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Investigation of Chocolate Matrix Interference on Cannabinoid Analytes

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Abstract:

The first known findings of chocolate matrix interference on cannabinoid analytes is reported. Stock solutions of four biogenic cannabinoids (Δ^9-THC, CBD, CBN, CBG) and one synthetic cannabinoid (CBDD) are subjected to milk chocolate, dark chocolate, and cocoa powder. A clear trend of matrix interference is observed, which correlates to several chemical factors. The amount of chocolate present is directly proportional to the degree of matrix interference, which yields lower percent recovery rates for the cannabinoid analyte. Structural features on the cannabinoid analytes are shown to affect matrix interference, as cannabinoids with fewer phenolic -OH groups suffer from increased signal suppression. Additionally, aromatization of the *p*-menthyl moiety appears to correlate with enhanced matrix effects from chocolate products high in cocoa solids. These findings represent the first known documentation of chocolate matrix interference in cannabinoid analysis, which potentially has broad implications for complex matrix testing in the legal *Cannabis* industry.

Keywords:

Cannabis - matrix interference - cannabinoid - chocolate - cocoa - HPLC - matrix effects - *Cannabis* testing – *Cannabis*-infused

Introduction:

Since the legalization votes in Washington and Colorado in 2012, a total of eleven states have legalized the recreational use of *Cannabis*, with another thirty-six allowing for some form of medical use.[1] This rapidly expanding legal market relies on third party *Cannabis* testing laboratories to test products and determine whether or not they are safe for public consumption. The standards for what is deemed 'safe' vary from state to state, as there is no federal input on best testing practices for *Cannabis*, which is still classified as a Schedule I narcotic. Thus, a patchwork of scientific testing protocols have emerged across legal *Cannabis* markets, where analyses, analytes, limits of detection, and even product types can differ drastically between regions.[2]

In California, where legalization went into effect January 1st, 2018, every legal *Cannabis* product must pass stringent testing requirements before it can be sold to consumers. In addition to contaminant testing (e.g. pesticides, heavy metals, microbials, *etc*.), all *Cannabis* products must be tested for the presence of six cannabinoids (Δ^9-THC, THCA, CBD, CBDA, CBN, CBG).[3] Cannabinoids are a class of biologically active compounds that may induce a psychoactive and/or medicinal effect on users.[4,5] Commercially relevant cannabinoids, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), and cannabinol (CBN), are typified by two core structural fragments: an oxygenated resorcinyl ring containing a pendant aliphatic chain, and a *p*-menthyl ring with varied positions of unsaturation[4,5] (*vide infra*). In the state of California, a label claim containing accurate potency is required on all commercial products; if the cannabinoid content of the product does not match the printed label claim within +/– 10%, the product must be relabeled, or in some cases destroyed.[3] To ensure accurate dosing information, third party testing laboratories must maintain highly precise, accurate, and rugged analytical methods for a large and ever-expanding number of *Cannabis*-infused matrices, with no standardized methods to reference.

With no prior literature on *Cannabis* testing, fractured scientific requirements from state to state, and a constant stream of new product types, it falls on the third party *Cannabis* testing laboratories to develop new methods for the analyses of these disparate matrices and establish scientific standards for *Cannabis* product testing. Our investigation of potency testing on complex *Cannabis*-infused matrices begins with *Cannabis*-infused chocolates, a ubiquitous product type that accounted for 15% of retail sales in 2018 for the combined legal markets of California, Washington, Oregon, and Arizona.[6] Chocolate is a notoriously difficult food matrix for analyte extraction and detection, as a high fat content and presence of polyphenolic compounds can frustrate precise analytical testing.[7–11] Relevant work from Khuda *et al.* showed that immunodetection of allergens was complicated by matrix interference from components of dark chocolate.[7,8] In addition to fats and sugars added during chocolate processing, cocoa solids are known to contain over seventy different organic flavoring compounds,[12] which could also have interactions with the little-studied cannabinoid analytes. The chemical complexity of the chocolate matrix, combined with its omnipresence in legal *Cannabis* markets, made it an ideal product type to begin our investigations.

Materials & Methods:

Analytical Materials: Milk and dark chocolate was supplied by Chill Chocolate (Oakland, California, USA; 42% fat by weight). Cocoa powder was purchased from the Ghirardelli Chocolate Company (San Leandro, California, USA; 100% unsweetened non-alkalized baking cocoa, 25% fat by weight). Δ^9-Tetrahydrocannabinol (Δ^9-THC) and cannabinol (CBN) were originally submitted to Cw Analytical in oil form for quality assurance testing and used as submitted. Cannabidiol (CBD) and cannabigerol (CBG) isolates were supplied by Vertosa Inc. (Oakland, California, USA). All cannabinoids used in this study were tested by Cw Analytical and confirmed to be free of pesticides, heavy metals, and residual solvents. CRM grade analytical standards of Δ^9-THC, CBD, CBN, and CBG were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). An

analytical standard of cannabidiol dimethyl ether (CBDD) was made by dissolving 100 mg of an analytically pure fraction in 100 mL of HPLC-grade acetonitrile. Acetonitrile was purchased from Emerald Scientific (San Luis Obispo, California, USA; ChemPure® HPLC-grade). Methanol was purchased from Emerald Scientific (TEDIA® LC-MS grade). Dionized HPLC-grade water was generated in-house utilizing a Millipore Milli-Q® Gradient A10 water purification system. Formic acid was purchased from Emerald Scientific (Sigma-Aldrich Suprapur® 98–100%). Testing vials were purchased from Nelson-Jameson (Marshfield, Wisconsin, USA; Capital Plastic Products polypropylene 40 mL vials).

Synthetic Materials: All glassware was flame-dried prior to use. Dimethylformamide (DMF) was degassed with argon and then passed through two 4 x 36 inch columns containing anhydrous neutral A-2 alumina (8 x 14 mesh; LaRoche Chemicals; activated under a flow of argon at 350 ºC for 12 hours) to remove residual H_2O. All other chemicals were purchased commercially and used as received. NMR were recorded on a Bruker DRX-400 (400 MHz ^{1}H) and CRYO-500 (125.7 MHz ^{13}C). Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.00). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), triplet of doublets (td), multiplet (m), broad multiplet (br m)], integration, coupling constants [Hz], part of the molecule). Carbon chemical shifts are reported in ppm (δ) relative to TMS with the solvent resonance as the internal standard ($CDCl_3$, δ 77.16 ppm). NMR data were collected at 25 ºC. Analytical thin-layer chromatography (TLC) was performed with Silca Gel 60 Å F254 precoated plates (0.25 mm thickness). Flash chromatography was performed utilizing a Teledyne Isco Combiflash® Rf+ automated flash chromatography system. High resolution mass spectrometry was performed by the University of California, Irvine Mass Spectrometry Center.

Cannabinoid Stock Solutions: All cannabinoid stock solutions used in this study were made in house at Cw Analytical. A concentration of 100 µg/mL in methanol was targeted for each stock

solution; this value is indicative of the concentration measured when testing *Cannabis*-infused edible products. Each stock solution was made in batches of 4 L at a time. All experiments for a given cannabinoid were performed using a single batch of stock solution. Each stock solution was tested [n = 10] for potency and the values averaged to determine the actual concentration. When not in use, stock solutions were stored at 4 ºC and warmed to room temperature before use.

Chocolate Testing Procedure: Chocolate was blended in a food processor until it became a fine powder, roughly 1 minute. Homogenized chocolate was then weighed into test vials in either 1 g, 2 g, or 3 g amounts, with [n = 10] for each and with no sample exceeding +/– 1% of the given testing value. An aliquot of 20 mL of cannabinoid stock solution in methanol was then added to each vial from a Dispensette® S brand bottle top dispenser, to serve as both cannabinoid delivery phase and extraction solvent. Vials were vortexed for 30 seconds, sonicated at 2200 W for 20 minutes, centrifuged at 1500 rpm for 5 minutes, and winterized at –20 ºC for 30 minutes. The solution was filtered (0.45 µm PTFE) into ALS vials and analyzed for cannabinoid content via HPLC-DAD. Recovery percentage was determined by dividing the average [n = 10] concentration of analyte (µg/mL) for a given amount of chocolate product by the average [n = 10] concentration of analyte (µg/mL) in the blank stock solution.

HPLC Method: HPLC separation was performed on an Agilent 1260 Infinity II using a Restek Raptor ARC-18 column (150 x 4.6 mm inner diameter, 2.7 µm particle, and 90 Å pore size) at 40 ºC. Mobile phases were 0.1% formic acid in HPLC-grade water [mobile phase A (MPA)] and 0.1% formic acid in HPLC-grade acetonitrile [mobile phase B (MPB)] at a flow rate of 1.5 mL/min on a gradient of 75% MPB for minutes 0–3, then 75%–100% MPB over minutes 3–7. The injection volume was 2.0 µL. The detector was an Agilent 1260 DAD HS measuring a signal wavelength of 218 nm. All results were quantitated against a five-point external calibration curve. All

calibration curves were linear-fit, set to include the origin, with a weighting factor of 1/*x* and correlation coefficients (R^2) of >0.999.

Synthesis of cannabidiol dimethyl ether (CBDD): CBDD was synthesized according to a modified procedure from Mechoulam.[13] CBD (5.00 g, 15.9 mmol) was added to a 250 mL round bottom flask equipped with stir bar, and dissolved in anhydrous DMF (90 mL). Next, K_2CO_3 (11.8 g, 85.9 mmol) and iodomethane (3.6 mL, 59 mmol) were added and allowed to stir for 24 h at room temperature under a flow of N_2. During the course of the reaction, the solution changed color from a deep purple hue to golden yellow. After 24 h, deionized water was added to the flask (150 mL) and the solution extracted with diethyl ether (3 x 150 mL). The organic layers were combined and washed with a saturated solution of NaCl (150 mL), dried over $MgSO_4$, and filtered. The organic solvent was removed via rotary evaporation, and the crude product was purified via flash column chromatography (5% diethyl ether/hexanes) to afford the title compound as a clear, pale yellow oil (4.25 g, 12.4 mmol, 78%). **TLC R_f** = 0.8 (5% diethyl ether/hexanes); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.34 (s, 2, $C_6\underline{H}_2$), 5.23 (s, 1, $CHC\underline{H}=C(CH_3)(CH_2)$), 4.46–4.42 (m, 2, $C\underline{H}_2=C(CH_3)(CH)$), 3.99 (m, 1, $(CH)_2C\underline{H}Ar$), 3.74 (s, 6, $OC\underline{H}_3$), 2.90 (td, 1, *J* = 10.7, 4.1, $(CH_2)_2\underline{H}C=CH_2$), 2.54 (t, 2, *J* = 7.8, $ArC\underline{H}_2CH_2$), 2.25–2.14 (br m, 1, $CH_2C\underline{H}_2C=C$), 1.98 (m, 1, $CH_2C\underline{H}_2C=C$), 1.67–1.60 (br m, 10, $CH_2C\underline{H}_2CH_2C\underline{H}_3$), 1.34 (m, 4, $CH_2C\underline{H}_2CH_2$), 0.91 (t, 3, *J* = 7.0, $CH_2C\underline{H}_3$); **^{13}C NMR** (125.7 MHz, $CDCl_3$) δ 158.9 (2C), 149.60, 141.97, 131.23, 126.11, 119.10, 109.72, 105.10 (2C), 56.03 (2C), 45.34, 36.55, 36.25, 31.84, 31.14, 30.89, 29.85, 23.56, 22.70, 19.18, 14.19; **HRMS** (TOF MS CI+) *m/z* calcd for $C_{23}H_{34}O_2H$ $[M + H]^+$ 343.2637; found 343.2632.

Results & Discussion:

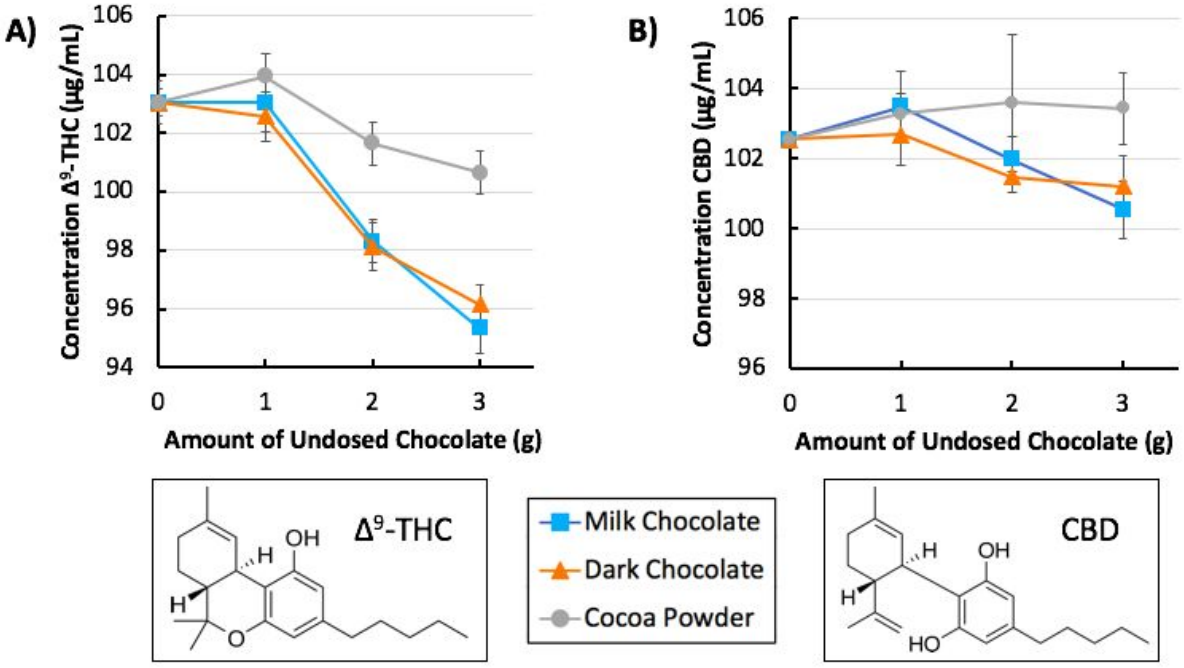
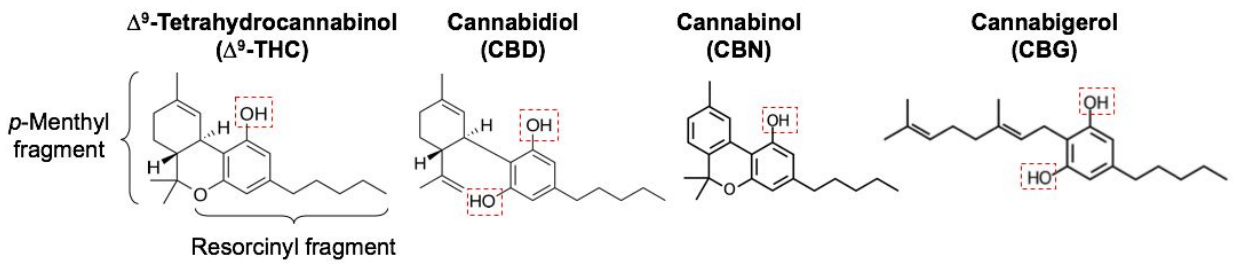


Figure 1: Investigation of matrix effects between various chocolate types and A) Δ^9-tetrahydrocannabinol (Δ^9-THC), B) cannabidiol (CBD), with chemical structures shown below. Blue squares represent milk chocolate, orange triangles represent dark chocolate, and grey circles represent cocoa powder.

Our investigation of potential matrix effects from chocolate centered on three different common chocolate sources: milk chocolate, dark chocolate, and unsweetened non-Dutch process cocoa powder. Milk and dark chocolates have very similar chemical compositions, but differ in the amount of cocoa solids and added milk fats. Conversely, cocoa powder has a minimal amount of fat present, and is mostly cocoa solids by weight.[14] In hopes of testing a purer source of cocoa, the cocoa used in this experiment has not undergone the 'Dutch process', a post-production step where alkaline compounds are added to preserve color and flavor. These three chocolate types were first subjected to a stock solution of Δ^9-tetrahydrocannabinol (Δ^9-THC), a ubiquitous cannabinoid in legal *Cannabis* edible products. For each chocolate type, three different sample loadings were tested (one gram, two grams, three grams chocolate, [n = 10] replicates) and plotted against the cannabinoid concentration, as measured by HPLC analysis. It is

hypothesized that if the recovery rate of a cannabinoid is lower at higher sample loadings, it might be indicative of matrix interference from the chocolate.

After subjecting the three chocolate types to a stock solution of Δ^9-THC, a clear trend of signal suppression emerges (Figure 1A). Both milk and dark chocolate show high recovery rates with 1 g loadings, but then decrease linearly as more chocolate is added. Cocoa powder displays a similar correlation between higher sample loadings and lower recovery rates of Δ^9-THC, but to a lesser degree than milk and dark chocolates. This initial experimentation suggests that the presence of chocolate may inhibit the complete recovery of cannabinoids in solution, presenting implications for finished product testing. To determine the scope of this potential matrix effect, we turned our focus to cannabidiol (CBD), another common cannabinoid in commercial *Cannabis* products. After performing the same recovery experiment with a stock solution of CBD, the negative correlation between sample loading and recovery rates of CBD was not as pronounced as with Δ^9-THC (Figure 1B). Neither milk chocolate nor dark chocolate had substantially diminished recoveries of CBD, and all permutations yielded recovery rates >98%, compared to ~93% Δ^9-THC recovery for milk and dark chocolates. While this difference in signal suppression is relatively subtle, it is surprising due to the structural similarity between Δ^9-THC and CBD (Scheme 1). CBD contains two phenolic -OH groups on the resorcinyl moiety, whereas Δ^9-THC has undergone electrophilic cyclization between the pendant allyl group and one of the phenolic -OH groups. We hypothesized that this difference in functional groups may account for the differing magnitudes of signal suppression seen in Figure 1.



Scheme 1: The structures of four common biogenic cannabinoids with *p*-menthyl and resorcinyl fragments annotated; phenolic -OH groups are outlined in red.

To test the hypothesis that structural features of cannabinoids can affect signal suppression rates, the recovery experiment was again repeated with two additional cannabinoids: cannabinol (CBN) and cannabigerol (CBG). While not commonly found in large quantities in edible *Cannabis* products, their varied structures can provide further insight into the relationship between cannabinoid structure and chocolate matrix effect. CBN is a derivative of Δ^9-THC that has undergone oxidative aromatization of the *p*-menthyl moiety, and like Δ^9-THC only contains one phenolic -OH group.[5] CBG is a biogenic precursor to both Δ^9-THC and CBD, and features a long, linear isoprenyl residue and two free phenolic -OH groups, similar to CBD (see Scheme 1).

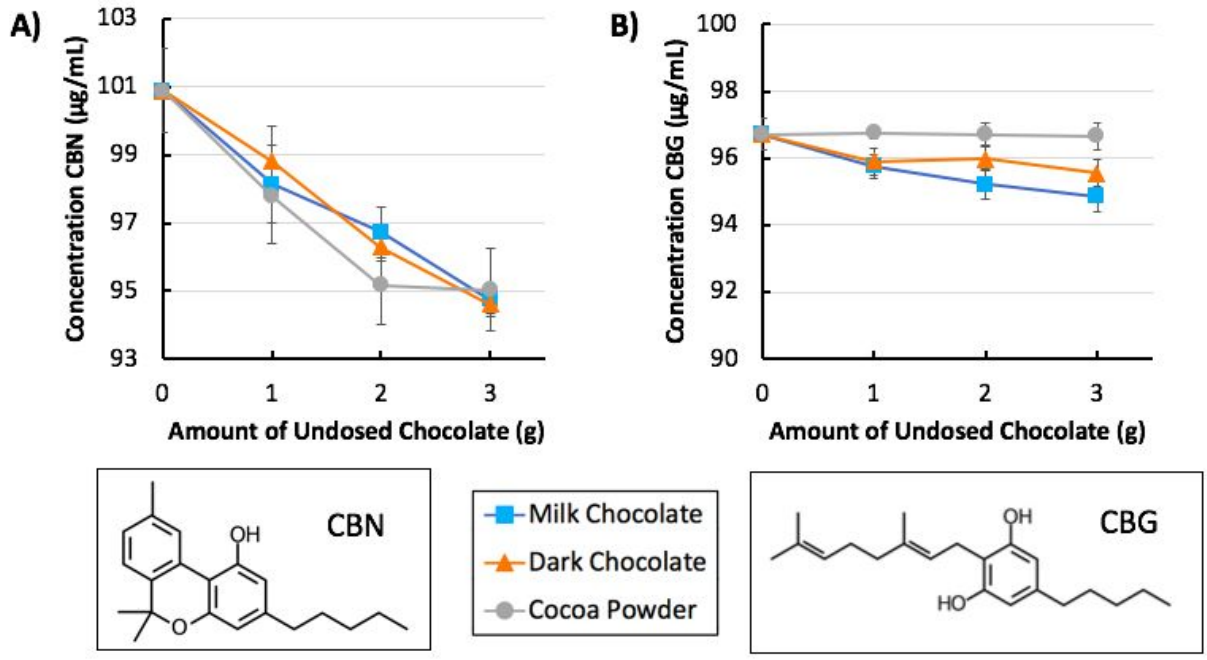
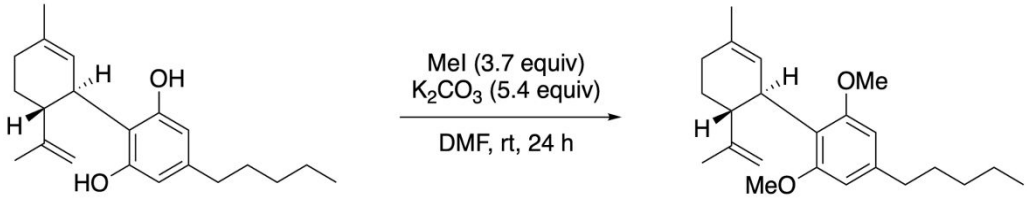


Figure 2: Investigation of matrix effects between various chocolate types and A) cannabinol (CBN), B) cannabigerol (CBG), with chemical structures shown below. Blue squares represent milk chocolate, orange triangles represent dark chocolate, and grey circles represent cocoa powder.

The results of the recovery experiments with CBN and CBG are seen in Figure 2. The recovery rates of CBN in the presence of all chocolate types decrease as the amount of chocolate

increases (Figure 2A), which roughly aligns with the trends seen for Δ^9-THC, which also contains only one phenolic -OH group. It is noted that the presence of cocoa powder substantially decreased the recovery of CBN, which suggests that the aromatized *p*-methyl moiety may cause additional interactions between the cannabinoid and cocoa solids (*vide infra*). Conversely, the recovery rates of CBG remained very high for all chocolate types at all sample loadings (Figure 2B). Similar to CBD, all recoveries for CBG were above 98%, which suggests that CBG and CBD have similar chemical interactions with the chocolate matrix in solution.

Viewed as a whole, the data in Figures 1 and 2 support the hypothesis that the intensity of chocolate matrix interference is related to structural features of the cannabinoids, specifically the number of phenolic -OH groups. Cannabinoids containing two phenolic -OH groups (e.g. CBD, CBG) result in a subtle matrix effect, and high recovery rates upon analysis. A cannabinoid with only a single phenolic -OH group (e.g. Δ^9-THC, CBN) will have more substantial matrix interference, with a concomitant drop in recovery rate. The pronounced effect of phenolic -OH moieties can likely be attributed to a change in equilibrium between the polar, protic methanol solvent and the less polar fat-rich chocolate matrix. Cannabinoids with two polar phenolic -OH groups will have an equilibrium favoring the solvent, and thus have higher recovery rates. A cannabinoid containing a single phenolic -OH functional group is less preferentially solvated by methanol, and thus at equilibrium will be distributed within both the chocolate and solvent phases, resulting in enhanced matrix interference and lower recovery rates. Extrapolating from these findings, a cannabinoid with no phenolic -OH groups could be expected to exhibit substantially lower recovery rates than even the singly phenolic -OH cannabinoids, as the lack of a polar functional group would drastically shift the equilibrium partitioning in favor of the chocolate matrix. Testing a cannabinoid without phenolic -OH groups could provide strong evidence in favor of this hypothesis.



Scheme 2: Synthesis of cannabidiol dimethyl ether (CBDD) from cannabidiol (CBD).

To determine if a cannabinoid without phenolic -OH groups would exhibit a strong matrix effect, a synthetic modification was made to CBD[13] (Scheme 2). By methylating the two phenolic -OH moieties of CBD, cannabidiol is converted to cannabidiol dimethyl ether (CBDD), a non-biogenic cannabinoid that has been shown to stimulate weight gain in (ApoE)-deficient BALB/c. KOR/Stm Slc-*Apoe*^{shl} mice.[15] CBDD contains no phenolic -OH groups, and thus direct comparison to CBD can elucidate the importance of free phenolic -OH functional groups on cannabinoid recovery rates.

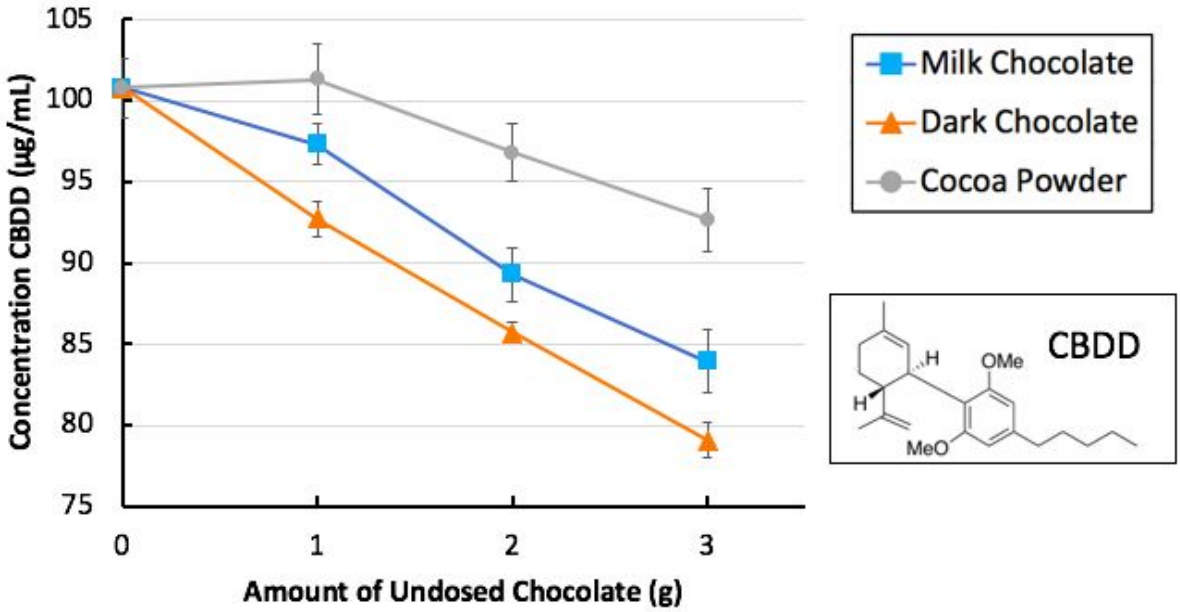
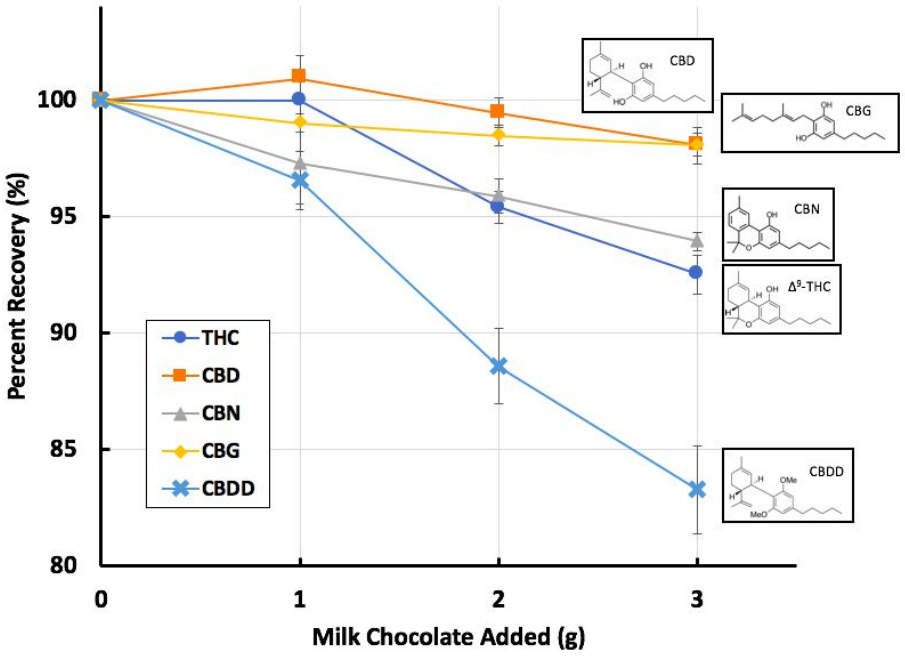


Figure 3: Investigation of matrix effects between various chocolate types and cannabidiol dimethyl ether (CBDD). Blue squares represent milk chocolate, orange triangles represent dark chocolate, and grey circles represent cocoa powder.

Consistent with our hypothesis, CBDD displayed a high degree of signal suppression (Figure 3). Similar to Δ^9-THC and CBN, the two cannabinoids containing one phenolic -OH group,

222 the decrease in recovery rates shows strong linearity, and is directly correlated to the amount of
223 chocolate present. However, the intensity of CBDD signal suppression is substantially higher than
224 that of either Δ^9-THC or CBN, consistent with the hypothesis that structural features of
225 cannabinoids play a significant role in determining if recovery rates will be altered by matrix
226 effects.
227
228 **A)**



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230 **B)**

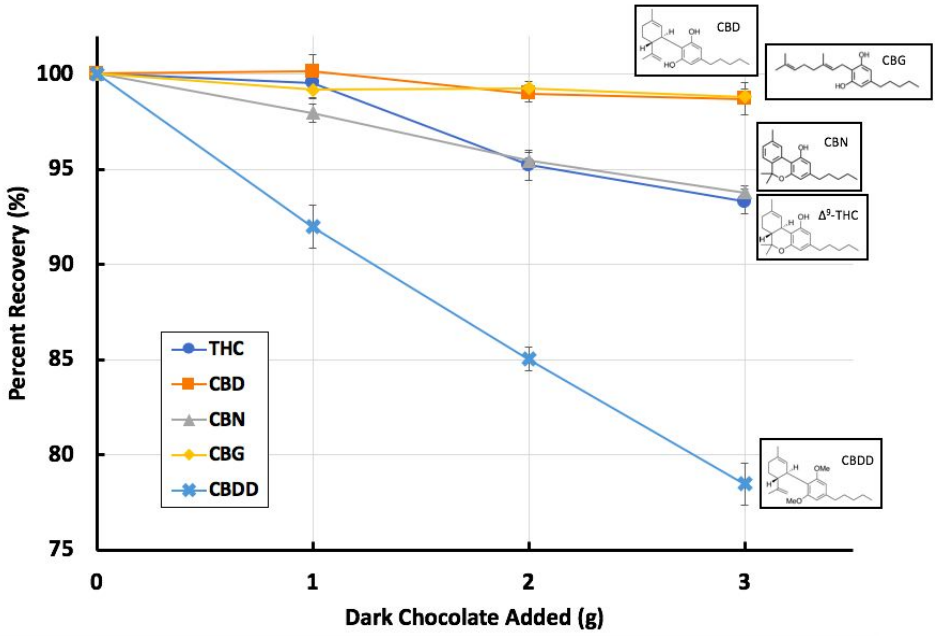

Figure 4: Percent recovery for five cannabinoids from A) milk chocolate and B) dark chocolate. Blue circles represent Δ^9-THC, orange squares represent CBD, grey triangles represent CBN, yellow diamonds represent CBG, and blue crosses represent CBDD.

When the data from Figures 1–3 are plotted as percent recovery, the importance of cannabinoid structure on recovery rate is made clear (Figure 4). For both milk and dark chocolate matrices, analytes with two phenolic -OH groups (CBD, CBG) have percent recoveries >98% for all quantities of chocolate tested. Δ^9-THC and CBN, which both contain one phenolic -OH group, behave similarly to one another, with recovery rates of 95% at 2 g chocolate added, and ~93% at 3 g chocolate. Synthetic CBDD, which bears no phenolic -OH group, exhibits dramatically lower recovery rates than CBD. The 88% recovery seen with 2 g milk chocolate and 85% recovery with 2 g dark chocolate are lower than the lowest recoveries seen on either monophenolic cannabinoid, and the percent recovery dropped <80% with 3 g dark chocolate loading.

The data presented in this study establishes for the first time an interaction between the chocolate matrix and cannabinoid analytes that can impact finished product potency testing. The degree to which the chocolate matrix interferes with cannabinoid analysis depends on two factors: the amount of chocolate present and the chemical structure of the cannabinoid analyte. The amount of chocolate present during testing is directly proportional to the degree of interference,

as the more chocolate present in the sample the lower the percent recovery of analyte. For each combination of chocolate matrix and analyte, the 3 g sample loading afforded the lowest percent recovery, followed by the 2 g loading, with 1 g of chocolate providing the highest percent recovery. This near linearity of this trend suggests that when present in large quantities, some component of the chocolate matrix alters the equilibrium state of the cannabinoid analyte, increasing partitioning within the chocolate matrix. This shift in equilibrium away from the liquid phase results in less cannabinoids in solution, and thus lower recovery rates.

The second major factor that dictates the magnitude of matrix interference is the cannabinoid's chemical structure. Despite the general structural similarities common to all cannabinoids, the effect of the phenolic -OH groups on analyte recovery is striking. If there are two phenolic -OH groups present on the resorcinyl fragment of a cannabinoid, there will be negligible interference from the chocolate matrix (recovery rates >98%). Monophenolic cannabinoids suffer from minor matrix interference (recovery rates >90%), and CBDD, which contains no phenolic -OH groups, has substantial interference from the chocolate matrix (recovery rates >75%). The pronounced effect of phenolic -OH moieties on analyte recovery rates likely stems from the analyte's equilibrium partitioning between liquid and solid phases. Compounds with more phenolic -OH groups are more polar, and likely have increased solubility in methanol, a polar protic solvent. An equilibrium favoring the liquid phase would result in higher recovery rates, such as those observed for both CBD and CBG (Figure 4). By decreasing the amount of phenolic -OH groups, there are fewer favorable interactions with the solvent, and thus equilibrium is expected to shift away from the liquid phase and towards the chocolate matrix. A cannabinoid without phenolic -OH moieties has substantially higher non-polar character, and is likely to have increased solubility in non-polar matrices. This decreased solubility would correlate with low recovery rates, as seen with CBDD in Figures 3 & 4.

Increasing non-polar character in cannabinoids would not only impact the solubility of the analyte in solution, but is also likely to increase interactions with the chocolate matrix. In addition

to added sugars and naturally occurring cocoa solids, chocolate is high in fat, with both milk and dark chocolates tested containing approximately 42% total fat by weight. Cannabinoids are known to be lipophilic and have a high degree of solubility in fats, a characteristic corroborated by the centuries old practice of extracting *Cannabis* with butter or ghee,[16] and the long-term accumulation of cannabinoids seen in human fatty tissue.[17] Thus, it is expected that cannabinoids dissolved in solution could be attracted to the chocolate matrix, due at least in part to its high fat content. This lipophilic interaction would be strongest for non-polar cannabinoids (i.e. containing no phenolic -OH groups) and weakest with polar cannabinoids (i.e. containing one or multiple phenolic -OH groups). This proposed interaction would result in lower recovery rates for non-polar cannabinoids compared to polar cannabinoids, a trend that is supported by the data in Figure 4, where CBDD has a substantially lower recovery rate than CBD when in the presence of chocolate.

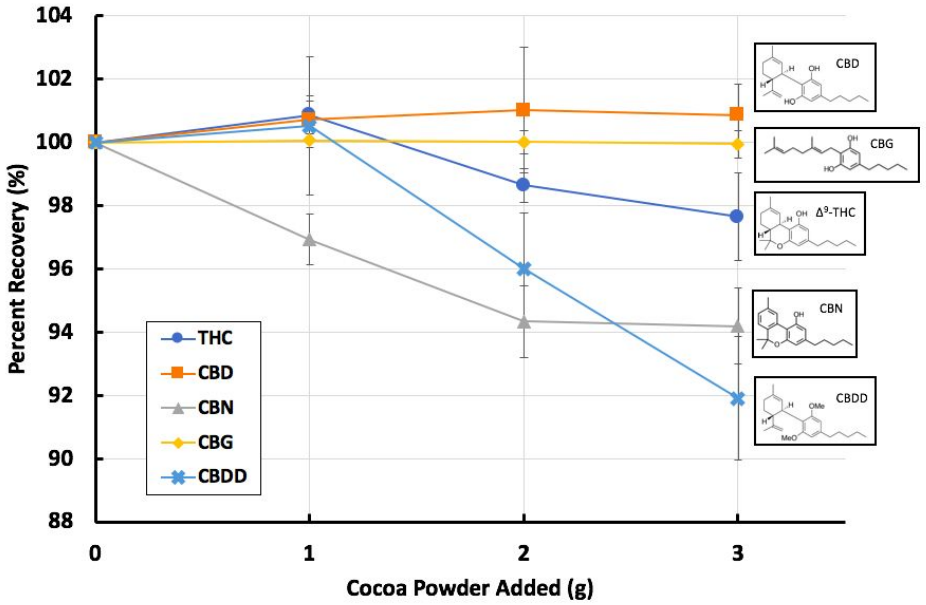

Figure 5: Percent recovery for five cannabinoids from cocoa powder. Blue circles represent Δ^9-THC, orange squares represent CBD, grey triangles represent CBN, yellow diamonds represent CBG, and blue crosses represent CBDD.

Our hypothesis that lipophilic interactions are at the heart of chocolate matrix interference is further bolstered by the data collected with the cocoa powder matrix (Figure 5). In almost all

instances, cannabinoid recovery rates from the cocoa powder matrix were higher than the analogous recovery rates from milk and dark chocolates. Unsweetened non-alkalized cocoa powder is pressed from raw cocoa liquor and thus is mostly cocoa solids, with a lower fat content than finished chocolate products.[12,14] The cocoa powder tested in this study was 25% fat by weight, compared to 42% fat by weight for the milk and dark chocolates. This comparatively lower fat content means there are less lipophilic interactions between the cannabinoid analytes and the cocoa matrix, which in turn would explain the higher recovery rates observed for cocoa powder (see Figures 1–3).

It is noted that this trend is valid for all cannabinoids except for CBN, which exhibits an unusually low recovery rate when extracting from the cocoa powder matrix. Even at 1 g of cocoa powder, recovery rates of CBN are lower than that of Δ^9-THC at 3 g cocoa powder. This sets it apart from the other four tested analytes, where the data matches the overall trends seen in Figure 4 (i.e. CBD/CBG recovery > Δ^9-THC recovery >> CBDD recovery). This suggests that there may be another chemical interaction between cannabinoids and the chocolate matrix at play, one that may be related to the overall amount of cocoa solids present and/or unique structural features of CBN. Specifically, CBN is the only tested cannabinoid with a fully aromatized *p*-menthyl fragment, which in turn makes the molecular skeleton of CBN almost entirely planar. Cocoa solids extracted from cocoa beans are rich with flavonoids, such as (+)-catechin, (–)-epicatechin, and (+)-gallocatechin, and on average can contain up to 8% flavonoids by weight.[12,18] These naturally occurring compounds are classified as flavan-3-ols, a class of molecules that have been shown to inhibit proteins via non-covalent London interactions between non-polar polarizable aromatic rings.[18] It is hypothesized that an analogous non-covalent London interaction between naturally occurring flavan-3-ols in cocoa and the highly π-conjugated CBN may account for the high degree of matrix interference observed between CBN and the cocoa matrix. Experiments testing this hypothesis are ongoing.

The results disclosed in this manuscript represent the early stages of modern *Cannabis* research, and underscore the need for further scientific investigation in the field of *Cannabis* analysis. In analogous fields such as pharmaceuticals, food, and agriculture testing, matrix effects and analyte suppression are well studied, leading to highly precise and accurate analyses. For *Cannabis* analytical testing, such research is scant, and specific molecular interactions that may affect precise testing are either understudied or presently unknown. Matrix interference from chocolate products on cannabinoid analytes appears to be quite nuanced, and has been shown to be dependent on quantity of chocolate analyzed, composition with respect to fat and cocoa solids, and multiple structural features of the cannabinoid analytes. Many of these factors are not limited to just chocolate matrices, as baked goods and topical products are high in fat and flavoring additives, and thus might interfere with analysis of cannabinoid content. Any scientific efforts to standardize *Cannabis* analytical methods must be based on detailed studies of the molecules and matrices involved. Further scientific advances in *Cannabis* testing will be required in order for the *Cannabis* industry to continue to make strides away from the black market and for the long-term stability of the *Cannabis* industry as a whole.

Abbreviations:

Δ^9-THC, Δ^9-tetrahydrocannabinol; THCA, Δ^9-tetrahydrocannabinolic acid; CBD, cannabidiol; CBDA, cannabidiolic acid; CBN, cannabinol; CBG, cannabigerol; HPLC, high pressure liquid chromatography; DMF, dimethylformamide; TMS, trimethylsilane; TLC, thin-layer chromatography; CBDD, cannabidiol dimethyl ether.

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Supporting Information: HPLC chromatograms of Δ^9-THC, CBD, CBN, CBG, and CBDD prepped with 3 g of dark chocolate (Figures SI1–SI5) (PDF). The supporting information is available free of charge on the ACS Publications website at DOI: **XXXXXXXXXXX.**

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