



# Development and validation of a LC-HRMS method for the quantification of cannabinoids and their metabolites in human plasma

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## ABSTRACT

The resurgence of *Cannabis* therapeutic discoveries have led to the need for sensitive and selective analytical methods for the detection of cannabinoids and their metabolites in biological matrices. High resolution mass spectrometry (HRMS) enables good sensitivity and provides more selectivity due to its accurate mass measurement of the targeted compounds. The aim of this study was to develop and validate a sensitive liquid chromatography high resolution mass spectrometry (LC-HRMS) method for the quantitative analysis of cannabidiol (CBD), cannabinol (CBN),  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) and its major metabolites 11-Hydroxy- $\Delta^9$ -THC and 11-Nor-9-carboxy- $\Delta^9$ -THC in human plasma. The method utilized a simple liquid-liquid extraction of the cannabinoids from plasma samples followed by an isocratic chromatographic separation and detection by ESI-HRMS Q-Exactive plus platform. The lower limit of quantification (LLOQ) was 0.2 ng/mL for the targeted cannabinoids and its metabolites with sample volume of 0.5 mL plasma. The method was linear from 0.2 to 100.0 ng/mL with an average correlation coefficient of  $>0.995$  using weighted ( $1/x$ ) linear least-squares regression. No significant carry-over was noticed for all analytes and the extraction recovery ranged from 60.4 % to 85.4 %. Dilution results indicated no influence on the accuracy of analysis. The method's intra-day and inter-day precision (CV %) ranged from 2.90 to 10.80 % and accuracy within -0.9 to 7.0 from nominal. Matrix effect ranged from 1.1 % to 49.8 %. The analytes were stable in the autosampler for 6 and 12 h, respectively. This method was sensitive and can be applicable to cannabinoids pharmacokinetics study.

## 1. Introduction

Cannabinoids are phytochemicals produced as secondary metabolites in *Cannabis sativa* plant. They are a group of around 70 terpenophenolic compounds formed mainly by decarboxylation of the corresponding acids in plant (Isvett Josefina and Robert, 2008). *Cannabis sativa* constituents are mainly analyzed for forensic purposes as *Cannabis* is widely abused and considered an illicit drug in many countries. Another purpose of their analysis is to study the pharmacokinetics and pharmacodynamics of the extract constituents and their metabolites to understand the therapeutic effects associated with each of these components.  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD) are the major pharmacologically active compounds present in *Cannabis*. While THC is responsible for the psychoactive effects and acts as sedative (Zuardi and Guimaraes, 2003), antiemetic (Parker et al., 2002) and antiepileptic (Karler and Turkans, 1981), CBD is devoid of

psychotropic effect (Zuardi et al., 1982). On the other hand, CBN also has anti-convulsion properties (Karler et al., 1973). The metabolism of THC has been previously characterized and two major metabolites were identified in human plasma, namely 11-Hydroxy- $\Delta^9$ -THC (11-OH-THC) and 11-Nor-9-carboxy- $\Delta^9$ -THC (THC-COOH) (Wall and Perez-Reyes, 1981). Many analytical methods have been developed for the analysis and quantification of cannabinoids, either in the plant extract (Mei et al., 2017) or in human biological matrices e.g. urine, plasma (Grauwiler et al., 2007), whole blood (Hubbard et al., 2019; Jagerdeo et al., 2009; Scheidweiler et al., 2016), oral fluids (Concheiro et al., 2013) and hair (Salomone et al., 2012).

Researchers face many challenges while developing analytical methods for the analysis of cannabinoids and their related metabolites in plasma and urine. Sensitivity of the method is a major limitation as well as selectivity and elimination of matrix effects associated with complex biological matrices. As human plasma is the matrix in focus for this

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study, various sample preparations, analytical methods and instrumentations previously used for this purpose will be discussed.

Gas Chromatography (GC) coupled to various detectors: electron capture detector (ECD), flame ionization detector (FID) and nitrogen-phosphorus detector (NPD) have been reported in the literature for the analysis of cannabinoids but these methods lacked either the sensitivity or specificity required for such analysis (McBurney et al., 1986). Gas chromatography coupled to mass spectrometry (GC-MS) has been successfully used for the analysis of THC, 11-OH-THC, THC-COOH, CBD, and cannabiol (CBN) in human plasma after C18 solid phase extraction (SPE) and trimethylsilyl-derivatization (Nadulski et al., 2005). Manual and automated liquid-liquid extraction with n-hexane / ethyl acetate mixture followed by silylation-derivatization and GC-MS analysis was also reported (Purschke et al., 2016). However, one major disadvantage of GC-MS is the time-consuming sample preparation due to the required derivatization step for thermolabile compounds.

Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has become the method of choice for the analysis of drugs of abuse including cannabinoids and their metabolites as it provides the required sensitivity and selectivity for detection and quantification of the compounds with less sample preparation. An LC-MS/MS method with atmospheric-pressure chemical ionization (APCI) has been developed for the same aforementioned target analytes while using mixed mode SPE Agilent Bond Elute Certify II™ with significantly less sample preparation (Grauwiler et al., 2007). Another method described the use of LC-MS/MS with electrospray ionization (ESI) for analysis of THC and its major metabolites, THC-COOH and 11-OH-THC in human plasma after C18 SPE (Maralikova and Weinmann, 2004). Phospholipids are a major concern when extracting and analysing biological matrices due to signal suppression in ESI, commonly used as ionization source in LC-MS. An interesting research suggested the application of Phree™ clean up extraction to eliminate phospholipids during sample preparation which leads to enhanced sensitivity (Palazzoli et al., 2018).

In literature, the most sensitive analytical method with lower limit of quantification (LLOQ) of 0.2 ng/mL for all targeted cannabinoids and specified metabolites (THC, CBD, CBN, THC-COOH and 11-OH-THC) in human plasma was reported by Grauwiler et al. (2007) (Grauwiler et al., 2007). In this study, high performance liquid chromatography (HPLC) coupled with atmospheric pressure chemical ionization (APCI) and tandem mass spectrometry in combination with costly and time consuming SPE method and higher sample volume of 1.0 mL human plasma was used. Hence, the focus of the current study is to achieve the same level of sensitivity (LLOQ of 0.2 ng/mL) for the same targeted cannabinoids and metabolites using ultra-performance liquid chromatography electrospray ionization high resolution mass spectrometry (UPLC-ESI-HRMS) in combination with cheaper liquid-liquid extraction (LLE) method and lower sample volume. Contrary to HPLC instrumentation, (UPLC) technology has been designed to withstand high pressure and also been demonstrated to be attractive option for trace level quantitative analysis due to its speed, sensitivity and resolution (Plumb et al., 2004), (Jim et al., 2006), (Churchwell et al., 2005). Analysis of cannabinoids by high resolution mass spectrometry (HRMS) have been reported in human oral fluid (Concheiro et al., 2013) and in human hair (Montesano et al., 2015) using Q-Exacte Orbitrap and in plant extracts using quadrupole time-of-flight (Q-TOF) (Aizpurua-Olaizola et al., 2014). The lower limit of quantitation (LLOQ) in oral fluid was 0.5 ng/mL for CBD, CBN, THC and 0.015 ng/mL for THC-COOH while in hair analysis LLOQ was 0.1 pg/mg for THC-COOH, 1 pg/mg for THC and, 2 pg/mg for CBD and CBN, respectively. HRMS gives the advantage of measuring the exact mass of the compounds to several decimal points. This allows discrimination between compounds that have the same nominal mass and enables researchers to accurately separate the signal of interest from the background, thus improve selectivity. .

Therefore the aim of this study was to develop and validate a sensitive detection method for the quantitative determination of  $\Delta^9$ -tetrahydrocannabinol (THC) and its metabolites, 11-Hydroxy- $\Delta^9$ -THC (11-

OH-THC) and 11-Nor-9-carboxy- $\Delta^9$ -THC(THC-COOH), as well as cannabidiol (CBD) and cannabiol (CBN) in human plasma using ultra-performance liquid chromatography electrospray ionization high resolution mass spectrometry (UPLC-ESI-HRMS). A future goal of this research is to apply the method to cannabinoid pharmacokinetic study.

Chemical structures of the target analytes are presented in Fig. 1.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Certified reference materials (CRMs) for cannabidiol (99.85 %), cannabiol (99.12%),  $\Delta^9$ -THC (97.81 %), 11-Hydroxy- $\Delta^9$ -THC (95.47%), 11-Nor-9-carboxy- $\Delta^9$ -THC(98.08 %) and  $\Delta^9$ -THC-D3 (97.93%) (1 mg/mL) were purchased from Cayman chemical (USA). HPLC grade acetonitrile and ethyl acetate were purchased from Sigma-Aldrich (Germany). Methanol HPLC grade, n-hexane analytical grade, pure HPLC grade water and potassium dihydrogen orthophosphate were purchased from Loba (India). LC-MS grade acetic acid was purchased from Fisher chemicals (UK) and blank EDTA human plasma was sourced from Divbio science (Netherlands).

### 2.2. Preparation of standard and quality control (QC) samples

Stock solutions of cannabidiol, cannabiol,  $\Delta^9$ -THC, 11-Hydroxy- $\Delta^9$ -THC, 11-Nor-9-carboxy- $\Delta^9$ -THC and  $\Delta^9$ -THC-D3 were prepared at 10  $\mu$ g/mL in methanol. Working concentrations of 1000 ng/mL, 100 ng/mL and 10 ng/mL were prepared for target cannabinoids in methanol for spiking of plasma calibrations and quality controls. Internal standard  $\Delta^9$ -THC-D3 was prepared at 100 ng/mL in methanol.

Plasma calibrators were spiked at 8 concentration levels; 0.2, 0.5, 1, 5, 10, 20, 50, 100 ng/mL. Quality controls (low, medium and high) were prepared at concentrations of 0.6, 50, 80 ng/mL, respectively.

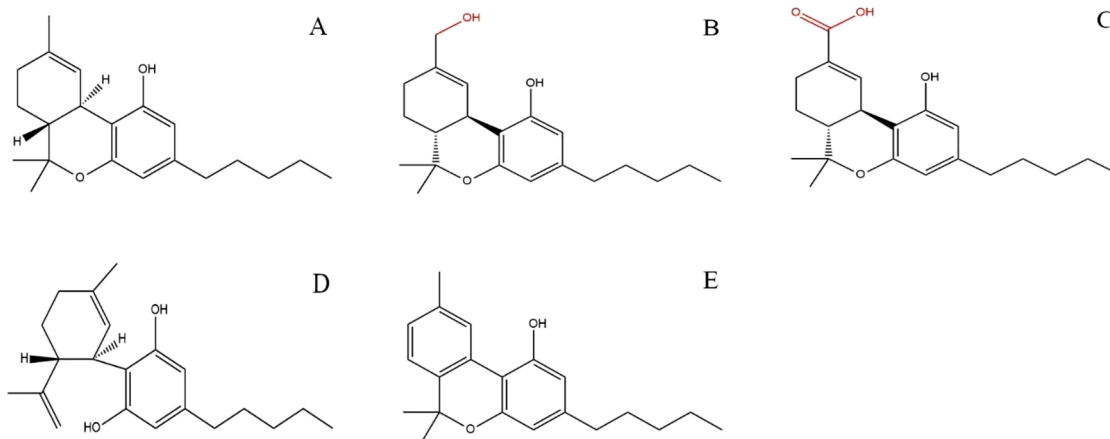
### 2.3. Human plasma extraction

Five hundred microliter human EDTA plasma was transferred into a 15 mL polypropylene centrifuge tube. Then 100  $\mu$ L of  $\Delta^9$ -THC-D3 internal standard solution was added followed by the addition of 0.5 mL phosphate buffer (1.5 M potassium dihydrogen phosphate, pH 4.5) and the sample was vortex-mixed for 10 seconds. Liquid-liquid extraction was performed by adding 5 mL n-hexane/ethyl acetate 8: 2 (v/v) followed by mixing by roller mixer for 30 min. The sample was then centrifuged for 10 min at  $3,506 \times g$ . The organic phase was transferred into glass reaction vial and evaporated to dryness under a stream of nitrogen at 40°C in a Ratek™ dry block heater. The dried sample was reconstituted in 100  $\mu$ L mobile phase (0.2 % acetic acid water/acetonitrile (35:65, v/v)), transferred to an autosampler vial with glass insert and 20  $\mu$ L was injected for LC-HRMS/MS analysis.

### 2.4. Liquid chromatography electrospray ionization high resolution tandem mass spectrometry

#### 2.4.1. Liquid chromatography

A robust and rapid chromatographic separation was established using Ultimate 3000 UPLC (Thermo Scientific, Germering, Germany) on a Zorbax Eclipse reverse phase C18 column (1.8 $\mu$ m, 50  $\times$  2.1 mm) fitted with Phenomenex C18 pre-column (4  $\times$  3.0 mm). Column oven was maintained at 40°C. Aqueous mobile phase (A) consisted of 0.2 % acetic acid in pure HPLC grade water while organic mobile phase (B) was acetonitrile. An isocratic program with a composition of 35 % mobile phase A and 65 % mobile phase B at a flow of 0.35 mL/min for 10 min was used. This chromatographic program allowed the essential separation of CBD and THC at retention times of 3.43 and 6.90 min, respectively. This separation is critical to differentiate between THC and CBD



**Fig. 1.** Chemical structure of cannabinoids (A)  $\Delta^9$ -tetrahydrocannabinol and its two main metabolites (B) 11-Hydroxy- $\Delta^9$ -THC and (C) 11-Nor-9-carboxy- $\Delta^9$ -THC (D) cannabidiol (E) cannabinal.

as they have the same  $m/z$  and the same product ions and thus, cannot be distinguished in the mass spectrometer.

#### 2.4.2. Mass spectrometry

For mass spectrometry detection, Q-Exactive plus high resolution hybrid quadrupoleOrbitrap mass analyser with Higher Energy Collisional Dissociation (HCD) collision cell was utilized. The MS acquired a targeted-MS/MS scan (Parallel Reaction Monitoring mode (PRM)) at 17,500 Full Width at Half Maximum (FWHM at  $m/z$  200) resolution. Single charged ions were generated using heated electrospray ionization (ESI) with negative/positive polarity switching. Electrospray ionization source and front end parameters optimization were carried using direct infusion of the target compounds into the mass spectrometer while maintaining the mobile phase flow from the column through a T connection. The optimized ESI and front end parameters is as follows; sheath gas and auxiliary gas flow rates were 55 and 10 arbitrary units respectively, spray voltage was 3.5 kV in both negative and positive mode, capillary temperature at 320 °C, heater temperature at 300 °C and S-lens RF level was 60. Nitrogen was used as auxiliary and sheath gas as well as in the HCD and the C-Trap. A full scan of all product ions was performed at different collision energies for each compound and optimized HCD values were selected. The mass spectra are available in **supplementary information Fig. 1**. Mass calibration was performed in positive and negative mode, mass accuracy was lower than 5 ppm. Ionization polarity, precursor ions, quantitation ions, qualifier ions, normalized collision energy (NCE) and retention time for each analyte are summarized in **Table 1**. The software used for instrument control, data acquisition and quantitation was XCalibur<sup>TM</sup> 4.0 and Tracefinder<sup>TM</sup> 4.1.

#### 2.5. Method validation

Validation was performed according to Food and Drug Administration (FDA) guidelines on bioanalytical method validation (Food and

drug administration, 2018). Validation parameters; selectivity, specificity, linearity, accuracy and precision, LLOD, LLOQ, carry-over, dilution integrity, stability, matrix effect and recovery were evaluated for the target analytes.

##### 2.5.1. Selectivity and Specificity

Selectivity was assessed by analysing six lots of blank human plasma samples. Specificity was evaluated by spiking the blank plasma with Caffeine, Ibuprofen, Diclofenac, paracetamol, aspirin and tramadol as examples of possible concomitant medications. Chromatograms at the retention time of the target compounds and internal standard were checked for presence of interference from these medications.

##### 2.5.2. Linearity

Linearity of the method was assessed by spiking all the target cannabinoids at 8 concentration levels; 0.2, 0.5, 1.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL in human plasma. Non-zero calibration levels accuracy should be  $\pm 15\%$  of nominal concentrations, except at LLOQ where the calibrator should be  $\pm 20\%$  of the nominal concentration. Seventy five percent and a minimum of 6 non-zero calibration levels should pass the aforementioned criteria. Moreover, the concentration – response relationship was fit with a simple weighting ( $1/x$ ) linear least-squares regression model for all analytes and kept uniform all over the study.

##### 2.5.3. Accuracy and precision

Accuracy and precision of the method were evaluated by running 3 different sample batches in 3 different days. Each batch consists of a calibration curve and 4 different quality control levels in 5 replicates; LLOQ (Lower Limit of Quantitation) at 0.2 ng/mL, QCL (low quality control) at 0.6 ng/mL, QCM (medium quality control) at 50.0 ng/mL, QCH (high quality control) at 80.0 ng/mL. All calibrator and quality controls were spiked in human plasma. Percent coefficient of variation (CV %) was calculated for intra-day and inter-day precision. Accuracy of the quality controls to the nominal concentration expressed as % was

**Table 1**

Ionization polarity, precursor ions, quantitation ions, qualifier ions, normalized collision energy and retention time for each analyte.

Analyte	Ionization polarity	Precursor ion( $m/z$ )	Quantitative product ion ( $m/z$ )	Qualitative product ion ( $m/z$ )	NCE (%)	RT (min)
CBN	[M-H] <sup>-</sup>	309.1860	279.13828	171.08012	65	5.25
CBD	[M+H] <sup>+</sup>	315.2318	193.12177	259.16962	45	3.43
THC-COOH	[M-H] <sup>-</sup>	343.1915	299.20097	245.15370	45	1.80
11-OH-THC	[M+H] <sup>+</sup>	331.2268	193.12241	201.09079	45	1.79
THC	[M+H] <sup>+</sup>	315.2318	193.12177	259.16931	45	6.90
THC-D3	[M+H] <sup>+</sup>	318.2507	196.14128	262.18683	45	6.80

NCE: Normalized collision energy, RT: Retention time.

addressed. Accuracy of QCL, QCM and QCH should be  $\pm 15\%$  of nominal concentrations while at LLOQ  $\pm 20\%$  is acceptable. Similarly for precision, CV% within inter-day runs and intra-day runs should be  $\pm 15\%$  for QCL, QCM and QCH,  $\pm 15\%$  for LLOQ.

#### 2.5.4. Lower limit of detection and lower limit of quantitation

Sensitivity of the method is expressed as its lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). LLOD is the concentration where the signal-to-noise ratio is  $\geq 3$ , while LLOQ is when the signal-to-noise ratio is  $\geq 5$ . The accuracy of the LLOQ should be within 20% of the nominal concentration and CV% for precision runs should be  $\pm 20\%$ .

#### 2.5.5. Carry-over

Carry-over was accessed by injecting 5 blank samples after the injection of the calibration standard at the upper limit of quantitation, as described in European Medicines Agency Guideline on Bioanalytical Method Validation (European Medicines Agency, 2011). Chromatograms were investigated for possible carry-over and the peak area of the analyte in the blank samples was expected to be less than 20% of the LLOQ peak area (FDA, 2018).

#### 2.5.6. Dilution integrity

Dilution integrity tests the possibility of diluting samples which exceed the upper limit of quantitation while maintaining accuracy and precision. A dilution factor of 3 was chosen and samples were spiked at 150 ng/mL and subsequently diluted 3 times with blank plasma. Five replicates were prepared and injected to evaluate accuracy and precision.

#### 2.5.7. Matrix effects

Matrix effects was evaluated at low and high levels using post-extraction spike approach (Matuszewski et al., 2003). Blank plasma samples were extracted and spiked after extraction with the reference standards of target compounds and internal standard. The absolute peak areas were compared against those of pure reference standards at the same concentration levels. The neat standards were prepared in n-hexane/ethyl acetate (8:2, v/v) then evaporated and reconstituted in mobile phase.

Matrix effect was then estimated as per the below equation:

$$\text{Matrix effect} = \left[ \left( \frac{\text{average absolute area of plasma spiked after extraction}}{\text{average absolute area of pure standard}} \right) - 1 \right] \times 100$$

#### 2.5.8. Recovery

Recovery of target analytes from plasma was estimated by comparing the QCL, QCM, QCH plasma quality controls to extracted blank plasma samples spiked post-extraction with the corresponding concentration as per below equation.

$$\text{Recovery} = \frac{\text{average absolute area of plasma spiked before extraction}}{\text{average absolute area of plasma spiked after extraction}} \times \%$$

Process efficiency was calculated by multiplying recovery by matrix effect and then divided by 100 (Matuszewski et al., 2003).

#### 2.5.9. Stability

The post-preparative stability of the cannabinoids and its metabolites was assessed in the autosampler using quality control samples; QCL (0.6 ng/mL) and QCH (80.0 ng/mL) for 6 and 12 h at room temperature. For each QC sample, four replicates were prepared and kept at the designated storage time in the autosampler prior to analysis. The results were compared with those of freshly analysed samples and the acceptable criterion used was 85 – 115% mean accuracy.

### 3. Results and discussion

#### 3.1. Chromatographic and mass spectrometric optimization

Firstly, separation of the compounds using different columns and mobile phases was evaluated. Initially, Phenomenex-Gemini-NX Reverse phase C18 3 $\mu$  110 Å 150  $\times$  2.0 mm was used with a gradient program. Methanol and acetonitrile were tested as organic mobile phase, whilst HPLC grade water with either formic acid or acetic acid as additive as the aqueous mobile phase. Both acetonitrile and methanol showed efficient separation of the cannabinoids; however, acetonitrile showed higher signal intensity. Acetic acid as an additive to aqueous phase gave superior signal intensity at a concentration of 0.2%. Retention time of the target compounds ranged from 12 to 14 min with a total run time of 18.0 min. Zorbax Eclipse reverse phase C18 column (1.8 $\mu$ m, 50  $\times$  2.1 mm) was then evaluated with a simple isocratic program. The optimized mobile phase composition was 35% of 0.2% acetic acid in water and 65% acetonitrile at a flow of 0.35 mL/min. This chromatography program with Zorbax Eclipse column allowed the efficient separation of the cannabinoids within 10 min runtime with the optimum signal intensity, and thus was chosen for the study.

Secondly, the cannabinoids reference standard solution was infused directly into the mass spectrometer through syringe infusion while maintaining mobile phase flow through a T-connection. This process enabled the optimization of the front end parameters of the mass spectrometer, including electrospray ionization (ESI) parameters, the heated capillary and S-Lens RF level. ESI parameters are responsible for the spraying of the sample carried in the mobile phase followed by either negative or positive ionization of the molecules. As those parameters are dependent on flow rate and mobile phase composition, T-connection was required to mimic actual working conditions. Sheath gas is the inner coaxial nitrogen that nebulizes the sample flowing from the needle into fine droplets, whilst auxiliary gas is the heated nitrogen flowing through the outer needle which aids the sheath gas in desolvation of the sample. The flow of these gases is optimized along with the heater temperature to ensure optimum desolvation of the sample. Sheath gas and auxiliary gas flow was adjusted to 55 and 10 arbitrary units respectively. Heater temperature was kept at 300 °C. The ionization was achieved by applying a spray voltage of 3.5 kV in negative and positive mode. The ion transfer capillary is responsible for transferring the ionized species produced by the ESI source to the S-lens while ensuring the remaining solvent is evaporated. The capillary temperature was maintained at 320 °C. Ions are then focused by the S-lens through the application of RF voltage. The RF amplitude level is mass dependent and optimized for the cannabinoids masses at 60%.

Negative and positive polarities as well as different collision energies were investigated and selection made based on signal stability and intensity. Negative ionization was chosen for CBN and THC-COOH while positive ionization was selected for THC, CBD, 11-OH-THC and THC-D3. Collision energies were varied to yield good intensity of the quantitative and qualitative fragment ions. Optimal parameters are presented in Table 1.

An attempt to include full scan negative and positive experiments in addition to the PRM experiments was evaluated; however, the acquired data points per peak were not enough for quantitation. To utilize the information-rich full scan feature of HRMS during method application to pharmacokinetics, samples can be re-injected using a modified method where only the full scan positive and negative experiments at 70,000 (FWHM at m/z 200) resolution are present. Different HRMS scan modes can be also utilized e.g. data-dependent or data independent acquisitions. This will allow to retrospectively analyze the data in case of discovery of new metabolites or other research purposes.

#### 3.2. Sample preparation optimization

Three different sample preparation approaches were evaluated;

liquid-liquid extraction (LLE), solid phase extraction (SPE) and direct protein precipitation followed by clean up with Phree™ columns.

LLE was performed using different solvents; diethyl ether, tertiary butyl methyl ether and hexane: ethyl acetate (8:2, v/v). Addition of phosphate buffer in the plasma prior to extraction was also evaluated. For SPE sample extraction, C18 and HLB SPE cartridges were tested. Protein precipitation using acetonitrile followed by Phree™ cleanup was also performed.

Both hexane/acetate (8:2, v/v) LLE and HLB SPE extraction methods achieved highest extraction recoveries. As the recovery was comparably similar, LLE was selected due to cost-effectiveness and less preparation time advantage. The addition of phosphate buffer (1.5 M potassium dihydrogen phosphate, pH 4.5) to the plasma sample prior to LLE improved the extraction recovery of the analytes.

### 3.3. Method validation

#### 3.3.1. Selectivity and specificity

The method was found to be selective and specific, with absence of interference peaks at the retention time of the target compounds and internal standard in extracted blank plasma samples and blank plasma samples spiked with concomitant medications. Chromatograms are shown in Fig. 2 and Fig. 3 in the supplementary information.

#### 3.3.2. Linearity

Linearity was established in a concentration range from 0.2 ng/mL to 100.0 ng/mL by spiking pooled blank human plasma with target analytes. Eight concentration levels were used; 0.2, 0.5, 1.0, 5.0, 10, 20, 50 and 100 ng/mL. Response ratios of the peak area of the analyte to the peak area of the internal standard were plotted against nominal concentrations to establish a linear calibration curve. Calibration standards

accuracy of all analytes were within  $\pm 15\%$  of the nominal concentrations except for LLOQ which was  $\pm 20\%$ . Weighting ( $1/x$ ) linear least-squares regression was used for all the analytes due to the simplicity of the model. The average correlation coefficient ( $r^2$ ) was higher than 0.995 for all target compounds. Regression data for each analyte is presented in Table 2.

#### 3.3.3. Accuracy and precision

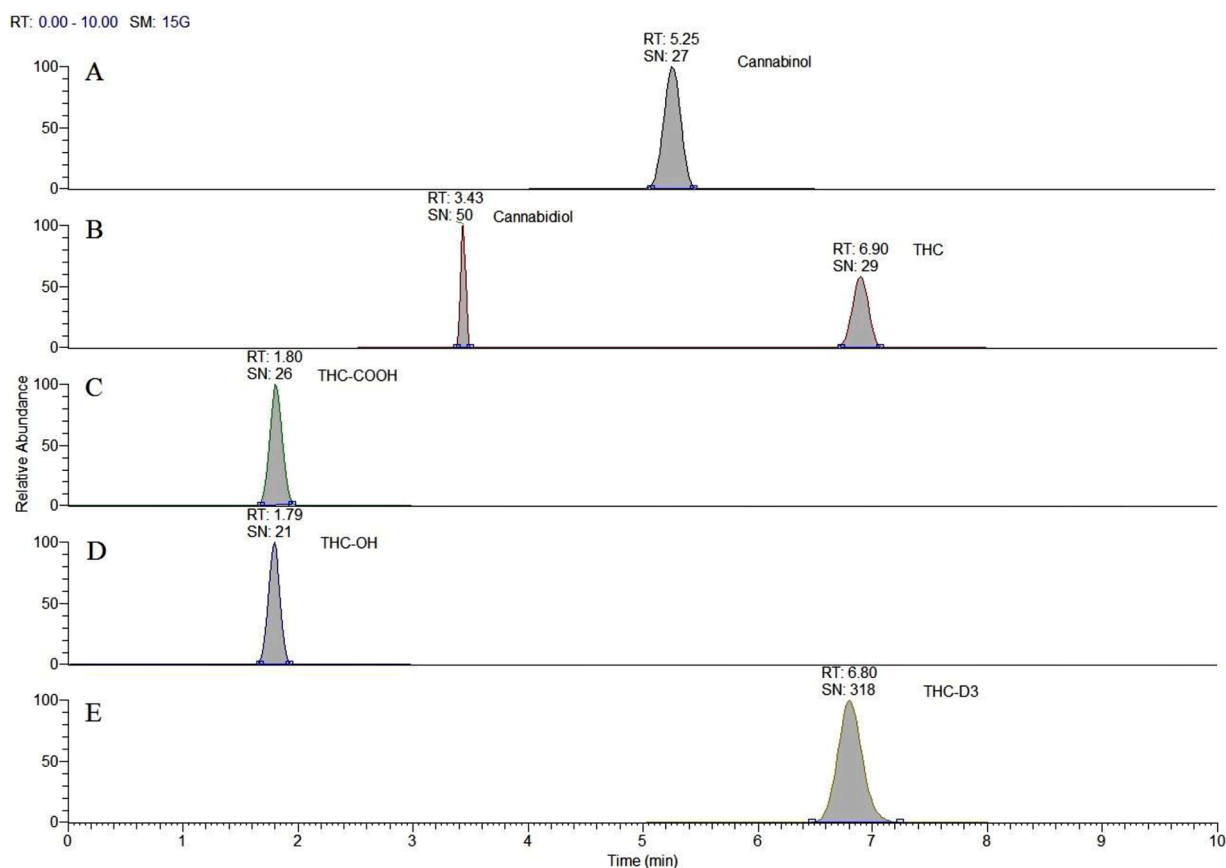
Quality control samples were used to evaluate accuracy and precision of the method. Intra-day and inter-day precision for the target analytes were determined at four different quality control levels and expressed as % CV. As shown in Table 3, intra-day precision ranged from 2.3 % to 9.5 % while inter-day precision ranged from 2.9 % to 10.8 %. Intra-day and inter-day accuracy runs were within  $\pm 15\%$  of the quality control nominal concentrations, except LLOQ which was  $\pm 20\%$ . The comprehensive results of the precision and accuracy data are presented in Tables 3 and 4.

**Table 2**

Linearity regression data for the target analytes.

Analyte	$r^2 \pm SD$ (n = 4)	Slope $\pm SD$ (n = 4)	Intercept $\pm SD$ (n = 4)
CBN	0.9974 $\pm$ 0.0027	0.0973 $\pm$ 0.0394	-0.0016 $\pm$ 0.0012
CBD	0.9987 $\pm$ 0.0007	0.0420 $\pm$ 0.0158	-0.0018 $\pm$ 0.0008
THC-COOH	0.9977 $\pm$ 0.0010	0.0616 $\pm$ 0.0186	-0.0016 $\pm$ 0.0014
11-OH-THC	0.9967 $\pm$ 0.0028	0.0293 $\pm$ 0.0229	0.0001 $\pm$ 0.0025
THC	0.9983 $\pm$ 0.0003	0.0484 $\pm$ 0.0108	-0.0011 $\pm$ 0.0019

Correlation coefficient ( $r^2$ ); number of replicate (n); standard deviation (SD)



**Fig. 2.** Extracted ion chromatogram for target compounds spiked at lower limit of quantification and internal standard (A) Cannabinol (B) Cannabidiol and  $\Delta^9$ -tetrahydrocannabinol (THC) (C) 11-Nor-9-carboxy- $\Delta^9$ -THC (THC-COOH) (D) 11-Hydroxy- $\Delta^9$ -THC (THC-OH) (E) Internal standard THC-D3.

**Table 3**

Intra-day and inter-day precision of cannabinoids in human plasma.

Analyte	Intra-day precision (% CV) (n = 5)				Inter-day precision (% CV) (n = 15)			
	LLOQ	QCL	QCM	QCH	LLOQ	QCL	QCM	QCH
CBN	7.5	4.1	5.2	3.0	10.1	2.9	7.1	6.3
CBD	6.5	3.1	4.6	7.1	7.5	3.4	4.8	4.6
THC-COOH	7.2	3.8	4.4	5.3	6.2	5.0	4.6	5.2
11-OH-THC	7.2	4.9	3.5	2.9	9.0	5.9	4.5	4.3
THC	9.5	2.3	4.7	4.5	10.8	6.8	3.9	2.9

% CV: Percent coefficient of variation, n: number of measurements

**3.3.4. Lower limit of detection and lower limit of quantitation**

Lower limit of detection (LLOD) was estimated to be 0.07 ng/mL for all target analytes with S/N ratio  $\geq 3$ , while LLOQ was 0.2 ng/mL with S/N ratio  $\geq 5$ . Moreover, the acceptance criteria was met for the LLOQ, where the accuracy was  $\pm 20\%$  of the nominal concentration and precision was within  $\pm 20\%$ . Extracted ion chromatograms of target compounds spiked at LLOQ (0.2 ng/mL) with the internal standard in plasma are shown in Fig. 2.

**3.3.5. Carry-over**

Chromatograms of blanks injected after high concentration of the target analytes showed no significant peaks at the retention time of the target analytes and the internal standard. Chromatograms are shown in supplementary information (Fig. 4 to Fig. 9).

**3.3.6. Dilution integrity**

Dilution integrity tested at a dilution factor of 3 times in five replicates indicated that integrity was maintained for all the target analytes as accuracy and precision of the replicates were within 15 % (Table 5).

**3.3.7. Matrix effect, recovery and process efficiency**

Matrix effects analysis indicated ion suppression ranging from -1.1 % for 11-OH-THC to -49.8 % for THC (Table 6). Thus, all targeted cannabinoids experienced some level of ionization suppression which could reduce the method sensitivity and also impair on precision and accuracy. This observed matrix effects, however, did not hinder the achievement of the expected method sensitivity level with acceptable precision and accuracy for all targeted cannabinoids. Recovery ranged from 60.4 % to 85.4 %. The overall process efficiency is a good indication to evaluate the method's sample preparation and analysis by combining both matrix effect and recovery. The data for recovery is presented along with matrix effect and process efficiency data in Table 6.

**3.3.8. Stability**

The stability study was carried out to determine the stability of the targeted cannabinoids and its metabolites in the post-preparative extract stored at room temperature in the autosampler for a period of 6 and 12 h, respectively. This study is expected to mimic the residence time of the samples in the autosampler during the batch run. As indicated in Table 7, acceptable accuracy within  $\pm 15\%$  of the nominal concentration was observed for all targeted cannabinoids and its metabolites.

**Table 4**

Accuracy of the assay for cannabinoids in human plasma with quality control samples at four concentration levels.

Analyte	Intra-day accuracy (% , n = 5)				Inter-day accuracy (% , n = 15)			
	LLOQ	QCL	QCM	QCH	LLOQ	QCL	QCM	QCH
CBN	94.1	107.3	93.9	89.8	95.4	109.2	100.8	97.0
CBN	97.4	108.0	100.9	99.0	93.0	106.8	105.2	98.8
THC-COOH	98.4	106.6	96.2	93.2	99.7	104.4	99.3	94.2
11-OH-THC	98.4	99.6	98.9	98.7	95.6	99.5	100.8	96.9
THC	99.1	106.7	103.7	98.8	97.0	105.1	105.7	99.7

n: number of measurements

**Table 5**

Dilution integrity accuracy and precision.

Analyte	Accuracy (%)	CV% (n=5)
CBN	106.9	2.8
CBD	100.6	7.5
THC-COOH	93.2	11.7
11-OH-THC	109.6	3.5
THC	97.9	2.4

n: number of measurements

**Table 6**

Recovery, matrix effect and process efficiency.

Analyte	Recovery (%)			Matrix effect (%)		Process efficiency (%)	
	QCL	QCM	QCH	QCL	QCH	QCL	QCH
CBN	69.7	74.5	66.8	-21.3	-29.9	54.9	46.8
CBD	73.2	74.5	77.3	-25.7	-26.4	54.3	56.9
THC-COOH	83.7	85.4	67.0	-23.5	-37.5	64.0	41.8
11-OH-THC	60.4	73.5	75.9	-7.4	-1.1	55.9	75.1
THC	65.9	69.4	63.7	-49.8	-48.9	33.0	32.6

**Table 7**

Autosampler stability for 6 h and 12 h expressed by accuracy (% , n = 4).

Analyte	6 h		12 h	
	QCL	QCH	QCL	QCH
CBN	109.3	108.7	105.4	103.2
CBD	98.1	100.1	99.0	104.2
THC-COOH	110	103.9	98.6	98.3
11-OH-THC	98.5	109.3	95.0	107.9
THC	105.8	104.7	104.0	106.8

**3.4. Comparisons with other reported methods**

Most cannabinoids are present at lower concentrations in plasma, requiring sensitive analysis techniques. For instance, in our previous work which involved the analysis of CBD in human plasma using liquid chromatography tandem mass spectrometry (LC-MS/MS) as part of CBD clinical study, it was found that, most of the measured CBD concentrations from unformulated CBD samples were below the LLOQ of 0.5 ng/mL, which prompted improvement in the analytical method's sensitivity for future studies (Unpublished). To date, the lowest LLOQ achieved in plasma (0.2 ng/mL) was only reported by Grauwiler et al. (2007)

**Table 8**  
Comparison of this LC-HRMS method with other reported methods.

Analytical instruments	Matrix	Extraction method		Target cannabinoids					Reference
				THC	THC-COOH	THC-OH	CBD	CBN	
LC-HRMS	Human plasma 0.5 mL	LLE	LLOQ (ng/mL)	0.2	0.2	0.2	0.2	0.2	This work
GC-MS-SIM	Human plasma 1.0 mL	C18 SPE + TMS derivatization	Recovery % LLOQ (ng/mL)	66.3 0.8	78.7 0.88	69.9 0.51	75 0.95	70.3 3.9	(Nadulski et al., 2005)
GC-MS-SIM	Human serum 1.0 mL	Automated and Manual LLE + MSTFA derivatization	Recovery % LLOQ (ng/mL)	50 0.6	85 1.1	95 0.8	90 N/A	43 N/A	(Purschke et al., 2016)
LC-APCI-MS/MS	Bovine serum 1.0 mL	Bond elute certify II SPE	Recovery % LLOQ (ng/mL)	N/A 0.2	N/A 0.2	N/A 0.2	N/A 0.2	N/A 0.2	(Grauwiler et al., 2007)
LC-ESI-MS/MS	Human plasma 1.0 mL	C18 SPE	Recovery % LLOQ (ng/mL)	77.5 0.8	50 4.3	77.6 0.8	71.4 N/A	47.7 N/A	(Maralikova & Weinmann, 2004)
			Recovery %	N/A	N/A	N/A	N/A	N/A	

(Grauwiler et al., 2007) for the targeted cannabinoids using LC-MS-APCI in combination with costly and time consuming SPE method and higher sample volume of 1.0 mL plasma. As shown in Table 8 other reported methods for analysis of cannabinoids by LC-MS/MS in human plasma showed significantly higher LLOQ  $\geq 0.8$  ng/mL (Maralikova and Weinmann, 2004). Compared to the previous method, this proposed method offered higher sensitivity (LLOQ of 0.2 ng/mL) with 0.5 mL plasma and simple and fast chromatographic separation (10.0 min compared to 25.0 min) using an isocratic program in combination with commonly used electrospray ionization and cost effective LLE extraction. A further attempt was made to reach even better sensitivity ( $< 0.2$  ng/mL), however, at this level the accuracy and precision did not meet the acceptance criteria. No retention shift was observed after long batch using isocratic program, whilst column cleaning method was incorporated at the end of each batch. Although the use of LC-HRMS for the analysis of cannabinoids in plasma is absent in literature; it has been reported for the analysis of cannabinoids in oral fluids with lower sensitivity than reported in this current study for THC, CBD and CBN (Concheiro et al., 2013); although plasma matrix is more complex than oral fluid. The enhancement in sensitivity in this study can be attributed partly to the polarity switching, which enabled efficient ionization of the compounds based on their polarity. Secondly, LLE method was effective for the targeted cannabinoids, which is simple, cheaper and quick as compared with the combined protein precipitation and solid phase extraction method used in the previous LC-HRMS method for oral fluids. The selection of this extraction method in the present study was based on the hydrophobic nature of the targeted cannabinoids (logP values; 5 -7). In LLE, non-polar solvents such as diethyl ether, tertiary butyl methyl ether, hexane, ethyl acetate or their mixtures are mostly utilized for extraction. The matrix (human plasma) pH which was adjusted by the addition of phosphate buffer before LLE allowed better recovery of the targeted cannabinoids. As indicated in Fig. 1, these targeted cannabinoids represent weakly acidic molecules with hydroxyl groups attached to unsaturated aromatic hydrocarbon rings and they exhibit pKa values above 9.00, with the exception of THC-COOH which has pKa value of 4.21 (Table 1, supplementary information). Thus, in a basic medium, cannabinoids are ionized due to deprotonation, thereby increasing in polarity. Hence, buffering of the matrix to acidic pH of 4.5 enabled the cannabinoids to be in the non-ionized form and enhanced their partitioning into the non-polar phase during LLE process, resulting in improved recovery.

The performance of tandem mass spectrometry on triple quad (QQQ)

after chromatography separation has been the gold standard for quantitation of small molecules in biological samples. Now LC-HRMS can offer similar linearity over a high dynamic range (Rochat, 2019). The method showed excellent linearity for the cannabinoids in the concentration range as well as satisfactory accuracy and precision. An advantage of LC-HRMS over QQQ is the ability to obtain information-rich full scan data that can be investigated retrospectively. This allows re-analysing the data qualitatively in case of a discovery of a new metabolite.

#### Study limitations

The use of only one internal standard (THC-D3) compensated for the ion suppression of THC, but it did not effectively compensated for the other cannabinoids. Hence, utilizing additional internal standards closer to the structure of the other targeted cannabinoids and its specified metabolites will certainly compensate for possible matrix effects. The use of one internal standard was cost effective and bypassed study delays caused by government regulation which required stringent import permits for the isotope labelled internal standards. Overall the precision and accuracy of the method was acceptable for the analysis.

Moreover, difficulty in obtaining commercially available CBD metabolites due to stringent import permit and government regulations explains their exclusion in this study. As the legalization of *Cannabis* use is on-going worldwide, we recommend researchers with easy access to CBD metabolites to include in their future studies.

One advantage of the HRMS over triple quadrupole is the ability to obtain information rich-full scan data that can be investigated retrospectively. But in the current application, it was observed that the full scan experiment of the precursor ions could not be performed, because if added to the targeted-MS/MS experiment, enough points per peak could not be obtained.

#### 4. Conclusion

The resurgence of *Cannabis* therapeutic discoveries have led to the need for sensitive and selective analytical methods. Comprehensive method development was performed using LC-HRMS to develop a sensitive and selective analytical method for the detection and quantitation of cannabinoids and its metabolites in human plasma. The method was able to quantify CBN, CBD, THC, and its two major metabolites; 11-OH-THC and THC-COOH from 0.2 ng/mL to 100 ng/mL. The method was

then challenged with a rigorous validation protocol which tested the methods' selectivity, specificity, carry-over, linearity, recovery, matrix effect, accuracy and precision. The validation parameters met acceptance criteria as per FDA guidelines for bioanalytical methods (Administration, 2018). The superior sensitivity (LLOQ of 0.2 ng/mL for all targeted cannabinoids and metabolites in 0.5 mL human plasma) and inherent selectivity of the method coupled with cheaper sample preparation will allow the method to be utilized in cannabinoids pharmacokinetic study.

### Declaration of Competing Interest

The authors declare no conflicts of interest.

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### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ejps.2021.105705](https://doi.org/10.1016/j.ejps.2021.105705).

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