HPLC-DAD Potency Assay for the Detection and Quantitation of Flavonoids in Hemp Flower

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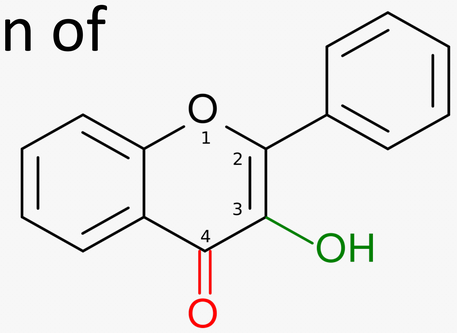

Abstract

Flavonoids are a group of compounds found in cannabis and many other plants that are receiving increased interest for their potential antioxidant, anti-inflammatory, neuroprotective, anxiolytic, and anti-cancer properties.[1] Over 20 different flavonoids have been identified in cannabis and are thought to work synergistically with other phytocannabinoids to increase the bioactivity of those cannabinoids in a process known as the "entourage effect".[2] These flavonoids are also thought to contribute to the unique aroma and color of cannabis.

Despite the contribution of flavonoids to the overall chemical profile of cannabis, few testing facilities have incorporated flavonoid testing into their workflow. Therefore, a reverse-phase HPLC-DAD Potency Assay was developed for the quantitation of flavonoids in Charlotte's Web Hemp derived products.

Target Flavonoids

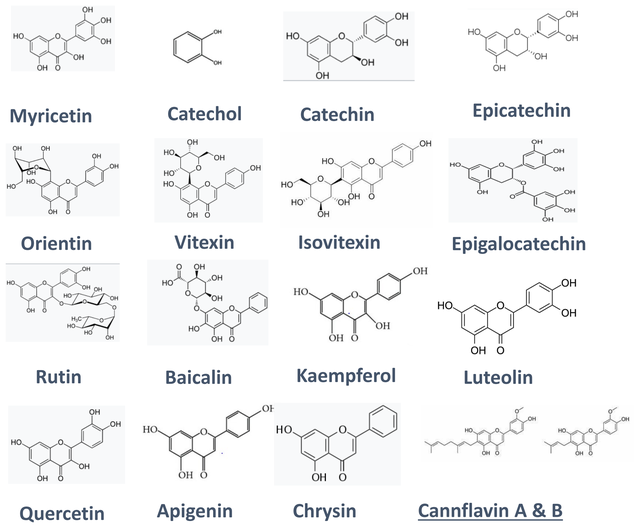

Figure 1. Target analytes and their chemical structures.

Method Conditions				
Column	Agilent Poroshell 120 EC-C18			
Dimensions	4.6*100mm			
Particle Size	2.7µm			
Temp	40 °C			
Diluent	100% Methanol			
Inj Vol	1 µL			
Mobile Phase				
A:	Water, 0.1% FA			
B:	Methanol 0.1% FA			
	Time (min)	**Flow (ml/min)**	**%A**	**%B**
	0	0.5	95	5
	19	---	30	70
	23	---	30	70
	25	---	12	88
	32	---	12	88
	32.01	---	0	100
	38	---	0	100
	38.01	---	95	5
	42	---	95	5
Detector	UV-DAD			
Wavelength	275 nm			
Instrument	1290 Infinity II			

Table 1. HPLC method conditions for flavonoid identification.

Flavonoid Detection

Analyte	Retention Time
Mycertein	8.377
Catechol	8.622
Catechin	9.801
Epichatechin	10.674
Epigalocatechin	12.791
Orientin	13.653
Vitexin	14.26
Isovitexin	14.676
Rutin	15.833
Baicalin	17.022
Quercertin	17.936
Kaempferol	18.522
Luteolin	19.68
Apigenin	19.953
Chrysin	22.998
Cannflavin B	27.812
Cannflavin A	31.356

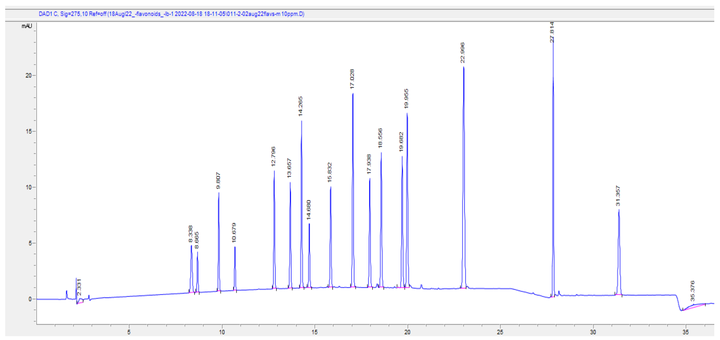

Figure 2. Chromatogram of 17 flavonoids in solvent at 30ppm.

Table 2. Respective retention times of 17 flavonoids in solvent.

Method Validation

- Linearity, Accuracy, Precision and Robustness were determined for the method using certified reference materials provided by Emerald Scientific.
- Per AOAC guidelines, Accuracy and Robustness were performed in triplicate (n=3) while Precision was evaluated at (n=6).
- Robustness was assessed using ± 1.0 °C differences from the set column oven temperature.

Compound		Mycertein	Catechol	Catechin	Epicatechin	Epigalocatechin	Orientin	Vitexin	Isovitexin
Linear Equation		y=2.107x-0.270	y=1.087x-0.169	y=2.510x-0.491	y=1.188x-0.154	y=3.442x+0.689	y=3.174x+0.557	y=4.760x+0.839	y=1.880x+0.347
R2		0.9929	0.99294	0.99287	0.99286	0.99811	0.99801	0.99806	0.99804
Limit of Detection	ppm	1.55504293	3.013582829	1.305233949	2.75828858	0.956891701	1.037675409	0.691969908	1.751452627
Limit of Quantitation	ppm	4.712251303	9.132069177	3.955254392	8.358450244	2.899671823	3.144470938	2.09687851	5.307432202
Accuracy (%Recovery)									
	80ppm spike	102.54%	101.30%	90.00%	94.23%	96.77%	89.90%	99.34%	88.99%
	30ppm spike	81.36%	113.52%	96.07%	108.19%	107.25%	111.71%	114.51%	98.51%
	10ppm spike	24.03%	94.19%	71.89%	98.22%	91.19%	92.47%	102.30%	74.58%
Precision (%RSD)									
	10ppm	±0.91%	±1.17%	±0.98%	±1.11%	±1.04%	±0.99%	±1.02%	±0.97%
Robustness (%Agreement)									
	-1° C	99.51648855	99.12688869	99.45435365	99.08763505	99.74047602	99.24866721	99.9486204	98.24118193
	+1° C	97.92%	97.83%	97.20%	97.68%	95.89%	97.96%	92.62%	99.01%

Compound		Rutin	Baicalin	Quercertin	Kaempferol	Luteolin	Apigenin	Chrysin	Cannflavin B	Cannflavin A
Linear Equation		y=3.121x-0.678	y=6.077x+1.22	y=3.575x+0.511	y=4.501x+0.650	y=4.415x+0.513	y=5.899x+1.053	y=10.064x+1.705	y=7.419x+0.288	y=4.214x+0.996
R2		0.99289	0.99814	0.99814	0.9982	0.99821	0.99859	0.99812	0.99991	0.99932
Limit of Detection	ppm	1.049988361	0.542058368	0.921304982	0.73187872	0.746054161	0.558696276	0.327277041	0.445127935	0.782626816
Limit of Quantitation	ppm	3.181782911	1.642601117	2.791833279	2.217814302	2.260770185	1.693019019	0.99174861	1.348872531	2.371596412
Accuracy (%Recovery)										
	80ppm spike	94.40%	56.29%	85.42%	83.22%	87.09%	88.27%	95.43%	94.21%	111.83%
	30ppm spike	93.68%	53.92%	97.72%	99.16%	100.90%	98.83%	102.88%	99.79%	109.26%
	10ppm spike	76.98%	40.16%	69.75%	71.48%	73.39%	78.82%	94.22%	123.06%	116.54%
Precision (%RSD)										
	10ppm	±1.14%	±1.83%	±1.07%	±1.27%	±1.05%	±1.94%	±1.01%	±1.02%	±0.95%
Robustness (%Agreement)										
	-1° C	99.36147999	99.74078856	99.52127236	98.99325335	99.60872127	99.53288285	98.9926272	99.98614949	99.45151074
	+1° C	98.99%	91.76%	87.46%	97.76%	98.09%	97.94%	97.22%	95.87%	97.85%

Table 3. All flavonoids were validated within the concentration ranges of 10-80ppm.

Testing of Hemp Flower

- An efficient and rapid sample preparation method was developed for the extraction of flavonoids in CW Hemp flower without the need for sub-dilution.
- 1-3 g of shredded and dried hemp was weighed in a 50ml centrifuge tube and dissolved in 10ml of methanol.
- The extraction mixture was vortexed (5 seconds), sonicated (10 minutes), and then centrifuged (5 minutes) at 4200 RPM. 0.5ml-2ml of supernatant was then filtered into in HPLC vial with a 0.2µm filter prior to analysis.

Figure 3. Shredded and dried hemp from Charlotte's Web's C1WAF-RR-12-18 cultivar.

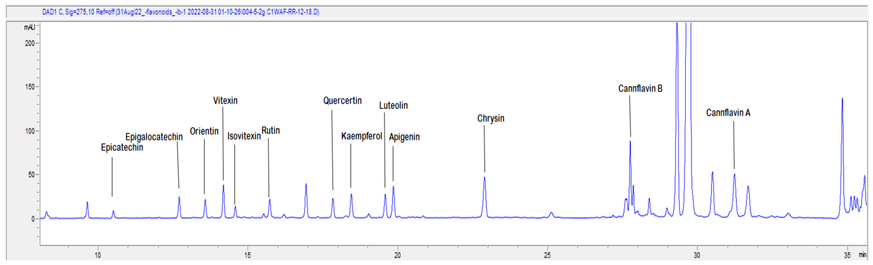

Figure 4. Chromatogram of C1WAF-RR-12-18 Hemp processed with the flavonoid method at 275nm.

Verification of Compound Identity

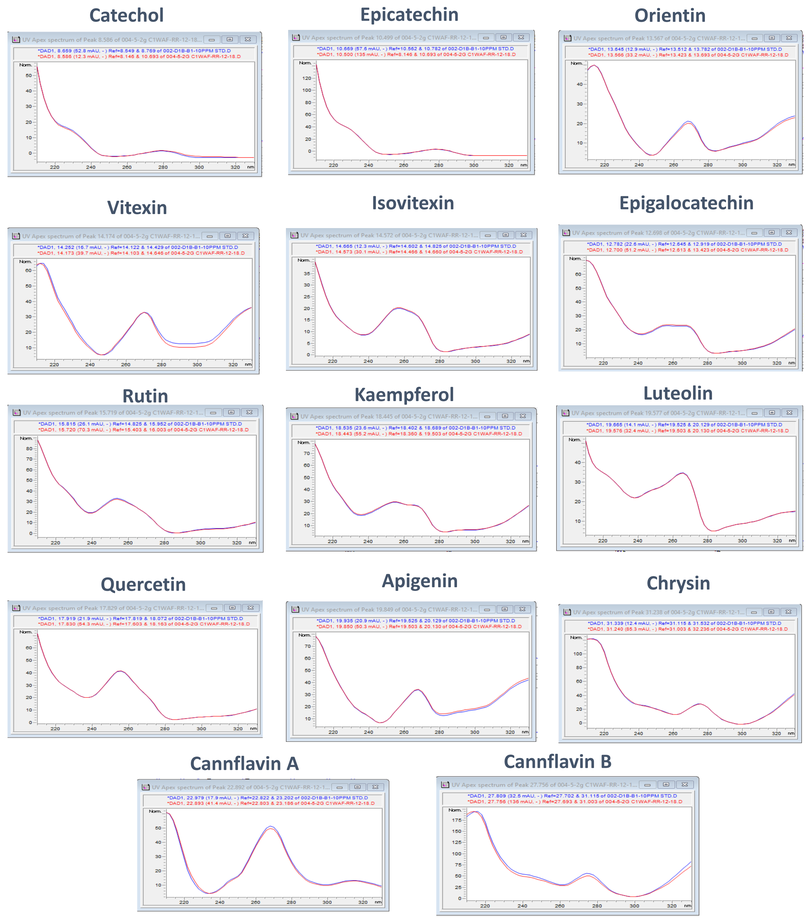

Figure 5. Spectral overlay of standard and C1WAF-RR-12-18 analytes after HPLC analysis. C1WAF-RR-12 spectra are in red and standard spectra are in blue.

Conclusions and Future Directions

A robust method was developed for the identification and quantitation of 17 different flavonoid analytes in Charlottes Web Hemp Flower on an Agilent Poroshell 120 EC-C18 column. 14 of the 17 analytes tested were found in quantifiable levels within the hemp, and percent recovery ranged from 70-111% for almost all analytes, suggesting this method is effective for the extraction of endogenous flavonoids. Future efforts should be aimed at shortening the run time of the method in order to scale it for quality testing and testing different extraction conditions for optimal analyte extraction.

Citations

(1) Baron, E. P. Medicinal Properties of Cannabinoids, Terpenes, and Flavonoids in Cannabis, and Benefits in Migraine, Headache, and Pain: An Update on Current Evidence and Cannabis Science. Headache J. Head Face Pain 2018, 58 (7), 1139–1186. https://doi.org/10.1111/head.13345.
(2) Bautista, J. L.; Yu, S.; Tian, L. Flavonoids in Cannabis Sativa : Biosynthesis, Bioactivities, and Biotechnology. ACS Omega 2021, 6 (8), 5119–5123. https://doi.org/10.1021/acsomega.1c00318.