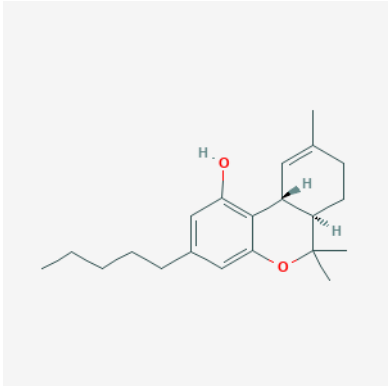
Quantitation of Cannabinoids

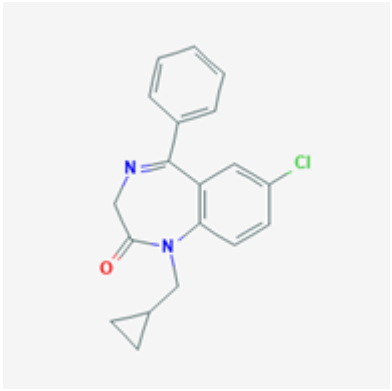
Titus K. Dowell, Tobias A. Konieczka, Caleb S. Temple, and Kevin M. Dunn

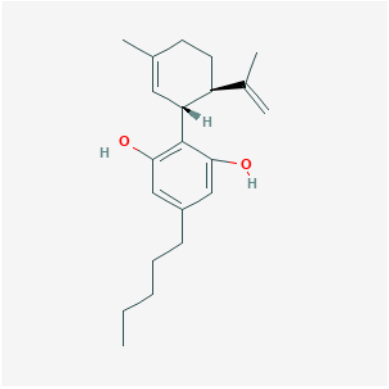
Department of Chemistry, Hampden-Sydney College, Hampden-Sydney, VA 23943

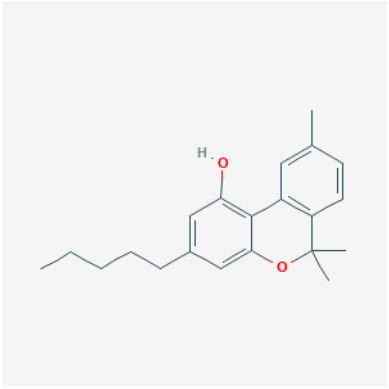
Introduction

In 2018 the United States Congress passed the 2018 Farm Bill legalizing the production of hemp and hemp-based products [1]. Hemp is a form of the plant *Cannabis sativa* which has a much smaller concentration of the psychoactive compound found in marijuana, delta-9-tetrahydrocannabinol [1]. The Farm Bill, which defines that hemp plants and its derivatives have no more than 0.3 % delta-9-tetrahydrocannabinol, created the first legal distinction between marijuana and hemp on the basis of the concentration of delta-9-tetrahydrocannabinol [1]. The compounds, which include delta-9-tetrahydrocannabinol, cannabidiol, cannabinol, and cannabigerol originate from a class of terpenophenolic derived from *Cannabis sativa* [2]. Terpenes are a family of naturally occurring aromatic compounds derived from Cannabis sativa, and terpenophenolic compounds are terpenes functionalized with alcohol groups. Researchers have estimated that there are over 500 cannabinoids [2,3]. These cannabinoids have been utilized in various medical treatments, specifically those that are yet deemed fully treatable. For example, CBD has been found to possibly benefit patients suffering from epilepsy and schizophrenia [4], while CBN has been found to display both powerful sedative and analgesic properties [5,6]. Another rather useful cannabinoid is Cannabigerol, (CBG). Although it has not received the same attention as CBD, it does have some medicinal purpose due to the fact that it is a non-psychotropic, which is rare in hemp. Cannabigerol is believed to help inflammatory bowel disease, glaucoma, Huntington's disease and even enhance the deletion of cancer cells [7]. CBG is often referred to as the “mother” cannabinoid because it happens to be the precursor from which all the cannabinoids are synthesized [7]. Due to the medicinal and supplemental applications of CBD and CBN it is important that reliable methods of determining the concentration of CBN, CBD, CBG and/or THC in products be developed. Reliable methods of quantitation for the relevant cannabinoids help to protect manufacturers from erring on the wrong side of the law while also protecting consumers from the unwanted side effects produced by compounds such as THC.

A. 

B. 

C. 

D. 

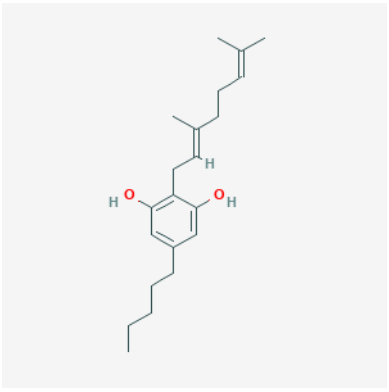
E. 

Figure 1: *Chemical structures of cannabinoids and internal standard [8-12].*
A. Delta-9-tetrahydrocannabinol. B. Prazepam. C. Cannabidiol. D. Cannabinol. E. Cannabigerol

The quantitation of cannabinoids has been studied extensively, and one of the most utilized methods is that of GCMS instrumentation [8]. This method of quantitation requires the development of a system of data points concerning samples of known concentrations and an internal standard, referred to as a calibration curve. The production of samples of known concentrations is of utmost importance in order to satisfy the parameters of the calibration curve. Quantitative techniques utilize an internal standard to remove a systemic source of error found in the injection volume of the GC/MS injector [9]. The use of an internal standard reduces quantitative error associated with the introduction of sample into GC because the ratio of the peaks of the two compounds, the internal standard and the compound of interest, within the mass spectrum will remain the same. Prazepam was used as the internal standard for the calibrations because it is non-native to *Cannabis Sativa* derivatives. Prazepam is also a good internal standard because its retention time is much later than that of the cannabinoids. The difference in retention times allowed us to easily determine the difference between CBD, CBG, and CBN peaks. Furthermore, in order to perform quantitative analysis we needed to determine target and reference ions for the internal standard and the compound of interest using a SIM scan. The SIM scan allowed us to look at single ions from the fragmentation pattern of each compound, and then determine the necessary parameters for quantitation. Defining the parameters for quantitation allowed us to ignore any compounds bleeding through the GC column and focus solely on the mass spectra of the cannabinoid and the internal standard. Knowledge of the fragmentation patterns of each cannabinoid allowed us to construct a compound table with the necessary parameters for quantitation of each cannabinoid. The calibration curves were developed using certified reference materials, which are pure samples of either the compound of interest or the internal standard dissolved in methanol.

The calibration curve determines the area ratio of the mass spectrum peaks for the internal standard and the cannabinoid of interest. This allowed us to determine the concentration of each cannabinoid in solution. By developing a calibration curve, we were able to create a system that could accurately quantitate the amount of a compound of interest within a given sample. During this course of study, the internal standard was chosen to remain constant within the series of samples when developing a calibration curve, and the volume of the compound of interest was increased incrementally. The certified reference materials used during this course of study were delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), and Prazepam (PZP).

Experimental

Certified Reference Materials ***(3.1)***

The certified reference materials were received in sealed ampules. Each ampule was broken, and the solutions were transferred to a clean vial via pipette so that they could be used for quantitation. The manufacturer and the batch codes of the standards are shown in the table below:

Compound	Manufacturer	Concentration	Batch Code
THC	Restek	1 mg/ml	A0153802
CBD	Cerilliant	1 mg/ml	FE10071912
CBN	Cayman	1 mg/ml	0584229
CBG	Cayman	1 mg/ml	A0567652
PZP	Restek	1 mg/ml	A0158093

Table 1: *CRM Specifications*

Preparation of Analytical Solutions of Cannabinoid Isolates ***(3.2)***

When analyzing samples of cannabinoid isolate, 0.100XX g of the isolate was weighed by difference using an analytical balance, and the isolate was quantitatively transferred to a 10 mL volumetric flask with analytical MeOH. The volumetric flask was filled to the fiducial mark with MeOH, and then XX uL of MeOH was added to the volumetric flask. By adding XX uL of MeOH to the volumetric flask, a solution with a concentration of 10 mg of isolate /1 mL of MeOH was produced. We called the 10 mg/ml solution "solution A". Solution A was then diluted by a factor of 10 to produce a final analytical solution with a concentration of 1 mg isolate/1 mL MeOH. The diluted solution, labelled as "solution B" was produced by transferring 1000 uL of Solution A into a second 10 mL volumetric flask and filling to the fiducial mark with MeOH.

Creating a Compound Table for Relevant Cannabinoids and Prazepam ***(3.3)***

Using an analytical solution method described in section **(3.2)** pertaining to the relevant cannabinoids (CBD, CBN, or CBG isolate) and the prazepam internal standard, three GC samples of the cannabinoid solution and prazepam in MeOH were produced. The samples contained 5 uL, 10 uL, and 20 uL of cannabinoid solution and Prazepam. The samples were then analyzed using a Single Ion Mode, or SIM, scan in order to determine the necessary target and reference ions for the relevant cannbinoid and prazepam.

Sample Preparation for CbxPzpZeroToTenAndFive ***(3.4)***

Using the CRM for the cannabinoid of interest and prazepam, 11 GC samples were produced. All of the samples contained 1 mL of MeOH, and 5 uL of prazepam. One vial received none of the cannabinoid, referred to as the blank. The other 10 samples were spiked with incremental amounts of cannabinoid, ranging from 1 uL CBN to 10 uL CBN. The samples were analyzed using a method that satisfied the parameters determined by the results from the SIM scan to produce a calibration curve for the relevant cannabinoid using 5 uL of internal standard.

Sample Preparation for CbxPzpZeroToTenAndTen ***(3.5)***

Using the CRM standards for the cannabinoid of interest and prazepam, 11 GC samples were produced. All of the samples contained 1 mL of MeOH, and 10 uL of Prazepam. 10 of the 11 samples were spiked with incremental amounts of CBN standard, ranging from 1 uL CBN to 10 uL CBN, while the other sample received no CBN standard, referred to as the "blank'. The sample was analyzed using a method developed that was in agreement with the parameters determined from the SIM scan, described in section **(3.3)**, to produce a calibration curve for CBN using 10 uL of internal standard.

Sample Preparation for CbxPzpZeroToTwentyAndTwenty ***(3.6)***

Using CRM for the cannabinoid of interest and prazepam we created 11 GC vials to run for potential calibration curves. Each of the samples contained 20 uL of prazepam and 1000 uL of methanol. Starting with the second vial again, we added 2ul of the cannabinoid of interest and increased the volume incrementally by 2 uL until 20 uL was reached in the 11th vial. This curve could then be used to analyze the CBG isolates discussed in sections **(3.10)** and **(3.11)**. This process was also repeated with CBD as the cannabinoid.

Analysis of CBN Isolate from Industrial Hemp Farmers (IHF) ***(3.7)***

A solution of CBN isolate was made using the method described in section **(3.2).** The solution of CBN isolate and the Prazepam standard were used to produce a GC sample with 10 uL of CBN isolate and 10 uL Prazepam in 1 mL MeOH. The sample was analyzed using the calibration curve CbnPzpZeroToTenAndTen. Furthermore, a 0.05 g sample of the isolate was dissolved in deuterated chloroform, and C^{13} NMR was performed on the sample.

Analysis of CBN Isolate from Hemp For Fitness (HFF) ***(3.8)***

An analytical solution of CBN isolate was made using the method described in section **(3.2)**. The analytical solution of CBN isolate and Prazepam standard were used to produce a GC sample with 10 uL of CBN isolate and 10 uL Prazepam in 1 mL MeOH. The sample was analyzed using the calibration curve CbnPzpZeroToTenAndTen. Furthermore, a 0.05 g sample of the isolate was dissolved in deuterated

chloroform, and C^{13} NMR was performed on the sample.

Analysis of CBG Isolate from Industrial Hemp Farmers. ***(3.9)***

An analytical solution of CBG was constructed using the same method as described in section 3.3. The 1mg/mL solution of CBG was then used to make a GC sample with 20 uL of CBG isolate and 20 uL of Prazepam in 1mL of MeOH. Additionally, we were then able to analyze the CBG isolate solution with the calibration curve described in section **(3.5),** and determine the percent concentration of CBG in the isolate.

Analysis of CBG Isolate from Hemp For Fitness ***(3.10)***

A solution of CBG isolate was made using the method described in section **(3.2)**. The solution constructed was then used to make a GC sample with 20 uL of CBG and 20 uL of prazepam in a 1mL solution of methanol. The sample was then analyzed using the calibration curve created using the CBG isolate from Cayman. Furthermore, we could then tell the percent concentration of CBG in the isolate by comparing it to the calibration curve described in section **(3.5)**.

Analysis of CBD Isolate from Essential Depot Lot # IL2003R-006B ***(3.11)***

To prepare a 1 mg/mL solution of CBD Isolate, 0.10040g of CBD Isolate was weighed by difference into a 10 mL volumetric flask using a microspatula. The isolate and the microspatula was rinsed with MeOH to ensure that all CBD was brought into solution. The volumetric flask was then filled to the fiducial mark, an additional 40 uL of MeOH was added, the volumetric flask was inverted 50 times, and labelled as Solution A. Then 1000 uL of Solution A was syringed into another 10 mL volumetric flask, which was then filled to the fiducial mark with MeOH and labelled Solution B. Solution B was inverted 50 times. 1000 uL of MeOH were inserted into an empty GC vial, after which 10 uL of Solution B and 10 uL of Prazepam were added. The vial was then analyzed with the GCMS using the same method file from CbdPzpZeroToTenAndTen.

Analysis of CBD Isolate from Essential Depot Lot # II1908I-045B-C4 ***(3.12)***

To prepare a 1 mg/mL solution of CBD Isolate, 0.10057g of CBD Isolate were weighed by difference into a 10 mL volumetric flask using a microspatula. The isolate and the microspatula were rinsed with MeOH to ensure that all CBD was brought into solution. The volumetric flask was then filled to the fiducial mark, an additional 57 uL of MeOH was added, the volumetric flask was inverted 50 times, and labelled as Solution A. Then 1000 uL of Solution A was syringed into another 10 mL volumetric flask, which was then filled to the fiducial mark with MeOH and labelled Solution B. Solution B was inverted 50 times. 1000 uL of MeOH were inserted into an empty GC vial, after which 10 uL of Solution B and 10 uL of Prazepam were added. The vial was then analyzed with the GCMS using the same method file from CbdPzpZeroToTenAndTen.

Results

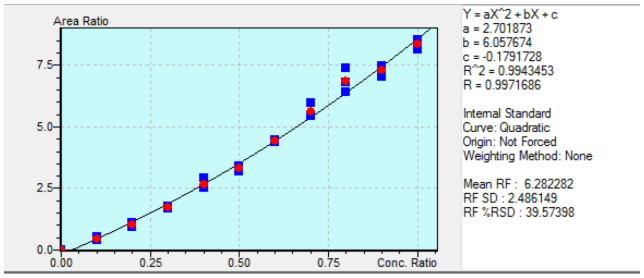


Figure 2: *CbnPzpZeroToTenAndTen*

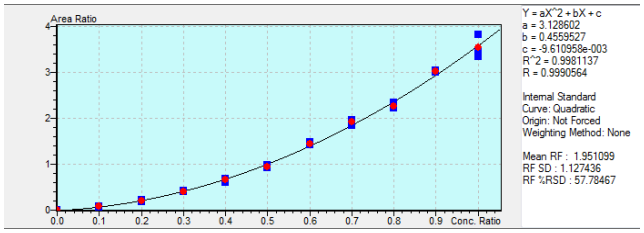


Figure 3: *CbdPzpZeroToTenAndTen*

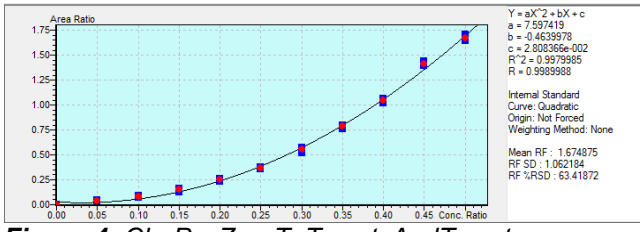


Figure 4: *CbgPzpZeroToTwentyAndTwenty*

Analysis of Various Cannabinoid Isolates

Analysis of CBN isolate from IHF:

Title	Cannabinol	Prazepam
Cbn_IHF_004	90.811	100.000
Cbn_IHF_005	92.135	100.000
Average	91.473	100.000
% RSD	1.024	0.000
Maximum	92.135	100.000
Minimum	90.811	100.000
Std. Dev	0.936	0.000

Table 2: *Concentration Summary of GCMS Analysis of CBN isolate from IHF.*

Moreover, analysis by C^{13} NMR indicated that the CBN isolate contained 21 distinct carbons. Two of the peaks overlapped each other, so the spectrum showed 19 distinct carbon peaks. The values of the PPM of each carbon peak were recorded as follows: 14.0, 21.5, 22.5, 27.0, 30.4, 31.4, 35.5, 108.6, 109.9, 110.7, 122.5, 126.4, 127.5*, 136.8*, 144.5, 153.0, 154.5 (*Denotes that two peaks overlap each other)

Analysis of CBN isolate from HFF:

Title	Cannabinol	Prazepam
Cbn_HFF_001	102.152	100.000
Cbn_HFF_002	95.211	100.000
Cbn_HFF_003	99.357	100.000
Average	98.906	100.000
% RSD	3.531	0.000
Maximum	102.152	100.000
Minimum	95.211	100.000
Std. Dev	3.493	0.000

Table 3: *Concentration Summary of GCMS Analysis of CBN isolate from HFF.*

Moreover, analysis by C^{13} NMR indicated that the CBN isolate contained 21 carbons. Two of the peaks overlapped each other, so the spectrum showed 19 distinct carbon peaks. The values of the PPM of each carbon peak were recorded as follows: 14.0, 21.5, 22.5 27.0, 30.4, 31.4, 35.5, 108.6, 109.9, 110.7, 122.5, 126.4, 127.5*, 136.8*, 144.5, 153.0, 154.5 (*Denotes that two peaks overlap each other).

Analysis of CBD from CBD Isolate from Essential Depot Lot # IL2003R-006B:

Title	Cannabidiol	Prazepam
Il2003R-006B003.qgd	164.784	100.000
Il2003R-006B002.qgd	164.042	100.000
Il2003R-006B001.qgd	165.733	100.000
Average	164.853	100.000
%RSD	0.514	0.000
Maximum	165.733	100.000
Minimum	164.042	100.000
Std. Dev.	0.848	0.000

Table 4: *Concentration Summary of GCMS Analysis of CBD Isolate from Essential Depot Lot # IL2003R-006B*

Analysis of Unknown CBD Isolate from Essential Depot Lot # II9081-045B-C4:

Title	Cannabidiol	Prazepam
II9081-045B-C4	94.767	100.000
II9081-045B-C4	94.997	100.000
II9081-045B-C4	93.655	100.000
Average	94.473	100.000
%RSD	0.760	0.000
Maximum	94.997	100.000
Minimum	93.655	100.000
Std. Dev.	0.718	0.000

Table 5: Concentration Summary of GCMS Analysis of CBG Isolate from Essential Depot Lot # II9081-045B-C4

Analysis CBG isolate from Industrial Hemp Farm:

Title	Cannabigerol	Prazepam
Cbg12345_003	105.417	100
Cbg12345_002	101.676	100.00
Cbg12345_001	103.386	100.00
AVERAGE	103.493	100.00
%RSD	1.810	0.000
Maximum	105.417	100.00
Minimum	101.676	100.00
Std Dev	1.873	0.000

Table 6: *Concentration Summary of GCMS Analysis of CBG Isolate from Industrial Hemp Farm*

Analysis of Cbg Isolate from Hemp For Fitness:

Title	Cannabigerol	Prazepam
Cbg6789_001	107.723	100.00
Cbg6789_002	100.825	100.00
Cbg6789_003	113.802	100.00
AVERAGE	107.450	100.00
%RSD	6.043	0.000
Maximum	113.802	100.00
Minimum	6.493	100.00
Std Dev	6.493	0.000

Table 7: *Concentration Summary of GCMS Analysis of CBG isolate from Hemp For Fitness*

Discussion

Certified Reference Materials

All certified reference materials were stored in brown amber 1.5 mL GC vials, and kept at 2℃ when not in use. When the certified reference materials were in use they were not left open to the atmosphere for any period of time longer than deemed necessary by the researcher.

Preparation of Analytical Solutions of Cannabinoid Isolate

An analytical balance with a precision of +/- 0.01 mg was used to measure the masses of cannabinoid isolate samples. The mass of the samples was determined through mass by difference techniques, and the isolate was transferred to a volumetric flask. In order to ensure a quantitative transfer of the isolate, the microspatula used during the measuring process was rinsed with MeOH into the flask.

Cannabinoid and Prazepam Compound Table

The target and reference ions for the cannabinoid of interest and prazepam were chosen based on the rate at which the fragmentations of the compound were readily ionizable. The most readily ionized fragment was chosen as the target ion because it was most prevalent within the fragmentation pattern of the compound. The reference ions were also chosen based on their prevalence within the fragmentation pattern. Typically, the reference ions were the 2nd and 3rd most prevalent ions in the fragmentation pattern. The target ion is used by the instrument to quantify how much of the compound is present while the reference ions are used to validate the presence of the target ion.

CbnPzpZeroToTenAndTen:

For the same reason we decided to increase the amount of internal standard when performing a THC calibration, to determine if the function would become more linear, we increased the amount of internal standard with the cannabinol calibration curve as well. A similar result was observed. The calibration curve did not increase greatly in its linearity when a larger volume of internal standard was used. The calibration curve was quadratic, and modeled by the following equation: $y=2.701873x^2+6.057674x-0.1791728$. Furthermore, the R^2 value of the function was 0.9943453, indicating that the calibration curve is accurately represented by the equation. Finally, the standard deviation of the GC sample containing 10 ul CBN and 10 ul prazepam was 1.112 which indicates that the calibration curve can be used to analyze samples containing 10 ul of a 1 mg/mL CBN solution and 10 ul of prazepam within +/- 1.112%.

CbdPzpZeroToTenAndTen:

Initially, we decided to increase the amount of internal standard to observe any change in the curve and to also observe the area ratios while trying to get them closer to one another. Going from 5ul of internal standard to 10ul of internal standard did not change the concentrations of the solutions but instead it

changed the scale of area ratios to the concentrations. The function of the calibration curve was quadratic, as modeled by the function $y=3.08704x^2+.4108094x-.008583889$. Furthermore, the R^2 the value of the function was .998606, demonstrating an accurate calibration curve shown by the equation. Another experiment involving the same concentrations of CBD and Prazepam, both from the same respective lots used previously, yielded a quadratic function with the function $y=3.128602x^x+0.4559527x-9.6100958e^{-003}$, with an R^2 value of 0.9981137, indicating that the curve can accurately be used to determine CBD concentration in future experiments that use the same concentrations of CBD and Prazepam as demonstrated in this experiment.

CbgPzpZeroToTwentyAndTwenty

The GC samples contained 20 uL of internal standard and incremental amount of CBG ranging from 0 uL to 20 uL. The function of the calibration curve generated was quadratic and can be represented by the following function: $y=1.398366x^2-0.1023896x-0.002849$. Additionally, the R^2value was 0.9957262, which demonstrates an accurate fit to the function of the calibration curve.

Analysis of Various Cannabinoid Isolates

Analysis of CBN Isolate from Industrial Hemp Farmers (IHF):

A 1mg/ml solution of CBN was analyzed using the calibration curve shown in figure 10. Analysis indicated that the isolate contained 91.473% CBN (+/- 1.112%) which does not agree with the reported value from the manufacturer. The sample was then analyzed using C^{13} NMR to determine the presence of any unevaporated solvents or other organic adulterants within the sample. Based on experimental data reported by Choi et. al., and the NMR spectra of the sample we determined that there were no other organic compounds within the sample [15]. However, we do not believe that this invalidates the findings from our GC-MS quantitation. The isolate may contain an inorganic solvent. For instance, a phosphorus based solvent could have been used during the manufacture and it had not yet fully evaporated at the time the product was sold. The presence of a phosphorus based solvent could be determined by P^{31} NMR. It is also plausible that the sample may contain a metal salt which could be determined through MP-AES analysis. However, analysis using C^{13} NMR and GC-MS quantitation indicate that the CBN isolate is one of relatively high purity.

Analysis of CBN Isolate from Hemp For Fitness (HFF):

A 1mg/ml solution of CBN isolate from the manufacturer Hemp For Fitness was analyzed using the calibration curve shown in figure 10. Analysis indicated that the isolate contained 98.906 % CBN (+/- 1.112%) which does not dissociate largely from the reported value by the manufacturer. The sample was then analyzed using C^{13} NMR to determine the presence of any unevaporated solvents or other organic adulterants within the sample. Based on experimental data reported by Choi et. al., and the NMR spectra of the sample we determined that there were no other organic compounds within the sample [15]. The results from GC-MS quantitation and C^{13} NMR analysis indicate that the CBN isolate is one of high purity.

Analysis of CBG isolate from Industrial Hemp Farm:

A 1mg/ml solution of CBG isolate from the manufacturer Industrial Hemp Farm was analyzed with the calibration curve CbgPzpZeroToTwentyAndTwenty. Through further analysis, we found that the isolate contained an average of 103.493 % CBG (+/- 1.810) which is above the 100% margin and that could be because the calibration curve has a margin of error and it will go above the 100% concentration. This CBG isolate most likely contained 100% CBG and the additional percentage is most likely within the margin of error of the calibration curve. Therefore, the results from this experiment show that the CBG isolate is one of high purity.

Analysis of CBG Isolate from Hemp for Fitness:

A 1mg/ml solution of CBG isolate from the manufacturer Hemp For Fitness was analyzed with the calibration curve CbgPzpZeroToTwentyAndTwenty. Throughout later analysis, we found the isolate contained an average of 107.450% CBG (+/- 6.493) which also happens to be above the 100% margin. Again, this is most likely due to the calibration curve and the error margin from the calibration curve. This sample can be determined to be 100% CBG and the solution can be considered a pure solution. This isolate had a high standard deviation, and we think that the standard deviation could be high because of the error range within the calibration curve.

Analysis of CBD Isolate from Essential Depot Lot # IL2003R-006B:

A 1mg/mL solution of Essential Depot CBD Isolate Lot # IL2003R-006B was analyzed through GCMS analysis, and yielded a total CBD concentration of 164.784%, a total PZP concentration of 100.000%, and a standard deviation of +/- 0.848%. The Prazepam concentration makes sense given the method used in the analysis, however the concentration of the CBD isolate solution is puzzling. The sample prep between the two CBD Isolate Lots followed the same procedure and were both conducted and analyzed on the same day. Both analytes were contained under the same storage conditions as outlined earlier. However, based on the advice given by Agricorp Labs [16], the results

should be accepted. With this acceptance, it appears that this concentration implies that CBD Isolate Lot # IL2003R-006B is marketable as pure CBD Isolate.

Analysis of CBD Isolate from Essential Depot Lot # II1908I-045B-C4:

A 1mg/mL solution of Essential Depot CBD Isolate Lot # II1908I-045B-C4 was analyzed through GCMS analysis, and yielded an average CBD concentration of 94.473% CBD and a total PZP concentration of 100.000%, and a standard deviation of +/- 0.718%. Both of these percentages make sense given the experimental conditions. Based on the data, it appears that CBD Lot # II1908I-045B-C4 is marketable as 94.5% CBD Isolate.

Conclusion

The work done this semester will help to pave the road for future experimentation done at Hampden-Sydney College regarding cannabinoid quantitation. Calibration curves with a high degree of precision were developed to analyze samples containing CBN. The calibration curves developed during this course of research will prove to be valuable in the quantitation of raw hemp, and other hemp products. The cannabinol isolates that were analyzed during this course of research may require further experimentation in order to determine their purity. However, based on the results it is believed that the isolates are specimens of high purity.

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