Analytical method validation for assay determination of cannabidiol and tetrahydrocannabinol in hemp oil infused products by RP-HPLC

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Abstract

A simple quantitative reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for assay determination of cannabidiol and tetrahydrocannabinol in hemp oil infused products. The RP-HPLC method was developed and optimized for the mobile phase composition, flow rate, column selection and detector wavelength. An isocratic elution of samples performed on SOLAS™ 100 Å C18 150 mm x 4.6 mm, 5 µm column with a mobile phase containing 75/25 acetonitrile/water v/v, delivered at a flow rate 1.5 mL/minutes to an Ultraviolet – Visible (UV/Vis) detector using 214 nm. The RP-HPLC method was validated to meet regulatory requirements and covers specificity, accuracy, range, linearity, precision, system suitability and robustness. The validated assay test method was applied successfully to quantify cannabidiol and tetrahydrocannabinol in commercial hemp oil infused products such as tablets, soft gel capsules, plant extract oils, oral drops, tincture, and beverage enhancers. All test results were found acceptable as per ICH guidelines, and this confirmed that the method is fit for its intended use in regular quality control and assay of cannabidiol and tetrahydrocannabinol in hemp oil infused products.

Introduction:

Testing of hemp oil infused products for its purity and quality, helps the consumers to make decisions upon purchasing hemp products, depends on the strength and composition. Moreover, for medicinal cannabis, accurate testing is important as the patients demand specific therapeutic effects. Cannabis is a plant belongs to the family *Cannabaceae* and contains various natural constituents. A total of 565 compounds have been isolated and identified from the *C. sativa* species where 120 belongs to the category of cannabinoids^{1,2}. Cannabinoids belongs to a group of terpenophenolic compounds with 21 carbon atoms and are further divided into 11 subgroups according to their chemical structure. This constitutes 7 compounds of Cannabidiol (CBD), 16 compounds of Cannabigerol (CBG), 23 compounds of Δ9-tetrahydrocannabinol (Δ9-THC), 5 compound of Δ8-tetrahydrocannabinol (Δ8-THC), 11 compounds of Cannabinol (CBN), 9 compounds of Cannabichromene (CBC), 5 compounds of Cannabielsoin (CBE), 2 compounds of Cannabinodiol (CBND), 3 compounds of Cannabicyclol (CBL), 9 compounds of Cannabitriol (CBT) types and remaining 30 compounds belongs to miscellaneous type³. CBD, THC, CBG, CBN, and CBC are neutral cannabinoids and are synthesised by decarboxylation reaction of their respective naturally occurring acid forms (CBDA, THCA, CBGA, CBNA, and CBCA). Out of 120 cannabinoids, CBD and THC are the two major biomarkers in commercial hemp oil samples⁴. CBD (Fig. 1a), the active pharmaceutical ingredient (API) found in the medicinal hemp oil products, shows several curative properties such as anti-psychotic, anti-anxiety, antiemetic, appetite-enhancing, analgesic, and muscle relaxant effects⁵. On the other hand, THC (Fig. 1b) is the principal psychoactive constituent of cannabis, contributes the use of cannabis as a recreational drug⁵. According to the US Farm bill 2018, hemp is defined as ‘the plant *cannabis sativa* L. and any part of that plant, including the seeds thereof and all derivatives, extracts, cannabinoids, isomers, acids, salts, and salts of isomers, whether growing or not, with a delta-9 tetrahydrocannabinol concentration of not more than 0.3 percent on a dry weight basis’⁶. That makes the need for a quantitative method for the simultaneous determination of both CBD and THC in hemp products because the composition of both CBD and THC levels have a strong influence on the effects on the final product.

Literature survey revealed that there have been several analytical methods to quantify cannabinoids and most methods utilise gas chromatography (GC) coupled with mass spectrometry (MS) detectors as an effective tool to analyse cannabis products^{7, 8, 9, 10, 11, 12, 13, 14}. The GC method limits the quantification of acidic analogues as a result of decarboxylation due to hot inlet and oven conditions. Moreover, the decarboxylation process was found to be incomplete which in turn makes in difficulty to quantitate the analysis. In GC, the determination of acidic cannabinoids requires extensive derivatization steps¹⁵. For the quantification of both acidic and neutral cannabinoids, methods based on high performance liquid chromatography (HPLC)¹⁶, thin layer chromatography (TLC)¹⁷, HPLC coupled with mass spectrometry (HPLC/MS)¹⁸, ultra-high performance supercritical fluid chromatography (UHPSFC)¹⁹, liquid chromatography coupled with MS (LC-MS)^{20, 21, 23}, and centrifugal partition chromatographic techniques²⁴ have been reported. Methods showing quantitative determination of CBD in pure isolate was also published earlier²⁵. Out of all the methods, the HPLC method found to be the simplest as it allows the determination of both acidic and neutral forms and also, the system not requires expensive MS detectors. A lot number of methods were published that uses HPLC system^{26, 27, 28, 29, 30, 31}, where most of these methods use buffer mobile phases and gradient elution techniques^{32,33}. Moreover, these methods demonstrate the separation of cannabinoid mixture where it includes several cannabinoids apart from CBD and THC^{28, 34, 35, 36}. The hemp industry demands the development of easy, robust, accurate and efficient novel analytical methodologies for the quantification of the main constituents such as CBD and THC, as the regulatory requirements changes so rapidly. Hemp oil is used to formulate several consumer goods such as food supplements in the form of liquid formulations, beverage enhancers, chocolates, capsules, topicals, baked goods, and as non-prescription medicines in the form of tablets, tinctures, capsules, oral drops, and sublingual drops.

Here, we demonstrate the feasibility of reverse phase HPLC (RP-HPLC) method for reliable and fast quantitative determination of CBD and THC in hemp oil products. The proposed method uses isocratic elution that makes the method feasible to perform in any standard HPLC system. Also, the method performs well in ambient temperature conditions and uses a mobile phase of organic modifier only. Specificity, linearity, accuracy, range, precision, and robustness of the analytical method were determined according to assay method validation requirements specified in the International council for harmonization (ICH) quality guideline Q2(R1), “Validation of Analytical Procedures: Test and Methodology” for quantitation of CBD and THC by liquid chromatography³⁷.

Materials And Methods:

Equipment:

RP-HPLC analysis was performed in Jasco Extrema (JASCO, Inc., 28600 Mary's court, Easton, Maryland 21601, USA) connected with in built autosampler (AS-4150), quaternary pump (PU-4180) with an on-line degasser, column oven (CO-4061), UV/Vis detector (UV-4075) and an interface box LC-NetII/ADC. Chromatographic data was collected and analysed using ChromNAV Ver.2 software. Second HPLC system (Agilent 1100 series) equipped with variable

wavelength detector (G1314A VWD), autosampler (G1313A) and isocratic pump system (G1310A) uses Chemstation (Agilent technologies) software was used for performing intermediate precision (repeatability) testing. Chromatographic separations were achieved by using SOLAS™ 100Å C18 150 mm x 4.6 mm 5 µm column (Glantreo limited, Ireland), and also, different HPLC columns such as SOLAS™ ODS C18 150 mm x 4.6 mm 5 µm, SOLAS™ ODS C18 150 mm x 4.6 mm 3 µm, SOLAS™ BDS C18 150 mm x 4.6 mm 5 µm, SOLAS™ C18 150 mm x 4.6 mm 3 µm and Eiroshell™ C18 150 mm x 4.6 mm 2.6 µm provided from Glantreo limited were used for method development purpose. All chemicals and test samples were accurately weighed using an analytical balance (Adventurer Pro AS214, Ohaus corporation, Pine Brook, NJ USA).

Chemicals and reagents:

All reagents and chemicals of HPLC or analytical grade were used, that includes acetonitrile from Alfa Aesar (Thermo Fisher scientific, Lancashire, United Kingdom) and distilled water from in-house facility. Cannabidiol (CBD) and Δ^9 -Tetrahydrocannabinol (Δ^9 -THC or THC) reference standards were commercially purchased from Cerilliant (Cerilliant Corporation, Round Rock, Texas, United States). Phytocannabinoid mixture 10 was purchased from Cayman chemical (Cayman chemical company, 1180 East Ellsworth Road, Ann Arbor, Michigan 48108 USA). Prism Science LLC, 30403 Kings Valley Dr., Conifer, CO 80433 kindly provided CBD tablets (Santeer LUV 5mg CBD Tablets-Medium) and the tablet placebo for testing. Millex-LCR PTFE 0.45µm membrane filters were purchased from Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland.

Chromatographic conditions used:

A mobile phase of acetonitrile and water 75/25 v/v with isocratic flow rate of 1.5 mL/minutes were used. Column temperature was maintained at 25°C and detection was made at 214 nm. 10 µL of standard and sample solutions were injected in the HPLC system, where a run time of 10 minutes for CBD standard and 20 minutes for system suitability and assay samples were used. For method development, 20 µL of phytocannabinoid mixture 10 working standard solution was injected in the HPLC system.

Preparation of stock standard solutions:

Stock standard solutions of 100 µg/mL CBD and 100 µg/mL THC was prepared by dissolving 1.0 mL of CBD and THC reference standards to 10 mL with acetonitrile in volumetric flasks separately. Diluted 1.0 mL of THC stock standard solution to 10 mL with acetonitrile and used as final standard stock containing 10 µg/mL of THC.

Method Development:

To optimise the chromatographic method parameters, working standard solution of 10 µg/mL CBD, prepared by diluting 1.0 mL of CBD stock standard to 10 mL with acetonitrile, and 25 µg/mL phytocannabinoid mixture 10 working standard solution, prepared by diluting 1.0 mL of 250 µg/mL standard solution to 10 mL with acetonitrile were used. Mobile phase varying from 60/40 v/v to 80/20 v/v acetonitrile/water were considered and flow rate of 1.5 mL/minutes and 2.0 mL/minutes were studied. After optimising the mobile phase and flow rate parameters, testing was conducted in different wavelengths such as 214 nm, 228 nm, 230 nm, 240 nm, 280 nm, and 305 nm. Chromatographic columns of 150 x 4.6 mm with different particle size and column chemistry were tested. The columns considered were SOLAS™ C18 5 µm, SOLAS™ ODS 5 µm and 3 µm, SOLAS™ BDS 5 µm and 3 µm, and Eiroshell™ C18 2.6 µm. The one with better efficiency and separation was chosen for final testing.

System suitability :

For assay method, mixed 1.0 mL each of CBD and THC stock standard solutions to 10 mL with acetonitrile in a volumetric flask (standard mixture of concentration 10 µg/mL CBD and 1 µg/mL THC each). This solution was used for system suitability testing where six injections were made in HPLC and determined the plate count, tailing factor, resolution, and reproducibility (percent RSD of retention time, peak area, and height for six injections).

Linearity:

Assay linearity was demonstrated by preparing five standard solutions within the range of 50–150% of the nominal sample concentration (0.1 mg/mL). Each solution was prepared by serial dilution from a single stock (5 µg/mL to 15 µg/mL for CBD and 0.5 µg/mL to 1.5 µg/mL for THC) and was injected in triplicate. Linear regression analysis was performed, excluding the origin as a point.

Specificity:

For specificity, prepared un-spiked placebo solutions by weighing placebo equivalent to one placebo blend tablet weight (weighed 20 tablets and calculated the average weight) and transferred into a 50 mL volumetric flask, added 35 mL of acetonitrile, and sonicated for 20 minutes. Diluted to volume with acetonitrile and mixed well (un-spiked placebo stock). Diluted 1.0 mL of this solution to 10 mL with acetonitrile, mixed well, filtered into auto sampler vial after discarding 2.0 mL of filtrate using 0.45 µm Millex PTFE filter unit. The placebo preparation was injected in duplicate. Prepared two separate spiked solutions containing the active at 100% by preparing un-spiked placebo stock separately and diluted 1.0 mL to 10 mL with acetonitrile, added 1.0 mL of CBD stock standard and 1.0 mL of THC final stock standard solutions, mixed well, filtered into auto sampler vial after discarding 2.0 mL of filtrate using 0.45 µm Millex PTFE filter unit. (Spiked concentration: CBD – 10 µg/mL and THC – 1 µg/mL each). Injected the spiked samples twice to confirm specificity.

Range:

Range for the assay method was demonstrated by analysing placebo solutions spiked in a range between 50% and 150% of the nominal method sample concentration of CBD (10 µg/mL) and THC (1 µg/mL). Three weights were prepared at each of five concentration levels and each solution was analysed in triplicate. Linear regression analysis was performed, excluding the origin as a point.

Accuracy:

Accuracy and recovery of the method for assay was validated by analysing data obtained from spiked placebo solutions during the range portion of the validation. The percent recovery of each individual sample weight and the average at each concentration level was determined.

Precision:

For precision, assay sample solutions are prepared as follows:

Accurately weighed a minimum of five tablets, calculated the average weight of each tablet and ground into fine powder. Weighed sample equivalent to 5 mg of CBD and transferred into a 50 mL volumetric flask. Added about 35 mL of acetonitrile and sonicated for 15 minutes with intermittent shaking. Diluted to volume with acetonitrile and mixed well. Diluted 1.0 mL of this solution to 10 mL with acetonitrile and mixed well. Filtered a portion through Millex PTFE 0.45 µm sample filtration kit into HPLC vials after discarding 2 mL of sample solution. Sample contains 10 µg/mL CBD in it. 10 µL of sample solutions were injected into HPLC system.

For method precision (repeatability), six assay samples prepared and percent label claim for CBD and THC were determined for each sample. Intermediate precision of the method was demonstrated by repeating the repeatability experiment with a second analyst. This analyst used different solution preparations, reagents, operating conditions, column, and HPLC systems and tested on different days from the first analyst.

Robustness:

Method robustness was established by variation of chromatographic parameters to indicate the reliability of the proposed analytical method during normal usage. The chromatographic parameters considered were variation in mobile phase composition, column oven temperature and mobile phase flow rate. In mobile phase composition increased the major component by 5% and 10%, column oven temperature was increased and reduced by 5°C and flow rate increased and decreased by 10% and 25% from the proposed method condition. System suitability standard solution was injected in duplicate with each parameter change.

Results And Discussion:

The assay method was developed and validated according to ICH guidelines with respect to system suitability, linearity, specificity, accuracy, range, precision (repeatability and intermediate precision) and robustness.

Method Development:

The focus of our research was to develop a new analytical method for the simultaneous determination of CBD and THC in hemp oil infused products. Moreover, the method proposed here can be utilized in commercial drug testing laboratories, pharmaceutical industries, and research laboratories as a standard quality control procedure. The simplest HPLC methods make use of isocratic elution techniques where the mobile phase consists of only organic modifiers were reported^{38,39}. Optimization of chromatographic conditions play a major role in the accurate detection and quantification of analytes in HPLC techniques. Mobile phases containing different percentage of acetonitrile (60, 70, 75 and 80%, v/v) were studied and noted the elution time for CBD and resolution between CBD and CBG. During the testing for different mobile phase compositions, a flow rate of 2.0 mL/minutes and detection wavelength of 214 nm were employed. Detection wavelength of 305 nm was used to identify the CBN in phytocannabinoid mixture as CBN generates intense peak at 305 nm. The test results lead to propose a mobile phase concentration of 75/25 v/v acetonitrile/water. Flow rate of 1.5 mL/minutes and 2.0 mL/minutes were compared and finalised the flow rate as 1.5 mL/minutes where CBD eluted with better efficiency. Different wavelengths (214 nm, 228 nm, 230 nm, 240 nm, 280 nm, and 305 nm) were checked and the peak intensity for CBD was noted. Different chromatographic columns with 150 mm x 4.6 mm column dimensions were tested for their ability to resolve CBD and THC from other cannabinoids. All the columns tested were manufactured by Glantreo limited using their in-house silica technology platform and it includes SOLAS™ 100Å C18 5 µm, SOLAS™ ODS 5 µm and 3 µm, SOLAS™ BDS 5 µm and 3 µm, and Eiroshell™ C18 2.6 µm. All the test results were tabulated in Table 1(a, b, and c).

Table 1
(a): Method development – Mobile phase and Flow rate optimization at 214 nm

Mobile phase composition (Acetonitrile/water v/v)	Flow rate (mL/minutes)	Retention time (CBD standard) minutes	Number of theoretical plates (N) CBD Standard	Symmetry (CBD Standard)	Resolution CBG/CBD (Cannabinoid mixture 10)	Retention time (cannabinoid mixture 10) minutes*
60/40	2.0	18.576	6894	1.09	CBG peak not detected	55.625
70/30	2.0	7.735	6704	1.14	0.76	21.123
70/30	1.5	10.292	7995	1.11	0.93	28.018
75/25	2.0	5.209	6317	1.21	1.12	13.372
	1.5	6.918	7644	1.19	1.23	17.546
80/20	2.0	3.654	5648	1.19	1.25	8.724
	1.5	4.848	6960	1.23	1.39	11.566

*CBC was eluted as the last peak in phytocannabinoid mixture 10 and was considered to choose the total run time for the samples.

Table 1(b): Method development – Optimization for detector wavelength where mobile phase was fixed as 75/25 v/v acetonitrile/water and flow rate 1.5 mL/minutes.

Table 1(c): Method development – Chromatographic column selection

UV wavelength (nm)	Peak Area (CBD standard)	Peak height (CBD standard)	N (CBD standard)	Columns used (150 mm x 4.6 mm 100Å)	Retention time (CBD Standard) minutes	N (CBD Standard)	Symmetry (CBD Standard)	Resolution (CBG/CBD)
214	374090	30108	7644	SOLAS™ C18 5 µm	6.918	7644	1.191	1.23
228	136453	10892	7441	SOLAS™ ODS C18 5 µm	3.129	3570	1.174	NR
230	122916	9842	7398	SOLAS™ ODS C18 3 µm	2.625	6819	1.009	NR
240	46056	3696	7385	SOLAS™ BDS C18 5 µm	2.841	6438	1.047	NR
280	10291	822	7324	SOLAS™ BDS C18 3 µm	2.863	7481	1.081	NR
**305	ND	ND	ND	EIROSHHELL™ C18 2.6 µm	3.123	4278	1.169	0.91

**305 nm was used for identifying the cannabinoid peaks CBN and CBC.

ND: Not detected; NR: CBG not resolved from CBD

From Table 1(a), it was clear that a mobile phase of 75/25 v/v acetonitrile/water with a flow rate 1.5 mL/minutes enables the CBD peak to elute with better efficiency and also, with comparatively better separation from CBG. Table 1(b) showed that detector wavelength of both 214 nm and 228 nm were suitable as in higher wavelengths the peak intensity for CBD has been reduced. In this proposed method 214 nm was considered as optimal wavelength as in this wavelength CBD elutes with higher peak intensity and better efficiency. Table 1(c) revealed that SOLAS™ C18 150 mm x 4.6 mm 5µm column shown well separation for phytocannabinoid mixture 10 with a superior resolution between CBG and CBD as compared to the SOLAS™ ODS 5 µm, BDS 5 µm and Eiroshell™ 2.6 µm C18 columns. Moreover, SOLAS™ C18 150 mm x 4.6 mm 5µm column demonstrated higher efficiency in terms of number of theoretical plates for CBD and also, with CBD peak symmetry ≤ 1.5 .

System suitability testing:

System suitability test performed for standard mixture containing 10 µg/mL CBD and 1 µg/mL THC and six replicate injections were made in both HPLC systems. The observed RSD values for retention time, peak area, and peak height, are well within the set limits of acceptance criteria of less than 1%. Theoretical plates (N), USP tailing factor (T), Capacity factor, USP resolution (Rs) between CBD and THC, and relative retention time (RRT) of THC were determined for assay. The results are summarized in Table 2(a) and all results are within the set acceptable limit mentioned in Table 2(a). A typical system suitability chromatogram was shown in Figure 2.

Table 2
System suitability and Validation results

Table 2(a): System suitability parameters for assay method validation				Table 2(b): Validation results of the RP-HPLC assay test method for CBD and THC							
System Suitability Parameter	Acceptance Criteria	Results		Compound	Amount of standard added (µg/mL)	Linearity (r ²)	Accuracy (% recovery)	Range	r ²	Precision (% RSD)	
		CBD	THC					% RSD (3 preparations / 9 injections)		Repeatability	Intermediate precision
Injection precision for retention time (min)	RSD ≤ 1%	0.42	0.53	CBD	5	0.9999	100.14	0.14	0.9997	0.23	0.14
					7		102.38	0.46			
Injection precision for peak area (n = 6)	RSD ≤ 1%	0.11	0.67		10		105.17	0.62			
					Injection precision for peak height		RSD ≤ 1%	0.62			
Resolution (Rs)	Rs ≥ 2.0	-	16.12								
USP tailing factor (T)	T ≤ 2.0	1.35	1.08		THC		0.5	0.9999			
Capacity factor (k)	k ≥ 2.0	10.3	21.8	0.7		98.85	0.65				
Theoretical plates (N)	N ≥ 3000	7970	9504	1.0		98.17	0.84				
Relative retention factor (RRT)	2.0	-	2.03	1.3		98.03	0.64				
				1.5		99.64	0.57				

Linearity:

The linearity of peak area response for CBD and THC was determined for assay method by analysing standard mixture with serial dilutions from 50 to 150% of the method concentration (10 µg/mL for CBD and 1 µg/mL for THC). Linear regression analysis was performed, excluding the origin as a point. For both CBD and THC, percentage RSD for the peak area for the triplicate injections were found to be less than 1.0% and exhibits linear responses with $r^2 > 0.999$ (Table 2(b)). A graph of concentration versus peak area response (intensity) is shown in Figure 3. A graph of the residuals versus analyte concentration is shown in Figure 3. No trend was observed from the residual plot. The y intercept did not show significant departure from zero.

Specificity:

Prepared placebo solutions by weighing an appropriate amount of placebo (the placebo remains 100% of method concentration), with respect to the concentration specified in the method being validated. The placebo preparation was injected in duplicate. Prepared two separate spiked solutions containing the active at 100%. Injected the spiked samples twice to confirm specificity. Example chromatograms of diluent blank, un-spiked placebo, and spiked placebo preparations are presented in the Figure 4. The method showed good specificity as no interfering peaks from the diluent and placebo were observed.

Range:

Placebo solutions spiked with sample concentration ranging from 50 to 150% of method concentration was prepared in triplicate. Three injections were made from each preparation, and linear regression analysis was performed, excluding the origin as a point. Both CBD and THC standards show linear responses with an $r^2 > 0.999$ (Table 2(b)). Also, percentage RSD for average area responses for the triplicate injections and for each concentration level (n=9, where n is number of injections made) was found to be less than 2.0% (Table 2(b)). A graph of concentration versus area response is shown in Figure 5. A graph of the residuals versus analyte concentration was plotted and no trend was observed from the residual plot. The y intercept did not show significant departure from zero.

Accuracy:

Percentage recovery at each individual spiked placebo samples and the average at each concentration level was calculated. The results were tabulated in Table 2(b). The percentage recovery of the spiked placebos was found to be 100-105% for CBD and 98-100% for THC (acceptance criteria: 90-110% as per ICH

guidelines) for the average of each set of three weights. Each individual sample recovery also found within the range of 90% –110%. After examination of the recovery data, the method was determined to have no bias and the method is demonstrating a high extraction efficiency for CBD and THC.

Precision:

Method precision (repeatability) was performed by preparing six sample solutions from the tablets for a concentration of 10 µg/mL CBD where equivalent of 5 mg CBD was weighed. Each preparation was injected twice, and the assay values calculated. Results including the percentage RSD values of CBD and THC for both repeatability and intermediate precision are presented in Table 2(b). Percentage RSD values for both CBD and THC are below 2.0%. The chromatograms of a typical standard and sample solutions (5 mg) were shown in Figure 6. The sample chromatogram was compared with the chromatograms of standard, placebo and blank. As CBG was appeared to be slightly overlapping with CBD in the hemp oil sample chromatogram, still the separation was distinct to allow a proper integration. Intermediate precision of the method was demonstrated by repeating the repeatability experiment with a second analyst. This analyst used different solution preparations, reagents, operating conditions, column, and HPLC systems and tested on different days from the first analyst. All acceptance criteria were met (The percentage RSD of the assay values should not be greater than 2.0%. The percentage RSD of the combined assay values from both analysts should be not more than 3.0% as per ICH guidelines).

Robustness:

Method robustness was proved by variation of the mobile phase composition, temperature, and flow rate, from the parameters specified in proposed method. A system suitability standard (10 µg/mL CBD and 1.0 µg/mL THC) was prepared and injected in duplicate with each parameter change. This solution was used to perform robustness for assay and each parameter change was tested one factor at a time. The retention time (RT), theoretical plates (N), Tailing factor (T), Capacity factor (k), resolution between CBD and THC (Rs), and the relative retention time (RRT) for THC are reported in Table 3. Examination of the results from Table 3 suggest that the variation in mobile phase concentration is a critical parameter as with a 5% and 10% decrease in major component (acetonitrile) in volume in the mobile phase composition results elution of THC not within the method run time. Besides, a 5% and 10% increase in major component in mobile phase composition results poor efficiency (reduced theoretical plate values). Variations in column oven temperature shows no variation in any of the system or column efficiency parameters.

Table 3 Robustness test for HPLC parameter variations _ CBD and THC assay method validation										
Parameter change	CBD				THC				Resolution (CBD/THC)	RRT (THC)
	RT (min)	N	T	K	RT (min)	N	T	K		
Method Conditions	7.03	6960	1.40	10.2	14.30	7974	1.22	21.8	14.90	2.03
Flow -25%	9.44	7223	1.45	13.2	19.12	7756	1.28	27.8	14.78	2.02
Flow -10%	7.82	6950	1.43	12.7	15.83	7707	1.19	26.8	14.64	2.02
Flow +10%	6.38	6361	1.42	10.2	12.91	7243	1.26	21.6	14.14	2.02
Flow +25%	5.59	6228	1.42	10.0	11.33	7316	1.28	21.2	14.14	2.03
Column temp: +5°C	6.92	6864	1.37	10.2	13.91	7711	1.18	21.4	14.48	2.01
Column temp: -5°C	7.12	6796	1.51	10.4	14.57	7850	1.32	22.3	14.89	2.05
MP +10%	4.45	4197	1.29	5.5	8.54	4634	1.26	11.6	10.55	1.92
MP +5%	5.69	4454	1.33	7.4	11.27	4777	1.20	15.5	11.27	1.98
MP -5%	9.58	7514	1.36	13.9	19.91	8567	1.07	29.9	15.91	2.08
MP -10%	12.42	8500	1.25	19.5	25.97	9849	1.02	41.8	17.13	2.09

Additionally, solution stability was established for 48 hours by testing system suitability standard solutions twice by using a freshly prepared standard, the solutions kept in room temperature for 24 hours, the solutions kept in 0-8°C for 24 hours and the standard solution kept in room temperature for 48 hours. The results show exceedingly small variation in peak area absorbance values and proved the standard solution stability of 48 hours in room temperature. Also, old column where > 500 injections made was tested and found a higher tailing and backpressure as compared to new column.

Application of final method to marketed hemp oil infused products:

The proposed validated method was applied for the analysis of various hemp oil infused products and presented⁴⁰. The samples tested were consist of tablets, capsules, tincture, oral drops, beverage-enhancers, and plant extract oils. Furthermore, Glantreo limited is employing this assay method for testing commercial hemp oil infused samples as a quality control procedure.

Conclusion:

The precise quantitation of compounds like cannabinoids where the products contain psychotic molecules such as THC and the presence and amount of THC makes legal restrictions for the product distribution, demands the laboratories for having affordable and easily applicable validated procedures. The assay

test method validated and presented here allows the quantitative and simultaneous determination of CBD and THC in Hemp oil infused products in 20 minutes run time. The method uses single wavelength of 214 nm, and the testing can be carried out using any traditional standard RP-HPLC and UV-Visible detectors. Moreover, the method utilizes only organic solvents for standard and preparation, and for mobile phase single organic modifier was involved with no pH adjustment and the testing follows an isocratic elution. Due to simplicity and consistency this method can be employed by any analyst with basic HPLC knowledge. The method was shown to be selective, precise, linear, and accurate within the range of 50–150% of the nominal concentration for tablet strengths. The method was shown to be robust with the mobile phase concentration changes and flow rate established as critical parameters.

Abbreviations

RP-HPLC, Reverse phase high performance liquid chromatography; CBD, Cannabidiol; THC, Tetrahydrocannabinol; RSD, Relative standard deviation; QC, Quality control; ICH, International council for harmonisation; USP, United states pharmacopeia.

Declarations

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Conflicts of interest / Competing interests:

The authors declare no competing interests.

Availability of Data and material:

The authors confirm that the data supporting the results of this study are available upon request from the corresponding author Dr. Eric Moore (e.moore@ucc.ie).

Authors contribution:

All authors contributed to the study conception and design. Author contribution according to CRediT taxonomy described below:

SA: Methodology, Validation, Formal analysis, Investigation, Writing - Original draft, Visualization, Data curation, Funding acquisition.

EM: Supervision, Writing- Review and Editing.

JH: Conceptualization, Supervision(Employment mentor), Writing - Review and Editing, Project administration.

VL: Methodology, Validation, Resources.

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Figures

Figure 1

(a) Cannabidiol and (b) Tetrahydrocannabinol - Structural representation of CBD and THC. Formula: $C_{21}H_{30}O_2$; Molecular weight: 314.464 g/mol

Figure 2

System suitability chromatogram of CBD and THC standard mixture (10 μ g/mL CBD and 1 μ g/mL THC) for assay

Figure 3

Linearity and residual plot diagrams for CBD and THC assay method validation (a) Residual plot for CBD (b) Residual plot for THC (c) Assay linearity plot for THC and (d) Assay linearity plot for CBD

Figure 4

Specificity chromatograms of (a) diluent blank (b) un-spiked placebo and (c) spiked placebo samples of CBD and THC assay

Figure 5

Concentration vs. area response graph of (a) CBD and (b) THC for accuracy and range testing for assay

Figure 6

(a) Standard and **(b)** sample chromatograms from precision testing for CBD and THC assay method validation