracy were evaluated using three quality-control levels spanning the linear range: a low concentration close to the quantification limit (Low QC, 5 µg/mL), an intermediate concentration (medium QC, 20 µg/mL), and a high concentration approaching the upper limit of the range (high QC, 80 µg/mL). Intra-assay precision was assessed from three consecutive measurements; intra-day precision from three measurements taken across a single day; and inter-day precision from measurements performed on three consecutive days. Additionally, accuracy was assessed for intra-day and inter-day precision experiments by evaluating all cannabinoids across the three quality control levels. For intra-assay precision, RSD were ≤ 5.09%; for intra-day experiments, RSD ≤ 7.49% with a bias ranging from -15.43 and 13.82%; and for inter-day experiments, RSD ≤ 6.03% with a bias between -14.22 and 14.86%. Only seven out of the 126 determined RSD values were above 5% but below 7.49%. Accuracy was within ±10% for all 252 determinations, except for thirteen, with the greatest bias being -15.43%. These results indicate strong precision and accuracy across the tested concentrations (Appendix 2Q). For QL determinations, this limit was established for the lowest concentration at which the values remained within ±15% for accuracy, while the RSD was ≤10% for precision.

3.3.5 Matrix Effect

Thus far, validation parameters have been assessed in solvent and indicate that the method is precise and accurate within established limits. Nevertheless, matrix effects (ME), that is, the

influence of the cannabis sample matrix on analyte measurements, should be evaluated to verify that the validation holds for the analysed sample. Matrix effects can significantly impact method robustness, linearity, precision and accuracy, resulting in inaccurate quantification [115].
Because the studied cultivar contains high levels of THCA and Δ^9-THC, performing spiking experiments on the extract dilutions intended for cannabinoid quantification is challenging. To avoid altering matrix effects through sample dilution, a surrogate cannabis matrix, consisting of a pooled extract from five CBD-dominant cultivars with low Δ^9-THC and THCA, was employed. The pooled sample contained only Δ^9-THC (2.9 µg/mL) and THCA (2.6 µg/mL) at the highest tested concentration. Recovery was determined using Equation 7,

$$\text{ME (\%)} = \frac{(\text{CS}-\text{CU})}{\text{CA}} \times 100 \quad \text{(Eq. 7)}$$

where "CS" is the concentration measured in the spiked sample (standard added plus endogenous cannabinoid), "CU" is the endogenous concentration measured in the unspiked sample, and "CA" is the concentration measured for the known standard solution added.
Matrix effect was evaluated for the most concentrated extract analysed by HPLC. Three concentrations (5, 10, and 80 µg/mL) were spiked, and recovery and precision for the spiked sample were assessed in intra-assay, intraday, and interday experiments.
The intra-assay relative standard deviation (RSD) values were consistently low, with a maximum of 3.04% (compound Δ^8-THC at a concentration of 5 µg/mL). Similarly, all intra-day and inter-day RSD values were ≤ 4.63%. The ME% values confirm a minimal interference from the cannabis matrix. For most cannabinoids analysed, the ME% fell within the defined range of ±10%. The lowest observed value was 93.9%, while the highest was 109.8% (Appendix 2R). These results collectively confirm the method's robustness and its suitability for the precise and accurate quantification of cannabinoids in complex hemp flower matrices.

3.4 Validation of the analytical method – CannProVar method B

CannProVar Method B was validated according to ICH guidelines using the same criteria as Method A, including specificity, limits of detection and quantification, linearity, range, accuracy, precision, and matrix effect (see Appendix 2S, 2T and 2U). Both robust methods, CannProVar A and B, successfully meet all validation criteria, demonstrating a bias within ±15%, a relative standard deviation (RSD) of ≤10%, and a linearity coefficient (R2) of ≥0.99.

4. Decarboxylation

Cannabis plants biosynthesise and accumulate cannabinoids in their acidic forms within glandular trichomes. The two major compounds, Δ^9-THCA and CBDA, are synthesised from CBGA by specific oxidoreductases, while CBGA itself is formed by alkylation of olivetolic acid [17,125]. Although CBDA and THCA have been associated with some pharmacological effects, neutral cannabinoids are generally regarded as the therapeutically active forms and have generally better bioavailability [126,127]. For example, THC-dominant *C. sativa* extracts do not produce psychoactive effects unless they are heated sufficiently to convert the acidic cannabinoids to their neutral form (Fig. 15). The neutral compounds (e.g., Δ^9-THC) arise from nonenzymatic decarboxylation of their acidic counterparts (e.g., THCA), a process that removes a carboxyl group and releases carbon dioxide (CO_2). Therefore, elevated levels of decarboxylated cannabinoids in flower samples may indicate improper storage or aging [17]. Decarboxylation proceeds slowly at ambient temperature but accelerates as temperature increases; other environmental factors such as light and oxygen also promote decarboxylation [19]. Furthermore, Δ^9-THC can also oxidise when exposed to the same environmental factors to CBN. Recreational users have traditionally induced decarboxylation by smoking, vaping, or baking. Decarboxylation is therefore an essential step in the cannabis industry to ensure high extract quality by maximising Δ^9-THC formation and minimising oxidation to CBN, while also trying to reduce the loss of other potentially important volatile plant constituents (e.g., terpenes).

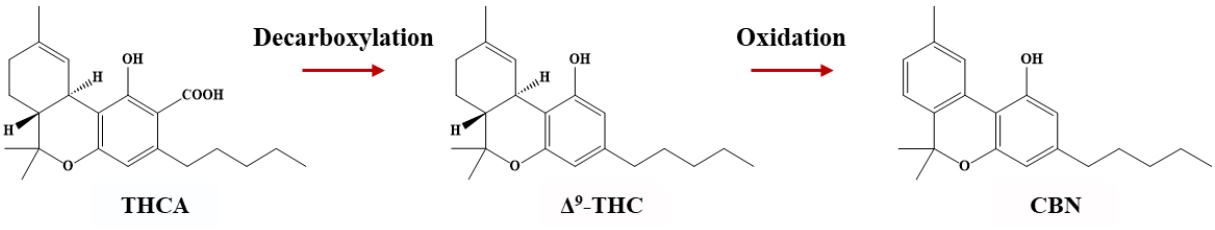


Figure 15. Schematic representation of THCA decarboxylation to Δ^9-THC and subsequent oxidation of Δ^9-THC to CBN.

The decarboxylation process was reported as early as 1967 by Nishioka et al. [128], and heating conditions were described in 1970 by Kimura and Okamoto [129], who applied heat (110 °C) to recently dried parts of the cannabis plant to determine THCA content by measuring Δ^9-THC levels. Veress et al. [130] conducted the first thermal decarboxylation kinetic study, examining

the decarboxylation of THCA and CBDA in an open oven, and characterised the reaction as first-order. Since then, several studies of THCA kinetics have been published. Using a vacuum oven, Perrotin-Brunel et al. [131] investigated decarboxylation in plant material from 90 °C to 140 °C, and Wang et al. [132] examined decarboxylation in extracts from 80 °C to 145 °C. More recently, Moreno et al. [133] investigated the decarboxylation of THCA, CBDA and CBGA in plant material (80–160 °C), both in the presence and absence of oxygen, and applied different kinetic models to predict the optimal decarboxylation conditions for achieving the highest Δ^9-THC or CBD concentration. They observed substantial losses of neutral cannabinoids at higher temperatures and longer reaction times, indicating byproduct formation or evaporation; these losses were markedly reduced under anoxic conditions, suggesting oxidation plays a role. In addition, the amount of plant material undergoing decarboxylation must also be considered, as it affects heat transfer and oxygen exposure in the oven. Larger sample loads can slow the process and produce apparently lower reaction rates. Avextra performs decarboxylation on the flowers before extraction using an autoclave at 120 °C.

This task aims to investigate THCA decarboxylation at temperatures near 120 °C (110 ºC – 130 ºC) to identify the optimal reaction times and temperatures that minimise Δ^9-THC loss and CBN formation. In addition, cannabis weight will also be monitored throughout the process, since smaller weight losses may indicate better preservation of thermally unstable and volatile constituents that enrich the resulting extracts.

Decarboxylation experiments were carried out at three temperatures (110 ºC, 120 ºC, 130 ºC) and THCA, Δ^9-THC, and CBN levels were monitored over time. These experiments were conducted after pulverisation (see section 8.4) and were followed by sample extraction (see section 8.6.2).

For the 120 °C decarboxylation experiments, seven parallel samples in Petri dishes were prepared. Each contained 0.50 g and taken from the same ground flower batch to reduce sampling variability. A non-decarboxylated sample was analysed to establish the baseline concentration for each cannabinoid of interest in the flower (Fig. 16A). The remaining six samples were placed in the oven at 120 °C for up to 1 h, with one plate removed every 10 minutes to monitor decarboxylation (Fig. 16B–G). The protocol was similar for the other two temperatures, differing in the time points studied since decarboxylation proceeds faster at higher temperatures: the protocol at 110 °C ran for up to 2 h, with samples removed every 15 minutes (nine experiments); at 130 °C, eight parallel experiments were performed to analyse time points of 0, 5, 10, 20, 30, 40, 50, and 60 min. Cannabinoid changes over decarboxylation time at the three studied temperatures are shown in Figure 17.

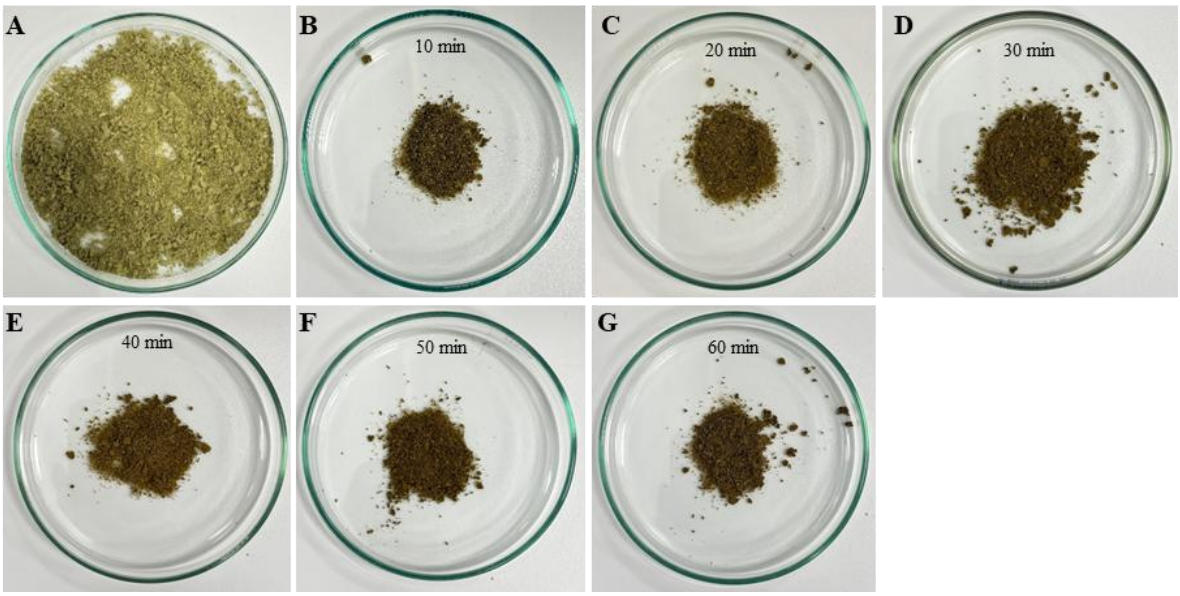


Figure 16. Decarboxylation experiments at 120 ºC (A) Illustrative image of ground sample (3.50 g) pre-decarboxylation. (B–G) Illustrative images of ground samples (0.50 g) after decarboxylation, according to time point experiment.

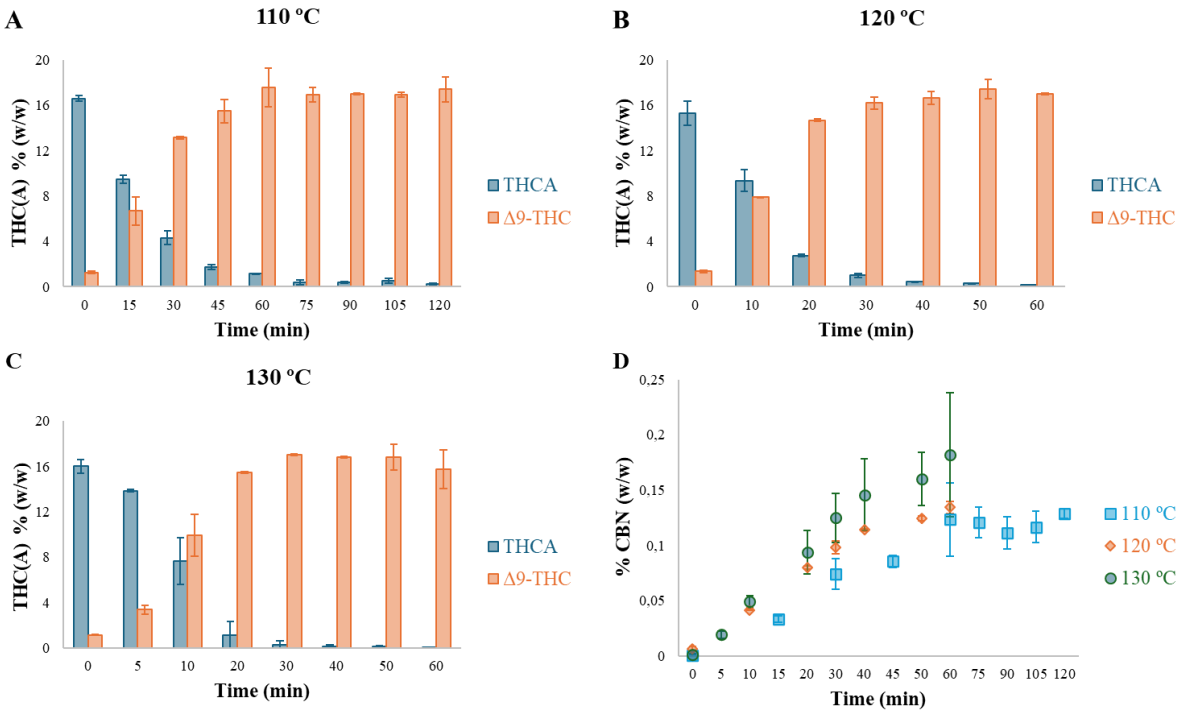


Figure 17. (A–C) Δ^9-THC and THCA content in the flower, expressed as % (w/w), over decarboxylation time at different temperatures: 110 ºC (A); 120 ºC (B), and 130 ºC (C). (D) CBN content in the flower, expressed as % (w/w), over decarboxylation time at 110 ºC, 120 ºC, and 130 ºC. Experiments were performed in duplicate; data corresponds to average ± standard deviation.

To help understand the impact of temperature and time in THCA decarboxylation, a simple kinetic model was applied. The relationship between the rate of the decarboxylation reaction and the concentration of THCA can be expressed by Equation 8 or, alternatively, by Equation 9

$$\frac{\mathrm{d}[THCA]}{\mathrm{d}[THCA]\mathrm{t}} = -k[THCA] \quad \text{(Eq. 8)} \qquad \ln\left(\frac{[THCA]0}{[THCA]\mathrm{t}}\right) = kt \quad \text{(Eq. 9)}$$

where K presents the rate constant, and $[THCA]_0$ and $[THCA]_t$ are the concentrations of THCA at time 0 and t min, respectively. In addition, the activation energy (E_A), which represents the minimum energy necessary for the reaction to occur, is obtained from the temperature dependence of the rate constants via the Arrhenius equation (Eq. 10).

$$\ln k = \ln k0 - \frac{Ea}{RT} \quad \text{(Eq. 10)}$$

where k_0 is the frequency factor, and R is the gas constant.

At a fixed temperature, first-order kinetics are observed when the logarithm of acid concentration varies linearly with time (Fig. 18A). Once the decarboxylation kinetic constants for each temperature are known (Table 5), plotting ln(1/k) versus 1/T allows the determination of E_a and K_0.

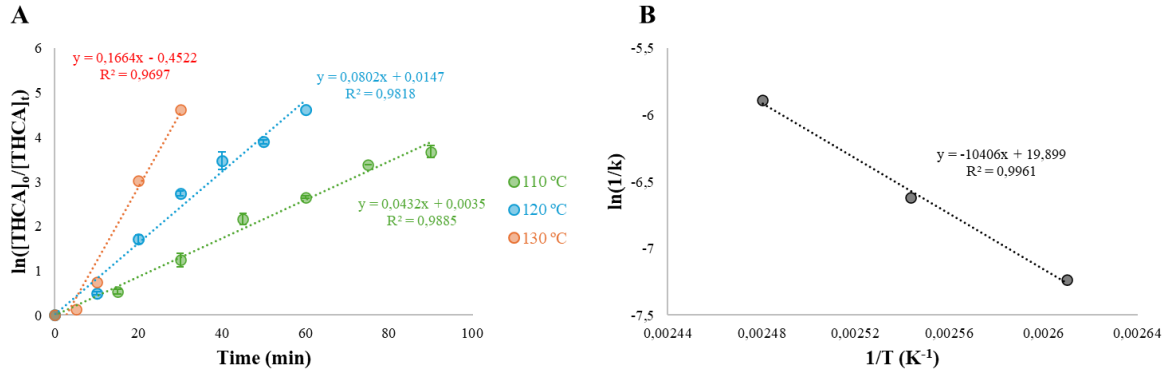


Figure 18. (A) THCA decarboxylation kinetics at different temperatures. Experiments were performed in duplicate; data corresponds to average ± standard deviation. (B) Arrhenius plots for THCA. K, rate constant, expressed in sec-1, T, temperature, expressed in Kelvin.

Table 5. Rate constants, k, and Activation energy, E_a, for the decarboxylation of THCA.

	***K* (sec^{-1})**				
	110 ºC	**120 ºC**	**130 ºC**	***E*$_a$ (kJ/mol)[a]**	***K*$_0$ (sec^{-1})**
THCA	0.00072	0.00134	0.00277	86.5	4.4 x 10^8

[a] Reported values in literature: 88 [132], 84 [131]

Kinetic equations are useful for estimating the time required to reach a desired decarboxylation endpoint based on the initial THCA concentration. For example, defining the end of decarboxylation as the point when flower THCA falls below 0.2% (w/w), a starting THCA level of ~16% (ZF cultivar studied) requires 54.6 minutes at 120 °C to reach that point (Table 6, Fig. 18). Experimentally, after 60 minutes the THCA level was 0.15 ± 0.02 % (w/w).

Table 6. Decarboxylation times required to reach flower THCA levels of 0.2% (w/w).

T (ºC)	Decarboxylation times (min)				
	14%	**16%**	**18%**	**20%**	**22%**
110	98.3	101.4	104.2	106.6	108.8
120	53.0	54.6	56.1	57.4	58.6
130	25.5	26.3	27.0	27.7	28.2

The range 14 – 22% (w/w) corresponds to THCA levels before decarboxylation ($[THCA]_0$) and represent Avextra's tested THC-dominant cultivars. Values were estimated using kinetic equations presented in Figure 18.

CBN formation should also be considered when selecting decarboxylation conditions, since oxidation of Δ^9-THC to CBN is accelerated at higher temperatures and in the presence of oxygen. Although Figure 17D indicates higher CBN levels at elevated temperatures, the maximum observed CBN under the harshest condition (130 °C, 60 min) was only 0.18%. Furthermore, when CBN concentrations are compared at the time points tested near the extrapolated decarboxylation time endpoints for flowers with THCA levels of 16% (w/w) (Table 6), the differences are negligible: 110 °C (105 min): 0.12 ± 0.01%; 120 °C (60 min): 0.13 ± 0.01%; 130 °C (30 min): 0.12 ± 0.02%. Consequently, any of the three conditions may be used with no meaningful difference in CBN formation. Furthermore, the levels of Δ^9-THC were consistently high for the three temperatures at time points near the expected decarboxylation endpoint (Fig. 17, A–C).

The release of carbon dioxide (CO_2) from the THCA molecule is critical for its conversion to Δ^9-THC. This release, along with evaporation of other volatile components such as terpenes, contributes to the reduction of extract mass. Mass loss was measured across the three temperature conditions to determine whether lower temperatures resulted in less mass loss (Fig. 19, A–C).

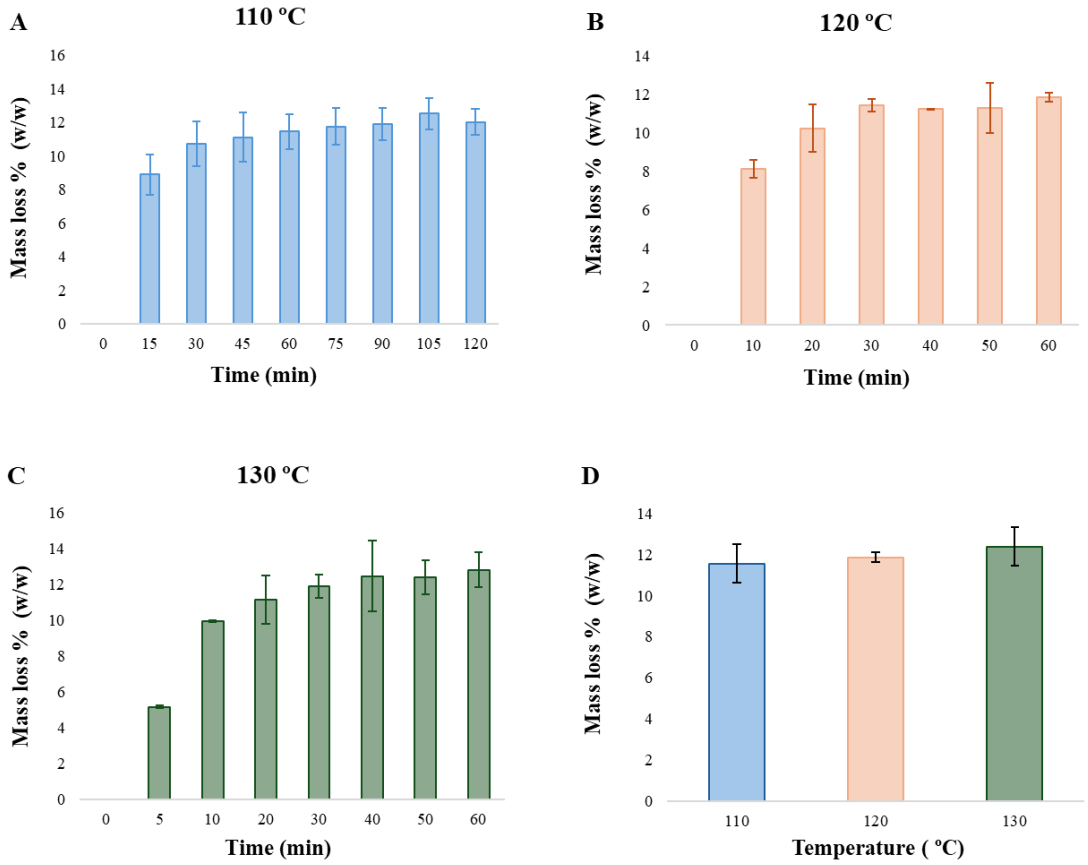


Figure 19. (A–C) Ground flower mass losses (%) over time at 110 ºC, 120 ºC, and 130 ºC (D) Mass loss at conditions: 110 ºC (105 min - 11.56 ± 0.93%), 120 ºC (60 min - 11.86 ± 0.23%) and 130 ºC (30 min – 11.89 ± 0.64%). Experiments were performed in duplicate; data corresponds to average ± standard deviation.

For the three conditions 110 °C (105 min), 120 °C (60 min) 130 °C (30 min) the mass loss was very similar between 11.56–11.89%.

Although all three conditions yielded excellent Δ^9-THC recoveries and minimal oxidative loss (CBN ≈ 0.12% w/w), and faster decarboxylation would generally be desirable, 120 °C was selected rather than 130 °C. This was because the 130 °C condition showed greater replicate variability: one replicate reached THCA levels below 0.2% only at 50 min, while the other reached that level at 30 min. Both replicates at 120 ºC showed very similar results. For this reason, the decarboxylation conditions were set at 120 ºC for 60 minutes for ZF cultivars containing 16% THCA.

5. Extraction

Extraction is a term used to describe a process that aims to isolate compounds of interest that are present in a sample matrix [134]. The use of an appropriate and optimised extraction methodology, taking into account multiple associated variables, is fundamental to the methodology's efficiency and facilitates the implementation of subsequent analytical methods for the separation and identification of the compounds [135]. It is also essential to understand the physicochemical properties of bioactive compounds and the distinctive characteristics of the matrix in order to select and optimise the most effective extraction technique [136]. Plant material, pulverisation, solvent-to-flower ratio, polarity, and temperature are factors that must be optimised. Finding the right balance between extraction time, operational parameters, and potential degradation of target compounds is essential for maximising extraction efficiency and yield (Fig. 20) [73].

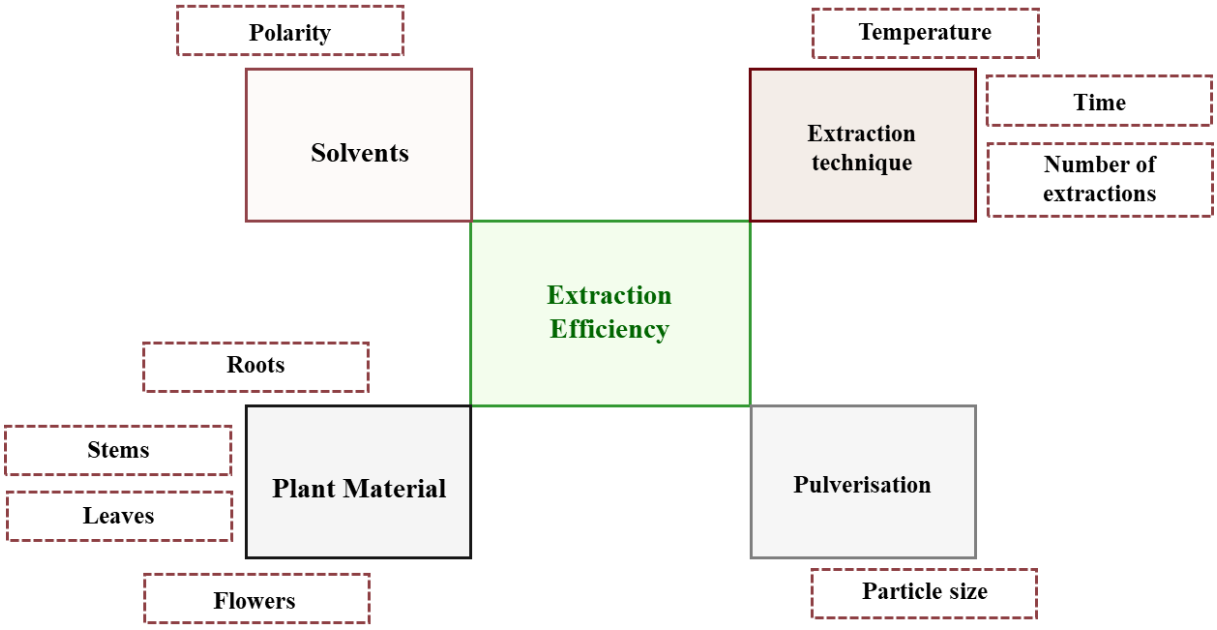


Figure 20. Factors influencing the extraction efficiency of phytocannabinoids.

Solvent extraction is a conventional method, designed to isolate compounds of interest from a matrix by leveraging a solvent for which these compounds have a selective affinity [137].
This study aims to achieve two primary goals: first, to optimise Soxhlet extraction technique on a small scale by examining the main factors that influence the process, and second, to establish a fast, reliable extraction method suitable for the routine analysis of incoming cannabis flower samples.

5.1. Plant Material

Cannabinoid concentration in the plant can be affected by the maturity of the plant, daily light, temperature and nutrient availability [138]. When plant material is analysed, extraction efficiency can vary significantly depending on which external plant structures are used —such as leaves, stems, roots, or flowers [135]. The highest percentages are in the secretory cells of glandular trichomes in flowers, while the lower concentrations are in the stems. No phytocannabinoids have been found in roots and seeds [126].

Dried flowers of the Z-Face cannabis cultivar were provided dried by Avextra Portugal's cultivation facilities. Before pulverisation, the inflorescences were cut at the base, leaving only minimal rachis to reduce variability between samples.

5.2. Pulverisation

Before implementing an extraction methodology, sample preparation is a fundamental step that directly affects the extraction, purification and detection of cannabinoids [74]. Techniques such as drying and grinding or milling play a significant role in the pulverisation and homogenization of samples [139]. By reducing particle size through pulverisation, the contact area between the matrix and the solvent is increased, ultimately leading to a more effective extraction of the target compounds [140].

The objective was to experimentally assess the influence of particle size on extraction efficacy, with the goal of improving recovery of targeted compounds. Two pulverisation equipment were tested on non-decarboxylated samples, which were then extracted according to the Ph. Eur. cannabis monograph (see section 8.6.1).

5.2.1. Retsch Knife Mill GRINDOMIX GM 200

The first equipment used in this study was the Retsch Knife Mill GRINDOMIX GM 200. Several pulverisation experiments were conducted on *Cannabis* flower, varying the frequencies (5,000–10,000rpm) and the number of cycles employed (1–6). To prevent sample overheating, each cycle was limited to 10 seconds. In a preliminary phase of the study, the degree of homogenization was assessed visually. As expected, the findings indicated that combining a higher frequency with an increased number of cycles produced a more finely pulverised and better-homogenised sample. To assess its impact on cannabinoid recovery, the two most

divergent conditions were compared: one cycle at 5,000 rpm (Table 7, entry 1) versus six cycles at 10,000 rpm (Table 7, entry 2). Furthermore, to reduce sampling variability, two replicate samples per procedure were collected from the same batch of ground flower. Entry 2 yielded higher total potential THC recovery (14.16 ± 0.20% vs. 13.44 ± 0.64%) with a lower RSD (1.39% vs. 4.74%), indicating better extraction efficiency and repeatability, most likely due to improved sample homogenization.

Table 7. Two pulverisation methods using the Knife Mill.

Entry	Frequency (rpm)	Cycle duration (seconds)	Number of cycles	%THC Total [a]			
				R1	R2	Mean ± SD	%RSD
1	5,000	10	1	13.89	12.99	13.44 ± 0.64	4.74
2	10,000	10	6	14.30	14.02	14.16 ± 0.20	1.39

[a] %THC Total = THCA × 0.877 + Δ^9-THC; SD: standard deviation; %RSD: residual standard deviation.

5.2.2. Retsch Mixer Mill MM 400

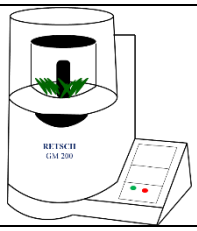
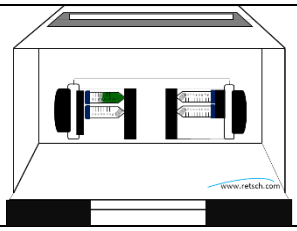
Because better extraction recoveries were obtained from the knife mill under conditions that produced smaller particles, a ball mill capable of achieving even finer ground cannabis was also evaluated.

In the Mixer Mill MM 400, samples are loaded into 50 mL microcentrifuge tubes with two 15 mm stainless-steel balls serving as grinding media. Tube oscillation causes the balls to tumble and collide, shearing the material to a finer size. The mixer can process up to eight cannabis samples simultaneously. Retsch has already established cannabis-grinding protocols [141,142], in which 4 g sample per tube were effectively pulverised for 3 minutes at a frequency of 30 Hz, yielding a particle size of 1–2 mm. They reported sample loss of only 4–5% and an RSD of 2% for Δ^9-THC recovery. Furthermore, the grinding balls are easy to clean, and the tubes are disposable.

Preliminary studies were conducted using the manufacturer's protocol [142] on 0.50 g of cannabis flower. To prevent sample overheating, the 3 minutes were divided into 12 cycles of 15 seconds each. However, some problems were encountered, including ruptured tubes that caused sample loss. To address these issues, the frequency was lowered to 25 Hz, which produced consistent, homogeneous pulverisation. This adjustment yielded an extraction of 15.11% total Δ^9-THC (w/w) from the sample, with a standard deviation of 0.59% (Table 8). Experiments in duplicate comparing the two pulverisation equipment were performed, by visual

assessment and cannabinoid quantification (Table 8). The results showed that Retsch Mixer Mill MM 400 produced a more homogeneous powder with a smaller particle size, delivered superior pulverisation efficiency, and was better suited to handling multiple samples.

Table 8. Comparison of optimised parameters and pulverisation efficiency between Retsch Knife Mill GRINDOMIX GM 200 and Retsch Mixer Mill MM 400.

	Retsch Knife Mill GRINDOMIX GM 200	**Retsch Mixer Mill MM 400**
Equipment		
Frequency	10,000 rpm	25 Hz
Cycle duration (seconds)	10	15
Number of cycles	6	12
Total run time (min)	1	3
Number of samples operation	1	8
Mean (%THC Total) ± SD [a]	13.39 ± 0.95	15.11 ± 0.59

[a] %THC Total = THCA × 0.877 + Δ^9-THC; SD: standard deviation.

5.3 Solvent extractor

Selecting the appropriate solvent is an essential parameter to achieve effective extraction, as it requires a careful balance between a strong affinity for target compounds, cost-effectiveness, and safety considerations [143]. The ability of a solvent to establish a strong molecular interaction and solubility is also a significant factor that should be considered during the solvent selection process [144]. For this project, ethanol (96% v/v) was chosen as the extraction solvent, in line with Avextra's Soxhlet extraction protocol and the Pharm. Eur. cannabis flower monograph [122]. Ethanol's lower toxicity and potential for being derived from renewable resources make it a more sustainable alternative to other organic solvents, aligning with the principles of green chemistry. This approach directly supports UN Sustainable Development

Goal 9 (Industry, Innovation, and Infrastructure), by advancing more efficient and safer processes, and Goal 12 (Responsible Consumption and Production), by minimising the use of environmentally harmful solvents and reducing the overall ecological footprint of the extraction and purification processes.

5.4. Extraction techniques

5.4.1. European Pharmacopoeia Cannabis monograph

The European Pharmacopoeia (Ph. Eur.) is a legally binding compendium of quality standards for medicines and their ingredients across Council of Europe member states, specifying how substances, dosage forms, and related materials must be prepared, tested, and labelled to ensure safety, quality and efficacy. Like many other medicinal products, the cannabis flower has its own monograph, officially published in July 2024 [70].

The monograph defines Cannabis flower as 'dried, whole or fragmented, fully developed female inflorescences of *Cannabis sativa* L. and applies to both raw material intended for extract production and the herbal medicinal product supplied for patient use. It describes a dynamic maceration extraction using ethanol (96% v/v) as the extracting solvent, and a HPLC–UV analytical method for quantifying five cannabinoids: CBD, CBDA, CBN, Δ^9-THC and THCA. No recommendations about the pulverisation procedure are made, except that only cut or milled herbal material that has not been sieved should be used. For simplicity, dynamic maceration was performed in this project using a magnetic stirrer. Briefly, 0.50 g of ground material was weighted into a 50 mL screw-cap centrifuge tube. 40 mL of ethanol (96% v/v) was added, and the mixture was agitated for 15 minutes. After centrifugation, the clear supernatant was transferred to a 100 mL volumetric flask. The extraction was repeated twice, using 25 mL of ethanol (96% v/v) each time. After combining all supernatants, the flask was filled to 100.0 mL with the same solvent (Fig. 21).

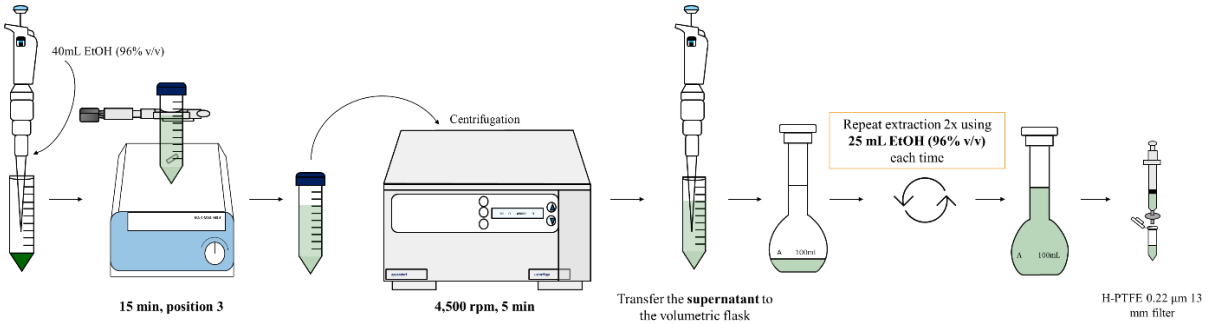


Figure 21. Illustration of the European Pharmacopoeia Cannabis extraction protocol.

In the present study, this protocol was used as the benchmark extraction method for analytical purposes and as a reference for newly developed extraction methods. The ZF cultivar was extracted with this method, and the results are shown in Table 9 (entry 1).

Table 9. Comparison of the cannabinoid profile of the ZF-cultivar obtaining from different extraction methods: Ph. Eur. extraction (entry 1), Ball Mill extraction (entry 2) and Soxhlet extraction (entry 3).

Entry	**1**	**2**	**3**
Compounds	**Ph. Eur. extraction** [a]	**Ball Mill extraction** [a]	**Soxhlet extraction** [a]
CBDV	<0.004% [b]	<0.004% [b]	<0.004% [b]
CBD	<0.004% [b]	<0.004% [b]	<0.004% [b]
CBG	0.03	0.04	0.11
THCV	<0.006% [b]	<0.006% [b]	<0.006% [b]
CBDA	0.01	0.02	0.06
CBGA	0.11	0.16	0.29
CBN	0.02	0.02	0.01
Δ^9- THC	0.87	1.19	1.13
Δ^8- THC	<0.006% [b]	<0.006% [b]	<0.006% [b]
THCVA	0.05	0.05	0.05
CBC	0.02	0.01	0.15
CBNA	0.14	0.08	0.19
THCA	15.2	16.5	15.8
CBCA	0.10	0.20	0.44

[a] Compound Extraction Yield = w compound /w flower sample × 100 (sample dilutions analysed by HPLC according to section 8.2.3). [b] %DL in the flower.

5.4.2 Ball Mill Extraction

Despite being an official method, the Ph. Eur. extraction procedure is quite time-consuming. Therefore, the goal was to create a quick extraction method to analyse flowers from new cultivars arriving at the laboratory, one that eliminates multiple extraction steps. If this method matched the efficiency of the Ph. Eur. procedure, it would become the standard approach, and it would be also used for decarboxylation studies (see section 4).

Since the best pulverisation results were obtained using a ball mill (Retsch Mixer Mill MM 400), an attempt was made to develop an extraction method using the same equipment. If successful, this would allow two consecutive operations in the same vessel (a 50 mL centrifuge

tube), requiring only a single weighing before pulverisation and eliminating a second weighing before extraction. This would reduce sample requirements and shorten total processing time. The optimisation process focused on the solvent-to-powder ratio, operational frequency, and extraction duration. Initial frequency experiments indicated that higher frequencies (25–30 Hz) raised the likelihood of centrifuge tube rupture; consequently, subsequent tests were conducted at a lower frequency of 5 Hz for 10 minutes to prevent visible damage. Extractions were performed in 50 mL centrifuge tubes with two 15 mm stainless-steel balls (as used for pulverisation). To prevent solvent leakage, tube lids were sealed with Parafilm®. With these conditions, no leakage or tube rupture was observed. Briefly, 0.50 g of ground flower was loaded into a 50 mL microcentrifuge tube containing two stainless-steel balls. 25 mL of ethanol (96% v/v) was added, and extraction was carried out at 5 Hz for 10 minutes (Fig. 22).

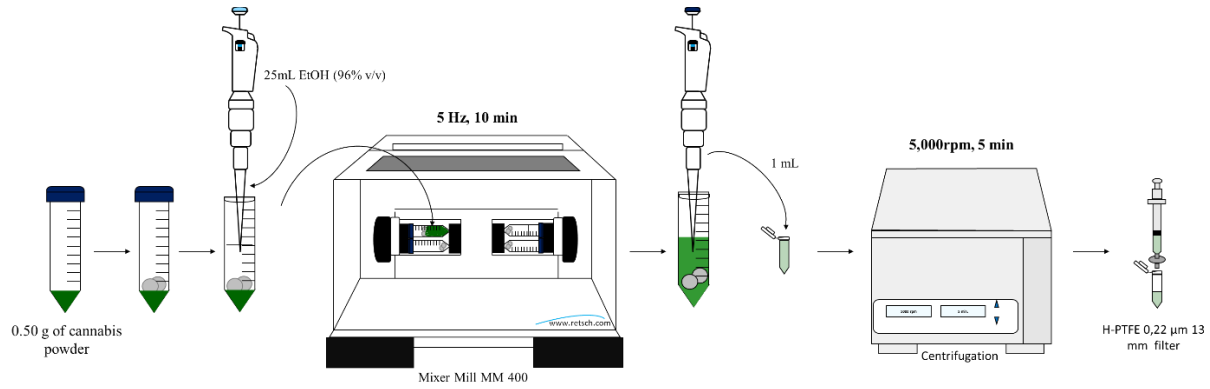


Figure 22. Illustration of the Ball Mill extraction protocol.

To assess whether the new extraction method was comparable to the Ph. Eur. cannabis flower monograph method, two parallel experiments were performed using decarboxylated samples from the same batch of pulverised cannabis to ensure that the extraction values were not affected by sample-related variability (Table 10). Ball-mill extraction yielded 16.43 ± 0.59% total THC (entry 2), compared with 15.16 ± 1.67% from the Ph. Eur. method (entry 1). The increased recovery of Δ^9-THC, along with a lower RSD from ball mill extraction, established it as the standard protocol for pulverisation and analysis of new samples. Additionally, this method also increased the recovery of minor cannabinoids (Table 9, entry 2 vs. entry 1).

Table 10. Comparative analysis of the Δ^9-THC and total THC percentages achieved with pharmacopoeia extraction versus ball mill technique.

Entry	Extraction Technique	Mean (%Δ^9-THC) ± SD [a]	Mean (%THC Total) ± SD [b]
1	Ph. Eur.	15.04 ± 1.98	15.16 ± 1.67
2	Ball Mill	16.20 ± 0.49	16.43 ± 0.59

[a] %Δ^9-THC = w compound / w sample × 100. [b] %THC Total = THCA × 0.877 + Δ^9-THC. SD: standard deviation.

5.4.3 Soxhlet Extraction

Soxhlet is a method that effectively solubilises compounds from a specific matrix using a selected solvent combined with a controlled temperature and a condenser [145]. There is a linear relationship between temperature and reaction kinetics: higher temperatures increase the solubility of target compounds in the solvent while reducing the viscosity and surface tension, thereby facilitating efficient extraction. Technically, the Soxhlet extraction apparatus consists of a distillation flask (Fig. 23 (4)) where the extracting solvent is added and heated in an oil bath (5); a thimble (3) placed in the main chamber of the Soxhlet extractor (2), loaded with plant material; and a condenser (1) on top of the extractor. The solvent evaporates and condenses continuously into the main chamber of the Soxhlet, gradually filling it while in contact with the plant material. When the freshly condensed solvent reaches the top of the siphon, it discharges the solution into the distillation flask, initiating a new cycle until the analyte of interest is completely extracted [146].

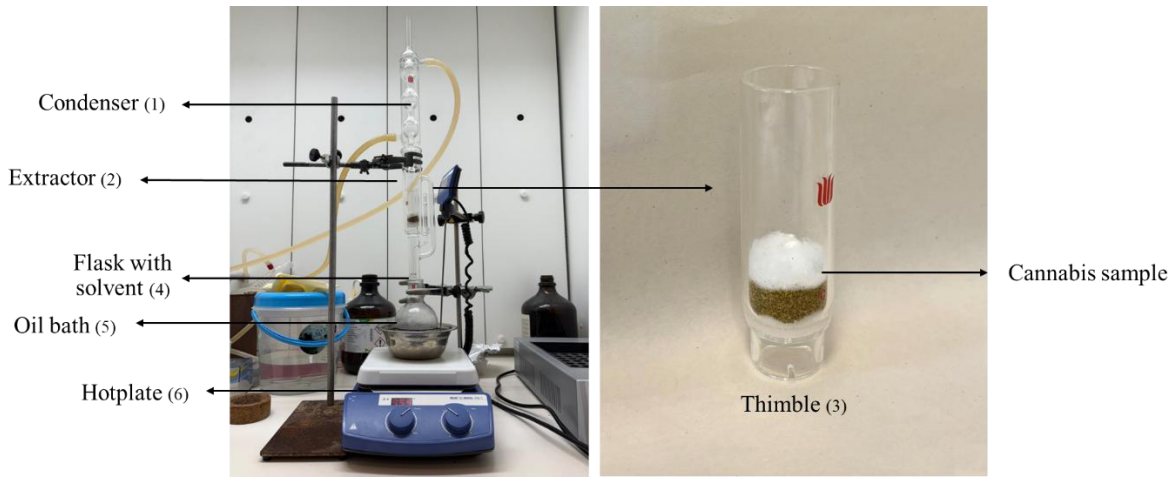


Figure 23. Components of the Soxhlet apparatus.

For optimisation of the Soxhlet extraction process at a small laboratory scale, the focus was on adjusting three crucial, interrelated parameters: oil bath temperature, cycle duration, and the total number of extraction cycles. Results were evaluated by both qualitative assessments (colour changes of the extracting solvent observed during each cycle) and quantitative analyses using HPLC to determine Δ^9-THC and THCA recoveries. The goal was to achieve an efficient cannabinoid extraction procedure from decarboxylated cannabis powder while reducing total extraction time.

The boiling point of the extraction solvent, ethanol (96% v/v), is approximately 78 °C at atmospheric pressure. Because this temperature cannot be changed, the only way to influence the Soxhlet system's thermal performance is by adjusting the oil-bath temperature. Although the temperature inside the extraction flask remains close to the solvent's boiling point regardless of the heating-plate setting, increasing the oil-bath temperature accelerates the onset of boiling and reflux, increases solvent flow through the system, and shortens each cycle. Furthermore, an oil bath on an agitation plate fitted with a temperature sensor was used rather than a conventional mantle because it provides substantially better temperature control (Fig. 23).

To assess the effect of temperature, a range of temperatures (110 °C–140 °C) was applied to analyse its impact on cycle duration and the number of cycles required for complete extraction. To simplify the procedure during optimisation, all experiments were conducted with 1.00 g of non-decarboxylated pulverised cannabis and extracted with 70 mL of ethanol (96% v/v).

For each temperature tested two sets of experiments were performed: one to assess cannabinoid recovery per cycle and another for continuous extraction to validate the first experiment. To evaluate cannabinoid recovery after each cycle, the Soxhlet extraction was stopped when the siphon discharged (signalling the end of the cycle). The extractor was washed, and the extraction was then restarted to run the next cycle with fresh ethanol (96% v/v) in the distillation flask. Figure 24 shows the four cycles require to achieve virtually complete cannabinoid recovery at 125 ºC.

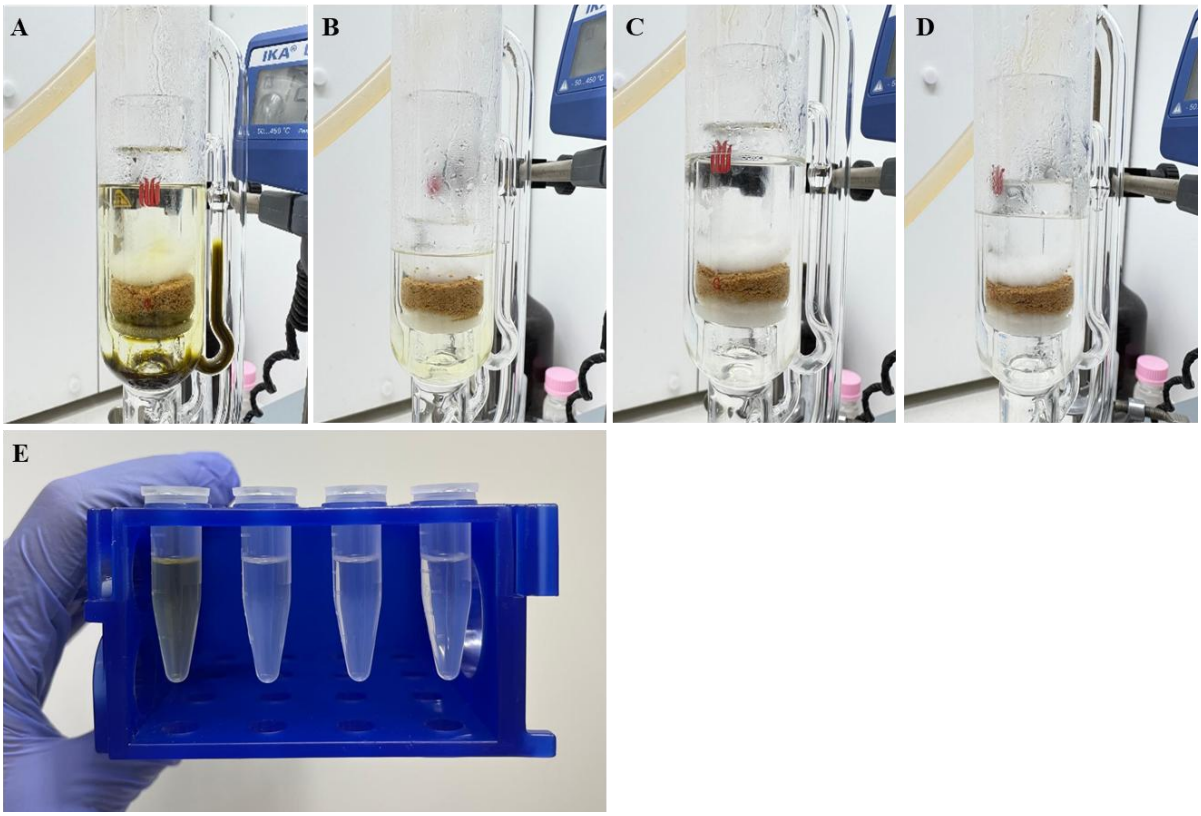


Figure 23. Qualitative assessment of extract colour from Soxhlet experiments at 125 °C: (A) first cycle, (B) second cycle, (C) third cycle and (D) fourth cycle. (E) Extracts from cycles 1–4, arranged left to right in 1.5 mL microcentrifuge tubes.

Table 11. Results of THC Total (%) and THC Recovery (%) per cycle at 125 ºC.

Entry	Number of cycles	Time of each cycle (approx.)	%THC Total [a]	%THC Recovery [b]
1	**1st**	29	15.35	99.62
2	**2nd**	28	0.04	99.90
3	**3rd**	28	0.01	99.98
4	**4th**	30	0.002	100.00

[a] %THC total = THCA × 0.877 + Δ^9-THC. [b] The recovery of THC is calculated as a percentage using the formula: % THC Recovery = % THC Total / % THC Total (condition 100% - sum of all cycles) × 100.

In the context of cycle optimisation, post-cycle washing of the extractor body was implemented to ensure thorough cleaning between cycles. Findings showed that two extraction cycles at 125 °C were sufficient for complete cannabinoid extraction (Table 11). However, during the continuous extraction process, it became clear that the discharge after each cycle was incomplete, which meant that additional cycles were needed for adequate washing of the extractor. Given the correlation between colour intensity and quantitative assessments, a total of four cycles was determined necessary to effectively remove the extracted compounds within

the syphon (Fig. 24, A–D). The qualitative assessments were corroborated by the HPLC results, showing 99.98% THC recovery from the extract in the 3rd cycle (Table 11, entry 3) and 100% with the last cycle of extraction (Table 11, entry 4). The time necessary to complete 4 cycles was found to be appropriate for setting up the continuous experiments. This correlation was observed and applied across the range of temperatures used.

Table 12 presents the results of a continuous Soxhlet extraction at 125 ºC and 140 ºC. Both extractions temperature required 120 minutes to complete, yielding similar THC recovery.

Table 12. Optimisation of Soxhlet extraction for cannabinoids from Cannabis sativa, comparing two methodologies based on temperature, cycles, time per cycle, total extraction time, and THC Total (expressed as % (w/w)).

Temperature	Number of cycles	Time per cycle (min)	Total time of extraction (min.)	%THC Total [a]
125 ºC	4	30	120	16.37
140 ºC	6	20	120	15.95

[a] %THC Total = THCA × 0.877 + Δ^9-THC.

Reported data confirmed that while a higher Soxhlet temperature (140 ºC) resulted in a faster cycle time (of 20 minutes), it did not lead to a reduction in the total extraction time, as more cycles were required to achieve complete total THC extraction. Since both required the same extraction time, the lower temperature of 125 ºC was used for purification studies. This temperature contributes to operational safety and component longevity by reducing thermal stress on the heating plate and the Soxhlet apparatus. Compounds characterization in the flower using Soxhlet extraction at 125 ºC are reported in Table 9 (entry 3).

6. Purification

Cannabis-based products, such as cannabis oils, vaporisation formulations, emulsions, food additives, and oral sprays, can utilise either oil or solvent extracts during their manufacturing process. Furthermore, depending on the intended formulation, the extracts may undergo additional purification steps before incorporation into the final product [147]. These processes enhance the quality of the final product, making it more appealing to consumers. In the context of extraction techniques, ethanol is a commonly used solvent, characterised by its polar properties and safety profile, and is effective in dissolving both cannabinoids and terpenoids. However, it also extracts undesired components from the cannabis plant, such as chlorophyll and waxes [148].

In the present work, after Soxhlet extraction of decarboxylated ground cannabis flower, the resulting extract underwent purification steps: winterization to remove lipids and waxes [149], and decolourisation with activated charcoal to improve the colour and purity of the extract by removing chlorophyll and other pigments [150].

6.1 Winterization

Winterization is a purification technique that focuses on removing unwanted lipids and waxes from an extract. It involves three key steps (Fig. 25). First, the extract is dissolved in a suitable polar solvent to maintain the solubility of cannabinoids while allowing waxes and other impurities to precipitate and/or solidify during the subsequent freezing step. Next, the extract is exposed to low temperatures, typically between -20 ºC [151] and -80 ºC [148]. Finally, it undergoes filtration to separate the impurities from the desired cannabinoid solution.

For this study, 0.3 g of dry extract in a 50 mL centrifuge tube was dissolved in 10 mL of ethanol (96% v/v) and then placed in a freezer at -80 ºC for 24 hours. This solvent-to-extract ratio is ten times lower than the ratio used during extraction. The reduction of this ratio, along with winterization, will help decrease the solubility of impurities, thereby enhancing efficacy. Additionally, using -80 ºC ensures that there is sufficient time for the filtration process to occur without significant resolubilisation. Nevertheless, all equipment and materials were kept on ice, and -80 ºC ethanol (96% v/v) was used to wash the system.

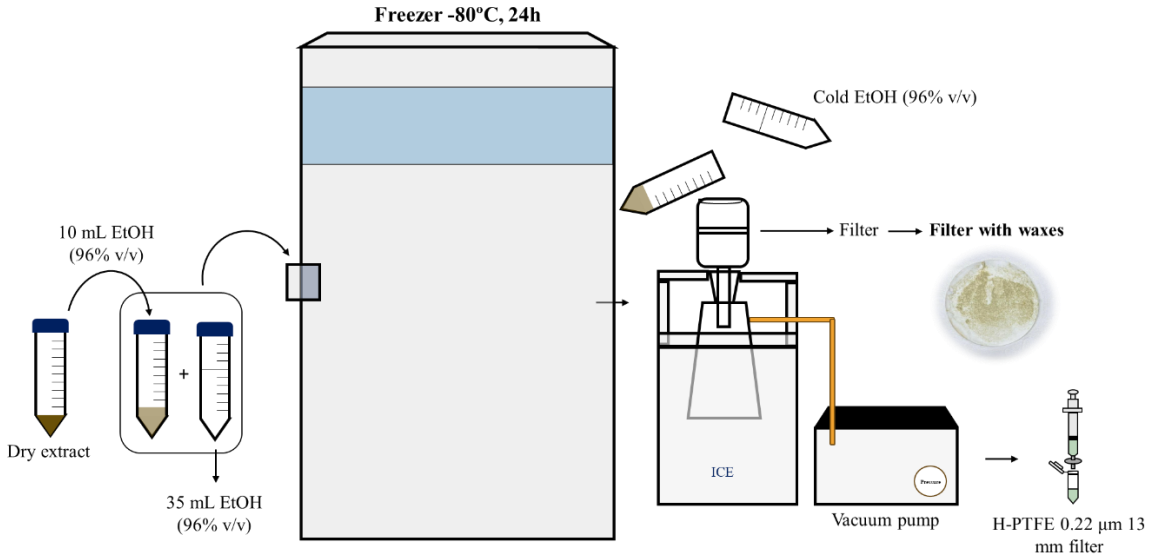


Figure 25. Illustration of the winterization protocol, including the filter paper obtained after filtration.

The effectiveness of winterization was evaluated on two dry decarboxylated extract samples - W1 and W2. To determine the mass and percentage of plant material removed from the extract, both the extract and the filter, before and after the winterization process, were weighed. Additionally, the percentage of Δ^9-THC and its relative increase in the extract were assessed using HPLC analysis (Table 13).

The winterization protocol led to a decrease in the weight of the cannabis extract, with Figure 25 providing a visual assessment of the extract components retained in the filter.

The data presented in Table 13 indicate that the winterization process increased Δ^9-THC concentrations by an extra 0.9–2%%, with an extract loss between 4.2% and 12.2%; however, this also corresponded to a decrease in total THC mass of 0.6% to 10.7%. Although more studies should be performed to assess this winterization process and understand the differences observed between experiments W1 and W2, this process led to the desired purification of the extracts. These differences are probably due to the small sample size, which complicates proper temperature control during filtration.

Table 13. Comparative analysis of %Δ^9-THC and %Δ^9-THC concentration increase and loss, before and after winterization, for samples W1 and W2.

Samples	Winterization	W extract (mg)	W paper filter (mg)	Δ^9-THC% [a]	W (Δ^9-THC) [b] (mg)
W1	Before	294.9	357.3	49.9	147.4
	After	282.5	380.6	51.9	146.5
	Difference	12.4 (-4.2%)	23.3	2	0.9 (-0.6% [c])
W2	Before	304.7	344.5	55.0	167.6
	After	267.6	362.4	55.9	149.7
	Difference	37.1 (-12.2%)	17.9	0.9	17.9 (-10.7% [c])

[a] %Δ^9-THC = w compound / w sample × 100. [b] W (Δ^9-THC) = w extract × Δ^9-THC Extraction Yield / 100. [c] Percentual Δ^9-THC loss = Difference W (Δ^9-THC) / Initial W (Δ^9-THC) × 100.

6.2 Activated Charcoal

One consequence of ethanol extraction is the formation of dark green extracts, primarily due to chlorophyll. In addition to altering the colour, chlorophyll imparts a bitter taste, making its removal a priority during post-extraction refinement [150]. This is frequently achieved by adding activated charcoal to the extract, followed by filtration after a specific period of exposure. This form of carbon, is characterised by a microcrystalline structure that provides a substantial surface area and high porosity, giving it powerful adsorptive properties [152]. The adsorptive characteristics of activated charcoal are influenced by its surface chemistry, featuring functional groups such as carbonyls and hydroxyls. Furthermore, factors including the percentage of activated charcoal, contact time, and temperature can significantly affect the efficiency of its adsorption performance [153]. Nevertheless, preserving cannabinoids while removing chlorophyll-induced colour remains a challenge due to the non-specific adsorptive nature of activated charcoal. In fact, depending on the conditions, its use can result in a significant reduction in cannabinoid content of about 50% [154].

Therefore, the primary goals of this extract purification step were to establish optimal activated charcoal conditions to promote decolourisation and minimise Δ^9-THC loss. To achieve this, three variables were investigated: activated charcoal percentage (w/w relative to dry extract), temperature, and contact time. Two extract solution (100 mL/0.322 g of dry non-decarboxylated extract and 100 mL/ 0.308 g of dry decarboxylated extract) were prepared in ethanol (96% v/v) and divided into several aliquots to allow for the testing of various conditions using samples from the same extracts. Activated charcoal was then added to 5 mL aliquots, and the mixture

was stirred or shaken during the experiments to enhance contact between the charcoal and the extract components. The removal of pigmented compounds was assessed by visual colour inspection (qualitative assessment), and the recovery of THCA and Δ^9-THC was monitored using HPLC analysis. Preliminary experiments were conducted using non-decarboxylated extract (temperature and the first study of activated charcoal percentages). Subsequently, for further fine-tuning experiments, decarboxylated extract was used (the second study of activated charcoal percentages and time). Both extracts were obtained through Soxhlet extraction.

6.2.1 Percentage of activated charcoal

A series of experiments was conducted at room temperature (20 °C) for 1 hour to determine the optimal amount of activated charcoal needed to lighten the extract colour.
For the first group of experiments, six 5 mL ethanolic non-decarboxylated extracts (0.016 g) were prepared, with varying percentages of activated charcoal (w/w): 10%, 50%, 100%, 200%, and 1000%. A reduction in the colour of the extract solution was observed as the percentages of activated charcoal increased, with 50% displaying a very light colour and virtually no colour was present at 100% for the tested dilutions (Fig. 26A). As expected, with the increase in the percentage of activated charcoal, the content of THCA and Δ^9-THC in the extract declined, with a more significant reduction observed at higher concentrations, particularly at 100, 200 and 1000% of activated charcoal (Fig. 26B). In Table 14 (entries 1–6), the condition with 1000% activated charcoal (entry 6) resulted in a significant decrease in total Δ^9-THC recovery with only 12% of the compound detected in the solution. In comparison, the 50% activated charcoal condition (entry 3) displayed a loss of 7.31% of total Δ^9-THC, while the 100% condition (entry 4) showed a higher loss of 14.93%. Therefore, since 50% and 100% activated charcoal promoted a significative visual decolourisation, a subsequent study was performed in duplicate using a decarboxylated extract sample, focused on a narrower range: 20%, 40%, 50%, 60%, and 70%. During the previous experiments, a residual amount of activated charcoal was still observed when higher percentages were used. Therefore, from this point forward, colour comparison was performed after a second filtration using an H-PTFE 0.22 µm filter. The results indicated that percentages of 20% and 40% were insufficient to remove chlorophyll, as the extract still exhibited a darker green colour (Fig. 26C), although less so compared to the 0% condition (Fig. 26A). In contrast, the higher percentages of 50%, 60%, and 70% revealed a very light colour in the qualitative assessment (Fig. 26C). Analytical results, presented in Table 14 (entries 7–12), showed a slightly decrease in the recovery of Δ^9-THC in the extract as the

percentage of activated charcoal increased (Fig. 26D). Additionally, the difference in the percentage loss of Δ^9-THC was minimal among conditions that exhibit a lighter green colour (2.6-7.0% for 50-70%). 50% activated charcoal, showing a Δ^9-THC recovery of 97.4 ± 0.42%, was the selected condition to proceed with another studies.

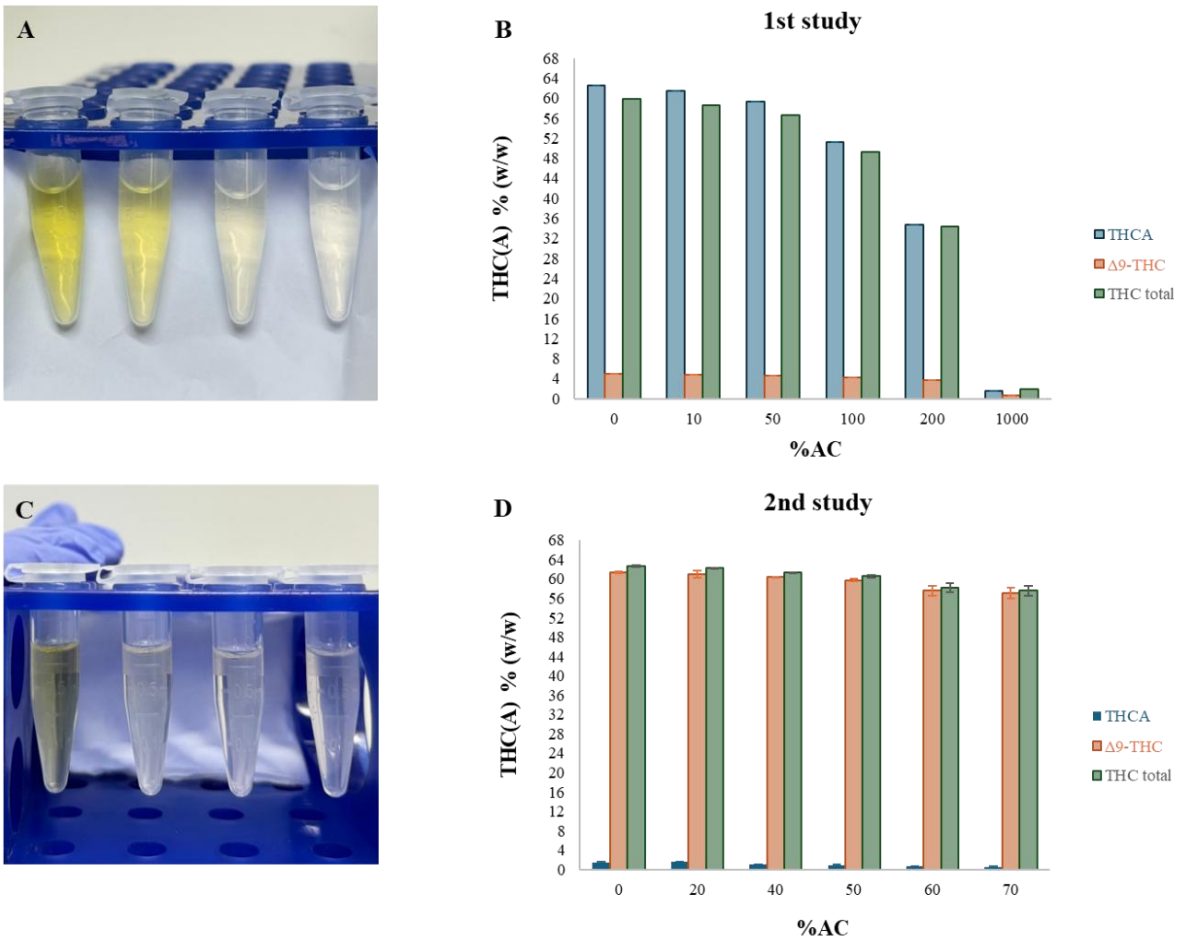


Figure 26. Effect of activated charcoal concentration on chlorophyll removal and in Δ^9-THC, THCA and THC total percentages. (A, C) Qualitative assessments of the 1st (0%, 10%, 50%, and 100%) and 2nd (40%, 50%, 60% and 70%) study, respectively, with different activated charcoal percentages. (B, D) Δ^9-THC, THCA and THC total content in the extract, expressed as % (w/w), over the percentage's conditions applied in the 1st and 2nd study, respectively. Experiments of the second study were performed in duplicate; data corresponds to average ± standard deviation.

Table 14. Total Δ^9-THC (%) and Δ^9-THC recovery (%) according to the percentage of activated charcoal used, for the 1st and 2nd studies.

1st study				2nd study			
Entry	**%AC**	**%THC Total** [a]	**%THC Recovery** [b]	**Entry**	**%AC**	**Mean (%Δ^9-THC) ± SD** [c]	**Mean (%Δ^9-THC Recovery) ± SD** [d]
1	0%	6.43	100	7	0%	61.38 ± 0.26	100
2	10%	6.06	94.1	8	20%	60.93 ± 1.19	99.2 ± 1.19
3	50%	5.96	92.7	9	40%	60.35 ± 0.17	98.3 ± 0.17
4	100%	5.47	85.1	10	50%	59.76 ± 0.42	97.4 ± 0.42
5	200%	4.88	75.8	11	60%	57.55 ± 1.66	93.7 ± 1.66
6	1000%	0.81	12.5	12	70%	57.09 ± 1.99	93.0 ± 1.99

[a] %THC Total = THCA × 0.877 + Δ^9-THC. [b] The recovery of THC is calculated as a percentage using the formula: %THC Recovery = %THC Total/ %THC Total (condition 0%) × 100. [c] % Δ^9-THC = w compound / w sample × 100. [d] The recovery of Δ^9-THC is calculated as a percentage using the formula: % Δ^9-THC Recovery = %Δ^9-THC/ % Δ^9-THC (condition 0%) × 100. AC: Activated Charcoal. SD: standard deviation.

6.2.2 Temperature

These experiments were conducted after the initial study of activated carbon percentage, in which a 100% w/w treatment for 1 hour at room temperature was effective in reducing the colour of the extract (Table 14, entry 4; Fig 26A). Using these conditions, the effect of temperature on THCA and Δ^9-THC recovery and chlorophyll removal was assessed by testing three different temperatures: room temperature (~20 ºC), 30 °C, and 40 °C.

Room temperature experiments utilised a stirrer on an agitation plate, while the 30 °C and 40 °C experiments were conducted in an orbital shaker-incubator. The 0% condition experiment, in which no activated charcoal was added, served as the baseline for THCA and Δ^9-THC content to calculate the percentage of total THC recovery (Table 15, entry 1). After 1 hour, all samples exhibited no colour, indicating the absence of pigmented compounds under all three conditions (Fig. 27A). HPLC analysis revealed that the recovery of total THC decreased as the temperature increased: recovery at 30 °C was 9.39% (Table 15, entry 3) lower than at room temperature and decreased by an additional 16.81% at 40 °C (Table 15, entry 4, Figure 27B). As the temperature increases, there is a decrease in the levels of THCA, Δ^9-THC and total THC (Fig. 27B). In conclusion, although visual inspection showed that all conditions led to decolourisation, the condition that promoted the least cannabinoid loss (room temperature) was used for the subsequent experiments.

Table 15. THC total (%) and THC recovery (%) according to room temperature, 30ºC and 40ºC conditions.

Entry	Temperature (ºC)	%THC Total [a]	%THC Recovery [b]
1	Condition 0	53.92	100
2	Room temperature (~20)	49.28	91.39
3	30	44.22	82.00
4	40	40.22	74.58

[a] %THC Total = THCA × 0.877 + Δ^9-THC% [b] The recovery of THC is calculated as a percentage using the formula: %THC Recovery = %THC Total / %THC Total (Condition 0) × 100. Condition 0: condition without activated charcoal treatment.

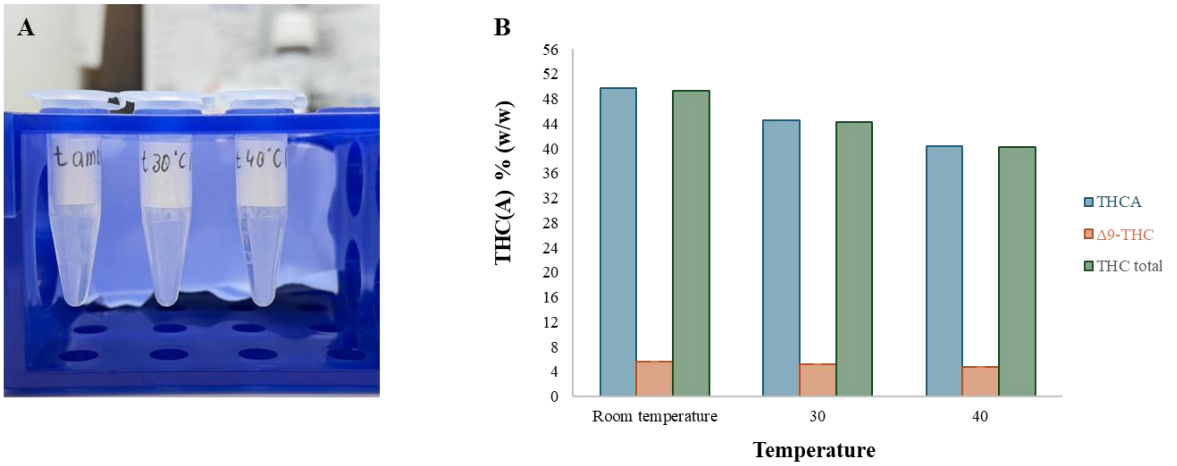


Figure 27. Effect of temperature on chlorophyll removal and in Δ^9-THC, THCA and THC total percentages. (A) Qualitative assessment at room temperature (~20 ºC), 30 ºC, and 40 ºC conditions, with transparent colour visualised for each extract solution. (B) Δ^9-THC, THCA and THC total content in the extract, expressed as % (w/w), over the temperature conditions applied.

6.2.3 Time

Based on previous findings, a 50% concentration of activated charcoal at room temperature for one hour resulted in an extract solution displaying a very light green-yellow colour for the specified extract concentration. To explore the influence of time on the removal of chlorophyll, a total of eight experiments were conducted, varying in duration from 0.5-hour to 4.5-hours, with 30-minute intervals in between.

Qualitative assessments, in accordance with prior tests, showed that in the one-hour condition, the extract solution exhibited a tenuous green-yellow colour, with forward conditions (0.5-4.5 hours) showing a gradual decolouration (Fig. 28A).

Interestingly, Δ^9-THC recovery was not substantially affected by increasing time exposure; it ranged between 94% and 100% recovery (Table 16). Based on the results, 50% activated charcoal for one hour at room temperature was selected as the optimal condition for effectively

removing pigment compounds from approximately 5mL of ethanolic extract solution (16.13 mg), with minimal loss of Δ^9-THC (0.8 ± 0.32%).

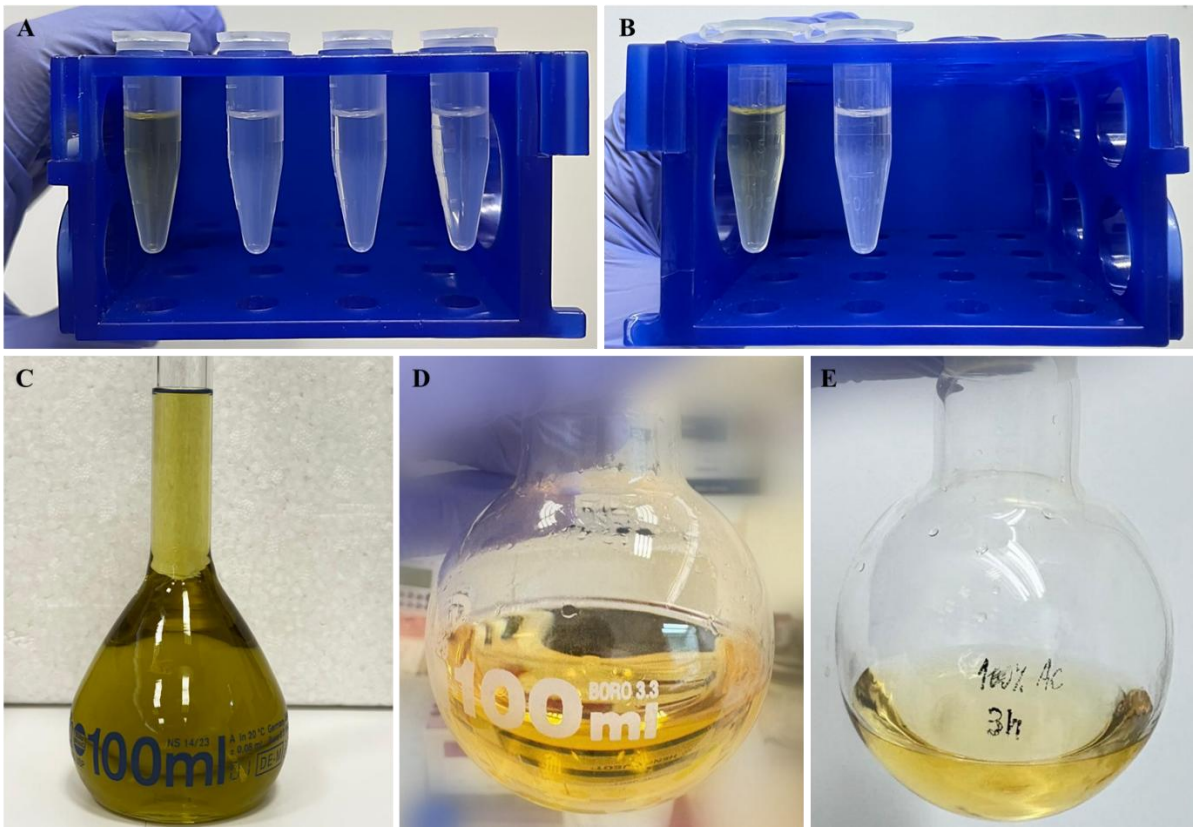


Figure 28. (A) From left to right: Extracts after 0.5, 1, 1.5 and 2 hours exposure to 50% activated charcoal, showing a darker green solution at 0.5 hours and already a tenuous colour at 1 hour. (B) Colour comparison between 0% activated charcoal (green, left microcentrifuge tube) and 100% activated charcoal after 3 hours (clear solution, right tube). (C) Extract solution (100 mL / 0.3 g) prepared post-winterization and before the addition of activated charcoal. (D) Concentrated extract solution after treatment with 50% activated charcoal for 1 hour. (E) Concentrated extract solution after treatment with 100% activated charcoal for 3 hours.

Table 16. Δ^9-THC (%) and Δ^9-THC recovery (%) results according to the time of extract solution exposure to 50% of activated charcoal.

Time (h)	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5
%Δ^9-THC [a]	61.2	59.8	60.6	58.0	59.7	58.3	58.8	59.0	60.1	59.3
%Δ^9-THC Recovery [b]	100	97.7	99.2	94.8	97.5	95.4	96.2	96.4	98.3	97.0

[a] %Δ^9-THC = w compound / w sample × 100. [b] The recovery of Δ^9-THC is calculated as a percentage using the formula: %Δ^9-THC Recovery = %Δ^9-THC / %Δ^9-THC (Condition 0) × 100.

All these experiments were conducted using a small amount of extract solution (5 mL per approximately 0.016 g of extract) in microcentrifuge tubes. Therefore, to determine wthether this level of colour reduction could be observed on a larger scale, the optimal conditions were applied to a scale 20 times greater, maintaining the same dilution (Fig. 28C). Additionally, after

filtration, the extract was concentrated to evaluate its colour. The findings indicated that this condition was successful in eliminating the green pigmentation associated with chlorophyll; however, it still displayed a distinct orange-yellow colour (Fig. 28D). To verify whether this colour could be reduced, a higher percentage of activated charcoal (100%) was tested with increasing exposure times (Table 17).

The results indicated only a slight decrease in the colour of the final extract solution (Fig. 28, D to E). Interestingly, for 100% activated charcoal, a reduction in Δ^9-THC was observed over time (Table 17), contrary to what was previously observed with 50% (Table 16). A decrease of 8.8% Δ^9-THC was observed during 1-hour experiment (Table 17, entry 3). Extended exposure times were associated with greater Δ^9-THC losses, recorded at 9.7% after 2-hour (entry 4) and rising to 19.2% after 3-hour (entry 5). The qualitative and quantitative analyses led to two primary conclusions: an increase in the proportion of activated charcoal (100% vs. 50%) and extended exposure time (2 and 3 hours vs. 1 hour) did not result in a significant improvement in the colour of the final extract. However, these changes were accompanied by a decrease in the concentration of Δ^9-THC. Therefore, the optimal conditions identified for this process are the application of 50% activated carbon for one hour at room temperature, resulting in less than 3% Δ^9-THC loss (entry 2). Although this study's optimisation established good condition for using activated charcoal in the purification of a THC-dominant cultivar, future research could explore the use of different types of activated carbon or filtering the extract through an activated carbon column.

Table 17. Δ^9-THC recovery (%) according to the different conditions tested in the complete extract.

Entry	Conditions	%Δ^9-THC Recovery [a]
1	Condition 0	100
2	50% AC – 1-hour	97.9
3	100% AC – 1-hour	91.2
4	100% AC – 2-hour	90.3
5	100% AC – 3-hour	80.8

[a] The recovery of Δ^9-THC is calculated as a percentage using the formula: %Δ^9-THC Recovery = %Δ^9-THC / %Δ^9-THC (Condition 0) × 100.

7. Conclusion and Future Perspectives

Cannabis sativa L. has become a widely cultivated plant. The inflorescences, resins, and oils derived from this plant are utilised for both medicinal and recreational purposes, primarily due to the effects associated with the cannabinoids CBD and the psychoactive Δ^9-THC. Nevertheless, the increase in cannabis abuse have led to regulatory restrictions in numerous countries. Consequently, research aimed at developing analytical and extraction techniques for cannabinoid profiling, particularly for Δ^9-THC, has intensified.

Alcoholic extraction techniques followed by HPLC-UV quantification are among the most utilised methodologies for analysing cannabis materials. These methods are critical in two key domains: the pharmaceutical industry, for the development of medicinal products, and forensic toxicology, for screening illicit psychoactive substances. This study aimed to optimise cannabis extraction and refinement processes, as well as analytical quantification techniques, to enhance the efficiency, sustainability, and reliability of cannabinoid analysis for both applications.

An accurate and robust HPLC-UV method – CannProVar method A – was successfully optimised for the characterisation of 14 cannabinoids in THC-dominant cannabis flower samples. Chromatographic separation was achieved on an InfinityLab Poroshell 120 EC-C18 (3.0 x 150 mm, 2.7 µm) column. The method utilised a gradient elution with a mobile phase consisting of MeOH with 0.05% FA and deionised H_2O with 0.1% FA mixture, at a flow rate of 0.5 mL/min, a column temperature of 30 ºC, and a run time of 30 minutes. Additionally, a rapid 12-minute HPLC method – CannProVar method B – was developed with the same stationary and mobile phases in a different gradient setting, with a flow rate of 0.75 mL/min and a column temperature of 50 ºC. This method was optimised for the detection of Δ^9-THC, THCA, and CBN, allowing for the evaluation of their variations throughout the sample processing stages, namely decarboxylation and refinement procedures. Both analytical methods were validated according to ICH Q2 guidelines, confirming their specificity, accuracy (within ±15% bias), precision (≤10% RSD), and linearity (R^2 ≥0.99).

The studied Z-face strain contains: 0.04% CBG; 0.02% CBDA; 0.16% CBGA; 0.02% CBN; 1.19% Δ^9-THC; 0.05% THCVA; 0.01% CBC; 0.08% CBNA; 16.49% THCA; and 0.20% CBCA. Additionally, CBDV, CBD, THCV, and Δ^8-THC were not detected in the flower.

Significant insights were gained throughout the entire cannabis sample processing, resulting in more efficient and sustainable methodologies. Most importantly, it was possible to understand how each variable can influence the process involved. The decarboxylation process was optimised at 120 °C for 60 minutes, resulting in a final extract containing only 0.15 ± 0.02% (w/w) THCA and minimal CBN formation (0.13 ± 0.01%) for cultivars with 16% THCA. The Soxhlet extraction method was optimised to achieve complete Δ^9-THC recovery within 2 hours at 125 °C. Increasing the hotplate temperature to 140 °C did not result in any improvement in total extraction time, thereby confirming the effectiveness of the optimised conditions. Furthermore, a rapid extraction method was also developed that combines pulverisation (3 minutes) and extraction (10 minutes) in a single vessel and equipment (ball mill) to facilitate analytical measurements, resulting in a higher total Δ^9-THC yield (16.43 ± 0.59%) compared to the reference method, Ph. Eur. (15.16 ± 1.67%). This novel approach, in comparison to Ph. Eur., also operated with a significant reduction in solvent extractor volume – 75% less – which aligns with green chemistry principles by lowering operational costs and minimising environmental impact. Finally, the optimised refinement process, which includes the application of winterization (-80 ºC, 24 h) and activated charcoal (50%, room temperature, 1h), successfully removed undesirable waxes and pigments from the extract.

After optimising all these processes, a final sequential procedure encompassing pulverisation, Soxhlet extraction, winterization, and decolourisation by activated charcoal was performed, resulting in a final extract with 58.90% Δ^9-THC (Figure 29).

In conclusion, this research has provided validated, efficient, and sustainable methodologies for cannabinoid analysis and extraction. The optimisations made throughout this project align with three United Nations Sustainable Development Goals (2030 Agenda): SDG 9, by promoting innovation in extraction and refinement methods; SDG 12, by providing insights into cannabinoid profile variation during cannabis extraction to ensure the consistent quality and safety of cannabis-based medicinal products; and SDG 17, by fostering partnerships between academia and industry to facilitate knowledge sharing and ensure that innovations are accessible and beneficial across diverse populations.

In the future, the application of the insights from this study regarding the variables affecting various processes of cannabis extraction and refinement should be evaluated in scaled-up environments to potentially enhance industrial processes. Furthermore, the rapid cannabis sample preparation and the HPLC-UV method developed for quantifying cannabinoids can be useful for screening illicit flower samples in forensic laboratories.

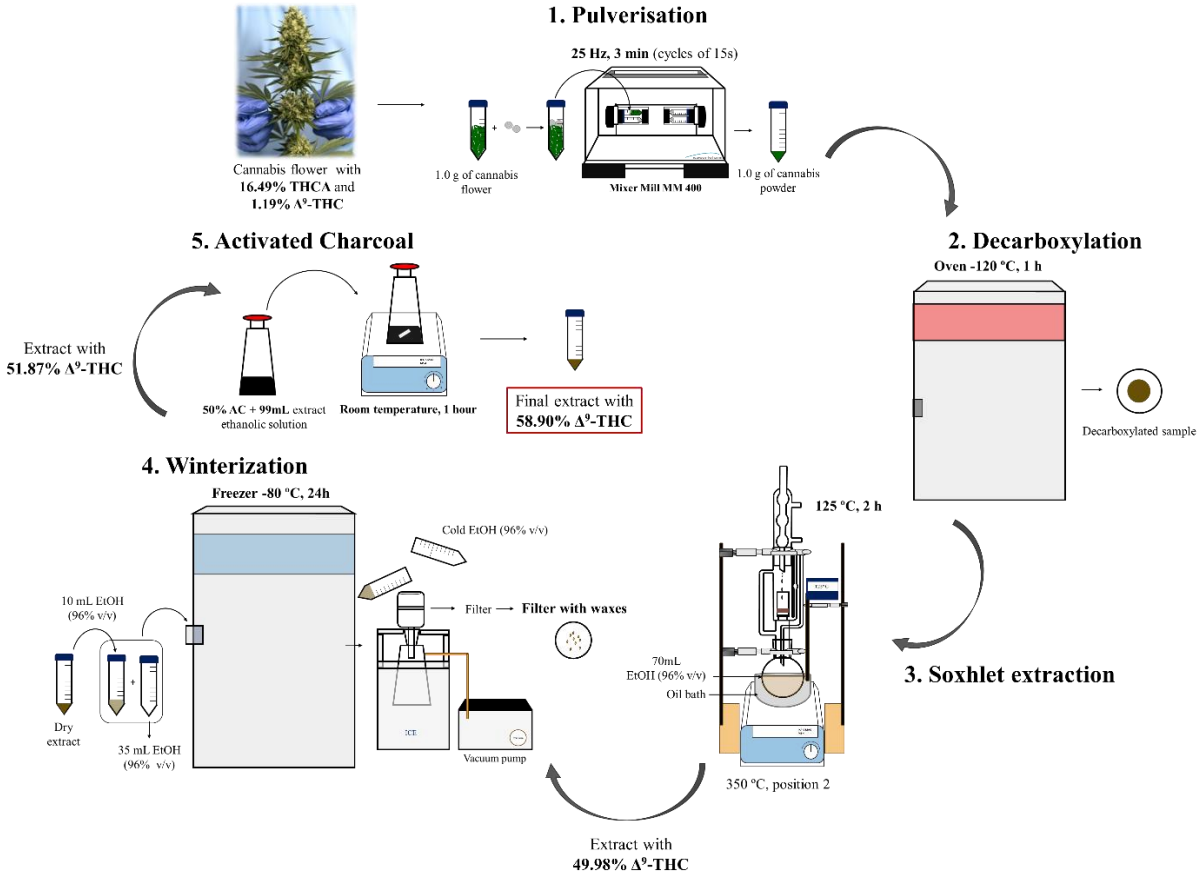


Figure 29. Illustration of all optimised protocol conditions from pulverisation (1) to the refinement with activated charcoal (5). It was possible to achieve a decarboxylated extract, from a Cannabis flower with 16.49% THCA and 1.19% Δ^9-THC, with 49.98% Δ^9-THC after Soxhlet extraction, 51.87% Δ^9-THC after winterization and 58.90% Δ^9-THC with the final refinement process – activated charcoal.

8. Materials and Methods

8.1 Reagents, standards and solvents

14 cannabinoids standards, from DR EHRENSTORFER, were purchased to LGC, including CBC, CBD, CBDV, CBG, CBN, CBNA, Δ^8-THC, Δ^9-THC, THCV, in a concentration of 1 mg/mL in methanol (MeOH), and CBCA, CBDA, CBGA, THCA, THCVA, in a concentration of 1 mg/mL in acetonitrile (ACN). Chemical purity of all compounds varies between 81-100%, which was confirmed by HPLC-DAD analysis. MeOH, ACN, 2-propanol (iPrOH) and Formic acid (≥ 99%), all VWR BDH Chemicals with HPLC purity grade, and Ethanol absolute (EtOH), were purchased from Avantor. Ultrapure water was obtained through the Purification System from VimatechLab Unip. Lda. For chromatographic purposes, the aqueous phase was filtered through a VWR Glass microfibres filter, 0.7 µm. Activated charcoal was purchased to Merck KGaA.

The materials and equipment's used in this project were:

- OHAUS balance;
- Retsch Knife Mill GRINDOMIX GM 200;
- Retsch Mixer Mill MM 400;
- Binder Drying and Heating chamber;
- IKA C-MAG HS7;
- Eppendorf 5804 R;
- Biofuge pico (Heraeus);
- Air Cadet Vacuum Pump;
- LABCONCO SpeedVac;
- BUCHI rotavapor (Heating Bath B-491; Vacuum Pump V-700; Huber minichiller 300);
- Ultrasonic Cleaner USC-T (VWR);
- Gilson P10/5mL pipette;
- Gilson P1000/200/20µL pipette.

8.2 Preparation of standard solutions and samples

8.2.1 Stock solutions

Stock solutions of the 14 cannabinoids standards were stored at -20 ºC until use.

8.2.2 Standard solutions

To optimise the CannProVar method A, a mixed stock solution containing all 14 cannabinoid, each at a concentration of 2.5 µg/mL was prepared in methanol.
For validation purposes, four stock solutions containing three or four cannabinoids each, at 100 µg/mL (corresponding to the highest concentration tested), were prepared. By serially diluting these stock solutions, eight additional calibration controls were obtained (0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL). To assess precision and accuracy, quality controls containing three or four cannabinoids at 5 µg/mL, 20 µg/mL, and 80 µg/mL in methanol were prepared. Similarly, to evaluate the quantification limit, a series of five quality controls ranging from 0.15 µg/mL to 3 µg/mL containing three or four cannabinoids were also prepared in methanol.
To assess the matrix effect, two sets of solutions were prepared: one in solvent (methanol) and the other in matrix extract at concentrations of 5 µg/mL, 20 µg/mL, and 80 µg/m. Additionally, they were assessed in the most concentrated extract dilution used for cannabinoid quantification. For the majority of cannabinoids, two pooled samples containing five CBD-dominant cultivar extracts were prepared: one non-decarboxylated to analyse neutral cannabinoids, and one decarboxylated to assess acidic cannabinoids. To diminish the interference of CBD and CBDA signals, a few cannabinoids were assessed in a THC-dominant cultivar: CBG and CBGA were analysed in decarboxylated extracts, while CBDV, CBD, and CBDA were assessed in decarboxylated extracts.
For the optimisation and validation of the CannProVar method B, the protocolfollowed was consistent with that of the CannProVar method A. Details regarding the concentrations utilised can be found in Appendix 2S, 2T, and 2U.

8.2.3 Sample solutions

Following each extraction process, 1 mL of the resultant solution was filtered using a H-PTFE 0.22 µm 13 mm syringe filter (VWR) to a 1.5 mL microcentrifuge tube prior to HPLC analysis.

Samples from each extraction method were diluted according to the following scheme: Pharmacopoeia extraction samples were analysed directly or diluted at ratios of 2x and 20x, Soxhlet extraction samples were diluted at 2x, 4x and 40x, and Ball Mill extraction samples were diluted at 4x, 8x, and 80x. The lower-ratio dilutions for each extraction method (directly, 2x, and 4x) were used for the quantification of minor compounds, while the higher-ratio dilutions (20x, 40x, and 80x) were used for the quantification of major cannabinoids: THCA in non-decarboxylated samples and Δ^9-THC in decarboxylated samples. All dilutions were prepared to a final volume of 1 or 2 mL

8.3 Plant material and samples preparation

Dry flowers of Cannabis sativa cultivar ZF-2023-4.1 were supplied by Avextra Portugal SA. The samples were stored at room temperature. Also, five CBD-dominant cultivars (Lemon Haze, Gorilla Glue, Blue Cheese, Bubble Gum and Fruit Cake) were purchased from Doctor CBD online store.

8.4 Pulverisation

For analysis, a pulverisation step was required. In the optimisation of the pulverisation process, two pieces of equipment were utilised, one of them the Retsch Knife Mill GRINDOMIX GM 200, which achieved optimal performance at 10,000 rpm for six cycles of 10 seconds each.
In the refined pulverisation protocol, an aliquot of 0.5g of the inflorescences was manually separated from the raiches and weighed into a 50mL centrifuge tube. The material was subsequently pulverised using a Retsch Mixer Mill MM 400, equipped with two 15 mm steel balls, operating at a frequency of 25 Hz for 12 cycles of 15 seconds each.

8.5 Decarboxylation

1.0 g of ground cannabis was transferred to a pre-weighed Petri dish and placed in the oven. For the final decarboxylation condition maintained at 120 ºC for one hour. Accurate temperature control was achieved using a thermometer placed inside the oven. After heating, the Petri dish was cooled and reweighed for mass-loss analysis.

8.6 Extraction from hemp inflorescences

8.6.1 Protocol 1 - Following European Pharmacopoeia Cannabis flower monograph Extraction [70]

0.50 g of ground cannabis was accurately weighed into a 50 mL centrifuge tube and extracted with 40 mL of ethanol (96% v/v) with the help of a stirrer. The extraction was carried out on an agitation plate at speed setting 3 at ambient temperature (≈20–22 ºC) for 15 minutes. Following agitation, centrifuge tube (with the stirrer removed) was centrifuged for 5 minutes at 4,500 rpm without temperature control. The resulting supernatant was then carefully transferred into a 100 mL volumetric flask. The extraction process was repeated twice, each time using 25 mL of ethanol (96% v/v). The volumetric flask was filled with ethanol (96% v/v), followed by vigorous shaking to ensure thorough homogenization. For analytical purposes, dilutions of 2x and 20x were prepared (see section 8.2.3). To obtain a dry extract, the solution was filtered through Whatman No. 1 filter paper (90 mm) using a vacuum pump setup, then concentrated to dryness using a rotary evaporator followed by a SpeedVac.
This protocol was used for decarboxylated and non-decarboxylated samples.

8.6.2 Ball Mill Extraction

0.50 g of ground cannabis was accurately weighed into a 50 mL centrifuge tube and extracted at room temperature with 25 mL of ethanol (96% v/v). The extraction was performed in a Retsch Mixer Mill MM 400 for 10 minutes, using two 15 mm stainless steel balls at a frequency of 5 Hz. The centrifuge tube was sealed with Parafilm to prevent leaks. A 1 mL aliquot of the extract was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 5,000 rpm for 5 minutes at ambient temperature. For analytical purposes, dilutions of 8x and 80x were prepared (see section 8.2.3). To obtain a dry extract, the solution was filtered through Whatman No. 1 filter paper (90 mm) using a vacuum pump setup, then concentrated to dryness using a rotary evaporator followed by a SpeedVac.
This protocol was used for decarboxylated and non-decarboxylated samples.

8.6.3 Soxhlet Extraction

1.0 g of ground cannabis was loaded into a Synthware 25 mm glass thimble and topped with a cotton plug to prevent powder loss. The extraction thimble was positioned inside the Soxhlet extractor (Synthware, top joint: 45/50, bottom joint: 24/40). The three components of the Soxhlet apparatus (flask, extractor body and condenser) were connected, with silicone lubricant applied to the joints. An oil bath was set up on an agitation plate at speed setting 2 and heated to 125 °C ± 2 °C. After 2 hours of extraction (or per cycle during optimisation), counted from the onset of ethanol condensation, the system was stopped, the thimble removed, and 60 mL of ethanol (96% v/v) added for a siphon wash. The 250 mL flask containing the extract was subsequently removed, and the solution was concentrated using a rotary evaporator at 40 ºC until the volume was less than 100 mL. After cooling, the concentrated extract was transferred to a 100 mL volumetric flask and filled with ethanol (96% v/v). For analytical purposes, dilutions of 4x and 40x were prepared (see section 8.2.3). To obtain a dry extract, the solution was filtered through Whatman No. 1 filter paper (90 mm) using a vacuum pump setup, then concentrated to dryness using a rotary evaporator followed by a SpeedVac.
This protocol was used for decarboxylated and non-decarboxylated samples.

8.7 Purification

8.7.1 Winterization

A dry extract decarboxylated sample (0.2949-0.3047 g) was reconstituted with 10mL of ethanol (96% v/v) in a 50 mL microcentrifuge tube and placed in a freezer at −80 °C for 24 hours alongside a centrifuge tube with ethanol (96% v/v). Then, the centrifuge tubes were removed and placed on ice. The extract solution was filtered through a pre-weighed Whatman No. 1 filter paper (90 mm) placed in a Büchner funnel, which was also kept on ice. The vacuum pump was connected to the filtration system. The centrifuge tube containing the extract was rinsed with cool ethanol (96% v/v). After filtration, the filter was left in the fume hood to dry completely and was finally weighed. The resulting solution was transferred to a 100 mL volumetric flask and filled with ethanol (96% v/v). For analytical purposes, dilutions of 4x and 40x were prepared (see section 8.2.3). The solution was then concentrated using a SpeedVac until it was completely dry, preparing it for subsequent activated charcoal experiments.

8.7.2 Activated charcoal

A dry extract decarboxylated sample (0.298-0.322 g) was reconstituted with 25 mL of ethanol (96% v/v) utilising ultrasound equipment for enhanced solubilization. The solution was transferred to a 100 mL volumetric flask and reconstituted to the meniscus with ethanol (96% v/v) followed by vigorous shaking to ensure complete homogenization. A 1 mL aliquot of the resulting solution was transferred to a transparent 1.5 mL Eppendorf tube and filtered using a H-PTFE 0.22 µm 13 mm syringe filter (VWR) for HPLC analysis, as the 0 time. The percentage of activated charcoal was weighed for a flask, and a stir was added. The 99 mL extract solution was added to the flask and stirred in an agitation plate. After agitation, 1 mL of the solution was transferred to transparent 1.5 mL microcentrifuge tube and subjected to centrifugation at 5,000 rpm for 5 minutes at ambient temperature. For analytical purposes, dilutions of 4x and 40x were prepared (see section 8.2.3). The solution that remained in the flask was filtered with a Whatman 1 Paper Filter (90mm) using vacuum pump connected to the filtration system. After filtering, the solution was concentrated using a rotary evaporator at 40 ºC until the volume of approximately 20 mL. The concentrated solution was filtered one more time with a TERUMO Syringe without a needle using a CA 0.22 µm 25 mm syringe filter (VWR).

8.8 HPLC analysis

Chromatographic conditions of the developed analytical methods – CannProVar method A and B – are described in Appendix 2N and 2O, respectively.

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Appendix I

Appendix 1A. Summary of extraction techniques conditions used in the reviewed articles.

Entry	Technique	Pulverisation (particle size, mm)	Time of each extraction (min)	Nº extractions	Temperature (°C)	Frequency (kHz) [a]	Solvent	Plant-to-solvent ratio (g/mL)	Ref
1	Maceration	Ground	4320	NR	RT		C_6H_{14}/EtOAc	1:20	[155]
2	DM - NS	NR	15	3	RT		EtOH	1:6	[156]
3	DM - NS	0.5 – 2.0	45	1	RT		MeOH:$CHCl_3$ (9:1)	NR	[95]
4	DM - NS	NR	20	NR	NR		MeOH:$CHCl_3$ (9:1)	NR	[157]
5	DM - Shaking	Ground	30	2	NR		EtOH	1:40	[96]
6	DM - Shaking	NR	15	3	NR		EtOH	1:30	[158]
7	DM - Shaking	NR	30	2	NR		MeOH	1:40	[159]
8	DM - Shaking	Ground	30	2	NR		MeOH	1:40	[160]
9	DM - Shaking	1.0	90	NR	NR		MeOH	1:100	[161]

Table Appendix 1A. *Cont.*

Entry	Technique	Pulverisation (particle size, mm)	Time of each extraction (min)	Nº extractions	Temperature (°C)	Frequency (kHz) [a]	Solvent	Plant-to-solvent ratio (g/mL)	Ref
10	DM - Shaking	1.0	30	NR	NR		EtOH	1:50	[97]
11	DM - Shaking	0.125-1.0	5	2	NR		MeOH	1:100	[162]
12	DM - Shaking	Ground	15	1	RT		EtOH	1:8; 1:40	[163]
13	DM - Shaking	Powder	15	1	NR		EtOH	1:40	[164]
14	DM - Shaking	Ground	15	1	RT		EtOH	1:8; 1:40	[165]
15	DM - Shaking	1.0	90	1	RT		MeOH	1:100	[166]
16	DM - Shaking	Ground	60	3	NR		EtOH (96% v/v)	1:33	[167]
17	DM - Shaking	Ground	15	NR	NR		EtOH	1:40	[168]
18	DM - Shaking	Ground	15	NR	NR		EtOH	1:40	[169]
19	DM - Shaking	NR	4	1	NR		EtOH	1:20	[170]

Table Appendix 1A. *Cont.*

Entry	Technique	Pulverisation (particle size, mm)	Time of each extraction (min)	Nº extractions	Temperature (°C)	Frequency (kHz) [a]	Solvent	Plant-to-solvent ratio (g/mL)	Ref
20	DM - Shaking	Ground	NR	3	NR		EtOH (96% v/v)	1:50	[171]
21	DM - Stirring	Ground	120	NR	NR		EtOH	1:6;1:2	[172]
22	DM - Stirring	Ground	15	3	RT		EtOH	1:40	[173]
23	DM - Stirring	Ground	60	3	RT		EtOH (96% v/v)	1:33	[98]
24	DM - Vortexing	NR	1	NR	NR		H_2O	1:100	[99]
25	DM - Vortexing	Ground	3	NR	NR		MeOH	1:3	[174]
26	Reflux	NR	NR	2	80		EtOH	1:15	[175]
27	Reflux	Powder	120	2	80		EtOH (70% v/v)	1:10	[176]
28	Hydrodistillation	Ground	300	NR	NR		Distilled H_2O	1:6	[177]

Table Appendix 1A. *Cont.*

Entry	Technique	Pulverisation (particle size, mm)	Time of each extraction (min)	Nº extractions	Temperature (°C)	Frequency (kHz) [a]	Solvent	Plant-to-solvent ratio (g/mL)	Ref
29	Hydrodistillation	NR	180	NR	NR		Distilled H_2O	1:6	[178]
30	Hydrodistillation	Powder	180	NR	NR		Distilled H_2O	1:10	[179]
31	Dexso extractor	NR	2880	NR	NR		BUT/DME	1:17	[180]
32	SE - NS	NR	60	1	NR		MeOH:$CHCl_3$	NR	[181]
33	SE - NS	NR	NR	NR	NR		EtOH	NR	[182]
34	UAE	Powder	30	NR	NR	NR	MeOH	1:100	[100]
35	UAE	Ground	15	NR	NR	NR	EtOH (99.8% v/v)	NR	[183]
36	UAE	Cut	10	NR	NR	NR	EtOH (96% v/v)	1:20	[184]
37	UAE	Powder	1-20	1-3	NR	40	ACN:EtOH (50:50)	1:5	[185]
38	UAE	<0.2	15	2	RT	NR	MeOH:$CHCl_3$ (9:1)	1:5	[186]

Table Appendix 1A. *Cont.*

Entry	Technique	Pulverisation (particle size, mm)	Time of each extraction (min)	Nº extractions	Temperature (°C)	Frequency (kHz) [a]	Solvent	Plant-to-solvent ratio (g/mL)	Ref
39	UAE	0.6-1.3	7	NR	IB	NR	EtOH:C_6H_{14} (1:1)	1:10	[187]
40	UAE	Powder	30	NR	NR	NR	MeOH	1:100	[101]
41	UAE	NR	20	NR	NR	NR	MeOH	NR	[102]
42	UAE	Cryo-milled	15	NR	IB	NR	$C_4H_8O_2$	1:85	[188]
43	UAE	0.180-0.250	10	2	25	NR	MeOH	1:200	[189]
44	UAE	Powder	60	NR	NR	40	MeOH	NR	[190]
45	UAE	1.0	15	NR	40	37	NR	1:98	[191]
46	UAE	Ground	15	NR	NR	NR	MeOH:$CHCl_3$ (9:1)/MeOH	1:10	[192]
47	UAE	Ground	NR	3	NR	NR	EtOAc	1:4	[193]
48	UAE	Ground	30	2	NR	NR	MeOH:CH_3Cl (9:1)	1:100	[194]
49	UAE	Powder	30	1	35	34 ± 3	ACN	1:10	[195]

Table Appendix 1A. *Cont.*

Entry	Technique	Pulverisation (particle size, mm)	Time of each extraction (min)	Nº extractions	Temperature (°C)	Frequency (kHz) [a]	Solvent	Plant-to-solvent ratio (g/mL)	Ref
50	UAE	1.0	10	2	RT	40	MeOH/ACE	1:50	[196]
51	UAE	Powder	30-40	2	20	NR	C_6H_{14}	1:10	[103]
52	UAE	0.5	30	1	50	NR	EtOH (96% v/v)	1:100	[197]
53	UAE	NR	5	3	NR	NR	EtOH	1:8	[198]
54	UAE	1.0	10	NR	25	40	H_2O:ES	1:20	[199]
55	UAE	100	15	NR	25	35	EtOH	1:50	[200]
56	UAE	Powder	5	4	NR	NR	MeOH	NR	[104]
57	UAE	Ground	15	NR	NR	NR	EtOH (99.8% v/v)	NR	[201]
58	UAE	Powder	60	NR	55	NR	ACN	1:7	[105]
59	UAE	0.710	30	NR	RT	NR	ACN:MeOH (4:1)	1:63	[202]

Table Appendix 1A. *Cont.*

Entry	Technique	Pulverisation (particle size, mm)	Time of each extraction (min)	Nº extractions	Temperature (°C)	Frequency (kHz) [a]	Solvent	Plant-to-solvent ratio (g/mL)	Ref
60	UAE	NR	15	NR	RT	NR	EtOH (96% v/v)	1:10	[203]
61	UAE	Powder	40	NR	NR	NR	MeOH:$CHCl_3$ (9:1)	1:40	[204]
62	UAE	Powder	30	3	<30	NR	CH_2Cl_2/EtOAc/ EtOH	1:10	[205]
63	UAE	1	15	NR	35	37	EtOH (96% v/v)	1:98	[206]
64	UAE	<0.2	20	NR	30	NR	MeOH	1:200	[106]
65	UAE	20	20	1	NR	NR	ACN	1:100	[207]
66	UAE	Ground	10	NR	NR	NR	MeOH	1:100	[107]
67	UAE	Powder	30	NR	RT	NR	CH_5H_{12}	1:5	[208]

[a] Frequency was only applied in articles with the UAE extraction technique. DM-NS: dynamic maceration-non specified; SE-NS: solvent extraction-non specified; NR: no reported; RT: room temperature; C6H14: hexane; EtOAc: ethyl acetate; MeOH: methanol; EtOH: ethanol; CHCl3: chloroform; H2O: water; BUT: butane; DME: dimethyl ether; ACN: acetonitrile; CH3Cl: chloromethane; H2O: water; ACE: acetone; ES: eutectic solvent; CH2Cl2: dichloromethane; CH5H12: pentane

Appendix 2A. Table presents the chromatographic conditions of Agilent Method.

Parameters	**Chromatographic Conditions**		
Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm		
Flow rate	1.0 mL/min		
Column temperature	50 ºC		
Injection volume	5.0 µL		
Analyse time	9.5 min		
Post-run time	1.5 min		
Autosampler temperature	Ambient		
Gradient	Time (min)	MeOH 0.05% FA	H_2O 0.1% FA
	0.00	60%	40%
	1.00	60%	40%
	7.00	77%	23%
	8.20	95%	5%

Appendix 2B. Table presents the chromatographic conditions of Method 1.

Column temperature	50 ºC		
Flow rate	1.0 mL/min		
Gradient	Time (min)	MeOH 0.05% FA	H_2O 0.1% FA
	0.00	60%	40%
	1.00	60%	40%
	14.00	77%	23%
	16.40	95%	5%
	17.70	95%	5%

Appendix 2C. Table presents the chromatographic conditions of Method 2.

Column temperature	50 ºC		
Flow rate	1.0 mL/min		
Gradient	Time (min)	MeOH 0.05% FA	10mM AF H_2O 0.1% FA
	0.00	60%	40%
	1.00	60%	40%
	14.00	77%	23%
	16.40	95%	5%
	17.70	95%	5%

Appendix 2D. Table presents the chromatographic conditions of Method 3.

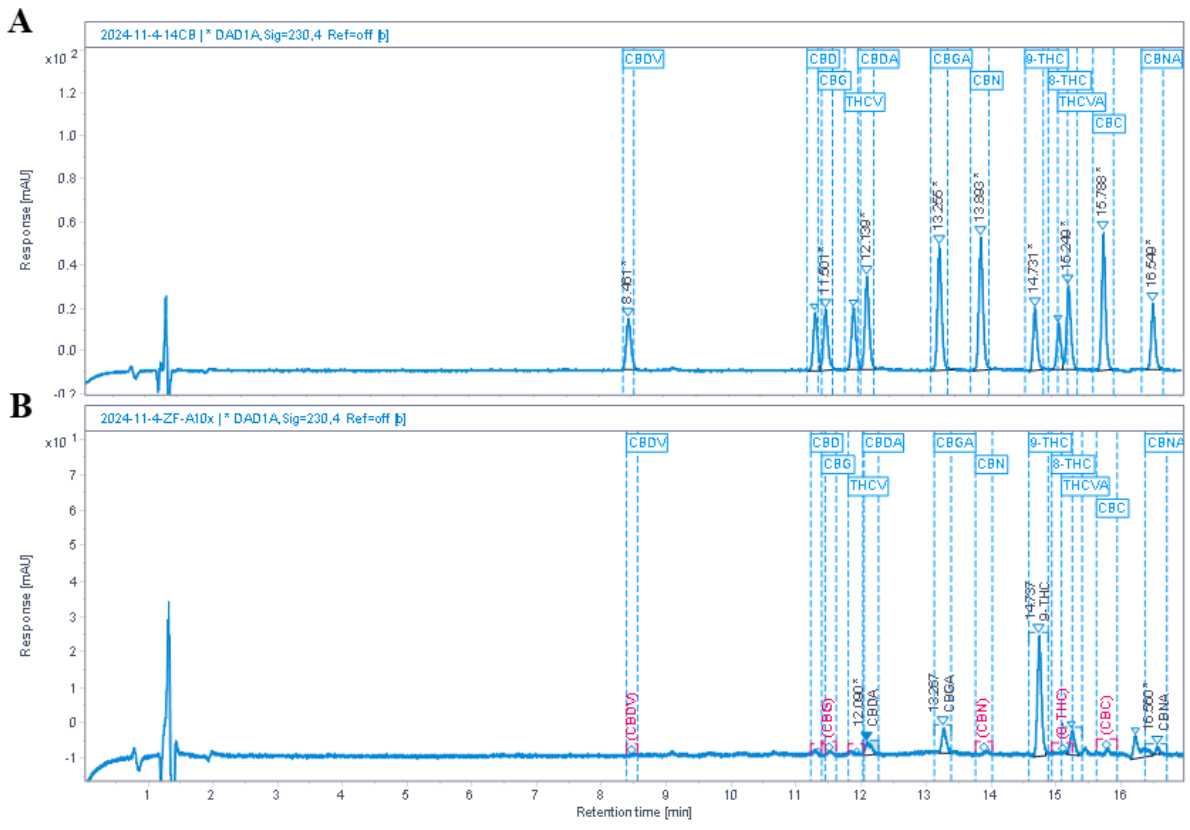
Column temperature	50 ºC		
Flow rate	0.7 mL/min		
Gradient	Time (min)	MeOH 0.05% FA	10mM AF H_2O 0.1%FA
	0.00	65%	35%
	1.00	65%	35%
	14.00	95%	5%
	17.70	95%	5%

Appendix 2E. Table presents the chromatographic conditions of Method 4.

Column temperature	30 ºC		
Flow rate	0.5 mL/min		
Gradient	Time (min)	MeOH 0.05% FA	10mM AF H_2O 0.1% FA
	0.00	65%	35%
	1.00	65%	35%
	14.00	95%	5%
	17.70	95%	5%

Appendix 2F. Table presents the chromatographic conditions of Method 5.

Column temperature	30 ºC		
Flow rate	0.5 mL/min		
Gradient	Time (min)	MeOH 0.05% FA	H_2O 0.1% FA
	0.00	65%	35%
	1.00	65%	35%
	14.00	95%	5%
	17.70	95%	5%

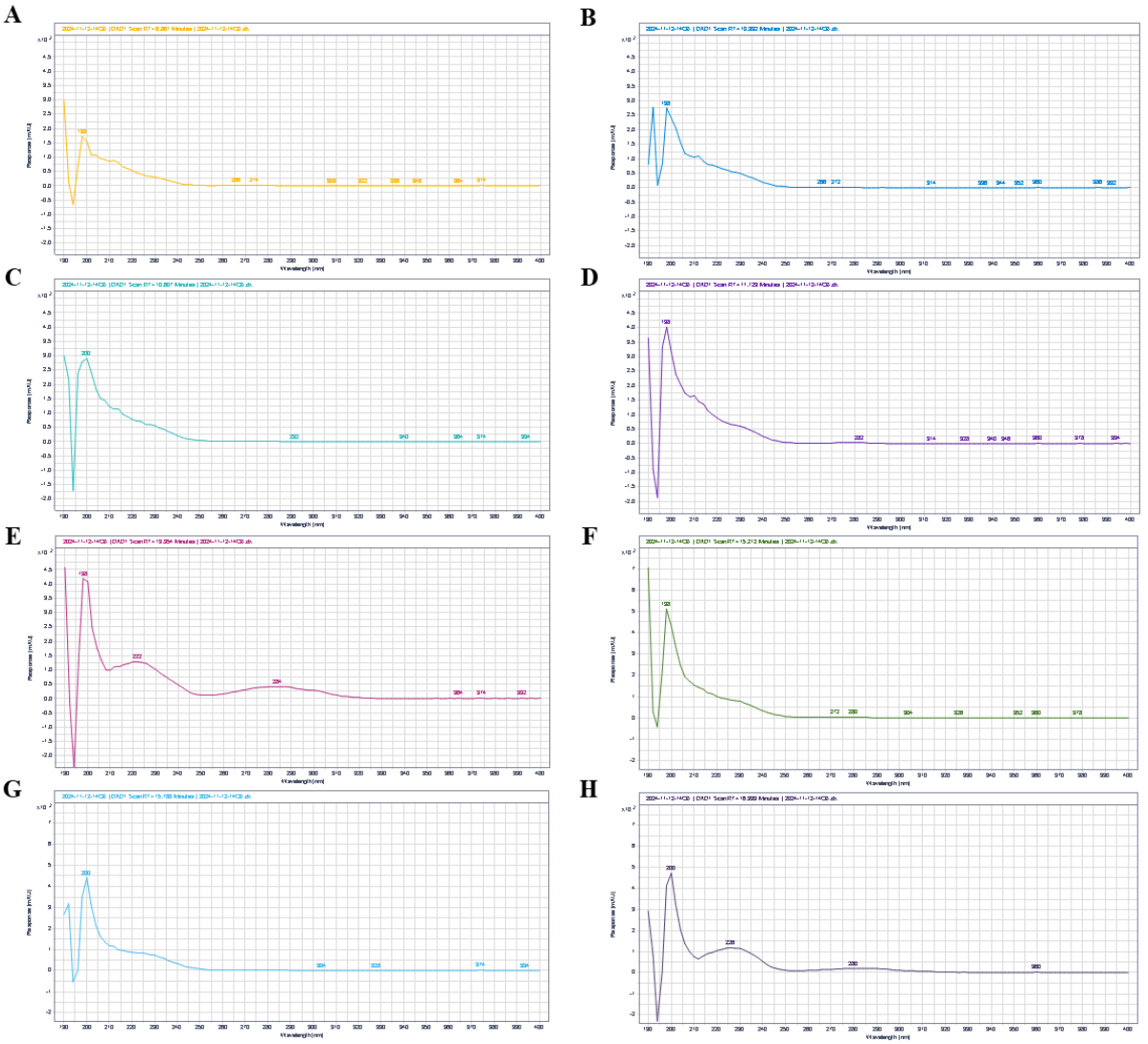


Appendix 2G. Representative chromatograms, using method 5 (Appendix 2F), of the (A) 14 standard cannabinoids mixture (2.5 µg/mL) and (B) Avextra's cultivar.

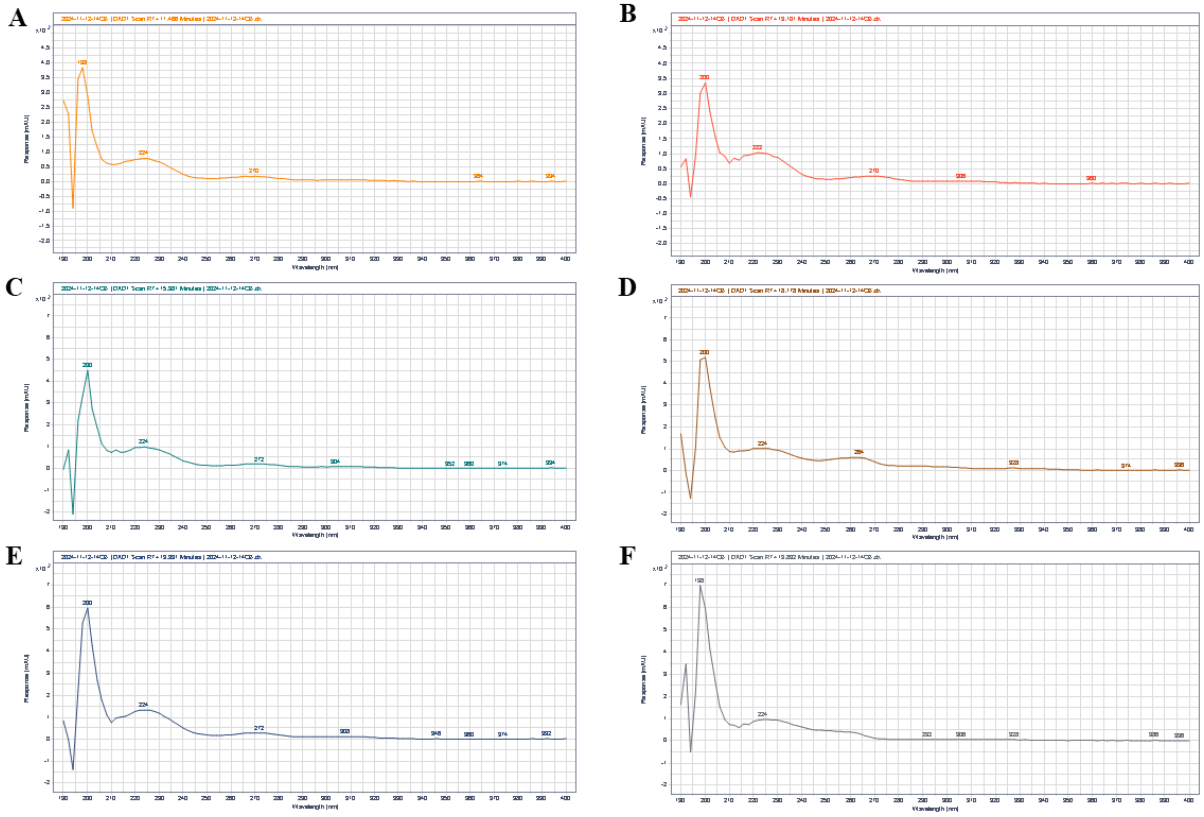
Appendix 2H. Table presents the chromatographic conditions of Method 6.

Column temperature	30 ºC		
Flow rate	0.5 mL/min		
Gradient	Time (min)	MeOH 0.05% FA	H_2O 0.1% FA
	0.00	70%	30%
	1.00	70%	30%
	18.00	95%	5%
	21.00	95%	5%

Appendix 2I. Absorbance spectrum of each neutral cannabinoid (A: CBDV; B: THCV; C: CBD; D: CBG; E: CBN; F: 9-THC; G: 8-THC; H: CBC).



Appendix 2J. Absorbance spectrum of each acidic cannabinoid (A: CBDA; B: CBGA; C: THCVA; D: CBNA; E: THCA; F: CBCA).



Appendix 2K. Table with the areas of each compound in standard (2.5µg/mL) and cannabis cultivar corresponding to the selected wavelengths.

	Area (mAU-s) – Standards													
	CBDV	**CBD**	**CBG**	**THCV**	**CBDA**	**CBGA**	**CBN**	**Δ^9-THC**	**Δ^8-THC**	**THCVA**	**CBC**	**CBNA**	**Δ^9-THCA**	**CBCA**
224 nm	162.56	147.008	145.085	142.384	249.046	249.046	324.462	125.617	97.782	229.407	232.87	142.925	283.502	86.054
230 nm	123.169	106.125	103.499	114.433	208.896	157.873	230.543	96.418	80.351	183.414	255.072	123.276	226.205	107.806
254 nm	<10	<10	<10	<10	58.35	50.278	40.681	<10	<10	53.861	25.993	235.286	71.779	195.783
264 nm	<10	<10	<10	<10	102.589	81.325	86.278	<10	<10	97.228	55.936	280.295	130.225	169.027
268 nm	<10	<10	<10	<10	110.366	89.529	116.137	15.208	<10	109.621	66.411	232.254	146.403	89.993
270 nm	<10	<10	<10	10.098	108.544	85.514	128.159	<10	<10	111.274	71.884	189.527	149.464	75.968
272 nm	11.58	<10	<10	12.724	110.384	86.011	139.838	<10	10.01	116.699	77.992	156.194	146.806	59.99
280 nm	10.171	10.335	<10	14.085	73.645	53.813	170.925	10.817	10.945	81.108	96.441	96.061	106.72	46.326
284 nm	<10	<10	<10	11.678	52.542	36.716	182.05	11.203	<10	55.209	18.516	92.053	73.791	44.487

	Area (mAU-s) – Avextras's cultivar													
	CBDV	**CBD**	**CBG**	**THCV**	**CBDA**	**CBGA**	**CBN**	**Δ^9-THC**	**Δ^8-THC**	**THCVA**	**CBC**	**CBNA**	**Δ^9-THCA**	**CBCA**
224 nm	12.162	69.913	72.335	<DL	107.946	414.872	30.434	1661.152	<DL	232.111	50.036	120.51	22162.38	709.084
230 nm	10.947	57.42	55.812	<DL	82.343	290.316	28.121	1285.434	<DL	178.955	54.693	97.015	19992.6	642.803
254 nm	<10	<10	<10	<DL	27.935	94.987	<10	48.042	<DL	54.592	<10	158.558	7344.464	624.336
264 nm	<10	<10	<10	<DL	40.202	154.711	12.487	89.915	<DL	100.64	11.083	193.917	13959.95	608.981
268 nm	<10	<10	<10	<DL	38.815	165.236	15.202	117.155	<DL	114.318	12.02	160.281	15613.78	458.674
270 nm	<10	<10	<10	<DL	39.305	162.798	15.564	129.381	<DL	114.132	12.889	134.887	15980.02	389.886
272 nm	<10	<10	<10	<DL	39.351	156.726	16.588	143.989	<DL	113.349	15.117	111.501	16222.13	348.058
280 nm	<10	<10	<10	<DL	27.826	93.321	16.959	156.287	<DL	89.916	20.928	72.093	1925.555	212.075
284 nm	<10	<10	<10	<DL	19.8	65.631	16.242	140.849	<DL	60.125	18.904	66.705	7734.801	161.722

<DL: lower than detection limit; highlighted in green are the areas corresponding to the specific wavelength for each cannabinoid

Appendix 2L. Table presents the chromatographic conditions of Method 7.

Column temperature	30 ºC		
Flow rate	0.5 mL/min		
Gradient	Time (min)	MeOH 0.05% FA	H_2O 0.1% FA
	0.00	70%	30%
	1.00	70%	30%
	57.00	70%	30%
	57.01	95%	5%
	60.00	95%	5%

Appendix 2M. Table presents the chromatographic conditions of Method 8.

Column temperature	30 ºC		
Flow rate	0.5 mL/min		
Gradient	Time (min)	MeOH 0.05% FA	H_2O 0.1% FA
	0.00	70%	30%
	1.00	70%	30%
	46.00	95%	5%
	49.00	95%	5%

Appendix 2N. Table presents the chromatographic conditions of Method 9 (CannProVar method A).

Parameters	**Chromatographic conditions**		
Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 x 150 mm, 2.7 µm		
Guard column	Poroshell 120 EC-C18 3.0 mm		
Flow rate	0.5 mL/min		
Column temperature	30 ºC		
Injection volume	5 µl		
Analyse time	30 min		
Post-run time	4 min		
Autosampler temperature	15 ºC		
UV detection	230, 260, 272, 280 nm		
UV quantification	230 nm		
Mobile phase	MeOH 0.05% (v/v) FA + H_2O 0.1% (v/v) FA		
Gradient	Time (min)	MeOH 0.05% FA	H_2O 0.1% FA
	0.00	74%	26%
	1.00	74%	26%
	5.00	74%	26%
	5.01	76%	24%
	9.00	76%	24%
	9.01	86%	14%
	15.00	86%	14%
	15.01	80%	20%
	26.00	90%	10%
	26.01	98%	2%
	30.00	98%	2%

Appendix 2O. Table presents the chromatographic conditions of CannProVar method B.

Parameters	**Chromatographic conditions**		
Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 x 150 mm, 2.7 µm		
Guard column	Poroshell 120 EC-C18 3.0 mm		
Flow rate	0.75 mL/min		
Column temperature	50 ºC		
Injection volume	10 µl		
Analyse time	12 min		
Post-run time	3 min		
Autosampler temperature	15 ºC		
UV detection	230, 260, 272, 280 nm		
UV quantification	230 nm		
Mobile phase	MeOH 0.05% (v/v) FA + H_2O 0.1% (v/v) FA		
Gradient	Time (min)	MeOH 0.05% FA	H_2O 0.1% FA
	0.00	72%	28%
	1.00	72%	28%
	11.00	95%	5%
	12.00	95%	5%

Appendix 2P. Table presents the linear range (µg/mL), calibration curve, R^2 and detection and quantification limits (µg/mL), and % flower (w/w) for CannProVar method A.

	Linear Range (µg/mL)	Calibration Curve	R^2	Detection Limit			Quantification Limit		
				Conc. (µg/mL)	S/N	%Flower (w/w) [a]	Conc. (µg/mL)	S/N	%Flower (w/w) [a]
CBDV	0.25-100	y = 22.184x + 2.375	0.999	0.10	> 3	0.004	2.0	> 10	0.08
CBD	0.25-100	y = 20.386x + 8.1923	0.999	0.10	> 3	0.004	2.5	> 10	0.10
CBG	0.5-100	y = 19.335x + 0.0267	1.0	0.10	> 3	0.004	1.0	> 10	0.04
THCV	0.25-100	y = 19.626x + 1.7399	0.999	0.15	> 3	0.006	2.0	> 10	0.08
CBDA	0.25-100	y = 37.784x + 20.034	0.999	0.10	> 3	0.004	1.5	> 10	0.06
CBGA	0.25-100	y = 37.304x + 1.9642	0.999	0.10	> 3	0.004	2.0	> 10	0.08
CBN	0.25-100	y = 49.208x + 4.0132	0.999	0.10	> 3	0.004	1.5	> 10	0.06
Δ^9- THC	0.5-100	y = 19.676x + 0.0252	1.0	0.10	> 3	0.004	1.0	> 10	0.04
Δ^8- THC	0.25-100	y = 18.263x + 9.893	0.999	0.15	> 3	0.006	2.5	> 10	0.10
THCVA	0.25-100	y = 35.273x + 20.257	0.999	0.10	> 3	0.004	3.0	> 10	0.12
CBC	0.25-100	y = 47.62x – 12.053	0.999	0.10	> 3	0.004	1.5	> 10	0.06
CBNA	0.25-100	y = 26.405x – 7.4841	0.999	0.2	> 3	0.008	2.5	> 10	0.10
Δ^9- THCA	0.5-100	y = 36.213x – 9.9239	0.999	0.10	> 3	0.004	2.0	> 10	0.08
CBCA	0.25-100	y =20.142x –17.284	0.999	0.10	> 3	0.004	2.5	>10	0.10

[a] %Flower (w/w) calculated in the most concentrated dilution (4x); S/N: signal-to-noise

Appendix 2Q. Table with precision and accuracy for the three quality controls for CannProVar method A.

	Conc. (µg/mL)	Precision (RSD%)			Accuracy (Bias%) [b]					
					Intra-day			Inter-Day		
		Intra-assay [a]	Intra-day	Inter-day [b]	ID1	ID2	ID3	Day 1	Day 2	Day 3
CBDV	80	0.15	0.49	1.34	3.15	3.40	2.47	3.01	2.36	0.06
	20	0.21	0.25	1.61	-0.82	-.0.98	-1.33	-1.04	-1.10	-4.74
	5	0.89	2.46	3.46	0.98	1.34	-3.34	-0.34	-7.80	-2.23
CBD	80	0.10	0.39	1.31	1.33	1.09	1.82	2.24	3.92	1.32
	20	0.07	0.58	1.99	4.05	5.27	4.71	4.88	8.09	4.64
	5	1.65	1.67	3.56	1.04	-2.15	2.22	7.09	0.15	-0.36
CBG	80	3.92	0.02	0.89	-1.50	-1.39	5.39	0.11	0.23	0.87
	20	5.09	0.15	1.16	-0.22	-0.71	8.60	1.13	1.40	2.57
	5	2.15	0.17	1.94	2.84	0.20	3.03	2.46	3.95	1.75
THCV	80	0.32	0.37	1.07	3.42	4.11	3.40	3.64	3.25	1.54
	20	0.72	0.46	1.60	-0.29	-1.08	-0.52	-0.63	-0.89	-3.36
	5	2.12	1.02	3.38	-3.23	-0.67	-0.99	-1.28	-9.23	-2.92
CBDA	80	0.34	1.56	3.38	1.89	0.05	-0.54	-2.68	3.94	0.47
	20	0.45	2.96	1.70	1.15	-0.001	-4.90	-0.30	3.17	-1.25
	5	1.07	0.45	6.03	0.40	-2.32	3.15	11.17	-0.60	0.41
CBGA	80	0.12	0.28	0.22	-4.82	-4.43	-5.01	-4.48	-4.93	-4.75
	20	0.02	0.58	0.58	1.96	2.21	1.18	2.44	1.30	1.75
	5	0.53	0.61	1.04	4.22	5.37	4.35	3.99	5.83	4.67
CBN	80	0.02	4.93	0.75	-1.19	-1.15	7.06	0.11	0.09	1.58
	20	0.14	5.42	0.56	-0.43	-0.42	8.82	0.66	0.19	2.66
	5	0.44	0.17	0.58	0.29	0.37	0.79	1.14	1.41	0.49
Δ^9- THC	80	0.05	4.69	0.65	-3.93	-3.82	4.19	-2.79	-2.76	-1.18
	20	0.04	4.94	0.55	-3.26	-2.98	6.29	-2.29	-2.29	0.02
	5	0.33	0.25	0.45	-1.92	-2.36	-1.59	-1.02	-1.61	-1.96

	Conc. (µg/mL)	Precision (RSD%)			Accuracy (Bias%) [b]					
					Intra-day			Inter-Day		
		Intra-assay [a]	Intra-day	Inter-day [b]	ID1	ID2	ID3	Day 1	Day 2	Day 3
Δ^8- THC	80	0.06	1.36	0.86	0.92	3.02	3.58	-0.92	-0.49	2.51
	20	0.16	1.48	2.21	4.16	4.31	7.00	-0.44	1.73	5.16
	5	0.94	5.02	0.47	-2.75	5.93	-4.39	-3.37	-3.74	-0.40
THCVA	80	0.32	0.05	0.03	-1.76	-2.21	-11.10	-1.38	-5.04	-6.57
	20	0.29	0.00	0.02	-3.82	-4.09	-3.38	-2.20	-4.03	-6.90
	5	0.99	0.09	0.01	-11.20	5.71	4.77	5.91	-0.30	4.01
CBC	80	0.32	0.57	3.55	-3.71	-2.54	-3.00	-3.08	2.67	4.07
	20	0.01	0.11	2.31	-4.55	-4.48	-4.33	-4.45	-0.95	-0.04
	5	0.26	0.68	1.35	0.96	2.22	1.50	1.56	2.73	0.39
CBNA	80	1.83	5.48	1.63	0.72	1.16	-10.861	1.10	3.44	2.77
	20	2.10	5.09	3.99	6.63	2.38	-6.63	1.48	3.63	0.56
	5	3.15	7.49	4.93	13.82	1.13	-15.43	2.34	10.13	6.43
Δ^9- THCA	80	0.01	0.83	0.28	-1.99	-1.97	-0.53	-0.49	-2.46	-1.95
	20	0.06	2.09	0.81	1.88	-0.25	-2.26	-0.21	1.10	1.06
	5	1.96	1.26	1.71	2.85	4.93	2.97	3.58	3.55	1.70
CBCA	80	0.02	1.43	0.78	-8.22	-5.53	-5.62	-9.14	-9.89	-6.46
	20	0.37	0.08	0.14	1.77	1.65	1.10	1.77	1.53	1.50
	5	0.93	0.53	2.27	11.98	11.46	11.48	14.86	-14.22	11.64

[a] n = 3; [b] Same QC analysed in three consecutive days; ID: Intra-day; RSD: relative standard deviation

Appendix 2R. Table with precision and accuracy percentages of ME, for spiked concentrations in the CannProVar method A.

	Conc. spiked (µg/mL)	Precision (RSD %)			Matrix Effects (ME %)				
					Intra-day		Inter-Day		
		Intra-assay [a]	Intra-day	Inter-day [b]	ID1	ID2	Day 1	Day 2	Day 3
CBDV	80	0.39	n.d.	0.27	n.d.	n.d.	102.9	103.0	103.0
	20	0.44	n.d.	1.66	n.d.	n.d.	99.9	99.7	99.9
	5	0.27	n.d.	0.95	n.d.	n.d.	99.7	99.2	99.7
CBD	80	0.18	n.d.	0.61	n.d.	n.d.	105.8	104.6	102.8
	20	0.17	n.d.	1.35	n.d.	n.d.	109.5	109.8	109.7
	5	0.73	n.d.	0.72	n.d.	n.d.	100.7	99.3	104.3
CBG	80	0.25	0.04	0.71	98.5	97.0	98.6	99.1	98.5
	20	0.27	3.95	0.07	101.6	106.8	104.4	101.7	101.6
	5	0.26	0.19	0.5	97.1	100.5	97.4	97.6	97.1
THCV	80	0.02	0.34	0.95	108.5	108.4	105.9	108.7	108.5
	20	0.87	2.03	2.49	98.5	103.7	97.6	99.3	98.5
	5	0.04	0.48	1.14	105.2	105.1	105.2	108.3	105.2
CBDA	80	0.01	n.d.	1.52	n.d.	n.d.	105.9	103.2	102.9
	20	0.05	n.d.	1.16	n.d.	n.d.	109.6	106.0	105.6
	5	0.05	n.d.	2.13	n.d.	n.d.	106.3	98.8	104.2
CBGA	80	0.33	0.36	1.06	97.0	99.2	98.7	98.6	97.0
	20	0.29	4.63	0.42	106.8	99.1	101.2	99.5	106.8
	5	0.08	1.27	1.84	100.5	103.0	100.3	102.9	100.5
CBN	80	0.16	0.74	1.01	101.6	101.2	101.6	101.6	100.8
	20	0.38	0.49	0.64	101.8	99.8	101.0	101.8	100.6
	5	0.10	0.77	0.87	107.0	107.6	105.7	107.0	107.3
Δ^9-THC	80	0.39	0.70	1.12	98.3	101.6	102.3	98.3	100.9
	20	0.45	0.86	0.70	101.8	99.0	99.3	101.8	99.1
	5	0.86	1.38	0.12	101.3	100.6	97.4	101.3	98.2

	Conc. spiked (µg/mL)	Precision (RSD %)			Matrix Effects (ME %)				
					Intra-day		Inter-Day		
		Intra-assay [a]	Intra-day	Inter-day [b]	ID1	ID2	Day 1	Day 2	Day 3
Δ^8-THC	80	0.93	0.37	1.57	100.7	101.2	102.0	100.7	101.2
	20	1.80	0.36	1.47	100.6	98.3	97.8	100.6	98.7
	5	3.04	0.11	1.80	95.4	99.4	93.9	95.4	98.2
THCVA	80	0.01	1.56	0.92	104.8	106.3	104.7	106.4	104.8
	20	0.87	1.91	0.67	95.4	96.9	97.0	95.7	95.4
	5	1.16	0.64	4.12	99.6	99.9	95.7	98.6	99.6
CBC	80	0.23	3.06	2.44	100.5	102.1	102.7	100.5	100.5
	20	0.16	0.08	1.02	100.4	99.7	101.7	100.4	100.4
	5	0.27	2.61	1.78	96.4	99.0	100.6	96.4	99.7
THCA	80	0.30	0.14	0.69	102.6	101.9	102.4	102.6	102.6
	20	0.41	0.47	0.58	100.2	98.0	101.1	100.2	99.2
	5	0.16	0.86	1.32	99.2	101.0	96.2	99.2	100.4
CBCA	80	0.13	1.10	0.86	107.0	107.3	105.5	107.5	107.0
	20	0.74	0.77	0.74	101.2	100.6	98.9	101.2	101.2
	5	0.82	2.31	3.11	107.2	107.1	106.2	104.1	107.2

[a] n = 3; [b] Same QC analysed in three consecutive days; n.d.: not determined; ID: intra-day; ME: matrix effect

Appendix 2S. Table presents the linear range (µg/mL), R^2 and detection and quantification limits (µg/mL) for CannProVar method B.

	Linear Range (µg/mL)	R^2	DL (µg/mL)	QL (µg/mL)
CBN	0.25 - 50	1.000	0.10	0.25
Δ^9-THC	0.50 - 50	1.000	0.10	0.50
THCA	0.25 - 50	1.000	0.10	0.25

Appendix 2T. Tables with precision and accuracy for the three quality controls for CannProVar method B.

	Conc. (µg/mL)	Precision (RSD%)		
		Intra-assay [a]	Intra-day	Inter-day [b]
CBN	30	0.61	1.65	1.19
	5	0.23	0.67	1.14
	1	0.42	0.64	0.82
Δ^9-THC	30	0.55	1.64	1.10
	5	0.50	0.47	0.78
	1	1.28	1.72	0.93
THCA	30	0.56	0.98	1.92
	5	0.87	1.33	0.99
	1	0.79	1.27	2.84

[a] n = 3; [b] Same QC analysed in three consecutive days; ID: Intra-day; RSD: relative standard deviation

	Conc. (µg/mL)	Accuracy (Bias%) [a]					
		Intra-day			Inter-Day		
		ID1	ID2	ID3	Day 1	Day 2	Day 3
CBN	30	-4.44	-1.41	-0.96	-3.02	-1.41	-0.64
	5	-2.86	-2.58	-1.46	-3.11	-2.58	-0.84
	1	-4.84	-3.86	-3.21	-4.20	-3.86	-1.92
Δ^9-THC	30	-1.14	2.00	2.40	0.99	2.00	3.28
	5	0.68	1.22	1.36	0.55	1.22	2.39
	1	1.78	-3.10	-0.58	-2.14	-3.10	-2.64
THCA	30	4.02	6.00	5.93	3.87	4.02	0.38
	5	-5.06	-3.59	-3.16	-4.40	-5.06	-2.38
	1	0.36	3.11	0.32	-2.26	0.36	2.62

[a] Same QC analysed in three consecutive days; ID: Intra-day

Appendix 2U. Tables with ME% for the diluted and concentrated samples; precision and accuracy percentages of ME, for spiked concentrations in cannabis sample - decarboxylation and purification step.

	Concentration spiked (µg/mL)	ME % (40x)	ME % (4x)
CBN	25	98,0	96,8
	5	100.4	98,3
	1	99.8	105.2
Δ^9-THC	25	97,2	96,7
	5	103,8	98,3
	1	109,8	108,2
THCA	25	98,0	96,2
	5	101,5	96,4
	1	106,7	97,6

ME: matrix effect

	Concentration spiked (µg/mL)	Precision (RSD %)		
		Intra-assay [a]	Intra-day [a]	Inter-day [a]
CBN	25	0,37	1,32	0,91
	5	0,30	1,22	1,14
	1	0,04	1,50	0,81
Δ^9-THC	25	0,12	0,60	1,05
	5	1,01	1,06	1,90
	1	0,03	0,54	5,16
THCA	30	0,20	0,11	0,69
	5	0,18	1,51	1,44
	1	0,08	0,78	0,77

[a] n=3; RSD: relative standard deviation

	Conc. (µg/mL)	ME (%)					
		Intra-day			Inter-Day		
		ID1	ID2	ID3	Day 1	Day 2	Day 3
CBN	25	97,0	97,3	97,3	97,0	97,0	97,3
	5	100,5	98,8	97,4	98,3	100,5	97,8
	1	109,3	108,9	109,6	105,2	109,3	108,8
Δ^9-THC	25	96,7	98,9	97,2	98,9	97,2	98,4
	5	98,3	96,3	96,1	96,3	98,5	97,4
	1	102,2	93,9	108,9	93,9	101,2	98,0
THCA	25	96,2	96,6	96,5	96,6	96,8	96,8
	5	97,6	96,6	99,2	96,6	100,3	97,3
	1	96,4	98,3	103,8	98,3	98,5	96,4

ID: intra-day; ME: matrix effect

OPTIMIZATION AND VALIDATION OF A HPLC-DAD METHOD FOR THE IDENTIFICATION OF 14 CANNABINOIDS: APPLICATION IN *CANNABIS SATIVA* EXTRACTS

avextra

Ana F. Morais[1,2,3], Robin Fertig[4], Ricardo J. Dinis-Oliveira[1,2,3,5] and Carlos J. A. Ribeiro[2,3,5,*]

[1] Department of Public Health and Forensic Sciences, and Medical Education, Faculty of Medicine, University of Porto, Porto, Portugal. [2] Associate Laboratory i4HB - Institute for Health and Bioeconomy, University Institute of Health Sciences - CESPU, 4585-116 Gandra, Portugal. [3] UCIBIO - Applied Molecular Biosciences Unit, Translational Toxicology Research Laboratory, University Institute of Health Sciences (1H-TOXRUN, IUCS-CESPU), 4585-116 Gandra, Portugal. [4] Avextra Innovations GmbH, 64625 Bensheim, Germany. [5] FOREN—Forensic Science Experts, 1400-136 Lisbon, Portugal. [6] Avextra Portugal SA., 7570-033 Grândola, Portugal.
*carlos.ribeiro@avextra.com

BACKGROUND

- **Cannabis sp. plant** has its **origins in East Asia**, where it was initially used for **recreational and religious purposes**.
- This plant encompasses **hundreds of chemical compounds**, including **phytocannabinoids, terpenoids and flavonoids**. The cannabinoids of **greatest medicinal interest** are **Δ^9-tetrahydrocannabinol** (Δ^9-THC) and **cannabidiol** (CBD), which are present in the plant in their acid form.
- **Sativex®** is an oromucosal spray containing standardized extract with **Δ^9-THC** and **CBD** already approved as a treatment option for **neuropathic pain associated with multiple sclerosis.**

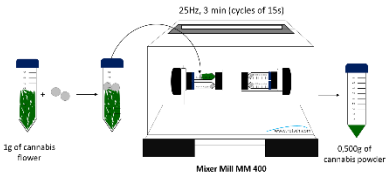
- With cannabis increasing recognition as a medicinal option, there are growing concerns over **how to extract, detect and quantify cannabinoids** properly and efficiently.
- **High-performance liquid chromatography** (HPLC) coupled with **ultra-violet** (UV) detection is considered the **gold standard for cannabinoid analytical assessment** included in cannabis monographs present in several pharmacopeias.

AIM

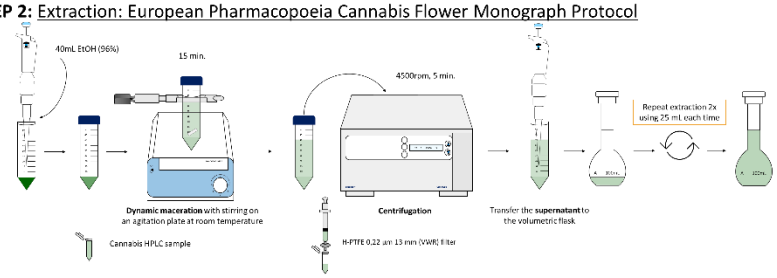
The main goal of this project was to **optimize and validate** an **HPLC-DAD analytical method** for the quantification of **14 cannabinoids** in cannabis extracts.

MATERIAL AND METHODS

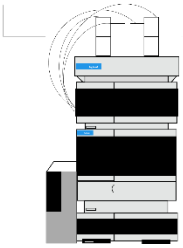
STEP 1: Pulverization



STEP 2: Extraction: European Pharmacopoeia Cannabis Flower Monograph Protocol



STEP 3: Optimization and Validation



Chromatographic Conditions:

Mobile phase:
- Solvents
- Gradient
- pH
- Flow rate (0,5-1mL/min)

Column:
- Length (50-150 mm)
- Temperature (30-50ºC)

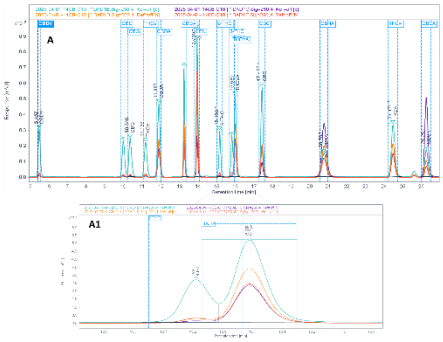
UV detection:
- 230 nm – other neutral cannabinoids
- 230, 260 nm – CBCA and CBNA
- 230, 272 nm – other acid cannabinoids
- 230, 280 nm – CBN and CBC

OPTIMIZED METHOD:
- ***COLUMN***: Agilent InfinityLab Poroshell 120 EC-C18, 3.0x150mm, 2.7µm; ***Guard column:*** Poroshell 120 EC-C18 3.0 mm, 2.7 µm
- ***FLOW RATE***: 0,5 mL/min.
- ***TEMPERATURE OF COLUMN***: 30ºC
- ***RUN TIME***: 30 min.
- ***POST-RUN TIME***: 4 min.
- ***UV DETECTION***: 230, 260, 272, 280nm
- ***MOBILE PHASE***: MeOH 0,05% FA + H_2O 0,1% FA

ICH Q2 Guidelines:
- Range
- Linearity (R>0,99)
- Detection Limit
- Quantitation Limit
- Specificity
- Accuracy (%Bias)
- Precision (Intraday, inter-day, %RSD)

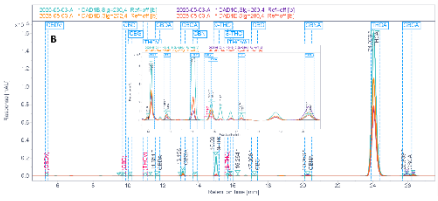
RESULTS

A Chromatogram presenting **14 cannabinoids** (2,5 µg/mL)



(A1) The resolution between **Δ^8-THC** and **THCVA** is **higher than 1.5** at 230nm, but without baseline separation. However, the two compounds can be clearly distinguished by their **absorbance wavelength spectra**. In contrast to Δ^8-THC, THCVA exhibits a strong absorbance peak at 272 nm.

B **Cannabis ZF cultivar extract** chromatogram



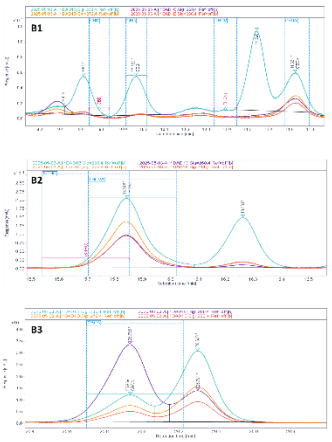
(B) 10 compounds were detected in the **cannabis ZF cultivar**: **CBG, CBDA, CBGA, CBN, Δ^9-THC, THCVA, CBC, CBNA, THCA, and CBCA.**

(B1) Good separation between CBD, CBG, THCV and CBDA and two additional matrix compounds was achieved.

(B2) No Δ^8-THC was detected, enabling quantification of THCVA at both wavelengths: 230 nm and 272 nm. Additionally, THCVA was baseline separated from a close by matrix compound.

(B3) Effective separation of CBCA from an unidentified compound was achieved with a **resolution greater than 1.5**. CBCA exhibits a maximum absorbance peak at **260 nm**, whereas the other matrix compound displays maximum absorbance at 230 nm.

B1, B2 and B3 Selective amplifications of chromatogram **B**



CONCLUSION

The developed method successfully separates **14 cannabinoids**, as well as other unknown compounds present in the cannabis ZF cultivar, which has a total THC content of 16.1%.
Full validation of the optimized method is currently in progress.

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1. Martinez, A.S. et al. Extraction techniques for bioactive compounds of cannabis. Nat Prod Rep, 40: 676-717, 2023, doi: 10.1039/d2np00059h
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Poster 44

Optimization and validation of an HPLC-DAD method for the identification of 14 cannabinoids: application in *Cannabis sativa* L extracts

Ana F. Morais [1,2,3], Robin Fertig [4], Ricardo Jorge Dinis-Oliveira [1,2,3,5] and Carlos J. A. Ribeiro [2,3,6,*]

[1] Associate Laboratory i4HB - Institute for Health and Bioeconomy, University Institute of Health Sciences - CESPU, 4585-116 Gandra, Portugal
[2] UCIBIO - Applied Molecular Biosciences Unit, Translational Toxicology Research Laboratory, University Institute of Health Sciences (1H-TOXRUN, IUCS-CESPU), 4585-116 Gandra, Portugal
[3] Department of Public Health and Forensic Sciences, and Medical Education, Faculty of Medicine, University of Porto, Porto, Portugal
[4] Avextra Innovations GmbH, 64625 Bensheim, Germany
[5] FOREN—Forensic Science Experts, 1400-136 Lisbon, Portugal
[6] Avextra Portugal SA, 7570-003 Grândola, Portugal

* Correspondence: carlos.ribeiro@avextra.com

Abstract

Background: *Cannabis* sp. plant has its origins in East Asia, where it was initially used for recreational and religious purposes. This plant encompasses hundreds of chemical compounds, including phytocannabinoids, terpenoids, and flavonoids. The cannabinoids of greatest medicinal interest are Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), which are present in the plant in their acid form [1]. Sativex® is an oromucosal spray containing standardized extract with Δ^9-THC and CBD already approved as a treatment option for neuropathic pain associated with multiple sclerosis [2]. With cannabis increasing recognition as a medicinal option, there are growing concerns over how to extract, detect and quantify cannabinoids properly and efficiently. High-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detection is considered the gold standard for cannabinoid analytical assessment included in cannabis monographs present in several pharmacopeias [3,4]. **Objective:** The main goal of this project was to optimize and validate an HPLC-DAD analytical method for the quantification of 14 cannabinoids in cannabis extracts. **Methods:** Flower pulverization was accomplished with a Retsch MM 400 ball mill. Extraction was performed according to the European Pharmacopoeia [4]. Chromatographic separation of cannabinoids was achieved on an Agilent 1260 Infinity II HPLC-DAD system, using an InfinityLab Poroshell 120 EC-C18 (3.0 x 150 mm, 2.7 µm) column protected with a Poroshell 120 EC-C18 3.0 mm, 2.7 µm guard column. The gradient elution was performed using methanol with 0,05% formic acid and deionized water with 0,1% formic acid mixtures, with a flow rate of 0,5 mL/min, run time of 30 min, and injection volume of 5 µL. **Results:** The optimized method resulted from adjusting chromatographic conditions: mobile phases (solvents, gradient, pH and flow rate: 0,5 – 1 mL/min), column length (50 – 150 mm) and temperature (30 – 50 °C). Diode array analysis was performed for specificity assessment and UV quantification was performed at 224, 230, 260, 272 and 280 nm. To demonstrate that the analytical method fits its purpose, accuracy, precision, linearity, and range were established based on regulatory guidelines – ICH Q2. **Conclusions:** The developed and validated method successfully separates 14 cannabinoids, as well as other compounds present in the cannabis extracts tested.

Keywords: cannabis; Δ^9-THC; HPLC-DAD

Acknowledgments/Funding

This work received financial support through the annual funding of 1H-TOXRUN of the University Institute of Health Sciences (IUCS-CESPU). The authors also acknowledge the funding provided by Avextra Portugal SA.

GC-MS-Based Study of 14 Cannabinoids Separation in *Cannabis sativa* L. Extracts using a Derivatization Approach

avextra

Bruna A. S. Nunes[1,2], Albertina M. Capita[1,2], Ana F. Rita[1,2,3], Ricardo J. Dinis-Oliveira[1,2,3,4], Carlos J. A. Ribeiro[1,2,5] and Eduarda M. P. Silva[1,2] *

[1] Associate Laboratory i4HB - Institute for Health and Bioeconomy, University Institute of Health Sciences - CESPU, 4585-116 Gandra, Portugal.
[2] UCIBIO - Applied Molecular Biosciences Unit, Translational Toxicology Research Laboratory, University Institute of Health Sciences (1H-TOXRUN, IUCS-CESPU), 4585-116 Gandra, Portugal. [3] Department of Public Health and Forensic Sciences and Medical Education, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal. [4] FOREN—Forensic Science Experts, 1400-136 Lisbon, Portugal. [5] Avextra Portugal SA., 7570-033 Grândola, Portugal.
*eduarda.silva@iucs.cespu.pt

Background

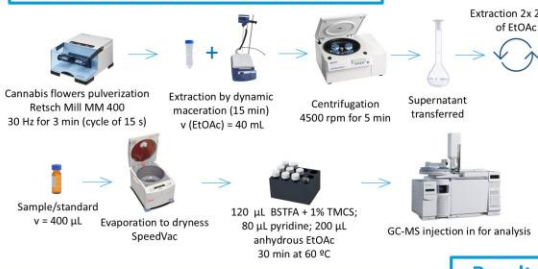
- Cannabinoids from *Cannabis sativa* L. are increasingly studied due to their potential as medicinal drugs, offering therapeutic benefits such as pain relief and anti-inflammatory effects, while also raising concerns as substances of abuse since Δ-9-tetrahydrocannabinol (Δ^9-THC) has psychoactive properties [1,2].
- Gas chromatography-mass spectrometry (GC-MS) is a powerful tool for identifying and quantifying cannabinoids, offering high sensitivity and specificity.

Objective

Development of a derivatization protocol and a GC-MS-based analytical method for cannabinoids detection in extracts of the Cannabis sp cultivar ZF plant.

Methods

Extract and standard preparation



Chromatographic conditions

- ✓ Capillary column containing 5% diphenyl 95% dimethylpolysiloxane (30 m x 0.25 mm x 0.25 µm)
- ✓ Injector temperature set to 280 ºC
- ✓ Injection volume of 1 µL followed by a temperature ramp from 180 up to 280 ºC at helium flow rate of 1 mL/min. The following oven temperature program was applied:
  - **Stage 1:** Initial temperature of 180 °C, held for 0.5 minutes.
  - **Stage 2:** Ramp at 8 °C/min to 250 °C, held for 10 minutes.
  - **Stage 3:** Ramp at 20 °C/min to 280 °C, held for 4.25 minutes.
- ✓ Total run: 25 min.

Results

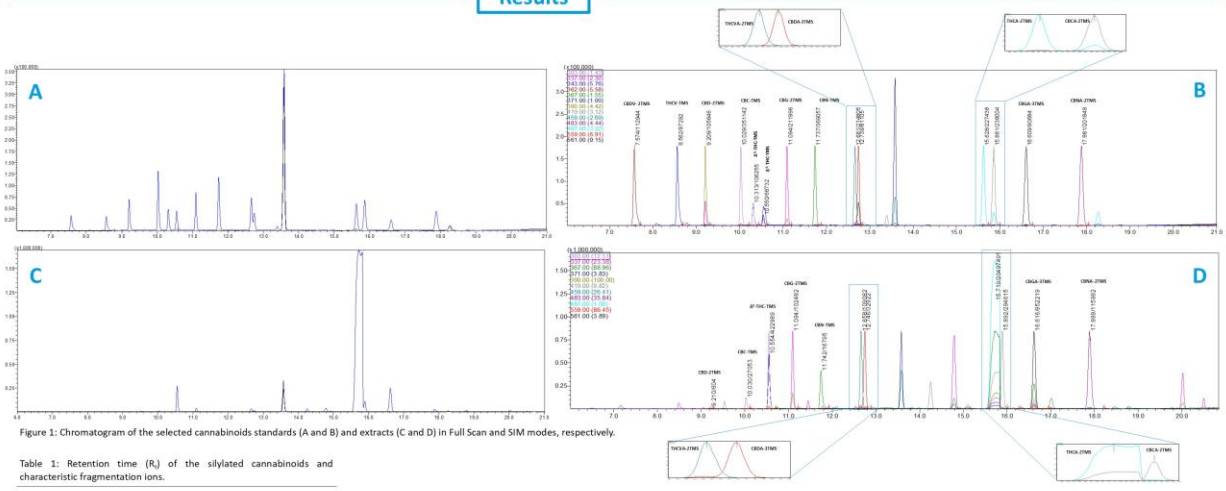


Figure 1: Chromatogram of the selected cannabinoids standards (A and B) and extracts (C and D) in Full Scan and SIM modes, respectively.

Table 1: Retention time (R_t) of the silylated cannabinoids and characteristic fragmentation ions.

Compounds	R_t(min)	Fragmentation (*m/z*)
CBDV- 2TMS	7.57	73, 309, 362, 430
THCV-TMS	8.56	73, 275, 343, 358
CBD-2TMS	9.21	337, 390, 458
CBC-TMS	10.03	73, 303, 371, 386, 73
Δ^8-THC-TMS	10.31	73, 303, 330, 386
Δ^9-THC-TMS	10.550	73, 315, 371, 386
CBG-2TMS	11.094	73, 337, 460
CBN-TMS	11.737	73, 310, 367, 381
THCVA-2TMS	12.661	73, 459
CBDA-3TMS	12.739	73, 453, 491, 559
THCA-2TMS	15.628	73, 487
CBCA-2TMS	15.861	73, 257, 419, 487
CBGA-3TMS	16.609	73, 417, 453, 561
CBNA-2TMS	17.881	73, 483, 498

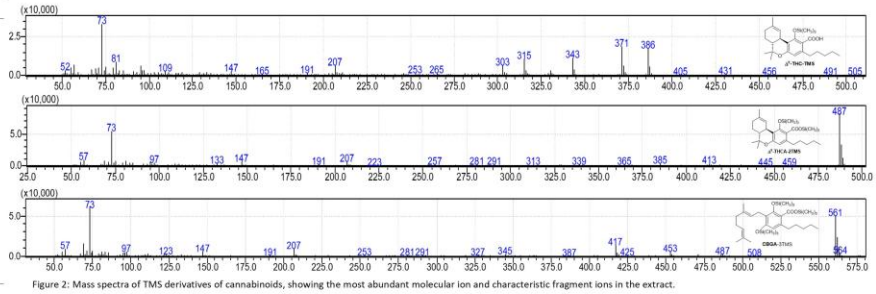


Figure 2: Mass spectra of TMS derivatives of cannabinoids, showing the most abundant molecular ion and characteristic fragment ions in the extract.

Conclusions

- The optimized derivatization conditions ensured the stability of the different cannabinoids avoiding the decarboxylation of the acidic forms and the formation of byproducts.
- The established chromatographic conditions provided an adequate separation and peak resolution of a total of 14 cannabinoids.
- The GC-MS-based analytical method was successfully applied to the identification and detection of these cannabinoids in cannabis extracts.

Acknowledgements: This work received financial support through the annual funding of 1H-TOXRUN of the University Institute of Health Sciences (IUCS-CESPU). The authors also acknowledge the funding provided by Avextra Portugal SA.

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1. Pellati, F.B. et al. Cannabis sativa L. and Nonpsychoactive Cannabinoids: Their Chemistry and Role against Oxidative Stress, Inflammation, and Cancer. BioMed Res. Int, 2018, doi: 10.1155/2018/1691428.
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Poster 47

GC-MS-Based Study of 14 Cannabinoids Separation in Cannabis sativa L. Extracts using a Derivatization Approach

Bruna A. S. Nunes[1,2], Albertina M. Capita[1,2], Ana F. Morais[1,2,3], Ricardo J. Dinis-Oliveira[1,2,3,4], Carlos J. A. Ribeiro[1,2,5] and Eduarda M. P. Silva[1,2] *

[1] Associate Laboratory i4HB - Institute for Health and Bioeconomy, University Institute of Health Sciences - CESPU, 4585-116 Gandra, Portugal.
[2] UCIBIO - Applied Molecular Biosciences Unit, Translational Toxicology Research Laboratory, University Institute of Health Sciences (1H-TOXRUN, IUCS-CESPU), 4585-116 Gandra, Portugal.
[3] Department of Public Health and Forensic Sciences and Medical Education, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal.
[4] FOREN—Forensic Science Experts, 1400-136 Lisbon, Portugal.
[5] Avextra Portugal SA, 7570-003 Grândola, Portugal.

* Correspondence: eduarda.silva@iucs.cespu.pt

Abstract

Background: Cannabinoids from *Cannabis sativa* L. are increasingly studied due to their potential as medicinal drugs, offering therapeutic benefits such as pain relief and anti-inflammatory effects, while also raising concerns as substances of abuse due to their psychoactive properties [1,2]. Gas chromatography-mass spectrometry (GC-MS) is a powerful tool for identifying and quantifying cannabinoids, offering high sensitivity and specificity. However, due to the thermal instability of cannabinoids, derivatization is a crucial step to improve their detectability and chromatographic behavior in GC-MS analysis. **Objective:** This study aims to develop a derivatization protocol and a GC-MS-based analytical method for cannabinoid detection in extracts of the *Cannabis* sp cultivar ZF plant. **Methods:** Extraction of cannabinoids from dried cannabis flowers was achieved following the European Pharmacopoeia protocol [3]. The standards and extracts were derivatized with 120 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane (BSTFA + 1% TMCS), 80 µL of pyridine in 200 µL of anhydrous ethyl acetate. The mixture reacted for 30 min at 60 °C, then cooled to room temperature and injected directly into the GC-MS for analysis. The chromatographic conditions were established using a capillary column containing 5% diphenyl 95% dimethylpolysiloxane (30 m x 0.25 mm x 0.25 µm), injector temperature set to 280 °C followed by a temperature ramp from 180 up to 280 °C at a helium flow rate of 1 mL/min to a total run of 25 min. **Results:** Several derivatization conditions were tested to allow high yields of the derivatized cannabinoids while preventing decarboxylation of the acidic forms. Hence, reaction time and temperature, the quantity of derivatizing agent, the use or not of pyridine, and the use of solvents like ethyl acetate, dichloromethane, and acetonitrile were studied. Chromatographic conditions were also optimized to allow the simultaneous separation and detection of 14 compounds in the same run. **Conclusions:** The optimized derivatization conditions ensured the stability of the different cannabinoids avoiding decarboxylation of the acidic forms and formation of byproducts. The established chromatographic conditions provided an adequate separation and peak resolution of a total of 14 cannabinoids. The GC-MS-based analytical method was successfully applied to the identification and detection of these cannabinoids in cannabis extracts.

Keywords: medicinal cannabis; phytochemical analysis; gas chromatography; mass spectrometry

Acknowledgments/Funding

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References

1. Pellati, F.B. et al. Cannabis sativa L. and Nonpsychoactive Cannabinoids: Their Chemistry and Role against Oxidative Stress, Inflammation, and Cancer. *BioMed Res. Int*, **2018**, doi: 10.1155/2018/1691428.
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3. Ph. Eur. 11.5. Cannabis Flower Monograph, 07/**2024**:3028.

Analysis of Terpenes in Cannabis by GC-MS: Method Development and its Application to *Cannabis sativa* extracts

avextra

Albertina M. Capita [1,2], Ana F. Morais [1,2,3], Ricardo J. Dinis-Oliveira [1,2,3,4], Carlos J. A. Ribeiro [1,2,5] and Eduarda M. P. Silva [1,2] *

[1] Associate Laboratory i4HB - Institute for Health and Bioeconomy, University Institute of Health Sciences - CESPU, 4585-116 Gandra, Portugal. [2] UCIBIO - Applied Molecular Biosciences Unit, Translational Toxicology Research Laboratory, University Institute of Health Sciences (1H-TOXRUN, IUCS-CESPU), 4585-116 Gandra, Portugal. [3] Univ Porto, Fac Med, Dept Publ Hlth & Forens Sci & Med Educ, P-4200450 Porto, Portugal. [4] FOREN Forens Sci Experts, Ave Dr Mario Moutinho 33-A, P-1400136 Lisbon, Portugal. [5] Avextra Portugal SA, 7570-003 Grândola, Portugal.
*eduarda.silva@iucs.cespu.pt

Background

- In addition to cannabinoids, key bioactive compounds in cannabis, terpenes and terpenoids also have therapeutic importance, working synergistically through their entourage effect for the medicinal efficacy of this plant [1,2].
- Terpenes and terpenoids are also responsible for the characteristic aroma of numerous varieties of cannabis, ranging from citrus to woody aromas [3].
- Gas Chromatography-Mass Spectrometry (GC-MS) has become a powerful and reliable analytical tool for the precise identification and quantification of terpenes in cannabis. GC-MS allows to characterize cannabis profile, essential for optimizing strain selection and the tailoring of treatments for specific medical conditions [4].

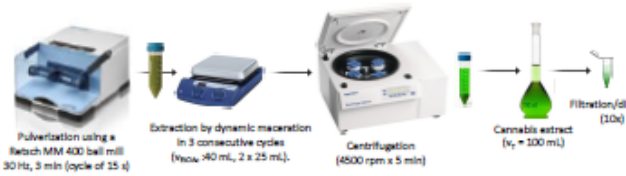
Objective

- Optimize an analytical method for the separation and quantification of 9 terpenes.
- Apply this method to the identification of terpenes in cannabis extracts.

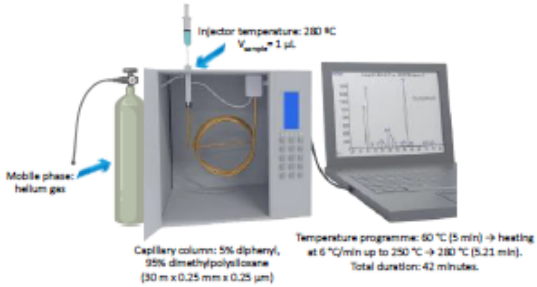
Methods

Extraction

- Dried cannabis flowers from Cannabis sp cultivar ZF plant were pulverized in a Retsch MM 400 ball mill and extracted using a modified European Pharmacopoeia method [5].
- Ethyl acetate (EtOAc) was added and the mixture stirred for 15 min and centrifuged at 4500 rpm for 5 min.
- Supernatant transferred to a volumetric flask and extraction was repeated twice. Final volume was adjusted to 100 mL followed by filtration.



GC-MS conditions



Results

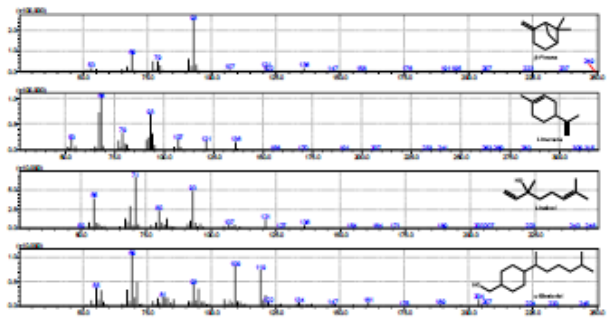


Figure 1: Mass spectra of selected terpenes showing the molecular ion (when detected) and characteristic fragment ions.

Table 1. Retention time (Rt) of terpene standards, characteristic fragmentation ions obtained and molecular ion.

Compounds	R$_t$(min)	Molecular ion (m/z)	Fragmentation (m/z)
α-Pinene	5.85	136	121 (M-15), 105, 93, 77, 67
β-Pinene	7.14	136	121 (M-15), 93, 79, 69
β-Myrcene	7.61	136	121 (M-15), 93, 79, 69
Limonene	8.75	136	121 (M-15), 107, 93, 79, 68
Terpinolene	10.65	136	136, 121 (M-15), 93, 79
Linalool	10.88	154	136 (M-18), 121, 93, 80, 71, 55
Tridecane	16.35	184	85, 71, 57
β-Caryophyllene	19.25	204	189 (M-15), 133, 93, 79, 69
α-Humulene	20.02	204	189 (M-15), 147, 121, 93, 80)
α-Bisabolol	24.77	222	204 (M-18), 119, 109, 93, 69, 55

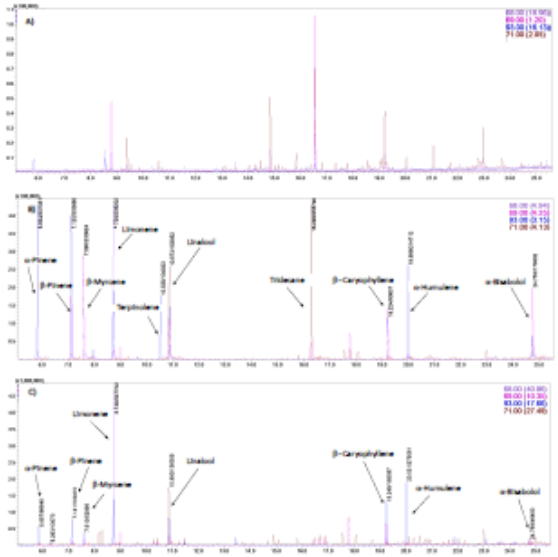


Figure 2: Chromatogram of the A) blank, B) terpene standards mixture and internal standard (tridecane) at 1 µg/mL, and C) extract in SIM mode.

- The separation and identification of the nine standards terpenes was possible. In the extract only terpinolene was not detected.

Conclusions

- A robust gas chromatography-mass spectrometry (GC-MS) method was established for analyzing terpenes.
- This method successfully separated and detected nine terpenes.
- Future work will involve method validation and terpene quantification.

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Poster 45

Analysis of terpenes in cannabis by GC-MS: method development and its application to *Cannabis sativa* L extracts

Albertina M. Capita [1,2], Ana F. Morais [1,2,3], Ricardo Jorge Dinis-Oliveira [1,2,3,4], Carlos J. A. Ribeiro [1,2,5] and Eduarda M. P. Silva [1,2,*]

[1] Associate Laboratory i4HB - Institute for Health and Bioeconomy, University Institute of Health Sciences - CESPU, 4585-116 Gandra, Portugal
[2] UCIBIO - Applied Molecular Biosciences Unit, Translational Toxicology Research Laboratory, University Institute of Health Sciences (1H-TOXRUN, IUCS-CESPU), 4585-116 Gandra, Portugal
[3] Department of Public Health and Forensic Sciences and Medical Education, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal
[4] FOREN—Forensic Science Experts, 1400-136 Lisbon, Portugal
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* Correspondence: eduarda.silva@iucs.cespu.pt

Abstract

Background: In addition to cannabinoids, key bioactive compounds in cannabis, terpenes and terpenoids, also have therapeutic importance, potentially working synergistically through their entourage effect for the medicinal efficacy of this plant [1,2]. Besides their therapeutic advantages, they are also responsible for the characteristic aroma of numerous varieties of cannabis, ranging from citrus to woody aromas [3]. Gas Chromatography-Mass Spectrometry (GC-MS) has become a powerful and reliable analytical tool for precisely identifying and quantifying terpenes in cannabis. Understanding the terpene profile is essential for optimizing strain selection, which may enable the development of targeted therapies for specific medical conditions [4]. **Objective:** This study aims to develop a GC-MS-based analytical method for the separation and quantification of terpenes in cannabis extract. **Methods:** Dried cannabis flowers were pulverized in a Retsch MM 400 ball mill and extracted using a modified European Pharmacopoeia method [5]. 40 mL of ethyl acetate was added to the ground cannabis sample, and the mixture was stirred for 15 minutes at room temperature and centrifuged at 4500 rpm for 5 minutes. The supernatant was transferred to a volumetric flask and extraction was repeated twice with 25 mL. The final volume was adjusted to 100 mL followed by filtration. The extract was diluted to 1:10 in ethyl acetate and analyzed by GC-MS. For the chromatographic separation, a capillary column containing 5% diphenyl 95% dimethylpolysiloxane (30 m × 0.25 mm × 0.25 µm) was used, and the injector temperature was programmed to 280 °C. A typical run started at a temperature of 60 °C, raising to 280 °C at a helium flow of 1 mL/min with a total run time of 42 min. **Results:** Several chromatographic parameters were studied to enhance the separation of terpenes, namely the starting run temperature, ramp profile, and running times. The extraction from cannabis flowers was performed using two solvents, dichloromethane and ethyl acetate, to evaluate the efficiency of the extraction. The chromatographic conditions established made it possible to separate and identify the nine compounds in the same run, both in a mixture of standards and in the extracts. **Conclusions:** A GC-MS analytical method was developed, allowing the separation, identification, and quantification of 9 terpenes simultaneously.

Keywords: medicinal cannabis; phytochemistry; mass spectrometry; gas chromatography

Acknowledgments/Funding

This work received financial support through the annual funding of 1H-TOXRUN of the University Institute of Health Sciences (IUCS-CESPU). The authors also acknowledge the funding provided by Avextra Portugal SA.

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1. Lee, S. et al. Identification of Terpene Compositions in the Leaves and Inflorescences of Hybrid Cannabis Species Using Headspace-Gas Chromatography/Mass Spectrometry. *Molecules* **2023**, *28*, 1-13, doi.org/10.3390/molecules28248082.
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