# A Validated Low-LOQ Study of Cannabinoid Content in Cold-Pressed Hemp Seed Oil (CPHSO) Manufactured in North America

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**Abstract**

A study of cannabinoid content in commercially available cold-pressed hemp seed oil (CPHSO) manufactured in North America and assayed using a validated low-LOQ analytical method with UHPLC-MS/MS quantitation was conducted. Thirty CPHSO samples from small, medium, and large-scale manufacturers were voluntarily submitted. Samples were produced from eleven known cultivars grown in three Canadian provinces and six US States, plus one sample from seeds imported from Poland and pressed in the USA. Oil density was measured for each sample, as were the content of sixteen cannabinoids with validated commercial reference standards, and reported in parts-per-million (ppm). Observational and statistical methods were used to examine variances in analyte concentrations, demonstrating significant differences in cannabinoid concentrations between samples. Several per-sample and per-analyte heatmaps aided in the visual examination of variances. A two-phase series of linear regressions were performed on normally distributed cannabinoids with raw and trimmed data sets to determine if content variations correlated to manufacturer cleaning, handling, and storage procedures, or if the variation was influenced more by cultivar. The research findings suggest that variance in cannabinoid content is likely most influenced by cultivar, but do not rule out contributions by supplier handling and processing techniques.

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A study of cannabinoid content in commercially available cold-pressed hemp seed oil (CPHSO) manufactured in North America and assayed using a validated low-LOQ analytical method with UHPLC-MS/MS quantitation was conducted. Thirty CPHSO samples from small, medium, and large-scale manufacturers were voluntarily submitted. Samples were produced from eleven known cultivars grown in three Canadian provinces and six US States, plus one sample from seeds imported from Poland and pressed in the USA. Oil density was measured for each sample, as were the content of sixteen cannabinoids with validated commercial reference standards, and reported in parts-per-million (ppm). Observational and statistical methods were used to examine variances in analyte concentrations, demonstrating significant differences in cannabinoid concentrations between samples. Several per-sample and per-analyte heatmaps aided in the visual examination of variances. A two-phase series of linear regressions were performed on normally distributed cannabinoids with raw and trimmed data sets to determine if content variations correlated to manufacturer cleaning, handling, and storage procedures, or if the variation was influenced more by cultivar. The research findings suggest that variance in cannabinoid content is likely most influenced by cultivar, but do not rule out contributions by supplier handling and processing techniques.

# List of Abbreviations

[?]8-THC [?]8 Tetrahydrocannabinol

AOAC The Association of Official Agricultural Chemists (a.k.a. AOAC International)

AOCS The American Oil Chemists' Society

CBC Cannabichromene

CBCA Cannabichromenic acid

CBD Cannabidiol

CBDA Cannabidiolic acid

CBDV Cannabidivarin

CBDVA Cannabidivarinic acid

CBG Cannabigerol

CBGA Cannabigerolic acid

CBL Cannabicyclol

CBN Cannabinol

CBNA Cannabinolic acid

CFR Unites States Code of Federal Regulations

CI Confidence interval for an observed mean

COA Certificate of Analysis

CPHSO Cold-Pressed Hemp Seed Oil (mechanically extracted at <50° C)

FAP Feed Additive Petition (as defined by 21 CFR §571)

FDA-CVM US Food and Drug Administration Center for Veterinary Medicine

GLP Good Laboratory Practices (as defined by 21 CFR §58)

LOD Level of detection specifies the minimum amount of detectable analyte

LOQ Level of quantitation specifies the minimum amount of quantifiable analyte

MS Mass Spectrometry

ppm Parts per million

THC [?]9 Tetrahydrocannabinol ([?]9-THC)

THCA [?]9 Tetrahydrocannabinolic acid ([?]9-THCA)

THCV Tetrahydrocannabivarin

THCVA Tetrahydrocannabivarinic acid

UHPLC-MS/MS Ultra High-Performance Liquid Chromatography with MS/MS Detection

# Figures and Tables



# Introduction

Many studies of the nutritional properties and a few studies oxidation of cold-pressed hemp seed oil (CPHSO) have been previously conducted . More recently, researches have started to investigate the cannabinoid content and decarboxylation properties of cannabinoids in CPHSO, but much of the research has been conducted outside North America .

This study is the first known investigation of CPHSO manufactured explicitly in North America. Production of CPHSO began in Canada many years before it was allowed in the United States, so American manufacturers generally have less experience than Canadian producers . Nevertheless, this researcher is not aware of any published data indicating significant differences in American vs. Canadian CPHSO.

The data and findings from this study apply to multiple areas of industry and future investigations, and the analysis provides insights and informs other researchers and the scientific community at large. Also, regulators interested in cannabinoid safety and toxicity require bonafide sources of credible information to make informed decisions. Finally, animal feed and human food formulators can consider these findings as they incorporate hempseed oils into their products.

One study suggests cannabinoids are present in the nut when tested after dissection. More recent studies suggest cannabinoids are not produced in the seeds of the plant, but small quantities are transmitted to the seed and oil by contact with other plant surfaces . Also, some researchers have postulated that seed cleaning methods and procedures may account for significant variances in cannabinoid content found in CPHSO, but there are conflicting opinions on this subject, and other studies primarily attribute differences to cultivar .

Current data suggests that the cannabis plant contains over one hundred (100) identifiable cannabinoids, but standardized tests and reference materials are lacking for most . While complete elimination of cannabinoids in CPHSO is unlikely, proper processing and seed cleaning steps will minimize them . Current research suggests that cannabinoids in cold-pressed hemp seed oil (CPHSO) be treated as naturally-occurring contaminants, much like those found in many other food products, since they are not germane to any nutritional value.

This study quantifies the cannabinoid content of commercially available CPHSO manufactured in North America (Canada and the United States). In addition, the investigator evaluates the observed variances in cannabinoid content to determine if such variances correlate to cultivars, suppliers, or both. The term supplier is analogous to the CPHSO manufacturer in this study.

Observational and statistical analysis was performed in two phases. In the first phase, statistical analysis was conducted without removing outliers. The second phase re-analyzed the data with outliers removed. Outliers for phase 2 are defined as those measurements more than the 95% confidence interval upper limit calculated in the phase 1 data set. This paper provides an analysis of the findings and sets forth the conclusions drawn from the findings.

# Materials and Methods

## Sample Collection and Description

The terms "supplier" and "suppliers" used in this document and the statistical data and charts is analogous with "manufacturer(s)." All suppliers are the original CPHSO manufacturer in this study. The researcher excluded distributors and resellers from the study to understand the characteristics of CPHSO manufactured in North America vs. what could be purchased on the open market and sourced from a wide variety of countries and locales.

Manufacturers voluntarily submitted CPHSO samples with supporting documents (e.g., COAs) directly to the analytical laboratory following the prescribed procedures for a GLP study. Eight firms provided a total of thirty-four samples for study. Four samples from a single manufacturer were excluded from the study due to excess solids in the oil. The final data set included thirty samples from two Canadian manufacturers and five American manufacturers. Table 1 provides a summary of the samples included in the study.

Table 1: Summary of samples investigated

| Total manufacturers providing samples | Total manufacturers providing samples | 7 |
|---|---|---|
| Total growing locations (excludes unknowns) | Total growing locations (excludes unknowns) | 11 |
| Total cultivars (excludes unknowns) | Total cultivars (excludes unknowns) | 12 |
| Total samples identified as conventional | Total samples identified as conventional | 19 |
| Total samples identified as organic | | 7 |
| Total samples without conventional/organic designation | | 4 |

Seeds for one sample were grown in Poland, the remaining seeds used to press the oils were grown North America. Five Canadian-sourced samples were grown in provinces the manufacturer did not disclose. Of the remaining, one was grown in Alberta, three in Manitoba, and two were grown in Saskatchewan. Samples grown in the United States include three from seeds grown in Indiana, two from Kentucky, one from Montana, three from North Dakota, three from New York, and five from Virginia.

The researcher did not investigate cannabinoid variances based on growing locale since most suppliers reported only a single cultivar grown in a single location. Therefore, analysis by growing locale would not provide any new or significantly meaningful information given the available data.

Suppliers identified the cultivar for twenty-six of the samples, which included a mix of eleven varietals. The number of different cultivars ranged from one to four varietals per supplier. Of the four samples of an unknown varietal, one supplier did not respond to follow-up requests to identify the cultivar(s), and one sample came from a US manufacturer that sourced seeds from Poland without the varietal data being available.

Nineteen of the samples were classified as "conventional" by the supplier, and seven were classified as "organic." The researcher did not attempt to verify compliance with any official organic certification. The conventional or organic status of four samples was not provided.

### Analytical Procedures and Sample Analysis

The researcher contracted with a third-party ISO 17205 accredited laboratory that is also DEA, FDA, and USDA licensed to develop a validated GLP method to assay sixteen (16) cannabinoids in CPHSO with a lower LOQ of 50 ppb (0.05 ppm) using UHPLC-MS/MS. The FDA-CVM reviewed the method before the laboratory conducted the GLP-compliant analysis. All analytical measurements were conducted using a Shimadzu 8050 LC-MS/MS instrument and are reported in ppm (except density).

The development of a low-LOQ analytical method was required to quantify cannabinoids that naturally occur in minimal quantities that commercial laboratories could not assay. Also, there is no commonly-available method or approved AOAC or AOCS method for testing cannabinoids in CPHSO at very low LOQs, and the AOCS draft method did not provide sufficiently-low LOQ for the study requirements. Finally, this research is part of a series of safety and efficacy investigations relating to hemp in animal feeds. Existing research indicates some animals have a lower tolerance to cannabinoids than humans, with CBDA and CBD hepatotoxicity documented in multiple species, so these cannabinoids are of particular interest.

The third-party laboratory assayed the acceptable samples using the validated method and provided the results to this researcher. Each sample's oil density and cannabinoid analytes were assayed multiple times per the GLP requirements. The mean values of density and each assayed cannabinoid content were reported in ppm and entered into a spreadsheet for further analysis. The analytical assay found no detectable levels of Δ8-THC, so this analyte was not included in the statistical analysis or reported herein. The analyte values (in ppm) were also summed for each sample.

Data were analyzed using Microsoft Office Professional® 2016 Excel®spreadsheets and IBM SPSS® version 26 statistical analysis software. The final documentation was constructed with Microsoft Office Professional® 2016 Word® and Adobe Acrobat Professional® 2017.

### Data preparation before statistical analysis

For the phase 1 investigation, a value of 95% of the LOQ was substituted for cannabinoid analytes detected below the lower LOQ. This substitution value was chosen because the investigator determined that this value may better represent the true data than zero (0.0) or treatment as missing values for the analysis. In many cases, only a few values are substituted for any given analyte. Notable exceptions are CBN (20% <LOQ replaced) and THCVA (46.7% <LOQ replaced). The <LOQ substituted values are highlighted with light-gray in Table 7.

Descriptive statistics were computed for all values, including a 95% confidence interval and the inter-quartile lower and upper range limits. Table 8 shows the descriptive statistics for the trimmed data. Light-red backgrounds and red text indicate values that exceed the upper limit of the 95% confidence interval in Table 7.

The raw data was copied to a second spreadsheet for phase 2, and all substituted <LOQ values and values higher than the upper limit of the initial 95% confidence interval were deleted. The descriptive statistics were recomputed for all values. Table 12 (a-b) shows the trimmed data with recomputed descriptive statistics.

## Results and Discussion

Statistical tests for normal distribution were conducted before any regression analysis. The Shapiro-Wilk method tests the null hypothesis that the data are normally distributed, and was selected due to the small

number of samples, particularly after removing upper outlier values in the phase 2 data set. Normality tests are essential since many other statistical tests presume a normal distribution of data, notably the dependent variables in linear regression. Table 2 summarizes the results of the Shapiro-Wilk normality tests for both raw and trimmed data.

In the phase 1 data set, only density and three of the fifteen (20%) cannabinoids (CBCA, CBDA, CBNA) had a statistically normal distribution ($p > 0.05$). Conversely, the distribution was statistically normal for nine of fifteen (60%) of the analytes in the phase 2 data set. CBCA, CBC, CBD, CBL, CBN, and THC were not normally distributed in the phase 2 data set. The Shapiro-Wilk test showed increases in significance ($p$ -values) for individual analytes in the phase 2 data set, but it was not enough to pass the normality test at 0.05 significance.

Table 2 (a-b): Shapiro-Wilk tests for normal distribution

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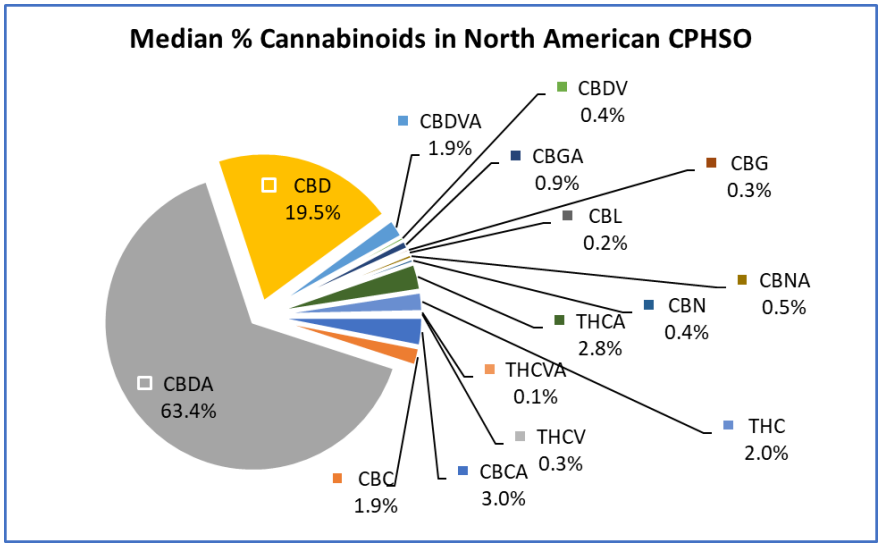
Note: Items in Green are normally distributed per the Shapiro-Wilk test ($p > 0.050$).

## Phase 1 Analysis (raw data)

Figure 1 shows the median percentage of each analyte in the raw data set. Most analytes represent a tiny fraction of the total cannabinoids. Notably, CBDA and CBD are significant contributors to total measurable cannabinoid content.

Figure 1: Median cannabinoid percentages found in CPHSO




In the phase 1 data set, the least amount of measured CBDA content was 11.80% of the total measured cannabinoid content. However, this sample also had unusually high levels of CBD, indicating extensive enzymatic or heat-induced decarboxylation. The highest amount of CBDA was 83.24% of the total cannabinoid content, and the mean CBDA content was 58.59% (SEM 2.84%, n = 30). CBD content is the next most abundant constituent and ranged from a low of 6.09% of the total measured cannabinoids to a high of 75.04% of the total, with the mean being 24.56% (SEM 2.89%, n = 30).

Histograms, Q-Q plots, and scatter plots by both supplier and cultivar were produced for all measured and computed analyte values. The supplemental data addendum contains all of these charts. Visual inspection shows many of the histograms indicate right-tailed skew, with the majority of values clustered on the lower end of the scales.

Visual inspection of each cannabinoid's content was conducted by applying color scales to the percentage-of-content table on a per sample (row-by-row) basis. The per-sample color-scaling creates a heatmap showing the analytes with the greatest to least percentage of the cannabinoid content, as shown in Table 9. Darker to lighter shares of red indicate the higher percentages, medium percentages are highlighted in shades of yellow, and the lowest percentages of cannabinoid content are in green. Note that some totals do not add up to 100% due to small rounding errors.

Differences in cannabinoid content by cultivar are also observable when samples are placed in a row-oriented heatmap and sorted by cultivar, as shown in Table 10. Differences are also observed when row-oriented heat-mapped data is sorted and categorized by the supplier, as in Table 11. The color-coding in Table 10 and Table 11 both follow the same scheme as described for Table 9, where red indicates more copious amounts of any specific cannabinoid, yellow being moderate levels, and green indicates lower levels of the analyte. Strong similarities were observed between cannabinoid content in the scatter plots by the supplier and by cultivar, but this relationship is not surprising since most manufacturers only supplied a single cultivar.

Each supplier was assigned a unique identifier, and these values were transformed and coded into a series of new dichotomous variables to investigate any supplier-related variance. A series of linear regression tests with 0.05 significance were performed on the analytes having statistically normal distribution using supplier-encoded dichotomous variables as coefficients.

For the phase 1 data set, the regression analysis was limited to CBCA, CBDA, and CBNA. The supplemental data addendum contains the model summary data, ANOVA, and coefficients for the regression of these four tests. A summary of each regression analysis done with the suppliers (n = 30) is provided in Table 1Table 3:

Table 3: Phase 1 regression of analytes and suppliers

*Analyte Adj. $R^2$ F p-value # Coefficients # Sig.*

CBCA 0.368 3.814 0.009 6 2

CBDA 0.602 8.309 <0.0005 6 5

CBNA 0.372 3.866 0.008 6 2

Each varietal was also assigned a unique cultivar identifier (CULID), and then the cultivar identifiers were transformed and coded into new dichotomous variables to test for variance potentially attributable to cultivar. The four samples with unknown cultivars were not coded or included in the analysis. The four samples of CFX, CFX2, and CRS1 cultivars were encoded as a single group. A linear regression analysis with 0.05 significance was performed on each of the normally distributed variables with the cultivar-encoded variables used as coefficients.

The supplemental data addendum contains the model summary data, ANOVA, and coefficients of the regression for CBCA, CBDA, and CBNA using the dichotomous cultivar variables. A summary of each regression analysis done with cultivar (n = 30) is provided in Table 4.

Table 4: Phase 1 regression of analytes and cultivars

*Analyte Adj. $R^2$ F p-value # Coefficients # Sig.*

CBCA 0.075 1.260 0.316 9 N/A

CBDA 0.246 2.052 0.087 9 N/A

CBNA 0.526 4.573 0.002 9 3

## Phase 2 analysis (trimmed data)

For phase 2 of the analysis, the <LOD substitution values and all values that exceeded the 95% confidence interval upper limit computed in the initial investigation were removed. The rationale here was to determine if data would exhibit a more normalized distribution once upper outliers and <LOQ values, which may have contributed to the observed right-tailed skew, were deleted.

While oil density retained its normal distribution, the normality of distribution for many of the analytes was noticeably improved from phase 1 (see Table 2). Histograms and Q-Q plots were constructed for oil density, and each cannabinoid in the phase 2 data set, as shown in the supplemental data addendum. The higher number of statistically normally distributed analytes allowed for an increased number of regression tests in phase 2.

The supplier and cultivar were coded into new dichotomous variables in phase 2 using the same organization as conducted in phase 1 to investigate the possible correlation between cannabinoid content variation and suppliers and cultivars. Not all suppliers or cultivars were included in the phase 2 regression analysis because some samples were excluded as a result of trimming data at the 95% CI upper limit.

Linear regression analysis was conducted using the phase 2 (trimmed) and coded supplier data. The supplemental data addendum shows the regression model summaries, ANOVA tables, and coefficient tables for the phase 2 supplier regression tests. Many of the regression models for supplier were significant and had a higher number of significant coefficients than in phase 1. A summary of the supplier regression analysis with suppliers is provided in Table 5.

Table 5: Phase 2 regression of analytes and suppliers

*Analyte Adj. $R^2$ F p-value # Coefficients # Sig.*

CBDA 0.350 3.150 0.039 5 1

CBDVA 0.631 6.699 0.002 6 3

CBDV 0.681 9.536 <0.0005 6 2

CBGA 0.574 5.035 0.008 6 1

CBG 0.332 3.287 0.028 5 0

CBNA 0.426 3.100 0.050 6 0

THCA 0.233 2.093 0.131 5 N/A

THCVA -0.236 0.332 0.732 2 N/A

THCV 0.888 28.697 0.002 2 2

The investigation of any variance attributable to cultivar was conducted via linear regression with 0.05 significance using the cultivar-encoded variables as coefficients. The supplemental data addendum contains the model summary data, ANOVA, and coefficients of the regression for the phase 2 data with the recoded cultivar variables. A summary of the phase 2 regression test results with cultivars is provided in Table 6.

Table 6: Phase 2 regression of analytes and cultivars.

*Analyte Adj. $R^2$ F p-value # Coefficients # Sig.*

CBDA 0.521 4.629 0.009 6 4

CBDVA 0.523 4.137 0.013 7 4

CBDV 0.707 9.289 <0.0005 7 3

CBGA 0.067 1.217 0.362 6 N/A

CBG 0.461 4.277 0.008 6 2

CBNA 0.156 1.450 0.287 7 N/A

THCA 0.001 1.003 0.478 7 N/A

# Conclusions

The small number of samples in the study limited in-depth conclusions from statistical analysis but did provide an opportunity to investigate certain aspects of research published before this study. The variety of cultivars aligned closely with suppliers and most manufacturers provided samples from only a single varietal, which indicates the manufacturers and growers may limit their cultivar selection to those best suited to the geographic area or to those the farmers are most experienced in growing.

The phase 1 (raw data) findings showed that the majority of cannabinoid analytes found in commercial cold-pressed hemp seed oil manufactured by producers in North America are of minor quantity. Notable exceptions include CBDA and CBD, which generally account for 50-90% of the total measured cannabinoid content.

Most assays revealed content concentrations at the lower end of the observed ranges, with a substantial amount of samples having higher than average value exceeding the upper limits of the 95% mean confidence interval (CI). In some analytes, more than 30% of the samples exceeded the upper limits of the CI for multiple analytes. In phase 2, after the data was trimmed to the upper limit of the CI, the range of cannabinoid content for most analytes was considerably lower.

These findings suggest that commercially available hemp seed oils can contain a wide range of cannabinoid concentrations. Measuring the cannabinoids and understanding their contribution to total cannabinoid content satisfies the first and third objectives of this study. The researcher makes no particular claims about the second objective, which relates to any safety and efficacy concerns, since any such determinations will likely be species-specific. Nevertheless, these findings should be informative to formulators wishing to incorporate CPHSO.

For the second objective, the researcher investigated whether the variations could be attributed to factors related to cleaning, handling, and processing, or are more cultivar-related due to natural cannabinoid content variances in specific breeds of the plant. The results of investigations into supplier-related variations in cannabinoid concentrations were inconclusive, with mixed and conflicting results.

Variations in cannabinoid content can be attributed to multiple factors, but the evidence seems to support these variations are related more to cultivar than the supplier. The investigations into cultivar-related cannabinoid concentrations showed significant correlations, and even though most suppliers provided only one cultivar, reciprocal relationships did not exist when the same data was regressed with suppliers. However, the researcher determined that there is insufficient data in this study to make reliable conclusions as to whether cannabinoid content differences are influenced by supplier-related issues.

While this study satisfied the stated objectives, it highlights areas needing additional research concerning cannabinoid content and variances. A more extensive data set, with a larger number of samples of each varietal, and higher supplier participation is recommended to produce reliable conclusions about factors contributing to cannabinoid variance in CPHSO.

## Acknowledgments

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Table 7: Phase 1 (raw) data with outliers highlighted

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Notes:

1. Light gray with black text indicates detection below the LOQ, which is a non-zero, unquantified observation. The substitution of a reported "< LOD" with a value equal to 95% of the LOQ supports mathematical and statistical comparison.
2. Red numbers in pink backgrounds indicate that the value exceeds the 95% Confidence Interval (CI) upper limit (see Table 2 for the CI).

Table 8: Phase 1 (raw) data descriptive statistics

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Table 9: Phase 1 (raw) data heatmap of cannabinoids as percentages of the total content

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Notes:

1. Color scales were applied on a row-by-row basis to highlight the most significant cannabinoids as a percentage of measured content.
2. Not all totals equal 100% due to small rounding errors.

Table 10: Phase 1 (raw) data heatmap of cannabinoids grouped by cultivar

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Table 11: Phase 1 (raw) data heatmap of cannabinoids grouped by supplier

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Table 12: Phase 2 data trimmed at the original 95% CI upper limit

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