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**Development of a simple and sensitive liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) method for the determination of cannabidiol (CBD),  $\Delta^9$ -tetrahydrocannabinol (THC) and its metabolites in rat whole blood after oral administration of a single high dose of CBD**

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## Highlights

- The conversion of CBD to THC was evaluated after a single oral high dose of CBD (50 mg/kg)
- A LC-MS/MS method was developed and validated according to Forensic Toxicology guidelines
- The extraction of cannabinoids was optimized in order to remove phospholipids
- CBD, THC and its metabolites were determined in rat whole blood
- No conversion of CBD into THC was observed in rat whole blood
- CBD concentration was higher when administered in olive oil than in ethyl alcohol

## Abstract

The investigation of the possible conversion of cannabidiol (CBD) into  $\Delta^9$ -tetrahydrocannabinol (THC) *in vivo* after oral administration of CBD is reported herein since recent publications suggested a rapid conversion in simulated gastric fluid. To this end, single high dose of CBD (50 mg/kg) was administered orally to rats and their blood was collected after 3 and 6 hours. A highly sensitive and selective LC-MS/MS method was developed and fully validated in compliance with the Scientific Working Group of Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. This method also involved the optimization of cannabinoids and their metabolites extraction in order to remove co-eluting phospholipids and increase the sensitivity of the MS detection. Neither THC nor its metabolites were detected in rat whole blood after 3 or 6 hours from CBD administration. After oral administration, the amount of CBD dissolved in olive oil was higher than that absorbed from an ethanolic solution. This could be explained by the protection of lipid excipients towards CBD from acidic gastric juice.

**Keywords:** *cannabidiol,  $\Delta^9$ -tetrahydrocannabinol, liquid chromatography, mass spectrometry, cannabinoids extraction*

## 1. Introduction

Cannabidiol (CBD; Fig. 1) is one of the 142 phytocannabinoids that have been isolated so far from hemp and its chemical structure was determined by Mechoulam and Shvo in 1963 [1]. Along with its isomer,  $\Delta^9$ -tetrahydrocannabinol (THC) represents the main cannabinoid in cannabis inflorescence.

Although CBD was isolated and characterized first, THC has been investigated more thoroughly since it is responsible for the unique psychotropic activity of cannabis. Indeed, THC is an internationally controlled substance, though widely used for recreational purposes or

medication [2]. Due to its unique psychotropic activity on the one hand and its therapeutic potential on the other hand, the pharmacokinetics and pharmacodynamics of THC are much better studied than those of the non-intoxicating CBD, which until few years ago has been a neglected phytocannabinoid [3]. In recent years, several pharmacological activities of CBD have been discovered, encompassing analgesic, anti-inflammatory, antioxidant, antiemetic, antianxiety, antipsychotic, anticonvulsant and cytotoxic effects (confined to malignant cell lines) [4-6]. These biological functions are mediated by a wide variety of signaling mechanisms including activity on cannabinoid receptors, 5-HT<sub>1A</sub>, GPR55, GPR18, TRPV1, and other transient receptor potential channels [7]. This has led to an exponential growth of the scientific contributions regarding this phytocannabinoid.

In particular, the promising results proved by CBD in treating cancer and drug-resistant epilepsy in children have recently brought this molecule to the attention of the scientific community, clinicians, the media, as well as politicians and regulatory agencies. Consequently, CBD preparations have received the “Orphan Drug” designation for use in the treatment of epilepsy in children (Dravet and Lennox-Gastaut syndromes) and neonatal asphyxia by the US Food and Drug Administration and the European Medicines Agency. Moreover, clinical trials sponsored by GW Pharma Ltd. have been started in these indication areas [8].

A claim that CBD could be converted into THC has gained some credibility especially after the publication of a recent article by Merrick *et al.*, in which it was demonstrated that this reaction could occur after prolonged exposure to ‘simulated’ gastric fluid [9]. Whilst this isomerization reaction has been known for decades since first reported with putative structures by Roger Adams in 1940, and with definitive structures by Yehiel Gaoni and Raphael Mechoulam in the 1960s, there is no reliable evidence that the reaction can occur *in vivo* in humans [3]. In contrast to the hypothesis of the conversion reaction Franjo Grotenhermen *et al.* presented various scientific papers and clinical trials to support their theory in a commentary article published straight after the one by Merrick *et al.* [10]. First, there is no knowledge of an enzyme that can catalyze such a bioconversion. In addition, this reaction is in complete contrast to any pharmacokinetic and metabolism study in human clinical trials [11].

Since THC is metabolized via cytochrome P450 (CYP) 2C9 and 2C19 isozymes to several phase I metabolites, the most important and common being 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (THCCOOH) [12], it is important also to test for these compounds. Moreover, THC and its phase I metabolites undergo UDP-glucuronosyltransferase-catalyzed phase II metabolism to form THCCOOH-glucuronide (THCCOOH-gluc) *in vivo*, favoring excretion [13]. Detection and quantification of THC, 11-OH-THC, THCCOOH and THCCOOH-gluc may provide scientific evidence for an accurate evaluation of the conversion of CBD into THC after oral ingestion of CBD itself.

The literature includes several different methods for the determination of blood concentrations of cannabinoids, mainly based on chromatographic techniques. Blood (whole blood, serum or plasma) cannabinoids concentrations can be determined with either gas chromatography (GC) or liquid chromatography (LC). Flame ionization detectors (FID) or MS detectors are generally interfaced to a GC system or a two-dimensional GC set up (GC×GC) [14]. However, LC coupled to UV, electrochemical, but especially MS detectors has become the method of choice for its superior sensitivity, accuracy and precision [15-19].

The determination in blood of very low levels of THC and its metabolites, which could be originated by CBD conversion in gastric fluids, requires the use of an extremely sensitive analytical method. Most certainly, LC-MS is among the most sensitive and specific analytical methods for the determination of cannabinoids in biological fluids [2, 20]. Anyway, the accuracy and sensitivity of all detection techniques are highly dependent on sample preparation, which should be sufficiently simple, rapid, and effective for multi-component analysis in complex matrices [20, 21]. The co-extraction of different types of bioorganic compounds, including lipids, proteins, carbohydrates and others, can decrease the method selectivity and generate complex undesired side effects, such as background noise, drift, false peaks, and strong suppression effects leading to inaccurate quantification results [22]. In fact, a recent work by Sørensen *et al.* indicates that the ionization of THC and other cannabinoids in the electrospray ionization (ESI) source is characterized by a marked suppression due to co-elution phospholipids (PLs) from blood extracts obtained after deproteination with organic solvents [23]. The most common strategy for extraction, purification and concentration proposed by the scientific literature involves the protein precipitation with acetonitrile and then a solid phase extraction (SPE). The main drawback of conventional SPE based methods is related to the difficulty in separating PLs from cannabinoids on reverse phase chromatography due to the broad elution profile of the former [24]. PL co-elution represents a problem also with ESI sources of MS detectors, as they are particularly sensitive to PLs, thus generating further decrease of sensitivity [24]. Hence, it becomes necessary to remove PLs from blood extracts in order to achieve the optimum efficiency of MS detection techniques [25].

A novel concept of rapid and highly selective SPE technologies to remove PLs based on tubes, plates, and related systems has been marketed for the last few years by Phenomenex® (Phree™), Sigma-Aldrich (HybridSPE™), Biotage® (ISOLUTE®), and other companies. This new technology has been widely employed for the determination of bioactive compounds in biological matrices and in pharmaceutical analysis and recently by Sørensen *et al.*, thus reaching a five-fold increase in sensitivity for the determination of THC with respect to previous methods [23].

The present study describes the development and full in-house validation of a sensitive and simple method based on an optimized rapid PL removal procedure combined with a robust and highly sensitive triple quadrupole (QQQ) MS detection technique. This analytical method has been designed for the occurrence studies of CBD, THC, 11-OH-THC, THCCOOH and THCCOOH-gluc (Fig. 1) in rat whole blood after oral administration of a single high dose of CBD.

## 2. Material and methods

### 2.1 Chemicals

THC, CBD, 11-OH-THC, 11-nor-THCCOOH and THCCOOH-gluc were supplied from Sigma-Aldrich (Milan, Italy). THC-*d*<sub>3</sub>, 11-OH-THC-*d*<sub>3</sub> and 11-nor-THCCOOH-*d*<sub>3</sub> were used as internal standards (IS) and were from Sigma-Aldrich (Milan, Italy). All solvents and chemicals for HPLC-MS/MS were of LC-MS purity grade (Baker-VWR, Milan, Italy), while other chemicals used for sample preparation were of analytical grade (Carlo Erba, Milan, Italy).

Phree™ Phospholipid Removal Tabbed 1 mL Cartridges were supplied from Phenomenex (Bologna, Italy).

## 2.2 Animals

Male Sprague Dawley rats (Charles River Laboratory, Lecco, Italy) weighing 250-300 g were used in the experiments. The animals were housed two for cage and were maintained under controlled environmental conditions ( $22\pm 1$  °C; 12:12 hour light-dark cycle; food and water *ad libitum*) for one week before use in the experiments. Animal care, maintenance and surgery were conducted in accordance with the Italian law (D.L. n.116/1992) and European legislation (EEC n.86/609). The experimental design and procedures received the approval of Bioethical Committee of the Italian Institute of Health.

Five rats/group received a single oral administration of vehicle (olive oil (oil) or 96% alcohol (EtOH)) or 50 mg/kg of CBD (Crystal Hemp SA, Lugano, Switzerland) dissolved either in oil or EtOH and were sacrificed 3 or 6 h after oral gavage administration. After deep anesthesia, whole blood sample was collected from each rat. Whole blood samples were immediately collected in polypropylene tubes and stored at  $-80$  °C until analysis.

## 2.3 Preparation of standard solutions

Stock solutions (1 mg/mL in methanol) of the analytes (THC, CBD, 11-OH-THC, THCCOOH and THCCOOH-gluc) were diluted in methanol to obtain working solutions at ten different concentration levels in the range 10-1000 pg/ $\mu$ L for THC, 11-OH-THC, 11-nor-THCCOOH, 5-1000 pg/ $\mu$ L for CBD, and 50-5000 pg/ $\mu$ L for THCCOOH-gluc analyte. An IS was also prepared by diluting a stock solution (0.1 mg/mL in methanol) of THC- $d_3$ , 11-OH-THC- $d_3$  and 11-nor-THC-COOH- $d_3$  (Fig. 1) with methanol up to the concentration of 50 pg/ $\mu$ L for THC- $d_3$  and 11-OH-THC- $d_3$ , and 100 pg/ $\mu$ L for 11-nor-THCCOOH- $d_3$ . All solutions were stored at  $-20$  °C until use.

## 2.4 LC-MS/MS conditions

LC analyses were performed on an Agilent 1200 LC system consisting of a binary pump, a thermostated autosampler, an on-line degasser and a thermostated column compartment (Agilent, Waldbronn, Germany). Samples were analyzed on a Kinetex EVO C18 column (100  $\times$  2.1 mm; 5  $\mu$ m particle size) (Phenomenex, Bologna, Italy). The mobile phase was composed of (A) 2.0 mM aqueous ammonium acetate and (B) acetonitrile using the following gradient program: 0.0–10.0 min, linear gradient from 30 to 90% (B); 10.0–15.0 min, isocratic at 90% (B), 15.0–18.0 min, linear gradient from 90 to 30% (B). A pre-equilibration period of 2.0 min was applied between each run. The flow-rate was 0.35 mL/min and the column temperature was 40 °C. The injection volume was 25  $\mu$ L and the injector needle was washed with methanol/0.05% formic acid in water (1:9 v/v); the autosampler was maintained at room temperature. The chromatographic conditions were optimized by analyzing the standard solutions and extracts of whole blood spiked with the target analytes.

Tandem mass spectrometry was performed using a SCIEX API 4000 QTRAP mass analyzer equipped with a Turbo Ion Spray source (SCIEX, Toronto, Canada) operating in electrospray ionization (ESI) positive/negative mode. The Analyst Software (version 1.5.2) was used for instrument control, data acquisition, qualitative and quantitative data analyses. Detection and

quantitation of all analytes were accomplished using multiple reaction monitoring mode (MRM) due to the achieved high selectivity and sensitivity. Optimized instrument settings were as follows: ionization mode, positive and negative; curtain gas, 10 psi; CAD gas, 4 psi; nebulizer gas (GS1), 35 psi; heater gas (GS2), 45 psi; ion spray voltage, 4000 V; temperature, 450 °C. The nitrogen flow was produced by a gas generation system (Nitrogen Generator model 75–72, Whatman Inc., MA, USA). A dwell-time of 100 msec was used for all transitions. MS/MS parameters were optimized by direct infusion of each individual analyte at 100 ng/mL in the initial LC mobile phase at a flow rate of 10  $\mu$ L/min. The validity of the chosen MRM transitions was also verified by LC-MS/MS analyses of blank whole blood samples spiked with the individual analytes at 100 ng/mL. The mass spectrometer was calibrated to <2.0 mDa mass error prior to each batch analysis. LC-MS/MS optimized parameters for cannabinoids and metabolites are reported in Table 1.

## 2.5 Whole blood extraction and sample preparation

Aliquots of whole blood (200  $\mu$ L) were transferred into a 1.5 mL polypropylene centrifuge tube. Then 20  $\mu$ L of IS solution and 900  $\mu$ L of 0.1% (v/v) formic acid solution in acetonitrile:methanol 70:30 (v/v) was added to each sample. The samples were vigorously shaken for 1.3 min using an automatic shaker and centrifuged for 7 min at 6000 rpm. The supernatant was collected and loaded for the purification onto a Phree™ phospholipid removal tube (1 mL). The extract obtained was evaporated to dryness under nitrogen stream at 55 °C. The residue was dissolved in 150  $\mu$ L of 2.0 mM aqueous ammonium acetate:acetonitrile (70:30, v/v). The samples were transferred to autosampler vials for HPLC analysis. A 25  $\mu$ L aliquot of each sample was injected into the chromatographic system.

## 2.6 Validation

The present study was validated in compliance with Scientific Working Group of Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology [26]. The following parameters were evaluated: selectivity, calibration model, limit of detection (LOD), lower limit of quantitation (LLOQ), precision, accuracy, carryover, matrix effect, recovery and dilution integrity.

### 2.6.1 Calibration and quality control samples

To aliquots of blank whole blood (200  $\mu$ L) were added 20  $\mu$ L of the IS solution and an appropriate volume of working solution. The samples were subjected to the described sample processing in order to have 6 calibration levels in the range 1-100 pg/ $\mu$ L for THC, 11-OH-THC, and THCCOOH, 0.5-100 pg/ $\mu$ L for CBD, and 5-500 pg/ $\mu$ L for THCCOOH-gluc analyte. Quality control (QC) samples were prepared by spiking blank whole blood (200  $\mu$ L) with 20  $\mu$ L of the IS solution and an appropriate volume of the working solution at two concentration levels. These QC samples were processed as described above; the final concentration in the samples was 2 and 50 ng/mL for CBD, THC, 11-OH-THC and THCCOOH, and 15 and 400 ng/mL for THCCOOH-gluc whole blood for low (LQC), and high (HQC) levels, respectively (n = 5 for each level).

### 2.6.2 Selectivity

Aliquots (200  $\mu\text{L}$ ) of blank whole blood specimens were added to 100  $\mu\text{L}$  of methanol and processed as described above. These blank samples were individually assessed for the presence of any interference across the retention window of each analyte and the IS.

### 2.6.3 Calibration model

The processed calibration samples were analyzed in triplicate (injection volume: 25  $\mu\text{L}$ ). Calibration curves were generated from the peak-area ratio of each analyte quantifier transition to the deuterated IS; the ratio was then plotted on the y-axis against the nominal analyte concentration to generate the standard curves by the method of least squares using a weighed (1/x) linear regression model.

### 2.6.4 LOD and LLOQ

Analyte identification criteria included a symmetric peak eluting within  $\pm 0.2$  min of average calibrator retention times with signal:noise of at least 3:1 ratio qualifier/quantifier MRM transition peak area ratio within  $\pm 20\%$  average calibrator ratios. The sensitivity of the developed analytical procedure was evaluated by determining the LOD and the LLOQ. Fortified samples at decreased concentrations ( $n=3$ ) were prepared and subjected to the described sample processing. LOD value for each analyte was estimated as the lowest concentration that achieved acceptable predefined detection and identification criteria. The LLOQ value was the lowest concentration fulfilling LOD criteria, while maintaining a bias of  $\pm 20\%$  and  $\text{CV} < 20\%$ .

### 2.6.5 Precision and accuracy

The precision and accuracy were evaluated at two levels, LQC (2 ng/mL for THC, CBD, 11-OH-THC and THCCOOH; 15 ng/mL for THCCOOH-gluc) and HQC (50 ng/mL for THC, CBD, 11-OH-THC and THCCOOH; 400 ng/mL for THCCOOH-gluc).

Method precision and accuracy were determined by replicate analyses of the QC samples spiked at low and high levels ( $n = 5$ , each). Each sample was analyzed repeatedly three times within a single day to determine the intra-day precision, and three times a day for five successive days ( $n = 15$ ) to determine the inter-day precision. The precision was expressed as the coefficient of variance (%CV).

Accuracy of the method was evaluated by comparing the levels found in whole blood samples after clean-up procedure with the nominal analyte concentration; the obtained values are expressed as percent of the estimated concentration (bias).

### 2.6.6 Carryover

Carryover effect was evaluated by injecting extracts of blank whole blood samples after analyses of calibration samples spiked at the upper limit of quantitation. For acceptance, the peak areas of the blank sample should not exceed 10% of the peak areas obtained for the lowest calibrator.

### 2.6.7 Matrix effect and recovery

Matrix effect was evaluated for each analyte analyzing the QC samples spiked at low, medium and high concentrations. ME values were calculated by dividing the analyte peak area in QC samples spiked after the clean-up procedure (post-spiked samples) to the response for neat standards. Matrix effect was calculated as matrix factor (*MF*) by the formula:

$$MF\% = \left( \frac{A_M - A_S}{A_S} \right) \times 100$$

Where  $A_M$  is the area of the peak in the matrix and  $A_S$  is the area of the peak of neat standards. Recovery values were determined by comparing the levels found in QC samples spiked before the sample processing with the levels found in the post-spiked samples.

### 2.6.8 Dilution integrity

**2.6.9 To validate the dilution integrity, blank whole blood samples (200µl) were spiked at 5 times the highest validation sample and mixed with additional blank blood to achieved a 5-fold (n=6), 10-fold (n=6), 15-fold (n=6) dilution; internal standard was added and samples were processed. The obtained samples were evaporated, reconstituted in the LC mobile phase and analyzed against the calculated calibration curves to assess if the performance criteria were still met.**Stability

The short-term stability was determined for LQC and HQC samples in processed samples for 24 hours at room temperature (n = 3). Stability of the drugs in processed samples was assessed after 24 hours in the autosampler at 10 °C (n = 3). The drugs were considered stable if the mean concentration was within ±15% of the nominal concentration.

## 3. Results and discussion

### 3.1 Liquid chromatography

One aim of this work was to develop a simple and rapid analytical method for the quantitative determination of CBD, THC, 11-OH-THC, THCCOOH and THCCOOH-gluc in rat whole blood. To this end, the best performance for the determination of cannabinoids in biological matrices have been achieved employing C18 columns with gradient elution [27, 28]. In this study, the chromatographic performance of different columns was evaluated. The presence of ammonium acetate (2 mM) in the mobile phase provided a better reproducibility of retention times, especially for THCCOOH. Given the better chromatographic performance (in terms of both resolution and sensitivity), a shorter analysis time, Kinetex 5u EVO (100 × 2.1 mm, 5 µm, Phenomenex) column was finally selected for this study.

Using the gradient elution described in the experimental section the observed retention times were: 10.52 min for THC, 9.44 min for CBD, 7.68 min for 11-OH-THC, 5.49 min for THCCOOH and 3.07 min for THCCOOH-gluc; the deuterated IS (THC- $d_3$ , 11-OH-THC- $d_3$ , and THCCOOH- $d_3$ ) presented the same chromatographic retention time of the corresponding

non-deuterated analytes. The relative standard deviation (RSD%) values less than 0.3% for all analytes and the IS indicated a good repeatability (Table 1).

The retention time ( $R_T$ ) was the mean of at least 50 different individual analyses.

### 3.2 Mass spectrometry

Multiple reaction monitoring (MRM) mode was used to carry out the quantitative analyses due to the achieved high selectivity and sensitivity. For each analyte, one precursor ion and one (or two) MRM transitions were set up, monitoring the more abundant product ion for quantitation (quantifier ion) and the less abundant product ions as qualifier ions for confirmation (Table 1). The LC-MS/MS chromatograms of a blank whole blood sample spiked with IS in positive and negative ionization mode are reported in figures S1 and S2 (Supplementary Material), respectively.

### 3.3 Clean up procedure

The present study describes the development and full in-house validation of a high throughput method based on an optimized rapid PL removal procedure combined with a robust and highly sensitive LC-MS/MS detection technique, designed for the simultaneous determination and quantification of CBD, THC, 11-OH-THC, THCCOOH and THCCOOH-gluc in rat whole blood.

A novel concept based on rapid and highly selective technologies of solid-phase PL removal tubes has been used. The efficiency of the proposed analytical procedure was confirmed and validated in compliance with SWGTOX standard practices for method validation in forensic toxicology [26]. The clean-up procedure was quick, efficient and reproducible as shown by the results of the method validation described in the following paragraph.

### 3.4 Validation

#### 3.4.1 Selectivity

Monitoring the MRM transitions shown in Table 1, no interfering peaks were observed in the extracts of blank whole blood samples at the retention times of each compound and deuterated IS. Moreover, no interferences deriving from either the deuterium labelled IS or from the matrix were detected. Therefore, the developed procedure was found to be selective for all the target analytes.

#### 3.4.2 Calibration model, LOD and LLOQ

Detailed information about the calculated calibration parameters is shown in Table S1 (Supplementary Material). The linearity was adequate in the range 1-100 ng/mL whole blood for THC, 11-OH-THC and THCCOOH, 0.5-100 ng/mL for CBD, and 5-500 ng/mL for THCCOOH-gluc with correlation coefficient values ( $R^2$ ) of at least 0.990. The LOD and LLOQ values for each analyte were estimated as described in the materials and methods section (paragraph 2.6.4), and then validated by analyzing blank whole blood samples fortified with analytes at the calculated LOD and LLOQ levels. The obtained data fulfilled the usual acceptance criteria, being the corresponding deviation from the expected concentration (% accuracy) less than 20%. The LLOQ values were in the range 0.5-5 ng/mL whole blood (Table

S1), confirming the satisfactory sensitivity of the developed procedure. The chromatograms of a mixture of analytical standards and IS at the LLOQ level in positive and negative ionization mode are reported in figures S3 and S4 (Supplementary Material), respectively.

### 3.4.3 Precision and accuracy

The within-run precision, between-run precision and accuracy were evaluated at the low (2 ng/mL for THC, 11-OH-THC and THCCOOH, and 15 ng/mL for THCCOOH-gluc;  $n = 5$ ) and high concentration (50 ng/mL for THC, 11-OH-THC and THCCOOH, and 400 ng/mL for THCCOOH-gluc;  $n = 5$ ) using spiked QC samples prepared and analyzed as described. The calculated data are shown in Table S2. The intra-batch and inter-batch precision values were satisfactory, being lower than 10% for all analytes at all concentration levels. As the precision data did not exceed 20%, the proposed method was considered precise for all analytes. Accuracy values were in the range 90–110%, which are within the accepted limits for this parameter [26].

### 3.4.4 Carryover

As suggested [26], the carryover value should not exceed 10% of signal of the lowest calibrator. The observed effects were negligible for all analytes, being the peak areas in the blank sample <5% of the peak areas found for the calibrator spiked at the LLOQ, which are within the proposed acceptance limits for this parameter.

### 3.4.5 Matrix effect and recovery

Concerning the matrix effect, there are often endogenous matrix components that may co-elute with the target analytes, which are often invisible to the MS detector at the monitored masses but that may significantly affect the efficiency and reproducibility of the ionization process. This may cause an ionization suppression/enhancement in the signal response, which is termed “matrix effect”. According to the proposed guidelines, the matrix effect should be between  $\pm 25\%$ . No marked matrix effect was observed in the QC samples spiked at low and high levels for all analytes, being the corresponding values within the prescribed range, except for THC and THC- $d_3$ , characterized by a considerable ionization suppression (ranging from -30.4% to -48.7%).

This fact indicates the presence in the final extracts of residual matrix components affecting markedly the ionization process only for the cited analytes. For quantitative analyses, the matrix effect has been compensated by the use of IS. The addition of IS characterized by similar retention time and ionization behavior of the target analyte, such as isotopically labelled ISs, can efficiently compensate the matrix effect.

Recovery was also calculated at low and high concentrations; the recovery values were higher than 60%, suggesting that the extraction efficiency was adequate for all analytes at all tested concentrations (Table S3).

### 3.4.6 Dilution integrity

In the dilution integrity studies, the precision was less than 10% and the accuracy within the range 90–110% for all analytes. These results proved that authentic samples with concentrations higher than the highest calibrator could be diluted and re-analyzed.

### 3.5 Analysis of authentic whole blood samples

The analytical method developed was applied to authentic whole blood samples from rats administered with a single oral high dose of CBD (50 mg/kg). Rats ( $n = 5$  per group) were administered with either the excipients (oil or EtOH) or CBD dissolved in either one of the excipients. A first group was sacrificed after 3 hours and another one after 6 hours according to CBD (and its metabolites) peak values in whole blood reported in the literature. Considering that the main aim of the application of the method developed was to evaluate the possible conversion of CBD to THC in the acidic environment of the stomach, a single high dose of CBD was administered orally to ensure the detection of very low whole blood concentrations of THC and/or its metabolites. To this end, it was necessary to have a highly sensitive analytical method that could reach limits of detection in the order of ng/mL. The results reported in Table 2 showed the complete absence of either THC or its metabolites, thus suggesting no conversion of CBD into THC at least in rats. Furthermore, the concentration of CBD in oil at both 3 and 6 hours after administration was significantly higher than that found after administration of CBD in EtOH ( $p < 0.01$  at 3 hours;  $p < 0.001$  at 6 hours)<sup>2</sup>. Moreover, the concentration of CBD in olive oil was higher even after 6 hours ( $307.03 \pm 28.3$  ng/mL) with values comparable to those at 3 hours ( $345.64 \pm 71.52$  ng/mL). Conversely, CBD levels abruptly decreased from  $66.66 \pm 25.89$  ng/mL at 3 hours to about  $8.37 \pm 2.03$  ng/mL at 6 hours when EtOH was used as excipient. A representative chromatogram of a whole blood sample at 3 hours after CBD administration is shown in figures 2 and 3 (positive and negative ionization mode, respectively).

## 4. Conclusions

In summary, we developed and validated a highly sensitive and selective LC-MS/MS method for the simultaneous determination of CBD, THC, 11-OH-THC, THCCOOH and THCCOOH-gluc in rat whole blood. This method also involves a rapid, simple and efficient Phree PL removal SPE. . The results obtained in rat whole blood suggest no conversion of CBD into THC since no peaks corresponding to either THC or its metabolites were detected. Moreover, the results indicate a marked influence of the excipient on the absorption of the active principle since the whole blood concentrations of CBD are significantly higher when it is administered in oil than in EtOH.

This finding is not surprising as lipid formulations have shown oral bioavailability enhancement for several lipophilic drugs [29]. Hence, the CBD absorption in the oil solution could occur via the intestinal lymphatic system similarly to other lipophilic drug [30]. On the contrary, given the scarce solubility of CBD in water, its oral administration in EtOH solution, which is completely soluble in the gastric juice, may lead to precipitation in the solid state of the drug in the stomach. Consequently, the lower bioavailability of CBD when administered in EtOH is most likely due to its poor solubility in water and precipitation in the stomach.

In conclusion, this work responds, at least in the animal model, to the ongoing discussion on the article by Merrick *et al.* where an *in vitro* rapid conversion of CBD into THC in simulated gastric fluid is reported. Since CBD is insoluble in the aqueous solution of acidic gastric juice

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<sup>2</sup> An independent sample t-test was run to compare CBD in EtOH vs CBD in oil at 3 or 6 h post-administration.

while it is extremely soluble in olive oil, its conversion into THC could be prevented by lipid excipients. On the contrary, alcoholic excipients are miscible in any ratio with the gastric juice leading to exposure of CBD to acid catalysis. Hence, if the lack of CBD conversion into THC can be explained by the oil protection from gastric juice, no shield could be hypothesised for the ethanolic solution.

## 5. References

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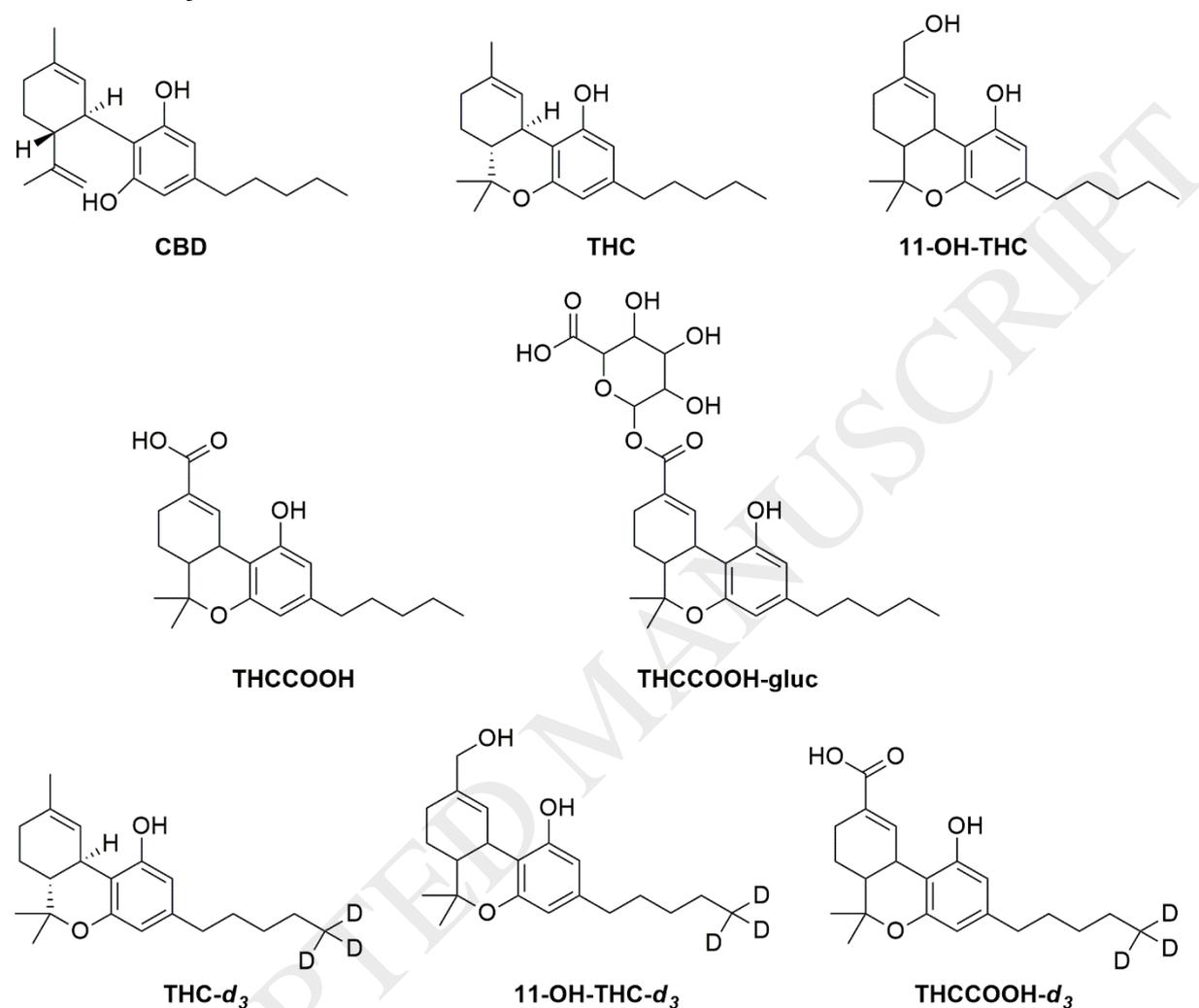
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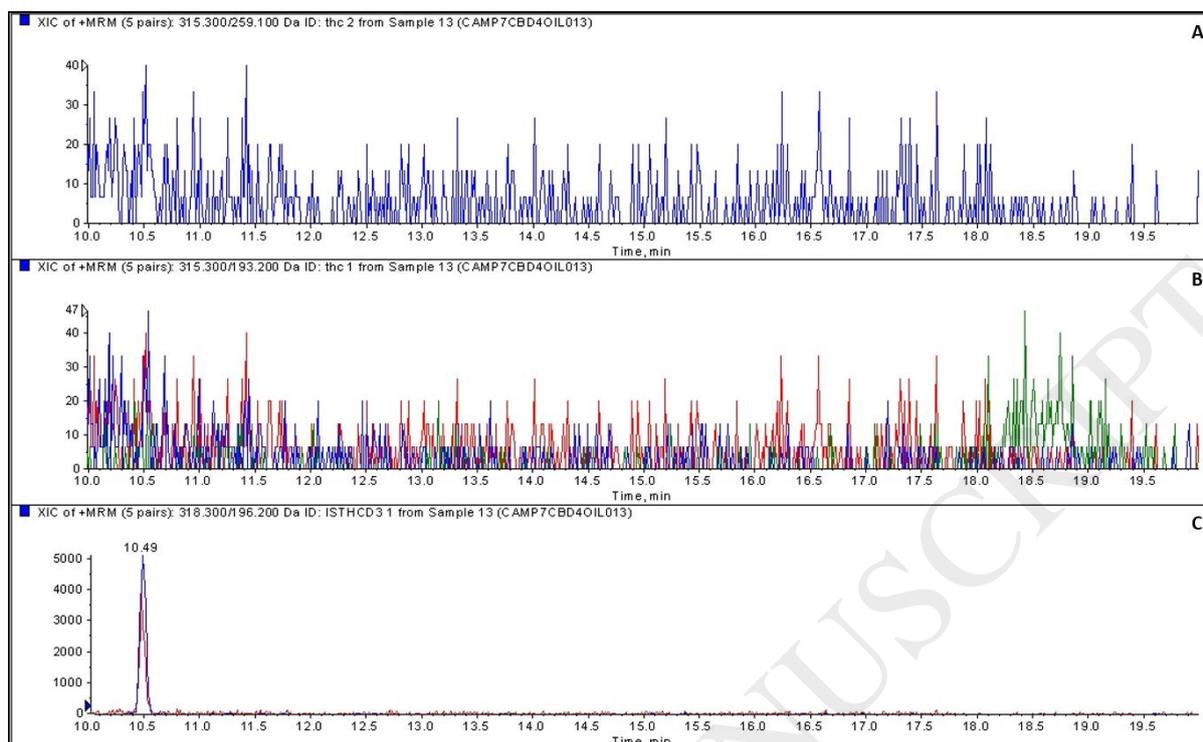
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## FIGURE CAPTIONS

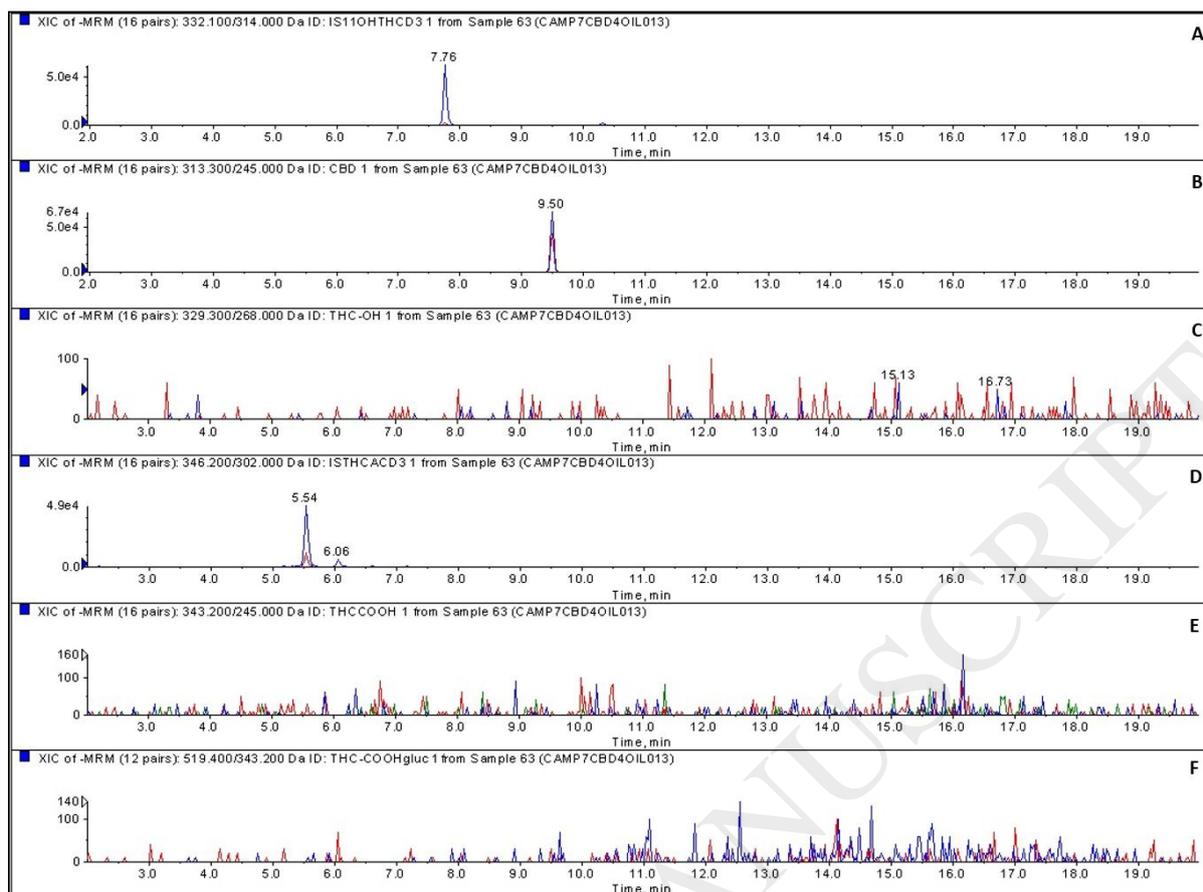
**Figure 1.** Molecular structure of cannabidiol (CBD),  $\Delta^9$ -tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), THCCOOH-glucuronide (THCCOOH-gluc) and of the deuterated internal standards THC- $d_3$ , 11-OH-THC- $d_3$  and THCCOOH- $d_3$ .



**Figure 2.** Chromatogram of a whole blood sample at 3 hours after CBD administration in positive ionization mode. **A)** Extraction of transitions 315.3→259.1 of THC ( $R_T=10.52$  min). **B)** Extraction of transitions 315.3→193.2, 315.3→259.1 and 315.3→135.1 of THC ( $R_T=10.52$  min). **C)** Extraction of transitions 318.3→196.2 and 318.3→135.1 of THC- $d_3$  ( $R_T=10.49$  min).



**Figure 3.** Chromatogram of a whole blood sample at 3 hours after CBD administration in negative ionization mode. **A)** Extraction of transitions 332.1→270.0 and 332.1→314.0 of 11-OH-THC- $d_3$  ( $R_T=7.76$  min). **B)** Extraction of transitions 313.3→245 and 313.3→107 of CBD ( $R_T=9.50$  min). **C)** Extraction of transitions 329.3→268.0 and 329.3→173.1 of 11-OH-THC ( $R_T=7.76$  min). **D)** Extraction of transitions 346.2→248.1 and 346.2→302.0 of THCCOOH- $d_3$  ( $R_T=5.54$  min). **E)** Extraction of transitions 343.2→245.0, 343.2→299.4 and 343.2→191.0 of THCCOOH ( $R_T=7.76$  min). **F)** Extraction of transitions 519.4→343.2 and 519.4→299.1 of THCCOOH-gluc ( $R_T=3.07$  min).



## 6. Tables

*Table 1. LC-MS/MS parameters optimized for the target analytes.*

Analyte	MRM transitions (m/z)	DP (V)	CE (v)	$R_T$ (min)	IS
THC	315.3→ <b>193.2</b> <sup>a</sup> , 259.1 <sup>b</sup> , 135.1 <sup>b</sup>	70	28, 31, 28	10.52	THC- <i>d</i> <sub>3</sub>
CBD	313.3→ <b>245</b> <sup>a</sup> , 107 <sup>b</sup>	-50	50, 50	9.44	11-OH-THC- <i>d</i> <sub>3</sub>
11-OH-THC	329.3→ <b>268.0</b> , 173.1	-50	38, 44	7.68	11-OH-THC- <i>d</i> <sub>3</sub>
THCCOOH	343.2→ <b>245.0</b> , 299.4, 191.0	-50	40, 30, 43	5.49	THCCOOH- <i>d</i> <sub>3</sub>
THC-COOH-gluc	519.4→ <b>343.2</b> , 299.1, 268.0	-90	33, 26, 47	3.07	THCCOOH- <i>d</i> <sub>3</sub>
THC- <i>d</i> <sub>3</sub>	318.3→ <b>196.2</b> , 135.1	70	32, 20	10.50	
11-OH-THC- <i>d</i> <sub>3</sub>	332.1→ <b>270.0</b> , 314.0	-50	25, 45	7.65	
THC-COOH- <i>d</i> <sub>3</sub>	346.2→ <b>248.1</b> , 302.0	-50	28, 38	5.46	

DP: declustering potential, CE: collision energy,  $R_T$  retention time. <sup>a</sup> Quantifier transitions are highlighted in bold characters, <sup>b</sup> Qualifier transitions are used for confirmation purposes.

*Table 2. Rat whole blood concentration of cannabinoids and cannabinoid glucuronide in the control group (ctrl), after 3 hours and 6 hours of CBD administration. Values are reported in ng/mL as mean ± standard error (n = 5).*

		CBD	THC	11-OH-THC	THCCOOH	THCCOOH-gluc
Ctrl	Oil	<LOD	<LOD	<LOD	<LOD	<LOD
	EtOH	<LOD	<LOD	<LOD	<LOD	<LOD
3 h	Oil	345.64±71.52	<LOD	<LOD	<LOD	<LOD
	EtOH	66.66±25.89	<LOD	<LOD	<LOD	<LOD
6 h	Oil	307.03±28.31	<LOD	<LOD	<LOD	<LOD
	EtOH	8.37±2.03	<LOD	<LOD	<LOD	<LOD