Potency, Terpenes and Pesticides Analysis Using High Resolution LC-MS/MS: Results and Comparison to Alternative Analytical Methodologies

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About Next Frontier Biosciences

• Colorado-based biotech company using advanced pharmaceutical methods to develop highly standardized and reproducible purified cannabinoid products
• Highly experienced group of biotech executives and research scientists with over 100 years of combined pharmaceutical drug development experience
• Leveraging proprietary formulations to develop purified cannabinoid products that provide accurate dosing, improved bioavailability, and optimized cannabinoid profiles for fast acting and consistent results
Why Hi-Res LC/MS?

• Accurately measure cannabinoids in variety of matrices
• Trace analysis of raw materials for contaminants (pesticides, aflatoxins etc.)
• Evaluate unknown or components of interest (flavonoids, lipids, terpenes etc.)
• Support bioavailability studies
Cannabinoid Potency
Common Cannabinoids

- $\Delta 9$ tetrahydrocannabinol (THC)
  - “Active ingredient” in cannabis
- Cannabidiol (CBD)
- Cannabichromene (CBC)
- $\Delta 8$ tetrahydrocannabinol
- Cannabigerol (CBG)
- Cannabinol (CBN)
- Cannabidivarin (CBDV)
- Tetrahydrocannabinidivarin (THCV)
- Acid analogues (CBDA, THCA, CBGA)
Cannabinoid Structures and molecular formula

THC - C21H30O2

CBD - C21H30O2

Note common backbone and formula
Challenges in MS quantitation of cannabinoids

Due to common structure and identical molecular formula of several cannabinoids mass spectrometry is NOT exclusively selective for individual cannabinoids

- High resolution has no intrinsic advantages for cannabinoids with identical formulas
- Product ion scans (MS/MS) do not have unique transitions
- Good chromatography is a necessity
## LC/MS Conditions

### Column
- Agilent Zorbax Bonus RP 4.6 x 150mm 3.5µm

### Mobile Phase
- A: 0.3% Formic Acid
- B: Methanol
- Flow 1.0 mL/ min

### DuoSpray™ Source (SCIEX)
- Temperature- 500°
- GS1- 40
- GS2- 70
- Curtain- 40
- ISV- 3000
- DP- 80
Example cannabinoid HPLC separation

CBDA
THCA

CBD
exo-THC
Δ9 THC
Δ8 THC
CBC
Advantages of TOF MS quantitation of cannabinoids

Interferences from non-cannabinoids is essentially absent with narrow m/z extraction window (0.01 Daltons)

Our methods acquire TOF scan data from m/z 100-700 with dependent spectra

We can extract signal(s) of interest and analyze accurate m/z or MS/MS for probable structure/molecular formula
The evolution of the Internal Standard

Internal standards not only minimize sample preparation errors but help normalize matrix effects in MS.

- **Methyl Paraben**
  - Stable response
  - Prolific in environment (personal care products)

- **2-Acetyl Biphenyl**
  - Stable response
  - Did not follow cannabinoid response in matrix

- **Genistein**
  - Current selection
  - Good response matching in matrix
Tips for Internal Standards

- Add internal standard to diluents instead of multiple small volume additions to samples and standards.
- Once a good chromatographic separation is established, compare results with and without ISTD, UV vs MS.
- Track internal standard response throughout runs; are there any trends?
- Confirm suitability with appropriate validation (accuracy in matrix).

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Example Internal Standard response throughout run

- $3\sigma$ plot of ISTD response over a 11hr analysis
## Accuracy study

- Select cannabinoids (CBD, CBC and CBD) were added to 3 individual samples of a high THC concentrate

<table>
<thead>
<tr>
<th>Sample</th>
<th>CBD</th>
<th>CBC</th>
<th>CBG</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>92.3</td>
<td>98.2</td>
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<td>2</td>
<td>94.7</td>
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<td>3</td>
<td>93.5</td>
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<td>Avg</td>
<td>93.5</td>
<td>97.9</td>
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<tr>
<td>RSD</td>
<td>1.3</td>
<td>0.2</td>
<td>0.1</td>
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</table>
LC/MS vs HPLC UV- Experimental Design

Common sample preparations in triplicate of concentrate oil, distillate and flower were analyzed by each technique.

Adjustments in sample dilution, gradient elution and injection volume were made accordingly.

Both systems use the same HPLC stationary phase and mobile phase.
LC/MS vs HPLC UV - THC Results

### Concentrate Oil

<table>
<thead>
<tr>
<th>% Assay</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Average</th>
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<tbody>
<tr>
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<td>84.00</td>
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<tr>
<td>88.00</td>
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#### Flower

<table>
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<th>% Assay</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Average</th>
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#### Distillate

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<td>88.00</td>
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</tbody>
</table>

### Notes

- The graphs show the comparison of THC assay results using LC/MS and HPLC UV for different samples (Concentrate Oil, Flower, Distillate).
- The data indicates that the assay results are consistent across samples for each method.
- The average % assay values for each sample and method are provided.

[Next Frontier Biosciences Logo]
**Calibration Standards**

Supplied as MeOH solutions typically 1mg/mL

- Special precaution should be taken when handling due to volatility of solvent
- Cerilliant used as primary source
  - Widest selection of cannabinoids with extensive characterization
- Restek used as second source
  - Agreement with primary standards +/- 5%
  - Used as QC for select cannabinoids
Working range for analysis

Selected based on anticipated sample types (i.e. concentrates)
- 1-100µg/mL THC
- 0.1-10µg/mL all other cannabinoids
- Typical sample preparation ~10mg/10mL diluted 2:100

Dilutions are modified if working with flower or other products

THC curve is non-linear (Wagner fit)

All other cannabinoids are linear fit
Calibration curves

Δ9 THC

CBD
Sample Preparation

Use solubility to your advantage

- Common solvents we use in sample preparation
  - Tetrahydrofuran (creams, salves)
  - Methanol (concentrates)
  - 10% Chloroform/ MeOH (flower)
  - Water (edibles)
- Final dilution should be compatible with your LC method (100% Methanol)
- Any precipitant should be filtered or centrifuged
Laboratory Control

THC

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<th>Date</th>
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<td>7/3/2017</td>
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CBD

<table>
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<td>7/3/2017</td>
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Selectivity - Sublingual Dosage Placebo

UV-225nm
Troubleshooting

- **Time to clean the source?**
  - Erratic Recovery
  - Low Sensitivity
  - $R^2 \leq 0.990$

- **Spray Quality**
  - Clogged Electrode
  - Adjustment

- **TOF calibration**
  - Calibrant Ion Sensitivity
  - CDS pump
Pesticides Quantitation in Cannabis Raw Materials
What are cannabis raw materials?

• Flower or leaf
  • Processed plant materials
  • Typically ingested by smoking
• Concentrates
  • Oils or waxes prepared by extraction/ concentration of flower/ leaf
  • Used in vape devices, edibles
• Contaminates are often concentrated with cannabinoids depending on method of extraction
Current Pesticide regulations

Colorado
- Executive order by governor 11/2015 prohibiting “off-label” usage
- Colorado Department of Agriculture published list of allowed pesticides

Washington State
- Pesticide use is allowed for “tolerance exempt” and “unspecified food crop” labeled pesticides

Oregon
- Oregon Department of Agriculture published list of allowed pesticides
Commonly used Fungicides/ Pesticides

- Myclobutanil
- Piperonyl Butoxide
- Avermectin
Sample Preparation

• Flower (Quechers)
  • Grind ~1g material in homogenizer with 5 mL ACN + 5mL H₂O
  • Add 1 packet Restek Q-sep Q110 (EN 15662 Method)
  • Vortex to mix then centrifuge
  • Recover supernatant and transfer to Restek Q-sep dSPE tube (150mg MgSO₄, 50mg PSA, 50mg GCB)
  • Vortex intermittently 1hr, centrifuge then transfer supernatant to HPLC vial- Can visually observe chlorophyll removal

• Concentrates
  • Dilute to 200mg/ mL in appropriate solvent (THF)
# LC/MS Conditions

## Column
- Restek ARC-C18 2.7µm 3.0 x 150mm

## Mobile Phase
- A: 0.05 M Ammonium Formate
- B: Methanol
- Flow 0.5 mL/ min
- Gradient: 5%B → 95%B in 13 minutes

## DuoSpray™ Source (SCIEX)
- Temperature- 300°
- GS1- 40
- GS2- 60
- Curtain- 40
- ISV- 4000
- DP- 70
## LC/MS Conditions (cont)

**TripleTOF® 5600 System (SCIEX)**

- **TOF MS**
  - Positive Polarity
  - scan 100-1000 m/z
  - Accumulation time 0.25 sec
- **IDA**
  - Candidate ions monitored/ cycle- 4
  - Exclusion- Target excluded after 6 sec
  - Dynamic Background Subtraction (DBS) enabled
Method Development

Source Optimization - Infuse select compounds (Myclobutanil, Avermectin and Spiromesifen)

Inject calibration including all compounds (7 initial) at concentrations 1-500ng/mL

Extract m/z signal from TOF/MS (typically +/- 0.005)

New compounds are added by calculating accurate m/z and extracting signal

Initial list was 7 compounds and has now been expanded to 19 compounds - no MRM transitions to optimize
250ng/mL Standard

- 250ng/mL - Metalaxyl (Standard) 280.1518 - 280.1568 - 051316 Calibration.wiff (sample 8)
- Area: 5283158.622, Height: 6.811e5, RT: 9.30 min
- Myclobutanil (289.1219 - 289.1221)
- Spiromesifen (371.2192 - 371.2242)
- Imidacloprid (256.0600 - 256.0602)
- Etoxazole (360.1774 - 360.1776)
- Bifenazate (301.1551 - 301.1553)
- Abamectin B1a (890.5160 - 890.5360)
- Monocrotophos (224.0657 - 224.0707)
- Fenoxycarb (302.1362 - 302.1412)
- Spirotetramat (374.1937 - 374.1967)
- Azoxystrobin (404.1216 - 404.1266)
- Boscalid (343.0375 - 343.0425)
- Piperonyl Butoxide (356.2407 - 356.2457)
- Imazalil (297.0531 - 297.0581)
- Malathion (331.0409 - 331.0459)
How Much Data Do We Really Have?
Example Calibration Curves
# Recovery (100ng/ mL spike)

## Flower

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Recovery</th>
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<tbody>
<tr>
<td>Myclobutanil</td>
<td>85.7</td>
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<tr>
<td>Spiromesifen</td>
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<tr>
<td>Imidaclorpid</td>
<td>87.4</td>
</tr>
<tr>
<td>Etoxazole</td>
<td>62.9</td>
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<tr>
<td>Binfenazole</td>
<td>79.0</td>
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<tr>
<td>Avermectin</td>
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<tr>
<td>Monocrotophos</td>
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<tr>
<td>Fenoxycarb</td>
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<tr>
<td>Spirotetramat</td>
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<tr>
<td>Azoxystrobin</td>
<td>87.6</td>
</tr>
<tr>
<td>Boscalid</td>
<td>68.3</td>
</tr>
<tr>
<td>Piperonyl Butoxide</td>
<td>--</td>
</tr>
<tr>
<td>Metalaxyl</td>
<td>87.7</td>
</tr>
<tr>
<td>Imazalil</td>
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<tr>
<td>Malathion</td>
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<tr>
<td>Permethrin</td>
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<tr>
<td>Spinosad (A/D)</td>
<td>41.5</td>
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<tr>
<td>Tebuconazol</td>
<td>53.0</td>
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## Concentrate

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Recovery</th>
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<td>Myclobutanil</td>
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<td>Spiromesifen</td>
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<td>Imidaclorpid</td>
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<td>Tebuconazol</td>
<td>45.4</td>
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Pesticide Quantitative Analysis by QTOF

Conclusions

TOF/ MS is acceptable for low level quantification

- Analyte responses are generally linear
- Adequate sensitivity for ppb detection

Limitations

- Polarity switching isn’t practical
- Other MS platforms offer additional sensitivity

Method Improvement

- Some optimization of extraction may be needed for improved analyte recovery
- Should consider more sophisticated sample prep for concentrates
- Use divert to limit contamination of source
Terpenes
What’s So Interesting About Terpenes?

<table>
<thead>
<tr>
<th>Major Constituent in Cannabis</th>
<th>Pharmacologically Active</th>
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<tbody>
<tr>
<td>• Characteristic smell</td>
<td>• Dermal penetration</td>
</tr>
<tr>
<td>• Strain unique</td>
<td>enhancers</td>
</tr>
<tr>
<td></td>
<td>• Binds to receptors</td>
</tr>
<tr>
<td></td>
<td>• Major biosynthetic</td>
</tr>
<tr>
<td></td>
<td>building block</td>
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Common Terpenes in Cannabis

Monoterpenes

Sesquiterpenes

Terpene alcohols lose $\text{H}_2\text{O}$ in source
LC/MS Conditions

**Column**
- Restek ARC-C18 2.7µm 3.0 x 150mm

**Mobile Phase**
- A: 0.3% Formic Acid
- B: Methanol
- Flow 0.5 mL/ min

**DuoSpray™ Source (SCIEX)**
- Temperature- 600°
- GS1- 40
- GS2- 60
- Curtain- 40
- NC- 5
- DP- 60
25µg/ mL Terpenes Standard (19 components)
Example Calibration Curves
Samples- Flower

- Monoterpenes < calibration range (0.5µg/mL → 0.1%)
- Consistent with decarboxylated materials
Samples- Concentrate
Other Techniques for Analysis of Terpenes

**GC/MS**
- Excellent sensitivity and selectivity for monoterpenes
- Reports of degradation of sesquiterpenes during analysis

**LC/UV**
- Terpenes do not have chromophore
- May respond in RI or ELSD
Terpenes by APCI QTOF Conclusions

APCI QTOF is a suitable method for analysis of terpenes
- Enhanced selectivity for sesquiterpenes
- UPLC would provide better overall selectivity

Terpene profile may differentiate strains of cannabis
- Nomenclature does not accurately describe strains
- May be useful in addition to genetic profiling

May identify products with non-endogenous infused terpenes
- Many products are infused with terpenes to appeal to consumers
Contact
Kris Chupka
kris@nextfrontierbio.com