University of Kent

INVESTIGATION AND ANALYSIS OF NATURALLY OCCURRING CANNABINOIDS

MSc Research Thesis

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Abstract:

There has been a recent proliferation of edible consumer products containing Cannabidiol (CBD), which is a closely related molecule to Tetrahydrocannabinol (THC), both of which are found in cannabis plants. It is thought that any products containing CBD are likely to have a calming effect on the consumer without the psychoactive effect's THC tends to induce. Due to this growing market it is becoming increasingly important to establish an analytical method which can quantify these substances and differentiate between them, as THC is a regulated compound.

Both CBD and THC can exist in acid form, CBDA and THCA respectively, which make such analyses challenging. A further complication is that certain analytical techniques such as GC, with high temperature at the inlet, can cause decarboxylation of the CBDA/THCA to CBD/THC.

In this project, use of NMR to initially identify and then assess purity of compound standards, was then followed up with method development of liquid chromatography – mass spectrometry (LC-MS) and gas chromatography - mass spectrometry (GC-MS) instruments to further identify and then quantify any cannabinoids present. A method was then developed for detecting and quantifying the presence of cannabinoids within foodstuffs labelled as containing CBD using the developed methods.

Qualitative results of investigations were consistent between GC-MS and LC-MC instruments suggesting these are reliable, but quantification optimisation on the methods is needed in future. Due to the lower temperatures used in the LC-MS, reducing decarboxylation reactions within the instrument, this allowed for the a more successful method to detect and identify cannabinoids regardless of acid form present to be achieved.

Key words:

Cannabinoid, Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography-Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC), ionisation, Nuclear magnetic resonance (NMR), Decarboxylation, McLafferty rearrangement Terpene, Phytocannabinoids, Endocannabinoid, Psychoactive, Entourage effect, Analgesic, Hemp, Strain. Cannabidiol (CBD), Cannabidiolic Acid (CBDA), Tetrahydrocannabinolic acid (THCA), Tetrahydrocannabivarin (THCV).

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction & Literature Review

Brief History

Historically, the cannabis plant has been grown and cultivated for several reasons including use in religious ceremonies/rituals, for fibre, for medicinal properties and recreational uses[1,2].

Interest for such a range of plant attributes means that properties of the plant have changed over time to meet the needs of the consumer. Focus varies from cannabinoid content to strong hemp fibres for construction, cannabis can be cultivated indoors or outdoors and depending on the strain of plant, which then requires different hours or light and quantities of water[3]. Over the years the increased recreational use, achieved from the psychoactive properties of THC within the plant, has meant the status plant has become recognised as a drug in society and led to laws preventing the growth of the plant, different countries each with their own regulations. These are now slowly changing as positive properties of the plant are once again being bought back to the attention of the media and public[4].

'Hemp' has become more popular as it is legally defined as a cannabis plant with less than 0.3% THC content[5]. Hemp fibres have been the focus for farmers and many other major parts of history, for many years with claims that in 1455 the first Gutenberg bible was printed on hemp paper[4,6]. This history of the plant shows initially it was ideal to be farmed for CBD content, or the plants fibres, as these have many uses including construction of ropes, clothing, insulation, textiles[4] (for example the boat used by Christopher Columbus to sail the seven seas used hemp as sails as well as make the boat watertight[2])and even use as a biofuel[7].[4,6]

The cannabis plant with a content of THC above 0.3% is known as a marijuana cannabis plant and this becomes a regulated plant in the UK. These are grown with more of the psychoactive cannabinoids present with varying ratios of THC to CBD present depending on the strain of plant[8]. The effect of THC on the consumer is a feeling of relaxing euphoria known as a high. This effect alters different processes such as feelings of pain, stress and anxiety and can increase appetite[9,10]. This results in a feeling of euphoria and can alter perception of time and reactions to stimuli, which is why driving while under the influence of cannabis is illegal. Increased use of cannabis has found that there are negative impacts including reduced cognition and reduced memory retention[9,11].

The effect of CBD is thought to be like that of THC in that anxiety is reported to be reduced, however there are no psychoactive properties or delay in reaction times so this is legal to consume[10]. CBD is also considered a sleep aid as the anti-anxiety effect helps to relax the consumer[12,13].

Total known cannabinoids with the exact number varies in literature from 110 to 120[14,15] however, the number of psychoactive cannabinoids has not yet been established. There has been some investigation into CBN and THCV to suggest these possess psychoactive properties, but these have not been sufficiently researched or studied to understand exactly what these psychoactive effects are[16]. This is interesting as although these are present in smaller quantities within the plant and are difficult to extract fully, there are currently no UK regulations on these specific cannabinoids[17–19].

Legal aspects of Cannabis

The laws in the USA for example, vary from state to state with some places completely legalising the growth and recreational use of the plant, and other states still completely outlawing even possession of the plant material[20,21]. UK law classes possession of cannabis or the THC cannabinoid in products past 0.3% weight for weight as illegal[5,22].

Due to the fact each plant contains varying ratios of each with THC and CBD, these are the most prominent and focussed on cannabinoids and are therefore far more publicised and debated in governments[20,23]. This has recently led to some UK regulations specifically regarding CBD and its change from an unregulated substance requiring no applications or licence from the producer to a 'Novel Food' status[24]. This novel food status is specific only to CBD as there is no known history of specifically human consumption of CBD extract before May 1997, whereas Hemp, a source of CBD, does have history of consumption before this[4,25].

Having a 'novel food' status now means that any company wishing to enter a new CBD food product to the market will need to submit an application to the Food Standards Agency (FSA), this change was officially approved in February 2019 however due the deadline for products on the market has officially been set as 31 March 2021[24]. Applications are required to provide information regarding details of the production process including the source of the CBD as well as absorption, distribution, metabolism, and excretion (ADME) research of the compound and a full specification of the product. Without this application approval from the FSA no new CBD products may be added to the market until approval. As of 24 May 2021 the FSA has stated "There are currently no authorised CBD extracts or isolates on the market."[26] There is a slight loophole to this

however, if a product was on the market in 13th Feb 2020, when the deadline was first announced, the product may continue sales as long as labels are correct with no product safety issues and no controlled substances are also present.

There are many reasons for the introduction and specific wording of the last requirement for sale of CBD products, the phrase 'broad spectrum' or 'full spectrum' is often placed before CBD in packaging, this simply means that CBD is not the only cannabinoids present and the presence of other naturally occurring cannabinoids such as CBG or CBC as the manufacturing process cannot or does not separate CBD as an isolate (pure). The main difference between full and broad spectrum is that full is more likely to contain up to 0.3% THC whereas broad will only usually contain a trace amount[5]. This is due to the type of hemp plant the CBD is extracted from, however the cumulative quantity of these cannabinoids is not considered additional to the CBD so limits on the 'spectrum' are not set in current legislation[27,28].

Cannabinoid structures and biosynthesis

The building block and initial structure of CBD and THC is known to be Cannabigerolic Acid (CBGA)[18,29]. CBGA has an molecular weight of 360 Da and is a non-psychoactive compound made up of Olivtolic Acid (OC) and Geranyl Diphosphate (GPP) within the plant and is thought to be the fundamental precursor to the cannabinoids we are interested in. The formation of CBGA is shown below in Figure 1, with the alkylation of Olivtolic acid with GPP, to form CBGA[30-32]. An alternative to Olivetolic acid can be Divarinic acid ($C_{10}H_{12}O_4$) which simply has a propyl group rather than the pentyl, attached to the benzene ring. The cannabinoid biosynthesis can alter between Divarinic acid and Olivtolic Acid with the difference being the production of Cannabigerovaric acid (CBGVA). Cannabinoids derived from either CBGA or CBGVA are broadly categorised as Phytocannabinoids, a naturally occurring group of cannabinoids in cannabis[30,33].

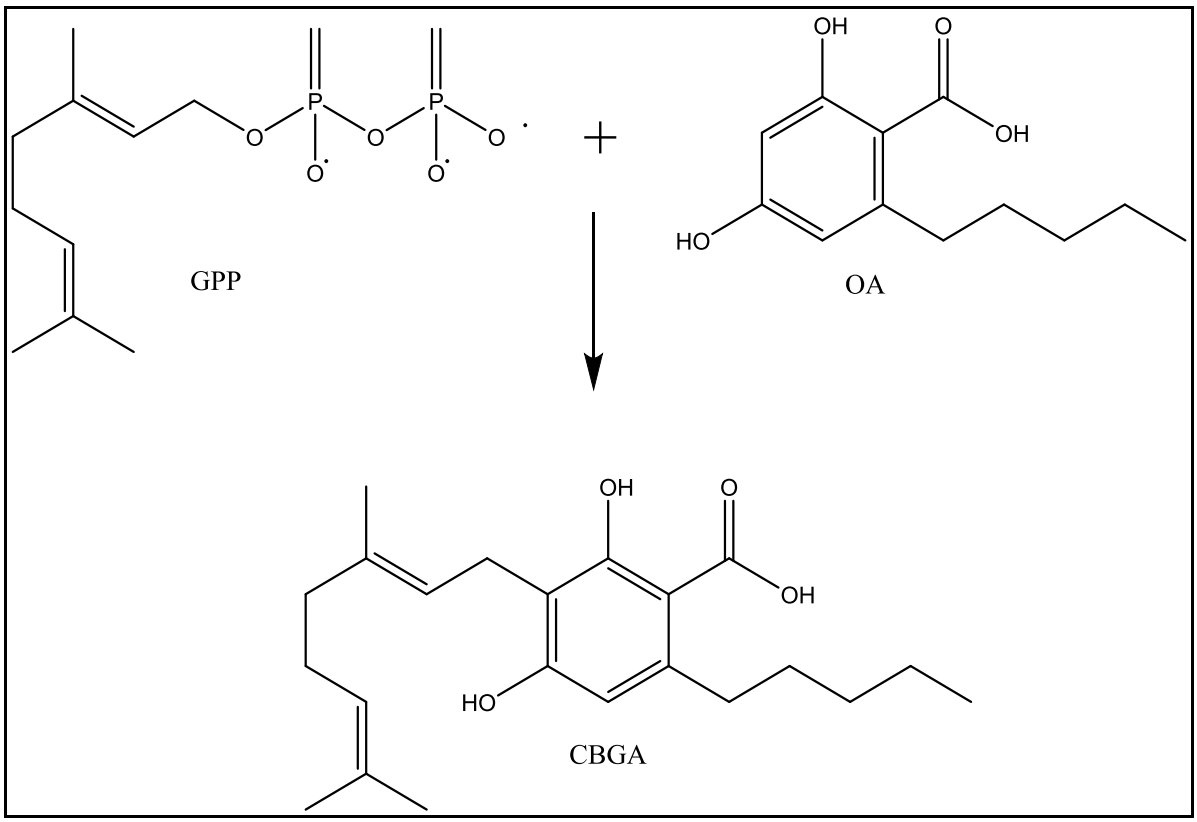


FIGURE 1: CBGA BIOSYNTHESIS

CBGA does not possess psychoactive effects to result in a 'high', so it is not a controlled substance in the UK although is known to interact with the Endocannabinoids System (ECS), explained below, in positive ways[34]. One major factor limiting research into these effects is the low quantity of the compound within the plant requiring complex extraction methods and an understating of the plants development and growth to obtain the highest quantity of the cannabinoid[35,36].

Conversion of CBGA to THC, THCA, CBD and CBDA are all similar in mechanism with the only difference being the initial enzyme that, processes the CBGA. It is also worth noting the THC and THCA, shown is the Δ 9 isomer, can occur as structural isomers where the double bond on the hexane ring can be at different positions. Of these variants the Δ 9 is the most stable and other isomer is present in far lower levels[33].

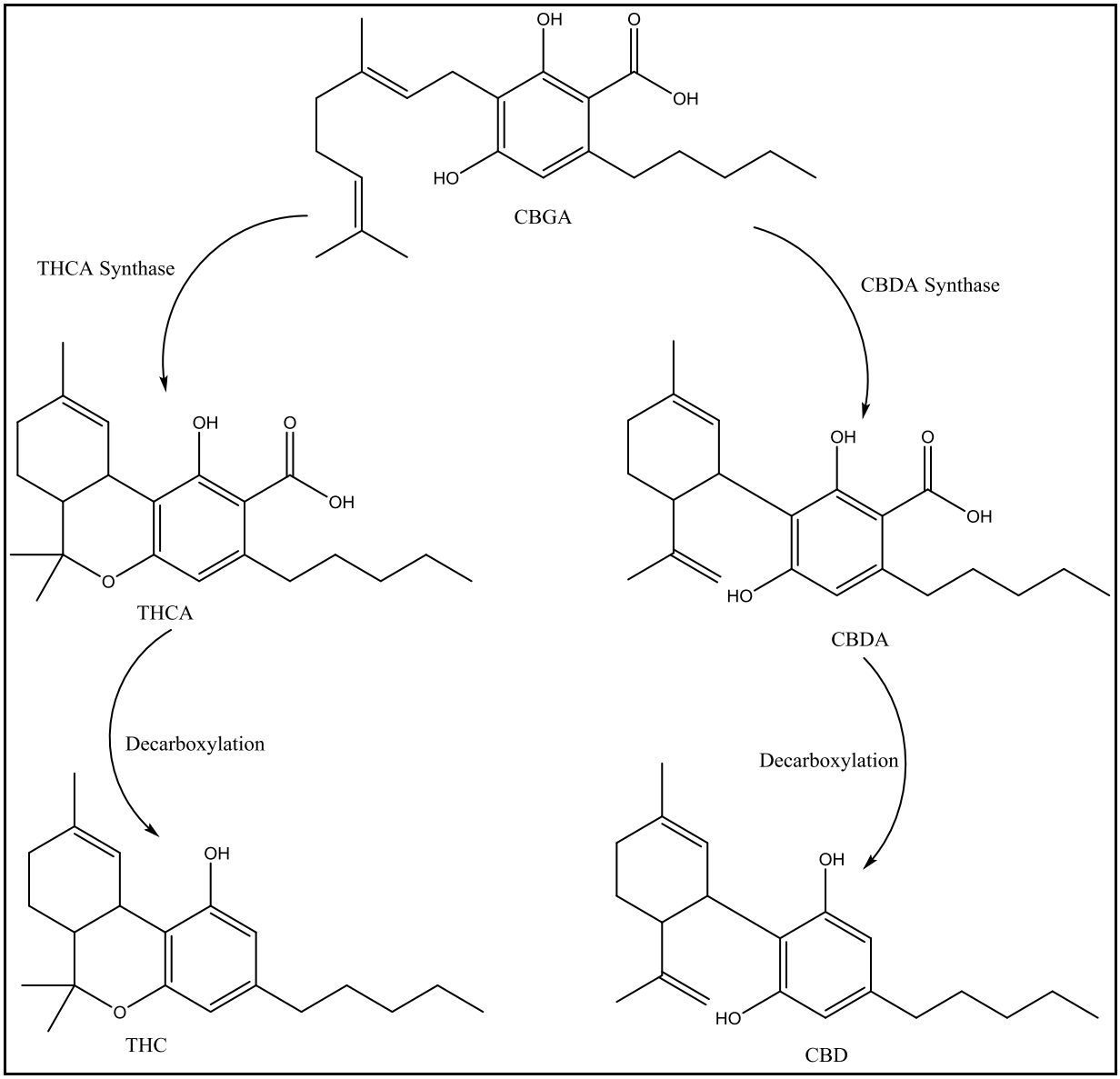


FIGURE 2: BIOSYNTHESIS AND DECARBOXYLATION OF THC AND CBD

There are many different enzymes responsible for the conversion of CBGA into other cannabinoids, two examples are CBDA synthase and THC synthase, see figure 2, which convert CBGA to CBD and THC respectively. Conversion to a neutral cannabinoid first requires the acid precursor as the carboxyl group is still present from GBCA and remains within the compound until decarboxylation occurs. The acid counterparts do not possess the same properties as their neutral partner as the carboxyl group needs to be removed for the cannabinoid to efficiently interact with the Endocannabinoid System, which can happen in several was but is usually done through smoking or introducing heat in some way[14].

The decarboxylation reaction from THCA to THC is shown below in figure 3, however this reaction is also thought to happen naturally over a long-time span if the material is left

exposed to oxygen over time and not appropriately stored[37]. The decarboxylated or neutral cannabinoid counterparts for CBGA and CBDA and the most abundant cannabinoids within the plant can be found in the Appendix A. This illustrates the vast number of cannabinoids we are aware of and have been identified within the plant[14,31].

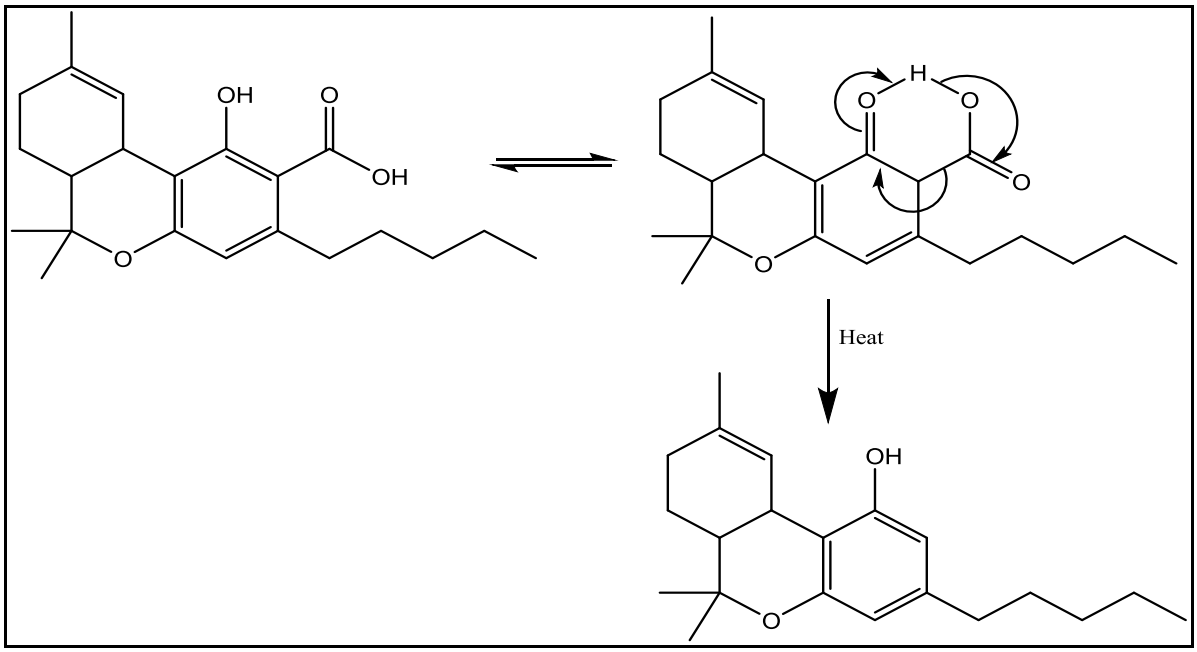


FIGURE 3: DECARBOXYLATION OF THCA

As mentioned above, there is a variation of Δ9-tetrahydrocannabinolic acid known as Δ8-tetrahydrocannabinolic acid. This Δ8-THCA, figure 3, has the difference of simply one double bond (8) a carbon to the left, best illustrated in comparison in figure 4 below, in their neutral counterparts, is becoming more popular with consumers as the compound shares many of the same properties of the Δ 9 but studies show may also provide less of a euphoric 'high' feeling in comparison. This is still a highly regulated compound as there is still a psychoactive effect but provides an alternative cannabinoid on the market and widens understanding of the way in which cannabinoids work and encourages funding to study the effects of these, perhaps one day in vivo. Due to the uncontrolled production of this Δ8-THCA however, the quantity and extraction is an issue here as pure extracts are difficult to obtain due to the very similar properties of the compounds and difficulty in distinguishing a difference between the two with strong separation and resolution in instruments[38,39].

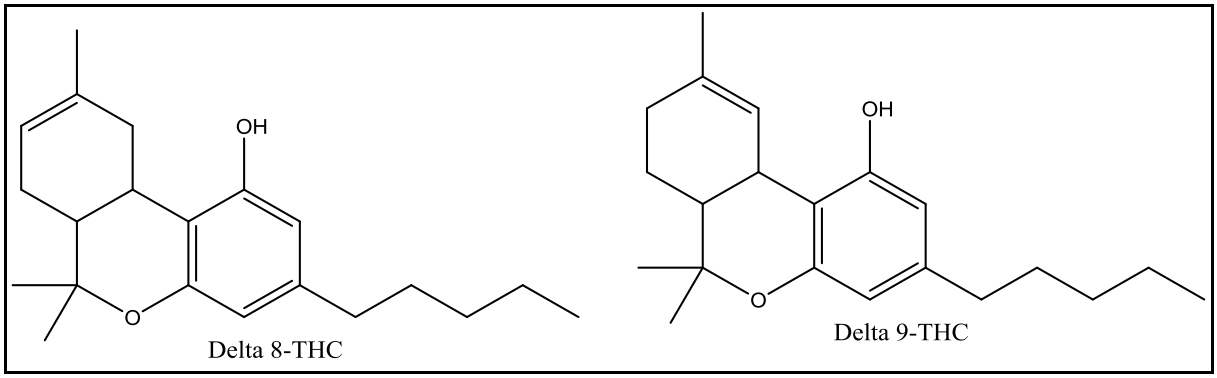


FIGURE 4: Δ 8 THC AND Δ 9 THC

This Δ8-THCA: Δ9-THCA ratio has also created a loophole for companies in the US looking to sell their product as listing only Δ 8 as present means it is not listed as a controlled substance in some states and without being tested to prove this claim, may be sold under the guise of THCA d8 which in fact growing a plant with one type of isomer present would not be possible to control on a commercial scale[33].

Synthetic production of THC is of course a very popular topic with methods widely available online to attempt this, for example by dissolving CBD in sulphuric acid/acetic acid and leaving it from 3 hours to 3-day time points. Theoretically, this will allow production of Δ-9-THC and Δ-8-THC mix as a powder. This method also states that after 3 hours, any CBD present will be converted into 52% Δ-9-THC and 2% Δ-8-THC[40].

The Endocannabinoid System (ECS)

The key to the psychoactive and therapeutic effects of the cannabinoids is knowing that while in their native form themselves they are present in acid form and do not interact with the ECS, and although THCA has been recognised as having medicinal properties such as an immune-modulating effect[41] , this is not through use of the endocannabinoid system. Instead, decarboxylation is required in order to have any effect on the consumer[16,34,42].

Generally, the Endocannabinoid system is a neuromodulator system within the central nervous system[15]. The ECS specifically regulates functions such as sleep, energy metabolism, regulates dopamine levels, cognition, and inflammation[43,44].

CB1 and CB2 are the two main receptors within the system responsible for a range of bodily functions from appetite and digestion to mood and sleep[43]. These receptors are present in different areas all around the body and can produce different cell responses depending on where they are, as necessary. For example, in the skeletal muscles CB1 can regulate energy metabolism and insulin resistance and in bones CB2 receptors help control bone formation and turnover[45,46].

This is an established link as these receptors are linked to depression chronic pain and do not require cannabinoids as we have two known endogenous cannabinoids (aka endocannabinoids) present anandamide (AEA) and 2-arachidonoylglyerol (2-AG). Structures of the endocannabinoids can be found in Appendix A highlighting the difference in structure[47,48]. These interact with the receptors to produce an effect or result in the body, depending on where the receptor is and what protein is interacting with the receptor. Once this interaction is carried out, there are two enzymes that can break down the endocannabinoid: for AEA there is fatty acid amide hydrolase (FAAH) and for 2-AG, monoacylglycerol acid lipase [44-46]

The introduction of Exo-cannabinoids or Phytocannabinoids to the ECS interaction with your body's ECS receptors and results in a higher or lower production of a reaction, depending on what receptors and cannabinoids are present.

Understanding the mode of action of cannabinoids is relatively new in relation to how long humans have been using and cultivating cannabis for a high THC content. Looking at how the cannabinoids interact with our ECS mean we can identify other potential cannabinoids in the plant that might have psychoactive effects. One other known psychoactive cannabinoid is Cannabinol (CBN), shown in figure 5 below, which has a very similar structure to THC but is a degradation product. The process of this degradation can happen over a long period of time, or if exposed to high levels of oxygen or heat[18,35,40].

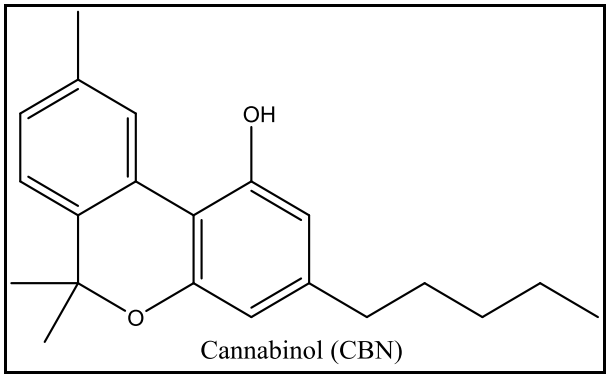


FIGURE 5: CANNABINOL (CBN)

Cannabinol is an oxidation product of THC and has a range of its own effects on the ECS. Studies on the combination of different THC and CBD ratios over time, as well as how these combinations effect the body have shown that this is a desirable cannabinoid in the plant and understanding more on how this can be isolated for use in medical or recreational purposes is currently being investigated and may be part of the future in cannabinoid drugs[17].

Both CB1 and CB2 are G protein-coupled receptors (GPCR), also members of the seven-transmembrane (7-TM) receptor or heptahelical receptors[49]. GPCR's are simply a large collective of cell surface receptors that are responsible for responding to external signals. This is usually carried out by the GPCR binding to the external signal, in this case an endocannabinoid or phytocannabinoids. This binding will activate a G Protein and trigger a cell response. The G proteins consist of three subunits: alpha, beta, and gamma, of which the gamma and alpha subunits are attached to the plasma membrane by lipid anchors. While inactive the alpha unit will bind to guanosine diphosphate (GDP) which attaches to the alpha subunit and is attached to the GPCR as a complex[33,44,50,51]. Once a signalling molecule interacts with the GPCR the GDP is replaced by guanosine triphosphate (GTP) and the G protein can then separate into its alpha and beta unit. This separation allows interaction with the cell membrane proteins as they are no longer bound to the GPCR. This activation will remain all while the GTP is joined to the alpha unit, until the GTP be hydrolysed back to GDP, when this happens the unit reconnects to the GPCR and becomes inactive again[43,52,53]. In this way the receptor acts as an on/off switch mechanism. This mechanism is illustrated in the figure 6.

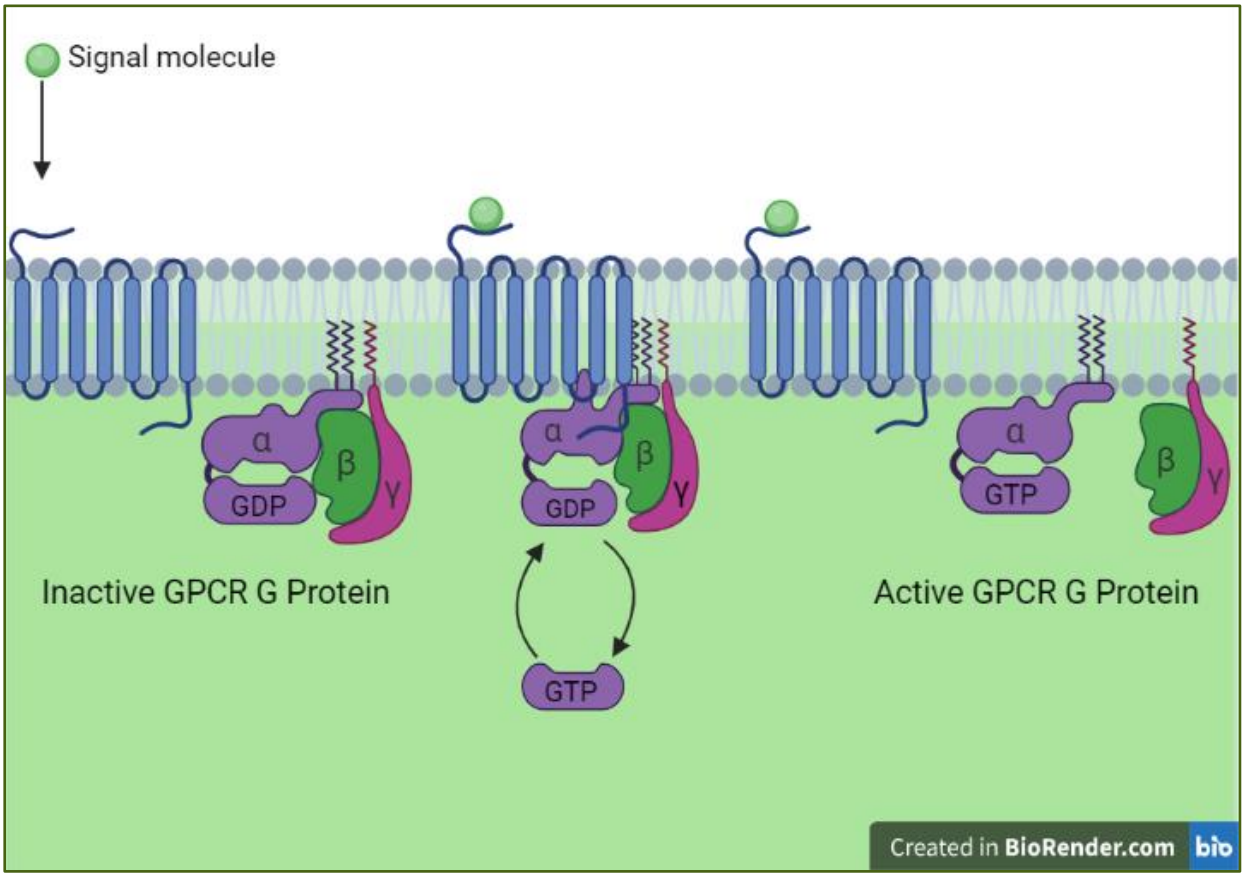


FIGURE 6: GPCR DIAGRAM

In relation to cannabinoids, the signal molecule (shown as a green sphere in the illustration above) is a cannabinoid such as THC or CBD, and the receptor (the blue coloured cylinder shapes) would most likely be either CB1 or CB2[53].

When considering this on/off switch mechanism, the Signal molecule is key to achieving the active G –protein, but there are two main types of Signal molecule this can be: an antagonist or an agonist, and the difference between the two is fundamental to any possible cell reaction to take place. An agonist will usually be a drug molecule that can occupy and activate the receptor, whereas the antagonist will occupy the receptor but does not activate any response. Instead, antagonists usually prevent and block any possible agonists from occupying the receptor themselves. The psychoactive and therapeutic effects form from the specific activation of the G protein in the CB1 and CB2 receptors, so for this to take place the molecules at the receptors need to be agonists[44,49].

A way in which we can compare how CBD and THC affect us is by comparing their K_i values. A K_i value is a measure of how of how well the drug can bind to receptors. A lower K_i value represents the concentration of the drug required to bind to that receptor, a lower concentration means the agonist or antagonist is strong as fewer signalling molecules/ drug molecules are required to achieve the inhibition of the receptor, if a higher concentration is required this is considered a poor agonist or antagonist[54].

At CB1, THC has a K_i value of 5.05- 80.3 nM and at CB2 has a K_i value of 3.13- 75.3 nM and is an agonist at both sites. These are low values and means THC is a strong agonist as a low concentration is required to achieve the GPCR activation[35,50].

CBD on the other hand is an antagonist and at CB1 has a K_i value of 4,350 - >10,000 nM and at CB2 has a K_i value of 2,399 ->10,000 nM[35,47,54]. This high value means that CBD is a poor antagonist, and a very high concentration is required in order for the receptor to be inhibited and the agonist prevented from accessing the receptor site.

CBN and Δ8-THC are both agonists at CB1 and CB2 receptors so these will encourage the entourage effect by easily binding to receptors as they both have low K_i values for both CB1 and CBD[47].

CBD has a lower K_i value at the CB2 receptor and CB1 so there is constant research into confirming these numbers and investigating why and how exactly CBD can have any positive effects at receptor sites without being an agonist[47].

Research has shown that instead CBD works by slowing down or preventing the FAAH enzyme from breaking down the anandamide endocannabinoid. This means that CBD

blocks any AEA from being broken down allowing the endocannabinoid to remain at a higher concentration and interact with the CB1 receptor for longer, which in turn is responsible for producing the anti-anxiety or analgesic properties CBD is known to have[50-52]. THC, as an agonist, has a stronger affinity to CB1 receptors which work in the same way as AEA, but instead produce a psychoactive response as an additional cell response.

Phytocannabinoids Today

There are many positive effects cannabis has been proven to possess, helping with side effects of glaucoma, weight gain, anxiety, liver function improvement, epilepsy, multiple sclerosis, and anti-nausea through chemo, as these can all be regulated through CB1 and CB2 receptors[36,52].

Lack of government regulation due to a absence of interest until the last few years, and no proven medical health benefits to taking CBD mean it cannot be advertised as a treatment for medical or health issues. CBD oil and CBD supplements are still presented in the media as having certain properties and abilities, majority of these have either been very recently researched and are not considered fact, or are not founded on fact but instead consumer reviews. Unfortunately, as this miscommunication and misrepresentation of CBD has made it very popular in the UK, resulting in a vast variety of products having CBD added wherever possible[5,40]. A study found that in 2016 a study by the FDA[55] found only 2 in 24 products contain CBD as advertised and in February 2021 a study by Leafreport in America found that over 77% of CBD products on sale did not contain the specified amount of CBD as advertised[56].

There are many variations of phytocannabinoids as mentioned above, cannabielsoin (CBE) for example, is a non-psychoactive and non-regulated as the impact on the body, both positive and negative, is not well researched as its own cannabinoid. Other than suggesting due to the structural similarities between other phytocannabinoids, it is likely CBE has a part in the entourage effect[57,58] and may be more likely to encourage calming and anti-anxiety effects than to promote stress or nausea is one conclusion.

The demand for CBD has meant that production of CBD had been unregulated and could be carried out in such a number of ways with many different outcomes, such as no CBD present at all which is perhaps a safer scenario, or could even result in containing a full spectrum of CBD depending on the extraction method, or, as a worst case, could contain THC and induce some psychoactive response without the consumer realising if present at low quantities[36].

Terpenes and The Entourage Effect

Examination of plant material does not only involve CBD and THC analysis, but there are also a range of terpenes found in the plants which give the plant a characteristic smell unique to the strain. For example, citrusy smells come from Limonene over woody/ earthy scents which Myrcene is responsible for[59]. Myrcene is one of the most common and therefore well-known cannabinoids presents, shown below in figure 7, responsible for producing a relaxing and anti-anxiety effect on the body[60,61]. Research into the different aspects including creating terpene profiles per strain, extraction of each terpene to analyse individually and possibly quantify, understanding how terpene content relates to THC content or ratios of Phytocannabinoids content, and investigating the effect of each terpene on the body in combination with other plant molecules[19,62].

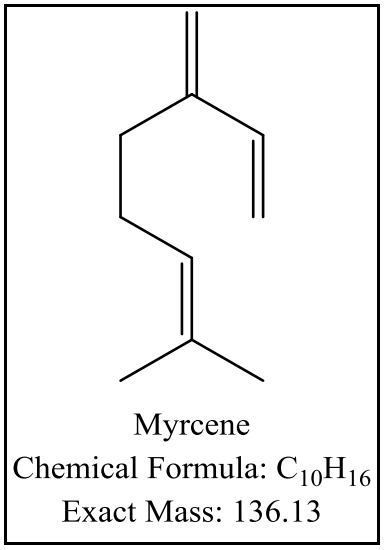


FIGURE 7: MYRCENE

Terpenes are recognised as having beneficial properties as they interact within the ECS with compounds such as THC resulting in enhancement of certain therapeutic properties such as relaxation or increased drowsiness to aid with sleep[19,63]. Terpinolene, Figure 8, commonly found in high THC stain cannabis, has been linked to an increased uplifting

feeling. There are so far over 200 terpenes that have been identified and extracted from cannabis[58,64].

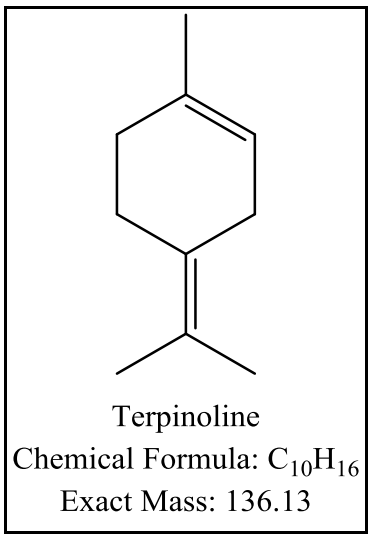


FIGURE 8: TERPINOLENE

Beta-Caryophyllene (BPC), shown in figure 9, has been shown to specifically target CB2 receptors and has links to improving serious medical conditions such as cancer and osteoporosis as well as links to improving symptoms of depression[63,64]. Access to terpenes such as these and research into isolation and extraction is key to improving treatment options for patients but requires an understanding of how each one works. The most common terpenes found in cannabis plants have all been linked to providing a vast range medical health benefits from depression to glaucoma[65].

Due to the variation of quantity and content of terpene per plant strain however, the terpenes are dubbed to be part of an entourage effect responsible for enhancing the Phytocannabinoids present and providing an overall supported medical benefit, rather than considered a 'cure' or even a treatment in the UK- other than specially regulated and approved cases such as severe cases of epilepsy[66,67].

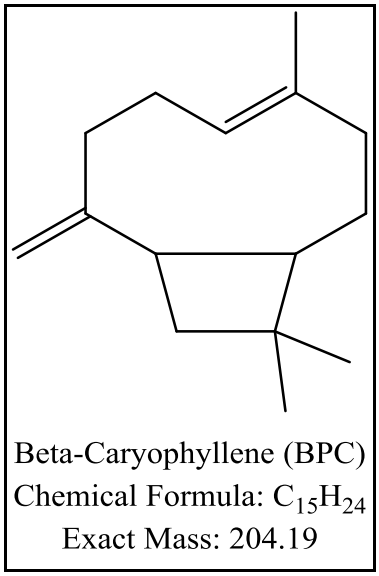


FIGURE 9: BETA-CARYOPHYLLENE (BPC)

Phytocannabinoid Research and Analysis

Considering the way UK law and US laws are changing towards legalisation of cannabis, and certain cannabinoids, having a market that meets standards or and a set of regulations is key to streamlining any future opportunities, for example regulating percentage content of each cannabinoid by weight. This would require a developed understanding of the cannabinoids present, identification of each cannabinoid and quantitation.

As mentioned above, the cannabinoid acids are what is present in the plant and are then decarboxylated in heat to their neutral counterparts. Looking at the molecule CBD and THC, these have the same molecular weight and only small differences between the molecules. This makes distinguishing between the two very difficult and necessary to involve different analytical equipment to prove identity[68,69].

This brings us onto the variety of analytical methods to achieve cannabinoid identification that have already been attempted or established, and what has been proven so far. The two main instruments that have proven to be successful are currently LC-MS and GC-MS. These are both chromatography techniques (liquid chromatography – LC, gas chromatography -GC) combined with mass spectrometry to first separate compounds using a detector and then identify using mass spectrometry[69-71].

The GC-MS consists of an inlet that is heated allowing the sample to be vaporised to a gas before entering a spiral column inside an oven. This then allows the sample to separate within the column and a detector, usually a Flame Ionization Detector (FID), at the end of the column is able to ionise compounds as they leave the column allowing identification.

Samples analysed can be in a solid, liquid or gas sate, but they need to be able to vaporise and withstand the high temperature for GC-MS analysis, making this ideal for volatile samples such as terpenes.

THC has a boiling temperature of 157°C[14] whereas CBD has a range of 160-180°C[40], which are ideal temperatures for use in a GC-MS analysis. When considering their acid counterparts in the plant, however, it is recognised that decarboxylation occurs at the lower temperature of 110 °C. This means that THCA, CBDA or any other acid counterparts present will be decarboxylated and presented as neutral in the chromatogram and TIC.

This presents a variety of issues in developing a GC method that can both detect and quantify specific cannabinoids: 1) quantification of the cannabinoid acids is not possible which would present legal issues if THCA not THC is present in the product. 2) Strong separation of the neutral counterparts is essential to quantify and find a ratio of what is present within the plant, ideal for legal use[32,72].

The Decarboxylation reaction, which has been described above figure 3, presents an issue for GC-MS analysis as it means an accurate quantity of the cannabinoids cannot be established as the acids will always be neutralised within the column and not present on the chromatogram or mass spectrometer. Relating this to use of fast analysis of products however this could be ideal for the UK market where THCA is considered a regulated compound so would require proper documentation and details of quantity which could be compared, or, if the reaction was taken into consideration and the cannabinoids were simply analysed for presence and detecting any range of cannabinoids present such as CBC or CBG per strain which would be useful in understanding different information about the samples tested, depending on what the sample is i.e. a face cream with a 'full spectrum' of CBD[27].

GC-MS is sometimes used as a confirmatory test on samples as part of performing more in-depth research such as on seized goods or workplace drug testing. The analysis is quick and does not require many consumables, other than instrument gasses and maintenance which makes it ideal when considering plans for long term studies.

As mentioned above, terpenes are ideal for GC-MS analysis due to their high volatility. This uses the headspace function of the instrument rather than liquid, with methods already specifically optimised for the function of detecting the cannabinoid terpene successfully[59,71,73]. SPME fibres have been especially effective in aiding with this, detecting full profiles from plant vapour for comparison between each other.

Overcoming the second issue of identification between samples however is possible to achieve using the mass spectrometry data, even if the chromatography presents the same retention times. For GC-MS this is possible based on the principle that THC will undergo a McLafferty rearrangement and present a 299 ion where CBD does not due to the cyclic ring on THC, where CBD has the hydroxyl group[33,60,74]. This is caused by the inlet and column temperatures heating the sample causing the fragmentation. Once this differentiation is achieved, the chromatography method can be optimised to achieve resolution between a mix of compounds and peaks can then be identified by their retention time. The McLafferty rearrangement occurs when molecules with a ketone functional group undergo a ß-cleavage gaining a hydrogen atom, as in figure 10 to illustrate how the 299 fragment is formed from THC.

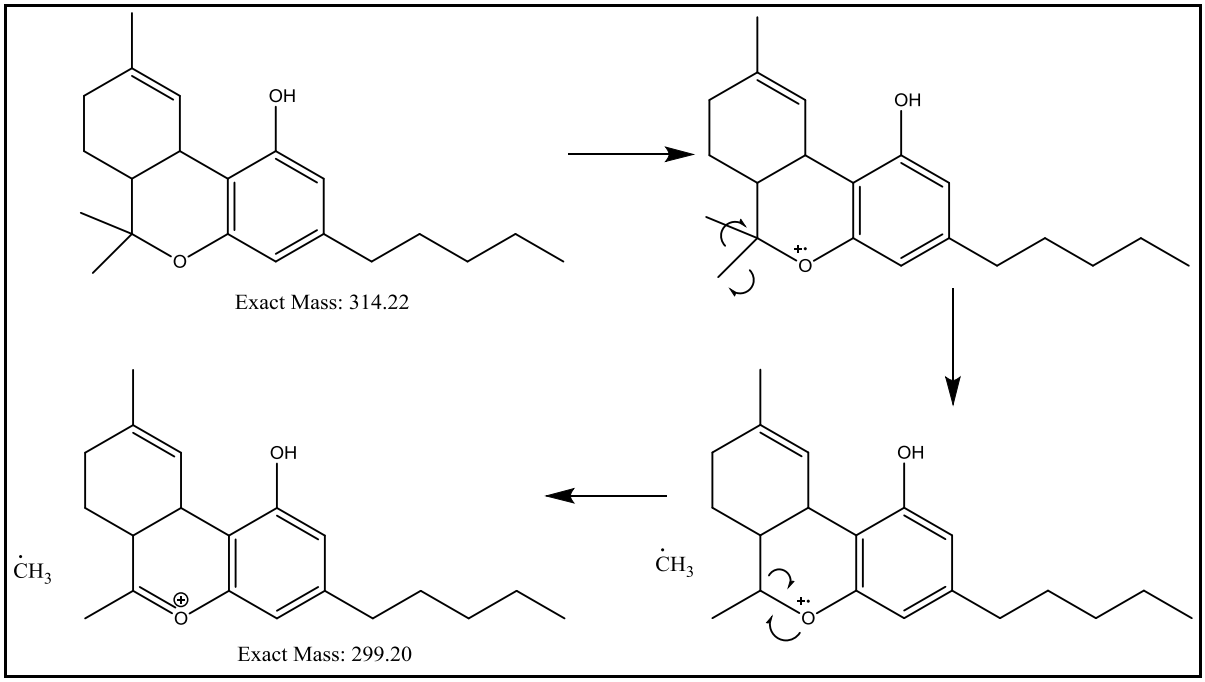


FIGURE 10: THC MCLAFFERTY REARRANGEMENT

This rearrangement makes identification between the two compounds far easier to achieve with certainty and presents GC-MS as a possible method for fast analysis of cannabinoids in samples with the possibility for other applications in future[75].

LC-MS instruments operate in a different way to GC-MS, at far lower temperatures with much shorter columns in comparison to the GC. LC requires a liquid mobile phase rather than a carrier gas to move and separate the sample along a shorter straight column. LM-MS stationary phases are contained within the column and can be altered to allow for better separation. The separation and elution of a depends on factors such as particle size, polarity and affinity to stationary phase. These additional variables in an LC-MS mean that

although the 299-ion identification is not possible, there are alternative options and ways to achieve identification[3,61,75].

The other major advantage use of LC-MS is the analysis of acid counterparts which can be detected as they do not fully decarboxylate within the instrument. Having this major advantage makes the instrument far more popular in the US where research into plant content is developing and having a full decarboxylation is a hindrance to studies. The LC-MS also presents more control over the retention time to achieve full separation between peaks and optimisation of the method depending on what cannabinoids you are looking for and the specific use.

Comparison of small physiochemical properties of each cannabinoids makes it possible to work out based on retention times and interaction with different columns and mobile phases, exactly what cannabinoid you have. There is a vast library of comparative chromatograms and TICs to support a conclusion, as well as standards to purchase which of course provide certainty to new research projects[60,73]. In relation to practical uses and concerns, there may be more sample preparation required in comparison to use of the GC-MS, as oils or other compounds that may damage an LC column.

An alternative technique to consider when looking at cannabinoid identification is NMR. This provides information in relation to specific atoms in a compound, for example an illustration of where carbons are in relation to each other through spectroscopy. NMR is a non-destructive technique so the sample can be recovered after the analysis and used in further studies making it perfect for finite samples[76]. NMR is limited in one way in that the technique works by placing the sample in a magnetic field to measure the specific resonant frequency. To achieve this the compound needs to be made up in an NMR tube "*it is recommended to dissolve between 2 and 10 mg in between 0.6 and 1 mL of solvent so that the sample depth is at least 4.5 cm in the* [NMR] *tube*"[77,78].

Many studies have utilised NMR as a method of identifying a range of cannabinoids and even using the technique for quantification. Studies in differentiating between cannabinoids that are very similarly structured have been completed as there are varieties of NMR studies to target specific molecules such as Carbon (Carbon or ^{13}CNMR) or Hydrogen (AKA proton or ^{1}H NMR) which have worked very well with cannabinoids[79], even the acid precursors[76,77].

NMR research completed confirming cannabinoids present in plant material, was carried out as it is an established technique that can be cross referenced in literature, homonuclear correlation spectroscopy (COSY) NMR and use of Heteronuclear Single

Quantum Coherence (HSQC). COSY works by detecting correlations that appear when spin-spin coupling between protons occur[76,78]. This is especially helpful to separate any multiplets and confirming correlation between protons which is important in compound identification. HSQC, however, is typically used in protein studies as it provides information on protein relations to a heteronucleus molecule other than protein, usually nitrogen[29,46,79]. In relation to cannabinoids the correlation of single bond spin-spin coupling reveals which proton and carbon groups are bonded together. With a combination of NMR techniques, it is possible to achieve confirmation of the cannabinoid structure, with the identification of any impurities or additional molecules is possible to a high standard. Knowledge that the standards used are pure is important in continuing with studies, especially in this project, as any impurities may degrade over time into other metabolites and impact quantitation data. Identification confirmation is also particularly useful as a non-destructive technique, allowing recovery of the compound once the analysis is complete[70,71,76].

Establishing analytical methods has been fundamental to building a library of cannabinoid data relating to each structure as well as any interesting properties they may hold. With chromatography and mass spectrometry combination techniques such as LC-MS and GC-MS available as well as NMR, the analysis of cannabinoids in products is becoming more efficient and accessible in labs. Increased knowledge through building on past research provides an optimistic outlook using this information practically and applying this to quantify and assess purity of a product correctly for medical use[18,77,80]. Purity of a compound is a particularly important feature of products, especially relevant to the health and safety of the consumer[81]. It helps track degradation products, gain information on stability of the compounds and allow reactions to be tested, for example in drug development, with a baseline and an established library of data to make comparisons between content reliably[82,83].

Stability of CBD has been validated up to one year, in Epidyolex[13] drug the degradation products do include THC, but this is under a very long time which has not been specified for obvious reasons and may also require conditions such as oxidation and high temperatures[84]. That said, the data and studies relating to further CBD degradation products suggests that Cannabielsoin is the more favourable compound formed and more likely to occur over time, with studies supporting CBE formed in the liver as a metabolite following an oxidation reaction with an enzyme called Cytochrome P450, otherwise known as CYP P450[85].

The exact time frame and conditions this occurs *ex vivo* is not fully established or understood as research has not been fully completed into long term studies, the reaction of this oxidation is shown in figure 11 below. As there is a lack of monetary value in establishing the stability over periods over and number of years as a limited sell by/ expiration date pushes consumers to re-purchase and replenish their stock of the drugs sooner. A 12-month expiration date also ensures that any other components such as binding agents within the drug, storage condition variations that may have accelerated or impacted degradation products are all accounted for and taken into consideration. These factors eliminate a need to validate stability studies past a practical threshold as research standard Cannabidiol is noted to have less than 1% degradation over 48 months, twice the time quoted on drugs containing the compound[84,86].

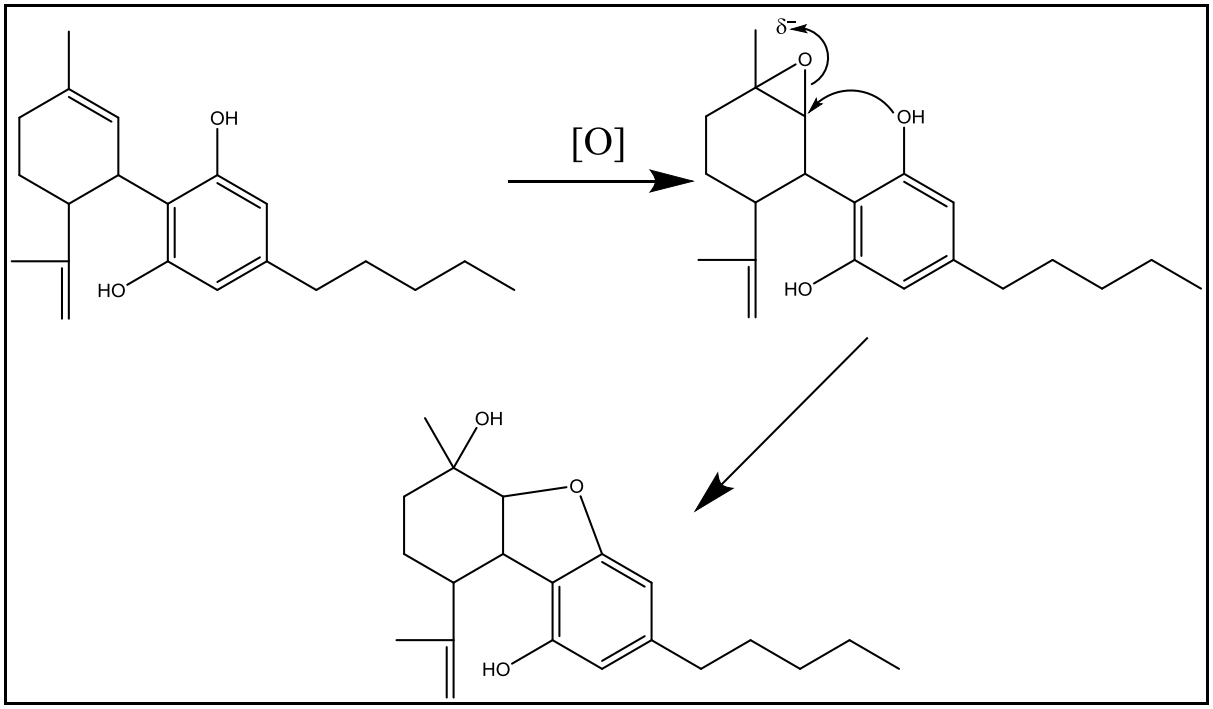


FIGURE 11: OXIDATION CONVERSION OF CANNABIDIOL TO CANNABIELSOIN

Project aims and Summary

The aim of the project is to develop a method that can identify cannabinoids and possibly quantify them too, in different samples. In order to achieve this, standards of cannabinoids and a method to successfully identify these as pure was developed followed by application and testing of these methods in chapter 3. This is divided into sub sections; first cannabinoid identification to confirm the samples are what they are said to be and are pure and fit for purpose. If deemed fit for purpose using NMR the next step, using LC-MS and GC-MS in parallel, is to develop the method for quantification and use the

approved standards to create a calibration curve that is to a high standard. These are compared to ICH standards[81] as if being developed to lab GMP standards for comparison.

Following the quantification method parameters for the LC-MS and GC-MS being optimised and confirmed as fit for purpose, there are a range of experiments that can apply the method with different specific areas of interest such as assessing extraction technique, looking at hemp seed content and ratios of specific plant parts as well as a consumer product chosen at random[74].

Looking at the data from each of these and how well the methods could be applied gives a comparison of how well each method is suited for specific purposes as well as any limitations or room for future improvement to help conclude if the aim has been achieved[3]. Considerations of factors such as budgets, assess to consumables and efficiency of each technique will provide an unbiased opinion of the usefulness of the data provided. Reliability of the data may also come into question here if there are major disagreement between the results for each experiment.

Chapter 4 focuses on a slightly different aim relating to terpene studies which were completed using the GC-MS on a SPME headspace method and is important looking into the entourage effect, giving an idea as to how easy creating profiles for plants are, as well as the different parts of a plant which may be of interest to law enforcement[71]. Although not strictly cannabinoid quantification, the aim of this chapter is to analyse terpenes present which as key to understanding content of samples and their effect, as explained in the section above. For example, if a stalk or any one part of the plant is available for analysis, a specific profile has not yet been established for what terpenes and cannabinoids to expect, but this is an area to apply knowledge of terpenes and provide an insight into this application. Terpenes are fundamental to the entourage effect resulting in such a larch impact to receptors within the body, one of the main reasons cannabis has had such a rich history and development into different strains which focus mainly on the terpenes present to achieve a desired effect[69]. The entourage effect is an understudied topic as there are so many varying factors to monitor, requiring time and far more experiments and data to explore quantitative and qualitative results[83].

The last chapter summarises all the key information and outcomes of each chapter and subsection, rounding up any major conclusions with the benefit of context from all that has been carried out. This is important when considering future work and where limitations on budget may have restricted areas of research and where new or interesting results have been obtained throughout the project.

Chapter 2: Experimental

Instruments:

NMR instrument details: Bruker AV2 400 MHz NMR. Control software IconNMR 4.7.7 and Bruker Topspin 4.1.1 software was used for raw data processing.

LC-MS instrument details: DIONEX UltiMate 3000 UHPLC^{+} focused with single quad MSQ Plus Chomeleon 7.2. for acquisition and analysis.

GC-MS instrument details: Agilent 5973N MSD. MassHunter GC/MS Acqusition B.07.06.2704 and MassHunter Workstation Software; Qualitative Analysis Navigator Version B.08.00

Prep HPLC instrument details: 1100 OpenLab

Ultrasonic Bath: Cole-Parmer Ultrasonic cleaner with timer, 1/2 gallon, 115 VAC. # WZ-08890-01

Stirrer plate: IKA 3692500 RO 15-Position Magnetic Stirrer; 100-240V

Centrifuge: IKA mini G; Ident. No.: 0003958000; 6000 - 6000 rpm

Consumables and materials:

All chemicals and acids used, including Formic Acid, Deuterated Chloroform, Water, Methanol, Hexane and Acetonitrile were purchased from Thermo Fischer Scientific and were used within expiry dates. Provided by the University of Kent.

CONFIDENTIALITY DISCLAIMER:

Samples were provided under an informal agreement of confidentiality by GW Pharma, understanding the relevant legislation and use for scientific research only.

Additional samples of plant material were also ordered online from a range of sources including Ice Headshop and Sigma Aldrich where possible. A range of other sites including eBay were also used to obtain samples where necessary which has not been listed as these did not appear to follow regulations. In order to increase anonymity between samples and sources the names of specific sources have been change to abbreviations where possible.

Methods:

As a large range of samples were analysed, and different extraction techniques were implemented, below are the generic methods used which were applied unless noted

otherwise. The specific methods with variations and exact instrument parameters varied per sample and per experiment, to account for this and allow for clear link to the experiment with the conditions used, the parameters of the instrument as well as the relevant method is listed with the details of the experiment.

Sample preparation for NMR analysis:

Specifics and variations to experimental information is provided within each study. The generic method used unless specified is as follows: In an NMR tube, 5 mg sample and 0.75 mL of deuterated chloroform as added. This is then assessed to ensure sample has dissolved and then taken to the NMR instrument where a Hydrogen and Carbon NMR analysis is completed. Recovery of sample is not attempted, once the NMR tube is returned the NMR tube is left in a fume hood to allow evaporation of the solvent and then rinsed out with methanol and then water according to UKC NATS/SPS guidelines.

Sample Preparation for LC-MS analysis:

Generic method: sample made up to 1 mg/mL Acetonitrile, cannabinoid content based on either packaging or extracted mass as appropriate, in a glass HPLC vial. Sample is vortexed for 2 mins to dissolve. The sample is labelled and placed in the LC-MS sample rack for analysis. Samples not left out for more than three days to prevent any evaporation or contamination due to lack of storage space.

Sample Preparation GC-MS analysis:

The analysis carried out on GC-MS using headspace required minimal sample preparation (including for SPME analysis).

Generic method: 3g sample weighted out in headspace sample vial and placed in autosampler. Extraction methods are not necessary here.

Liquid injection GM-MS required specific compound preparation depending on the sample analysed to allow for specific concentration analysis.

Chapter 3: Development of Cannabinoid Quantification Methods

With an understanding of what cannabinoids are and the methods of detection available, the aim of this chapter will be to develop a method to quantify cannabinoids, specifically CBD, CBDA, THC and THCA, and to apply this in practical uses with the instruments and budget provided.

When considering the aim to focus on specific cannabinoids, this was done for several reasons. Project budget and cost of purchasing standards as well as time management to achieve this to a high standard meant focusing on THCA and THC for the extraction and purification process providing more control over method development processes, use of skill set and use of 'world class equipment' available. CBDA is also able to be extracted in this way providing efficient use of time and reducing costs overall, despite adding additional steps to reach the useable compound.

The extracted CBD compound is readily available online to purchase to a high purity standard from a variety of sources. The availability of a previous sample removed delays due to delivery time, and purification processes were not a limitation, that said there are several parts to this chapter:

Confirming the identity of the compounds:

This involves the extraction and purification steps differing relating to source of the sample. These are NMR for initial confirmation, and the Mass Spec data from the GC-MS and LC-MS. MS data are secondary and more of a supportive set of data that will be used depending on the cannabinoid, as THC can be found on the GC-MS with the 299-fragment present, unlike CBD, and the precursor acids are no longer present after decarboxylation in the GC oven making the identification of these impossible. THC and CBD LC-MS analysis relies more on the retention times and elution in the chromatogram as these are easier to alter and separate rather than using the mass spec of compounds which will only differ with minor changes between THCA & CBDA, and CBD and THC.

The biosynthesis of CBDA and THCA stems from one main 'building block' which is CBGA figure 1, which is essential to cannabinoid production and through THCA synthase which is the enzyme responsible for catalysing the formation of THCA. This is the same with CBDA synthase which means both cannabinoids will likely have some trace of CBGA if they have not been separated and purified. CBGA can also decarboxylase into CBG making this an additional cannabinoid to be aware of due to the similarities with the target cannabinoids.

Identifying the CBGA is not part of this project and will be a mild hindrance if present in high concentrations or may delay separation if the peaks co-elute in the prep HPLC during the sample preparation step. If there is some CBGA present this will be noticeable on the NMR as there will be protons or carbons that do not resonate with the structure of the expected compound.

LC-MS Method development:

Once the identity of the cannabinoids has been confirmed, the second step is to develop an LC-MS method that can detect the cannabinoids with strong resolution, in one run and have them ionise to cross reference identification with literature available. This secondary identification will be completed during the method development stage as part of the process depending on how retention times vary, or conditions needed for a suitable elution.

This method will need to be validated with comments on all factors and considerations for use of the method once complete, including any limitations or issues that may arise. This is something that will not be rushed and take a substantial amount of time to achieve to prefect each factor and consider many variations to achieve the optimum conditions for the project. Due to the global pandemic and instrument technical issues, there was some delay in achieving this as efficiently possible.

GC-MS Method development:

In the same way, the GC-MS method will then be developed to detect the cannabinoids. This will be different form the LC-MS method as complete decarboxylation is expected so THCA is expected to present as THC making separating THC and CBD by retention times necessary to allow for easier quantitation. This means CBDA and THCA will not be included or attempted to be identified and only CBD and THC will be the target cannabinoids.

Method validation:

Studies into method applications in ‘real world’ uses to test how well the methods work, as well as answer some simple questions such as label claim content will then be carried out. This will involve more online purchases from unregulated sources with claims of CBD content as this is very popular now to add to items from health care to beauty.

This section will be split into each experiment carried out with the established GC-MS and LC-MS method, comparing results from both instruments to assess differences between the methods as well as look for similarities and any uses that may be more tailored to the instrument. A comparison of the content of the cannabinoids within the plant material itself will also be carried out as some analysis into the study of cannabinoids present in every part of the plant or perhaps just the bud where cannabinoids are known to be present in very high concentrations. Specific focus on cannabinoids in the seeds of the plant, dry and germinated, will be carried out. This analysis is specifically relevant to the current market of hemp seeds that are available in supermarkets and have no restrictions on purchase. A question of the possibility of cannabinoids present as biosynthesis is not

expected to occur at this early stage, however some studies have noted that there are traces present and it is possible to detect and quantify these. Investigations such as these are not complex and simply require accurate lab skills to complete the analysis and obtain useful results. In future methods such as these may be easier to establish and transfer onto other instrument models/ makes as needed to test products meet regulations.

CBD Identification

Two samples of CBD extract were purchased online from the same provider one year apart. A CoA was attached and confirmed by the provider to be applicable to both samples purchased. Due to the limited quantity of CBDA available however, an NMR analysis was unable to be completed as the concentration of 5 mg / 0.75 mL could not be made up. This means identification will be completed through literature comparison of the mass spectrometry results through the LC-MS.

NMR was the chosen method of identification as a non-destructive technique allowing for sample recovery if necessary. The data gained from NMR aids to assessment of purity based on proton and carbon spectra provided ensures samples used for quantification have been properly verified[81,82]. Each full spectra obtained can be found in Appendix B to cross reference peak placement.

Sample preparation/ method:

Each sample was analysed in separate in a separate NMR tube made up 5 mg CBD sample in 0.75 mL of deuterated chloroform. A Hydrogen and Carbon NMR analysis was then completed on each sample.

Data & Results:

CANNABIDIOL REFRENCE STRUCTURE:

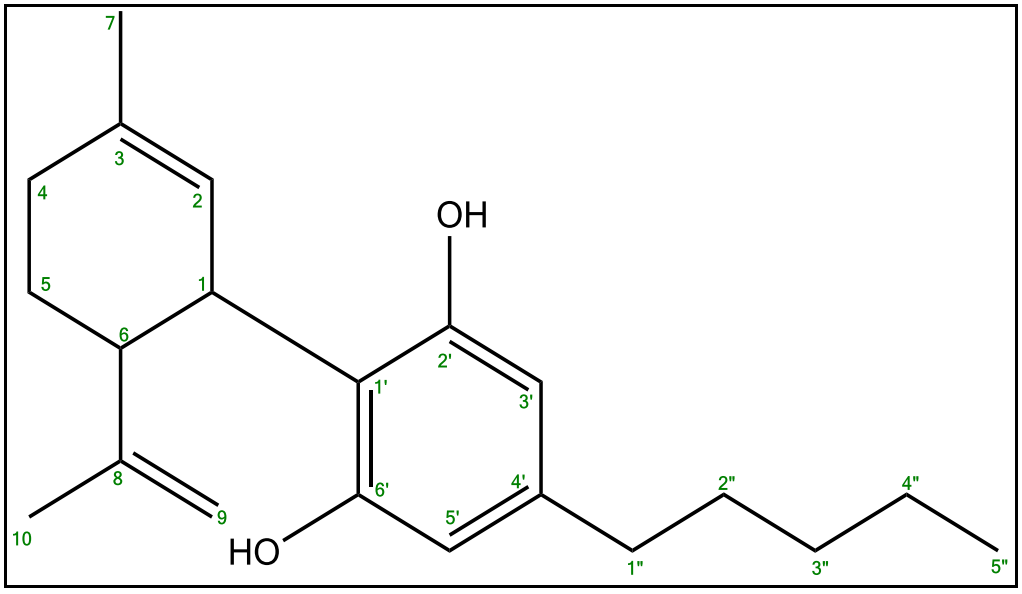


FIGURE 12: CANNABIDIOL REFERENCE STRUCTURE

CANNABIDIOL NMR ASSIGNMENT DATA:

Key: singlet=s, m= multiplet, triplet of doublets= ToD, broad singlet= brs, triplet= triplet

Compound ID: CBD1 1H-NMR (400 MHz, 298.15 K, CDCL3): δ: 6.2582 (1H, brs), 6.1716 (1H, brs), 5.9571 (s), 5.5606 (1H, s, j=7.5), 4.6551 (1H, m), 4.553 (1H, m), 3.8328 (1H, s), 2.4301 (2H, t), 2.3915 (m, ToD), 2.1069 (1H, m), 2.0622 (1H, m), 1.7913 (3H, s), 1.8119 (m), 1.7913 (3H, s), 1.5495 (2H, m), 1.2903 (m), 0.8678 (3H, t).

Compound ID CDB1 13C-NMR: δ: 143.2533, 140.2540, 124.3014, 28.6032, 23.8709, 149.6243, 113.9313, 111.0162, 46.3259, 37.5000, 35.664, 31.6766, 30.592, 30.8183, 22.7275, 20.7557, 14.2235.

Compound ID: CBD2 1H-NMR (400 MHz, 298.15 K, DMSO-d6): δ: 6.2392 (1H, brs), 6.1787 (1H, brs), 5.96 (s), 5.5659 (1H, s), 4.9629, 4.6438, 4.5441, 3.8563 (m), 2.4313 (t), 2.372 (m), 2.2179 (1H, m) 2.0852 (1H, m), 1.8183 (m), 1.7855 (3H, s), 1.6539 (3H, s), 1.5538 (qui), 1.2963 (m), 0.8725 (m)

Compound ID CDB2 13C-NMR: δ: 149.434, 143.175, 140.1277, 124.3616, 113.9435, 110.9954, 46.3549, 37.3036, 35.6617, 31.6698, 30.5751, 29.1082, 28.5726, 23.8478, 22.7156, 20.5627, 14.2063.

These values tabulated to corresponding position on CBD (Figure 12) can be found in Appendix B.

IMPURITIES:

TABLE 1: IMPURITIES OF CBD2 SAMPLE

CBD2 unassigned peaks	
^{1}H-NMR ppm	^{13}C-NMR ppm
7.7033	131.071
7.5252	128.9874
4.2149	68.3627
3.6592	51.05
3.4848	38.9197
3.3058	30.8161
1.083	11.1348

Discussion:

CBD1 had been purchased 14 months prior and used in projects without issue, however analysis on CBD degradation to THC and stability studies on CBD isolate past 12 months is rare and suggests that a new standard should be purchased for reliability and purity reassurance, hence CBD2.The hydrogen and carbon NMR spectra shows that CBD remains a pure and stable isolate appropriate for quantitation analysis and is still suitable for use in my studies, however the new CBD2 isolate contained some impurities that could not be explained. These results suggest that despite both samples having the same CoA reference sheet, it is possible they may have some key differences and have different origins.

As noted in the assignment data, in CBD2 there are two multiplets at present which could not be assigned to protons at 7.0733 ppm and 7.5252 ppm present, and at 131.0710 ppm and 128.9874 ppm, which could not be assigned to a carbon in the cannabidiol structure, these could potentially correspond to each other however identification of this impurity itself was not possible. The full specra of these can be found the in appendix B to provide a more complete view.

The aromatic hydrogens responsible for the singlets at 6.1716 ppm and 6.2582 ppm are clearly visible as broad singlet peaks, helping confirm identification as these are specifically known to be present within the aromatic ring and indicative of cannabidiol. In the case of CBD1, and CBD2, they have merged and caused some overlap due to how broad the peaks are.

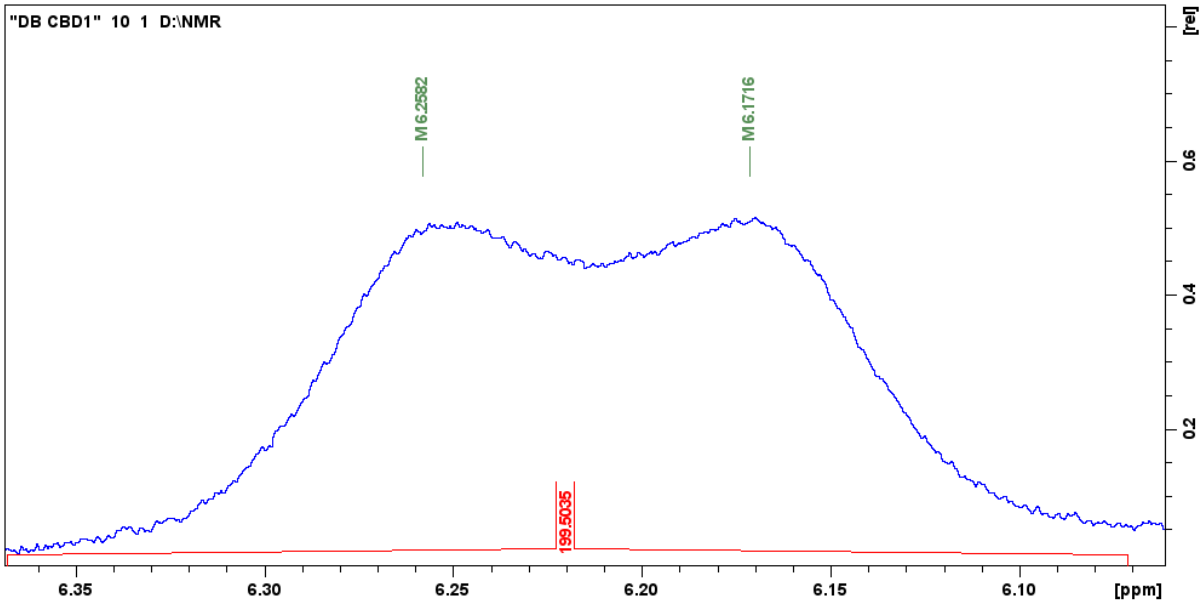


FIGURE 13: CBD1 BROAD HYDROGEN SINGLETS

In CBD2 the peaks at 4.5441 ppm and 4.6438 ppm, Figure 13, could not be accurately described as multiplets as they appear to be broad singlets or perhaps a triplet for the 4.6438 ppm, quite like the peaks present at the CBD1 comparison, Figure 14. This is inconsistent with some literature however this could be a matter if difference in interpretation or be the result of some difference in the protons presence and positioning in relation to each other.

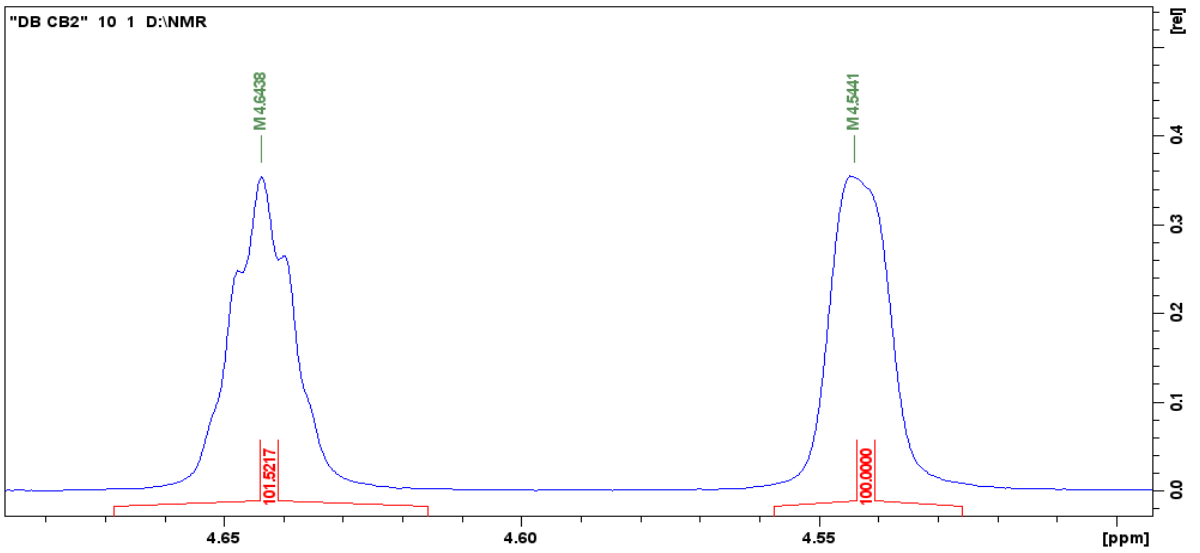


FIGURE 14: CBD2 HYDROGEN NMR PEAKS AT CARBON 9

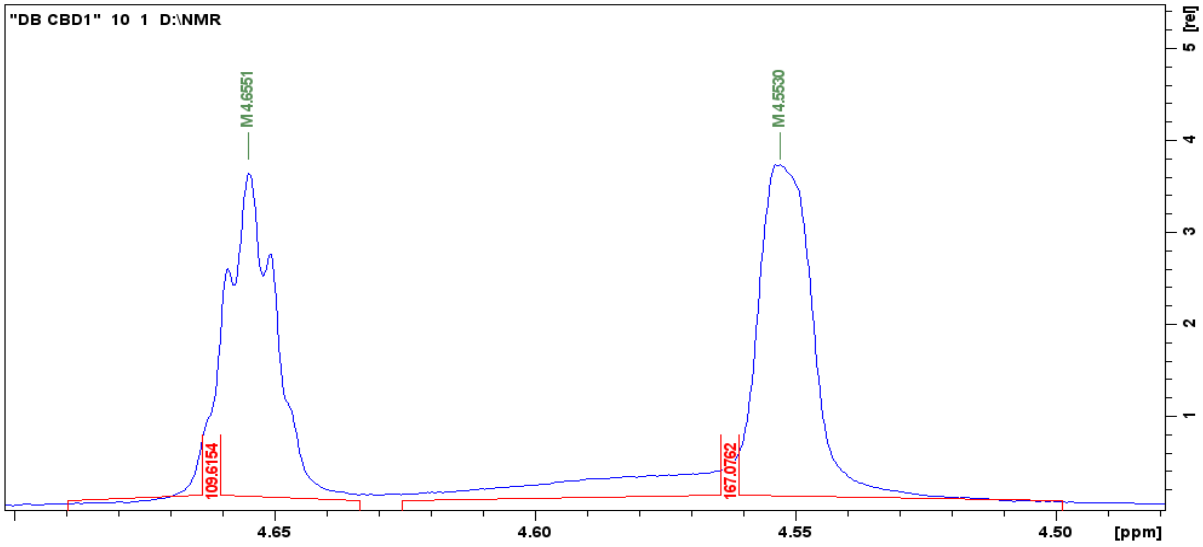


FIGURE 15: CBD1 HYDROGEN NMR PEAKS AT CARBON 9

One of the features of CBD1 that helped with identification was the singlet that has been overlapped by the multiplet, and is identified through an understanding that the three hydrogens present at position 7 (1.7913 ppm) are responsible for the singlet but are overlapped by the multiplet at protons in position 5 (1.8119 ppm), as shown below in figure 16.

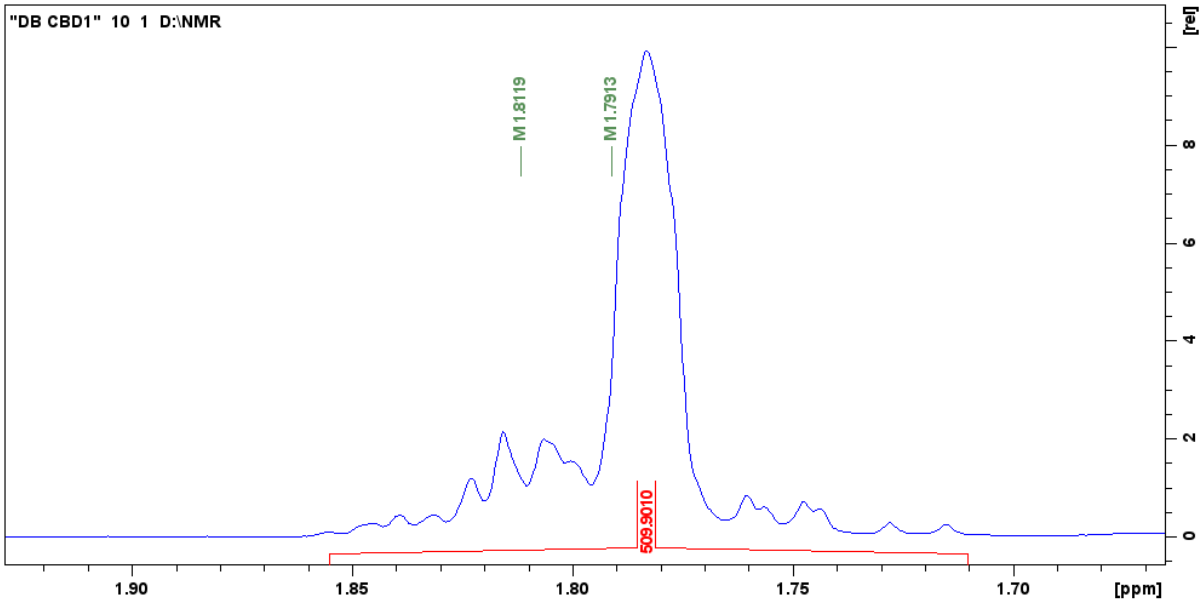


FIGURE 16: CBD1 OVERLAPPING SINGLET

The proton peaks at position 2 in the structure for CBD2 and CBD1 also differ as CBD2 looks closer to a quintet, figure 17, shape at 1.5538 ppm and in CBD1 sample presents more obviously as a multiplet, shown in figure 18.

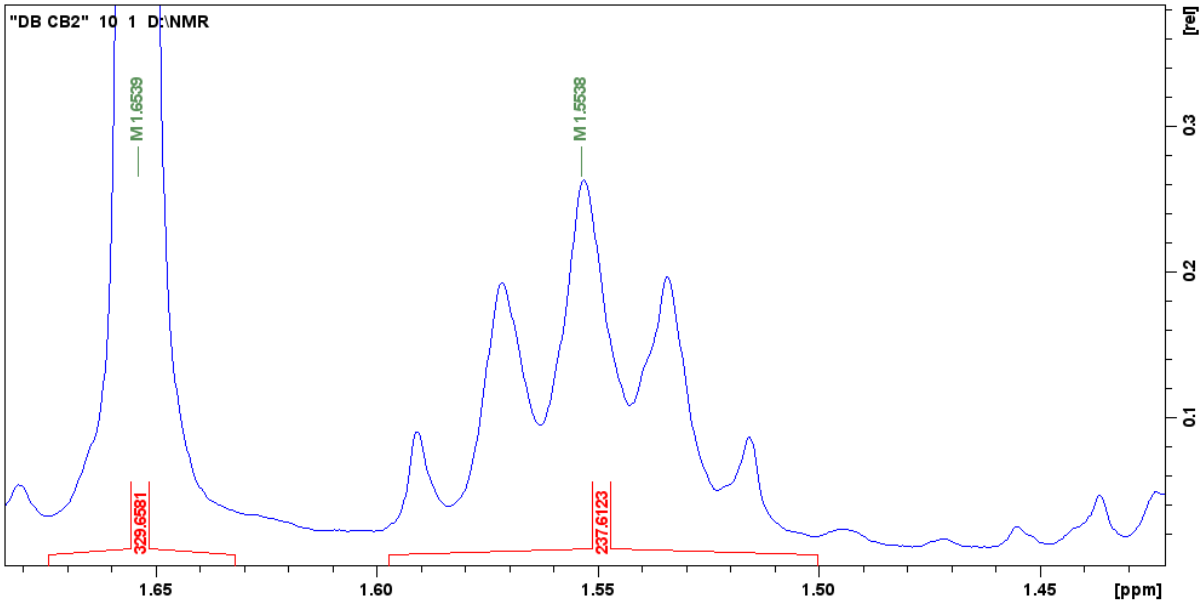


FIGURE 17: CBD2 PROTON NMR 1.5538 PPM

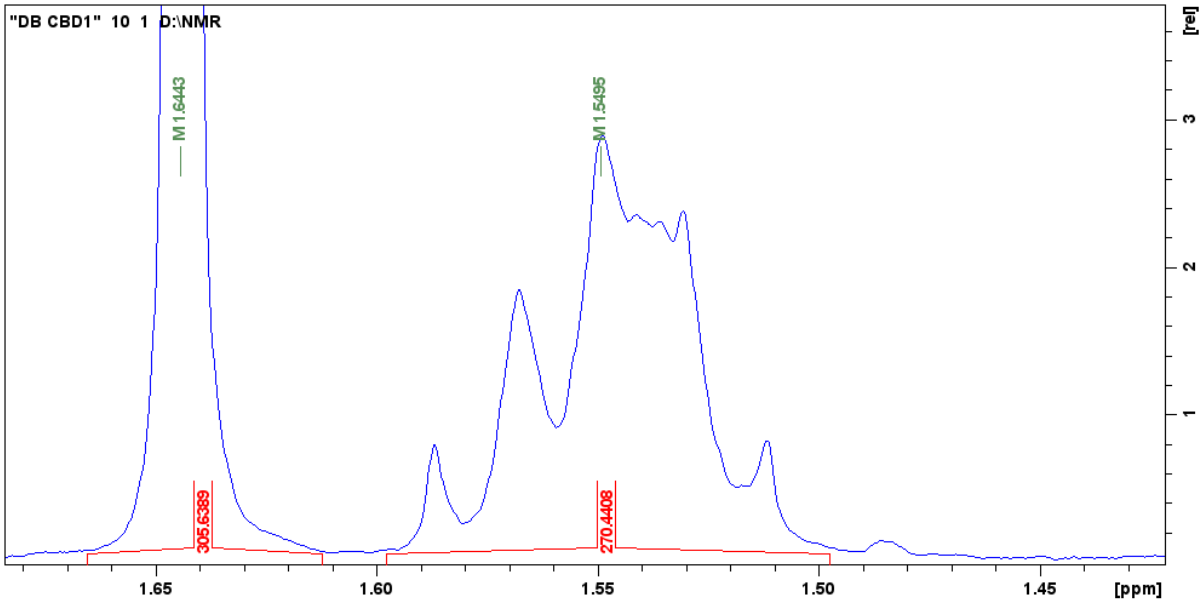


FIGURE 18: CBD1 PROTON NMR 1.5495PPM

One other major difference between the CBD1 and CBD2 samples is the peaks in 5.9600 ppm and 4.9629 ppm.

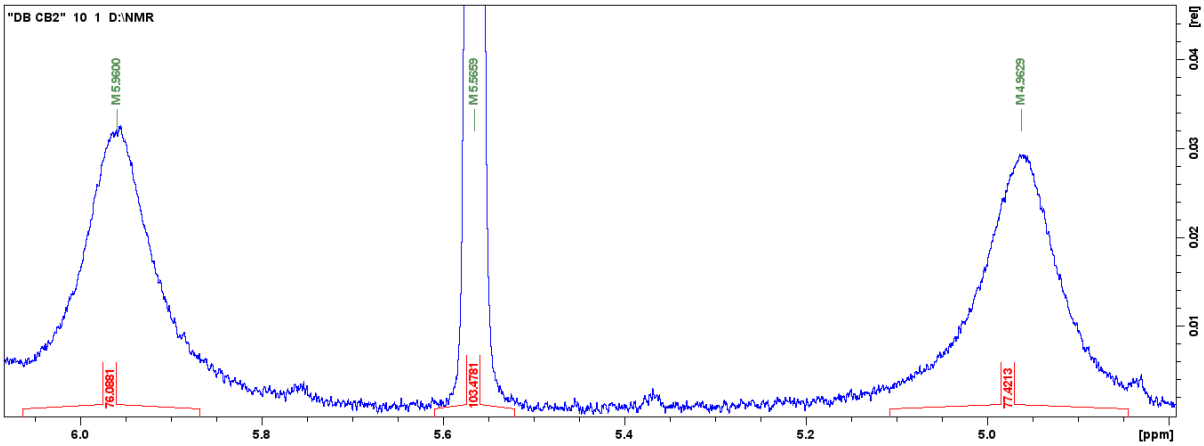


FIGURE 19: CBD2 HYDROXY GROUPS

The peaks shown in figure 19 are indicative of the hydroxy groups, due to how broad they are (caused by exchange of protons from the solvent to the hydroxy groups) and they are quite downfield of the spectrum which would be due to the electronegative oxygen group, however these are not present in the CBD1 sample which is not entirely surprising as these hydroxyl groups are not always detected at all using NMR simply due to the water present in the solvent and concentration of hydroxyl groups.

A COSY and HSQC NMR were completed with results supporting the Proton and Carbon NMR analysis. This supports the positive identification of CBD present within the samples and that CBD1 is of a higher purity with CBD2 containing some unknown impurities.

The CoA accompanying the CBD samples tested provides a list of the possible trace cannabinoids present however reference NMR for these were found in literature and confirmed these were not present in the sample or responsible for the additional peaks.

The overall result from the CBD1 and CBD2 study is that both isolates consist of cannabidiol, but the CBD2 sample contains some impurities whereas CBD1 only contains methanol as an impurity which means it is of a higher standard and fit for purpose in this study.

THCA identification

Sample preparation:

Plant material was taken and soaked in hexane overnight to allow for extraction of cannabinoids. The plant material was then removed, and the vial was left to allow the hexane to evaporate. Once the hexane has evaporated a dark oil remained, 5 mL methanol was then added to this and left to sonicate to allow for the cannabinoids to make up a homogenous solution ready to separate the cannabinoids.

The next step was then to separate the cannabinoids within the mixture. This is done using the prep HPLC producing two main peaks which can be separated and collected. Each fraction should be run on the LC-MS to confirm THCA presence, in fraction one Δ9-THCA isomer was found with the second peak to be Δ8-THCA.

The Δ8-THCA was co-eluting with some Δ9-THCA so the decision to collect the Δ9-THCA separately and then the combination of both Δ8-THCA and some Δ9-THCA to increase quantity of cannabinoids collected and prevent any potential loss was made.

The two THCA fractions collected were kept in separate evaporating dishes and left for two days in a fume hood to prevent dust contamination and allow for the solvent to fully evaporate.

Method:

Analysis was carried out on each sample using NMR to confirm the purity and identity of the compound to proceed with quantitation studies confidently. A Hydrogen (1H) and Carbon (13C) NMR run was completed. 5 mg sample mixed with 0.75 mL deuterated chloroform.

Data & Results:

The spectra obtained from each analysis can be found for reference in Appendix C.

TETRAHYDROCANNABINOLIC ACID REFRENCE STRUCTURE:

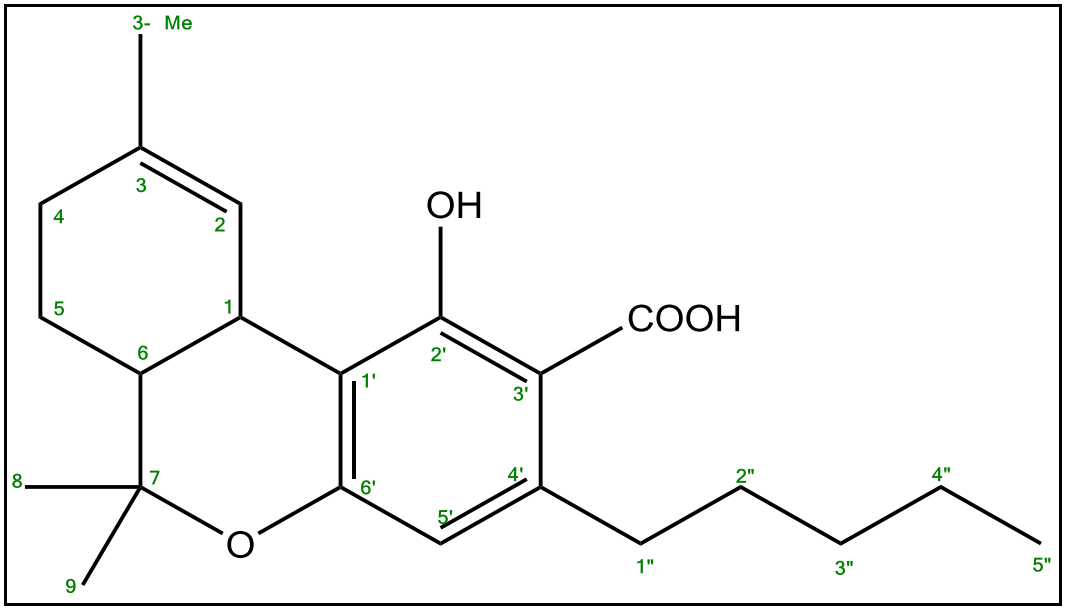


FIGURE 20: Δ 9 THCA NMR REFERENCE STRUCTURE

Δ9 THCA SAMPLE 1H AND 13C NMR DATA:

Compound ID: Δ9 THCA 1H-NMR (400 MHz, 298.15 K, CDCL3): δ: 12.2042 (s), 6.2543 (1H, s), 6.392 (m), 3.2278 (dm), 2.9359 (1H, m), 2.781 (1H, m), 2.1759 (m), 1.9205 (m), 1.6832 (3H, s), 1.6624 (m), 1.5718 (2H, m), 1.4440 (3H, s), 1.3437 (m), 1.1092 (3H, s), 0.8992 (t).

Compound ID: Δ9 THCA 13C-NMR: 175.8716, 164.8919, 159.9482, 147.0347, 134.0409, 123.8002, 112.8006, 110.054, 102.3916, 79.0391, 45.7908, 36.7126, 33.6547, 32.2172, 31.4875, 31.4071, 27.5761, 25.1823, 23.5293, 22.7084, 19.7092, 14.2514.

Compound ID: THCA Mix 1H-NMR (400 MHz, 298.15 K, CDCL3): δ: 12.2160 (s), 6.3916 (m), 6.2525 (1H, s), 3.2277 (dm), 2.9286 (1H, m), 2. 7744 1H, m), 2.1716 (3H, s), 1.9237 (m), 1.6825 (3H, s), 1.6626 (m), 1.5714 2H (m), 1.4432 (3H, s), 1.3433 (m), 1.1083 (3H, s), 0.8981 (t)

Compound ID: THCA Mix 13C-NMR: 27.576, 175.7403, 164.8851, 159.9224, 147.0023, 123.8029, 134.0379,112.7832, 110.0467, 102.3916, 79.0317, 45.7948, 36.7124, 33.6539, 32.217, 31.4948, 31.4071, 25.1823, 23.529, 22.7083, 19.7089, 14.2502.

These values tabulated to corresponding position on THCA (Figure 24) can be found in Appendix B.

IMPURITIES

TABLE 2: THCA MIX IMPURITIES

^{1}H-NMR ppm THCA Mix
3.4985
0.0705

Discussion:

The impurities of THCA Mix sample as shown below in figure 21, specifically the 3.4985 ppm singlet, appears to possibly methanol as there are two smaller peaks (spinning sidebands) either side as with the CBD2 sample which are only identified on closer inspection and may require further verification. The samples were extracted from methanol before purification which could be the source of the peaks and something to keep in consideration for future method development if this becomes a higher quantity.

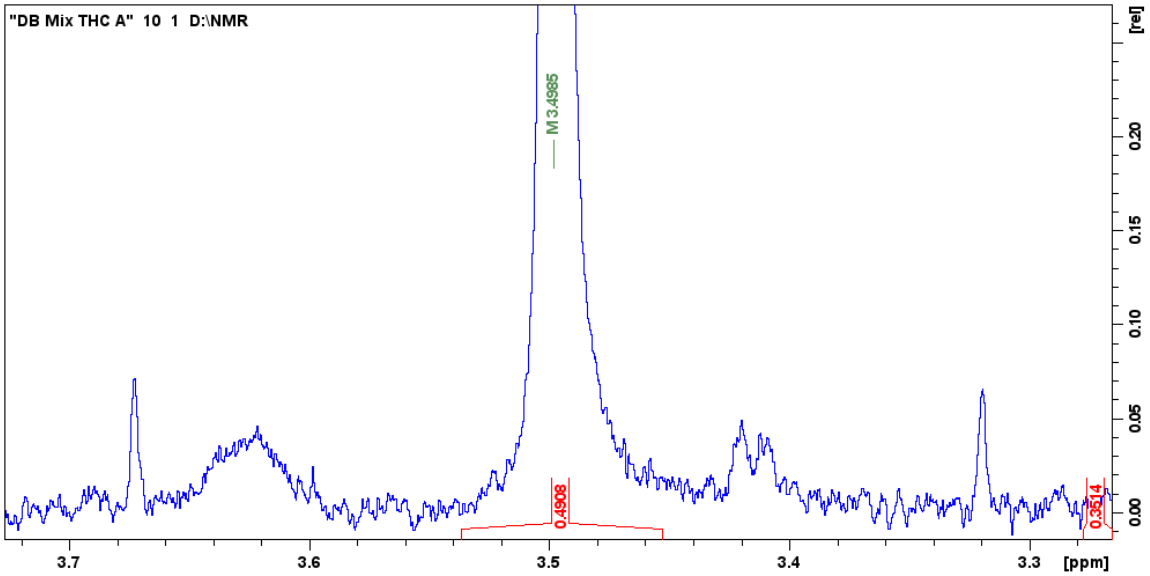


FIGURE 21: SUSPECTED METHANOL IN THCA MIX SAMPLE

The 0.0705 ppm peak, in figure 22, is relevant to considering possible contamination as it appears to possibly be a multiplet or broad singlet depending on the interpretation and context making identification of this impurity difficult as literature suggests silicone grease could be responsible for this as this has a very low external magnetic field and requires a very low frequency to achieve resonance resulting in a very low ppm.

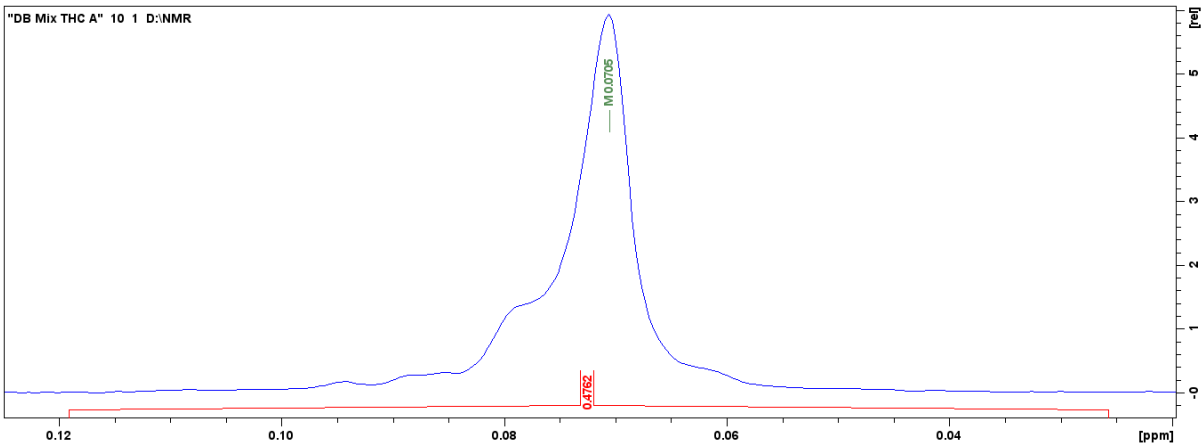


FIGURE 22: THCA MIX UNKNOWN IMPURITY

Other than this the two samples show strong consistencies between each other with very few differences in peak appearance. For example, the triplet at carbon 5” for both THCA Δ 9, shown in figure 23 and THCA mix, shown in figure 24, both have a j (Hz) value of exactly 7 and are consistent in broadness and ppm suggesting as they are from the methyl group at the end of the pentyl chain on the THCA molecule this is a correct placement and interpretation of the spectra.

An example of a consistency between the two samples is the sets of multiplets shown in figures 25 and 26 present at 1”, these are consistent in ppm value and shape, and are expected when compared to reference spectra found in literature with the comparison of THCA Mix and Δ 9 THCA shown below. This also supports that the compounds analysed are pure as there is no interference or additional peaks presenting that could be from impurities if they were present.

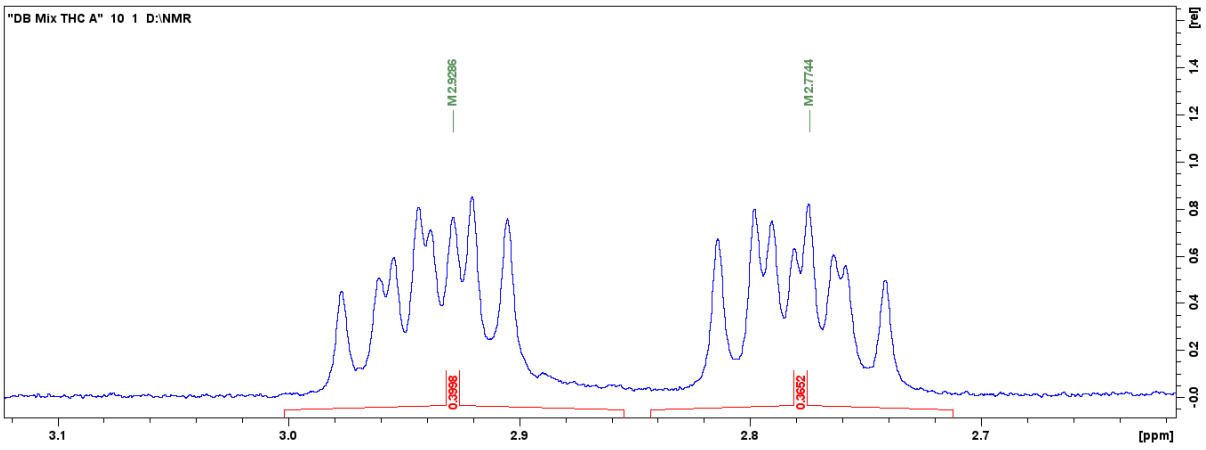


FIGURE 23: THCA MIX PROTON NMR MULTIPLETS

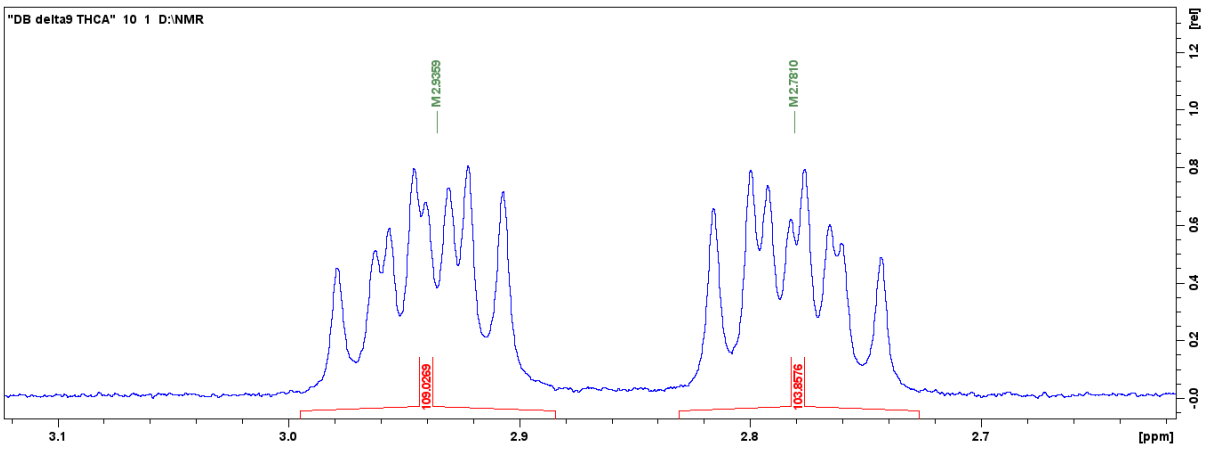


FIGURE 24: Δ 9 THCA PROTON NMR MULTIPLETS

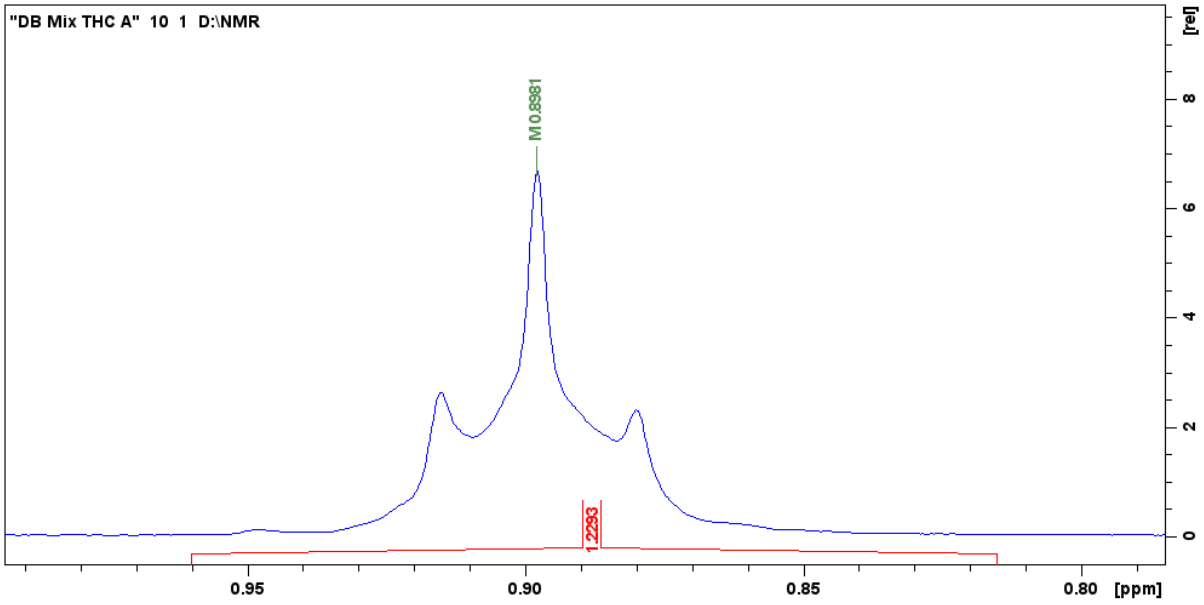


FIGURE 25: THCA MIX TRIPLET

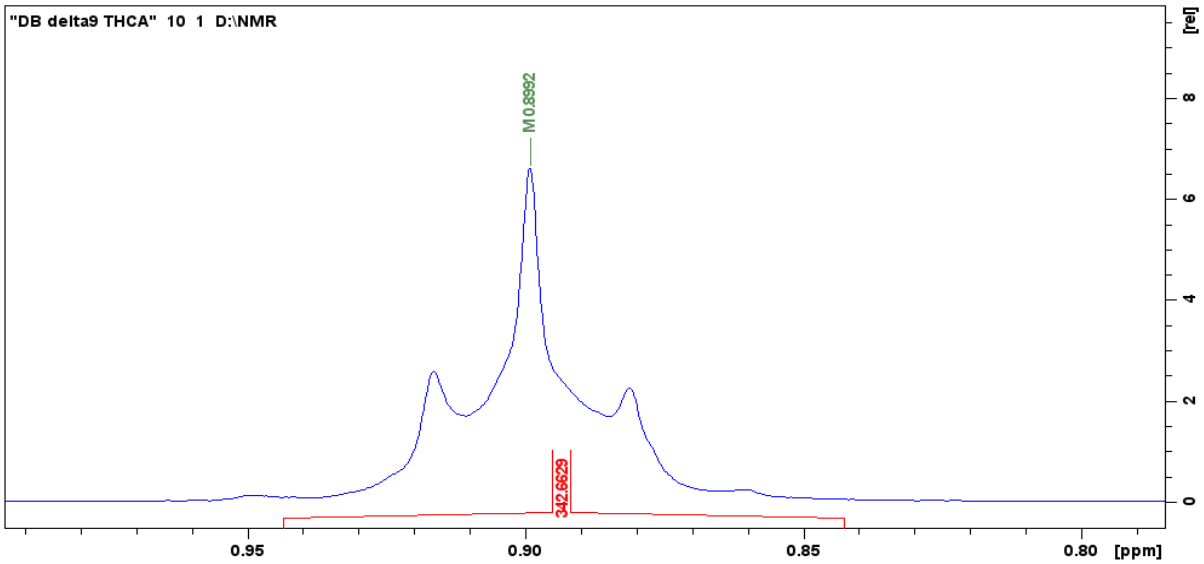


FIGURE 26: Δ9 THCA TRIPLET

Both samples' spectra confirm the presence of pure Δ9THCA, however, in the 'THCA Mix' sample there does appear to be some possible Δ8THCA present as the proton NMR included some impurities as listed in table 2. This requires further analysis to confirm as the additional peaks could also be due to another cannabinoid entirely as the starting material would provide a *full spectrum* of cannabinoids.

One interesting feature of the two samples spectrum is the presence of the OH peak at 12.2160 ppm in the THCA Mix and 12.2042 ppm in the Δ 9 THCA. These are broad suggesting interference with the solvent over time but are clearly singlets as expecting with a high electronegative bond. The Δ 9 THCA hydroxy group peak is shown in figure 27.

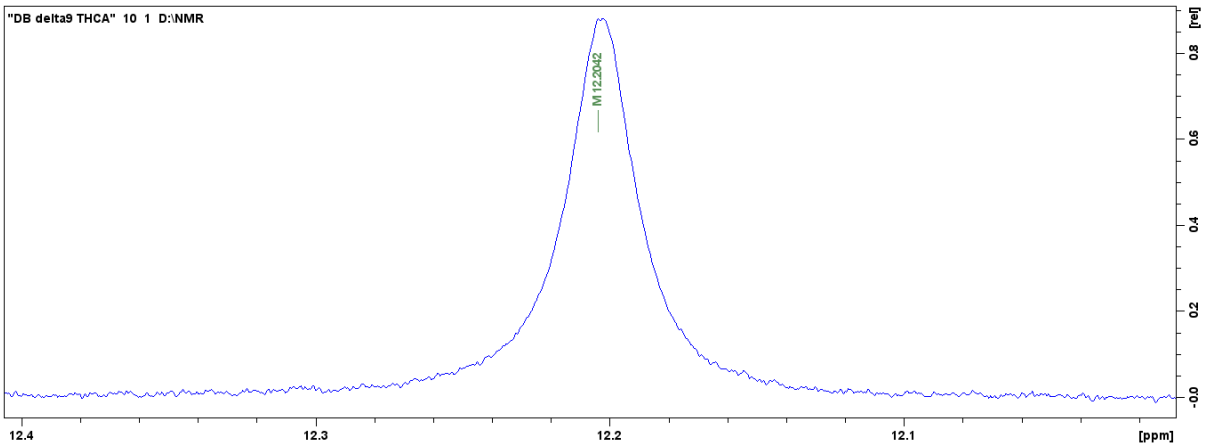


FIGURE 27: Δ 9 THCA HYDROXY GROUP

This contamination in THCA Mix appears to be of a low-level quantity and is confirmed present in the additional COSY and HSQC NMR completed, the full spectra for these are present in the Appendix C.

The Δ9THCA sample analysed conformed to the literature comparison and did not present any peaks to indicate impurities were present in the material. The comparison of the THCA samples analysed confirm the presence of Tetrahydrocannabinolic acid to a pure standard in the 'Δ9THCA' sample tested making it fit for purpose and use into the project for future use. Henceforth 'THCA' will refer to Δ9THCA purified unless specifically stated otherwise, therefore in GC-MS studies THC will refer to Δ9THC from THCA decarboxylation, rather than Δ8THC.

Quantification method development

Sample preparation method:

CBDA was extracted and purified from material in the same way in which THCA was obtained, as described above. This ensured CBDA, CBD and THCA each consisted of a fine white powder texture.

To ensure consistency between the GC-MS and LC-MS all calibration standards were made using the following method:
Stock solution 1 mg/ mL: Take 10 mg standard in 10 mL acetonitrile and mix until dissolved. This will be 1000 ppm.
0.75 mg/ mL Calibration: take 75 µL stock solution and add to 25 µL in HPLC vial and mix. This is 750 ppm.
0.50 mg/ mL Calibration: take 50 µL stock solution and add to 50 µL in HPLC vial and mix. This is 500 ppm.
0.25 mg/ mL Calibration: take 25 µL stock solution and add to 75 µL in HPLC vial and mix. This is 250 ppm.
0.10 mg/ mL Calibration: take 10 µL stock solution and add to 90 µL in HPLC vial and mix. This is 100 ppm.

LC-MS Method development

Beginning with LC-MS for quantification to determine quantity of CBD, CBDA, THC and THCA in one method. Several factors were specifically focused on to optimise the LC-MS method: solvent for sample extraction, mobile phases, column, flow rate, column temperature, injection volume, MS probe, and MS cone voltage.

These were assessed systematically so the best results could be determined for both the acid and non-acid cannabinoids, which included polarity and ionisation to be incorporated into consideration on how each factor may affect the outcome.

Chromatography was initially focussed on looking for strong UV absorbance at 254 nm as this is supported in literature to be appropriate for the cannabinoids[88].

A range of solvents were used including hexane, acetonitrile, methanol, dichloromethane, and heptane[17]. The results from this showed they each worked well at extracting cannabinoids overall with acetonitrile and dichloromethane being consistently successful. As a compromise between environmental and practical issues acetonitrile was chosen to proceed.

The mobile phases were then changed as these would interact with the solvent, for mobile phase B 0.1% formic acid in Methanol (MeOH) was compares with 0.1% formic acid in Acetonitrile (MeCN) with little improvement on chromatography so 0.1% formic acid in MeOH was selected.

The column was altered between a 150 mm C8, C18 and 50 mm C18 through the below parameters. This was due to an issue preventing the CBD to ionise, and therefore be detected, that was only properly corrected when the C18 short was found to ionise all compounds in a mixture of CBD spiked with THCA at same concentrations. Flow rate and gradient were interchanged to achieve the best possible result. The optimum conditions for the CBD to leave the column was very different to the THCA environment, and a 'wash' of the organic mobile phase ensured that there was no carryover between runs.

The MS probe was altered to an APCI and tested at both negative and positive, and then a series of ESI negative and positive runs were completed to compare ionisation, ESI- was the most successful with both samples as supported by literature. This was done to ensure the best possible detection and separation of peaks was achieved.

MS cone voltage and probe temperature were compared each at high, medium, and low parameters, and then proceeding to alter this in smaller increments to achieve optimum ionisation and ideal parameters in the MS. This was supported by other literature as the most suitable set of MS parameters for both cannabinoids. This ensures the method is properly suited to detect the cannabinoids.

Three columns used:

1) Penomenex® Gemini® 3 µm C18 110 Å, LC Column 50 x 4.6 mm, 00B-4439-E0, 33768-3
2) GRACE™ Alltech™ Platinum™ EPS C8, 5µm Analytical 4.6 x 150mm 32420, 1198-98, 26/108
3) Penomenex® Luna® 5 µm C18(2) 100 Å, LC Column 150 x 4.6 mm, 00F-4252-E0, 385834-48

Method Validation:

Specificity: investigation into the specificity of the method began with NMR analysis to independently confirm and support the identity of the compound and impurities were acknowledge and investigated appropriately. Following this, the ions observed in CBD2 TIC support impurities present but far too low to quantify. This verifies the LC-MS method is able to detect and present impurities present in the chromatogram and TIC despite

similarities in structure to the cannabinoids selected. With the comparison data obtained with CBD1 showing no impurities present, this supports previous studies carried out with the sample and discrimination between a pure sample and impurities is possible.

Accuracy: Due to the budget restrictions, accuracy is inferred from agreement between linearity, precision and specificity which have been established.

Precision:

Calculation of RSD to establish repeatability from triplicate repeats:

TABLE 3: LC-MS RSD DATA

Name	Mean Area	SD	RSD%
CBDA	48.36985	0.199204	0.411836
Δ9 THCA	16.9338	0.513809	3.034224
CBD	1.352375	0.020623	1.524949

Linearity and Range:

The linearity and range have been confirmed fit for purpose for all standards analysed using the calibration curve. The R values obtained are suitable for the research carried out and range established has been determined by the necessary parameters.

Robustness: Robustness of the method has been analysed and tested during method development; altering column type could cause severe alteration of results, factors such as flow rate, mobile phase pH and column change me be altered ±5% without impacting resolution or retention time of the compounds peak. Solvent for sample preparation can also vary as necessary without impacting retention times for example methanol and acetonitrile are both appropriate for LC-MS analysis.

Limit of Detection (LoD):

The limit of detection of this method is dependent upon the Standard Deviation of the Response and the Slope, this may be expressed as Equation 1.

EQUATION 1

$$DL = \frac{3.3\sigma}{S}$$

σ = the standard deviation of the response

S = the slope of the calibration curve

CBDA= $\frac{3.3 \times 0.1992}{0.0497}$ = 13.33 mg/ L

THCA = $\frac{3.3 \times 0.5139}{0.178}$ = 9.53 mg/ L

CBD $= \frac{3.3 \times 0.0206}{0.0131}$ = 5.19 mg/ L

Quantitation Limit (QL):

With units as above, the quantitation limit is determined using Equation 2.

Equation 2

$$QL = \frac{10\sigma}{S}$$

CBDA $= \frac{10 \times 0.1992}{0.0497}$ = 40.080 mg/ L

THCA $= \frac{10 \times 0.5139}{0.178}$ = 28.870 mg/ L

CBD $= \frac{10 \times 0.0206}{0.0131}$ = 15.725 mg/ L

LC-MS Method:

TABLE 4: LC-MS METHOD CONDITIONS

MS Conditions	
ESI-	75v Cone
350 °C Probe Temperature	FS Mass Range 100-1000 Da
LC Conditions	
UV Wavelength	254 nm
Column Temperature	50 °C
Injection Volume	10 µL
Flow Rate	2 mL/min
Mobile Phase A	0.1% Formic Acid in Water (v/v)
Mobile Phase B	0.1% Formic Acid in MeOH (v/v)

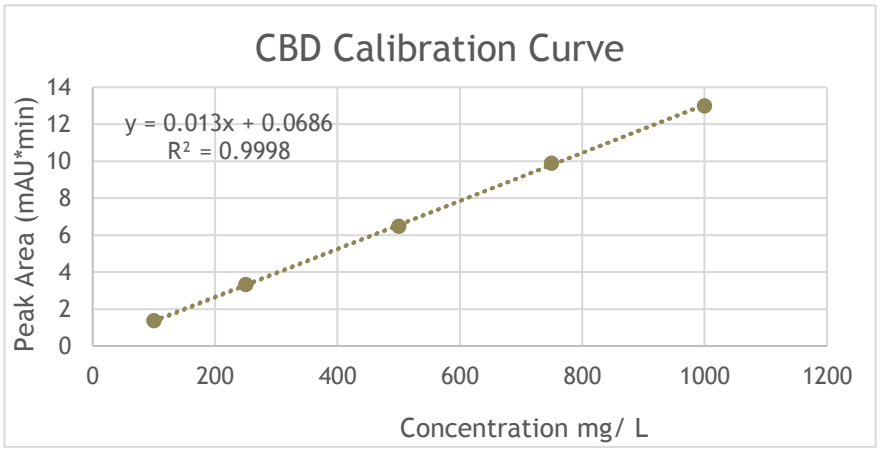
Gradient:

Time (min)	%A	%B
0.0	20	80
0.5	20	80
3.0	0	100
3.5	0	100
3.51	20	80
4.5	20	80

Results

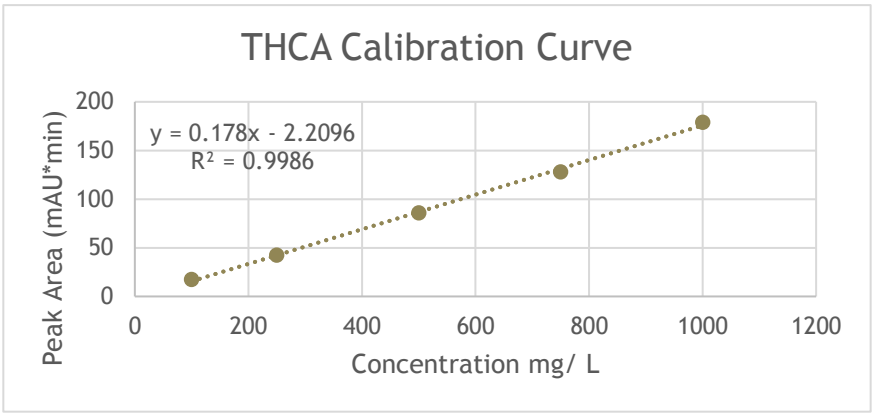
CBD

mg/ L	Area
100	1.3622
250	3.3308
500	6.4891
750	9.8899
1000	13.0037



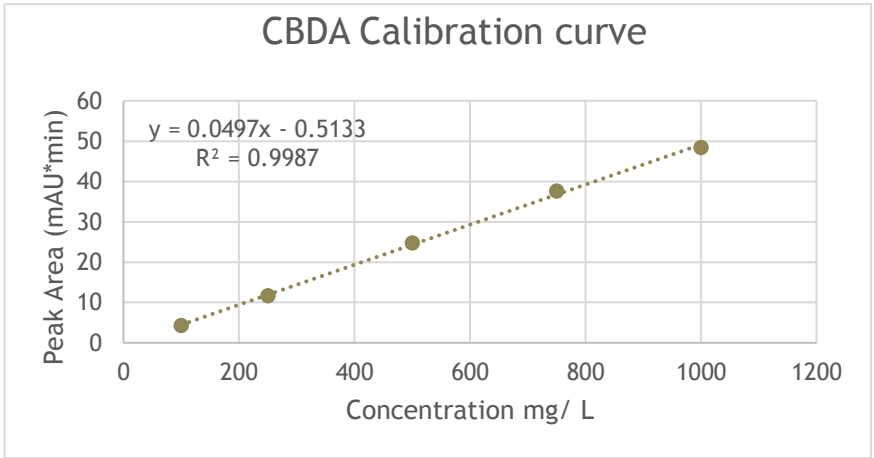
THCA

mg/ L	Area
100	17.1618
250	42.2283
500	85.7601
750	127.8839
1000	178.7212



CBDA

mg/ L	Area
100	4.2352
250	11.5988
500	24.7424
750	37.6178
1000	48.3969



Discussion:

The method conditions can accurately detect and quantify CBD, CBDA, THC and THCA in one run up to a concentration of 1000 ppm. Conditions selected apply to the LC-MS instrument listed and employ features from alternative methods in literature, suggesting the conditions are suitable and could be applied in other laboratory settings where supported. Regarding project progress this now means the acid forms of the cannabinoids as well as non-acids can be separately quantified and detected as needed, depending on the sample available.

The CBDA compound tested in this study was identified successfully by comparison of the TIC with use of literature studies. Looking at the ionisation of the compound, with the CBD1 sample already confirmed, a comparison on retention time studies in the chromatography additionally confirmed the extract is CBDA. The additional presence of a cannabinoid, with a mass of 360, was also detected. The quantity of this is very low and has the same retention time as CBDA suggesting the compound may be Cannabigerolic Acid (CBGA). Separation is not possible as it would require further complex method development to reach CBDA isolated, followed by fractional collection using a prep HPLC. These additional steps would result in product loss, which is an issue as total cannabinoid recovery is already very low. With more starting material and a larger time frame this should be completed to ensure quantification is completed with pure standard, or alternatively with a higher budget to purchase the standard from a reputable source. This is not considered a major hindrance to the project due to the low quantity of the impurity but does mean that the purity is considered compromised and may have some impact on quantifying CBDA in later studies.

The positive detection of each cannabinoid, was completed first with the chromatography conditions to achieve clearly resolved peaks with no tailing or fronting issues. Given the time frame and similarities between each molecule this is a considerable achievement, with the primary goal to detect each cannabinoid in one method being the largest time consumer. This was achieved with an understanding of the conditions necessary and consideration towards factors such as injection volume and column choice as these are vital to achieve resolution.

Mass spectrometry condition optimisation meant time consumption became a factor, ionising all compounds under one set of conditions was a challenge and achieving this required consideration of possible decarboxylation of the acids to be tested for under a range of settings, results of these findings supported the outcome without altering the acid as a concern and can be found summarised in a table in the Appendix D.

This method may be suitable for other cannabinoids such as CBN or CBC as well as their acids, which would be very useful as these are often presented as present in trace quantities or are included in the umbrella of broad or full spectrum of products. This applies to the GC-MS method below (minus the acids) but would require authentic standards of each cannabinoid to assess this properly.

GC-MS Method development

The GC-MS method development began while completing a previous project on cannabidiol studies, comparing content to label claims on e-liquids. The parameters for successful detection of CBD were therefore already carefully considered and meant any improvements were made where possible.

[89]Backflash, which was prominent in these studies, was checked for and overcome during the current selection of solvents and instrument conditions[89]. This was done because backflash is a phenomenon that occurs when the solvent used to prepare the sample expands in the injection port of the GC component as it is vaporised. This expansion is larger than the capacity of the injection port and results in some of the sample and solvent 'flashing' or flushing to any exit available for example the split valve, septum purge or even through the injection port[89,90].

Due to the high temperatures needed for vaporisation in the injection port, the volume that the vaporised solvent and sample occupies is a function of the column head pressure setting and the molecular weight of the solvent. The carrier gas head pressure is required to force the sample vapour into the column to complete the analysis. If there are backflash issues in the GC method, then there is very little consistency between chromatographic results as differences in sample quantity varies between each run[91].

The key signs of backflash are the highly inconsistent peak in chromatography without any degradation of the sample seen, which can be verified with a MS to check fragments produced are consistent within runs. To overcome this a different solvent should be considered, however there are settings such as injection volume and split ratio which can also relieve symptoms[60,87,92].

During this project one of the major adaptions to optimise the method is the column change. Changing from the non-polar Zebron™ ZB-5MS to the Thermo Scientific™ TraceGOLD TG-35MS ensured that the increase of polarity in the stationary phase was able to fully separate CBD and THC while still working to a high enough temperature for all cannabinoids to elute. This is a consideration when working with samples with a mixture of cannabinoids or looking to develop a method to detect and identify all compounds within. Cannabichromene (CBC) is known to co-elute with CBD so having the sufficient polarity for allow for full resolution between compounds greatly improves reliability and data from raw / non GMP test materials with unknown content[91,93].

This change also improved a peak shape issue relating to tailing of the cannabinoids. This was not a major issue but with combined or mixture tests made quantitation sometimes

more difficult to establish precisely and accurately. An improved peak shape with symmetry helps establish single compound identity and method quality when applying to quantification studies.

Specificity: With the LC-MS method, impurities in CBD2 were able to be detected whereas CBD1, in comparison, presented as a pure sample highlighting the ability to detect these impurities. Regarding decarboxylation and acid detection the run of CBDA fully converted to CBD as well as all THCA standards converted to THC and analysed and regarded as such has meant adapting the study when making observations of quantities. In this way the specificity and suitability of the method has been fully assessed and is appropriate for research.

The NMR spectra and TIC from the LC-MS analysis confirm that the presence of impurities can be detected and confirmed as present using the method conditions.

Accuracy:

Due to the budget restrictions, accuracy is inferred from agreement between linearity, precision and specificity which have been established.

Precision:

Calculation of RSD to establish repeatability:

	Mean	SD	RSD%
CBD	13711603.94	213057.5522	1.553848501
THC	16586189.12	349970.0032	2.110008518

TABLE 5: GC-MS RSD DATA

Linearity and Range:

The linearity and range have been confirmed fit for purpose for all standards analysed using the calibration curve. The R values obtained are suitable for the research carried out and range established has been determined by the necessary parameters.

Robustness: Factors of this method including column and temperature settings are specific to the cannabinoids detected. Altering the column to the ZB5-MS may increase tailing issues and lead to co eluting peaks if overloaded sample however this is unlikely to occur and can be resolved by looking at the MS and altering sample concentration.

Limit of Detection (LoD):

The limit of detection of this method is dependent upon the Standard Deviation of the Response and the Slope. This may be expressed as Equation 1, as noted above, with the units as listed below.

σ = the standard deviation of the response

S = the slope of the calibration curve

CBD $= \frac{3.3 \times 213057.5}{13517}$ = 52.0 mg/ L

THC $= \frac{3.3 \times 349970}{18226}$ = 63.4 mg/ L

Quantitation limit (QL):

This is determined in the same way as with the LC-MS method using Equation 2.

CBD $= \frac{10 \times 213057.5}{13517}$ = 157.622 mg/ L

THC $= \frac{10 \times 349970}{18226}$ = 192.017 mg/ L

GC-MS conditions:

TABLE 6: GC-MS METHOD CONDITIONS

Carrier gas	Nitrogen			
Inlet	250 °C	47.7 psi	24.4 mL/min	
Split	10:1	20 mL/min		
Column flow	2 mL/min			
Equilibration time	1 min			
Injection volume	0.5 µL			
Gradient:				
Oven	Rate °C/min	Value °C	Hold Time	Run time min
Initial		150	1	1
Ramp 1	15	300	0	11
Ramp 2	20	320	2	13.167

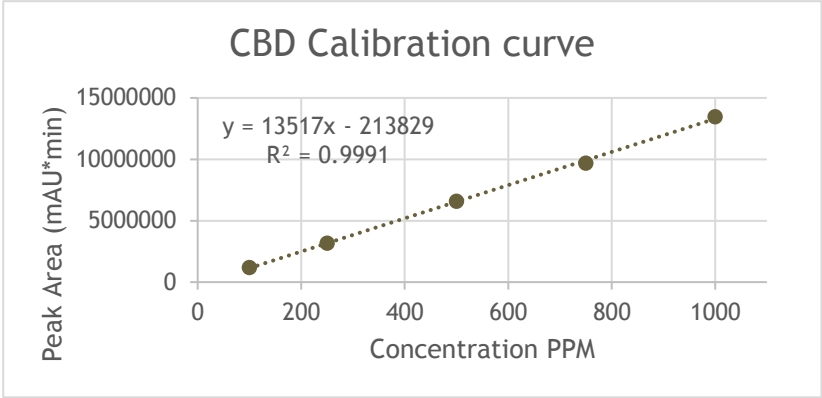
Column 1: Zebron™ ZB-5MS, GC Cap. Column 10 m x 0.10 mm x 0.10 µm. Composition: 5% Phenyl-Arylene, 95% Dimethylpolysiloxane.

Column 2: Thermo Scientific™ TraceGOLD TG-35MS. 0.25 um Thickness; 0.25 mm ID; 30 m Length. 35% diphenyl/65% dimethyl polysiloxane.

Results:

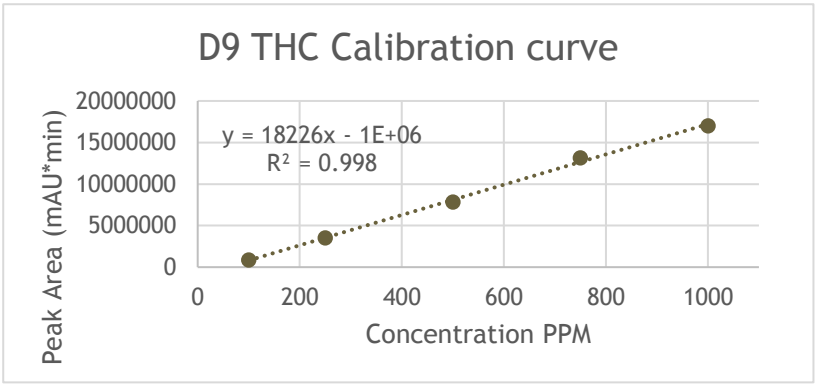
CBD

Concentration ppm	Peak area
100	1192140.17
250	3159484.82
500	6577877.97
750	9679991.59
1000	13465602.4



THC

Concentration ppm	Peak area
100	858094.51
250	3497392.01
500	7837520.75
750	13136475.85
1000	16988259.66



Discussion:

The calibration curve for both CBD and THC achieved a strong R^2 value, very close to 1, suggesting the sample preparation was completed to a high standard and the pipettes have high accuracy and precision when used.

As expected, no THCA was detected as present in the sample, only THC as any THCA or CBDA is decarboxylated during analysis. The process for this, as explained in Chapter 1, meets the conditions of the high temperatures involved in the GC which is why in this study only CBD and THC are identified and quantified. To confirm this is the case a sample of CBDA was also analysed on the GC-MS presenting as CBD, the mass spectrometry for this sample run can be found in the Appendix E.

In consideration of how this effects analysis it has previously been stated that any material known or thought to contain acid cannabinoids would not be accurately quantified or identified on the GC-MS as the quantity would combine with the neutral counterpart and give a false result. If acid quantities are likely to be totalled with their neutral counterparts, or acids are known not to be present within the sample tested the GC-MS is

more than suitable to complete the study and is fit for purpose, specifically for THC and CBD, under the developed method conditions.

The fragmentations at a mass of 299 through the McLafferty rearrangement is a great advantage when developing new studies on the GC-MS as this knowledge ensures positive identification of THC without relying on retention time comparison which may be difficult to achieve. This is very helpful when considering applications to legal checks or quick analysis for a single regulated compound.

During the analysis of THC and CBD no other cannabinoids or other impurities were noted in either standard, further confirming that the samples used to develop the method are pure and fit for purpose. The data supporting this can be found in Appendix E, is can also be noted the GC-MS instrument has an internal library for identification of compounds which has been used to support independent findings and conclusions as this is unavailable on the LC-MS.

Practical testing and application of method:

All quantitation was used by taking the line of best fit equation from the calibration experiments and replacing 'y' with the relevant peak area that is identified as the cannabinoid in question, with 'x' giving the concentration value in ppm. The exact area used for each calculation can be found in the Appendix F with the relevant chromatogram.

TABLE 7: PRACTICAL TESTING EQUATIONS TABLE

Instrument	CBDA	CBD	THC	THCA
LC-MS	$x = \frac{y - (-0.5133)}{0.0497}$	$x = \frac{y - 0.0686}{0.013}$		$x = \frac{y - 2.2096}{0.178}$
GC-MS		$x = \frac{y - 13517}{213829}$	$x = \frac{y - 1E + 06}{18226}$	

Gummy label claim analysis

The aim of this practical test is to confirm the quantity of CBD present in a gummy sweet and compare this to the label claim and packaging.

CBD gummies were purchased from an online store (IceHeadshop) with a label claim of 10 mg CBD present per gummy. These are tested to check that the content matched the claim and check for any presence of THC or THCA and these was not marked as present on the label despite claiming to be made from 'broad spectrum distillate' which implies the CBD contains compounds other than pure CBD. If the other cannabinoids present are part of the 10 mg/ gummy or additional to, is not stated.
Average weight of gummy sweet was taken to be 4424.63 mg.
This means there is a concentration of 10 mg / 4424.63 mg gummy is CBD.

Sample preparation:

611.48 mg of the gummy was sliced off taken and dissolved in 5ml DCM. 5 mL water was then added, and the gummy was left to mix overnight. The organic (DCM) layer was removed using a pipette to extract the cannabinoid mixture. The concentration of this is
Gummy slice CBD content= (611.48/ 4424.63) x 10 = 1.38 mg
Sample concentration = 1.38 / 5 mL = 0.277 mg/ mL or 277 ppm

Method:

The CBD extracted in the DCM from the gummy mix has been made up to a concentration of 277 ppm. This is separated from the water layer using a glass pipette and transferred to a HPLC vial which can be directly analysed on the LC-MS and GC-MS under the stated developed method conditions for each instrument.

Results and Discussion:

The experiment completed followed the method as planned with no need for adaptations or modifications. Considering the use of DCM as solvent for the cannabinoids rather than acetonitrile, this supports the 'robustness' aspect of the method validation as this alteration does not affect the function of the method or its analytical purpose.

Using the concentration of the gummy taken from the label claim, the sample for analysis was made up to 227 ppm as this was well within range of the calibration curve for quantification and provided more opportunity for full CBD extraction from the smaller gummy sweet, due to the size of the vials available and sweet surface area ratio.
The gummy concentration of CBD using the LC-MS was found to be: 141.320 ppm.
The gummy concentration of CBD using the GC-MS was found to be: 385.203 ppm.
The LC-MS data shows the content to be 0.623 % of the label claim which is significantly lower than expected. The quantitation of cannabidiol in this case was particularly difficult as it appeared to co elute with another cannabinoid; likely cannabigerolic acid. This is probable because the mass of 359 is present with cannabigerolic acid[93,94] having a mass of 360 and would present in the 'broad spectrum' as the label states. The decision to take the full peak area in this study was done as the separation of the two peaks was not possible despite altering injection volume in hopes of achieving some resolution. The addition of this cannabinoid did not have large effect on the concentration result however, as manually splitting the peak to higher 313:360 ratio resulted in an insignificant change to the peak area and concentration remained ~140 ppm, as shown below in figures 28 and 29 showing the two different manual peak separation variants highlighted pink.

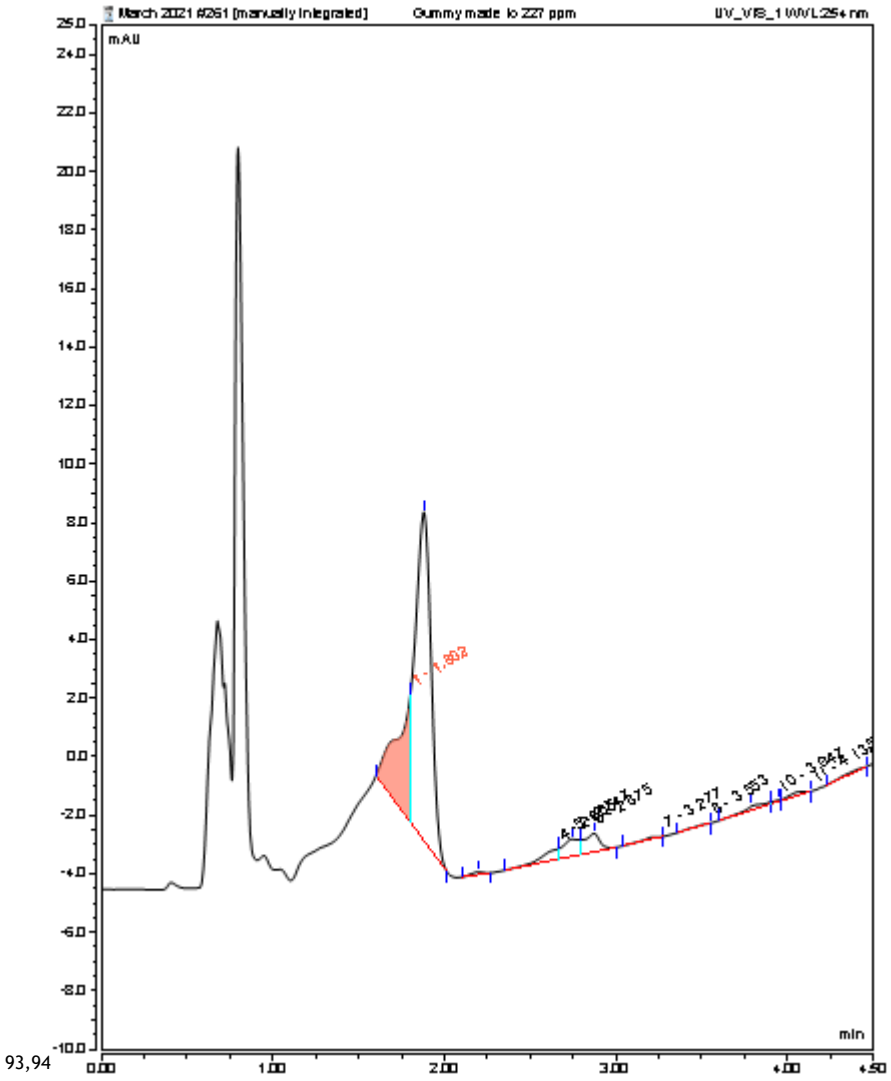


FIGURE 28: GUMMY ALTERED SPLIT 1 OF 2

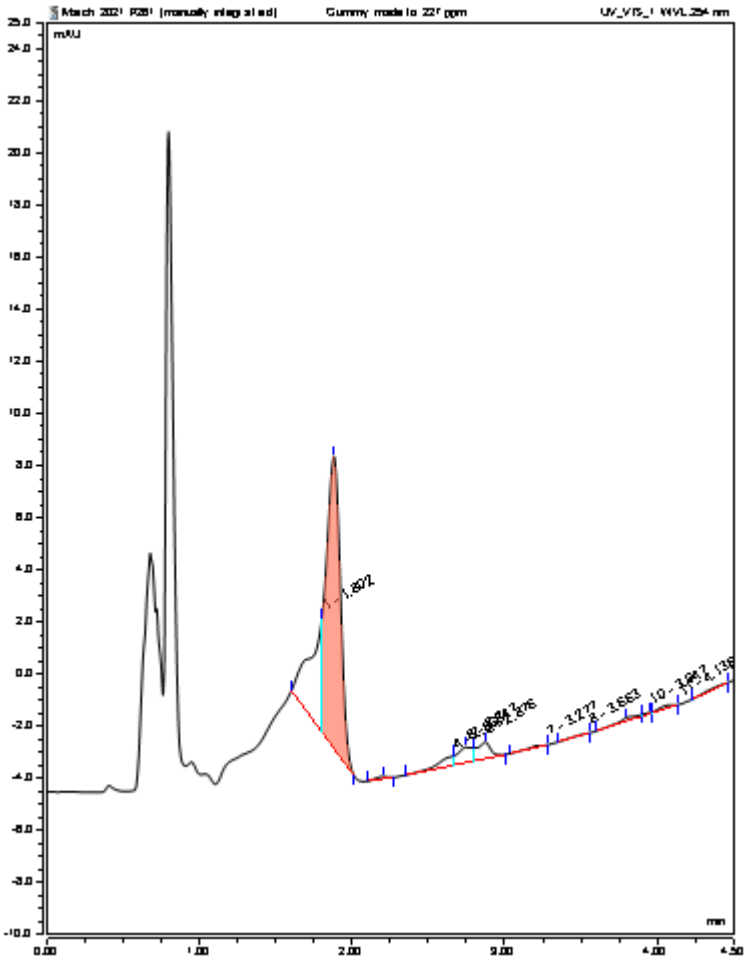


FIGURE 29: GUMMY ALTERED SPLIT 2 OF 2

The full chromatogram without peak split and the TIC's and peak mass spectrometry data has been included in Appendix G, the possible CBGA can also be seen here however is questionable and may require further research into CBD and limitations on quantitation with the presence of additional cannabinoids.

The GC-MS chromatogram appeared to contain only present one peak identified as CBD with no traces of any other cannabinoids present, also in Appendix G. This made quantification easier, however, one reason for this could be that if the CBGA was present as shown in the LC-MS data, it is possible that CBGA could have decarboxylated and metabolised into CBG under the high temperatures which would be difficult to distinguish between CBD in the mass spectrometry. The high quantity obtained of 385.204 ppm is 1.69 times the suggested amount of CBD present in the sweet, a considerable amount that the consumer is not aware of. The GC-MS data showing the single peak in the chromatogram and the relating mass spectra can be found in the Appendix G to support this claim.

Initial concentration of the gummy was found by weighing all 10 gummies to find the average mass of each gummy which should contain 10 mg CBD, to make up a more specific concentration and understand what the label claim meant numerically.
The gummy sweet preparation was chosen due to the sugar content of the gummy sweets interacting and likely be present in considerably higher quantity in comparison to the CBD content if not extracted separately. The polar water layer is necessary to dissolve the sugars, whereas the DCM layer ensures the CBD can be extracted separately. The layers separate due to the different densities, having DCM on the bottom as the denser solvent, 1.33 g/cm^3, and the water at 1 g/cm^3 with the sugars dissolving in the top phase. It is possible that any CBDA present may be dissolved with the water due to its polarity however this is not the focus of the investigation and would further disagree with the label claim if present. All concentrations were derived from the calibration curve Y equation as relevant.

The difference between instrument results shows that quantitation of CBD within the gummy sweet was not possible to confirm due to the difference in values so a conclusion on if the gummy sweet meets the label claim is not possible with confidence. From the LC-MS data it would be reasonable to conclude a shortage of CBD in the gummy whereas the GC-MS would lead to a conclusion of far more CBD than stated on the label which could be potentially far worse than under dosing the product.

Conclusion:

The conclusion of this practical experiment is that each sweet does contain some amount of CBD with no trace of THC present so no risk of psychoactive effects.

More research is necessary to establish if this is perhaps an issue with the sample side or extraction method, for example is one day not enough time for full CBD extraction and in fact the LC-MS results were accurate but clouded by the presence of additional cannabinoids.

The higher result obtained could then be explained through the combination of cannabinoids being presented, or uneven distribution of CBD within the sweet itself which could be possible and would need to be assessed by looking the manufacturing method considering each sweet is meant to be consumed as whole and CBD could be introduced at any stage of the production.

Future work would include analysis of different sweets within the same pack and perhaps a longer extraction time of two days rather than one to assess if higher quantities of CBD are detected in the LC-MS. The separation of CBD is also something to consider however

this would require method development and assessing the possible presence of contaminants and how these could affect the peak shape and chromatography. In terms of ease and efficiency of data processing, the GC-MS chromatogram is far clearer to interpret as only one cannabinoid is present. The lack of peaks suggests this method may not be able to separate the possible other cannabinoids present with the same retention time, which the LC-MS results support.

A possible alternative route of investigation is to contact the supplier and understand what the ‘broad spectrum’ of cannabinoids could include. This may give an idea of what to expect in future and be important to regulation enforcement as the additional cannabinoids may alter the total content of CBD detected, making quantification difficult for companies carrying out the analysis, and enforcing these regulations. Looking specifically at cannabinoid sources and tracing back contamination and methods of production is key to establishing a reliable LC-MS quantitation method.

Hemp seed analysis

The focus on hemp seeds is rapidly increasing in popularity as they become available in most supermarkets and integrated into regular items. The aim of this experiment is to first identify any cannabinoids present within the seeds and then quantify any CBD or THC present (or their precursor acids if present) to assess if these seeds breach any regulations as a food product.

Sample Preparation:

Separate hemps seeds onto sterile surface, transfer to mortar and pestle and grind to create consistent fine powder.
Weigh 235.38 mg sample into dram vial and add 2 mL acetonitrile. Mix for three hours to allow for full cannabinoid extraction.
Transfer mixture into centrifuge vials and centrifuge 6000 rpm for 5 minutes until solid material is separated at bottom of vial. Remove as much liquid possible without disturbing solids and label as plant seed acetonitrile mixture 117.69 mg/mL or 117690 ppm (w:v). Alternatively filter vial can be used to ensure no solid material is carried over.

Method:

Given the uncertainty of the cannabinoid identities or any quantity present in the hemp seeds, at an initial stage the sample is analysed at the stated concentration of plant material to allow for an initial assessment of any cannabinoids present and then adapt sample preparation as needed.

Analysis on LC-MS and GC-MS using the developed method conditions for each instrument are used.

Results and discussion:

Hemp seeds were first separated and cleaned from their shell and any other plant material present and then pulverised with a mortar and pestle to produce a powder. This was done to allow for a more efficient extraction process of any cannabinoids present and ensure consistency in extraction. Any cannabinoids extracted from the seeds should have a reasonable concentration present within detection and quantification range, but this is initially assessed on the LC-MS to confirm presence to assess if dilution is required. There is no risk of decarboxylation using sample preparation techniques although loss of cannabinoids is possible as a recovery assessment has not been carried out as sample yield is not a factor being assessed.

LC-MS: The chromatography presented a peak eluting at 2.457 min with a mass of 360 however the identity of this is unknown although it is likely a cannabinoid such as CBGA as this has a molecular weight of 360. The chromatogram with the peak of interest highlighted pink, along with the mass spectrometry is presented in figure 30 and 31.

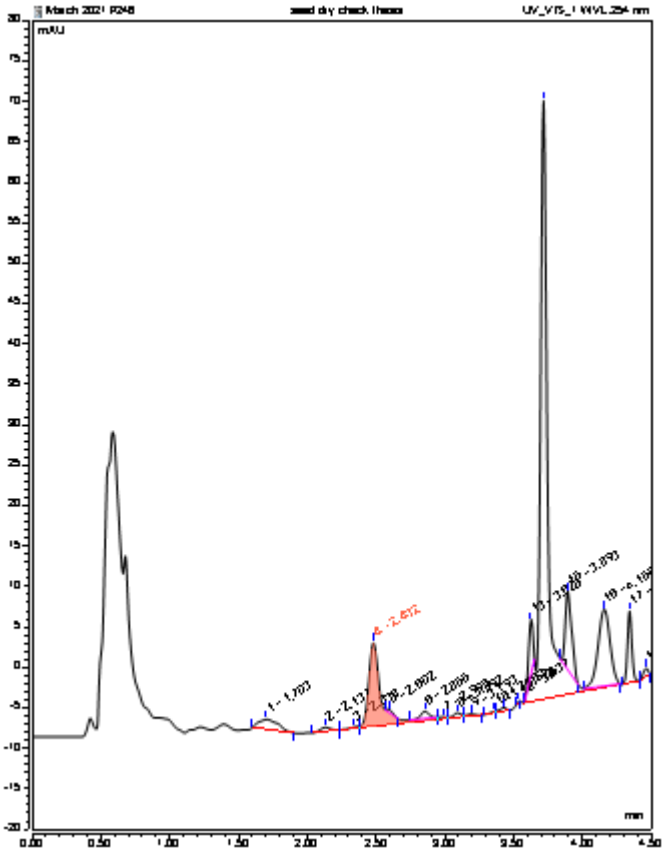


FIGURE 30: HEMP SEED CHROMATOGRAPHY

The next peak of interest identified has a mass of 353 suggesting this could be CBNA eluting at 3.625 min and present at a very low quantity, preventing any chance of purification or fraction separation collection. Δ9THCA was identified next to this peak at a much higher quantity, with a smaller peak eluting just after, likely Δ8-THCA, however this is unconfirmed and requires further analysis for confirmation for example purchasing standards of each.

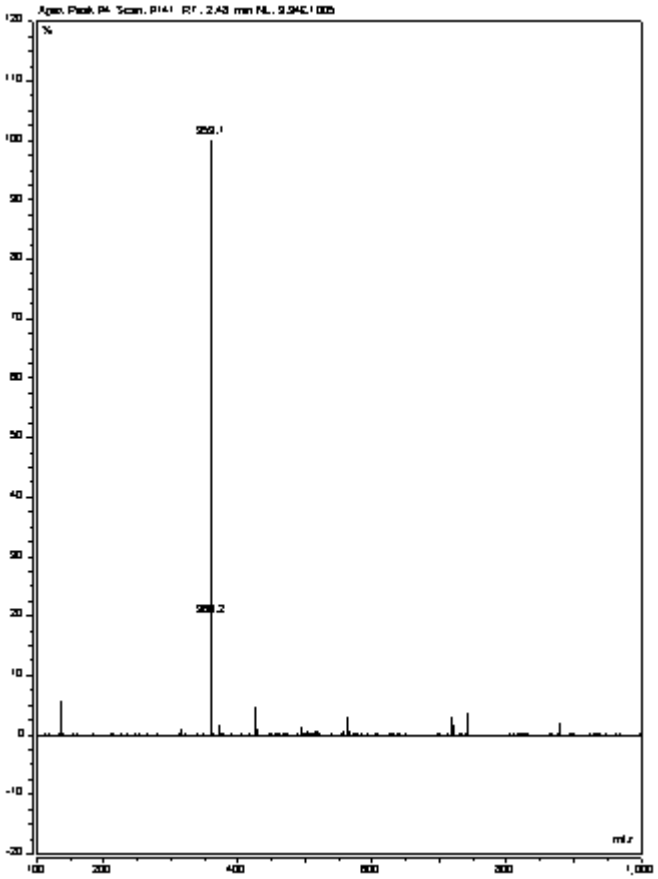


FIGURE 31: HEMP SEED MASS SPECTROMETRY FOR FIGURE 34 HIGHLIGHTED PEAK

The Δ9THCA present was quantified at 39.4354 ppm which is 0.029% by weight, well below the legal limit. This is interesting as no CBDA or CBD was detected in the seeds, suggesting perhaps these were high THC content plants or the CBD had yet to form. The experiment was repeated with a standard blank between runs to prevent any contamination within the extraction solvent, the results were consistent with the data obtained however with no qualitative difference within the LC-MS results.

LC-MS: THC present at a concentration of 39.35243 ppm which presents as 0.029% of the hemp seed content.

The data obtained from the GC-MS results was consistent with the LC-MS data in that the chromatogram was able to identify CBG, CBN and CBC, an example of this given in figure 32, suggesting these are all present but perhaps at different quantities or CBDA perhaps coeluted with CBGA as is known to sometimes happen, which would also explain the broadness of the peak.

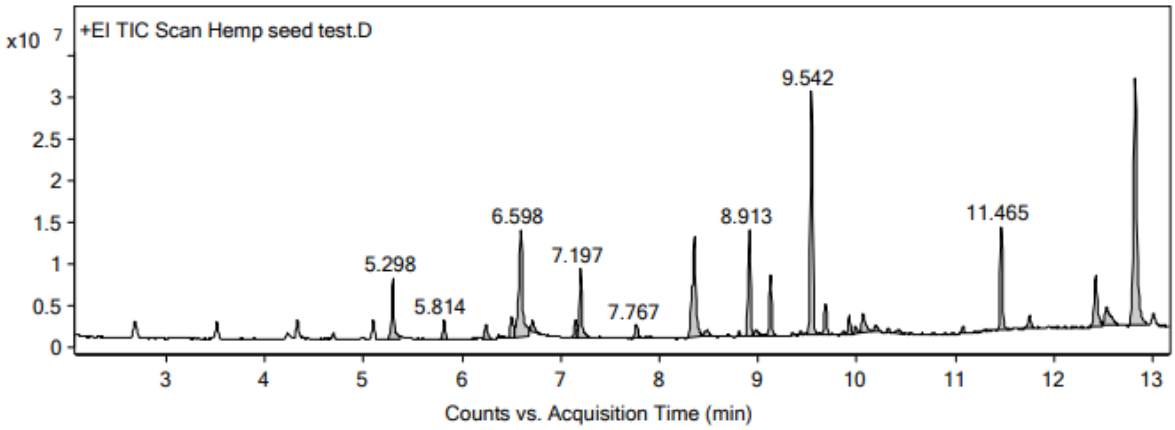


FIGURE 32: GC-MS CHROMATOGRAM FOR HEMP SEED

GC-MS: THC was present at a concentration of 22.073 ppm and CBD was present at 17.041 ppm. The quantitative results are not as consistent with the LC-MS and suggest there may be an error of some sort, however neither result suggests a legal issue or risk to the consumer of psychoactive effects.

The difference of quantitative results from the GC-MS to LC-MS could be due to a range of factors including degradation of the cannabinoids in the heat of the GC-MS which is always a possibility when there are a range of cannabinoids present and the sample analysed is not pure.

The LC-MS could also have some cannabinoids or fatty acids, as detected via the GC-MS, which could co-elute and combine to increase the peak area. Further investigation into what is possibly causing these discrepancies is needed to ensure this is perhaps an operator or instrument error.

Ideally this experiment would also be repeated with a variety of hemp seed brands and sources, with full knowledge of the storage conditions of each seed and date of harvest to study if the composition changes over time or remains constant, and if at any point the seed does contain higher levels of cannabinoids. This would also be studied across the different instruments to confirm consistency between the values obtained and assess the likely hood of the decarboxylation having such an effect on quantity or if this relates more to possible coelution / impurities altering quantitation studies.

Comparison of Plants

An initial study to establish if the extraction method achieved the best results via stirring of plants or use of a sonication bath is carried out as these have been the most popular techniques between past studies carried out but do not have a consistent 'best' approach. For consistency and to check for any decarboxylation in results a preliminary study on which to use is followed by a study comparing ratios of cannabinoid content per plant part, using the ideal extraction method. This will be a qualitative study to support literature suggesting certain plant parts may contain on cannabinoids or most cannabinoids using quantitation.

Extraction method confirmation:

Sample Preparation:

300 mg~ Fan leaf plant material prepared by cutting sample into equal sizes, roughly 1 cm^2 surface area and separated equally into two 10 mL dram vials. 5 mL pentane was then added to each vial.

In one dram vial a magnetic stirrer bar was added and left to stir at 300 rpm for one hour.

The second dram vial was left to sonicate for an hour.

Method:

An aliquot of 1 mL was taken from each vial to a 100 mL volumetric flask and diluted to volume with pentane. This was then analysed on the LC-MS and GC-MS using the validated method on each instrument.

Data:

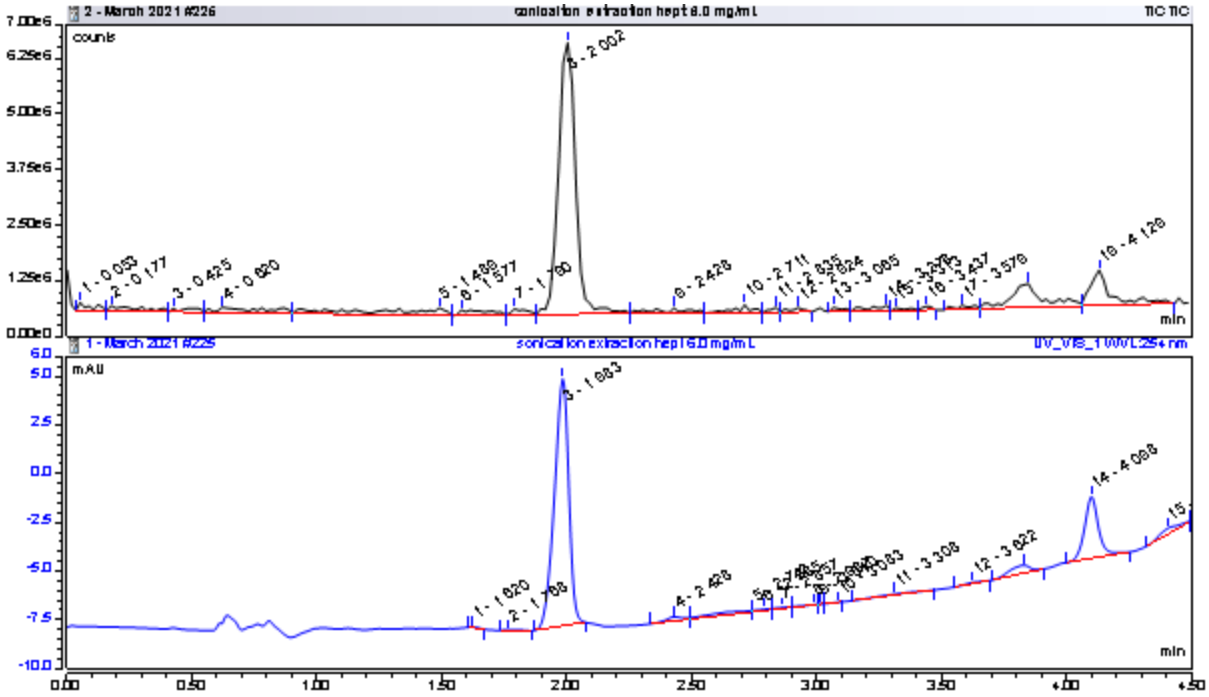


FIGURE 33: TIC AND CHROMATOGRAM FOR SONICATION METHOD

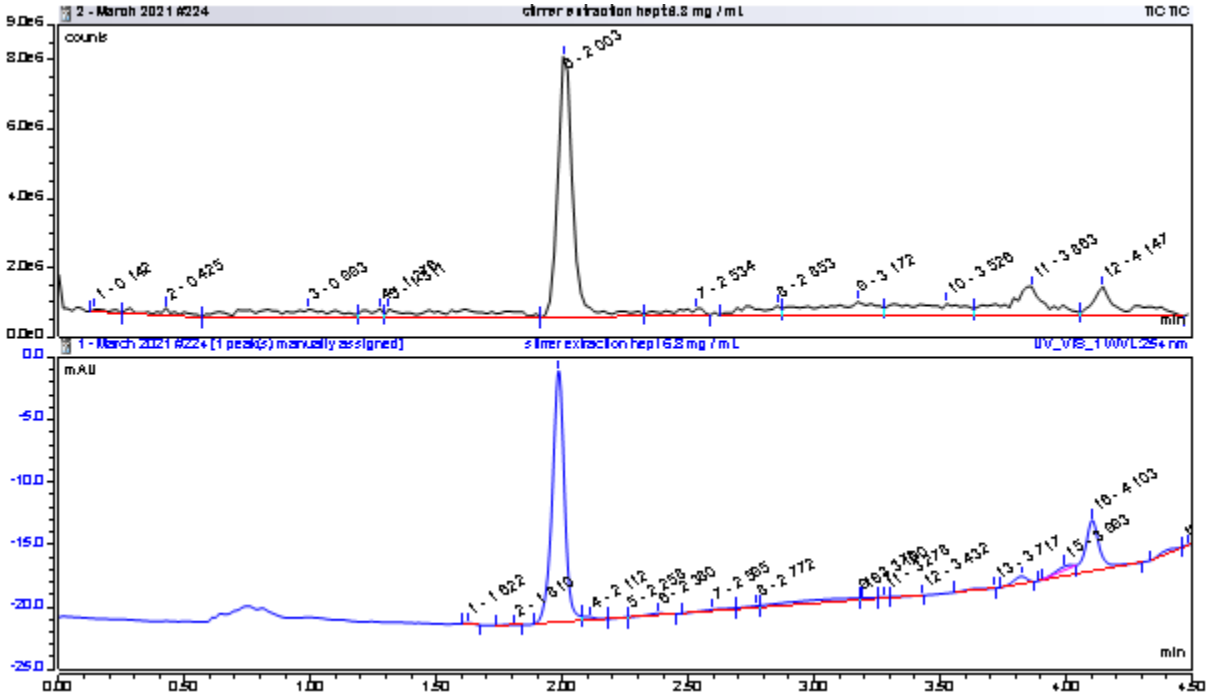


FIGURE 34: TIC AND CHROMATOGRAM FOR STIRRING METHOD

Both sets of data showed the same standard of quality and content tested to confirm there was no contamination or additional compounds present that could affect quantity of CBD to be analysed.

TABLE 8: DATA OBTAINED FROM SONICATION AND STIRRER EXPERIMENT

Method	Plant mass	Start Concentration	Aliquot concentration tested	Recorded concentration from LC-MS quantitation
Stirrer bar	343.31 mg	68.66 mg/ mL	6.8 mg/ mL	0.033 mg/ mL
Sonication	300.85 mg	60.17 mg/ mL	6.0 mg/mL	0.027 mg/ mL

Results and Discussion:

The two methods show a very similar recovery of cannabinoids with the stirrer bar proving to be more successful with a higher concentration extracted in total. The sonication method did not show any signs of decarboxylation or to have any impact of the sample structure as confirmed by the mass spectrometry data.

This was a simply preliminary analysis to ensure best practice of sample preparation was achieved, the additional analysis on GC-MS was not deemed necessary as the LC-MS showed reliable data relevant to moving forward with the next step in the experiment.

This could be developed into a far more in-depth study in future altering the solvent used for extraction, time frame and different surface area of the plant material to give an understanding of what has a larger impact on increasing the extraction ability. The RPM of the stirrer bar, make of the ultrasonic bath, and wave frequencies could also be altered as factors. Pentane was used due to availability of the solvent, as well as the understanding that as it is an extremely non- polar solvent, pentane can dissolve cannabinoids within plant matter[17,28,48].

Cannabinoid content Analysis

Sample preparation:

Separated plant material into part and label in a beaker accordingly. Using a scalpel, or something sharp, break material into equally sized pieces as possible to achieve an equal surface area between each sample as possible.

Transfer the material into a vial with the specific plant part and mass labelled.

Add in solvent as necessary to allow for the material to be submerged. See table 10 below for exact quantities used.

TABLE 9: QUANTITIES OF SAMPLE AND SOLVENT USED

plant part concentration	mass of plant material mg	solvent added mL	mg/ mL
Seedling 1	47.81	2.5	19.124
Stems	2093.44	10	209.344
Fan leaf	787.82	15	52.521
Sugar leaf	372.77	10	37.277
Bud	917.03	10	91.703

Add in the stirrer bar and leave to extract for 1 hour at 300 rpm.

Using a glass pipette, a 2 mL aliquot was taken and placed in a centrifuge 3000 rpm for 3 minutes.

Method:

Using a new glass pipette transfer as much of the top later with no solid material possible to a HPLC vial for analysis without disturbing the solid material at the bottom. Label the vial accordingly.

This was then analysed on the LC-MS and GC-MS using the validated method on each instrument.

Results and Discussion:

Below is a table 10 containing the qualitative results which represent the outcome of the study. This was achieved following calculations of the concentration in relation the mass of the material. A full table of quantities and calculations to support this can be found in Appendix H.

TABLE 10: PLANT PART CANNABINOID CONTENT RESULTS (HIGHEST CONTENT AT TOP)

LC-MS	GC-MS CBD	GC-MS THC
Bud	bud	bud
Sugar leaf	sugar leaf	seedling 1
Seedling 1 & 2	fan leaf	Sugar leaf
Fan leaf	stem	fan leaf
stem	seedling	stem

This was initially a quantitative study with use of quantitative techniques to achieve an understanding of the plant parts and the differences between their cannabinoid content.

There were however several interesting outcomes to this study and issues to overcome. This first being that the 'bud' sample had to be diluted to half the concentration to then accurately take a measurement as initially the LC-MS results were off scale, hence 'bud halved' in Appendix H.

The 'seedling' sample was also made up in a second vial to support the outcomes and ensure there were no major discrepancies. This was done as a precaution as a single seedling was used in each sample which could potentially lead to a unique result if only one was to be analysed, this is referenced in Appendix H table as 'Seedling 2'. This results from each seedling were consistent with each other supporting results found.

Data obtained from this study has excluded THCA as this was not present above the LOQ or LOD on the LC-MS for all plant samples.

The order of higher plant parts with a higher cannabinoid content ratio was consistent with what literature suggests, specifically with bud containing far more THC than other parts of the plant, followed by the sugar leaves having the second highest THC content. This is likely due to the higher content of trichomes present on the sugar leaves and bud, trichomes on the plant are tiny mushroom or hair shaped growths on the plant, originating from the Greek work Tríchōma meaning growth of hair[72,95]. These are gown on the plant as defence mechanisms as they contain very high concentrations of cannabinoids to deter insects from eating them[95].

The stems specifically contained what appeared to be mostly CBDA with some THC (below LOQ) in the LC-MS results whereas the GC-MS data showed there was more THC present than CBD. Conflicting results such as these suggest more investigations into how CBDA degrades is needed before relying completely on one instrument only. Having two sets of data to compare mean that conclusions made can be done with full context, however, in this case, pose more questions as to why this occurred. One reassuring feature is that the data was consistent in that the cannabinoids detected with GC-MS included traces of CBC, CBG and occasionally CBN, and the LC-MS presented possible traces of CBGA, CBNA and THCA Δ8. These corresponding compounds support the possibility for developing each method to perhaps detect and quantify these. Below in figure 35 is an example of the CBC

found in the analysis of bud in the GC-MS that was not present in the LC-MS results[72,95].

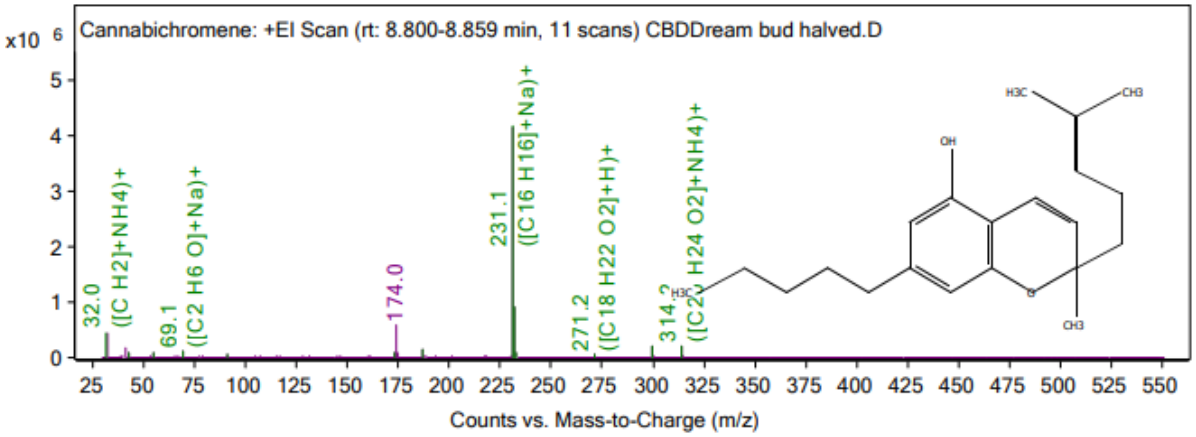


FIGURE 35: EXAMPLE OF CBC DETECTED IN BUD SAMPLE

These are all unquantified and unconfirmed however from literature comparisons and knowledge of cannabinoids these are the most likely results. Figure 36 is the LC-MS Chromatogram and TIC for the Stem plant part to illustrate this, and figure 37 the GC-MS Chromatogram from the same stem plant sample.

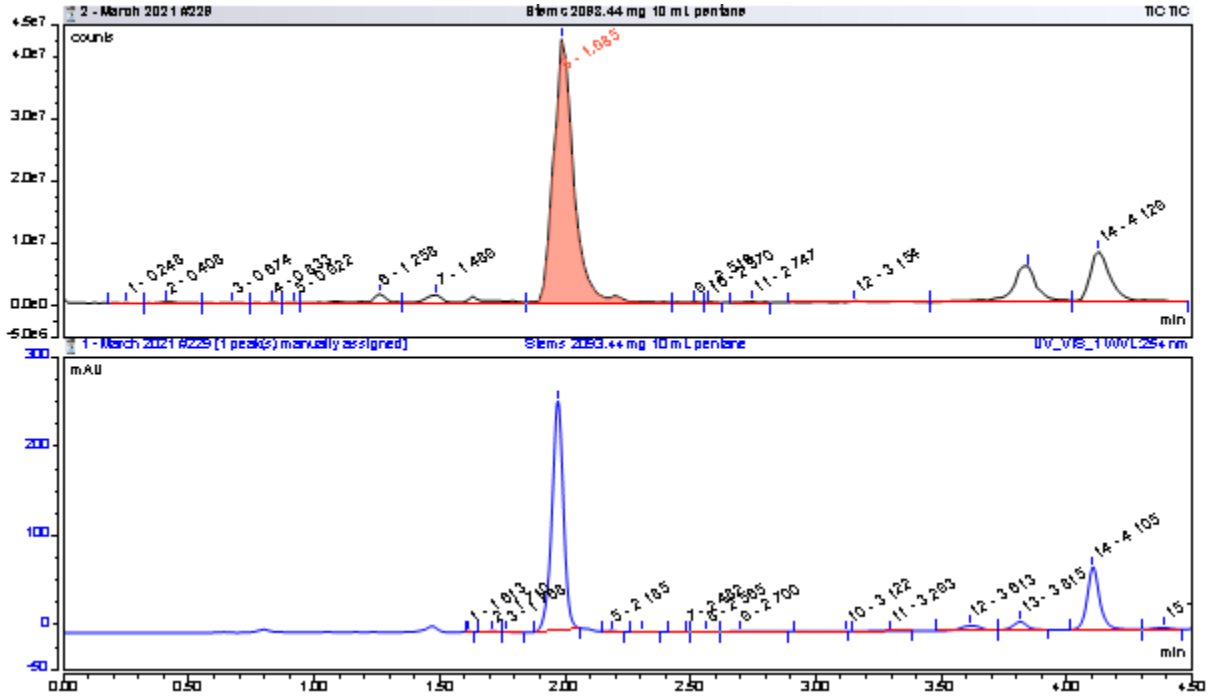


FIGURE 36: (LC-MS) TIC AND CHROMATOGRAM OF STEM PLANT PART

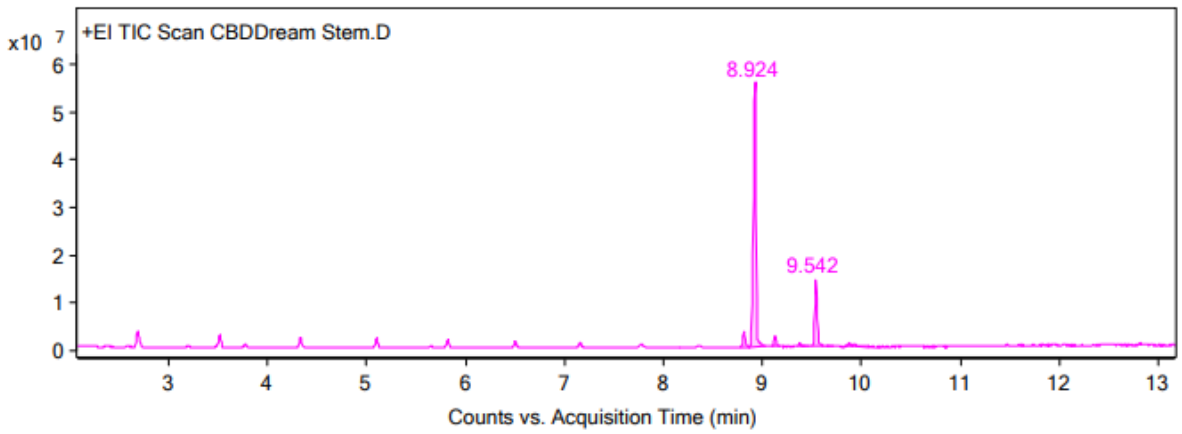


FIGURE 37: (GC-MS) CHROMATOGRAM OF STEM PLANT PART

This practical test is simply an analysis to gauge what the content of cannabinoids are, within different plants of the plant, to compare with literature where it is suggested cannabinoids would not be present at all. The results from this experiment are supported by literature in that plant parts with large numbers of trichomes contained the greatest number of cannabinoids whereas parts such as the stalk contained very little cannabinoid quantity.

It should be noted that cannabinoids other than those specifically able to be quantified were possibly present, such as the fan leaf which has some smaller additional peaks that have molecular ion masses ranging from 301-390. These suggest a presence of a range of other cannabinoids possibly in transition stages and require further identification.

Chapter 4

Terpene analysis:

Introduction

The aim of this study was simply to produce a profile for some plant material by using GC-MS Headspace, with use of a SPME fibre to specifically identify the terpenes present. Analysis of the data to list the terpenes present in each sample and see how this effects the smell and note any differences between the results. Smells and volatile and profile for each strain. Having two high THC content samples, a comparison between these and the CBD content samples would be interesting to compare as well as looking at the difference of terpenes in each plant part.

Sample profile experiment

Sample Preparation:

For each sample, no preparation was necessary as the plant material was analysed as receive with conditions noted on table.

Plant masses were weighed out to 3 g each in headspace vials as this is a qualitative rather than quantitative study.

Method:

The GC-MS conditions remain the same as liquid sample injection with the addition of the Headspace parameters:

TABLE 11: SPME HEADSPACE CONDITIONS

Headspace conditions	
Fibre:	polydimethylsiloxane / Divinylbenzene (PDMS/DVB), 65 µm, Fused silica SS
Pre-Incubation Time	30 min
Incubation Temperature	50 °C
Vial Penetration	22 mm
Extraction time	20 min
Injection penetration	54 mm
Desorption time	3 min
GC Run time	30 min

Results:

Results, fill table in appendix I, have shown that the profiles were simple enough to make, however very specific variations of terpene structures were identified making identifying

differences between samples harder to spot. It was expected that Myrcene would be present in each sample which was found as true. That said this work study would have many variables which were out of my control due to the source of my samples including specific information of the material analysed. The full table of these results are available in the Appendix I.

Age of the plant, conditions of growth, strain type (heavily CBD or THC) and repeatability between experiments would need to be achieved. This was only an initial study to understand that there are such a range of terpenes present and these all effect the smell and likely encourage a range of effects if inhaled due to the entourage effect. The investigation in future work is vast and could be very useful to police as there were differences between samples so a profile, even a generic one, could in theory be made up.

Determination of Terpenes in Plant Structures

Introduction:

As above looking ratios of cannabinoids present in different parts of the plant, a study into how there are different terpenes available in different parts of the plant was completed. This could provide significant information as terpenes are vital to achieve the entourage effect properly and receive as many benefits form the plant properly. The extraction of specific terpenes and sale is already being monetised in the USA with focus on Myrcene and the main set of terpenes known to have a specific set of effects on the body.

Coupling this with the cannabinoid study, if there are specific cannabinoids present in parts of the plant where there are low levels of cannabinoids this could result in development or interest in other parts of the plant that have previously only been utilised in hemp for structural and functional uses.

Sample preparation:

Material was separated into plant parts and labelled, before being weighted to 3g and placed in a headspace vial. The surface area was not altered and was simply analysed directly on the instrument.

Method:

The same method conditions as with the profile terpene study was applied with no alterations necessary.

Results and Discussion:

A full table of all plant parts and their terpene content detected is available in the Appendix J. The most common terpenes were found present in all parts of the plant which was expected: pinene, myrcene, limonene, linalool, beta-caryophyllene, alpha-bisabolol, eucalyptol, trans-nerolido, humulene, Δ 3 carene, borneol, terpineol, valencene, geraniol and ocimene. There were of course some variations between beta and alpha presence of the cannabinoid, but this was expected.

The overall result was that every part of the plant did contain some terpenes which was very interesting as parts such as the stalk which do not have a potent smell were not expected to contain large amounts. This could also be due to the SPME fibre able to detect very small quantities present. In future a quantitative study could be useful to support possible opportunities for extraction of terpenes and an understanding on how the content of varies between plant parts.

Chapter 5: Complete results and discussion

Experimental outcomes

The first step of the quantification process was to confirm identification of the cannabinoids to be involved in the project using NMR as the main analytical instrument for this, incorporating H^1 NMR, C^{13} NMR, COSY and HSQC. This allowed for a successful and reliable confirmation that the samples carried forward for the project were of a purity fit for purpose. The experimental procedures were carried out quickly and efficiently allowing the spectrometry and data produced to be processed over time.

The method development of the LC-MS quantification technique took far longer than the GC-MS technique due to many factors. There was a delay in the middle of the project just as parameters were being established due to the COVID-19 pandemic closing the lab space. To avoid any possible degradation due to storage conditions or factors yet unknown samples were re-made on return despite stability studies suggesting they would still be appropriate to use. Ongoing technical issues with the LC-MS instrument itself causing further delay in progressing with the method development. Once these were overcome parameters of the method were fully optimised for the specific cannabinoids. While purifying the cannabinoids on the prep HPLC, method development was involved to ensure the cannabinoids could be successfully collected with as little contamination possible. It is a concern that transfer between the fraction collection containers and evaporating bowl may have resulted in some product loss, as well as initial studies on the LC-MS requiring sample collected to confirm the fraction collected was correct, although this was a low volume of less than 1 mL and was required before proceeding with further prep HPLC method development to prevent extraction of an irrelevant compound.

Use of LC and GC independently was considered during the research phase as this has proven successful when retention times and order of elution is known based on compound properties. In this case this was not successful as co-elution and initial identification of CBDA required the mass spectrometer to identify the compound and was vital in spotting the cannabinoid impurities present.

The mass spectrometry is also vital in gas chromatography studies to prevent confusion between THC and CBD as they have such similar retention times. Altering the method could provide different results in comparison to literature studies and lead to confusion over identity which would not be good if applied to products or for checking content.

Including the mass spectrometry in this project has been vital to ensure the identity of the compound and ensure its purity, which is key to tracking any degradation throughout as

any new products would be quickly identified and the integrity of the project could remain intact. As there were no degradation products identified throughout the study, this suggests storage conditions of a closed dram vial in an 18 °C drawer (dark) was appropriate for at least 2 years for the CBD extract and one year on THC, THCA and CBDA, as this was the duration of analysis and storage.

The use of a variety of analytical instruments has been successful overall to develop methods of quantitative and qualitative parameters. NMR was particularly successful in analysis with proton, carbon, COSY and HSQC completed with no conflicting results or differences between studies. Although not included in the identification section an additional proton NMR analysis was carried out at the same time as the HSQC and COSY to ensure no changes to the sample had occurred, this was confirmed with the proton NMR and presented a very reliable view on use of NMR for cannabinoid identifications. It may be appropriate, in future to carry out a quantification study on the NMR, but this technique requires large sample quantities to allow for complete analysis and rateability studies. This would also be successful as acids are not decarboxylated within the instrument so although they are not clearly identified in every analysis the oxygen groups can still cause enough of a difference in shift to successfully differentiate between acids and neutral cannabinoids, as was possible in this case with THC and THCA.

Use of the SPME fibre in the GC-MS headspace analysis was also particularly successful with results supported by literature and interesting find through the profile study and comparison of plant parts. These supported Myrcene as being the most common terpene, which is well established in literature, however this does not validate the data found to be applicable to every study. Terpene development in plants has many variables so a future study into establishing what these variables could be, followed by quantification comparisons could encourage studies into the entourage effect and how this competition for CB1 and CB2 receptors works when different compounds are present in different quantities, and how best to apply this to medial uses.

A full decarboxylation study looking into mild conditions over longer time periods could also be completed as this could help understand what point a plant contains mostly or even only, CBN, which would make the plant material entirely legal but lead to perhaps a whole revision of cannabinoid laws in the UK, and US. This would be suggested as a positive step regardless as regulations on CBD in foods are coming into place and becoming more and more popular in the media. Glamorisation of cannabinoids has already become commonplace in media, exaggerating the positive effects and capabilities of the compound leading to misconceptions between consumers. A situation such as this, if left

unregulated, is potentially dangerous to the public when considering that this plant has such a long and rich history of use, and extortion has been fundamental in different ways to civilisations.

Beginning with stability studies and branching out into specific areas of interest, such as terpenes, plant part studies, comparison of extraction methods and total identification and quantification of cannabinoids present, are just some of the future studies this project has completed as an initial undertaking. The results from these studies have collectively been positive in terms of instrument capabilities, overcoming and understanding limitations. The clear detection of the chosen cannabinoids allowed for detection of the selected cannabinoids to confirm against a label claim. Extraction methods within plant parts confirmed the methods were successful in their detection and analysis of cannabinoids as part of the aim.

With a method for quantification of selected cannabinoids developed on both the GC-MS and LC-MS being validated and tested out on real world samples successfully, the next steps could involve use of a triple quadrupole mass spectrometry to allow for a more definitive result when analysing a mixture of cannabinoids.

Some issues that need to be addressed upon progressing any of the experiments is sample preparation method dependent on the sample being analysed. This is because the extraction method may need to vary depending on what may be present in the sample and consider any limitations that altering the extraction method may have on recovery of the cannabinoid analysed.

Analysis of the products with set label claims of cannabinoid content proved that the technique used to quantify may produce a different result, varying from over to under the claim. This suggests that there may be a sampling issue, recovery issue or perhaps the products do not meet their requirements. This is true for the gummy sweet specifically, so should be considered a major issue in foods claiming to contain CBD, although no THC was detected in the samples analysed in this study, as products reach the market untested this lack of consistency poses a huge risk the public suggesting investigations such as these are necessary and should be further developed for regular use. In conclusion more carrying out analysis of cannabinoids in foods requires more work and development in order to be achieved and may have more factors to consider in comparison to content analysis of other samples.

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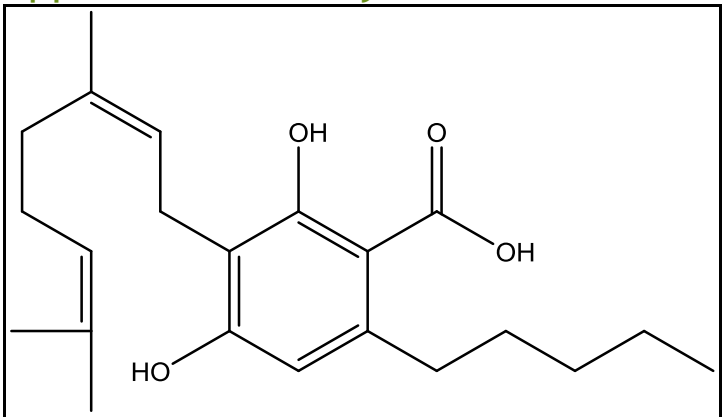
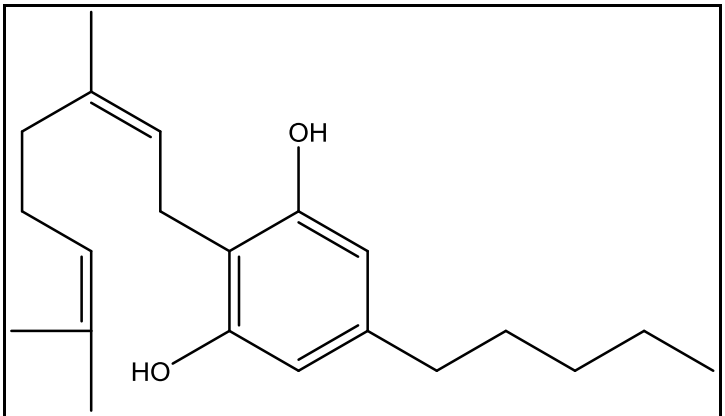
Appendix

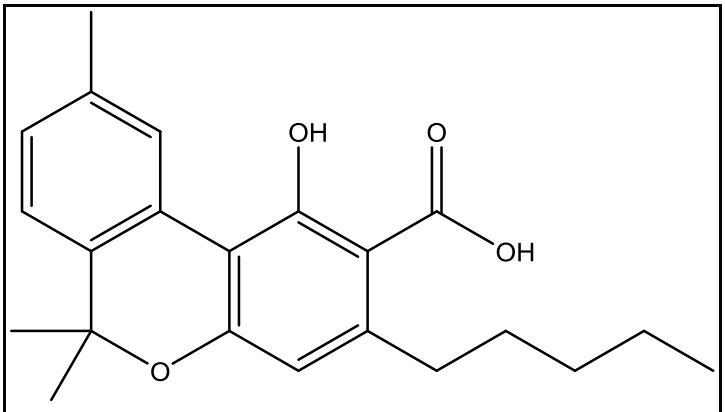
Appendix A: Library of Cannabinoids



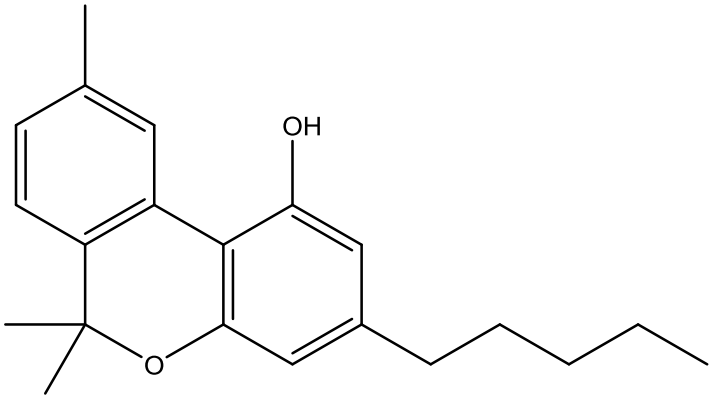
Cannabigerolic Acid (CBGA)
Exact Mass: 360.23



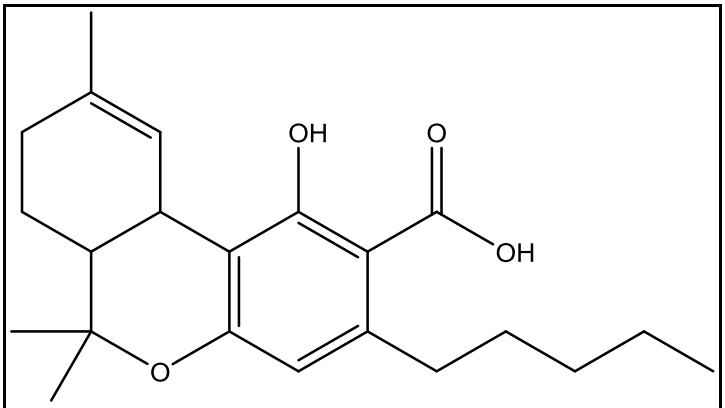
Cannabigerol (CBG)
Exact Mass: 316.24



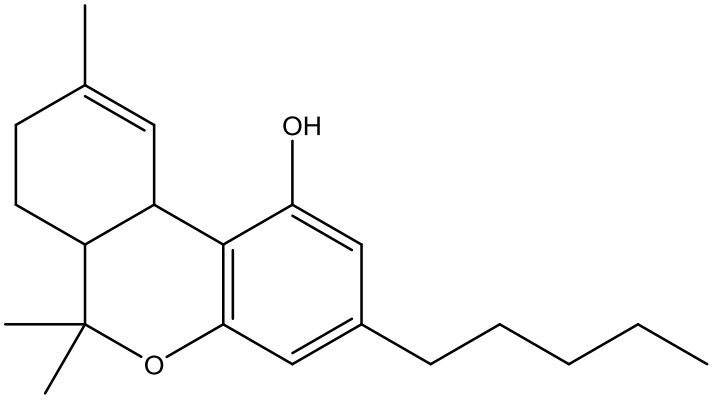
Cannabinolic Acid (CBNA)
Exact Mass: 354.18



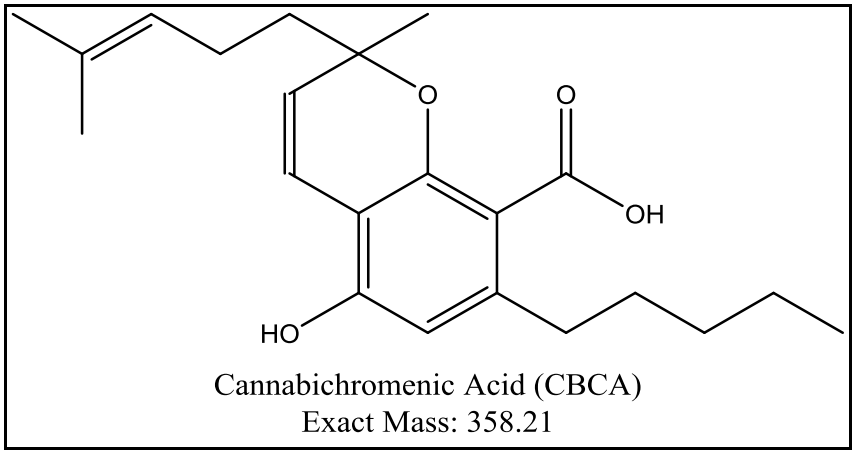
Cannabinol (CBN)
Exact Mass: 310.19



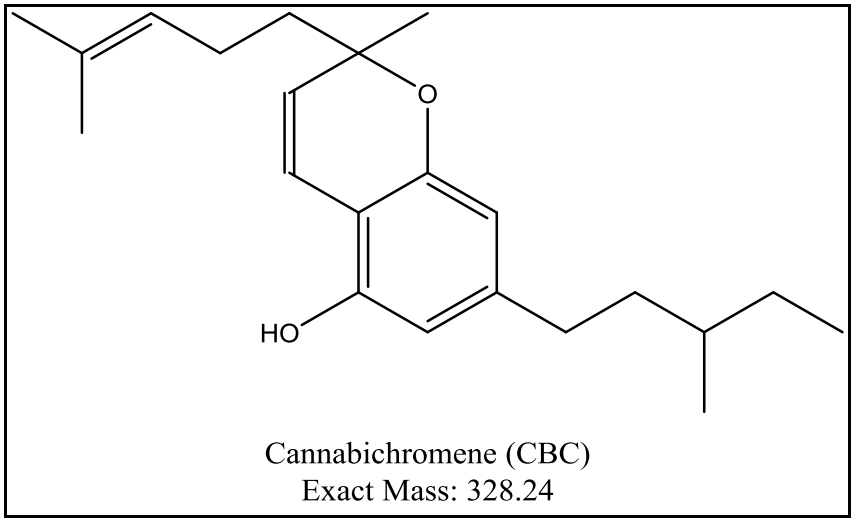
Tetrahydrocannabinolic Acid (THCA)
Exact Mass: 358.21



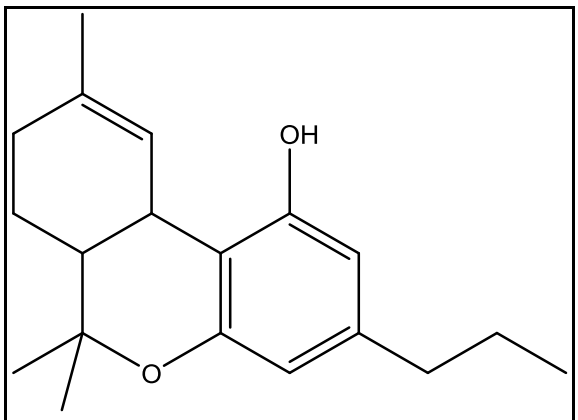
Tetrahydrycannabidiol (THC)
Exact Mass: 314.22



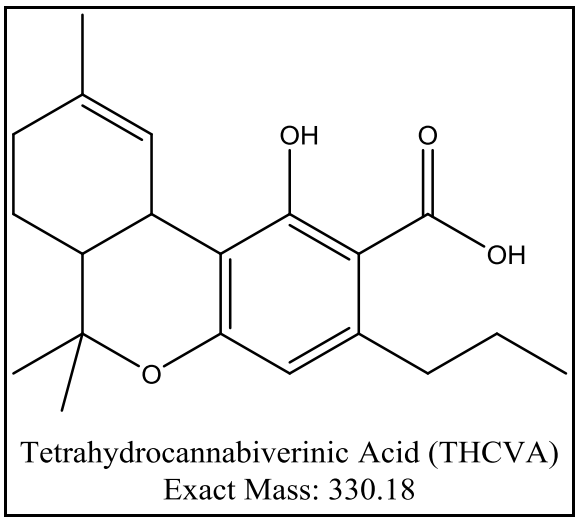
Cannabichromenic Acid (CBCA)
Exact Mass: 358.21



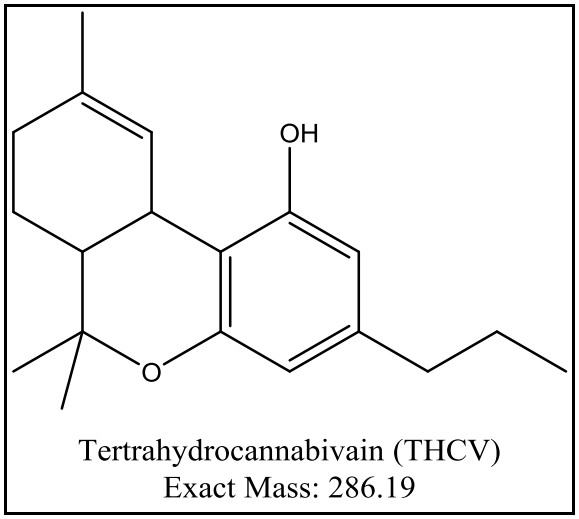
Cannabichromene (CBC)
Exact Mass: 328.24



Tertahydrocannabivarin (THCV)
Exact Mass: 286.19



Tetrahydrocannabiverinic Acid (THCVA)
Exact Mass: 330.18



Tertrahydrocannabivain (THCV)
Exact Mass: 286.19

Appendix B: Full NMR Spectra for CBD1 and CBD2 and Tables

CBD1 AND CBD2 1H AND 13C ASSIGNMENT TABLE

Position	CBD1		CBD2	
	^{1}H-NMR ppm	^{13}C-NMR ppm	^{1}H-NMR ppm	^{13}C-NMR ppm
1	3.8328 1H, s	37.5000	3.8563 m	37.3036
2	5.5606 1H s (j=7.5)	124.3014	5.5659 1H s	124.3616
3		140.2540		140.1277
4	2.0622 1H m		2.2179 1H m	31.6698
4	2.1069 1H m	31.6766	2.0852 1H m	
5	1.8119 m	28.6032	1.8183 m	28.5726

6	2.3915 m (ToD)	46.3259	2.372 m	46.3549
7	1.7913 3H s	23.8709	1.7855 3H s	23.8478
8		149.6243		149.4343
9	4.553 1H m	20.7557	4.5441	20.5627
9	4.6551 1H m		4.6438	
10		111.0162		110.9954
1'	1.6443 3H s	113.9313	1.6539 3H s	113.9435
2'				
3'	6.2582 1H brs	111.0162	6.2392 1H brs	110.9954
4'		143.2533		143.175
5'	6.1716 1H brs	111.0162	6.1787 1H brs	110.9954
6'				
1"	2.4301 2H t	35.664	2.4313 t	35.6617
2"	1.5495 2H m	30.592	1.5538 qui	30.5751
3"	1.2903 m	30.8183	1.2963 m	29.1082
4"	1.2903 m	22.7275	1.2963 m	22.7156
5"	0.8678 3H t	14.2235	0.8725 m	14.2063
2' OH	5.9571 s		5.96 s	
6' OH			4.9629	

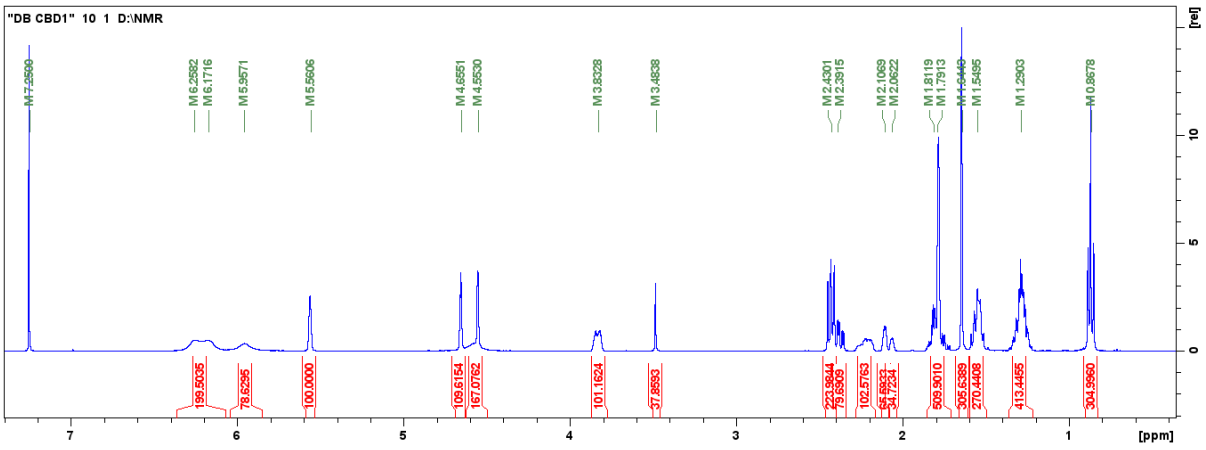
Δ9 THCA AND THCA MIX 1H AND 13C ASSIGNMENT TABLE

Position	**Δ9 THCA**		**THCA Mix**	
	^{1}H-NMR ppm	**^{13}C-NMR ppm**	**^{1}H-NMR ppm**	**^{13}C-NMR ppm**
1	3.2278 dm	33.6547	3.2277 dm	33.6539
2	6.392 m	123.8002	6.3916 m	123.8029
3		134.0409		134.0379
3-me	1.6832 3H s	23.5293	1.6825 3H s	23.529
4	2.1759 m	31.4071	2.1716 3H s	31.4071
5	1.9205 m	25.1823	1.9237 m	25.1823
5	1.3437 m		1.3433 m	
6	1.6624 m	45.7908	1.6626 m	45.7948

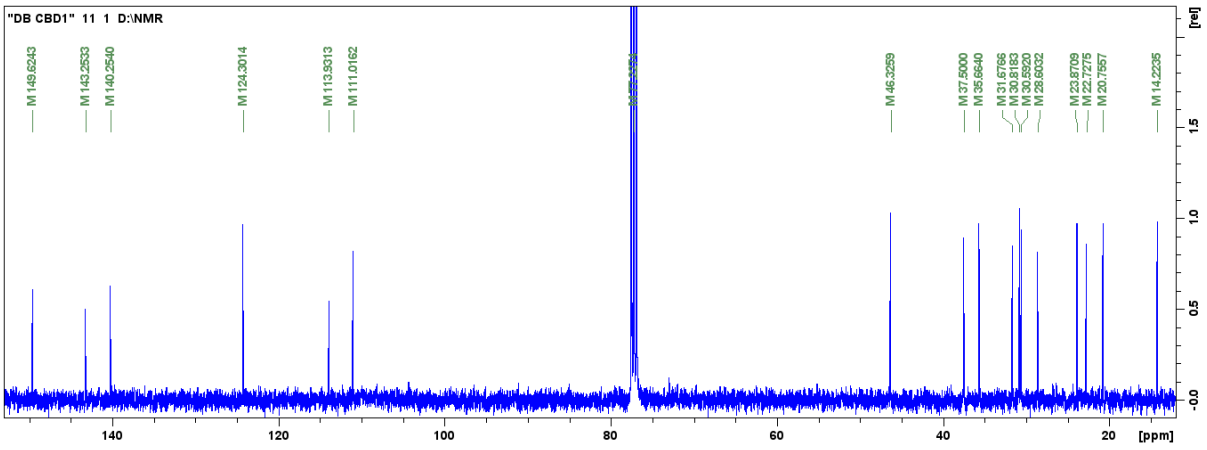
7		79.0391		79.0317
8	1.4440 3H s	27.5761	1.4432 3H s	27.576
9	1.1092 3H s	19.7092	1.1083 3H s	19.7089
1'		110.054		110.0467
2'		164.8919		164.8851
3'		102.3916		102.3916
4'		147.0347		147.0023
5'	6.2543 1H s	112.8006	6.2525 1H s	112.7832
6'		159.9482		159.9224
1"	2.9359 1H m	36.7126	2.9286 1H m	36.7124
1"	2.781 1H m		2.7744 1H m	
2"	1.5718 2H m	31.4875	1.5714 2H m	31.4948
3"	1.3437 m	32.2172	1.3433 m	32.217
4"	1.3437 m	22.7084	1.3433 m	22.7083
5"	0.8992 t	14.2514	0.8981 t	14.2502
2'-OH	12.2042 s		12.2160 s	
COOH		175.8716		175.7403

Full spectra collected for CBD1 Sample:

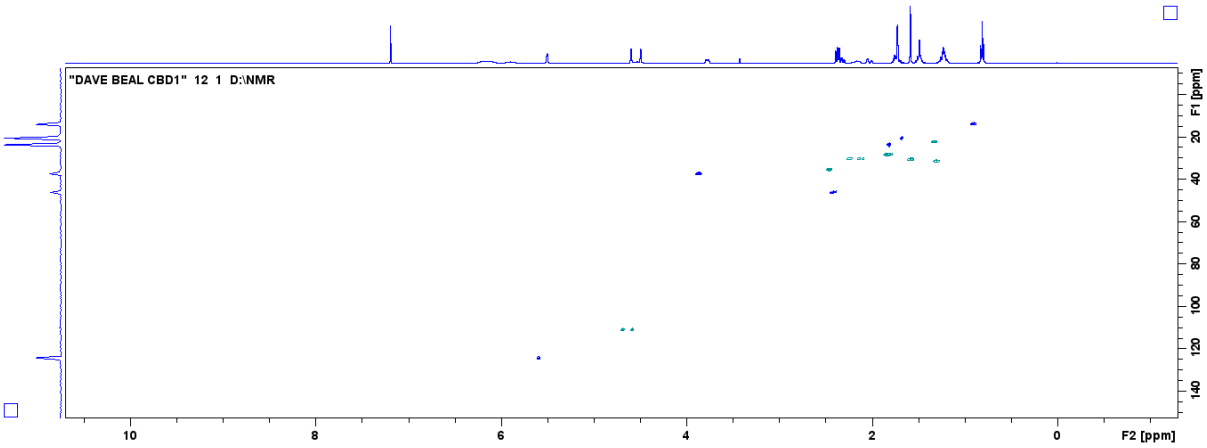
Proton NMR:



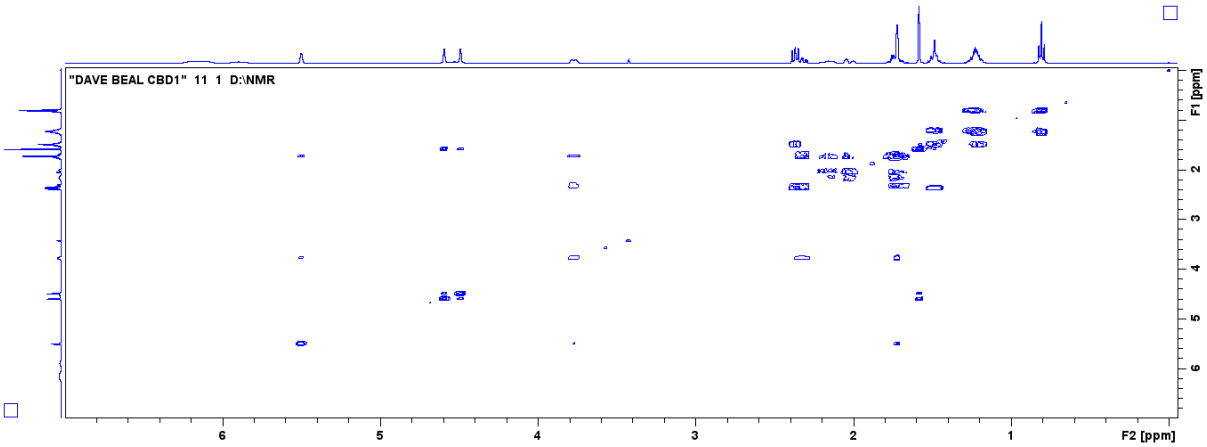
Carbon NMR:



HSQC:

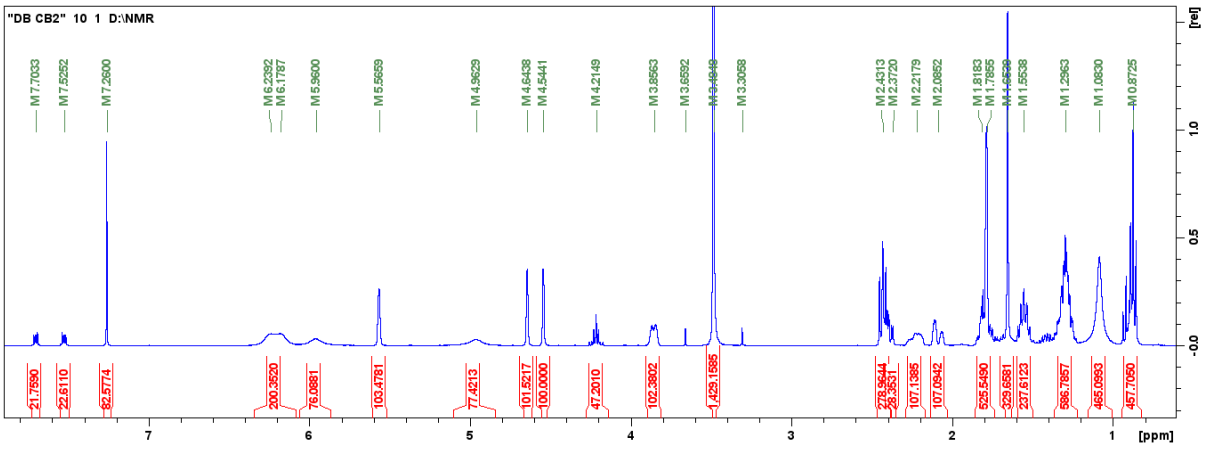


COSY:

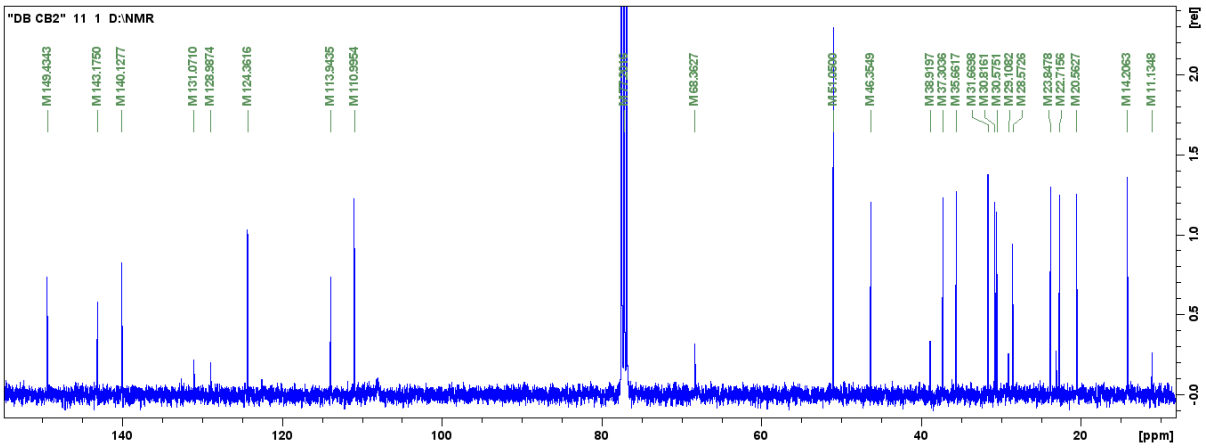


Full spectra collected for CBD2 Sample:

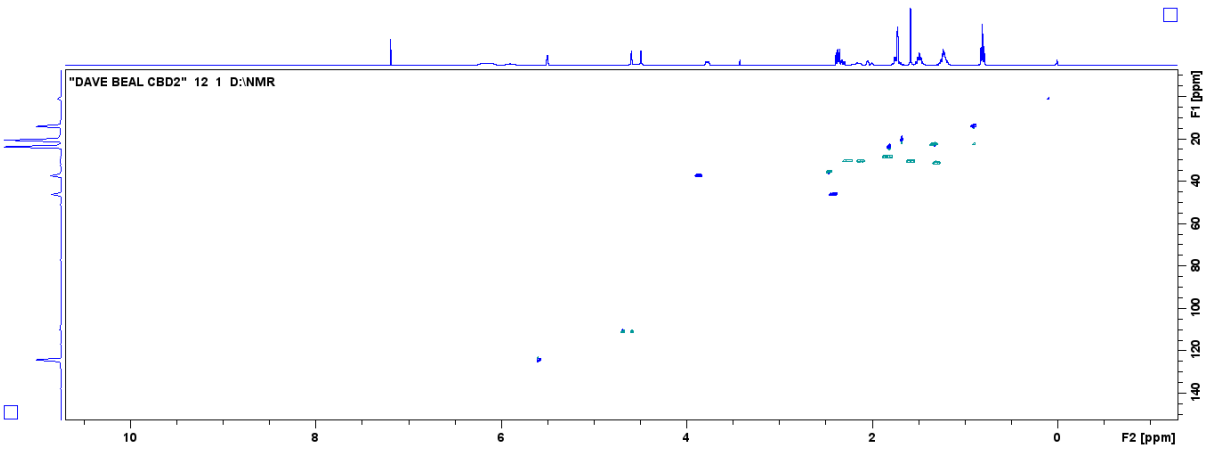
Proton NMR:



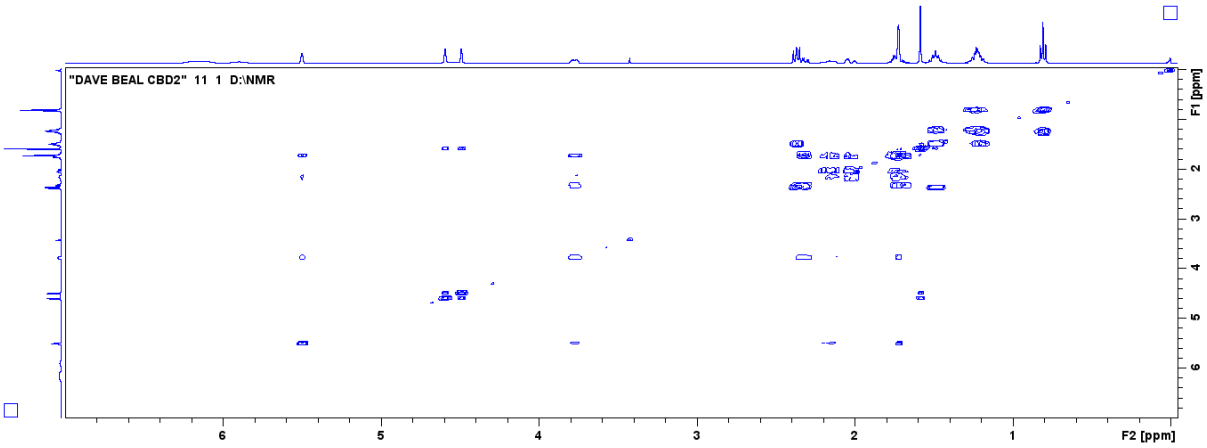
Carbon NMR:



HSQC:



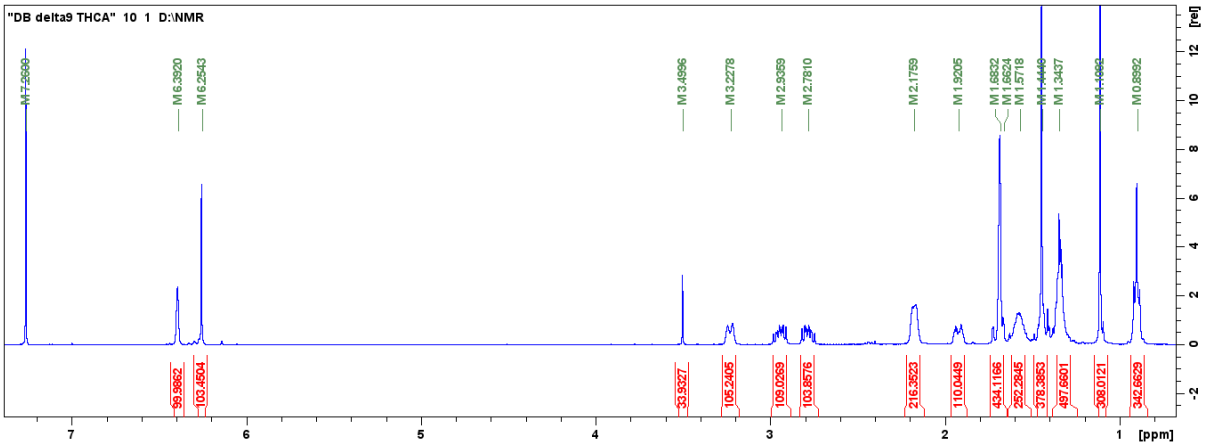
COSY:



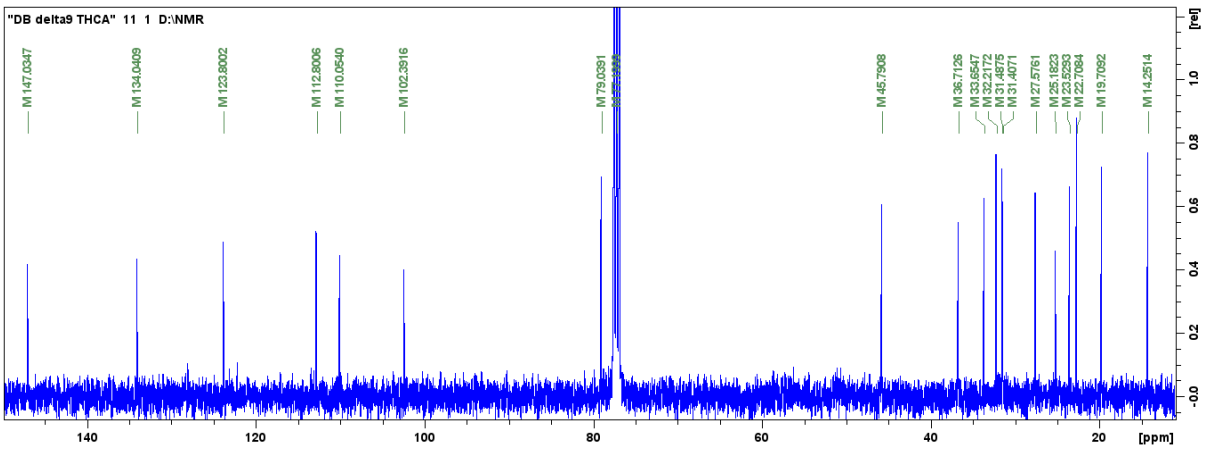
Appendix C: Full NMR Spectra for THCA9 and THCA Mix

Full spectra collected for Δ9THCA Sample:

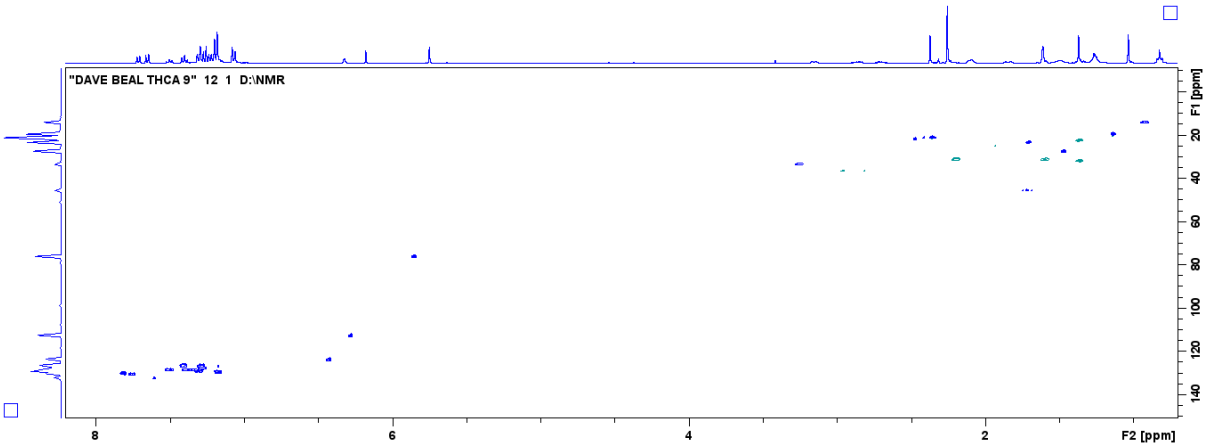
Proton NMR:



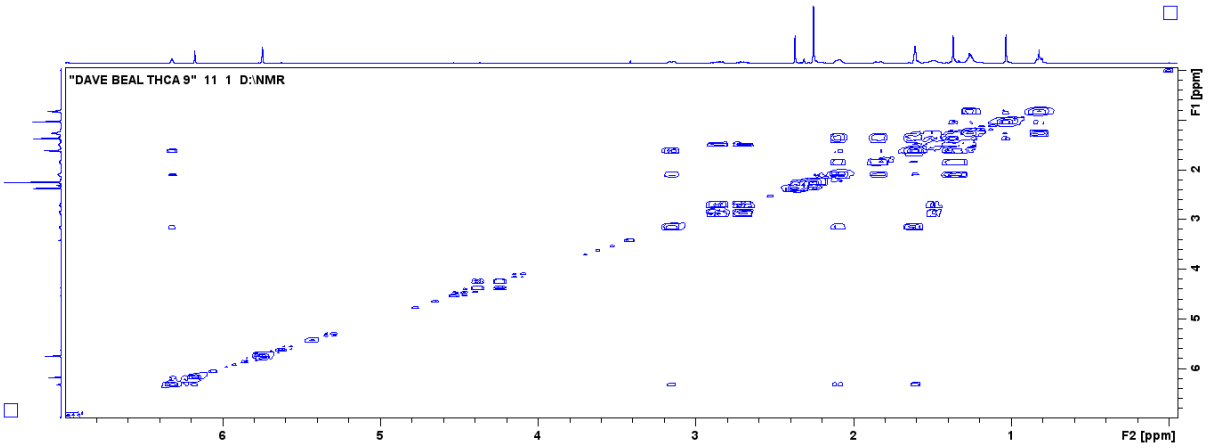
Carbon NMR:



HSQC:

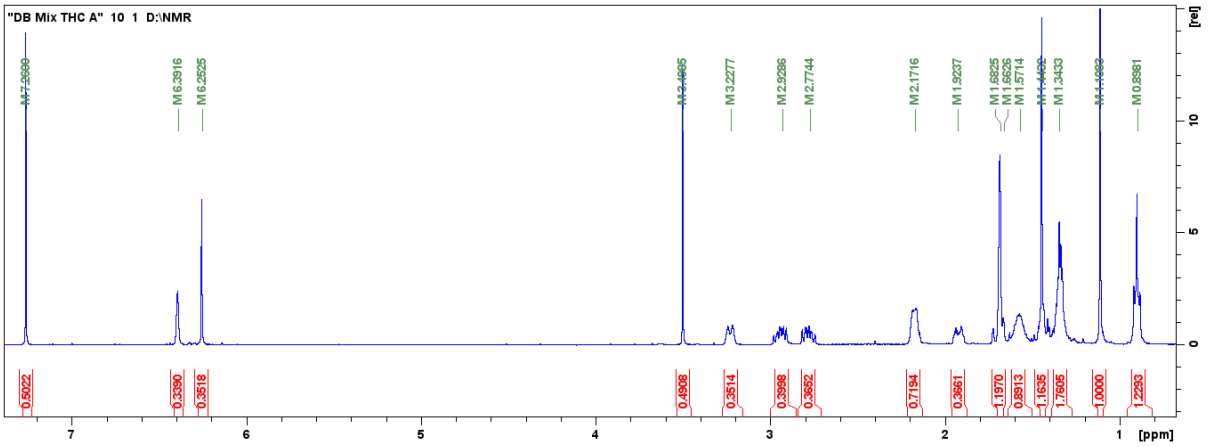


COSY:

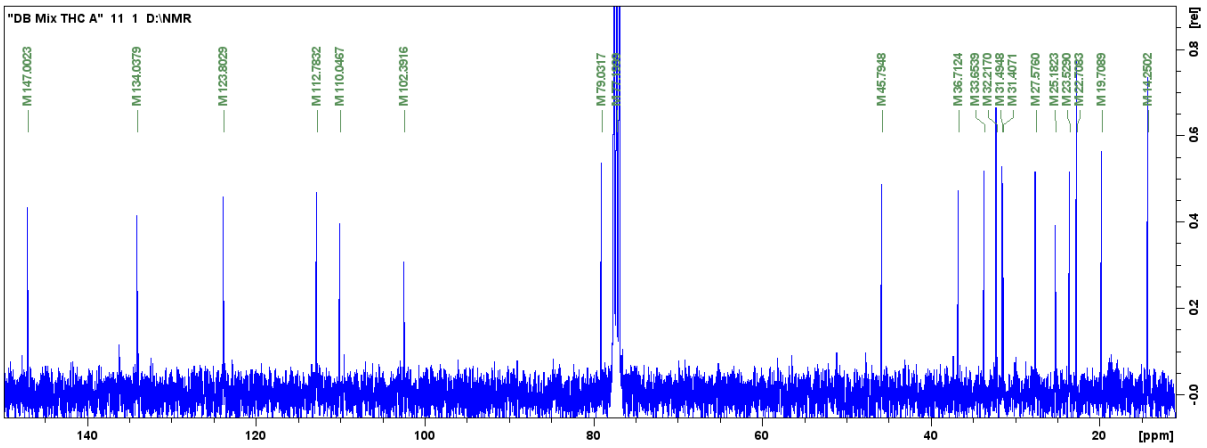


Full spectra collected for THCA Mix Sample:

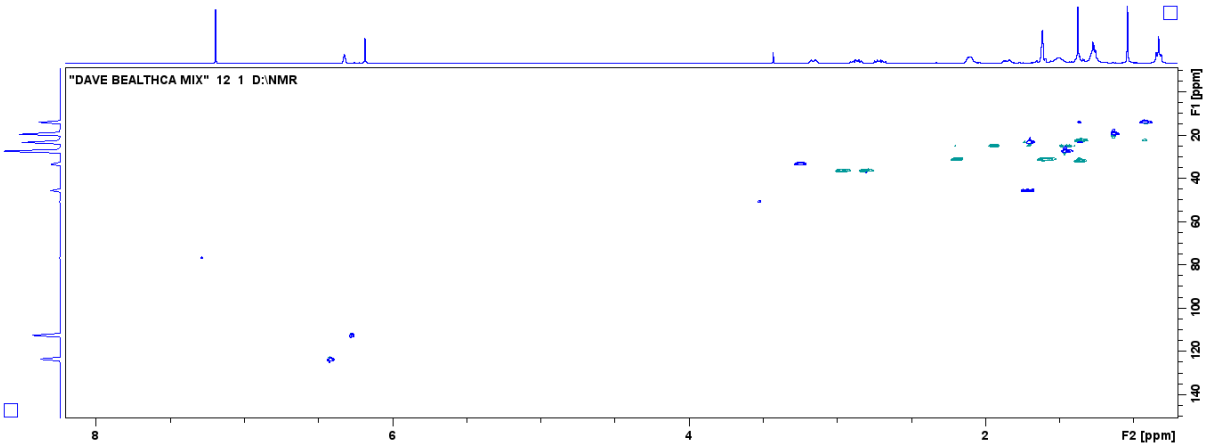
Proton NMR:



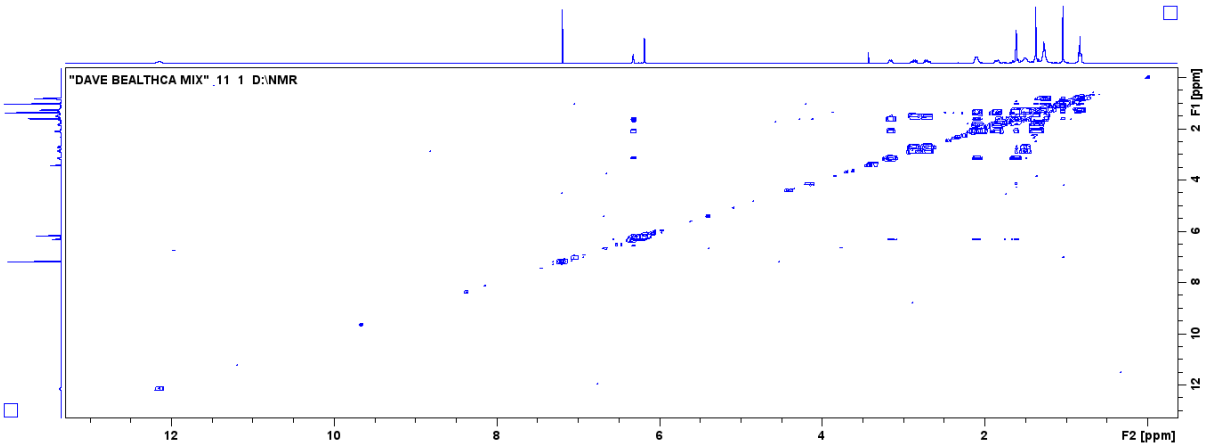
Carbon NMR:



HSQC:



COSY:



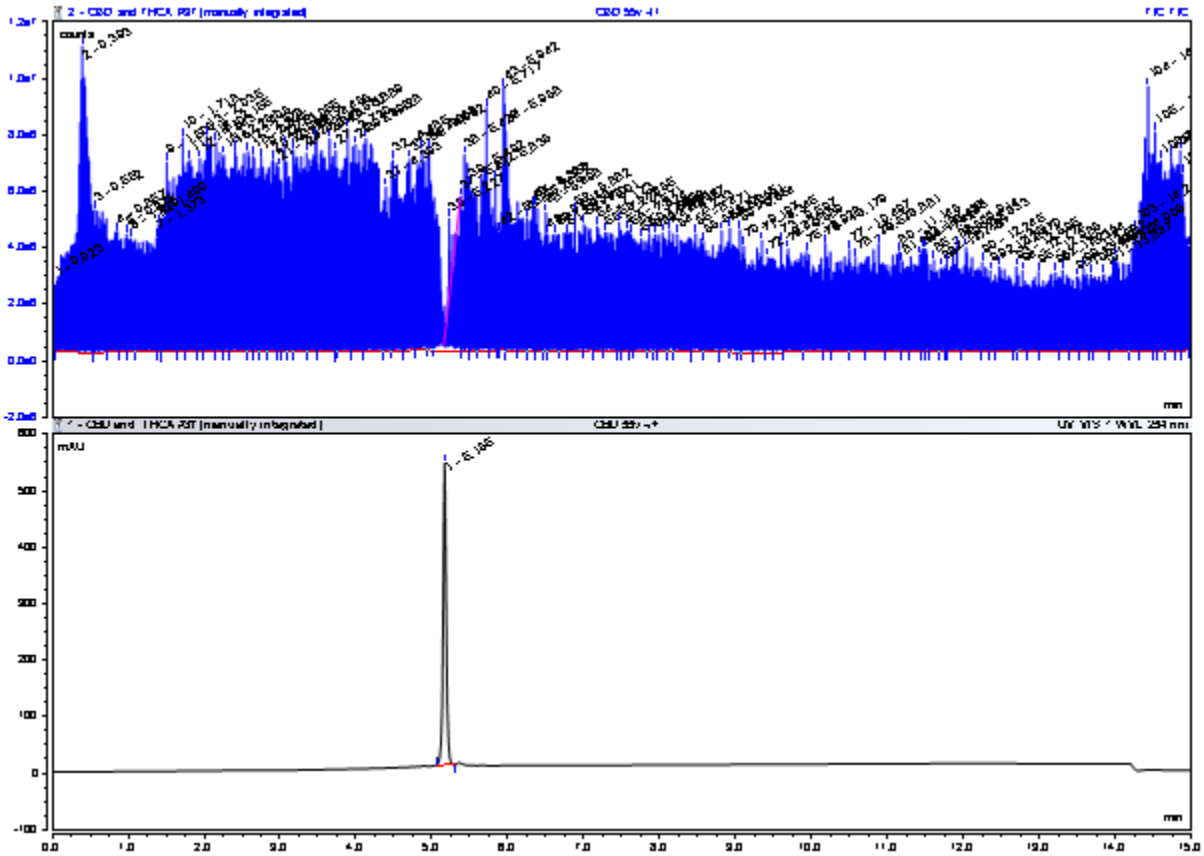
Appendix D: LC-MS data and Decarboxylation Studies (voltage changes)

Method development data table of different Mass Spectrometry conditions for LC-MS use to assess any decarboxylation or ionising issues within the LC-MS instrument.

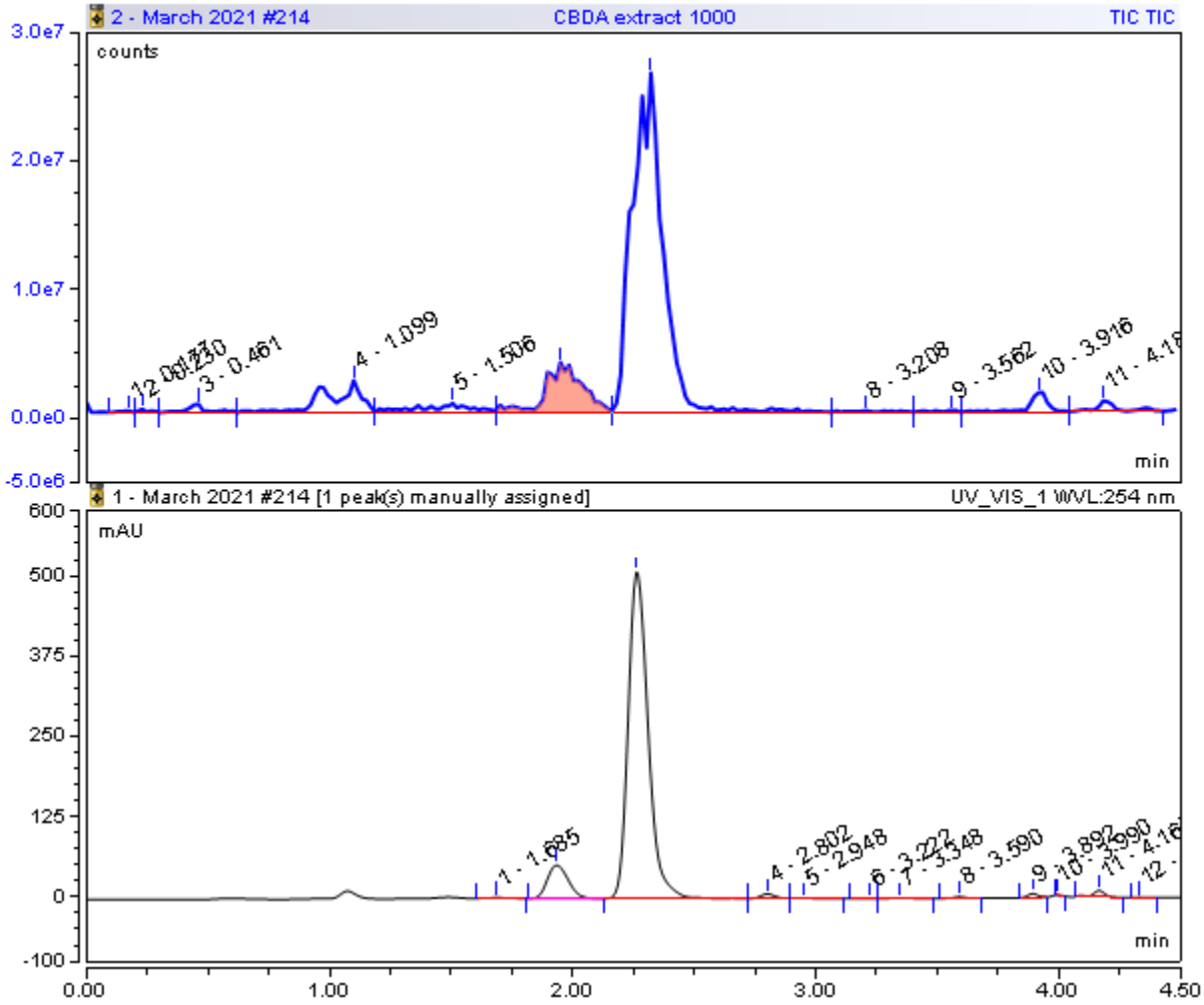
Name	Cone Voltage	Temperature	MM 313	MM 357	Ratio 313/357
LL ES- 150/50	50	150	108316	12510334	0.009
LL ES- 150/50	50	150	84828	11843038	0.007
LL ES- 150/50	50	150	89,654	9,980,308	0.009

Name	Cone Voltage	Temperature	MM 313	MM 357	Ratio 313/357
LL ES- 150/50	50	150	89654	9980308	0.009
ML ES- 350/50	50	350	166,730	16,042,662	0.010
HL ES- 550/50	50	550	223814	19297018	0.012
LM ES- 150/75	75	150	3317506	8303796	0.400
MM ES- 350/75	75	350	5,293,410	10,929,300	0.484
HM ES- 550/75	75	550	5,351,796	17363914	0.308
LH ES- 150/100	100	150	2090542	679956	3.075
MH ES- 350/100	100	350	3,620,164	3,058,634	1.184
HH ES- 550/100	100	550	3,783,942	3,189,384	1.186

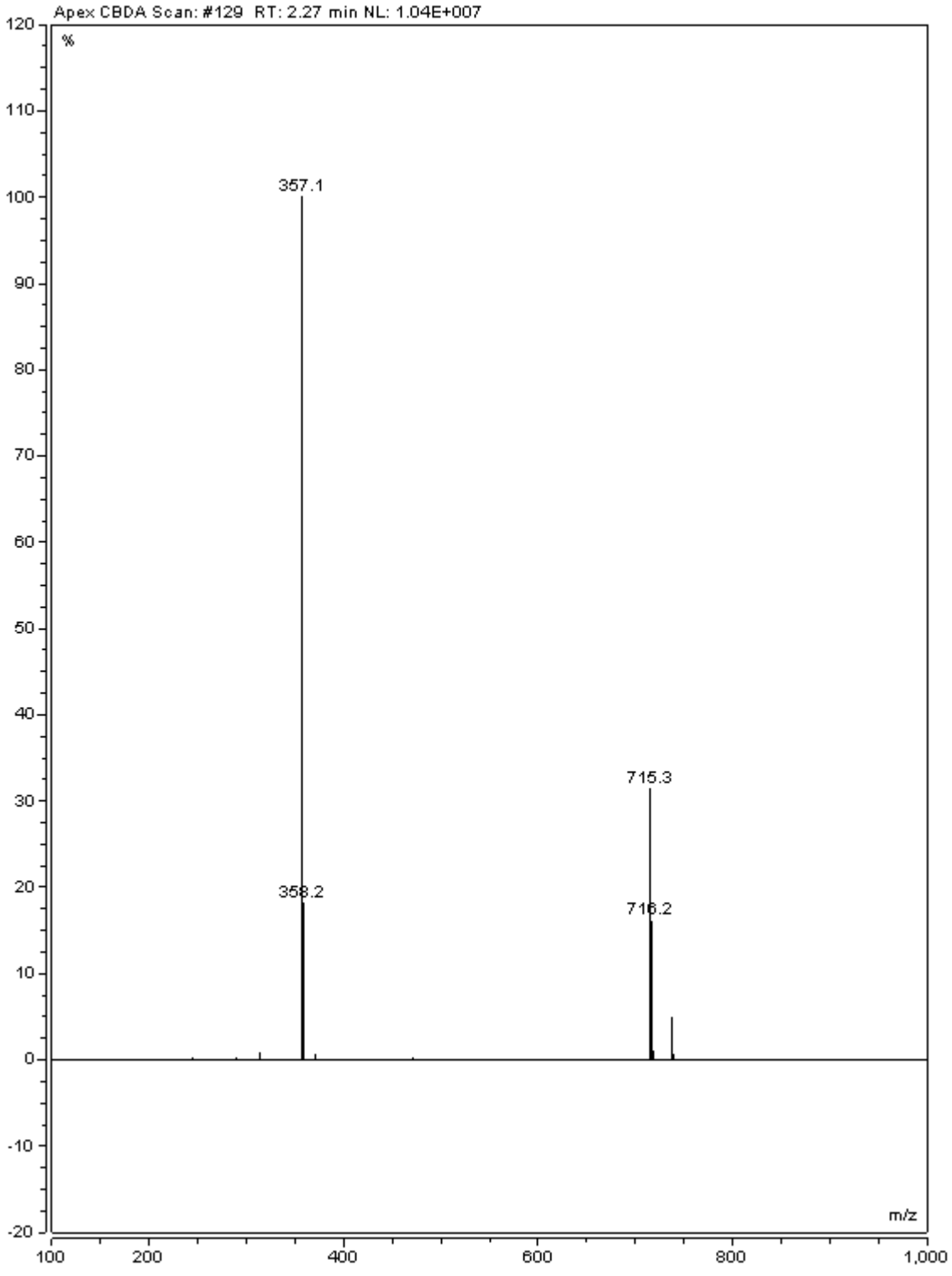
Below: One set of chromatography and TIC obtained during method development while on a positive and negative ESI setting to establish how CBD was ionising and what conditions best suited to progress



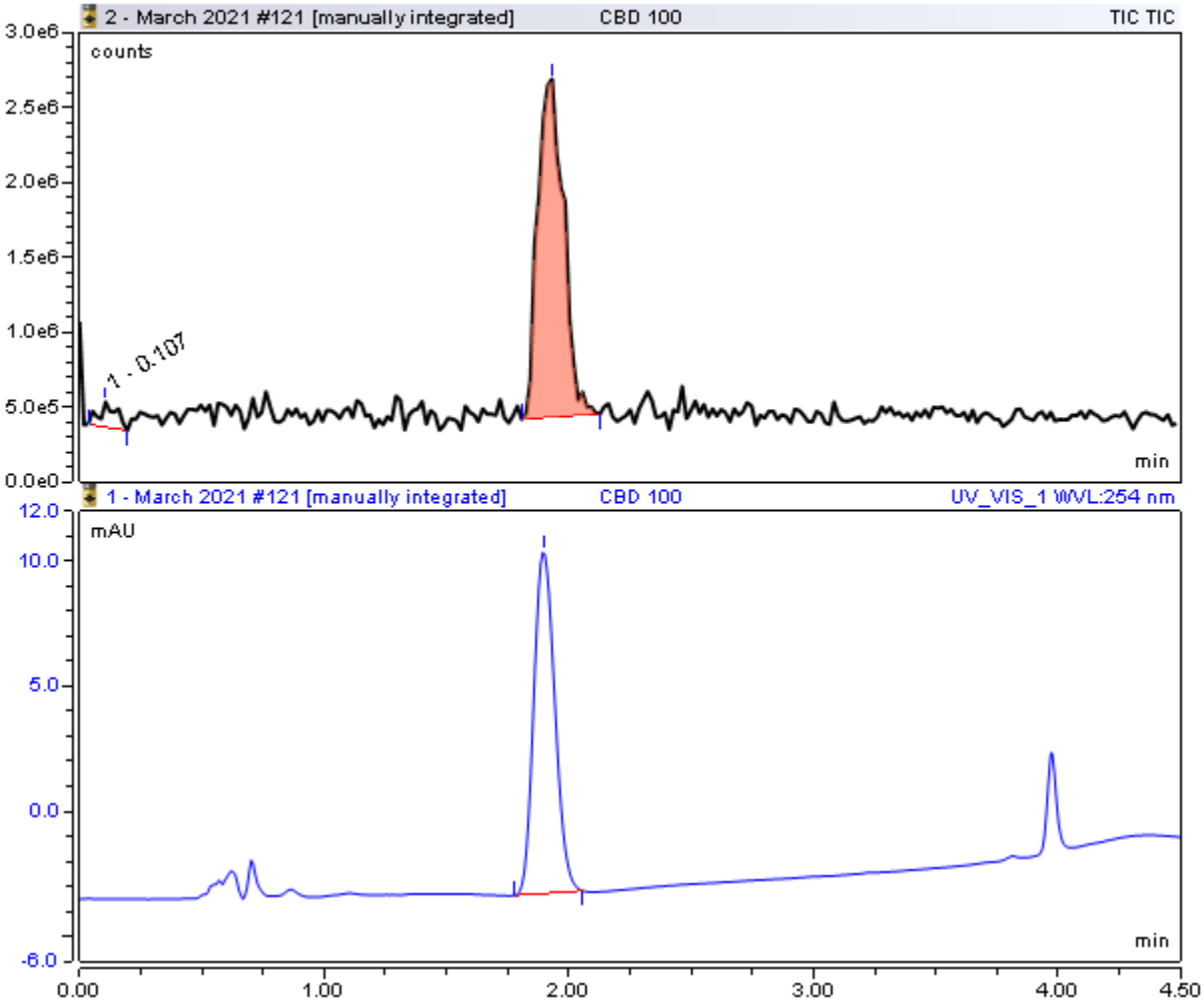
Chromatography and TIc from 1000 ppm of CBDA:



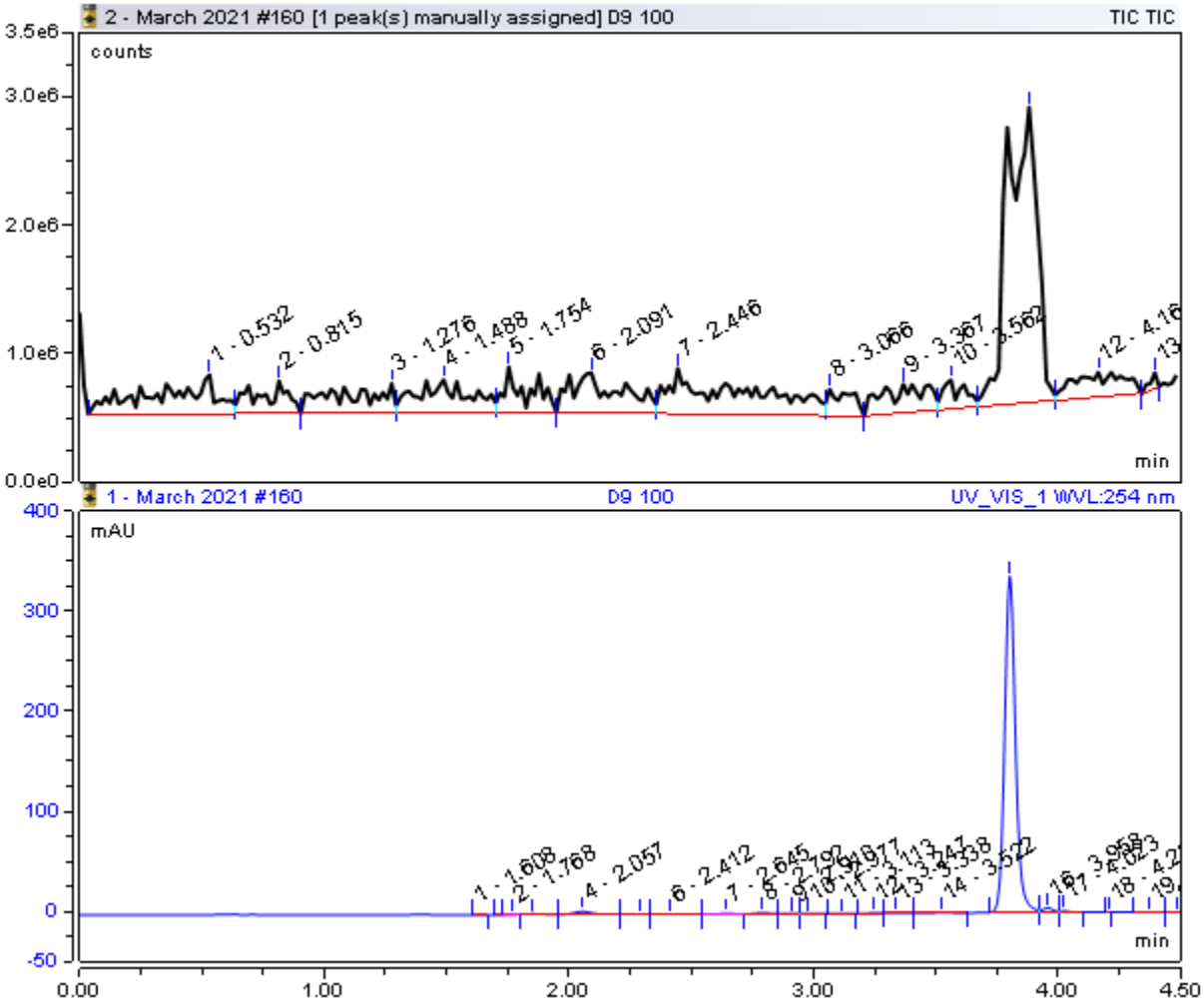
Highlighted pink peak is the referenced possibly minor impurity of most likely CBGA, however this is at such a low-level quantity in comparison to the CBDA content that the sample was deemed fit for purpose for this study. The mass spectrometry for the CBDA peak is below showing the compound has a mass of supporting this theory.



CBD 100 PPM Chromatogram and TIC

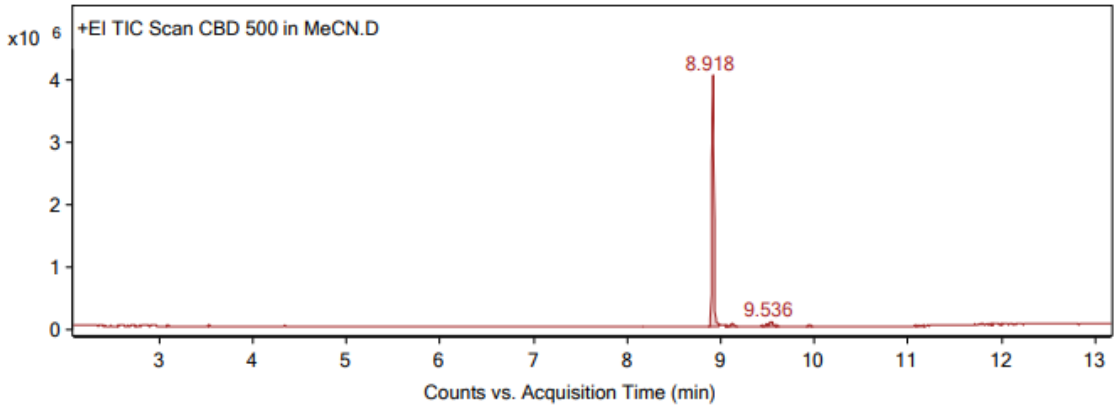

2 - March 2021 #121 [manually integrated]
CBD 100
TIC TIC
counts
3.0e6
2.5e6
2.0e6
1.5e6
1.0e6
5.0e5
0.0e0
1 - 0.107
min
1 - March 2021 #121 [manually integrated]
CBD 100
UV_VIS_1 WVL:254 nm
mAU
12.0
10.0
5.0
0.0
-6.0
min
0.00
1.00
2.00
3.00
4.00
4.50

THCA 100 ppm TIC and chromatogram data



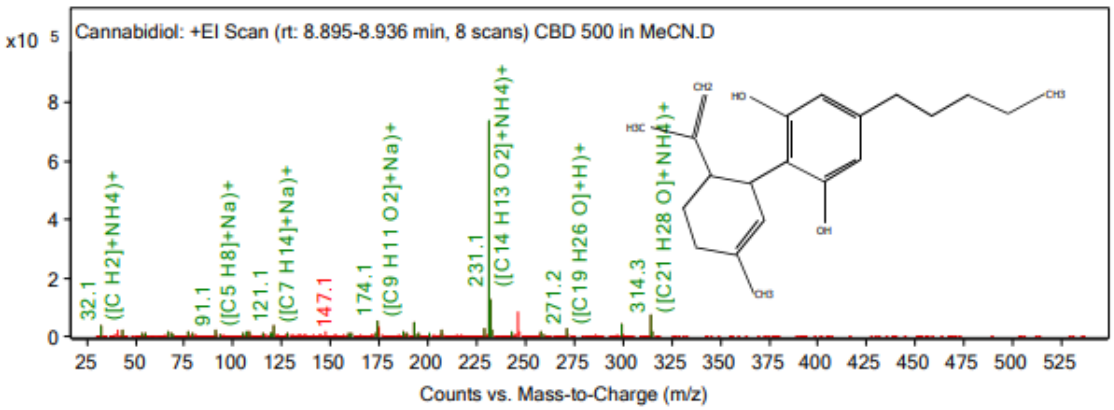
Appendix E: GC-MS data from Method development and Calibration. Data Extracted from full analysis reports.

CBD 500 PPM Calibration Chromatogram and Mass Spectrometry from CBD Peak



Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	8.871	8.918	8.99	4022368.38	6577877.97	100
2	9.073	9.126	9.185	44698.04	98694.39	1.5
3	9.461	9.494	9.506	39063.11	53711.89	0.82
4	9.506	9.536	9.613	75354.99	177684.9	2.7
5	9.91	9.933	9.979	12274.31	24956.46	0.38

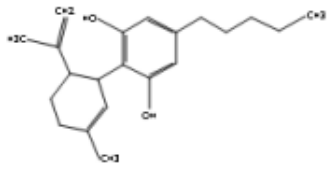


Peak List

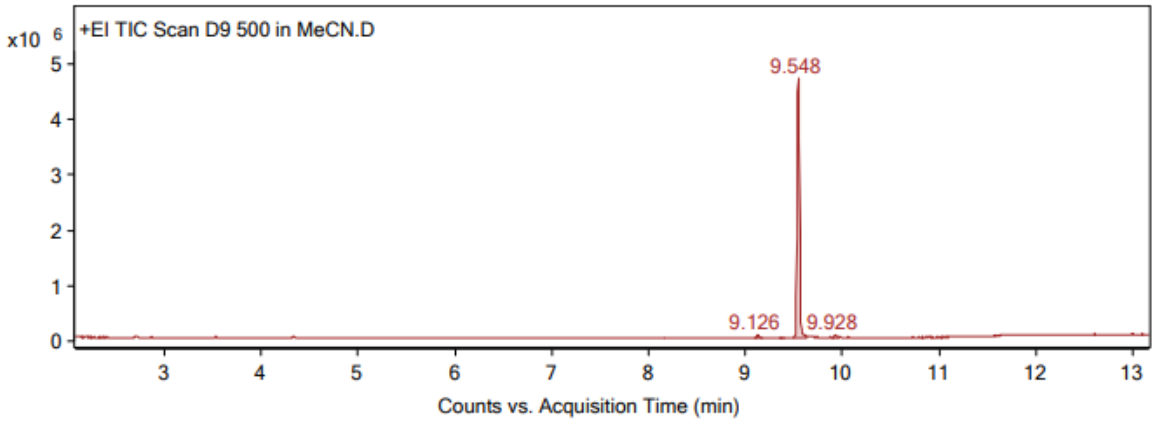
m/z	z	Abund	Formula	Ion
32.1		38276	C H2	(M+NH4)+
121.1	1	35546.88	C7 H14	(M+Na)+
174.1		52400.75	C9 H11 O2	(M+Na)+
175.1		30387.5		
193.1	1	48976.13	C15 H12	(M+H)+
231.1	1	735648	C14 H13 O2	(M+NH4)+
232.1	1	124952	C14 H13 O2	(M+NH4)+
246.2	1	84884.75		
299.2	1	39712.5	C20 H26 O2	(M+H)+
314.3	1	70536.25	C21 H28 O	(M+NH4)+

Spectrum Structure

Cannabidiol

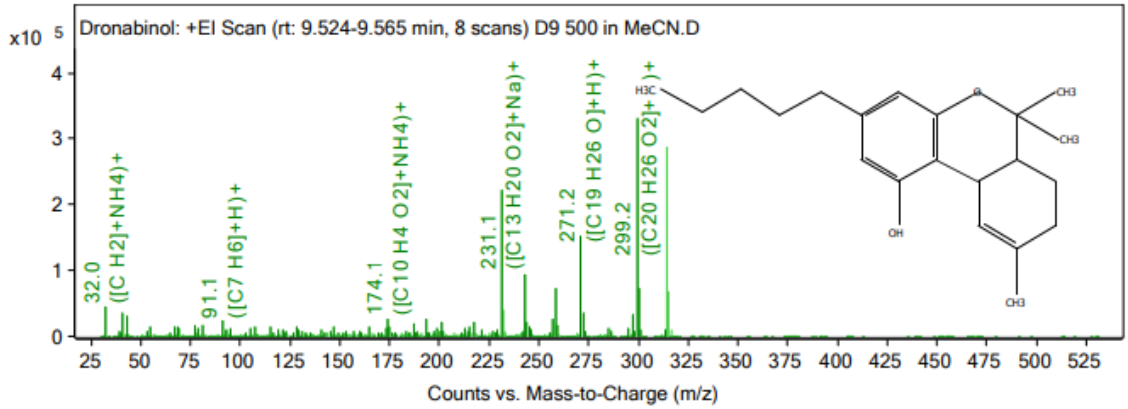


THC 500 PPM Chromatogram and Mass Spectrometry from THC Peak



Integration Peak List

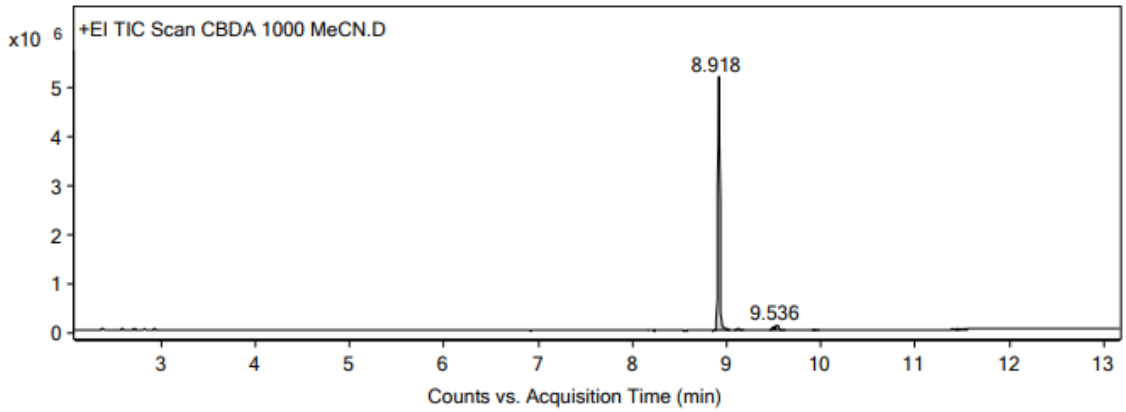
Peak	Start	RT	End	Height	Area	Area %
1	9.093	9.126	9.174	41095.33	76786.89	0.98
2	9.352	9.376	9.399	11773.41	17012.85	0.22
3	9.501	9.548	9.625	4687970.29	7837520.75	100
4	9.901	9.928	9.963	40477.94	67845.04	0.87



Peak List

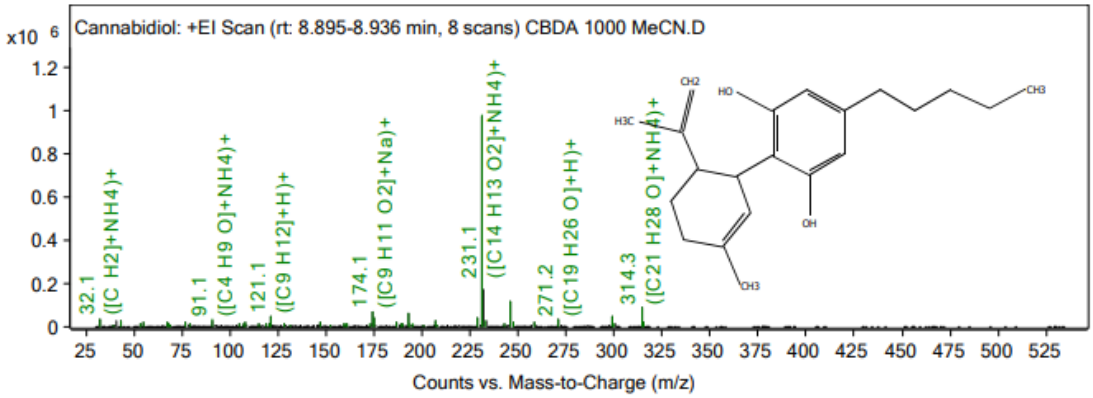
m/z	z	Abund	Name	Formula	Ion	Score (DB)	Hits (DB)
32		43419		C H2	(M+NH4)+		
231.1	1	221416		C13 H20 O2	(M+Na)+		
232.2	1	39620					
243.1	1	93705		C18 H9	(M+NH4)+		
258.2	1	72177		C16 H27 O	(M+Na)+		
271.2	1	152418		C19 H26 O	(M+H)+		
299.2	1	330406	Norethynodrel	C20 H26 O2	(M+H)+	99.95	1
300.2	1	72156.38		C20 H26 O2	(M+H)+		
314.2	1	286188					
315.3	1	66945					

Analysis from CBDA in GC-MS to confirm full identification and decarboxylation within instrument:



Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	8.876	8.918	9.031	5184855.14	8708765.89	100
2	9.085	9.126	9.18	42398.45	99216.68	1.14
3	9.458	9.494	9.506	62748.78	92073.58	1.06
4	9.506	9.536	9.619	94894.2	225065.29	2.58
5	9.91	9.933	9.975	14939.09	34553.03	0.4



Peak List

m/z	z	Abund	Formula	Ion
121.1	1	46922.13	C9 H12	(M+H)+
174.1		69839.5	C9 H11 O2	(M+Na)+
175.1	1	39816.88	C10 H16 O	(M+Na)+
193.1	1	63913.25	C14 H7	(M+NH4)+
229.2	1	38851.75	C16 H20 O	(M+H)+
231.1	1	975104	C14 H13 O2	(M+NH4)+
232.2	1	169557		
246.2	1	114659	C16 H20 O	(M+NH4)+
299.2	1	48376.75	C19 H21 O2	(M+NH4)+
314.3	1	88991.25	C21 H28 O	(M+NH4)+

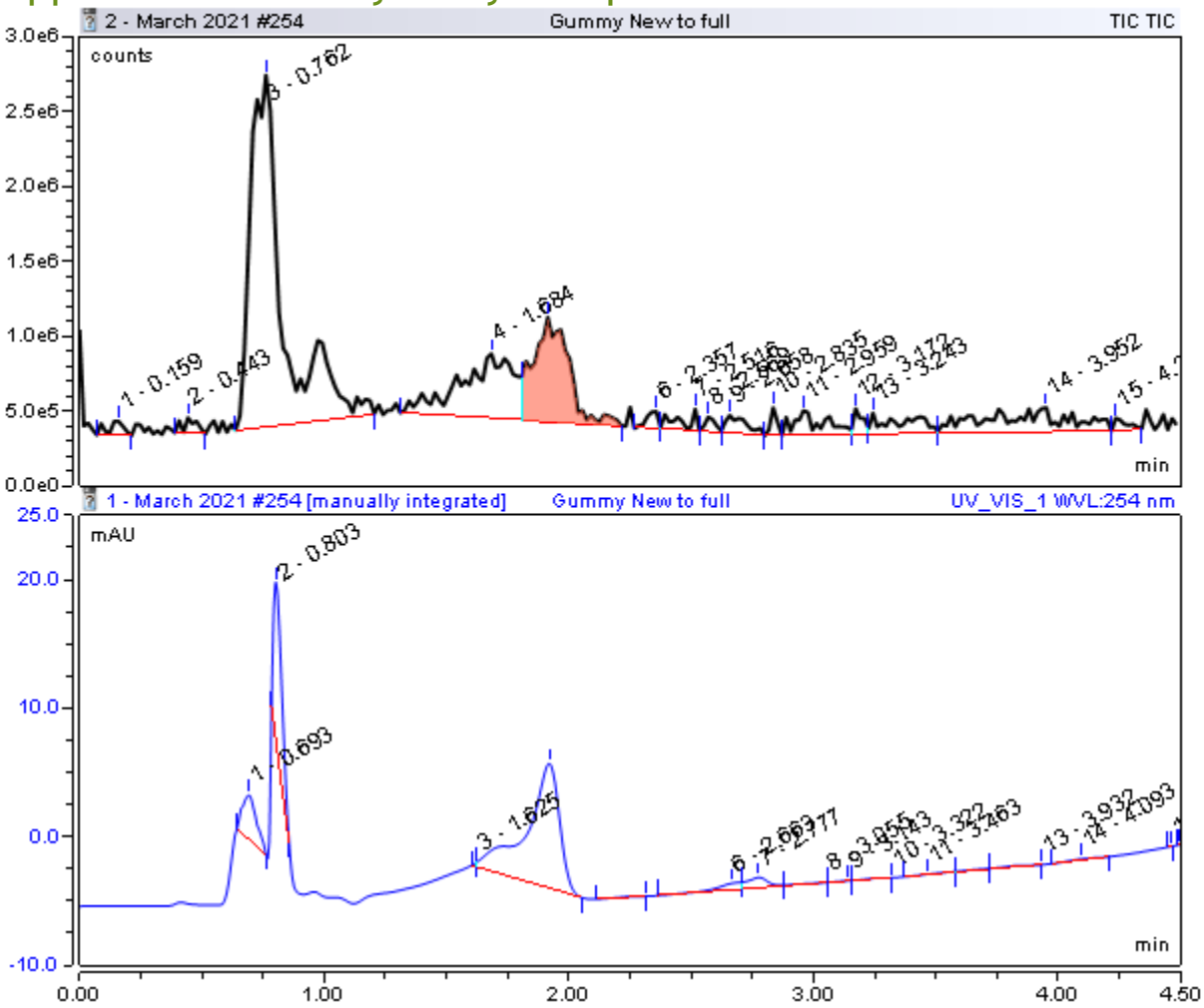
Appendix F: Area used for calculations in practical Experiment with Gummy and Hemp Seed analysis

Gummy Sweet Analysis Area

GC-MS (CBD)	40854350
LC-MS (CBD)	0.622556828
Hemp Seed Analysis Area	
GC-MS (CBD)	2089646.892
GC-MS (THC)	5023118.463

LC-MS (CBD)	4.1347

Appendix G: Gummy analysis reports



Appendix H: Plant part data

plant part concentration	mass of plant material mg	mg/ L	Concentration mg/ L	mg/ L plant material to content ratio	Concentration mg/ L2	mg/ L plant material to content ratio	Concentration mg/ L	mg/ L plant material to content ratio
Seedling 1	47.81	19124.00	10.39	0.0005	7.70	0.000	1032.35	0.05
Stems	2093.44	209344.00	23.99	0.0001	821.30	0.004	1251.45	0.01
Fan leaf	787.82	52521.33	17.54	0.0003	427.71	0.008	573.86	0.01
Sugar leaf	372.77	37277.00	29.99	0.0008	885.04	0.024	846.91	0.02
Bud (overloaded)	917.03	91703.00	241.88	0.0026	3790.10	0.041	3453.99	0.04

Seedling 2	47.81	19124 .00	10.40	0.0005	8.12	0.000	226.75	0.01
Bud halved		45851 .50	113.38	0.0025	2364.90	0.052	2131.25	0.05
Bud actual (2 x halved)		91703 .00	226.76	0.0025	4729.81	0.052	4262.50	0.05

Appendix I: Terpenes from plant profile study

These are the qualitative results of the plant profile study identifying the presence of the terpene with a 'yes', or absence of the terpene with 'no'.

Terpene	**Dry SK1**	**Dry SH1**	**Dry CBD1**	**Fresh CBD1**
(-)-Aristolene	No	Yes	No	No
.alpha.-Bisabolol	Yes	Yes	No	No
.alpha.-Guaiene	Yes	Yes	No	No
.beta.-Bisabolene	No	Yes	No	No
.beta.-Myrcene	Yes	Yes	Yes	Yes
1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-	No	Yes	No	No
2,6-Dimethyl-1,3,5,7-octatetraene, E,E	No	Yes	No	No
3,5,11-Eudesmatriene	No	Yes	No	No
3-Carene	No	Yes	Yes	No
Aromandendrene	No	Yes	No	No
Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4- dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]-	No	Yes	No	No
Benzene, (2-methyl-1-propenyl)-	Yes	no	No	No
Bicyclo[2.2.1]heptane, 2,2-dimethyl-3- methylene-, (1S)-	Yes	no	Yes	No
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2- methylene-, (1S)-	Yes	Yes	Yes	Yes
Caryophyllene	Yes	Yes	Yes	Yes
cis-.alpha.-Bergamotene	No	Yes	No	No
Copaene	Yes	Yes	No	No
Cyclohexane, 1-methylene-4-(1-methylethenyl)-	Yes	Yes	Yes	No
Cyclopropane, 1,1-dimethyl-2-(3-methyl-1,3- butadienyl)-	No	Yes	No	No
D-Limonene	Yes	no	Yes	Yes
endo-Borneol	Yes	no	No	Yes
Fenchol	Yes	no	Yes	Yes
GuaioL	No	no	No	Yes
Humulene	No	Yes	No	No

Linalool	Yes	no	Yes	Yes
Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8adimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.alpha.)]-	No	Yes	No	No
Selina-3,7(11)-diene	No	Yes	No	No
trans-.beta.-Ocimene	No	Yes	Yes	No
trans-2-Pinanol	Yes	no	Yes	Yes
Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	Yes	Yes	Yes	Yes
Tricyclo[4.2.2.0(1,5)]dec-7-ene	No	Yes	No	No
Ylangene	Yes	no	No	Yes

Appendix J: Terpenes form Plant part comparison study

During the plant part comparison study, sample preparation necessary was carried out, as stated in the table below to ensure the condition of the material was as expected.

Plant part	Starting mass (g)	1.5 hours at 50˚C	2 hours at 50˚C	overnight on radiator	2 hours at 75˚C	3 hours at 75˚C	4 hours at 75˚C
Stem	18089.82	18272.18	17703.05	16829.86	15931.06	15608.04	15603.61
Leaf	15973.78	16324.41	15750.75	15445.86	15439.71	15439.32	15439.91
Bud	15463.67	15702.55	15318.53	15157.46	15153.38	15153.21	15153.83

The table below specifically identifies the terpenes present with the boxes left blank if the terpene was not identified. The key (d) is given next to the dried out plants.

Terpene	Bud	Leaf	Stem	Bud (d)	Leaf (d)	Stem (d)
Alpha.-Pinene	Present	Present		Present		
alpha-Bergamotene	Present	Present	Present	Present		
Alpha-Himachalene			Present			
Aromandendrene		Present	Present			
Beta-Bisabolene	Present	Present	Present	Present		
Beta-caryophyllene	Present	Present	Present	Present		
Beta-Pinene	Present	Present	Present	Present		
Beta-Sesquiphellandrene	Present	Present	Present	Present		
Beta-Thujene	Present	Present		Present		
Camphene	Present			Present		
Carene	Present			Present		
Curcumene			Present			
Cyclofenchene	Present		Present	Present		
Cyclosativene	Present					
Famesene	Present	Present	Present	Present		
Fenchol	Present					
Humulene	Present	Present	Present	Present		
Limonene	Present	Present	Present	Present		
Linalool	Present			Present		
Muurolene	Present		Present	Present		
Myrcene	Present	Present	Present	Present	Present	
Ocimene						
Santalene	Present			Present		
Terpinene			Present			
Terpinol	Present			Present		