Analytical method development based on the ESI ionization behaviour of cannabinoids

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Abstract

Regulatory frameworks for medical and recreational cannabis programs have been established for the cannabis market in a number of countries and states across the world. Cannabis testing facilities are increasingly needed to identify the concentration of various cannabinoids as well as dangerous pollutants such as pesticides, mycotoxins, and heavy metals for the safety of consumers in this burgeoning business.

To address these concerns, two distinct high performance liquid chromatography (HPLC) procedures were developed, with method (A) being able to fully resolve 19 cannabinoids in a short period of time. The second method (B) was capable of analyzing acidic cannabinoids with great linearity; the entire analysis duration was 13 minutes, with samples eluting between 3 and 12 minutes. Calibration curves for each analyte achieved R^2 values greater than 0.999. For all analytes, a linear dynamic range of 0.0001 – 1 μg/mL (9 levels) was chosen, providing the best linearity results, particularly for acidic cannabinoids.

After evaluating ten various approaches to improve sensitivity, separation, and linearity, we determined that the pH of the mobile phase was the most critical element in achieving greater linearity and separation. We discovered that the pH of our previous mobile phase (acetonitrile/water 7:3 with the addition of 0.1 percent formic acid and 5 mM ammonium formate) was 4.5, which is the exact pKa of carboxylic acid, making it a potential cause of quadratic behavior, so we decided to lower the pH of the mobile phase using less ammonium formate and a higher amount of formic acid. This attempt resulted in the second approach (acetonitrile/water 7:3 with 0.3 percent formic acid and 2mM ammonium formate in pH 3.5).

The second method produced the best linearity findings when 9 different sample concentrations were considered (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 μg/mL). We were able to separate 18 cannabinoids using this approach (except CBLA due to co-elution of CBLA and CBCA).

These methods will enable regulatory laboratories, cannabis enterprises, and other facilities working on cannabis analysis to detect a greater number of cannabinoids in a single run, with more precision and in less time. Future research is needed to create techniques that can identify additional cannabinoids with high linearity and accuracy, given the increased interest in cannabis cultivation, cannabis testing, and the discovery of novel cannabinoids from this ever popular plant.

Résumé

Des cadres réglementaires pour les programmes de cannabis médical et récréatif ont été établis pour le marché du cannabis dans un certain nombre de pays à travers le monde. Des installations d'analyse du cannabis sont de plus en plus nécessaires pour identifier la concentration des divers cannabinoïdes ainsi que des polluants dangereux tels que les pesticides, les mycotoxines et les métaux lourds pour la sécurité des consommateurs dans ce secteur en plein essor.

Pour répondre à ces préoccupations, deux procédures distinctes de chromatographie liquide à haute performance (HPLC) ont été développées, la méthode (A) étant capable de résoudre complètement 19 cannabinoïdes en peu de temps. La seconde méthode (B) était capable d'analyser les cannabinoïdes acides avec une grande linéarité ; la durée totale de l'analyse était de 13 minutes, les échantillons éluant entre 3 et 12 minutes. Les courbes d'étalonnage pour chaque analyte ont atteint des valeurs R2 supérieures à 0.999. Pour tous les analytes, une plage dynamique linéaire de 0.0001 - 1 g/mL (9 niveaux) a été choisie, fournissant les meilleurs résultats de linéarité, en particulier pour les cannabinoïdes acides.

Après avoir évalué dix approches différentes pour améliorer la sensibilité, la séparation et la linéarité, nous avons déterminé que le pH de la phase mobile était l'élément le plus critique pour obtenir une plus grande linéarité et séparation. Nous avons découvert que le pH de notre phase mobile précédente (acétonitrile/eau 7:3 avec l'ajout de 0.1 % d'acide formique et de formiate d'ammonium 5 mM) était de 4.5, ce qui est le pKa exact de l'acide carboxylique, ce qui en fait une cause potentielle de comportement quadratique. Nous avons donc décidé d'abaisser le pH de la phase mobile en utilisant moins de formiate d'ammonium et une plus grande quantité d'acide formique. Cette tentative a abouti à la deuxième approche (acétonitrile/eau 7:3 avec 0.3 % d'acide formique et 2 mM de formiate d'ammonium pour un pH 3.5).

La deuxième méthode a produit les meilleurs résultats de linéarité lorsque 9 concentrations d'échantillon différentes ont été prises en compte (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 g/ml). Nous avons pu séparer 18 cannabinoïdes en utilisant cette approche (sauf CBLA en raison de la co-élution de CBLA et CBCA).

Ces méthodes permettront aux laboratoires de réglementation, aux entreprises de cannabis et à d'autres installations travaillant sur l'analyse du cannabis de détecter un plus grand nombre de cannabinoïdes en une seule analyse, avec plus de précision et en moins de temps. Des recherches futures sont nécessaires pour créer des techniques capables d'identifier des cannabinoïdes supplémentaires avec une linéarité et une précision élevées, compte tenu de l'intérêt accru pour la culture du cannabis, l'analyse du cannabis et la découverte de nouveaux cannabinoïdes dans cette plante très populaire.

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Chapter 2 was authored by Mohammad Reza Khan Bolouki, and he reviewed the literature and was responsible for gathering the information and writing the manuscript. Mark Lefsrud, Valérie Orsat, Stéphane Bayen provided critical review of the manuscript.

Chapter 3 was authored by Mohammad Reza Khan Bolouki and he was responsible for the analytical method development, experimental design, data collection, interpretation of data, analysis and writing the manuscript. Mark Lefsrud was involved in the supervision of the experiments and provided critical review of the manuscript. Valérie Orsat and Stéphane Bayen were co-supervisor and advisor, provided feedback on experimental results and provided critical review of the manuscript.

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Abbreviations

HPLC	High performance liquid chromatography
R^2	Coefficient of determination
LC	Liquid chromatography
GC	Gas chromatography
FID	Flame ionization detector
MS	Mass spectrometry
HPLC-MS	High-performance liquid chromatography-mass spectrometry
ESI	The electrospray ionization
ECS	The endocannabinoid system
THC	Tetrahydrocannabinol
IHR	The Industrial Hemp Regulations
MMAR	Medical Marijuana Access Regulations
MMPR	The Marijuana for Medical Purposes Regulations
ACMPR	The Access to Cannabis for Medical Purposes Regulations
OTC	Over-the-counter
CBD	Cannabidiol
FDA	The Food and Drug Administration
NSAIDs	Nonsteroidal anti-inflammatory medicines
CBG	Cannabigerol
CBN	Cannabinol
CBC	Cannabichromene
CBL	Cannabielsoin

CBT	Cannabitriol
CBDA	Cannabidiolic acid
THCA	Delta-9-tetrahydrocannbinolic acid
NMR	Nuclear magnetic resonance
TMS	Trimethyl silyl
DB-1	Dimethylpolysiloxane
DB-5	Dimethyl-polysiloxane
DC	Direct current
RF	Radio frequency
m/z	The mass-to-charge ratio
CEM	The channel electron multiplier
SRM	Selected reaction monitoring
UHPLC	Ultra-high-performance liquid chromatography
ICH	The International Conference on Harmonization
EC	The European Community
USP	The United States Pharmacopoeia
LOD	Limit of detection
LOQ	Limit of quantitation
S/N	Signal to noise ratio
RSD	Relative standard-deviation
CAN	Acetonitrile
CBE	Cannabinol

1. General Introduction

1.1. Thesis Motivation

The absence of consensus for the methods used for cannabis testing is a challenge, but presents opportunity for analytical chemists in the field because it enables the incorporation of the newest technologies and best practices without the restrictions imposed by legacy approaches that often impede method development in other industries. Liquid chromatography (LC) is proving to be a valuable complementary technique to gas chromatography (GC) in cannabis testing for the analysis of cannabinoids, mycotoxins, and pesticides. In the study of cannabinoids, the main compounds of cannabis species are normally determined using gas chromatography (GC) paired with both a flame ionization detector (FID) and mass spectrometry (MS). Due to the high temperature obtained, however, decarboxylation of the native components (acids) to their neutral forms happens using GC techniques. As a result, accurate cannabinoid metabolite profiling in plant material is not feasible (1–3). Since high-performance liquid chromatography-mass spectrometry (HPLC-MS) combines strong chromatographic separation with the excellent qualitative advantages of mass spectrometry, it is an efficient way of distinguishing unknown substances (4). To assist in structural elucidation, there is an emphasis placed on obtaining structural knowledge from HPLC-MS techniques (5,6). Mass spectrometers have been commonly used to identify synthetic cannabinoids in complex matrices due to their superior resolution and sensitivity (7). Current research is attempting to develop methods based on the electrospray ionization (ESI) behaviour of cannabinoids, for detecting a higher number of cannabinoids with the highest possibility of linearity in a short period of time, especially for acidic cannabinoids.

1.2. Research Problem

Most of the method development studies have focused on changing the flow rate or column to get better results in separation. However, to date these studies has highlighted a loss of linearity for acidic cannabinoids. According to a review of the recent literature, other important aspects, including the pH of the mobile phases, the column temperature or other parameters related to the HPLC analysis of cannabinoids, are less frequently considered. We realized that these factors can potentially have a determinant effect on the separation of cannabinoids and the linearity of acidic cannabinoids due to their impact on the ionization of cannabinoids.

1.3. Objective and Specific Aims

Objective: Analyzing and determination of reasons for losing signals overtime during the cannabinoids detection in multiple matrices with LC/MS-ESI method.

Aim 1: Detection and elucidation of the non-linearity in THCA, THC, CBD and CBDA concentration-response curve

Based on the received information from Agilent Technologies company, there are two major challenges occurring during the analysis of cannabinoids: their complete separation and the linearity of the signals over a wide range of concentration. Examples of calibration curve with different detection approaches are shown in Figure 1.1. and Figure 1.2. Non-linearity is obvious for acidic cannabinoids which shows the possibility of impact of carboxylic acid functional group on linearity in acidic cannabinoids. The results of Figure 1.1. and 1.2. obtained from Agilent 6470 LC/TQ and the instrument condition and mobile phase are shown in Table 1.1. and Table 1.2. The 9 different sample concentrations were considered (0.0005, 0.001, 0.005, 0.01, 0.05, 0.1 μg/mL).

Table 1.1. Mobile phase program and source conditions used in the Agilent laboratory for testing cannabinoids.

Timetable

Time	Mobile Phase A	Mobile Phase B
0 min	99	1
4 min	99	1
8 min	40	60
9 min	0	100
11 min	0	100

Source Conditions– 6470 LC/TQ

- Source: Jet Stream ESI
- Drying Gas Flow: 12 L/min
- Sheath Gas Temperature: 350 °C
- Nebulizer Pressure: 40 psi
- Drying Gas Temperature: 350 °C
- Sheath Gas Flow: 11 L/min
- Polarity: Positive
- Capillary Voltage: 3500 V
- Nozzle Voltage: 1000 V

Table 1.2. Chromatographic conditions used in the Agilent laboratory for testing cannabinoids.

Instrument	• Infinity II Prime pump G7104C • Infinity II Vial sampler G7129C with Cooling option • Infinity II Column oven G7130A • Infinity II DAD G7115A • 6470 LC/TQ, with AJS in either positive or negative polarity
Column	Agilent InfinityLab Proshell 120 EC-C18, 3.0 × 100mm, 1.9 µm
Mobile Phase	• Mobile Phase A: *modifier* (0.1% Formic Acid + 5 mM ammonium formate) in (acetonitrile:water7:3) • Mobile Phase B: *modifier* (0.1% Formic Acid) in methanol
Column temperature	30˚C
Wavelength	230 nm
Injection volume	5 µL
Flow rate	0.5 mL/min
Sample concentrations	(0.0005, 0.001, 0.005, 0.01, 0.05, 0.1 µg/mL)

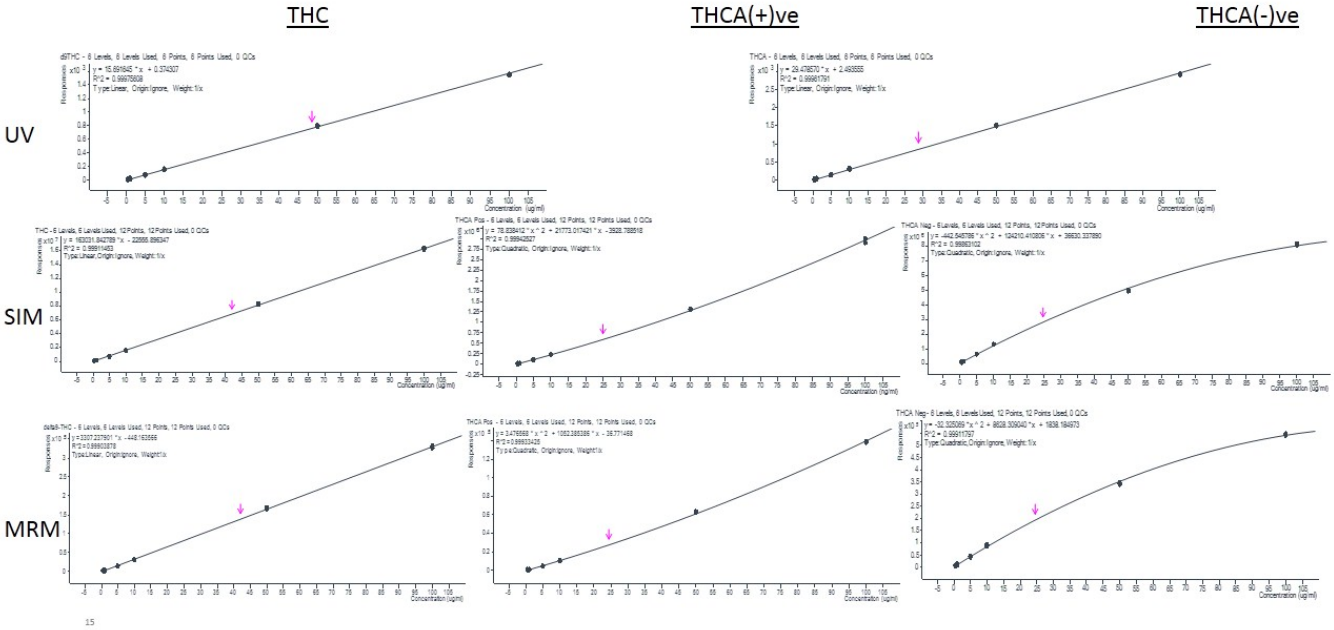

Figure 1.1. Calibration Curves (in methanol, no matrix) – THC vs THCA
(Provided from Agilent Technologies, Inc based on the conditions provided on Table 1.1 &1.2)

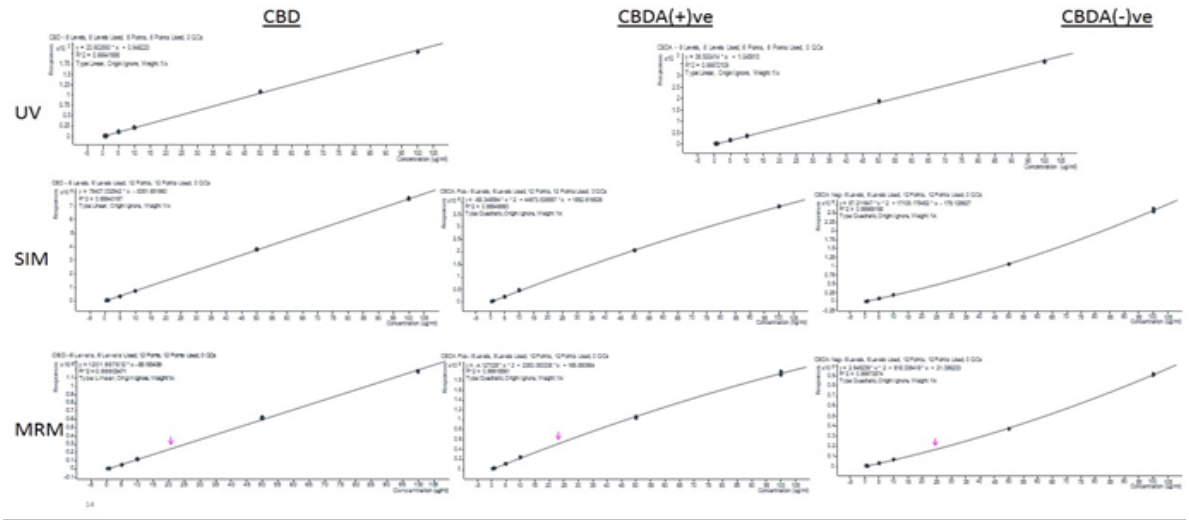

Fig 1.2. Calibration Curves (in methanol, no matrix) – CBD vs CBDA
(based on the conditions provided on Table 1.1 &1.2)

- As can be seen in Figures 1.1 and 1.2, there are poor results for THCA and CBDA in case of linearity.
- In contrast with MS source, LC/UV giving us a linear signal which means that there is an issue with MS detector and the ESI ionization.
- There are multiple important factors that can cause the non-linearity of the received signals from the LC/MS-ESI system, some of the factors that can cause this effect are:

- Source temperature
- Drying gas flow rate
- Nebulizer pressure
- The quality of initial products
- Mobile phase pH
- Voltage (entrance & fragmentation)
- Analyte concentration
- System tuning period
- Proton-bound dimerization
- Mobile phase additives
- Analyte/IS ratio (concentration of IS)

Aim 2: Reproducibility of collected data

The received data from Agilent company showed that the results were not reproducible from injection to injection with their common method that they used for cannabinoids testing which mentioned in Table 1.1. and Table 1.2. Obtained data from this method in different replications showed on stability in retention times and linearity. Consequently, developing a proper method for reproducibility and stability of the system result is the goal.

2. Literature Review

2.1. Background

Cannabis sativa, popularly known as marijuana, weed, and pot, has been used for decades for cultural, medical, and recreational purposes (8). It originated and was domesticated in Central Asia, where it was originally used for fibre, food, and medicine (8). Cannabis has only recently become associated with recreational usage (9). Cannabis is produced in practically every corner of the world, and the plant is either male, female, or hermaphrodite, with the latter bred for fibre production (9). Cannabis attracted scientific interest around the start of the 20th century when cannabis' pharmacokinetic and pharmacodynamic effects were investigated (10,11). The discovery of the endogenous cannabis system opened up a new area of study (12). This approach progressively contributed to a better understanding of the molecular mechanisms behind cannabis's effects. It is now feasible to connect to various modulating or regulatory systems in our bodies (13). The endocannabinoid system (ECS) is a broad neuromodulatory system that plays critical functions in central nervous system development, synaptic plasticity, and the response to endogenous and environmental stressors (14). The ECS consists of cannabinoid receptors, endogenous cannabinoids (endocannabinoids), and the enzymes involved in endocannabinoid synthesis and degradation (15). CB1 cannabinoid receptors are the most prevalent; nevertheless, CB2 cannabinoid receptors, transient receptor potential channels, and peroxisome proliferator-activated receptors are activated by cannabinoids (16,17). Exogenous cannabinoids, such as tetrahydrocannabinol (THC), exhibit their biological effects via interactions with cannabinoid receptors. As a result of the widespread social use of cannabis and the participation of endocannabinoids in a wide range of biological processes, much has been discovered about the physiologic and pathophysiologic activities of cannabis (18).

The cannabis plant and its products include a wide range of compounds. Some of the 572 chemicals identified are unique to cannabis, such as the more than 125 cannabinoids, also terpenes which have around 120 components and are found throughout the plant tissues (19–21). Table 2.1 shows the number of known components in the cannabis plant regarding their chemical class (19–22). The compounds of interest in cannabis research are often referred to as phytocannabinoids (23).

Table 2.1 Cannabis constituents

Chemical Class	Known
Cannabinoids	125
Nitrogenous compounds	27
Amino acids	18
Proteins, glycoproteins, and enzymes	11
Sugars and related compounds	34
Hydrocarbons	50
Simple alcohols	7
Simple aldehydes	12
Simple ketones	13
Simple acids	21
Fatty acids	22
Simple esters and lactones	13
Steroids	11
Terpenes	120
Non-cannabinoid phenols	42
Flavonoids	34
Vitamins	1
Alkaloids	2
Elements	9
TOTAL	572

2.2. Overview of the Canadian hemp industry

2.2.1. History of cannabis legalization

Cannabis legislation has been a challenging topic since 1923, when Parliament deemed the plant illegal (24). Access to medical cannabis was not permitted to medical patients under extreme circumstances until the early 1990s (25). After recognizing the crop's potential to diversify the Canadian agriculture industry, the Canadian government modified regulations to allow hemp production by the mid-1990s (26). Under the CDSA, the Industrial Hemp Regulations (IHR) were created in 1998 to allow for the commercial cultivation, processing, and sale of industrial hemp (27). With the implementation of the Medical Marijuana Access Regulations (the MMAR) in 2001, marijuana consumption for medicinal purposes first became legal (28). The MMAR was quickly superseded by the Marijuana for Medical Purposes Regulations 2013 (MMPR), which was quickly followed by the Access to Cannabis for Medical Purposes Regulations 2016 (ACMPR), which is still in effect today for establishing a legal framework that allows people to obtain cannabis for

medical reasons (28). The ACMPR re-established the MMAR's prior "personal production programme," which is confirming personal allowances under The Cannabis Act (29,30). Medical cannabis must be obtained from a federally licenced producer and transported by a postal services carrier on a national level. The Liberal Party of Canada committed in 2015 to legalise and regulate recreational cannabis consumption (29). As a result, the Liberal Party's initial legalisation policy included professional assistance from law enforcement and attempted to implement a carefully regulated profit and distribution system in a government-controlled setting (30). In this regard, the government introduced The Cannabis Act. The Cannabis Act, first proposed in 2016 as Bill C-45, was subjected to considerable legislative examination and discussion before being passed in 2018 and making cannabis legal on October 17th of that year (31). It was amended in October 2019 and expanded based on the first year's findings (30). The Cannabis Act, for example, regulates the types of products that can be produced, such as dried cannabis and cannabis oil, and more recently in 2019, subcategories such as topicals, extract for eating, and extract for inhaling were included (32). Only a few responsibilities are delegated to provinces and territories under The Cannabis Act, such as determining the age at which cannabis can be purchased, the number of plants that can be grown and used, workplace safety regulations, the penalty for youth offences, and home cultivation and possession regulations (30). Provinces are specifically responsible for developing a license, distribution, and marketing plans (31). The Cannabis Act, via regulation, ultimately accords provinces the same responsibility they have with regard to alcohol (30).

2.2.2. History of industrial hemp

Industrial hemp is primarily used for fibre production (33). Industrial hemp was the primary catalyst for the development of the hemp industry in Canada (34). But the hemp fibre market opportunities have never fully materialized, and the industry has suffered during this starting stage (34). The hemp industry has since shifted its focus to food applications (35). Omega-3 and omega-6 levels in hemp seeds are high, and the seeds are processed into oils, protein powders, and hulled or shelled seeds with limited claims for health benefits (36). Food products like salad dressing and body care products such as lip balm and massage oil can be made with hemp oil (37). In 2016, hemp exports from Canada exceeded $145 million (38). The prairie provinces of Canada account for the majority of hemp acres (39). The Canadian hemp industry originally imported varieties from Europe, but more recently, several hemp breeding programs have been developed locally to

breed for the local environment (40). Globally, the plant-based and environmentally friendly product industry is experiencing an upward trend due to people's demand for such products (41). Because of modifications in cannabis regulation, hemp-derived products have become a prominent commercial commodity, increasingly utilised for medicinal or therapeutic purposes (42). Over a thousand hemp-derived products are already available in Canada, ranging from oil extracts to hemp-infused drinks (43,44). As a result, the industry has been significantly consolidated (45).

2.3. Canadian cannabis related products

Over-the-counter (OTC) medicines containing cannabinoids found in both hemp and cannabis have emerged in recent years (46). The federal government of Canada proposed regulations for the legalisation of cannabis edibles and beverages (47). This legislation was successfully drafted, with the goal of legalising these new products by October 2019 (47). While smoking is still the most common way to consume cannabis, other methods, like edibles and vaping, are gaining popularity (48). Marijuana edible production has been and continues to be a roaring trade (49). Consumer preferences are shifting for a variety of reasons. First, people believe edibles are a safer and healthier way to ingest cannabis than smoking or vaping (50). Similarly, some physicians who prescribe medicinal cannabis to Canadian patients have advised them to use capsules and oils as an alternative to smoking (47). Second, unlike smoking, edibles are a handy and inconspicuous way of consuming cannabis, and there is no preparation needed to ingest the product (51). Because of their mobility, these items are convenient for customers to utilise outside of their homes, which may appeal to those wishing to consume during social gatherings or outdoor activities (52). Finally, consuming THC-containing edibles provides a notably different euphoric experience than inhaling, which some people may prefer (53). Cannabis-infused beers are being produced by a number of Canadian breweries (54). According to recent study on these products, the sales of CBD products alone will exceed $20 billion (55). According to ABD Analytics and ArcView Market Research, the market will be worth $42.7 billion dollars by 2024 (56). They estimated a total market value of $57 billion for cannabis by 2027 (57). The global cannabis industry is projected to be worth up to $130 billion USD by 2029 (58). Commercial analytical laboratories will require adequate quality control protocols. To ensure that the cannabinoid content of these products is compatible with regulations, they need to be measured and detected using a variety of techniques.

2.4. Medical and recreational use of cannabis

Canada became the second country in the world to legalise the production, distribution, sale, and nonmedical use of cannabis for adults on October 17, 2018 (30). This came roughly two decades after cannabis was legalised for medicinal purposes (28), corresponded with significant growth in both legal retail sales and volume sold (60). Since 2016, the number of registered medical cannabis users in Canada has more than quadrupled (60). The medicinal cannabis economy has expanded increasingly competitive, with dispensaries promoting cannabis for a variety of medical disorders ranging from mental diseases to physical conditions (61). One of the most recent topics, related to cannabis medical use, is cannabinoids' effects on the human body and their therapeutic effects (62). Some of these cannabinoids have a profusion of suggested or purported medicinal applications (63). Some of the recognized disorders that have shown improvement with cannabidiol (CBD) therapy include: Crohn's disease, glaucoma, Alzheimer's, Parkinson's, hepatic encephalopathy, chronic traumatic encephalopathy, autism, ADHD, migraines and cancer (64,65). Treatment for nausea in patients receiving chemotherapy is one of the positive applications (66). Reduced muscular spasms in people with multiple sclerosis is another medicinal application (67). Cannabinoids have proven to be effective in the treatment of anorexia nervosa (68). Cannabinoids have long been used to treat weight loss in HIV and nerve pain in chronic patients (69). Cannabinoids have been reported to reduce tolerance in chronic opiates users, allowing for a consistent dosage for pain relief (70). Dronabinol (commonly known as Marinol) and Nabilone are the only two synthetic THC analogues authorised by the Food and Drug Administration (FDA) in the United States (71). Both Dronabinol and Nabilone are used to treat nausea and vomiting caused by chemotherapy, and Dronabinol is used to treat anorexia and wasting in HIV patients (72). Because of its psychoactive properties, Dronabinol is no longer accessible in Canada (73). Nabiximols, an oral spray containing a combination of CBD and THC, is another cannabinoid medicine (74). In Canada, Nabiximols has been licensed for the treatment of treatment-resistant cancer pain, multiple sclerosis spasms, and central pain (72).

Cannabis may be divided into two categories: medicinal and recreational. For medical use a healthcare provider, such as a physician or nurse practitioner, must approve medicinal cannabis use (75,76). After receiving authorization, a person can purchase medical cannabis from a licenced

producer, register to cultivate it themselves, or select someone to grow it for them (77). Recreational cannabis is available without a prescription and is utilised for a number of purposes, including medical purposes. In a Canadian survey on cannabis usage, for example, 12 percent of respondents said they used cannabis for therapeutic purposes, although the majority (71 percent) said they didn't have permission to do so (78). There are two types of recreational cannabis retailers: licenced and non-licensed (79). A licenced retailer is defined as “a regulated retailer or licensed dispensary; regulated by each province and territory, as government-operated, privately licensed stores, or online” (79).

2.5. Chemical constituents of cannabis

2.5.1. Non-cannabinoid constituents

2.5.1.1. Terpenes

Terpenes, which are the plant's principal aromatic molecules which are made up of different combinations of C5 isoprene subunits, are a major class of cannabis compounds (80,81). Monoterpenes (C10) and sesquiterpenes (C15) are two separate families of volatile and semi-volatile variations based on the number of carbon atoms in their structure (82). Waxes and resins, as well as oxygenated terpenoids, include larger terpenes. The word "terpenes" is used here to refer to a group of volatile and semi-volatile terpenes and terpenoids that are of particular interest (83).

Terpenes have a wide range of therapeutic and pharmacological characteristics (84). There is currently no comprehensive list of terpenes present in cannabis, however, it has been stated that a single cultivar can contain up to 100 distinct terpenes and terpenoids (84). Figure 2.1 illustrates the structures of the main terpenes present in cannabis obtained from Chem Space application.
The most common terpenes are:

- **Pinene**: has a pine fragrance and has antiseptic properties (85).
- **Myrcene**: has a musky fragrance and can exhibit anti-oxidant and anti-carcinogenic properties (86).
- **Limonene**: has a citrus fragrance and is an antifungal and anti-carcinogenic compound (87).

- **Caryophyllene**: has a pepper fragrance and has gastroprotective and anti-inflammatory properties (88) .
- **Linalool**: can help with anxiety and convulsions, and displays a floral fragrance (89).

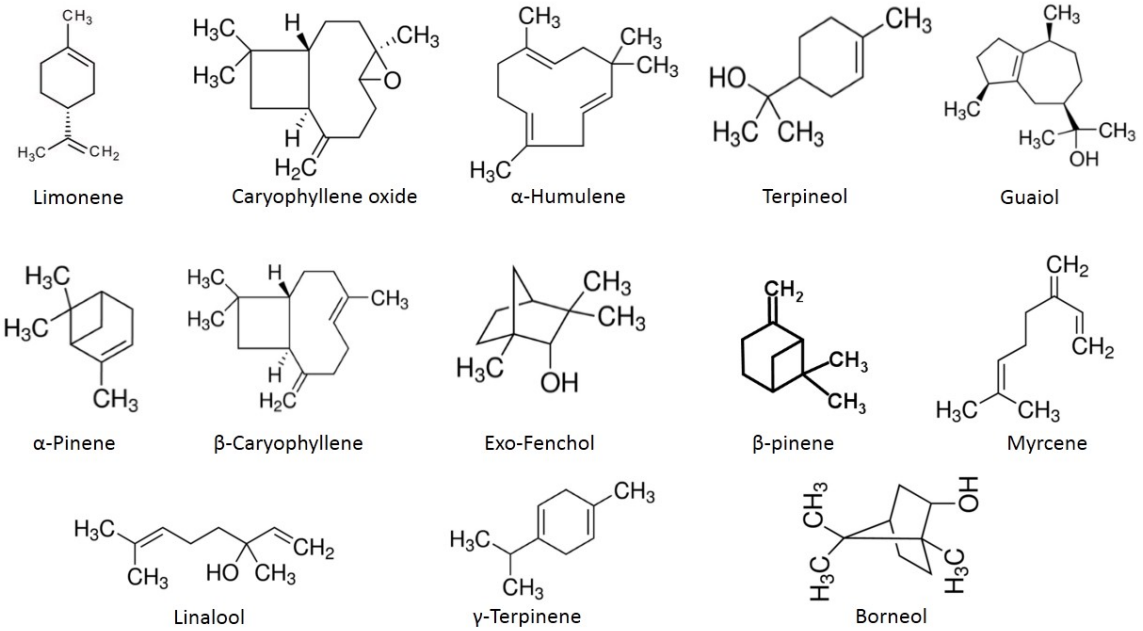

Figure 2.1 Structures of a selection of common terpenes and terpenoids found in *C. sativa.*

2.5.1.2. Flavonoids

Cannabis flowers, leaves, and pollen grains have at least 20 different flavonoids (90–94). There are three types of flavonoid compounds that have been reported:

- Apigenin, luteolin, quercetin, and kaempferol O-glycosides (95).
- Orientin and vitexin C-glycosides (95).
- Cannaflavin A and B prenylated flavonoids (91).

Cannaflavins are being studied to separate them from more prevalent flavonoids. It was recently discovered, for example, that the cannabis flavonoid "cannaflavin-A" suppresses "PGE-2," a prostaglandin responsible for inflammation that responds well to nonsteroidal anti-inflammatory medicines (NSAIDs) such as aspirin. The study found that cannaflavin-A decreases inflammation and is far more effective than aspirin (96).

Cannaflavin-B and cannaflavin-C are also being studied, while researchers are still learning how the presence of more common flavonoids in cannabis, such as -sitosterol, vitexin, isovitexin, apigenin, kaempferol, quercetin, luteolin, and orientin, work in tandem with—or as resistance to—cannabis cannabinoids and terpenes (97,98) .

Apigenin for example is very beneficial for organ transplant patients, particularly those who have had renal injury and require a kidney transplant. Apigenin might thus be used with CBD to aid in the treatment of kidney transplant patients, perhaps reducing the requirement for strong immunosuppressant medicines (99).

Many flavonoids have significant antioxidant characteristics that aid in the detoxification of tissue-damaging molecules. flavonoid intake is often (but not always) linked to a lower risk of various cancers, most notably lung and breast cancer (100,101).

2.5.1.3. Alkaloids

Alkaloids are a type of heterocyclical organic molecules with one or more nitrogen atoms. They may have an atom of oxygen, sulfur, chlorine, bromine, or phosphorus bonded to the molecule (102). From the nitrogenous components of *C. sativa*, ten alkaloids have been found, including many significant pseudo-alkaloids and related precursors such as choline, trigonelline (a pyridine), muscarine (a protoalkaloid), isoleucine betaine, and neurine (102,103). The components can be extracted from cannabis leaves, stems, pollen, roots, and seeds (104). They are most associated with plants, although microorganisms and animals can also generate them (105). Alkaloids are a type of chemical defence in plants against herbivores (106). A large number of alkaloids are pharmacologically active. Alkaloids account for over 60% of plant-derived medications (107). On a related note, endogenous indole alkaloids have been found in hemp (108,109). Alkaloids have a wide range of therapeutic uses, including analgesics, antibacterial, anticancer, antiarrhythmics, antiasthmatics, antimalarials, anticholinergics, bronchodilators, laxatives, miotics, oxytocics, vasodilators, psychotropics, and stimulants. Among the chemicals in this class are morphine, cocaine, nicotine, caffeine, quinine, and ephedrine (110). *C. sativa* produces alkaloids known as cannabinaceous alkaloids. Cannabisativine and anhydrocannabisativine are examples of these (111,112,113).

2.5.2. Cannabinoids

Cannabinoids are the biologically active components that impact humans from the cannabis plant, and they are the compounds that give the cannabis plant and hemp commercial products their distinctive medicinal characteristics (112,113).

There are no qualitative variations in the cannabinoid spectrum across plant sections, but quantitative differences in the cannabinoid or other component substances (114). The bracts of the flowers and fruits have the highest concentration of cannabinoids (in terms of dry-mass plant material) (115). The concentration of cannabinoids in the leaves is lower, and it is much lower in the stems and roots (115).

Dr. Raphael Mechoulam and his colleagues discovered the chemical composition of tetrahydrocannabinol (THC) and cannabidiol (CBD), the major cannabinoids in the cannabis plant, in 1963-1964 (116,117). They are the compounds that provide the medicinal characteristics of the cannabis plant and hemp commercial products (118). The cannabis plant can create up to 100 distinct cannabinoids (95). While THC and CBD are the most well-known cannabinoids, the cannabis plant contains numerous additional cannabinoids that have human health properties: annabigerol (CBG), cannabinol (CBN), and cannabichromene (CBC) are a few examples (95).

The female flower heads ("buds") that stay unfertilized during the plant's development have the highest level of THC in the cannabis plant (119). Industrial cannabis (industrial hemp) refers to a variety of cannabis plant cultivars used for agricultural and industrial applications (119). Industrial hemp is farmed for its seeds and fibres, industrial cannabis has a low THC level and a high CBD content. Consequently, the CBD-to-THC ratio is larger than one (119). The amount of THC in the plant has been utilized by policymakers and regulators all around the globe to differentiate between hemp and marijuana. The current upper legal limit for production in most European nations is >0.2 % THC, 0.3 % in Canada, and 0.3 % in the United States (120).The THC level varies depending on the plant part: 10-12% in pistillate flowers, 1-2% in leaves, 0.1-0.3% in stalks, and 0.03 percent in roots (119). THC, CBD, CBN, CBG, and CBC are the major cannabinoids found in cannabis plants and may be found in every cannabis breeding accession (115). Based on their chemistry, phytocannabinoids may be divided into three major classes (115).

- Acidic cannabinoids as a result of metabolism of the plant (115)
- Neutral cannabinoids resulting from decarboxylation (115)
- Cannabinoids resulting from degradation (oxidation, isomerization, UV-light) (115)

Cannabinoids are biosynthesized to an acidic (carboxylated) form in the cannabis plant. Δ9-tetrahydrocannabinolic acid A (THCA-A), cannabidiolic acid (CBDA), and cannabigerolic acid (CBGA) are the most frequent acidic cannabinoids (121). The major form is THCA-A, which will be referred to as THCA. CBGA is a direct precursor to THCA, CBDA, and cannabidiol (CBCA) (122). The carboxyl group is unstable and simply loses CO_2 when exposed to heat or light, resulting in the corresponding neutral cannabinoids: THC, CBD, CBG, and CBC (115,123,124). Cannabinoids are a category of terpenophenolic C21 (or C22 for neutral forms) chemicals (125). About 100 meroterpenoids (prenylated polyketides) have been identified so far, predominantly in glandular trichomes from *Radula marginata* (126). Cannabinoids are categorised into distinct categories based on their basic structure (127):

- Cannabigerol, CBG type
- Cannabichromene, CBC type
- Cannabidiol, CBD type
- Δ9-tetrahydrocannabinol, THC type
- Cannabicyclol, Δ8-THC type
- Cannabielsoin, CBL type
- Cannabinol and cannabinodiol, CBE type
- Cannabitriol, CBND and CBT type
- Miscellaneous cannabinoids

The chemical structure of each type is shown in Figure 2.2. obtained by Chem Space application. Immunoassays and chemical analyses have found cannabinoids in cannabis leaves, flowers, stems, pollen, seeds, and roots (127,128). More recently a total of eleven different cannabinoids have been measured in cannabis plant materials and the list may grow as new developments occur (129).

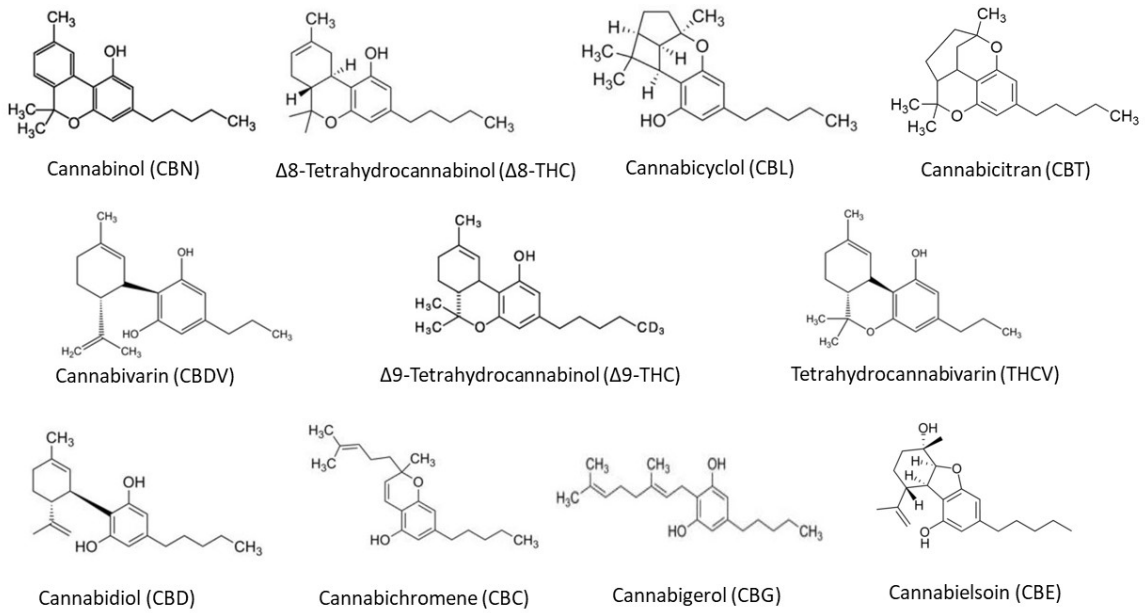

Figure 2.2 Structures of the 11 most prevalent cannabinoids in cannabis.

Some of these cannabinoids have numerous potential or purported medicinal applications (130). The following are the five primary cannabinoids and their properties.

THC is the psychoactive ingredient in the cannabis plant. Consequently, it is either removed or present in low concentrations in hemp oils and extracts (116). It is classified as a Schedule 1 drug in several nations, making it illegal (131). THC has analgesic, muscle relaxant, antispasmodic, and anti-inflammatory properties (116,132).

CBD is the most frequent cannabinoid found in most hemp plants, and it has the most potential therapeutic benefits (133). Many scientific articles and anecdotal accounts discuss CBD's potential pain alleviating, neuroprotective antioxidant, anti-inflammatory, anticancer, antianxiety, anticonvulsant, anti-seizure, and anti-nausea properties (134).

CBN is a by-product of aged THC that has been shown to have anticonvulsant and anti-inflammatory properties (135). When exposed to light or heat, THC in the cannabis plant degrades and changes its molecular structure to become CBN (136). CBN has been discovered to have potent antibacterial effects by scientists (116).

CBC may have anti-inflammatory, pain-relieving, and antidepressant effects, which may be attributed to its ability to inhibit the breakdown of the body's cannabinoids (137). CBC may have antifungal and anticonvulsant effects (116).

CBG concentrations are typically less than 1% in the cannabis plant, but can be somewhat greater in hemp (138). CBG has been shown to have therapeutic benefits such as analgesic, mildly antifungal, and antidepressant effects, as well as muscle relaxant and slightly antihypertensive effects (113,116).

When studying each cannabinoid in the plant, it is critical to understand how they are chemically connected to one another. Some cannabinoids may undergo changes or degradation as a result of storage conditions (139).

2.6. Introduction to detection and quantification of cannabinoids in cannabis

Potency is one of the most popular measurements for cannabis and hemp plant components (140). The word "potency" refers to the amount of THC and/or CBD (cannabidiol) in the plant material, although at least three additional cannabinoids, including THCA (delta-9-tetrahydrocannbinolic acid), CBDA (cannabidiolic acid), and CBN (cannabinol), are commonly examined (141,142). They have remarkable similarities, with same molecular weight yet with very different pharmacological effects (143).

When it comes to commercial "enthusiasm" for new cannabis products, it is wise to note that different cannabis cultivars have a wide range of additional chemical ingredients, which are present in varied ratios relative to one another depending on the cultivar (144–146). As a result, it is important to be cautious about assuming that the characteristics of various cultivars are the same, because the ratios of numerous elements fluctuate across similar cultivars, necessitating a chemical study to determine the composition and ensure the desired therapeutic effects and benefits (147,148).

The "potency" of the plant is a crucial consideration for a cannabis producer (149). The composition and concentration of specific chemical ingredients of the plant can have a significant influence on the product's value and expected price (149). The "specificity" or "selectivity" of the detector utilized is a critical component of accurate and precise analysis (150).

Different analytical techniques are used to determine the components present, or quantitative to determine the concentrations of the specific components of interest (151). Methods must examine these components with sufficient precision and accuracy to guarantee that they are suitable, which is validated by comprehensive method optimization and validation studies (148).

2.6.1. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a quick screening method for cannabinoid profile and quantification (152). It detects protons in organic molecules and the intensity of the signal is proportional to the molar concentration. As a result, without complicated sample preparation or chromatographic separation, NMR spectroscopy analyses and quantifies analytes in plant extracts. For example, a group of researchers compared the signal of an internal standard anthracene to unique singlet proton signals for five cannabinoids ranging from 4.40 to 6.44 (153). The main cannabinoids were quantified using NMR analysis in under 5 minutes (153). Semi-quantitative analysis of THC and THCA in water and ethanolic extracts was performed in order to differentiate between tinctures from various cannabis cultivars without any evaporation or separation steps (154). This method is recommended for a direct examination of plant tinctures (154).

2.6.2. Colour test

Colour tests (Duquenois-Levine test) have historically been the most used methods of cannabinoid identification. For nearly 80 years, the Duquenois-Levine test for tetrahydrocannabinol (THC) has been utilised in the forensic scientific field (155). Colorimetric testing is the process of mixing unknown compounds with certain sets of reagents to obtain clear coloured results (156). Each collection of reagents produces colours that are exclusive to various sorts of substances (156). As a result, depending on the tests chosen and the colours generated, the identification of the unknown

substance can be determined. These tests are beneficial for a variety of reasons (157). First, they operate with little amounts of unknown substance and only a few drops of reagent (158). Second, the tests are rapid, with results in as little as a few minutes (158). Third, the reagents are reasonably priced on a per-use basis (158). Colorimetric tests are utilised by the police for on-site drug analysis for these reasons (156). However, the capability of these tests is restricted by their limitations. One of the tests used to confirm the presence of THC is a three-part colour test (155). There have been complaints in recent years that this test is not precise enough to be used as the only method of conviction and there is no confirmation that the colour generated is due to the presence of THC or other cannabinoids (155,159). For example, for each test, a variety of molecules unrelated to the target molecule can generate the same colour (160). Colorimetric tests are used in police investigations as a sort of presumptive analysis because of the inaccuracies caused by false positives (156). In this manner, the data are utilised to evaluate whether or not an unknown material should be sent to the lab for a more thorough examination due to the inaccuracy of this method. Gas chromatography mass spectroscopy (GC-MS) and high-performance liquid chromatography (HPLC) are two examples of such additional examinations.

2.6.3. Gas chromatography

The measurement of THC, CBD, and CBN concentrations in cannabis was first done using gas chromatography (GC) (161). GC analysis necessitates decarboxylation of cannabinoids or derivatization of acids with trimethyl silyl (TMS) or TMS-containing reagents, such as N methyl-N-trimethyl silyl tri fluoroacetamide, because cannabinoids are naturally present in plants as non-volatile acids (162,163). Decarboxylation of dried extracts prior to analysis resulted in cannabinoid losses due to neutral cannabinoids volatilization (164,165). Cannabinoids should be decarboxylated at the GC injector port, thus reducing sample preparation losses but restricting quantitation of total cannabinoids (166).

Extraction solvents used in quantitative GC procedures for cannabinoids vary from ethanol to petroleum ether, while methanol or methanol:chloroform 9:1 % v/v are the most prevalent employed solvents in solvent-based extraction method (167,168). 100% dimethylpolysiloxane (DB-1) or 5% diphenyl- and 95% dimethyl-polysiloxane (DB-5) columns are commonly used,

and GC temperature gradients generally vary from 170 to 280°C (168). FID (flame ionisation detection) or mass spectrometry (MS) are the most prevalent methods of detection (169,170).

Due to the high-temperature conditions required in the inlet and column oven, derivatization is essential to detect acidic cannabinoids by GCMS, converted to their neutral analogues (168,171,172). THC acid (THCA) is thermally labile and converts to THC by decarboxylation during heating of the sample in the hot GC injector port (173,174). For this reason, it is not appropriate to detect the acidic cannabinoids by gas chromatography. In contrast, all cannabinoids can be detected by HPLC method (175). As a result, for identification and quantification, several laboratories have used liquid chromatography in combination with tandem mass spectrometers, allowing for the analysis of both acidic and neutral cannabinoids without derivatization. With columns with particle sizes of less than 2µm, UHPLC has recently been employed for the quantification of cannabinoids, offering improved resolution and sensitivity (176).

2.6.4. Liquid chromatography

Extraction methods for cannabis analysis in LC are inconsistent and use the same sample preparation as for GC samples. The extraction solvent employed in previous approaches for analysing qualitative cannabinoid characteristics was 80% methanol (171,174), while methanol:chloroform (9:1) is the most often used extraction solvent (169,177–180).
Because cannabinoids are often present in high concentrations in cannabis-based products, UV absorbance has been used to detect them in the past (177). This makes HPLC-UV or HPLC-PDA more appropriate for cannabinoid detection than mass spectrometry (177,180,181). However, for cannabis analysis, photodiode array (PDA) detection, fluorescence, and mass spectrometry have been combined with HPLC. Cannabinoids' chromophores have a maximum UV absorbance between 210 and 220 nm, whereas acidic cannabinoids have an additional UV peak at 274 nm, which is less sensitive than at 220 nm (173,177,182).

2.6.4.1. Reverse phase liquid chromatography

Physicochemical properties and interactions with the mobile phase and stationary phase are used in liquid chromatography to separate analytes from endogenous/exogenous components or other

analytes contained in the matrix (183). To avoid possible interferences in quantitative analysis, it is critical to divide analytes from other components or from each other in the matrix (184).

The most frequent type of liquid chromatography is the reverse phase, which involves the employment of a polar mobile phase and a nonpolar stationary phase (185). The nonpolar stationary phase of the column interacts with and adsorbs to the analyte (185). The retention time of the molecule on the non-polar column is affected by polarity (186). Analytes that are nonpolar, such as THC and CBC, interact with the column for a longer period of time, resulting in prolonged retention time, whereas polar substances interact less with the column and elute more quickly (187). The mobile phase parameters must be modified, and a suitable column must be chosen, to achieve optimal conditions for cannabinoid separation.

The mobile phase is made up of two solvents: mobile phase A (aqueous) and mobile phase B (organic). In LC-MS/MS analysis, the type of solvents and pH modifiers that are being used is critical. The organic mobile phase plays an important role in the elution of the analyte. The two most popular organic solvents for the mobile phase are acetonitrile and methanol. Acetonitrile can give us shorter retention times due to having a higher elution strength than methanol (185). When analysing numerous analytes, however, this method may result in overlapping peaks, making it inappropriate. The degree of ionisation of the analyte depends on the pH of the environment. When the pH falls below the analyte's pKa, the analyte may stay nonionized, resulting in higher retention time (188). Ionized forms of the analyte are produced at pH values above the pKa (188). Because ions have little or no interaction with the stationary phase, they will elute from the column sooner (188). pH values within 2 units of the pKa can cause the analyte to switch between ionised and nonionized states, causing a significant shift in retention time (188). The pH of the mobile phase is critical for maintaining stable retention times of acidic cannabinoids (177).
Ammonium acetate, ammonium formate, acetic acid and formic acid are some of the most often used pH modifiers/buffers. Controlling the pH with pH modifiers or buffers can improve the linearity of the concentration curve, separation, peak shape, and retention time of analytes in a method (189).

2.6.4.2. Triple quadrupole mass spectrometry

The method of mass spectrometry is widely utilised for the measurement and detection of pharmaceuticals and biological materials (190). Due to its sensitivity and selectivity for both high and low mass molecules, triple quadrupole mass spectrometry (Figure 2.3) is regarded as the optimum instrument for quantitative analysis (189). It consists of two mass analysers that are used to select and scan for specified mass-to-charge ratios (191).

The ionisation of the analyte is the first step in mass spectrometry. Desolvation and ionisation of the analyte are required for the detector to create a signal (185). Electrospray Ionization (ESI), a commonly used atmospheric pressure ionization electrospray ionisation (ESI) method, ionises the analyte in the condensed phase by providing a voltage to the sample that we are willing to analyses (185). As it passes through the capillary of the electrospray probe, it creates either positively or negatively charged ions (185). Because of greater desolvation and lower surface tension, ESI requires polar, volatile solvents (185). A nebulizer gas is delivered down the probe, causing the mobile phase containing the analyte to nebulize, resulting in a fine aerosol of charged droplets (192). The desolvation of the analyte suspended in condensed phase will be aided by temperature and a heater gas, resulting in a gas phase ion (193). After being transformed to a gas phase ion, now called as a precursor ion, it will pass through the curtain plate used with curtain gas, preventing solvents and neutral molecules from reaching the quadrupoles (193). A declustering potential is given to the precursor ions as they flow through the orifice plate, allowing gas phase ion to separate from the charge droplets (192). Finally, an ion guide helps the precursor ion into a narrower channel, increasing the sensitivity of the detected ions (192). The two mass analysers, the first and third quadrupoles, have a direct current (DC) and radio frequency (RF) potential that alternates between positive and negative potential, generating a spiral trajectory path (194). The mass-to-charge (m/z) ratio of the precursor ion is chosen in the first quadrupole, enabling only related analytes to pass down the quadrupole (195).

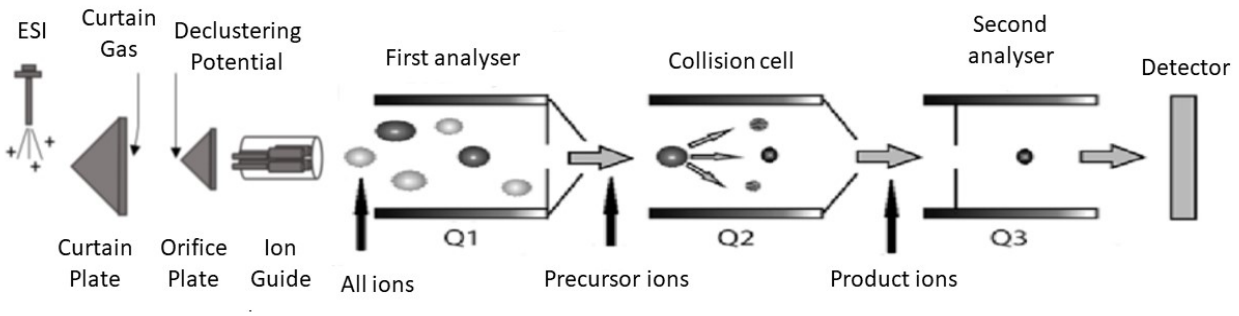

Figure 2.3. General schematic diagram displaying a triple quadrupole mass spectrometer, consisting of electrospray ionization (ESI).

Collision energy is used to establish a potential difference between Q1 and Q2, which causes the precursor ion to transform that energy into internal vibrational energy (192). The excited, unstable precursor ion goes to the collision cell, where it collides with a collision-activated dissociation gas (usually nitrogen or argon gas) and fragments into product ions (192). After scanning in the third quadrupole for the desired product ions, the ions are accelerated out of the third quadrupole and into the detector by applying a cell exit potential (192). Then, the channel electron multiplier (CEM), a frequently used mass spectrometer detector, amplifies the electrical signal produced by the product ions (196). On a chromatogram, peak intensity represents the electrical signal produced by the ions (192). Multiple reaction monitoring is a technique that is often used in triple quadrupole, in this technique by scanning for many product ions from a single or multiple precursor ion, improves the selectivity of the LC-MS/MS method (192). To quantify the response and concentration of the analyte, the most abundant product ion, known as the quantifier ion, is usually utilised (191). The qualifier ion, the second product ion chosen, is usually employed to ensure that the mass spectrometer is scanning for the desired analyte (197).

As mentioned, MS enables to identify cannabinoids based on mass-to-charge (*m/z*) ratios, which allows more selectivity. In terms of analytical method validation, selectivity is defined as a method's ability to measure and differentiate targeted analytes in the presence of other components that may be expected to be present (129). The high selectivity eliminates background noise, leading to higher sensitivity. An approach with high selectivity and specificity is required for multiple cannabinoid analysis. Reverse phase liquid chromatography (C-8 and C-18 columns) is the most widely employed method for HPLC cannabis analysis (198,199). The use of a nonpolar stationary phase allows for the effective separation of cannabinoids, which are a group of compounds with

varied polarity (187). Reverse phase columns do not allow chiral separation, which is a difficulty for many cannabinoids that exist as enantiomers (187). Specificity between enantiomers can be provided using chiral columns.

The current popular analytical technique used in support of the cannabis industry for determining potency is HPLC/PDA, or HPLC using a photodiode array detector. This technology is accepted by the cannabis analytical community because it is relatively easy and inexpensive to employ. Although some may suggest that a PDA detector is relatively selective as an absorbance detector, there is growing evidence that it may not reveal potential co-eluting or potentially interfering endogenous plant chemicals since it does not provide the combined sensitivity and selectivity of a mass spectrometer detector (200). Several chemical constituents are present in crude extracts of plant substances. This extract probably contains coeluting chemical constituents that are visible as chromatographic peaks under HPLC/PDA. When this occurs, the area under that peak will be larger than it would be if only the targeted compound was contained in it. Due to the increased peak area, a higher amount of cannabinoid is reported than is present. This incorrectly reports the cannabinoid level. The sample's selectivity can be increased using SIM LC/MS without interfering with chromatographic components. This is related to SIM LC/MS methods' enhanced selectivity, which monitor only the protonated molecules of the targeted cannabinoid(s). If LC SRM (selected reaction monitoring) MS/MS methods, high-resolution time of flight, or Orbitrap mass spectrometry techniques were used, even better selectivity and mixture analysis capacity may be achieved. When HPLC/PDA techniques are employed, the possibility for unknown interferences related to co-elution can be reduced by using SIM LC/MS techniques.

Despite the prospect of increasing separation efficiency by utilising 1.7-µm ultra-high-performance liquid chromatography (UHPLC) columns, the PDA detector's weak selectivity would still make distinguishing between targeted and untargeted compounds difficult (201). In addition, several of these interferences have yet to be identified in detail and there are no commercially available certified reference standards for them (202). The significantly higher selectivity of either LC/MS or LC/MS/MS techniques, in contrast to HPLC/PDA, can contribute to the qualitative identification and quantification of unknown compounds while decreasing the risk of unexpected chemical interferences (203).

2.7. Method development and validation

Method development for LC-MS is task-specific, based on the customer's or research's needs. Sample preparation, chromatography, and mass spectrometric detection are the three procedures that go into developing a technique (204). The assessment of what analytes to be tested, the concentration range of the analytes of interest, and the matrix to be analysed is the beginning of method development (205). After the determination of the above parameters, the next step is conducting a literature search on the analyte and prior techniques. The completed study aids in determining the optimal MS starting conditions, the best analysis column, and the most efficient sample preparation procedure (204). The injection of analytes into the MS is the first experiment that has to be carried out. The development of the chromatography technique is the next series of investigations. Finally, a procedure for preparing samples must be devised (206).

Following method development, methods should be validated to include consideration of characteristics specified in the International Conference on Harmonization (ICH) guidelines addressing analytical method validation (207,208). Analytical procedures that are not covered by the ICH guidelines should always be verified. The International Conference on Harmonization (ICH) is concerned with the harmonisation of technical standards for product registration among the three major geographical markets of the European Community (EC), Japan, and the United States (U.S.) of America (207,208). The most current FDA methods validation guidance paper (209–211), as well as the United States Pharmacopoeia (USP), both relate to ICH guidelines (212). The most widely applied typical validation characteristics (Table 2.2) for various types of tests are accuracy (bias), precision, carryover, selectivity and specificity, matrix effect, limit of detection (LOD), limit of quantitation (LOQ), linearity (calibration model), range, recovery (RE), reproducibility, repeatability, ruggedness, stability and dilution integrity (54).

Table 2.2. General definitions of parameters that need to be tested for method validation

Parameter	Definition
Accuracy (Bias)	Measurement of closeness of the calculated value for the measurand and to the true value of a measurand.
Precision	The closeness of agreement of repeated measurements from multiple samples of same homogenous sample.
Linearity (calibration model)	A mathematical model that demonstrates relationship between the analyte signal and its concentration.
Range	The concentration that can be adequately determined.
Carryover	The appearance of analyte signal in a subsequent sample after analysis of positive sample.
Matrix effect	Suppression or enhancement of analyte signal due to interferences from the matrix.
LOD	The lowest concentration of the analyte that can be reliably differentiated from background noise.
LOQ	The lowest concentration that can be reliably measured.
Selectivity and specificity	The ability to detect, differentiate the analyte of interest, when there are other non-targeted analytes present, other drugs, impurities.
Recovery	The percentage of analyte response after sample preparation compared to solution of neat analyte of same concentration.
Ruggedness	The susceptibility of a method to small changes that might occur during day-to-day analysis, for example small temperature or pH variations.
Reproducibility	The preparation of samples by more than one analyst on separate days in same laboratory.
Repeatability	Testing the sample on different days to test the closeness of the values to one another on different days.
Dilution integrity	Testing the sample accuracy when using smaller volumes of sample than in the method.
Stability	Testing the variability of sample concentration when they are kept frozen, are not analysed on same day when they are prepared.

Selectivity

Selectivity is defined as "the ability of the bioanalytical method to measure unequivocally and to differentiate the analyte(s) in the presence of components, which may be expected to be present. Typically, these might include metabolites, impurities, matrix components, etc." (213). Method selectivity is established by proof of the lack of response by analysing blank matrices from different lots (213,214). Signals interfering with the signal of an analyte, or an internal standard must be avoided (213).

The limit of detection (LOD)

LOD is the lowest concentration where an analyte can be detected. There are several approaches to assess LOD, it can be calculated by dividing the area of the signal on the area of the noise, and this ratio (signal to noise ratio, S/N) should be 3:1(207,215). The signal of the noise is the height of the baseline (215).

The limit of quantification (LOQ)

LOQ is the lowest concentration where an analyte can be quantified. It can be calculated in the same way as LOD, but the S/N should be ≥ 10 (215,216). If the signal to noise ratio is low, it is difficult to say how much of the signal is due to the analyte, and how much is due to the matrix, thus a reliable quantification would be difficult (216). There are no requirements for the LOQ or LOD value in doping analysis, but the value should fit the purpose (217,218).

Linearity

Linearity is the ability of the method to give a linear calibration curve in a given concentration range (219). The ratio is given by the response of the analyte which is divided by the response of the internal standard, and allows a plot at different concentrations (215). The linearity of the equation is described by R, the regression coefficient (215). R^2 should be as close to 1 as possible, but a value above 0.995 is satisfactory for external calibration (219).

Specificity

The specificity is the ability of the method to detect and quantify the analyte in presence of contaminations in the sample (220). The signal of the analyte should not be interfered by these contaminations (221).

Linear range

This is the interval between the lower and the upper concentration where the method can quantify the analyte with a suitable accuracy, precision and linearity (221,222).

Precision

This parameter describes the diversity of the results, and is expressed by a relative standard-deviation (RSD), which is the standard deviation of the results divided by the mean value of the same results, and multiplied by 100 (223). A low RSD indicates a good precision. The analyzed concentrations should reflect the concentration range (223).

Accuracy

Accuracy represents the closeness between the theoretical value and the calculated value (207,224). Hence, this parameter considers the uncertainty and the precision of the method (225). The uncertainty can be determined by calculation of the theoretical values in the sample by using a calibration curve (225). The calculated and the theoretical value are plotted in a curve (207). The linearity and the slope of the curve demonstrate the correlation between these values; a linear curve with a slope of 1 suggests a good correlation between these values (207,224).

Robustness

The robustness is an assessment of the ability of a method to stay unaffected by minor changes in the procedure, i.e. small variations in pH (215). This is to make sure that the analysis is not affected by variations that might occur during a sample preparation (215,216).

Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system (226). Stability procedures should evaluate the stability of the analytes during sample collection, handling and storage, as well as after going through freeze-thaw cycles and the analytical process (213). Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis (226).

Connecting Statement

Chapter 3, Developing an analytical method based on the ESI ionization behaviour of cannabinoids, authored by Mohammad Reza Khan Bolouki, Mark Lefsrud, Valérie Orsat and Stéphane Bayen.

Chapter 3 covers the methodology, results, and discussion of developing an analytical method for getting better results of separation in less operating time based on the ionization behaviour of cannabinoids. The chapter tackles specific parameters discussed in the literature review of this thesis by presenting a new method based on changing the mobile phase, column temperature, and pH of the system.

Chapter 3: Developing an analytical method based on the ESI ionization behaviour of cannabinoids

3. Abstract

In several countries throughout the world, regulatory frameworks for medicinal and recreational cannabis programs have been introduced to the cannabis industry. For the protection of consumers in this developing industry, cannabis testing laboratories are increasingly needed to detect the concentration of different cannabinoids as well as harmful contaminants such as pesticides, mycotoxins, and heavy metals. We designed two different HPLC methodologies to address these concerns, the method (A) being able to fully resolve 19 cannabinoids within a short period of time. The second method (B) was able to analyze acidic cannabinoids with high linearity and the total analysis time was 13 minutes with samples eluting from 3 to 12 minutes. Calibration curves for each analyte had acceptable R^2 values > 0.999. A linear dynamic range of 0.0001 – 1 µg/mL, and 9 levels were used for all analytes giving the best linearity results specifically for acidic cannabinoids. After testing 10 different methods to reach better sensitivity, separation, and linearity, we concluded that the most important factor to result in better linearity and separation was the pH of the mobile phase. As we have realized with our past mobile phase (acetonitrile/water 7:3 with the addition of 0.1 % formic acid and 5 mM ammonium formate) the pH was 4.5 which is the exact pk_a of carboxylic acid functional group, making it the potential cause of quadratic behaviour, so we decided to decrease the pH of the mobile phase using less ammonium formate and a higher amount of formic acid. This attempt gave us the second method (acetonitrile/water 7:3 with the addition of 0.3 % formic acid and 2 mM ammonium formate in pH of 3.5). The second method gave us the best linearity results, considering 9 different concentrations for the samples (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 µg/ml). With this method, we were able to separate 18 cannabinoids (except CBLA due to co-elution of CBLA and CBCA).

Development of these methods will help regulatory laboratories, cannabis companies, and other facilities that are working on cannabis analysis to detect a higher number of cannabinoids in one run, in less time and with high accuracy.

Due to growing interest in cannabis production, cannabis testing, and the identification of new cannabinoids in this plant, future work is needed to develop methods that are able to detect more cannabinoids with high linearity and accuracy.

3.1. Introduction

Following the discovery of cannabinoid receptors and the endogenous endocannabinoid system (227), attempts are currently being made to conduct clinical trials and research on cannabis for therapeutic applications such as the treatment of epilepsy, pain, and chemotherapy-induced nausea and vomiting (228–230). After the legalisation of recreational and medical cannabis in Canada and many other countries, a considerable number of analytical laboratories working in the cannabis market have developed to meet the increasing need for cannabis testing. Due to a lack of standardized methods in cannabis testing, a broad range of methodologies has been utilized, which has contributed to substantial variability of findings between testing facilities (231,232). As a result of the lack of a standardized method, that can be employed in most applications, few studies have been conducted to meet this demand. Due to the difficulties of testing cannabinoids using gas chromatography (GC), acidic cannabinoids undergo decarboxylation during transit through the column by higher column temperatures, high-performance liquid chromatography (HPLC) is one of the most commonly used instruments for method development in cannabis testing as an alternative. Consequently, there have been a variety of chromatographic methods published for the separation and detection of cannabinoids, with reversed HPLC (176,233–235). Without the necessity for derivatization, HPLC analysis of cannabinoids allows for the identification of both neutral and acidic cannabinoids (236). Reverse Phase HPLC (RP-HPLC), separates analytes based on polarity differences and is preferred by the pharmaceutical industry (237–239). Various parameters must be studied while creating analytical HPLC methods in order to obtain a good resolution, specificity, peak shape, retention time, and total run time (240,241). HPLC can be used with a variety of detectors, but MS detectors give excellent sensitivity and selectivity (242). One of the most important parameters of optimising the cannabinoids separation is to choose the right stationary and mobile phase properties. It is critical to control the pH of the buffer in the mobile phase for ionisable analytes. Ionization alters the relative distribution of analytes between the mobile and stationary phases, affecting the process's retention time (243). In isocratic reversed-

phase HPLC, the most typically employed buffers are methanol and acetonitrile. When compared to methanol, acetonitrile generates less pressure increase and shorter run times (244).

Shorter retention times can be accomplished by raising the temperature of the stationary phase, although overly high temperatures might affect the stationary phase stability (245). Peak shape, retention time, and resolution can be affected by the type and quantity of organic modifier, buffer pH, and mobile phase flow rate (246–249). Chromatographic run durations should ideally be not too long for more efficient analysis, but not too short for resolution and selectivity to be compromised (243). The pH values in the range of 2–8 are used in the majority of reversed-phase chromatographic studies (250).

The aim of the study was to develop two different analytical methods based on considering the effects of varying pH, mobile phase composition, the flow rate of the mobile phase, and column temperature.

3.2. Material and Methods

The method development was initiated with the goal of developing two robust methods that optimize the cannabinoid separation with high sensitivity and selectivity with the highest possible linearity. Some of the physical and chemical properties that can be controlled include pK_a values, polarity, ultraviolet absorption wavelength, electrical charge, molecular weight, molecular size, solubility, and chemical reactivity. The appropriate modified C18 stationary phase, column length, and particle size for successful separation of these phytocannabinoids was determined based on their chemical and physical properties, in addition to a review of the literature. Various mobile phase alternatives and their composition, including pH and ionic strength, were studied to optimize the chromatographic parameters which affect selectivity. Various chromatographic method parameters including column temperatures, solvent strengths, gradient time, gradient steepness, and flow rate were evaluated and optimized.

3.2.1. Mobile phase preparations

Different organic modifiers (methanol and acetonitrile) were used to screen reversed-phase conditions in both isocratic and gradient modes. In addition, formic acid was found to be important in achieving good peak shape for carboxylated species (e.g. THCA, CBCA, CBDA, etc.). For preparing the mobile phase for the method (A), HPLC, USP/ACS or Optima grade solvents were used. They consisted of methanol, acetonitrile, formic acid, ammonium formate and deionized water. The deionized water was obtained from a HPLC grade filtration system.

The mobile phase consisted of 150 mL of deionized water and 350 mL of acetonitrile (7:3 ACN/Water) prepared in the mobile phase container. 500 µL of 5 mM ammonium formate was added to a final volume of 500 mL, the solution was stirred and the pH was adjusted to 4.5 by adding 500 µL of concentrated formic acid. For the organic mobile phase, 500 mL of methanol was added with 500 µL of formic acid. The pH of the organic phase was measured at 2.76.

For preparing the mobile phase for the method (B), the procedure was the same except adding 200 µL of ammonium formate (5 mM) instead of 500 µL and adding more formic acid (1.5 mL) to reduce the pH of mobile phase to the value of 3.5. For the organic phase all procedures were the same.

3.2.2. Standard preparation

Four standard cannabinoid mixes from Agilent (Santa Clara, CA, USA) containing 11 cannabinoids were used in place of the standards. Eight other analytical reference cannabinoid standards (CBDVA, THCVA, CBNA, EXO-THC, CBL, CBCA, HU-210, CBLA) were acquired from Agilent Technologies (Santa Clara, CA, USA) and combined to create a final component concentration of approximately 10 µg/mL in acetonitrile. Table 3.1. shows the combined volume of each vial with 780 µL of can to get to the final concentration of 10 µg/mL for the 19 cannabinoids mixture to prepare the 1 mL sample. Then 10 µg/mL of the stock solution was diluted to the concentration of 1 µg/mL for analysis.

Acetonitrile was used to dilute the stock solutions in order to achieve intermediate fortifying solutions to make appropriate calibrators within the expected analytical performance of the chosen

detector. After serial dilution, 9 different level of concentrations for the samples (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 µg/ml) were prepared for testing the linearity.

Table 3.1. Standard samples of 19 cannabinoids used in this experiment

	Cannabinoids	Concentration	Part number	Used volume
Cannabinoid Mix A	CBD, CBN, Δ9 -THC	1.0 mg/mL	5190-9430	10 µL
Cannabinoid Mix B	THCA, CBDA, CBG	1.0 mg/mL	5190-9429	10 µL
Cannabinoid Mix C	CBDV, CBGA, CBC	1.0 mg/mL	5190-9428	10 µL
Cannabinoid Mix D	THCV, Δ8 -THC	1.0 mg/mL	5190-9427	10 µL
	CBDVA	1.0 mg/mL		10 µL
	THCVA	1.0 mg/mL		10 µL
	CBNA	1.0 mg/mL		10 µL
	EXO-THC	1.0 mg/mL		10 µL
	CBL	1.0 mg/mL		10 µL
	CBCA	1.0 mg/mL		10 µL
	HU-210	100 µg/mL		100 µL
	CBLA	500 µg/mL		20 µL

The molecular structures of these 19 cannabinoids are shown in Figure 3.1.

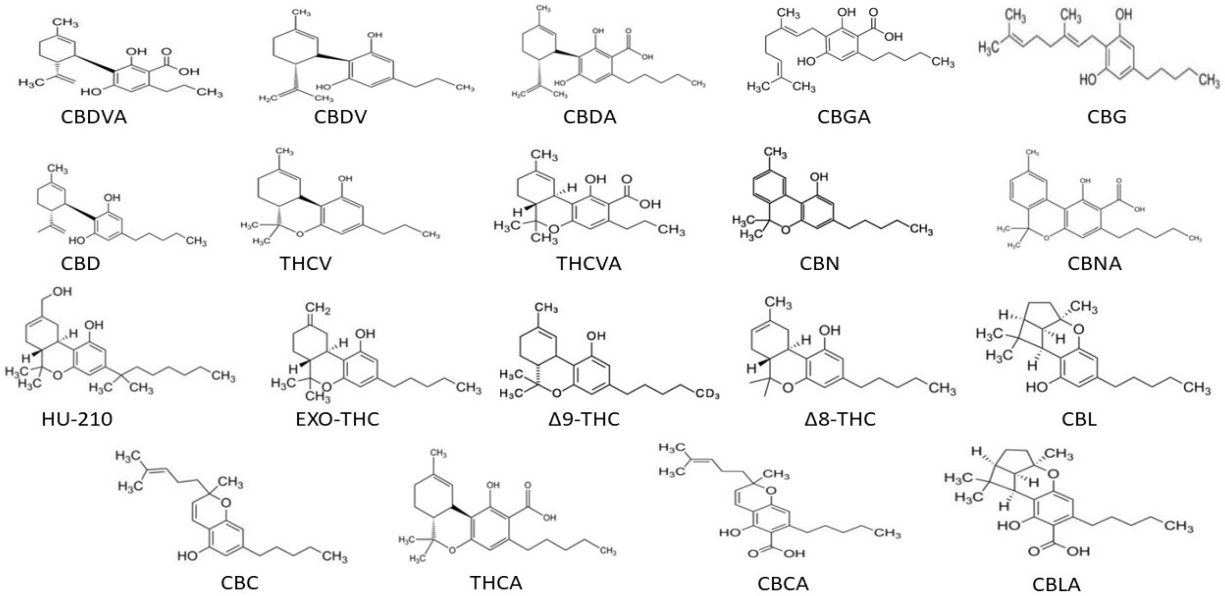

Figure 3.1. Molecular structures of the 19 cannabinoids separated in this application.

3.2.3. Instrumentation

Chromatographic method development was performed on an Agilent 6545 LC/DAD/Q-TOF (Santa Clara, CA, USA) using an Agilent InfinityLab Proshell 120 EC-C18, 3.0 × 50 mm, column packed with 2.7 µm fully porous particles from Agilent Technologies, Inc (Santa Clara, CA, USA).

3.2.4. Tested chromatographic conditions and final methods

Testing different column temperatures to get the best separation of 19 cannabinoids was performed on this instrument and column to detect the effect of column temperature. The results of different temperatures are shown in figure 3.2. Conditions were outlined that caused the best baseline peaks and served as the basis for further method development in Table 3.2, the source conditions and mobile phase gradient program are listed in Table 3.3.

- Mobile Phase A: 0.1% formic acid and 5 mM ammonium formate in 30:70 water: acetonitrile (pH 4.5)
- Mobile Phase B: 0.1% formic acid in methanol (pH 2.76)

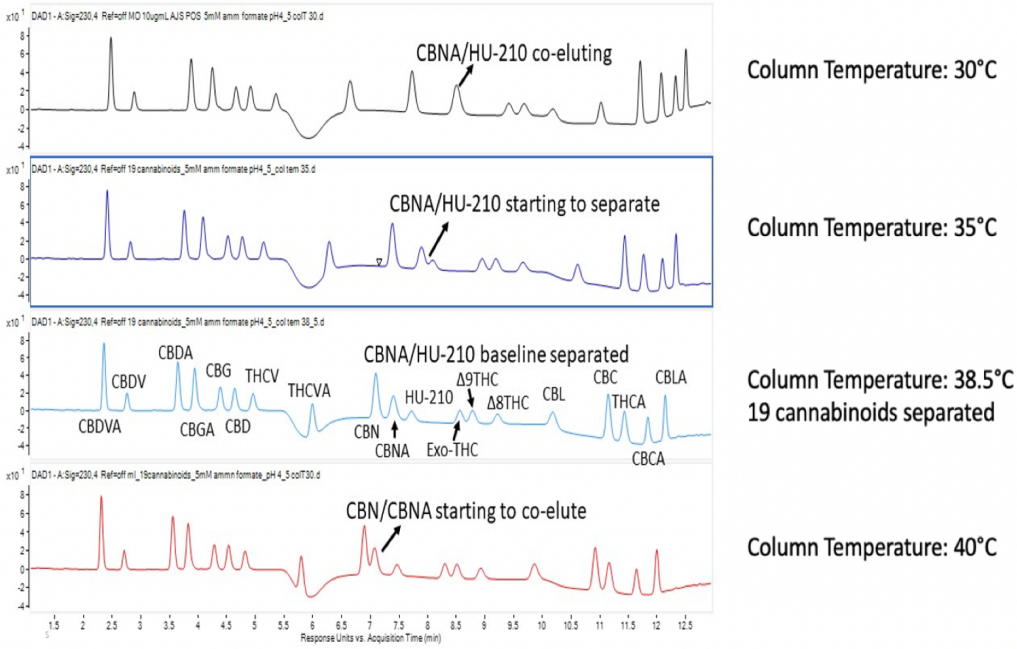

Figure 3.2. Attempts for separation of CBNA based on the column temperature variation.

Table 3.2. Chromatographic conditions were used in the development of the method (A) to separate 19 cannabinoid analytical reference standards.

Instrument	• Infinity II Prime pump G7104C • Infinity II Vial sampler G7129C with Cooling option • Infinity II Column oven G7130A • Infinity II DAD G7115A • 6545 QTOF used in TOF mode, with AJS in either positive or negative polarity
Column	Agilent InfinityLab Proshell 120 EC-C18, 3.0 × 150 mm, 2.7 µm
Mobile Phase	• Mobile Phase A: *modifier* (0.1% Formic Acid + 5 mM ammonium formate) in (acetonitrile:water7:3) • Mobile Phase B: *modifier* (0.1% Formic Acid) in methanol
Column temperature	38.5˚C
Wavelength	230 nm
Injection volume	5 µL
Flow rate	0.8 mL/min

Table 3.3. Mobile phase program and source conditions used in the development of the method (A) to separate 19 cannabinoid standards.

Timetable

	Time	Mobile phase A	Mobile phase B
1	4:00 min	99.00 %	1.00 %
2	4:50 min	75.00 %	25.00 %
3	8:50 min	75.00 %	25.00 %
4	10:50 min	25.00 %	75.00 %
5	11:00 min	00.00 %	100.00 %
6	13:00 min	00.00 %	100.00 %

Source Conditions– 6545 QTOF

- Source: Jet Stream ESI
- Drying Gas Flow: 12 L/min
- Sheath Gas Temperature: 350 °C
- Nebulizer Pressure: 40 psi
- Drying Gas Temperature: 350 °C
- Sheath Gas Flow: 11 L/min
- Polarity: Positive
- Capillary Voltage: 3500 V
- Nozzle Voltage: 1000 V

Table 3.4. Attempts of separation of cannabinoids with high linearity based on changing the mobile phase.

Mobile phase A	Mobile phase B	Notes
1 mM ammonium fluoride in 30:70 water:acetonitrile (pH 6.97)	0.1% formic acid in methanol (pH 3.02)	- Earlier elution of acidic cannabinoids most probably due to higher pH of mobile phase A - Co-elution of CBNA/THCVA
1 mM ammonium fluoride in 30:70 water:acetonitrile (pH 6.97)	1 mM ammonium fluoride in methanol (pH 7.21)	- Higher pH of mobile phase B makes acidic cannabinoids elute earlier compared to System 1 - Co-elution of CBDA/CBGA/THCVA
5 mM ammonium formate in 30:70 water:acetonitrile, adjusted to pH 3.02 with formic acid	5 mM ammonium formate in methanol, adjusted to pH 3.02 with formic acid	-Large amount of formic acid negatively affect the sensitivity - Co-elution of CBN/THCVA
0.1% formic acid in 30:70 water:acetonitrile (pH 3.30)	0.1% formic acid in methanol (pH 2.76)	-The absence of ammonium formate mostly impacts the retention time of THCVA and CBNA. - Co-elution between THCVA/CBN
1mM ammonium formate, 0.18% formic acid in 30:70 water:acetonitrile (pH 3.50)	0.1% formic acid in methanol (pH 2.76)	- Co-elution between THCVA/CBN
5mM ammonium formate, 0.7% formic acid in 30:70 water:acetonitrile (pH 3.50)	0.1% formic acid in methanol (pH 2.76)	-The greater the formic acid concentration, the higher the background at 230 nm, the lower the sensitivity -18 cannabinoids separated
2mM ammonium formate, 0.3% formic acid in 30:70 water:acetonitrile (pH 3.5)	0.1% formic acid in methanol (pH 2.76)	-18 cannabinoids separated perfectly with high sensitivity and good linearity (Considered as proper mobile phase)

Based on obtained results from mobile phase testing it can be concluded that the greater the formic acid concentration, the higher the background noise at 230 nm, the lower the sensitivity. Above 0.3% formic acid, the (0.5 ug/mL) calibrant does not show any peak for some cannabinoids; this phenomenon gets progressively worse as formic acid concentration is increased. Presence or absence of ammonium formate greatly influences retention time of CBNA and THCVA. Ammonium formate seems essential into separation of CBN/THCVA pair when pH is between 3 and 3.5. pH greatly influences retention times of all acidic cannabinoids, lower pH can increase retention time of acidic cannabinoids. Also, pH needs to be below 3.5 or above 5.5 for acidic

cannabinoids to behave in a linear fashion by mass spectrometry detection. Column temperature could be slightly increased or decreased to optimize separation.

Table 3.5. Chromatographic conditions used in the development of method (B)

Instrument	• Infinity II Prime pump G7104C • Infinity II Vial sampler G7129C with Cooling option • Infinity II Column oven G7130A • Infinity II DAD G7115A • 6545 QTOF used in TOF mode, with AJS in either positive or negative polarity
Column	Agilent InfinityLab Proshell 120 EC-C18, 3.0 × 150 mm, 2.7 µm
Mobile Phase	• Mobile Phase A: *modifier* (0.3% Formic Acid + 2 mM ammonium formate) in (acetonitrile:water 7 :3) • Mobile Phase B: *modifier* (0.1% Formic Acid) in methanol
Column temperature	40˚C
Wavelength	230 nm
Injection volume	5 µL
Flow rate	0.8 mL/min

Table 3.6. Mobile phase timetable and source conditions were used in the method (B) development

Timetable

	Time	Mobile phase A	Mobile phase B
1	4:00 min	99.00 %	1.00 %
2	4:30 min	70.00 %	30.00 %
3	5:50 min	70.00 %	30.00 %
4	6:30 min	65.00 %	35.00 %
5	8:50 min	65.00 %	35.00 %
6	9:00 min	50.00 %	50.00 %
7	11:50 min	50.00 %	50.00 %
8	12:00 min	0.00 %	100.00 %
9	13:00 min	0.00 %	100.00 %

Source Conditions– 6545 QTOF

- Source: Jet Stream ESI
- Drying Gas Flow: 12 L/min
- Sheath Gas Temperature: 350 °C
- Nebulizer Pressure: 40 psi
- Drying Gas Temperature: 350 °C
- Sheath Gas Flow : 11 L/min
- Polarity: Positive
- Capillary Voltage: 3500 V
- Nozzle Voltage: 1000 V

3.2.5. Statistical Analysis

Univariate analysis was performed using XLSTAT software (Addinsoft, Long Island City, NY, USA). Experimental results were expressed as means ± standard deviations. Statistical significance was assessed using analysis of variance (ANOVA) with the Tukey HSD (honestly significant difference) multiple comparison analysis. The criterion for statistical significance of differences was $P < 0.05$ for all comparisons. The results and data summary of multiple comparison analysis with Tukey HSD is shown in Table 3.7 and 3.8.

Table 3.7. Summary of data for Tukey HSD test

Summary of Data			
	Treatments		
	Method (B)	Method (A)	Total
N	23	23	46
∑X	22.9834	22.9456	45.929
Mean	0.9993	0.9976	0.998
$\sum X^2$	22.9668	22.8914	45.8582
Std.Dev.	0.0005	0.0012	0.0012

Table 3.8. The results of statistical comparison between two methods

	Result Details			
Source	*SS*	*df*	*MS*	
Between-treatments	0	1	0	$F = 38.314$
Within-treatments	0	44	0	
Total	0.0001	45		

Based on provided data from analysis of variance (ANOVA) with the Tukey HSD the f-ratio value is 38.31417 and the p-value is < .00001. Consequently, the result of this comparison between two methods is significant at $p < .05$.

3.3. Results and discussion

Testing different column temperatures showed determinant effects on separation of cannabinoids specifically in Method (A) on separation of CBNA. Figure 3.2 shows the results when changing the column temperature regarding separation of CBNA. This result was obtained at 38.5 °C (Figure 3.2). There is a slight shift to the left by increasing the column temperature due to the reduction in viscosity. Based on Method (A) conditions (Table 3.2 and 3.3), Figure 3.3a shows the chromatogram with the baselines subtracted for the separation of the 19 cannabinoids mixture. It was found that the addition of ammonium formate in a concentration of 5 mM to mobile phase (A) improved peak resolution. In the presence of ammonium formate, formic acid mobile phases become more ionic and the pH increases slightly (251,252). With 0.1 % formic acid and ammonium formate concentration of 5 mM, the pH values of mobile phase (A) were measured to be 4.5.

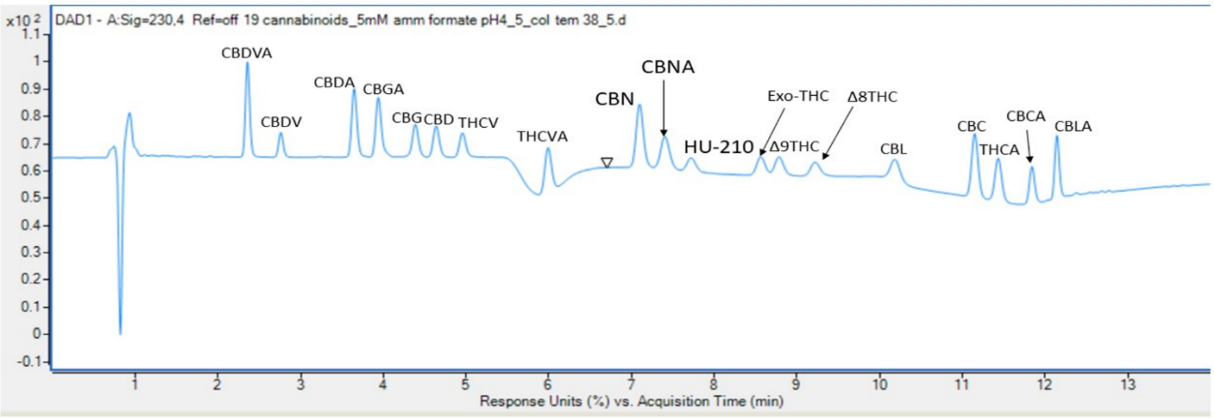

Figure 3.3a. The baseline chromatogram for the separation of the 19 cannabinoids test mixture using final conditions listed in Tables 3.2 & 3.3 (Method A)

After testing different mobile phase additives and changing the column temperature to improve sensitivity, separation, and linearity, we concluded that the most important factor that could cause better linearity and separation was the pH of the mobile phase. Based on earlier research testing, the mobile phase (acetonitrile/water 7:3 with the addition of 0.1 % formic acid and 5 mM ammonium formate) with a pH of 4.5, which is the exact pKa of carboxylic acid, may have caused the quadratic behavior. The assumption was we have kind of competition between the ionizable groups within an acidic cannabinoid at pH 4.5: hence we will have more deprotonated carboxylic

acid functional group in ph 4.5, this could cause the total net charge of zero for acidic cannabinoid, resulting no detection by positive mode. But in pH 3.5 due to the protonation of acidic group we have less competition between the carboxylic acid functional group and the rest of the molecule which could resolve the non-linearity issue in high concentrations. The deprotonation of the carboxylic acid group at pH 4.5 was confirmed by the detection of acidic cannabinoid in negative mode.

Therefore, the pH of the mobile phase was decreased using less ammonium formate (2 mM) and a higher amount of formic acid (0.3 % v/v). This attempt resulted in the second method: (acetonitrile/water 7:3 with the addition of 0.3 % formic acid and 2 mM ammonium formate at a pH of 3.5). Other information regarding this method's conditions is presented in Tables 3.5. and 3.6.

The second method, Method B, gave the best linearity results, considering 9 different concentrations (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 µg/mL). With this method, 18 cannabinoids were able to be separated (except CBLA due to co-elution of CBLA and CBCA) as is shown in Figure 3.3b.

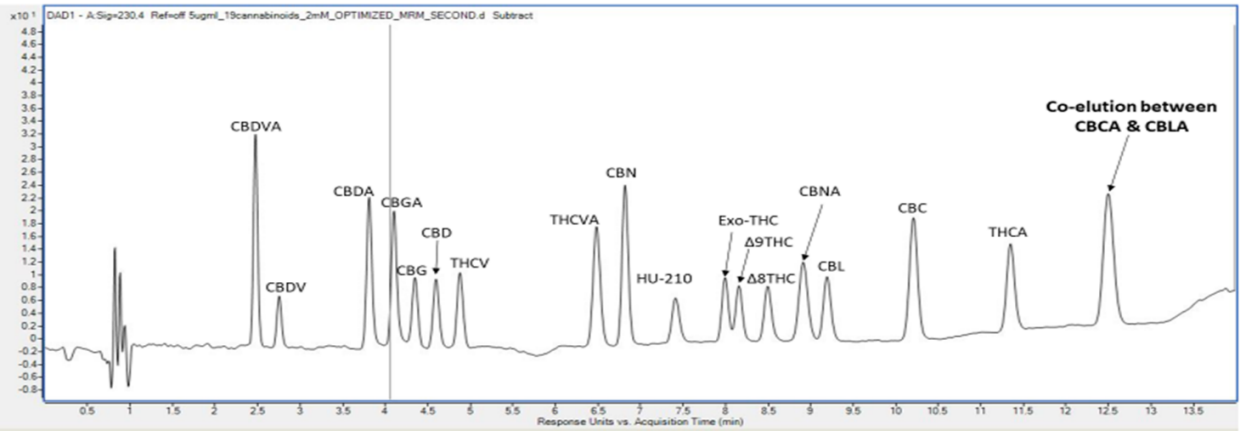

Figure 3.3b. The baseline chromatogram for the separation of the 19 cannabinoids test mixture using conditions listed in Tables 3.5 & 3.6 (Method B)

Based on calibration curves, from 9 level of concentrations which are shown in Figure 3.4 and 3.5, Method (B) resulted in a better linearity (R^2 values > 0.999) compared to Method (A) with (R^2 values > 0.997) in average. As a result, the second method was able to improve linearity for acidic cannabinoids as well as neutral cannabinoids.

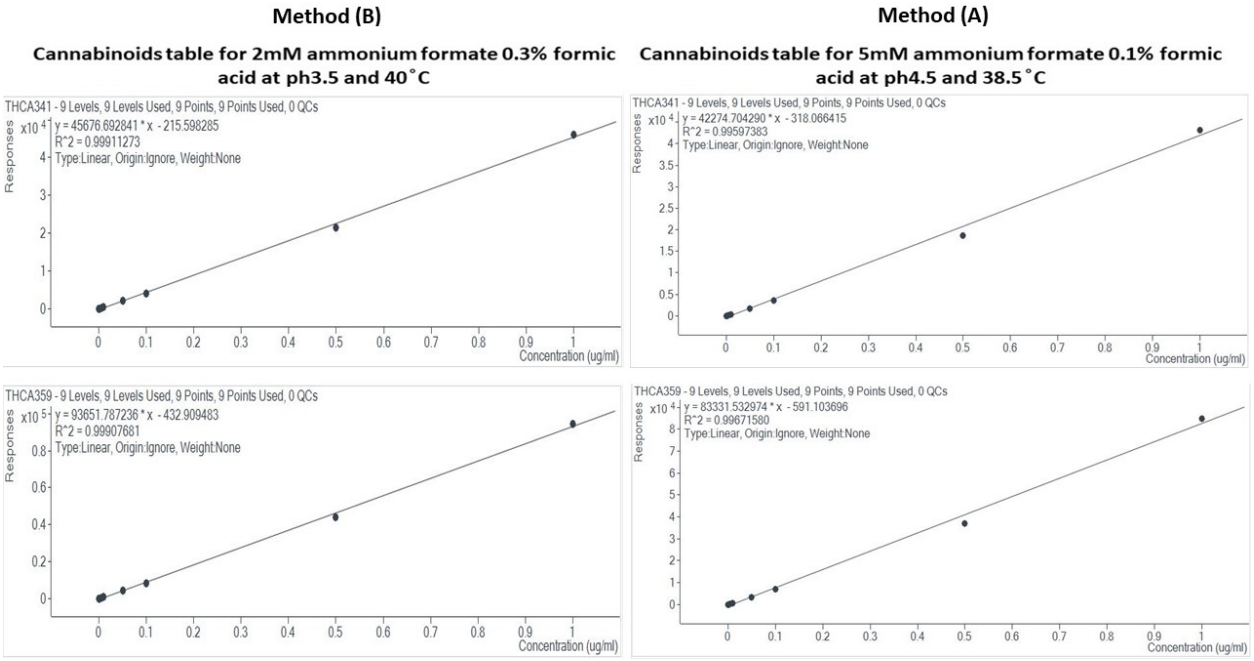

Figure 3.4. Calibration curves for THCA (341 (top) and 359 (bottom) *m/z*) obtained for the two proposed methods

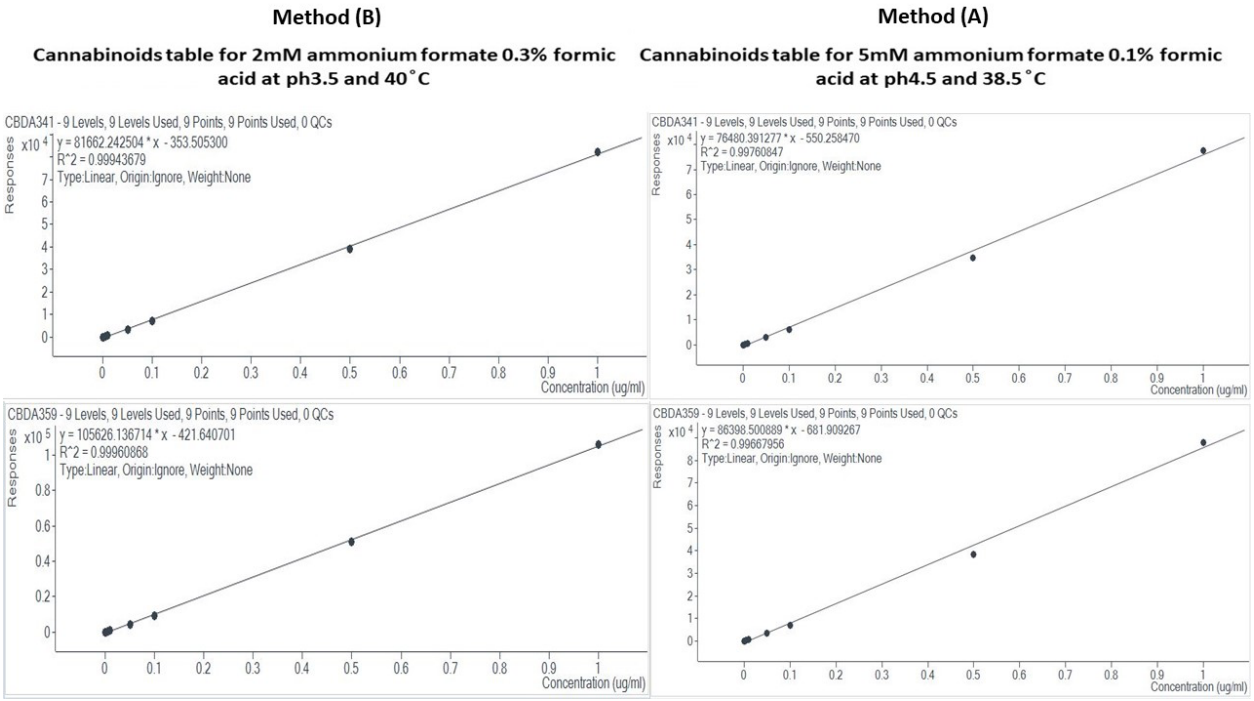

Figure 3.5. Calibration curve for CBDA (341 (top) and 359 (bottom) *m/z*) obtained for the two proposed methods

Comparing two different methods in terms of retention time of individual cannabinoid compound, the results showed that the retention time of some compounds changed and their order of elution was impacted. This observation was made for all of the tested methods. After changing the pH of the mobile phase, the shifting of the peaks was attributed to the pH of the mobile phase and the additives. Retention times of each cannabinoid regarding each method are shown in Table 3.9. CBNA, one of the carboxylated species, was investigated for interaction of buffer concentration with stationary phase and pH (between the two developed methods), as shown in Figure 3.6, it can be seen that the retention time of the carboxylated, ionisable cannabinoids is a complex function of eluotropic strength, pH, and buffer concentration/ionic strength.

Table 3.9. The retention time of each cannabinoid as a function of the method used

Cannabinoids table for 5mM ammonium formate 0.1% formic acid at ph4.5 and 38.5°C

	COMPOUND NAME	CHEMICAL FORMULA	RT	POSITIVE MODE M/Z	NEGATIVE MODE M/Z
1	CBDVA	$C_{20}H_{26}O_4$	2.389	331.1904 313.1798	329.1758
2	CBDV	$C_{19}H_{26}O_2$	2.777	287.2006	285.1060
3	CBDA	$C_{22}H_{30}O_4$	3.647	359.2217 341.2111	357.2071
4	CBGA	$C_{22}H_{32}O_4$	3.927	361.2373 343.2268	359.2228
5	CBG	$C_{21}H_{32}O_2$	4.353	317.2475	315.2330
6	CBD	$C_{21}H_{30}O_2$	4.598	315.2319	313.2173
7	THCV	$C_{19}H_{26}O_2$	4.906	287.2006	285.1860
8	THCVA	$C_{20}H_{26}O_4$	5.935	331.1904 313.1798	329.1758
9	CBN	$C_{21}H_{26}O_2$	6.982	311.2006	309.1860
10	CBNA	$C_{22}H_{26}O_4$	7.278	355.1904 337.1642	353.1758
11	HU-210	$C_{25}H_{38}O_3$	7.582	387.2894	385.2748
12	EXO-THC	$C_{21}H_{30}O_2$	8.392	315.2319	313.2173
13	Δ9-THC	$C_{21}H_{30}O_2$	8.604	315.2319	313.2173
14	Δ8-THC	$C_{21}H_{30}O_2$	9.024	315.2319	313.2173
15	CBL	$C_{21}H_{30}O_2$	9.970	315.2319	313.2173
16	CBC	$C_{21}H_{30}O_2$	11.034	315.2319	313.2173
17	THCA	$C_{22}H_{30}O_4$	11.336	359.2217 341.2111	357.2071
18	CBCA	$C_{22}H_{30}O_4$	11.821	359.2217 341.2111	357.2071
19	CBLA[1]	$C_{22}H_{30}O_4$	12.133	359.2217 341.2111	357.2071

1. CBLA separated perfectly

Cannabinoids table for 2mM ammonium formate 0.3% formic acid at ph3.5 and 40°C

	COMPOUND NAME	CHEMICAL FORMULA	RT	POSITIVE MODE M/Z	NEGATIVE MODE M/Z
1	CBDVA	$C_{20}H_{26}O_4$	2.512	331.1904 313.1798	329.1758
2	CBDV	$C_{19}H_{26}O_2$	2.795	287.2006	285.1060
3	CBDA	$C_{22}H_{30}O_4$	3.855	359.2217 341.2111	357.2071
4	CBGA	$C_{22}H_{32}O_4$	4.15	361.2373 343.2268	359.2228
5	CBG	$C_{21}H_{32}O_2$	4.40	317.2475	315.2330
6	CBD	$C_{21}H_{30}O_2$	4.664	315.2319	313.2173
7	THCV	$C_{19}H_{26}O_2$	4.937	287.2006	285.1860
8	THCVA	$C_{20}H_{26}O_4$	6.55	331.1904 313.1798	329.1758
9	CBN	$C_{21}H_{26}O_2$	6.903	311.2006	309.1860
10	HU-210	$C_{25}H_{38}O_3$	7.507	387.2894	385.2748
11	EXO-THC	$C_{21}H_{30}O_2$	8.093	315.2319	313.2173
12	Δ9-THC	$C_{21}H_{30}O_2$	8.254	315.2319	313.2173
13	Δ8-THC	$C_{21}H_{30}O_2$	8.605	315.2319	313.2173
14	CBNA	$C_{22}H_{26}O_4$	9.012	355.1904 337.1642	353.1758
15	CBL	$C_{21}H_{30}O_2$	9.293	315.2319	313.2173
26	CBC	$C_{21}H_{30}O_2$	10.31	315.2319	313.2173
27	THCA	$C_{22}H_{30}O_4$	11.438	359.2217 341.2111	357.2071
18	CBCA[1]	$C_{22}H_{30}O_4$	12.576	359.2217 341.2111	357.2071
19	CBLA[1]	$C_{22}H_{30}O_4$	12.576	359.2217 341.2111	357.2071

1. CBLA and CBCA eluted at same time

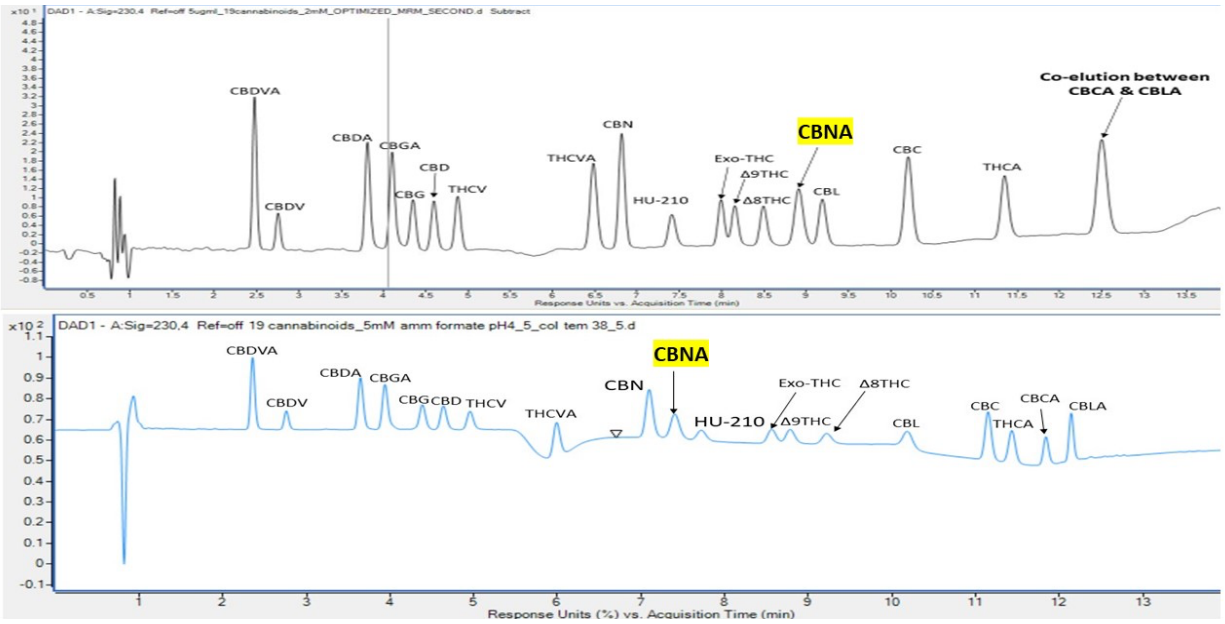

Figure 3.6. Comparison of retention time due to changing pH and buffer concentrations

Since ammonium formate is only added to the aqueous component of the mobile phase, the total ionic strength varies during gradient runtime. The run-to-run results, however, were reproducible after approximately 3 minutes of re-equilibration for both methods. Samples from plant extracts, may be affected by matrix effects. Analysts should examine whether other endogenous compounds of cannabis, such as terpenes and terpenoids, may interfere with cannabinoid identification.

3.3.1. Analytical Procedure Validation

Limit of quantification (LOQ), limit of detection (LOD), linearity, precision and accuracy were evaluated in the analytical procedure validation. For each analyte a calibration curve was built with 9 points, repeated in triplicate (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 µg/mL) of standard solutions. The LOQ were obtained from the minimum concentration that had acceptable accuracy between 80 - 120 %, and LOD were obtained by dividing LOQ by 3.3, based on analyzing different diluted standard samples. For interday relative standard deviation (RSD), samples were analyzed for three consecutive days with new mobile phase preparation for each day (interday precision or with reproducibility) at five different standard concentrations. The results of the validation experiments for method (A) and (B) are shown in the Tables 3.10 – 3.14.

Table 3.10. Limit of quantification (LOQ), limit of detection (LOD), and correlation coefficient obtained in analytical procedure validation.

	2mM ammonium formate, pH 3.5 at 40 °C			5mM ammonium formate, pH 4.5 at 38.5 °C		
Analyte	LOQ (µg/mL)	LOD (µg/mL)	R2	LOQ (µg/mL)	LOD (µg/mL)	R2
CBDVA313	0.005	0.002	0.9999	0.01	0.003	0.9983
CBDVA331	0.01	0.003	0.9996	0.01	0.003	0.9973
CBDV	0.005	0.002	0.9999	0.01	0.003	0.9993
CBDA341	0.01	0.003	0.9994	0.05	0.02	0.9976
CBDA359	0.01	0.003	0.9996	0.05	0.02	0.9966
CBGA361	0.01	0.003	0.9978	0.05	0.02	0.9950
CBGA343	0.01	0.003	0.9988	0.05	0.02	0.9974
CBG	0.01	0.003	0.9992	0.05	0.02	0.9982
CBD	0.01	0.003	0.9992	0.01	0.003	0.9988
THCV	0.01	0.003	0.9996	0.01	0.003	0.9988
THCVA313	0.01	0.003	0.9989	0.05	0.02	0.9966
THCVA331	0.01	0.003	0.9992	0.05	0.02	0.9972
CBN	0.01	0.003	0.9996	0.01	0.003	0.9981
EXO-THC	0.01	0.003	0.9991	0.05	0.02	0.9984
Δ9-THC	0.005	0.002	0.9997	0.01	0.003	0.9986
Δ8-THC	0.01	0.003	0.9991	0.01	0.003	0.9987
CBL	0.01	0.003	0.9996	0.01	0.003	0.9984
CBC	0.005	0.002	0.9996	0.05	0.02	0.9966
THCA359	0.01	0.003	0.9991	0.05	0.02	0.9967
THCA341	0.01	0.003	0.9991	0.05	0.02	0.9959
CBNA337	0.01	0.003	0.9994	0.01	0.003	0.9982
CBNA355	0.01	0.003	0.9998	0.01	0.003	0.9991
HU-210	0.01	0.003	0.9982	0.05	0.02	0.9958

Based on the provided data in Table 3.10. the limit of quantification, the limit of detection and the correlation coefficient calculated based on the accuracy and peak shape that gained from the MassHunter Quantitative Analysis software. The LOQ and LOD are higher in the method which had 5mM ammonium formate, so we can conclude that the method with 2mM ammonium formate is more precise and accurate to detect the lower concentration of cannabinoids. But having more baseline noise is also should be considered for 2mM method. The linearity of cannabinoids in 9 different concentration is better in comparison to the 5mM method. Based on unpaired t test results the two-tailed P value is less than o.ooo1. By conventional criteria, this difference is considered to be extremely statistically significant. Most of the correlation coefficients are higher than 0.999.

Table 3.11. The accuracy results, reported in percentage (%), obtained in analytical procedure validation of method (A).

Analyte	**Accuracy %**				
	0.01 µg/mL	0.05 µg/mL	0.1 µg/mL	0.5 µg/mL	1 µg/mL
CBDVA313	124.3	96.6	95.6	93.2	101.7
CBDVA331	113.5	94.5	94.8	91.4	102.2
CBDV	122.7	94.1	92.3	95.8	101.1
CBDA341	122.1	94.3	88.3	92.3	102.1
CBDA359	128.4	94.4	89.8	90.6	102.5
CBGA361	127.1	99.3	94.3	88.1	103.0
CBGA343	122.8	96.1	92.8	91.7	102.2
CBG	124.7	95.2	91.6	93.3	101.8
CBD	122.2	95.0	95.9	94.5	101.4
THCV	125.2	91.8	93.2	94.6	101.4
THCVA313	121.4	96.8	90.5	90.5	102.5
THCVA331	122.3	98.4	90.0	91.6	102.2
CBN	129.8	96.8	94.9	92.8	101.9
EXO-THC	111.0	93.9	92.2	93.6	101.7
Δ9-THC	127.1	95.1	93.8	94.0	101.6
Δ8-THC	127.0	93.2	93.2	94.2	101.5
CBL	119.4	96.1	91.2	93.7	101.7
CBC	118.9	99.2	97.3	90.2	102.5
THCA359	128.7	95.5	93.2	90.5	102.5
THCA341	125.7	93.3	91.8	89.3	102.7
CBNA337	92.1	97.4	99.7	92.9	101.8
CBNA355	129.5	93.7	97.7	95.1	101.3
HU-210	125.8	97.9	93.6	89.1	102.8
CBCA341	125.0	94.0	94.9	92.2	102.0
CBCA359	110.4	100.6	99.0	93.0	101.8
CBLA359	126.2	93.9	97.6	93.3	101.7

Table 3.12. The accuracy results, reported in percentage (%), obtained in analytical procedure validation of method (B).

Analyte	**Accuracy %**				
	0.01 µg/mL	0.05 µg/mL	0.1 µg/mL	0.5 µg/mL	1 µg/mL
CBDVA313	110.6	93.8	95.3	98.9	100.3
CBDVA331	113.7	94.2	94.3	97.1	100.8
CBDV	105.9	98.4	92.3	99.4	100.2
CBDA341	109.2	91.4	92.3	96.6	100.9
CBDA359	118.4	92.9	89.9	97.6	100.7
CBGA361	103.6	105.0	86.6	95.0	101.9
CBGA343	116.0	92.1	87.9	92.8	101.4
CBG	112.6	91.9	91.5	96.1	101.1
CBD	114.8	97.9	92.9	95.8	100.8
THCV	112.6	94.4	93.0	97.3	100.8
THCVA313	111.6	95.8	93.1	94.9	101.3
THCVA331	118.3	98.4	92.9	95.5	101.2
CBN	111.1	95.8	92.4	97.2	100.8
EXO-THC	118.8	92.9	93.9	95.4	101.2
Δ9-THC	116.8	95.4	92.1	98.2	100.5
Δ8-THC	110.0	91.0	91.8	95.8	101.2
CBL	114.2	93.1	90.8	97.6	100.7
CBC	123.9	98.2	96.3	97.1	100.3
THCA359	110.9	97.5	91.9	95.2	101.3
THCA341	115.7	96.3	91.8	95.4	101.2
CBNA337	118.2	92.4	89.7	96.8	100.9
CBNA355	107.6	94.7	90.7	98.5	100.5
HU-210	106.8	93.9	90.2	93.4	101.8

In terms of accuracy both methods showed good results. For the method (B) the range of accuracy were obtained between (86.6 % - 123.9 %), and for the method (A) the range of accuracy were between (88.1% - 129.8) for 5 different concentrations of each cannabinoids.

Table 3.13. The precision results, obtained in analytical procedure validation for method (A).

Analyte	**Precision**				
	Peak area (RSD)				
	(0.01 µg/mL)	(0.05 µg/mL)	(0.1 µg/mL)	(0.5 µg/mL)	(1 µg/mL)
CBC	3.25	6.98	4.80	11.00	10.91
CBCA341	8.95	18.57	10.63	20.71	22.50
CBD	1.24	0.16	2.27	1.01	2.01
CBDA341	7.51	0.44	3.99	3.17	1.01
CBDA359	13.63	13.45	14.36	11.06	12.41
CBDV	9.48	12.83	9.52	9.21	12.20
CBDVA313	1.22	6.42	2.09	2.83	4.99
CBDVA331	16.56	21.59	14.81	16.58	17.49
CBG	2.88	2.69	0.01	1.78	2.52
CBGA343	5.97	2.90	2.33	0.12	1.91
CBGA361	3.14	21.85	0.48	9.23	12.34
CBL	1.74	4.03	1.71	1.03	2.24
CBLA359	40.66	53.48	23.99	22.63	19.62
CBN	1.22	1.91	1.14	2.16	3.81
CBNA337	43.51	13.95	14.21	1.77	7.27
CBNA355	19.23	5.42	3.24	0.64	4.70
DELTA8-THC	3.20	1.85	3.53	3.29	7.26
DELTA9-THC	0.85	0.62	1.71	2.05	4.52
EXO-THC	12.11	3.92	3.29	1.58	2.64
HU-210	4.56	6.41	5.10	8.71	13.49
THCA341	16.61	24.56	17.29	25.93	25.43
THCA359	34.11	31.48	28.43	31.29	33.67
THCV	2.83	0.65	1.93	1.00	4.14
THCVA313	12.25	10.21	10.59	10.83	10.02
THCVA331	9.36	18.01	15.12	16.94	18.18

Table 3.14. The precision results, obtained in analytical procedure validation for method (B).

Analyte	**Precision**				
	Peak area (RSD)				
	(0.01 µg/mL)	(0.05 µg/mL)	(0.1 µg/mL)	(0.5 µg/mL)	(1 µg/mL)
CBC	6.43	12.53	9.83	9.72	6.69
CBD	6.09	1.92	0.49	1.35	1.45
CBDA341	3.72	1.10	4.75	6.40	1.10
CBDA359	10.58	7.82	4.59	10.32	4.82
CBDV	1.22	3.60	0.86	1.56	0.31
CBDVA313	0.26	2.51	2.58	2.16	0.59
CBDVA331	5.62	7.34	8.49	6.99	6.22
CBG	2.38	0.86	0.55	2.80	0.14
CBGA343	1.80	0.53	1.21	4.41	1.47
CBGA361	36.66	13.96	1.94	1.76	3.47
CBL	5.55	9.45	7.84	9.41	3.77
CBN	3.52	6.63	2.89	5.15	1.45
CBNA337	15.33	2.46	4.98	3.62	7.30
CBNA355	32.99	1.77	1.11	1.75	4.74
DELTA8-THC	8.98	5.42	7.94	7.40	4.01
DELTA9-THC	6.73	9.46	7.07	7.18	3.38
EXO-THC	4.32	7.48	9.61	6.88	4.22
HU-210	4.03	1.62	0.25	0.19	1.96
THCA341	7.88	6.63	11.25	8.87	10.52
THCA359	1.24	0.67	6.19	4.15	6.94
THCV	2.73	0.87	2.52	3.36	0.04
THCVA313	5.50	7.01	8.62	4.32	6.95
THCVA331	7.50	1.59	4.22	1.34	4.71

The precision of each method calculated based on the peak area of each compound in different concentrations with three replications for each compound and each method in three different days. The variety of RSD percentage could be due to the period between each testing, because samples and mobile phase properties could have been changed and affected by the laboratory temperature in summer during the three days period.

3.4. Conclusion

To summarize, two HPLC methods were developed that resolve 19 and 18 cannabinoids, respectively, by employing screening runs that changed the quantities of organic and acid modifiers, laying the foundation for future research. The addition of ammonium formate to mobile phase A allowed for the shift of carboxylated species retentions relative to neutral species. Addition of more formic acid and less ammonium formate which resulted less pH of mobile phase, improved the linearity of the calibration curve specially for acidic cannabinoids in method (B). Also, we conclude that the column temperature plays an important role to separate the CBNA from CBN in method (A).

4. General Summary

4.1. General Conclusion

The objective of this research was to develop proper methods for analyzing cannabinoid compounds in cannabis products. Current literature shows that the pH of the mobile phase and the column temperature are the two most important factors that can have a critical effect on the separation of cannabinoids and the linearity of the cannabinoids curve. After testing 10 different methods to reach better sensitivity, separation, and linearity we conclude and confirm the literature findings that the most important factors that cause better linearity and separation are the pH of the mobile phase and the column temperature.

The mobile phase which was used at the Agilent Technologies had acetonitrile/water in 7:3 (v/v) ratio with the addition of 0.1 % formic acid and 5 mM ammonium formate) with a pH of 4.5, which is the theoretical pk_a of carboxylic acid, thus this was assumed as the cause of the quadratic behavior. This is explained by the 1:1 protonated and unprotonated ratio of carboxylic acid functional groups, so decision was made to decrease the pH of the mobile phase using less ammonium formate and higher formic acid to keep the acidic group in neutral form which helps us to prevent deprotonation of the carboxylic acid functional groups. It is assumed that there is a kind of competition between the ionizable groups within an acidic cannabinoid at pH 4.5, hence more deprotonated carboxylic acid functional groups are expected in pH 4.5, which could cause the total net charge of zero for acidic cannabinoid, resulting in no detection by positive mode. While under the condition of pH 3.5, due to the protonation of the acidic group, there should be less competition between the carboxylic acid functional groups and the rest of the molecules which could resolve the non-linearity issue in high concentrations. The deprotonation of the carboxylic acid group at pH 4.5 was confirmed by the detection of acidic cannabinoids in negative mode (because by negative mode we can only detect acidic cannabinoids). Our results showed that the column temperature plays an important role in the separation of CBNA in method (A). Based on what is presented in Figure 3.2. even a 1.5 °C change in column temperature could cause co-elution in the separation of cannabinoids (CBNA with CBN).

4.2. Future Suggested Work

Suggested future work include development of a method which able cannabis testing laboratories to detect more cannabinoids in a short time runs. There are lots of synthetic cannabinoids that can be added to the sample for the detection of even more than 19 cannabinoids in one run which could be harder to separate without co-elution in short run time. Consequently, other effective factors should be considered to reach that goal. Our focus was on mobile phase pH and changing the column temperature, but there are other parameters that can have an impact on the separation of cannabinoids like drying gas flow rate, nebulizer pressure, quality of initial products, voltage (entrance & fragmentation), system tuning period, analyte/IS ratio, gas phase salt bridges, etc. As well as developing a proper method for separation of cannabinoids, operation cost is also important so, trying different organic solvent instead of acetonitrile to decrease the cost of operation could be beneficial.

References

1. Thomas BF, ElSohly MA. The Analytical Chemistry of Cannabis: Quality Assessment, Assurance, and Regulation of Medicinal Marijuana and Cannabinoid Preparations. The Analytical Chemistry of Cannabis: Quality Assessment, Assurance, and Regulation of Medicinal Marijuana and Cannabinoid Preparations. 2015.
2. Tzimas PS, Petrakis EA, Halabalaki M, Skaltsounis LA. Effective determination of the principal non-psychoactive cannabinoids in fiber-type Cannabis sativa L. by UPLC-PDA following a comprehensive design and optimization of extraction methodology. Anal Chim Acta. 2021;
3. Qamar S, Torres YJM, Parekh HS, Robert Falconer J. Extraction of medicinal cannabinoids through supercritical carbon dioxide technologies: A review. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 2021.
4. Pasin D, Cawley A, Bidny S, Fu S. Current applications of high-resolution mass spectrometry for the analysis of new psychoactive substances: a critical review. Analytical and Bioanalytical Chemistry. 2017.
5. Wang F, Zeng H, Wang J. Characterization of nineteen impurities in roxithromycin by HPLC/TOF and ion trap mass spectrometry. Chromatographia. 2013;
6. Horimoto S, Mayumi T, Aoe K, Nishimura N, Sato T. Analysis of β-lactam antibiotics by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry using bromoform. J Pharm Biomed Anal. 2002;
7. Zeng H, Wang F, Zhu B, Zhong W, Shan W, Wang J. Study of the structures of photodegradation impurities and pathways of photodegradation of cilnidipine by liquid chromatography/Q-Orbitrap mass spectrometry. Rapid Commun Mass Spectrom. 2016;
8. Bonini SA, Premoli M, Tambaro S, Kumar A, Maccarinelli G, Memo M, et al. Cannabis sativa: A comprehensive ethnopharmacological review of a medicinal plant with a long history. Journal of Ethnopharmacology. 2018.
9. Chandra S, Lata H, ElSohly MA, Walker LA, Potter D. Cannabis cultivation: Methodological issues for obtaining medical-grade product. Epilepsy and Behavior. 2017.
10. Vučkovic S, Srebro D, Vujovic KS, Vučetic Č, Prostran M. Cannabinoids and pain: New insights from old molecules. Front Pharmacol. 2018;
11. R.G. P. Cannabinoid pharmacology: The first 66 years. British Journal of Pharmacology.

2006.
12. Clarke RC, Merlin MD. Cannabis Domestication, Breeding History, Present-day Genetic Diversity, and Future Prospects. CRC Crit Rev Plant Sci. 2016;
13. Marzo V Di, Petrocellis L De, Di Marzo V, De Petrocellis L. The endogenous cannabinoid signalling system: chemistry, biochemistry and physiology. Internet J Sci Biol Chem. 1997;
14. Zou S, Kumar U. Cannabinoid receptors and the endocannabinoid system: Signaling and function in the central nervous system. International Journal of Molecular Sciences. 2018.
15. Zerrin. A. Cannabis, a complex plant: Different compounds and different effects on individuals. Therapeutic Advances in Psychopharmacology. 2012.
16. Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, Hipkin RW. Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: Antagonism by anandamide. Mol Pharmacol. 2000;
17. Luk T, Jin W, Zvonok A, Lu D, Lin XZ, Chavkin C, et al. Identification of a potent and highly efficacious, yet slowly desensitizing CB1 cannabinoid receptor agonist. Br J Pharmacol. 2004;
18. Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacological Reviews. 2002.
19. Mechoulam R, Gaoni Y. Recent advances in the chemistry of hashish. Fortschritte der Chemie organischer Naturstoffe. Progress in the chemistry of organic natural products. Progrès dans la chimie des substances organiques naturelles. 1967.
20. Degenhardt L, Coffey C, Carlin JB, Swift W, Moore E, Patton GC. Outcomes of occasional cannabis use in adolescence: 10-year follow-up study in Victoria, Australia. Br J Psychiatry. 2010;
21. Hall W, Degenhardt L. Prevalence and correlates of cannabis use in developed and developing countries. Current Opinion in Psychiatry. 2007.
22. Radwan MM, Chandra S, Gul S, Elsohly MA. Cannabinoids, phenolics, terpenes and alkaloids of cannabis. Molecules. 2021.
23. Hodgson E. Toxicology and Human Environments. Burlington: Elsevier Science; 2014.
24. Carstairs C. Jailed for Possession: Illegal Drug Use, Regulation, and Power in Canada,

1920–1961. University of Toronto Press; 2006. 243 p.

25. Fischer B, Kuganesan S, Room R. Medical marijuana programs: Implications for cannabis control policy - Observations from Canada. International Journal of Drug Policy. 2015.
26. Fike J. Industrial Hemp: Renewed Opportunities for an Ancient Crop. CRC Crit Rev Plant Sci. 2016;
27. Health C. Hemp and the hemp industry Frequently Asked Questions [Internet]. 2022. Available from: https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/producing-selling-hemp/about-hemp-canada-hemp-industry/frequently-asked-questions.html
28. Health Canada. Understanding the New Access to Cannabis for Medical Purposes Regulations [Internet]. 2022. Available from: https://www.canada.ca/en/health-canada/services/publications/drugs-health-products/understanding-new-access-to-cannabis-for-medical-purposes-regulations.html
29. Elliott J. "Liberals 'Committed' to Legalizing Marijuana: Trudeau," CTV News, September 30, 2015 [Internet]. Available from: https://www.ctvnews.ca/politics/election/liberals-committed-to-legalizingmarijuana-trudeau-1.2588260.
30. Health Canada. Cannabis Act (S.C. 2018, c. 16) [Internet]. Available from: https://laws-lois.justice.gc.ca/eng/annualstatutes/2018_16/FullText.html
31. Mead A. Legal and regulatory issues governing cannabis and cannabis-derived products in the United States. Frontiers in Plant Science. 2019.
32. Cox C. Implications of the 2018 Canadian Cannabis Act: Should regulation differ for medicinal and non-medicinal cannabis use?⋆. Health Policy (New York). 2021;
33. Carus M, Karst S, Kauffmann A. The European Hemp Industry : Cultivation , processing and applications for fibres , shivs and seeds. Eiha. 2016;
34. eGuide - Canadian Hemp Trade Alliance [Internet]. Available from: https://www.hemptrade.ca/content.aspx?page_id=22&club_id=950211&module_id=402335
35. Cherney JH, Small E. Industrial hemp in North America: Production, politics and potential. Agronomy. 2016.
36. Alagawany M, Elnesr SS, Farag MR, Abd El-Hack ME, Khafaga AF, Taha AE, et al.

Omega-3 and omega-6 fatty acids in poultry nutrition: Effect on production performance and health. Animals. 2019.

37. Pless P, Leson G. Assessing the impact of thc uptake from hemp oil cosmetics on workplace drug testing. Leson Environ Consult. 2001;94709(March).
38. Expansion Stratégies Inc. Development of an Industrial Hemp Strategy for the Province of New Brunswick - Final Report. 2019; Available from: https://www.agnb-vgnb.ca/content/dam/gnb/Departments/10/pdf/Agriculture/IndustrialHemp-ChanvreIndustriel/Hemp-Consultants-Report.pdf
39. Fisher M. Organic Management in the Prairie Provinces. Crop Soils. 2017;
40. Small E, Marcus D. Hemp: a new crop with new uses for North America. In: J Janick and A Whipkey (eds) ASHS Press, Alexandria, VA. 2002.
41. Schippmann U, Leaman DJ, Cunningham AB. Impact of cultivation and gathering of medicinal plants on biodiversity: global trends and issues. Biodivers Ecosyst approach Agric For Fish. 2002;
42. Maurer GE, Mathews NM, Schleich KT, Slayman TG, Marcussen BL. Understanding Cannabis-Based Therapeutics in Sports Medicine. Sports Health. 2020.
43. Crini G, Lichtfouse E, Chanet G, Morin-Crini N. Traditional and New Applications of Hemp. In 2020.
44. Crini G, Lichtfouse E, Chanet G, Morin-Crini N. Applications of hemp in textiles, paper industry, insulation and building materials, horticulture, animal nutrition, food and beverages, nutraceuticals, cosmetics and hygiene, medicine, agrochemistry, energy production and environment: a review. Environmental Chemistry Letters. 2020.
45. Walker LA, Koturbash I, Kingston R, ElSohly MA, Yates CR, Gurley BJ, et al. Cannabidiol (CBD) in Dietary Supplements: Perspectives on Science, Safety, and Potential Regulatory Approaches. J Diet Suppl. 2020;
46. Bruce D, Brady JP, Foster E, Shattell M. Preferences for medical marijuana over prescription medications among persons living with chronic conditions: Alternative, complementary, and tapering uses. J Altern Complement Med. 2018;
47. Blake A, Nahtigal I. The evolving landscape of cannabis edibles. Current Opinion in Food Science. 2019.
48. Knapp AA, Lee DC, Borodovsky JT, Auty SG, Gabrielli J, Budney AJ. Emerging Trends

in Cannabis Administration Among Adolescent Cannabis Users. J Adolesc Heal. 2019;
49. Doggett A, Battista K, Leatherdale ST. Modes of cannabis use among Canadian youth in the COMPASS study; using LCA to examine patterns of smoking, vaping, and eating/drinking cannabis. Drugs Educ Prev Policy. 2021;
50. Resko S, Ellis J, Early TJ, Szechy KA, Rodriguez B, Agius E. Understanding Public Attitudes Toward Cannabis Legalization: Qualitative Findings From a Statewide Survey. Subst Use Misuse. 2019;
51. Ashton L. CBD for Energy, Focus and Concentration: Can It Help?
52. Cats L, Cat VM, Life D, Training D, Breed RY. Best CBD Oil for Dogs with Arthritis– Top Brands Reviews in 2021.
53. MacCallum CA, Freitas L de, Lo LA, Boivin M. Cannabinoid-Based Medicines: Dosing, Titration & Monitoring. In: Cannabinoids and Pain. Springer; 2021. p. 167–78.
54. Charlebois S, Music J, Sterling B, Somogyi S. Edibles and Canadian consumers' willingness to consider recreational cannabis in food or beverage products: A second assessment. Trends in Food Science and Technology. 2020.
55. Sholler DJ, Schoene L, Spindle TR. Therapeutic Efficacy of Cannabidiol (CBD): a Review of the Evidence From Clinical Trials and Human Laboratory Studies. Current Addiction Reports. 2020.
56. Schiller M. Global Cannabis Market to Hit $42.7 Billion by 2024, According to Updated Report from Arcview Group, BDS Analytics [Internet]. Available from: https://www.cannabisbusinesstimes.com/article/global-cannabis-market-to-hit-42-billion-by-2024-arcview-group-bds-analytics/
57. Pellechia T. Legal Cannabis Industry Poised For Big Growth, In North America And Around The World. Forbes. 2018.
58. George-Cosh D. Global cannabis market could be worth up to US$130B by 2029: Jefferies [Internet]. 2019. Available from: https://www.bnnbloomberg.ca/global-cannabis-market-could-be-worth-up-to-us-130b-by-2029-jefferies-1.1219522
59. Rotermann M. What has changed since cannabis was legalized? Heal Reports. 2020;
60. Canada VA. Cannabis for Medical Purposes [Internet]. Available from: https://www.veterans.gc.ca/eng/about-vac/research/research-directorate/publications/reports/cmp

61. Cavazos-Rehg PA, Krauss MJ, Cahn E, Lee KE, Ferguson E, Rajbhandari B, et al. Marijuana Promotion Online: an Investigation of Dispensary Practices. Prev Sci. 2019;
62. Groce E. The Health Effects of Cannabis and Cannabinoids: The Current State of Evidence and Recommendations for Research. J Med Regul. 2018;
63. Braithwaite I, Bhagavan C, Doppen M, Kung S, Oldfield K, Newton-Howes G. Medicinal applications of cannabis/cannabinoids. Current Opinion in Psychology. 2021.
64. Shannon S, Opila-Lehman J. Effectiveness of Cannabidiol Oil for Pediatric Anxiety and Insomnia as Part of Posttraumatic Stress Disorder: A Case Report. Perm J. 2016;
65. Stella B, Baratta F, Della Pepa C, Arpicco S, Gastaldi D, Dosio F. Cannabinoid Formulations and Delivery Systems: Current and Future Options to Treat Pain. Drugs. 2021.
66. Parmar JR, Forrest BD, Freeman RA. Medical marijuana patient counseling points for health care professionals based on trends in the medical uses, efficacy, and adverse effects of cannabis-based pharmaceutical drugs. Research in Social and Administrative Pharmacy. 2016.
67. Wade DT, Makela PM, House H, Bateman C, Robson P. Long-term use of a cannabis-based medicine in the treatment of spasticity and other symptoms in multiple sclerosis. Mult Scler. 2006;
68. Rosager EV, Møller C, Sjögren M. Treatment studies with cannabinoids in anorexia nervosa: a systematic review. Eating and Weight Disorders. 2021.
69. Woolridge E, Barton S, Samuel J, Osorio J, Dougherty A, Holdcroft A. Cannabis use in HIV for pain and other medical symptoms. J Pain Symptom Manage. 2005;
70. Lucas P. Cannabis as an adjunct to or substitute for opiates in the treatment of chronic pain. J Psychoactive Drugs. 2012;
71. Spanagel R, Bilbao A. Approved cannabinoids for medical purposes – Comparative systematic review and meta-analysis for sleep and appetite. Neuropharmacology. 2021;
72. Strouse TB. Cannabinoids in Medical Practice. Cannabis and Cannabinoid Research. 2016.
73. Kramer JL. Medical marijuana for cancer. CA Cancer J Clin. 2015;
74. Überall MA. A review of scientific evidence for THC:CBD oromucosal spray (nabiximols) in the management of chronic pain. Journal of Pain Research. 2020.

75. Balneaves LG, Alraja A, Ziemianski D, McCuaig F, Ware M. A National Needs Assessment of Canadian Nurse Practitioners Regarding Cannabis for Therapeutic Purposes. Cannabis Cannabinoid Res. 2018;
76. Valleriani J. Identity narratives in the face of market competition: the emerging legal medical cannabis market in Canada. Drugs Educ Prev Policy. 2020;
77. Health Canada. (2020). Accessing cannabis for medical purposes. [Internet]. Available from: https://www.canada.ca/en/health-canada/topics/accessing-cannabis-for-medical-purposes.html
78. Statistics Canada. (2017). Canadian cannabis survey 2017 - Summary - Canada.ca [Internet]. Available from: https://www.canada.ca/en/health-canada/services/publications/drugs-health-products/canadian-cannabis-survey-2017-summary.html#a4
79. Kamrul R, Bunka D, Crawley A, Schuster B, LeBras M. Navigating cannabinoid choices for chronic neuropathic pain in older adults Potholes and highlights. Can Fam Physician. 2019;
80. Brenneisen R. Chemistry and Analysis of Phytocannabinoids and Other Cannabis Constituents. In: Marijuana and the Cannabinoids. 2007.
81. Chen F, Tholl D, Bohlmann J, Pichersky E. The family of terpene synthases in plants: A mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. Plant J. 2011;
82. Gaggiotti S, Palmieri S, Della Pelle F, Sergi M, Cichelli A, Mascini M, et al. Piezoelectric peptide-hpDNA based electronic nose for the detection of terpenes; Evaluation of the aroma profile in different Cannabis sativa L. (hemp) samples. Sensors Actuators, B Chem. 2020;
83. Hamm S, Bleton J, Connan J, Tchapla A. A chemical investigation by headspace SPME and GC-MS of volatile and semi-volatile terpenes in various olibanum samples. Phytochemistry. 2005;
84. Elsohly MA. Marijuana and the Cannabinoids Edited by. 2007. 333 p.
85. Gertsch J, Leonti M, Raduner S, Racz I, Chen J-Z, Xie X-Q, et al. Beta-caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci U S A. 2008 Jul;105(26):9099–104.
86. Russo Ethan B. Taming THC: Potential cannabis synergy and phytocannabinoid-terpenoid

entourage effects. British Journal of Pharmacology. 2011.

87. Fine PG, Rosenfeld MJ. The endocannabinoid system, cannabinoids, and pain. Rambam Maimonides Med J. 2013;4(4):e0022.
88. Sharma C, M. Al Kaabi J, M. Nurulain S, N. Goyal S, Amjad Kamal M, Ojha S. Polypharmacological Properties and Therapeutic Potential of β-Caryophyllene: A Dietary Phytocannabinoid of Pharmaceutical Promise. Curr Pharm Des. 2016;
89. Nosková K, Dovrtělová G, Zendulka O, Řemínek R, Juřica J. The effect of (-)-linalool on the metabolic activity of liver CYP enzymes in rats. Physiol Res. 2016;
90. Paris M, Boucher F, Cosson L. The constituents of Cannabis sativa pollen. Econ Bot. 1975;
91. Barrett ML, Scutt AM, Evans FJ. Cannflavin A and B, prenylated flavones from Cannabis sativa L. Experientia. 1986;
92. Vanhoenacker G, Van Rompaey P, De Keukeleire D, Sandra P. Chemotaxonomic features associated with flavonoids of cannabinoid-free cannabis (Cannabis sativa subsp. sativa L.) in relation to hops (Humulus lupulus L.). Nat Prod Lett. 2002;
93. Choi YH, Hazekamp A, Peltenburg-Looman AMG, Frédérich M, Erkelens C, Lefeber AWM, et al. NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of Cannabis sativa. Phytochem Anal. 2004;
94. Ross SA, Elsohly MA. The volatile oil composition of fresh and air-dried buds of Cannabis sativa. J Nat Prod. 1996;
95. McPartland JM, Russo EB. Cannabis and Cannabis extracts: Greater than the sum of their parts? In: Cannabis Therapeutics in HIV/AIDS. 2012.
96. Jin D, Dai K, Xie Z, Chen J. Secondary Metabolites Profiled in Cannabis Inflorescences, Leaves, Stem Barks, and Roots for Medicinal Purposes. Sci Rep. 2020;
97. Baron EP. Medicinal Properties of Cannabinoids, Terpenes, and Flavonoids in Cannabis, and Benefits in Migraine, Headache, and Pain: An Update on Current Evidence and Cannabis Science. Headache. 2018.
98. Radwan MM, Ross SA, Slade D, Ahmed SA, Zulfiqar F, Elsohly MA. Isolation and characterization of new cannabis constituents from a high potency variety. Planta Med. 2008;
99. Puglisi I, Barone V, Sidella S, Coppa M, Broccanello C, Gennari M, et al. Biostimulant

activity of humic-like substances from agro-industrial waste on Chlorella vulgaris and Scenedesmus quadricauda. Eur J Phycol. 2018;53(3):433–42.

100. Batra P, Sharma AK. Anti-cancer potential of flavonoids: recent trends and future perspectives. 3 Biotech. 2013;
101. Kopustinskiene DM, Jakstas V, Savickas A, Bernatoniene J. Flavonoids as anticancer agents. Nutrients. 2020.
102. Turner CE, Elsohly MA, Boeren EG. Constituents of cannabis sativa l. xvii. a review of the natural constituents. J Nat Prod. 1980;
103. Ross S, EISohly M. Constituents of cannabis sativa l. xvii. a review of the natural constituents. 1980-1994. Zagazig J Pharm Sci. 1995;
104. Elsohly MA, Turner CE, Phoebe CH, Knapp JE, Schiff PL, Slatkin DJ. Anhydrocannabisativine, a new alkaloid from Cannabis sativa L. J Pharm Sci. 1978;
105. Roberts Wink, Michael., MF. Alkaloids : Biochemistry, Ecology, and Medicinal Applications [Internet]. Boston, MA: Springer US; 1998. Available from: https://doi.org/10.1007/978-1-4757-2905-4
106. Lowe H, Steele B, Bryant J, Toyang N, Ngwa W. Non-cannabinoid metabolites of cannabis sativa l. With therapeutic potential. Plants. 2021;
107. Gowda, L.; Venkategowda A. Isolation and Characterization of Antimicrobial Alkaloids from Plumeria alba Flowers against Food Borne Pathogens. Am J Life Sci. 2014;2(6):1.
108. Yan X, Zhou Y, Tang J, Ji M, Lou H, Fan P. Diketopiperazine indole alkaloids from hemp seed. Phytochem Lett. 2016;
109. Jambois A, Ditengou FA, Kawano T, Delbarre A, Lapeyrie F. The indole alkaloids brucine, yohimbine, and hypaphorine are indole-3-acetic acid-specific competitors which do not alter auxin transport. Physiol Plant. 2004;
110. Dey P, Kundu A, Kumar A, Gupta M, Lee BM, Bhakta T, et al. Analysis of alkaloids (indole alkaloids, isoquinoline alkaloids, tropane alkaloids). In: Recent Advances in Natural Products Analysis. 2020.
111. Mechoulam R, Hanuš L. A historical overview of chemical research on cannabinoids. Chemistry and Physics of Lipids. 2000.
112. Citti C, Pacchetti B, Vandelli MA, Forni F, Cannazza G. Analysis of cannabinoids in commercial hemp seed oil and decarboxylation kinetics studies of cannabidiolic acid

(CBDA). J Pharm Biomed Anal. 2018;
113. Fine PG, Rosenfeld MJ, Ph D. The Endocannabinoid System ,. 4(4):1–15.
114. Small E, Cronquist A. A practical and natural taxonomy for cannabis. Taxon. 1976;
115. Hazekamp A. Cannabis; extracting the medicine. Department of Pharmacognosy, (IBL), Faculty of Science, Leiden University; 2007.
116. Pereira I, Severino P, Santos AC, Silva AM, Souto EB. Linalool bioactive properties and potential applicability in drug delivery systems. Colloids and Surfaces B: Biointerfaces. 2018.
117. Zgair A, Wong JCM, Sabri A, Fischer PM, Barrett DA, Constantinescu CS, et al. Development of a simple and sensitive HPLC-UV method for the simultaneous determination of cannabidiol and δ9-tetrahydrocannabinol in rat plasma. J Pharm Biomed Anal. 2015;
118. Hartsel JA, Eades J, Hickory B, Makriyannis A. Cannabis sativa and Hemp. In: Nutraceuticals. Elsevier; 2016. p. 735–54.
119. Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products: Manual for Use by National Drug Analysis Laboratories. New York: United Nations; 2009.
120. Mead A. The legal status of cannabis (marijuana) and cannabidiol (CBD) under U.S. law. Epilepsy and Behavior. 2017.
121. De Backer B, Maebe K, Verstraete AG, Charlier C. Evolution of the Content of THC and Other Major Cannabinoids in Drug-Type Cannabis Cuttings and Seedlings During Growth of Plants. J Forensic Sci. 2012;
122. Golub V, Reddy DS. Cannabidiol Therapy for Refractory Epilepsy and Seizure Disorders. Adv Exp Med Biol. 2021;1264:93–110.
123. Pattnaik F, Nanda S, Mohanty S, Dalai AK, Kumar V, Ponnusamy SK, et al. Cannabis: Chemistry, extraction and therapeutic applications. Chemosphere. 2022 Feb;289:133012.
124. Park S-H, Pauli CS, Gostin EL, Staples SK, Seifried D, Kinney C, et al. Effects of short-term environmental stresses on the onset of cannabinoid production in young immature flowers of industrial hemp (Cannabis sativa L.). J cannabis Res. 2022 Jan;4(1):1.
125. Page JE, Nagel J. Chapter eight Biosynthesis of terpenophenolic metabolites in hop and cannabis. In: Recent Advances in Phytochemistry. 2006.

126. Appendino G, Chianese G, Taglialatela-Scafati O. Cannabinoids: Occurrence and Medicinal Chemistry. Curr Med Chem. 2011;
127. Alves VL, Gonçalves JL, Aguiar J, Teixeira HM, Câmara JS. The synthetic cannabinoids phenomenon: from structure to toxicological properties. A review. Critical Reviews in Toxicology. 2020.
128. Ross SA, Mehmedic Z, Murphy TP, Elsohly MA. GC-MS analysis of the total delta9-THC content of both drug- and fiber-type cannabis seeds. J Anal Toxicol. 2000;24(8):715–7.
129. Rigdon, A., Sweeney, C., King, C., Cassidy, B., Kowalski, J., Dorman F. Method Validation for Cannabis Analytical Labs: Approaches to Addressing Unique Industry Challenges. In: Cannabis Science Conference. Portland, OR; 2017.
130. Levinsohn EA, Hill KP. Clinical uses of cannabis and cannabinoids in the United States. Journal of the Neurological Sciences. 2020.
131. Bifulco M, Pisanti S. Medicinal use of cannabis in Europe: The fact that more countries legalize the medicinal use of cannabis should not become an argument for unfettered and uncontrolled use. EMBO Rep. 2015;
132. Russo EB. Cannabinoids in the management of difficult to treat pain. Therapeutics and Clinical Risk Management. 2008.
133. Atalay S, Jarocka-karpowicz I, Skrzydlewskas E. Antioxidative and anti-inflammatory properties of cannabidiol. Antioxidants. 2020.
134. Kanabus J, Bryła M, Roszko M, Modrzewska M, Pierzgalski A. Cannabinoids—characteristics and potential for use in food production. Molecules. 2021.
135. Russo EB, Marcu J. Cannabis Pharmacology: The Usual Suspects and a Few Promising Leads. In: Advances in Pharmacology. 2017.
136. Aizpurua-Olaizola O, Omar J, Navarro P, Olivares M, Etxebarria N, Usobiaga A. Identification and quantification of cannabinoids in Cannabis sativa L. plants by high performance liquid chromatography-mass spectrometry. Anal Bioanal Chem. 2014;
137. Maurya N, Velmurugan BK. Therapeutic applications of cannabinoids. Chemico-Biological Interactions. 2018.
138. Chandra S, Radwan MM, Majumdar CG, Church JC, Freeman TP, ElSohly MA. New trends in cannabis potency in USA and Europe during the last decade (2008–2017). Eur

Arch Psychiatry Clin Neurosci. 2019;

139. Berman P, Futoran K, Lewitus GM, Mukha D, Benami M, Shlomi T, et al. A new ESI-LC/MS approach for comprehensive metabolic profiling of phytocannabinoids in Cannabis. Sci Rep. 2018;
140. McLaren J, Swift W, Dillon P, Allsop S. Cannabis potency and contamination: A review of the literature. Addiction. 2008.
141. Mudge E, Paley L, Schieber A, Brown PN. Optimization and single-laboratory validation of a method for the determination of flavonolignans in milk thistle seeds by high-performance liquid chromatography with ultraviolet detection. Anal Bioanal Chem. 2015;
142. Mudge EM, Jones AMP, Brown PN. Quantification of pyrrolizidine alkaloids in North American plants and honey by LC-MS: single laboratory validation. Food Addit Contam - Part A Chem Anal Control Expo Risk Assess. 2015;
143. Mudge E, Chan M, Venkataraman S, Brown PN. Curcuminoids in Turmeric Roots and Supplements: Method Optimization and Validation. Food Anal Methods. 2016;
144. Mudge EM, Brown PN. Determination of mitragynine in Mitragyna speciosa raw materials and finished products by liquid chromatography with UV detection: Single-laboratory validation. J AOAC Int. 2017;
145. Hazekamp A, Fischedick JT. Cannabis - from cultivar to chemovar. Drug Test Anal. 2012;
146. Mudge EM, Liu Y, Lund JA, Brown PN. Single-Laboratory Validation for the Determination of Flavonoids in Hawthorn Leaves and Finished Products by LC-UV. Planta Med. 2016 Nov;82(17):1487–92.
147. AOAC International. Appendix K: Guidelines for Dietary Supplements and Botanicals - Part I AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. Off Method Anal AOAC Int. 2013;
148. Brown PN, Lister P. Current initiatives for the validation of analytical methods for botanicals. Current Opinion in Biotechnology. 2014.
149. Nie B, Henion J, Ryona I. The Role of Mass Spectrometry in the Cannabis Industry. J Am Soc Mass Spectrom. 2019;
150. Brown PN, Chan M, Betz JM. Optimization and single-laboratory validation study of a high-performance liquid chromatography (HPLC) method for the determination of

phenolic Echinacea constituents. Anal Bioanal Chem. 2010;
151. Mudge EM, Betz JM, Brown PN. The importance of method selection in determining product integrity for nutrition research. Adv Nutr. 2016;
152. Araneda JF, Chu T, Leclerc MC, Riegel SD, Spingarn N. Quantitative analysis of cannabinoids using benchtop NMR instruments. Anal Methods. 2020;
153. Hazekamp A, Choi YH, Verpoorte R. Quantitative analysis of cannabinoids from Cannabis sativa using 1H-NMR. Chem Pharm Bull. 2004;
154. Politi M, Peschel W, Wilson N, Zloh M, Prieto JM, Heinrich M. Direct NMR analysis of cannabis water extracts and tinctures and semi-quantitative data on Δ9-THC and Δ9-THC-acid. Phytochemistry. 2008;
155. Jacobs AD, Steiner RR. Detection of the Duquenois-Levine chromophore in a marijuana sample. Forensic Sci Int. 2014;
156. Zaglul J. An Analysis of Froehde's and Duquenois-Levine Colorimetric Tests, in Biological Sciences. Wilkes Honors College of Florida Atlantic University. p. 23; 2013.
157. Butler WP. Duquenois-Levine Test for Marihuana. J AOAC Int. 1962;
158. Duquenois, P.; Moustaha HN. Identification and Assay of Cannabis Indicia. J Egypt Med Assoc, 1938, 21, 224.
159. F. Kelly J. The Non-Specificity of the Duquenois-Levine Field Test for Marijuana. Open Forensic Sci J. 2012;
160. Sharma P, Srinivas Bharath MM, Murthy P. Qualitative high performance thin layer chromatography (HPTLC) analysis of cannabinoids in urine samples of Cannabis abusers. Indian J Med Res. 2010;
161. Pourseyed Lazarjani M, Torres S, Hooker T, Fowlie C, Young O, Seyfoddin A. Methods for quantification of cannabinoids: a narrative review. J Cannabis Res. 2020;
162. Fish F. Gas chromatography of cannabis constituents. Chromatographia. 1974.
163. Fetterman PS, Doorenbos NJ, Keith ES, Quimby MW. A simple gas liquid chromatography procedure for determination of cannabinoidic acids in Cannabis sativa L. Experientia. 1971;
164. Kanter SL, Musumeci MR, Hollister LE. Quantitative determination of Δ9-tetrahydrocannabinol and Δ9-tetrahydrocannabinolic acid in marihuana by high-pressure liquid chromatography. J Chromatogr A. 1979;

165. Veress T, Szanto JI, Leisztner L. Determination of cannabinoid acids by high-performance liquid chromatography of their neutral derivatives formed by thermal decarboxylation. I. Study of the decarboxylation process in open reactors. J Chromatogr A. 1990;
166. Raharjo TJ, Verpoorte R. Methods for the analysis of cannabinoids in biological materials: A review. Phytochemical Analysis. 2004.
167. Chan KW. Validating a gas chromatography-mass spectrometric method and sample classification procedure for cannabis profiling using cannabinoids from case samples. Aust J Forensic Sci. 2014;
168. Barni Comparini I, Centini F. Packed column chromatography, high-resolution gas-chromatography and high pressure liquid chromatography in comparison for the analysis of cannabis constituents. Forensic Sci Int. 1983;
169. Mehmedic Z, Chandra S, Slade D, Denham H, Foster S, Patel AS, et al. Potency trends of Δ9-THC and other cannabinoids in confiscated cannabis preparations from 1993 to 2008. J Forensic Sci. 2010;
170. ElSohly MA, Ross SA, Mehmedic Z, Arafat R, Yi B, Banahan BF 3rd. Potency trends of delta9-THC and other cannabinoids in confiscated marijuana from 1980-1997. J Forensic Sci. 2000 Jan;45(1):24–30.
171. Smith RN. High-pressure liquid chromatography of cannabis. Identification of separated constituents. J Chromatogr A. 1975;
172. Turner JC, Mahlberg PG. Simple high-performance liquid chromatographic method for separating acidic and neutral cannabinoids in cannabis sativa L. J Chromatogr A. 1982;
173. Lehmann T, Brenneisen R. High Performance Liquid Chromatographic Profiling of Cannabis Products. J Liq Chromatogr. 1995;
174. Wheals BB, Smith RN. Comparative cannabis analysis. A comparison of high-pressure liquid chromatography with other chromatographic techniques. J Chromatogr A. 1975;
175. Noestheden M, Friedlander G, Anspach J, Krepich S, Hyland KC, Zandberg WF. Chromatographic characterisation of 11 phytocannabinoids: Quantitative and fit-to-purpose performance as a function of extra-column variance. Phytochem Anal. 2018;
176. Elkins AC, Deseo MA, Rochfort S, Ezernieks V, Spangenberg G. Development of a validated method for the qualitative and quantitative analysis of cannabinoids in plant biomass and medicinal cannabis resin extracts obtained by super-critical fluid extraction. J

Chromatogr B Anal Technol Biomed Life Sci. 2019;
177. De Backer B, Debrus B, Lebrun P, Theunis L, Dubois N, Decock L, et al. Innovative development and validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in cannabis plant material. J Chromatogr B Anal Technol Biomed Life Sci. 2009;
178. Swift W, Wong A, Li KM, Arnold JC, McGregor IS. Analysis of Cannabis Seizures in NSW, Australia: Cannabis Potency and Cannabinoid Profile. PLoS One. 2013;
179. Gul W, Gul SW, Radwan MM, Wanas AS, Mehmedic Z, Khan II, et al. Determination of 11 cannabinoids in biomass and extracts of different varieties of cannabis using high-performance liquid chromatography. J AOAC Int. 2015;
180. Patel B, Wene D, Fan Z (Tina). Qualitative and quantitative measurement of cannabinoids in cannabis using modified HPLC/DAD method. J Pharm Biomed Anal. 2017;
181. Kyriakou C, Marchei E, Scaravelli G, García-Algar O, Supervía A, Graziano S. Identification and quantification of psychoactive drugs in whole blood using dried blood spot (DBS) by ultra-performance liquid chromatography tandem mass spectrometry. J Pharm Biomed Anal. 2016;
182. Upton R, Craker L, ElSohly M, Romm A, Russo E SM. Cannabis Inflorescence: Cannabis spp. Standards of identity, analysis and quality control. In: American Herbal Pharmacopeia. Scotts Valley, CA; 2014.
183. Kafle A. Development and application of Differential Mobility-Mass Spectrometry as a rapid analytical platform. Northeastern University; 2014.
184. Cappiello A, Famiglini G, Palma P, Trufelli H. Matrix effects in liquid chromatography-mass spectrometry. Journal of Liquid Chromatography and Related Technologies. 2010.
185. Snyder LR, Kirkland JJ, Dolan JW. Introduction to Modern Liquid Chromatography. Introduction to Modern Liquid Chromatography. 2010.
186. Hamilton RJ, Sewell PA. Introduction to high performance liquid chromatography. In: Introduction to high performance liquid chromatography. 1982.
187. Citti C, Braghiroli D, Vandelli MA, Cannazza G. Pharmaceutical and biomedical analysis of cannabinoids: A critical review. Journal of Pharmaceutical and Biomedical Analysis. 2018.
188. Kazakevich Y, LoBrutto R. HPLC for Pharmaceutical Scientists. HPLC for

Pharmaceutical Scientists. 2006.
189. Köfeler HC, Fauland A, Rechberger GN, Trötzmüller M. Mass spectrometry based lipidomics: An overview of technological platforms. Metabolites. 2012.
190. Kostiainen R, Kotiaho T, Kuuranne T, Auriola S. Liquid chromatography/atmospheric pressure ionization - Mass spectrometry in drug metabolism studies. Journal of Mass Spectrometry. 2003.
191. Honour JW. Development and validation of a quantitative assay based on tandem mass spectrometry. Annals of Clinical Biochemistry. 2011.
192. Ho CS, Lam CWK, Chan MHM, Cheung RCK, Law LK, Lit LCW, et al. Electrospray ionisation mass spectrometry: principles and clinical applications. Clin Biochem Rev. 2003;
193. Glish GL, Vachet RW. The basics of mass spectrometry in the twenty-first century. Nature Reviews Drug Discovery. 2003.
194. Vaclavik L, Lacina O, Hajslova J, Zweigenbaum J. The use of high performance liquid chromatography-quadrupole time-of-flight mass spectrometry coupled to advanced data mining and chemometric tools for discrimination and classification of red wines according to their variety. Anal Chim Acta. 2011;
195. Haag AM. Mass analyzers and mass spectrometers. Mod Proteomics–Sample Prep Anal Pract Appl. 2016;157–69.
196. Arain M, Haque M, Johal L, Mathur P, Nel W, Rais A, et al. Maturation of the adolescent brain. Neuropsychiatric Disease and Treatment. 2013.
197. Wooding KM, Auchus RJ. Mass spectrometry theory and application to adrenal diseases. Molecular and Cellular Endocrinology. 2013.
198. Hazekamp A, Peltenburg A, Verpoorte R, Giroud C. Chromatographic and spectroscopic data of cannabinoids from Cannabis sativa L. J Liq Chromatogr Relat Technol. 2005;
199. Protti M, Brighenti V, Battaglia MR, Anceschi L, Pellati F, Mercolini L. Cannabinoids from Cannabis sativa L.: A New Tool Based on HPLC-DAD-MS/MS for a Rational Use in Medicinal Chemistry. ACS Med Chem Lett. 2019;
200. Kopec RE, Schweiggert RM, Riedl KM, Carle R, Schwartz SJ. Comparison of high-performance liquid chromatography/tandem mass spectrometry and high-performance liquid chromatography/photo-diode array detection for the quantitation of carotenoids,

retinyl esters, α-tocopherol and phylloquinone in chylomicron-rich fr. Rapid Commun Mass Spectrom. 2013;

201. Casetta C. Study of the composition of biologically active fractions from plant extracts by advanced techniques of HPLC and UHPLC/MS. Université de Genève.; 2013.
202. Kinyua J, Negreira N, Ibáñez M, Bijlsma L, Hernández F, Covaci A, et al. A data-independent acquisition workflow for qualitative screening of new psychoactive substances in biological samples. Anal Bioanal Chem. 2015;
203. Meng Q, Buchanan B, Zuccolo J, Poulin MM, Gabriele J, Baranowski DC. A reliable and validated LC-MS/MS method for the simultaneous quantification of 4 cannabinoids in 40 consumer products. PLoS One. 2018;
204. Sulyok M, Berthiller F, Krska R, Schuhmacher R. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Commun Mass Spectrom. 2006;
205. Polettini A. Applications of LC-MS in Toxicology. Pharmaceutical Press; 2006.
206. Hartmann C, Smeyers-Verbeke J, Massart DL, McDowall RD. Validation of bioanalytical chromatographic methods. J Pharm Biomed Anal. 1998;
207. European Medicines Agency. International Conference on Harmonisation - ICH Q2 (R1) Validation of analytical procedures: text and methodology. Encycl Toxicol Third Ed. 1995;
208. ICH. ICH Q2B - International Conference on Harmonization. Q2B Validation of Analytical Procedures: Methodology. Int Conf Harmon Tech Requir Regist Tripart Guidel. 1996;
209. Meesters RJ, Voswinkel S. Bioanalytical Method Development and Validation: from the USFDA 2001 to the USFDA 2018 Guidance for Industry. J Appl Bioanal. 2018;
210. U.S. FDA DHHS 21 CFR Parts 210 and 211. Current Good Manufacturing Practice of Certain Requirements for Finished Pharmaceuticals [Internet]. Proposed Rule. Available from: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=211
211. Wells M, Dantus M. Validation of chromatographic methods. Anal Instrum Handbook, Third Ed. 2004;(November):1015–33.
212. Breaux J, Jones K, Boulas P. Understanding and Implementing Efficient Analytical

Methods Development and Validation. Pharm Technol [Internet]. 2003;6–13. Available from: www.pharmatech.com

213. Peters FT. Stability of analytes in biosamples-an important issue in clinical and forensic toxicology? Analytical and Bioanalytical Chemistry. 2007.
214. Lindner W, Wainer IW. Requirements for initial assay validation and publication in J. Chromatography B. Journal of Chromatography B: Biomedical Applications. 1998.
215. Tome T, Žigart N, Časar Z, Obreza A. Development and Optimization of Liquid Chromatography Analytical Methods by Using AQbD Principles: Overview and Recent Advances. Organic Process Research and Development. 2019.
216. Sanap GS, Zarekar NS, Pawar SS. Review on method development and validation. Int J Pharm Drug Anal. 2017;5(5):177–84.
217. Shah VP, Midha KK, Findlay JWA, Hill HM, Hulse JD, McGilveray IJ, et al. Bioanalytical method validation - A revisit with a decade of progress. In: Pharmaceutical Research. 2000.
218. Lavanya G, Sunil M, Eswarudu MM, Eswaraiah MC HK and SB. Analytical Method Validation: An Updated Review. Int J Pharm Sci Res 2013; 4(4); 1280-1286.
219. Karnes HT, March C. Calibration and validation of linearity in chromatographic biopharmaceutical analysis. J Pharm Biomed Anal. 1991;
220. International Conference on Harmonisation (ICH). Validation of Analytical Methods: Definitions and Terminology Q2A. ICH Top Q2A London. 1995;
221. Ravisankar P, Naga Navya C, Pravallika D, Sri DN. A review on step-by-step analytical method validation. IOSR J Pharm. 2015;
222. Whitmire M, Ammerman J, Lisio P de. LC-MS/MS Bioanalysis Method Development, Validation, and Sample Analysis: Points to Consider When Conducting Nonclinical and Clinical Studies in Accordance with Current Regulatory Guidances. J Anal Bioanal Tech. 2011;
223. Analytical Procedures and Methods Validation for Drugs and Biologics Guidance for Industry. U.S. Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research (CDER). 2015;
224. Francotte A, Esson R, Abachin E, Vanhamme M, Dobly A, Carpick B, et al. Development and validation of a targeted LC-MS/MS quantitation method to monitor cell culture

expression of tetanus neurotoxin during vaccine production. Talanta. 2022;
225. González AG, Herrador MÁ. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. TrAC Trends Anal Chem. 2007;26(3):227–38.
226. Food and Drug Administration (FDA). Guidance for Industry Bioanalytical Method Validation. Vet Med. 2001;
227. Zuardi AW. History of cannabis as a medicine: a review. Rev Bras Psiquiatr. 2006 Jun;28(2):153–7.
228. Elliott J, DeJean D, Clifford T, Coyle D, Potter B, Skidmore B, et al. Cannabis for pediatric epilepsy: protocol for a living systematic review. Syst Rev. 2018 Jul;7(1):95.
229. Klumpers LE, Thacker DL. A brief background on cannabis: From plant to medical indications. J AOAC Int. 2019;
230. Lafaye G, Karila L, Blecha L, Benyamina A. Cannabis, cannabinoids, and health. Dialogues Clin Neurosci. 2017;
231. Jikomes N, Zoorob M. The Cannabinoid Content of Legal Cannabis in Washington State Varies Systematically Across Testing Facilities and Popular Consumer Products. Sci Rep. 2018;
232. Smith BC. Inter-lab variation in the cannabis industry, part I: problem and causes. Cannabis Sci Technol. 2019;12–7.
233. Gallo-Molina AC, Castro-Vargas HI, Garzón-Méndez WF, Martínez Ramírez JA, Rivera Monroy ZJ, King JW, et al. Extraction, isolation and purification of tetrahydrocannabinol from the Cannabis sativa L. plant using supercritical fluid extraction and solid phase extraction. J Supercrit Fluids. 2019;
234. Dei Cas M, Casagni E, Saccardo A, Arnoldi S, Young C, Scotti S, et al. The Italian panorama of cannabis light preparation: Determination of cannabinoids by LC-UV. Forensic Sci Int. 2020;
235. Burnier C, Esseiva P, Roussel C. Quantification of THC in Cannabis plants by fast-HPLC-DAD: A promising method for routine analyses. Talanta. 2019;
236. Mandrioli M, Tura M, Scotti S, Toschi TG. Fast Detection of 10 Cannabinoids by RP-HPLC-UV Method in Cannabis sativa L. Molecules. 2019;
237. Nahar L, Onder A, Sarker SD. A review on the recent advances in HPLC, UHPLC and

UPLC analyses of naturally occurring cannabinoids (2010–2019). Phytochemical Analysis. 2020.

238. Nikolin B, Imamović B, Medanhodzić-Vuk S, Sober M. High perfomance liquid chromatography in pharmaceutical analyses. Bosn J basic Med Sci. 2004 May;4(2):5–9.
239. Rafferty JL, Siepmann JI, Schure MR. Mobile phase effects in reversed-phase liquid chromatography: a comparison of acetonitrile/water and methanol/water solvents as studied by molecular simulation. J Chromatogr A. 2011 Apr;1218(16):2203–13.
240. Wilson RE, Groskreutz SR, Weber SG. Improving the Sensitivity, Resolution, and Peak Capacity of Gradient Elution in Capillary Liquid Chromatography with Large-Volume Injections by Using Temperature-Assisted On-Column Solute Focusing. Anal Chem. 2016 May;88(10):5112–21.
241. Locatelli M, Melucci D, Carlucci G, Locatelli C. Recent HPLC strategies to improve sensitivity and selectivity for the analysis of complex matrices. Instrumentation Science and Technology. 2012.
242. Vékey K. Mass spectrometry and mass-selective detection in chromatography. J Chromatogr A [Internet]. 2001;921(2):227–36. Available from: https://www.sciencedirect.com/science/article/pii/S0021967301008251
243. Vella J, Mifsud M, Bartolo NS, Ferrito V, Serracino-Inglott A, Azzopardi LM, et al. The combined effects of ph and acetonitrile composition on the separation of two lincosamide antibiotics. Asian J Pharm Clin Res. 2014;
244. Deidda R, Avohou HT, Baronti R, Davolio PL, Pasquini B, Del Bubba M, et al. Analytical quality by design: Development and control strategy for a LC method to evaluate the cannabinoids content in cannabis olive oil extracts. J Pharm Biomed Anal. 2019 Mar;166:326–35.
245. Yang Y. High-Temperature Liquid Chromatography. LCGC Suppl [Internet]. 2008;0(0). Available from: https://www.chromatographyonline.com/view/high-temperature-liquid-chromatography
246. Hendriks G. Theoretical models in LC based bioanalytical method development. Journal of Pharmaceutical and Biomedical Analysis. 2009.
247. Lopez Marques RM, Schoenmakers PJ. Modelling retention in reversed-phase liquid chromatography as a function of pH and solvent composition. J Chromatogr A. 1992;

248. Galaon T, David V. The influence of mobile phase ph on the retention and selectivity of related basic compounds in reversed-phase liquid chromatography. Rev Roum Chim. 2012;
249. Buhagiar LM, Scorpiniti M, Bartolo NS, Szijj JV, Ferrito V, Inglott AS, et al. Implications of mobile phase composition and pH on the chromatographic separation of amitriptyline and its metabolite nortriptyline.. Int J Pharm Pharm Sci. 2018;
250. Ahuja S. 1 Overview of HPLC method development for pharmaceuticals. In: Separation Science and Technology. Elsevier; 2007. p. 1–11.
251. McCalley D V. Effect of buffer on peak shape of peptides in reversed-phase high performance liquid chromatography. J Chromatogr A. 2004;
252. Johnson D, Boyes B, Orlando R. The use of ammonium formate as a mobile-phase modifier for LC-MS/MS analysis of tryptic digests. J Biomol Tech. 2013;