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Method Optimization for the Determination of Cannabinoids in Blood by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

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Method Optimization for the Determination of Cannabinoids in Blood by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

A thesis presented in partial fulfillment of the requirements for the degree of

Master of Sciences in Forensic Science.

John Jay College of Criminal Justice

City University of New York

Kevin D. Carrera

August 2022

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A thesis has been presented to and accepted by the Office of Graduate Studies, John Jay College of Criminal Justice in partial fulfillment of the requirements for the degree of Master of Sciences in Forensic Science.

John Jay College of Criminal Justice

City University of New York

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Abstract

Delta-9-tetrahydrocannabinol (THC) continues to be one of the most popular drugs in the USA. Along with THC, other cannabinoids such as cannabidiol (CBD), are on the rise due to an increase in medicinal usage as well as the passage of different legislations removing these products from their current schedule I status. Thus, an urgency exists to develop robust and sensitive analytical methods to determine cannabinoids, especially THC, CBD and metabolites, in biological samples. The purpose of this study was to investigate different analytical procedures to determine the best method to identify and quantify CBD, THC and their metabolites in whole blood by liquid chromatography tandem mass spectrometry (LC-MS/MS). In LC-MS/MS, we found that most cannabinoids worked better in electrospray (ESI) under negative mode except THC, which showed higher intensity under positive mode. Regarding extraction procedures, we optimized four different methods including liquid-liquid extraction (LLE), supported-liquid extraction (SLE), solid phase extraction (SPE) and QuEChERS. The optimal LLE employed hexane:ethyl acetate (70:30) as extraction solvent; the optimal SLE eluted the samples with dichloromethane and hexane; and the optimal SPE procedure employed mixed mode cation exchange cartridges. The procedure that yielded the best process efficiency was LLE (28.5-121.2%), and the poorest was QuEChERS (7.3-27.5%). Although the evaluated methods showed different matrix effect, extraction efficiency and process efficiency, all of them were able to achieve low limits of quantification (LOQs) between 0.5 and 5 ng/mL. Compared to currently published literature, the present methods were more sensitive in identifying THC-COOH and CBD metabolites in blood with LOQs of 0.5 ng/mL.

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1. Introduction

The cannabis plant contains over 60 cannabinoids, the major ones being delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Atakan, 2012). However, the plant does not directly synthesize these cannabinoids. Instead, it produces cannabinoid acids, delta-9-tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), that are decarboxylated to THC and CBD by heat. THC and CBD have close related chemical structures (Fig. 1 and 2) but produce vastly different pharmacological effects. THC is a psychoactive compound, which produces, in a dose-dependent manner, hypoactivity, hypothermia, and spatial and verbal short-term memory impairment (Hayakawa et al., 2008). CBD does not produce these effects, and several studies indicate that CBD has anxiolytic properties (Crippa et al. 2011) as well as anti-inflammatory, anti-psychotic, and antioxidant effects (Malaca et al., 2021; Ujvary & Hansun, 2016; Taylor et al., 2018; Moorthy et al., 2019). However, there are adverse effects that CBD causes if taken at high dosages, such as greater than 1500 mg per day (Iffland & Grotenhermen, 2017). If one takes CBD at this dosage, it has been shown to cause diarrhea, weight loss, and lethargy. Additionally, taking high dosages of CBD may increase the risk of liver damage (Cerne, 2020).

Cannabis (THC) is the most commonly used Schedule I controlled substance in the United States. According to the Substance Abuse and Mental Health Services Administration (SAMHSA), 48.2 million people have used it at least once in 2019 (Substance Abuse and Mental Health Services Administration, 2019). Medicinal use of cannabis is legal in 37 states and the District of Columbia (D.C.), and its recreational use has been legalized in 19 states and D.C.

With the passing of the farm bill of 2018, the United States removed hemp derived products (contain less than 0.3% THC), such as CBD products, from Schedule I status drugs

under the Controlled Substance Act (Marcoux, Holmes & Vogenberg, 2019). This has led to the rampant rise of THC alternative products, most notably products containing CBD. CBD is commonly encountered in different types of products. These products claim to have anti-anxiety, and anti-inflammatory properties. In a 2020 survey performed by SingleCare®, 33% of Americans have used some form of CBD. Recently, a CBD drug named Epidolex was approved by the FDA to treat epilepsy (Malaca et al., 2021).

Both THC and CBD are lipophilic substances that accumulate in fatty tissue. In the liver, THC undergoes phase I metabolism resulting in the formation of 11-hydroxy-THC (THC-OH) via hydroxylation. THC-OH undergoes further oxidation resulting in the formation of 11-Nor-9-carboxy (THC-COOH). See Fig. 1. THC-COOH is not pharmacologically active, and it is the major metabolite; THC-OH is a minor metabolite but is pharmacologically active (Jamwal et al., 2017). In a similar manner, CBD undergoes phase I metabolism in the liver resulting in the formation of different hydroxylated metabolites. The predominant one is 7-hydroxy-CBD (7-OH-CBD), which undergoes further oxidation resulting in the formation of 7-carboxy-CBD (7-COOH-CBD) (Sempio et al., 2022). Similar in nature to THC metabolites, CBD-COOH is not pharmacologically active and it’s the major metabolite, while CBD-OH is active and a minor metabolite (Fig. 2).

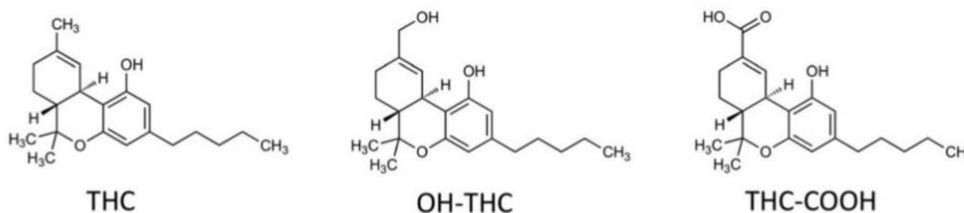

Figure 1. Chemical structures of THC and its metabolites (THC-COOH and THC-OH)

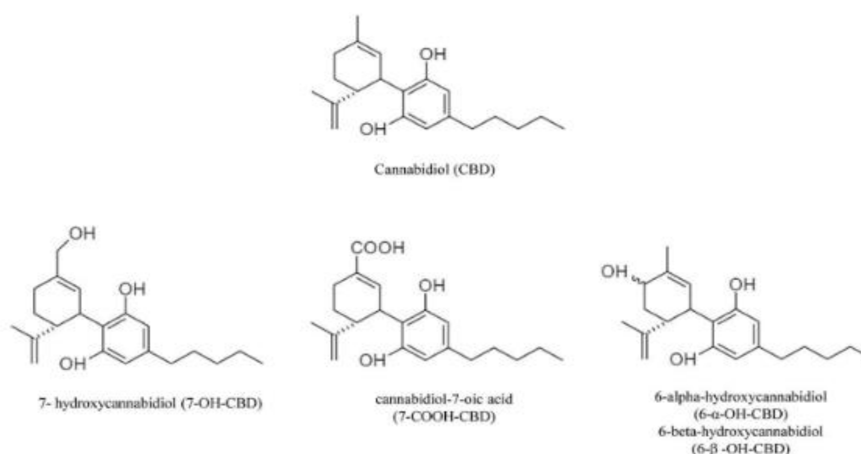

Figure 2. Chemical structure of CBD and its hydroxylated and carboxylic metabolites (Pichini et al., 2021).

In forensic toxicology, blood is the biological matrix of reference. Drug concentrations in blood correlate with the degree of impairment and intoxication, and therefore are critical for the interpretation of the analytical results. Blood is a complex matrix which contains phospholipids, blood cells, cholesterol, proteins, and clotting factors, among others. Because of this, it is necessary to clean up the sample using different techniques before performing instrumental analysis. There are multiple extraction techniques or clean up methods that are used in forensic toxicology. Jones et al. (2022) recently reviewed the most common extraction procedures, including liquid-liquid extraction (LLE), supported liquid extraction (SLE) and solid phase extraction (SPE). All of them have been employed for the extraction of THC and metabolites from blood; however, publications dealing with the extraction of CBD metabolites are scarce. THC and its metabolites have been extracted from blood using LLE (del Mar Ramirez Fernandez., 2008; Tiscione et al., 2016), SLE (Kevin et al., 2020), QuEChERS (Dybowski & Dawidowicz, 2018), and mostly SPE (Koenig et al.,2011; Sorensen & Hasselstrom, 2017; Scheidweiler et al., 2016; Schwope et al., 2011; Frei et al. 2022; Teixeira et al., 2007; Sempio et

al., 2022). Among SPE procedures, the most common cartridges used were reversed phase cartridges (Koenig et al., 2011; Schwope et al., 2011; Ferreiros et al., 2013; Frei et al., 2022; Teixeira et al., 2007). CBD has been extracted from blood using LLE (Palazzoli et al., 2018) and SPE (Schwope et al., 2011; Sorensen & Hasselstrom, 2017; Scheidweiler et al., 2016; Frei et al., 2022; Sempio et al., 2022). CBD metabolites have been extracted from blood using LLE (Malaca et al., 2021; Pichini et al., 2021), SLE (Kevin et al., 2020), and SPE (Sempio et al., 2022). Among these publications, only two of them developed a method that analyzed THC, CBD, and their metabolites together (Kevin et al., 2020; Sempio et al., 2021).

Gas chromatography mass spectrometry (GC-MS) has been the most popular instrument used to identify and quantify cannabinoids in different types of biological matrices, including blood. However, with the development and expansion of liquid chromatography tandem mass spectrometry (LC-MS/MS), researchers have been increasingly employing this technique (Ferreiros et al., 2013; da Silva et al., 2020; Sempio et al., 2022; Sanchez-Gonzalez et al., 2016; Dzaidosz et al., 2017; Koenig et al., 2011; del Mar Ramirez Fernandez et al., 2008; Haedener et al., 2016; Palazzoli et al., 2018; Scheidweiler et al., 2016; Sorrensen & Hasselstrom, 2017; Kim et al., 2017; Taylor et al., 2018, Teixeira et al., 2007; Tiscione et al., 2016; Schwope et al., 2011; Kevin et al., 2021; Pichini et al., 2021; Malaca et al., 2021) over GC-MS (Frei et al., 2022; Dybowski & Dawidowicz, 2018; Gray et al., 2010, Purschke et al., 2016). The problem that arose with using the GC-MS when working with cannabinoids is that it required a derivatization step. This would increase the time of analysis and would make the procedure more complex leaving a possibility of error (Sorensen & Hasselstrom, 2017). The benefit of using LC-MS/MS is that no derivatization step is necessary, therefore resulting in direct detection of cannabinoids and reducing the time of analysis.

Although LC-MS/MS offers several advantages, it also poses different challenges. Studies have shown that electrospray ionization (ESI) of cannabinoids such as THC in LC-MSMS may result in signal suppression due to coeluting blood phospholipids (Sorensen & Hasselstrom, 2017). Thus, it is imperative that a protein precipitation and other extraction procedures are conducted to cleanup blood samples prior to instrumental analysis by LC-MS/MS. THC, CBD, and their metabolites are lipophilic acidic compounds that may work under both positive and negative mode in ESI. Much of the research that has been conducted in analyzing cannabinoids in blood by ESI-LC-MS/MS has been done in positive ionization mode (Frei et al., 2022; Sorensen & Hasselstrom, 2017; Tiscione et al., 2016; Haedener et al., 2016; del Mar Ramirez Fernandez, 2008; Dybowski & Dawidowicz, 2018; Koenig et al., 2011; Sempio et al., 2022; Jamwal et al., 2017; Ferreiros et al., 2013; Kevin et al., 2021). However, research has shown that it may be better to analyze metabolites of cannabinoids and CBD in negative ionization mode (Scheidweiler et al., 2016; Dziadosz et al., 2017; Malaca et al., 2021; Jensen et al., 2019). Using this information, some publications have chosen to use both positive modes, to detect the parent drug (THC), and negative mode, to detect THC metabolites, CBD and its metabolites (Teixeira et al., 2007; Schwope et al., 2011; Palazzoli et al., 2018; da Silva et al., 2020).

The purpose of the present study was to investigate different analytical procedures and determine which of these would be the best method to determine THC, CBD, and their metabolites in whole blood samples by LC-MS/MS.

2. Materials and Methods

2.1 Reagents and Supplies

The following reference materials were purchased from Cerilliant (Round Rock, TX) at a concentration of 100 µg/mL or 1 mg/mL in methanol: 7-hydroxy-cannabidiol (CBD-OH), 7-carboxy-cannabidiol (CBD-COOH), delta-9-tetrahydrocannabinol (THC), 11-hydroxy-delta-9-tetrahydrocannabinol (THC-OH), 11-Nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH), cannabidiol (CBD), and cannabinol (CBN).The internal standards THC-d_3, THC-COOH-d_3, CBD-d_3, THC-OH-d_3, and CBN-d_3 were also purchased from Cerilliant at 100 µg/mL in methanol.

Glacial acetic acid, acetonitrile, ammonium hydroxide, dichloromethane, ethyl acetate, formic acid, hexane, hydrochloric acid, isopropanol, methanol, methyl-tert-butyl ether (MTBE), and sodium acetate trihydrate were purchased from Fisher Scientific (Fair Lawn, NJ). Strata X-C 60 mg/3 mL polymeric strong cation exchange solid phase extraction (SPE) cartridges were purchased from Phenomenex (Torrance, CA). Styre Screen THC 60 mg/3 mL and Clean Screen THC 200 mg/3 mL SPE cartridges were purchased from United Chemical Technologies (UCT, Bristol, PA). Novum supported liquid extraction (SLE) 3 mL cartridges were purchased from Phenomenex. Isolute supported liquid extraction (SLE+) 1 mL cartridges were purchased from Biotage (Charlotte, NC). Fifteen-mL QuEChERS 800 mg $MgSO_4$/200 mg sodium chloride tubes and 1-mL dSPE (dispersive SPE) CEC18 tubes were purchased from UCT. The SPE and SLE cartridges were mounted on a 24-port vacuum manifold from Fisher Scientific. 10-mL Sarstedt tubes and 350-µL fused insert LC-MS/MS vials were purchased from Fisher Scientific as well.

2.2. Blood Samples

Pooled sheep blood samples were purchased from Carolina Biological Supplies Company (Burlington, NC) and tested for the absence of drugs. The blood samples were stored at 4°C, and they were used in preparing calibrators and quality control (QC) samples.

2.3 Preparation of Working Solutions

Working solutions containing all 7 analytes were prepared at concentrations of 1 µg/mL, 0.1 µg/mL, and 0.01 µg/mL in methanol. Working solutions containing 5 internal standards were prepared at 0.1 µg/mL in methanol. All working solutions were stored in amber vials at -20°C.

2.4 Preparation of Calibrators and Quality Control Samples

Five hundred µL of blood was transferred to a clean Sarstedt tube. The blood was spiked with the corresponding working solutions shown in Table 1 to yield the concentrations of 0.5, 1, 5, 10, 50, and 100 ng/mL. Each tube was spiked with 100 µL of internal standard mixture at 0.1 µg/mL. The tubes were capped and vortexed for 30 s. Further sample preparation was dependent on the type of extraction preformed on the samples.

Table 1. Volume of working solution at 1, 0.1 or 0.01 µg/mL needed for each calibrator using 500 µL of blood.

Calibrators (ng/mL)	**1 µg/mL**	**0.1 µg/mL**	**0.01 µg/mL**
100	50 µL	-	-
50	25 µL	-	-
10	-	50 µL	-
5	-	25 µL	-
1	-	-	50 µL
0.5	-	-	25 µL

2.5 Sample Extraction Procedures

2.5.1 Liquid-Liquid Extraction

Five hundred µL of deionized H_2O (diH_2O) was added to the blood samples, followed by the addition of 200 µL of 10% acetic acid in water. The tubes were vortexed for 30 s. After vortexing, 4 mL of a mixture of hexane: ethyl acetate (70:30) was added and the tubes were shaken by mechanical shaker for 30 min. Following shaking, the tubes were centrifuged for 10 min at 10°C at 7,830 rpm. The supernatant was collected and dried under nitrogen in a Biotage Turbovap LV at 50°C for approximately 20 min. Dried extracts were reconstituted in 200 µL of mobile phase A:B (50:50) solution. Mobile phase A consisted of 5 mM ammonium formate and mobile phase B consisted of acetonitrile. The solutions were transferred to insert vials and 20 µL were injected into the LC-MS/MS.

2.5.2 Supported-Liquid Extraction

Five hundred µL of 1% formic acid in water was added to the blood samples, the tubes were vortexed for 30 s, and centrifuged for 10 min at 10°C at 7,830 rpm. The supernatant was loaded by gravity to the SLE cartridges. After waiting 5 min, the elution was performed by 2 x 900 µL of dichloromethane and 2 x 900 µL of hexane. The eluents were evaporated at 50°C under nitrogen stream for about 20 min using the Biotage Turbovap LV. Dried extracts were reconstituted using 200 µL of mobile phase A:B (50:50) solution. Following reconstitution, tubes were centrifuged for 10 min at 10°C at 7,830 rpm. The supernatants were transferred to insert vials and 20 µL were injected into the LC-MS/MS.

2.5.3 Solid Phase Extraction

Blood samples were first submitted to protein precipitation. Cold acetonitrile (1 mL) was added dropwise to 0.5 mL of blood while vortexing. After centrifugation, the supernatant was

transferred to a clean Sarstedt tube and evaporated to dryness under nitrogen. The extract was reconstituted in 3 mL of 1% formic acid in diH_2O:acetonitrile (70:30). The Strata X-C cartridges (mixed mode cation exchange) were conditioned using 2 mL methanol, followed by 2 mL diH_2O. Following this, the sample was loaded onto the cartridge. The cartridge was washed with 2 mL 1% formic acid in diH_2O, followed by 2 mL 1% formic acid in diH_2O:methanol, 45:55. After drying for 10 min under vacuum, the elution was performed using 3 mL solution dichloromethane: isopropanol (50:50). Eluents were evaporated and reconstituted in 200 µL of mobile phase A:B (50:50). Twenty µL were injected into the LC-MS/MS.

2.5.4 QuEChERS

A volume of 1.5 mL of diH_2O was added to the blood samples and vortex in a Sarstedt tube. Two mL of acetonitrile was added to 15-mL QuEChERS tubes. The samples were transferred to the QuEChERS tubes and they were vortexed for 1 min, followed by shaking for 5 min. The tubes were centrifuged for 10 min at 10°C at 7,830 rpm. After centrifugation, 1 mL of supernatant was transferred to dSPE tubes. These tubes were vortexed for 1 min and centrifuged at 12,000 rcf for 5 min. The supernatant was transferred to clean Sardtedt tube and evaporated in the Turbovap at 50°C. Following reconstitution, the extract was centrifuged for 10 min. The supernatant was transferred to insert vials and 20 µL was injected into the LC-MS/MS.

2.5.5 Extraction Procedure Optimization

Different solvents, volumes and type of cartridges were evaluated in the development of the LLE, SLE and SPE procedures. For each attempt, triplicates of blood samples fortified at 10 and 100 ng/mL were evaluated and compared to neat samples to estimate the process efficiency. The procedure that yielded the best process efficiencies was further investigated.

In LLE, hexane: ethyl acetate was chosen as the solvent mixture based on previous research involving THC and its metabolites in blood (del Mar Ramirez Fernandez, 2008). The hexane: ethyl acetate ratios 90:10, 70:30, and 50:50, were compared using the procedure described in section 2.5.1.

In SLE, two different types of cartridges, Novum SLE 3cc cartridges and Biotage Isolute SLE+ cartridges, were investigated. Regarding elution solvents, different solvents and combinations were compared to determine which one yielded the best process efficiency. Elution solvents that were evaluated were hexane, MTBE, ethyl acetate, and their combinations hexane:ethyl acetate (3:1), hexane:ethyl acetate (1:3), hexane:ethyl acetate (1:1), hexane:MTBE (1:3), and hexane:MTBE (1:1). Elution volumes of 900 µL and 2x900 µL were also investigated. Regarding loading conditions, blood samples previously treated with 1% formic acid, acetonitrile or methanol were evaluated. The loading volume of the sample onto the SLE cartridges was tested as well to determine if the volume added to the cartridge influenced the results. Sample volumes of 0.5, 0.6, 0.7 and 0.8 mL of supernatant were loaded onto SLE cartridges.

In SPE, three different SPE cartridges, Styre Screen (anion exchange), Strata X-C (mixed mode cation exchange), and Clean Screen THC (mixed mode anion exchange) were evaluated to determine which provided the optimal results. Due to the different retention mechanism of the cartridges, the same procedure could not be conducted on all of them (anion exchange, cation exchange and reversed phase). Instead, procedures recommended by the UCT manufacturer (Styre Screen and Clean Screen THC) or from already established literature (Strata X-C mixed mode cation exchange) (Concheiro et al., 2021) were tested, and the process efficiency of all three cartridges were compared to determine the best cartridge to use. Using the Styre Screen cartridges (anion exchange), no preconditioning of the cartridges occurred. Fortified blood samples were

submitted for protein precipitation. Cold acetonitrile (1 mL) was added dropwise to 0.5 mL of blood. After centrifugation the supernatant was transferred to a clean Sarstedt tube and evaporated under nitrogen gas to dryness. The extract was reconstituted in 200 µL of acetonitrile followed by 2 mL of deionized water. The extract was loaded onto the cartridge and eluted out via gravity. The cartridge was washed with 2 mL diH_2O:acetonitrile:ammonium hydroxide (84:15:1) and dried for 15 min under vacuum. Collection tubes were placed inside the manifold and a 3 mL solution of hexane:ethyl acetate:glacial acetic acid (49:49:2) was employed as elution solvent.

Utilizing the Clean Screen THC (mixed mode anion exchange cartridges), the cartridges were conditioned using 3 mL methanol, 3 mL diH_2O, and 1 mL of 100 mM sodium acetate buffer (pH 3). Fortified blood samples were submitted for protein precipitation. Cold acetonitrile (1 mL) was added drop wise to 0.5 mL of blood. After centrifugation the supernatant was transferred to a clean Sarstedt tube and evaporated to dryness. The extract was reconstituted using 200 µL of acetonitrile and 2 mL 100 mM sodium acetate buffer (pH 3). The sample was loaded onto the cartridge and washed with 2 mL diH_2O, followed by 2 mL 100 mM HCl: acetonitrile (95:5). The cartridges were dried for 15 min under vacuum. Collection tubes were placed inside the manifold and 2 mL hexane followed by 3 mL ethyl acetate: hexane (50:50) were used for elution.

2.6 Instrumentation

Samples were analyzed on an LC-MS/MS 8050 triple quadrupole liquid chromatography tandem mass spectrometer instrument from Shimadzu (Columbia, MD). The Nexera high-performance liquid chromatography (HPLC) system consisted of a binary LC-20AD XR pump, an online degassing unit (DGU-20A 3R), a cooled autosampler (SIL-20A XR) and a column oven (CTO-20AC). Two different chromatographic separations were performed depending on the method was run in positive or negative mode. In positive mode, a Kinetex F5, 2.6 µm, 2.1 x 100

mm, 100 Å pore size chromatographic column from Phenomenex was employed. Mobile phase A was composed of 0.1% formic acid in water and mobile phase B was composed of 0.1% formic acid in acetonitrile, with a flow rate of 0.5 mL/min. The column oven temperature was 40°C. The gradient used was previously described by Kim et al. (2018) and was the following: initial composition of 30% B increased to 78.5% over 8.5 min, then increased to 98% over 0.2 min, held for 3 min, and then returned to initial composition over 0.2 min and held for 2.1 min for a total run time of 14 min. In negative mode, a Kinetex C18, 2.6 µm particle size, 2.1 x 100 mm, 100 Å pore size, chromatographic column from Phenomenex was utilized. Mobile Phase A was composed of 5 mM ammonium formate in water, and Mobile Phase B was composed of acetonitrile. The flow rate was 0.4 mL/min and the column oven operated at 50°C. The method was in gradient mode. The initial composition 5% B was held for 0.25 min, then increased to 100% at 5.3 min, held for 0.7 min, and returned to initial conditions in 0.5 min. The equilibration time was 1.5 min, and the total run time was 8 min.

The triple quadrupole mass spectrometer operated with an electrospray ionization (ESI) source in positive or in negative mode. The following parameters were employed for the source in both modes: drying gas flow at 5 L/min, nebulizing gas flow at 2 L/min, heating gas flow at 15 L/min, desolvation line temperature at 250°C, interface temperature at 300°C, and heating block temperature at 400°C. An optimization procedure was conducted to identify the correct multiple reaction monitoring (MRM) transitions needed for each analyte in positive and in negative mode. Each analyte was injected into the mass spectrometer at 0.1 µg/mL without using a column. A list of various fragment ions was obtained at different intensities. From this list, the two most intense transitions for each analyte were selected. Based on these two MRM transitions, Q1 voltage, Q3

voltage and collision energy (CE) values were collected as depicted in Table 2 (positive mode) and Table 3 (negative mode).

Table 2. Molecular weight (MW), MRM transitions, Q1 and Q3 voltages and collision energy (CE) for each analyte and deuterated standard in positive mode.

Analyte	MW (g/mol)	MRM Transition Quantifier	Q1 (V)	CE (V)	Q3 (V)	MRM Transition Qualifier	Q1 (V)	CE (V)	Q3 (V)
CBD-COOH	344.4	345.1>299.3	-24	-20	-20	345.1>193.1	-10	-28	-12
CBD-OH	330.4	332.0>193.3	-12	-25	-20	332.0>202.3	-17	-24	-23
THC-COOH	344.4	345.0>327.3	-23	-18	-21	345.0>299.3	-17	-21	-30
THC-OH	330.5	331.0>193.3	-30	-25	-29	331.0>200.9	-22	-25	-20
CBD	314.5	315.2>193.2	-30	-23	-28	315.2>123.0	-11	-30	-21
CBN	310.4	311.2>194.9	-24	-30	-21	311.2>222.9	-14	-23	-23
THC	314.4	315.2>193.0	-24	-26	-19	315.2>123.3	-20	-34	-20
CBD-d_3	317.4	318.2>196.2	-12	-24	-13	318.2>123.2	-16	-37	-24
CBN-d_3	313.4	314.1>223.0	-13	-23	-10	314.1>208.1	-25	-34	-13
THC-d_3	317.4	318.2>196.1	-11	-21	-20	318.2>123.1	-22	-37	-12
THC-COOH-d_3	347.4	348.0>330.3	-23	-18	-11	348.0>302.3	-30	-20	-20
THC-OH-d_3	333.4	334.0>196.1	-13	-28	-21	334.0>201.2	-25	-23	-29

Table 3. Molecular weight (MW), MRM transitions, Q1 and Q3 voltages and collision energy (CE) for each analyte and deuterated standard in negative mode.

Analyte	MW (g/mol)	MRM Transition Quantifier	Q1 (V)	CE (V)	Q3 (V)	MRM Transition Qualifier	Q1 (V)	CE (V)	Q3 (V)
CBD-COOH	344.4	342.9>231.2	16	26	10	342.9>179.2	22	21	30
CBD-OH	330.4	329.2>261.2	11	24	12	329.2>245.2	15	25	12
THC-COOH	344.4	343.2>245.2	12	30	11	343.2>191.1	23	31	30
THC-OH	330.5	329.2>173.2	22	32	17	329.2>267.2	23	35	19
CBD	314.5	313.5>245.2	11	24	11	313.5>107.2	21	33	10
CBN	310.4	308.9>279.2	10	33	12	308.9>222.1	15	47	10
THC	314.4	313.0>245.0	19	27	12	313.0>191.3	30	29	22
CBD-d_3	317.4	316.2>248.2	15	24	12	316.2>107.1	15	33	11
CBN-d_3	313.4	312.0>282.2	11	33	13	313.0>222.1	15	45	10

THC-d_3	317.4	316.0>248.1	21	30	14	316.0>194.3	15	30	20
THC-COOH-d_3	347.4	346.1>248.2	17	29	11	346.1>194.2	24	33	30
THC-OH-d_3	333.4	332.2>271.1	22	29	18	332.2>173.2	23	31	21

2.7 Sample Preparation Comparison and Evaluation

In this study, different sample preparation methods for the determination of cannabinoids in whole blood were optimized and compared in terms of extraction efficiency, process efficiency, matrix effect and sensitivity. During the method optimization, different procedures using a certain technique (LLE, SLE or SPE) were compared in terms of process efficiency at 10 and 100 ng/mL (n=3). Once the best procedure was selected for each technique, the different extraction procedures were compared in terms of sensitivity (estimated limit of quantification, LOQ) and extraction efficiency, process efficiency, and matrix effect at 10 and 100 ng/mL (n=9).

To investigate the extraction efficiency, process efficiency and matrix effect, three different sets of samples were prepared: before, after and neats. The before blood samples were fortified with the standard and internal standard prior to the extraction procedure. The after blood samples underwent the extraction procedure and were fortified with the standard and internal standard just before the evaporation step. The neats were prepared adding 100 µL of the internal standard working solution at 0.1 µg/mL and 50 µL of the standard working solution (1 µg/mL for 100 ng/mL and 0.1 µg/mL for 10 ng/mL) to a clean Sarstedt tube, evaporated and reconstituted in the initial conditions of the mobile phase.

To determine the extraction efficiency, mean peak areas from the before samples were compared to mean peak areas from the after samples. To determine matrix effect, mean peak areas obtained from the neat samples and after samples were compared. Finally, process efficiency was

determined by comparing mean peak areas obtained from the neat samples to the before samples. The formulas that were utilized to determine extraction efficiency, matrix effect and process efficiency are summarized in Table 4. Based on the ASB guidelines (SWGTOX, 2019), for the matrix effect to be accepted, the coefficient of variation (CV) among different sources must be less than 20% and the ionization suppression/enhancement must be less than 25%. The ASB guidelines do not make any recommendation for extraction efficiency neither for process efficiency values.

Table 4. Formulas utilized to determine matrix effect, extraction efficiency and process efficiency.

Parameter (%)	Formula
Matrix Effect	(Mean Peak Area$_{After}$ – Mean Peak Area$_{Neat}$ / Mean Peak Area$_{Neat}$) x 100
Extraction Efficiency	(Mean Peak Area$_{Before}$ / Mean Peak Area$_{After}$) x 100
Process Efficiency	(Mean Peak Area$_{Before}$ / Mean Peak Area$_{Neat}$) x 100

The sensitivity of the different procedures was evaluated through a calibration curve with calibrators at 0.5, 1, 5, 10, 50, and 100 ng/mL. The possible limit of quantification (LOQ) was determined as the lowest concentration that could be quantified within ±20% bias, and that showed both MRM transitions with a S/N > 10.

3. Results and Discussion

3.1 Ionization Mode and Chromatographic Separation

The negative mode method yielded a better signal, better peak separation and peak shape compared to the positive mode method for most of the compounds, except for THC as previously indicated (Texieria et al., 2007; Schwope et al., 2011; Palazzoli et al., 2018; da Silva et al., 2020). Therefore, the negative ionization mode method was chosen as the instrumental method

for the evaluation of the different sample preparation procedures. Fig. 3 shows a MRM chromatogram of a neat sample at 50 ng/mL in positive and in negative mode.

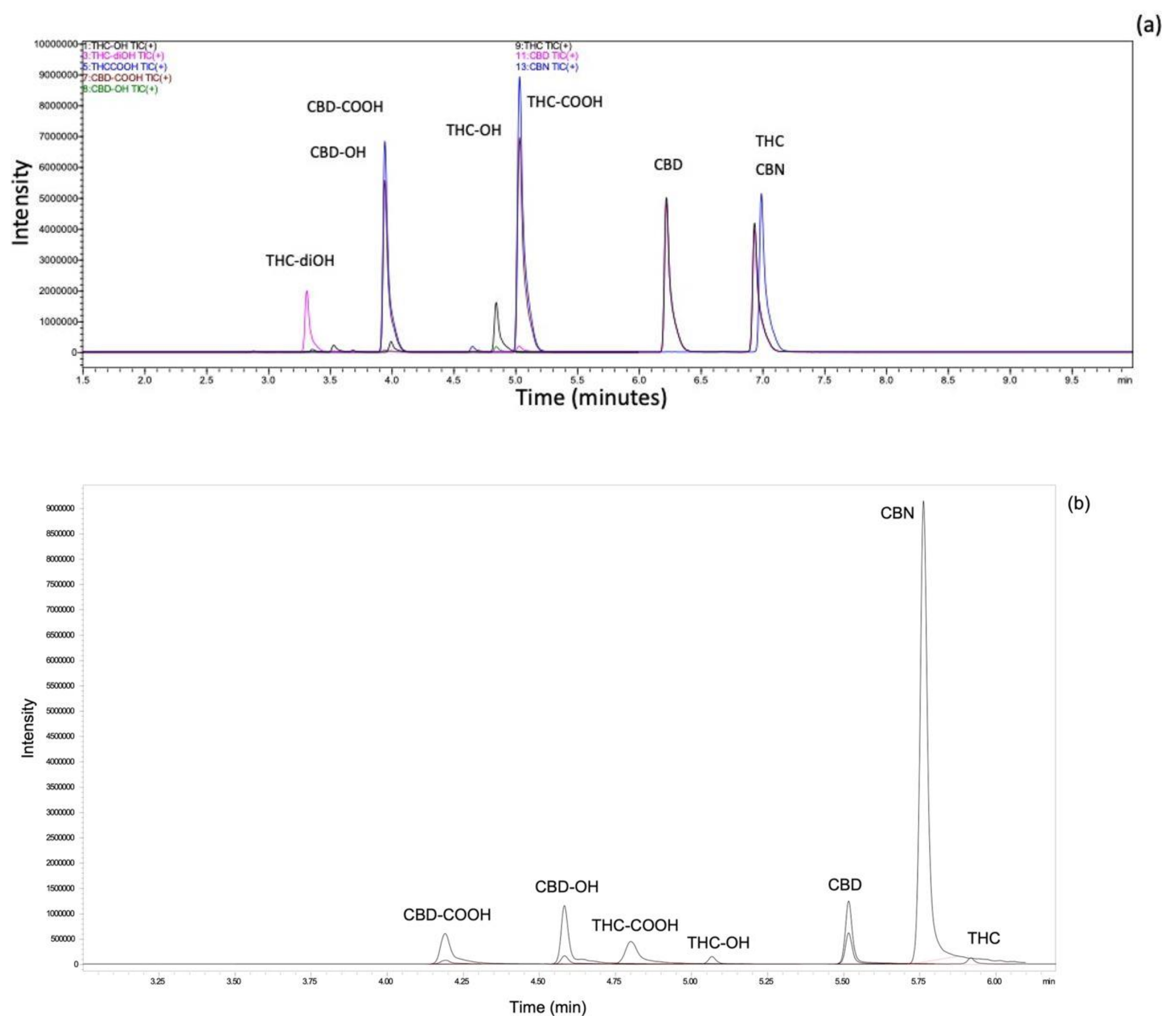

Figure 3. MRM Chromatogram for all 7 cannabinoids (a) Positive mode using MP A:B 0.1% formic acid in water: 0.1% formic acid in acetonitrile and Kinetex F5, 2.6 µm, 2.1 x 100 mm, 100 Å pore size chromatographic column from Phenomenex (b) Negative mode using MP A:B 5 mM ammonium formate: acetonitrile and Kinetex C18, 2.6 µm particle size, 2.1 x 100 mm, 100 Å pore size, chromatographic column from Phenomenex. The 7 target analytes eluted in the following order: CBD-COOH, CBD-OH, THC-COOH, THC-OH, CBD, CBN, THC.

3.2 Sample Preparation Optimization

Different LLE procedures were investigated, using different ratios of hexane:ethyl acetate as organic phases. Table 5 compares the preliminary process efficiencies obtained using the different ratios. According to previous research, hexane:ethyl acetate (90:10) provided high recovery rates for THC and its metabolites (Fernandez et al., 2008). However, our results indicated that hexane: ethyl acetate (70:30) produced better results than hexane:ethyl acetate (90:10). Thus, hexane: ethyl acetate (70:30) was chosen for all further LLE experiments.

Table 5. Comparison of preliminary process efficiencies obtained from LLE using different ratios of organic phases. Low Qc was 10 ng/mL and High QC 100 ng/mL.

Analyte	Hexane:Ethyl Acetate (90:10)		Hexane:Ethyl Acetate (70:30)		Hexane: Ethyl Acetate (50:50)	
	Low QC	High QC	Low QC	High QC	Low QC	High QC
CBD-COOH	38.4	38.4	109.3	92.3	80.1	77.9
CBD-OH	69	62.6	80.3	58.5	68.7	51.3
THC-COOH	46	42.5	74.6	59.9	68.1	59.8
THC-OH	45	50.9	56	52.9	57	45.9
CBD	75.2	75.4	68	58.7	64.8	75.6
CBN	94.2	105.3	97.2	87.9	87.8	93.7
THC	52.4	45.5	61.1	41.2	58.7	45.1

In SLE, evaluating the two different types of SLE cartridges resulted in Novum SLE 3cc cartridges being chosen to be used in all further SLE experiments. Different elution solvent mixtures were also evaluated to determine which would produce the best results. Based on the recommendations of the manufacturer (Brusius & Spurgin., 2017), hexane: MTBE (1:3) provided the best extraction recovery. However, our results indicated that dichloromethane followed by hexane produced better results, especially for CBD metabolites. Regarding the loading conditions, 1% formic acid provided the most optimal data in SLE extraction using Novum SLE 3cc cartridges. Changing the loading volumes didn't improve the results, and the

lowest volume, 0.5 mL, was chosen to be the loading volume for all further SLE experiments. It was determined that overloading the SLE cartridge with 0.8 mL of supernatant produced poor results. Table 6 compares the process efficiencies of these different procedures.

Table 6. Comparison of preliminary process efficiencies obtained from SLE using different loading conditions, elution solvents and volume. Low Qc was 10 ng/mL and High QC 100 ng/mL. ACN: acetonitrile; DCM: dichloromethane; EA: ethyl acetate; FA: formic acid; MeOH: methanol

Analyte	Loading Conditions					
	FA		MeOH		ACN	
	Low QC	High QC	Low QC	High QC	Low QC	High QC
CBD-COOH	19.6	26.3	7.1	8	15.7	17.1
CBD-OH	27.8	23.6	4.4	4	6.6	9.1
THC-COOH	24	24.4	15	13.4	23	14.3
THC-OH	21.9	23.1	5.5	5.1	7.9	6.4
CBD	14.8	14.8	0.3	0.3	0.4	0.9
CBN	20.7	20.6	0.6	0.5	0.4	1.1
THC	14.3	16.1	N/A	N/A	N/A	N/A
Analyte	Different Elution Solvents					
	EA-Hexane		Hexane-Hexane		DCM-Hexane	
	Low QC	High QC	Low QC	High QC	Low QC	High QC
CBD-COOH	16.4	15.4	0.1	N/A	17.2	15
CBD-OH	19.9	17.1	0.9	0.7	27.5	22.2
THC-COOH	20.1	15.7	N/A	N/A	22.5	19.3
THC-OH	26.7	12.3	N/A	N/A	20	15.1
CBD	12.4	15.5	32.3	31.2	23.5	29.2
CBN	18.3	16.8	50.8	36.7	38.8	35.2
THC	14.5	7.6	31.8	16.9	26	18.4
Analyte	Doubling Elution Volume					
	DCM-Hexane		DCM x2-Hexane x 2			
	Low QC	High QC	Low QC	High QC		
CBD-COOH	17.2	15	20.6	20.8		
CBD-OH	27.5	22.2	30.5	32.9		
THC-COOH	22.5	19.3	19.3	24		
THC-OH	20	15.1	24.7	20.1		
CBD	23.5	29.2	27.6	24.1		
CBN	38.8	35.2	42.6	37.3		
THC	26	18.4	21.3	16.5		

In SPE, the data indicated that mixed mode cation exchange cartridges produced the best process efficiency and were chosen for all further SPE experiments. Table 7 compares the process efficiencies of the different SPE procedures.

Table 7. Comparison of preliminary process efficiencies obtained from SPE using different cartridges (Ion exchange or reverse phase).

Analyte	Styre-Screen		Strata XC		Clean Screen THC	
	Low QC	High QC	Low QC	High QC	Low QC	High QC
CBD-COOH	32.9	31.8	224.1	107.2	40.4	32.5
CBD-OH	18.9	23.6	54.9	37	39.4	27.8
THC-COOH	41.9	42.3	47.2	38.3	40.8	35.7
THC-OH	30.7	27.9	46.3	33.6	34.2	28.7
CBD	4.7	3.6	24.2	24.7	8.6	6.3
CBN	7.7	6.9	26.4	47.4	13.3	11.3
THC	4.6	3.9	7.2	7.5	6.1	10.8

3.3 Sample Preparation Comparison

The best LLE, SLE and SPE procedures and QuEChERS methodology were compared in terms of matrix effect, extraction efficiency, process efficiency and sensitivity. Figures 4 to 9 shows the MRM chromatograms of fortified blood samples at 10 ng/mL obtained with all these procedures.

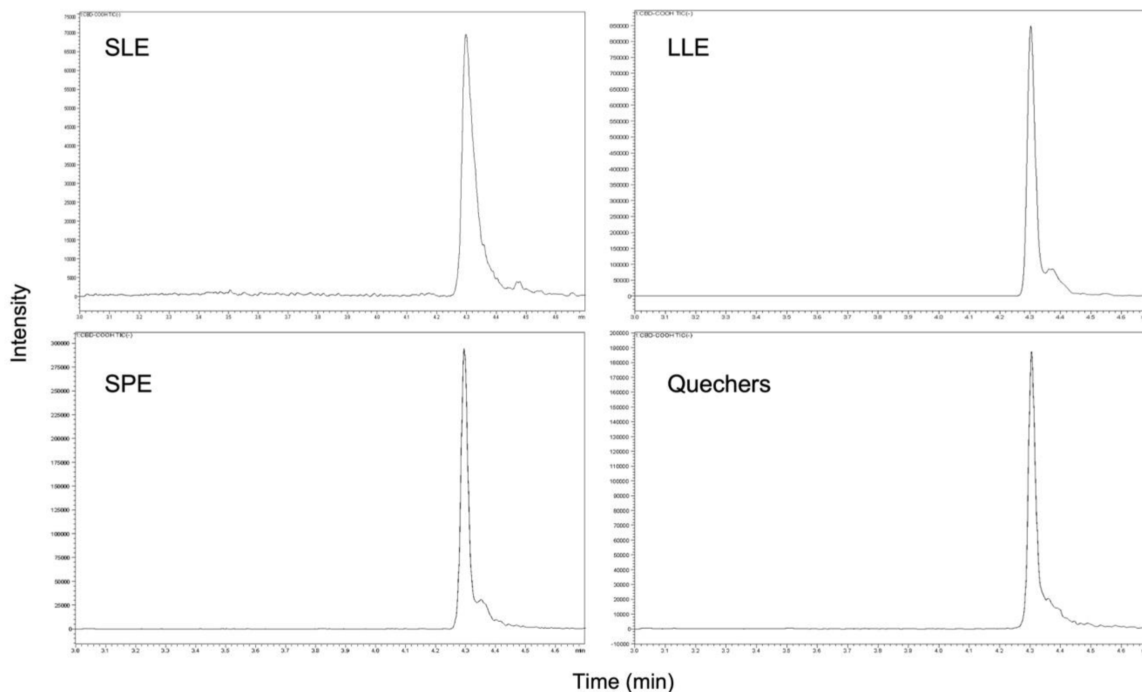

Figure 4. Total Ion MRM Chromatogram (TIC) for CBD-COOH in blood at 10 ng/mL after undergoing SLE, LLE, SPE and QuEChERS extraction procedures.

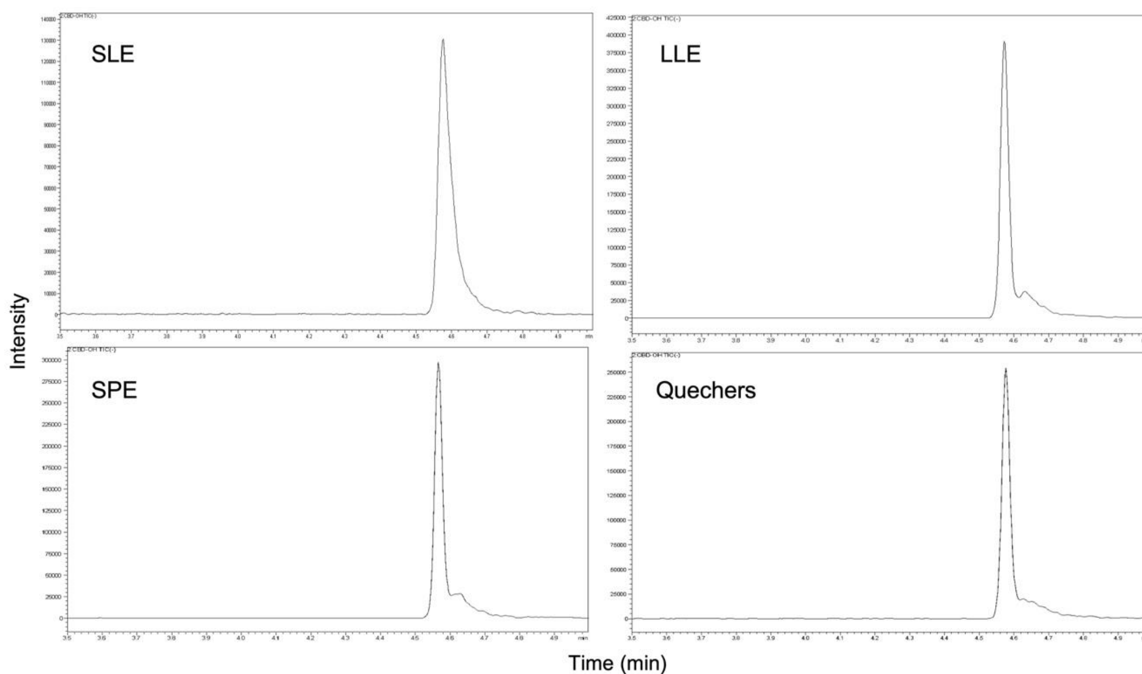

Figure 5. Total Ion MRM Chromatogram (TIC) for CBD-OH in blood at 10 ng/mL after undergoing SLE, LLE, SPE and QuEChERS extraction procedures.

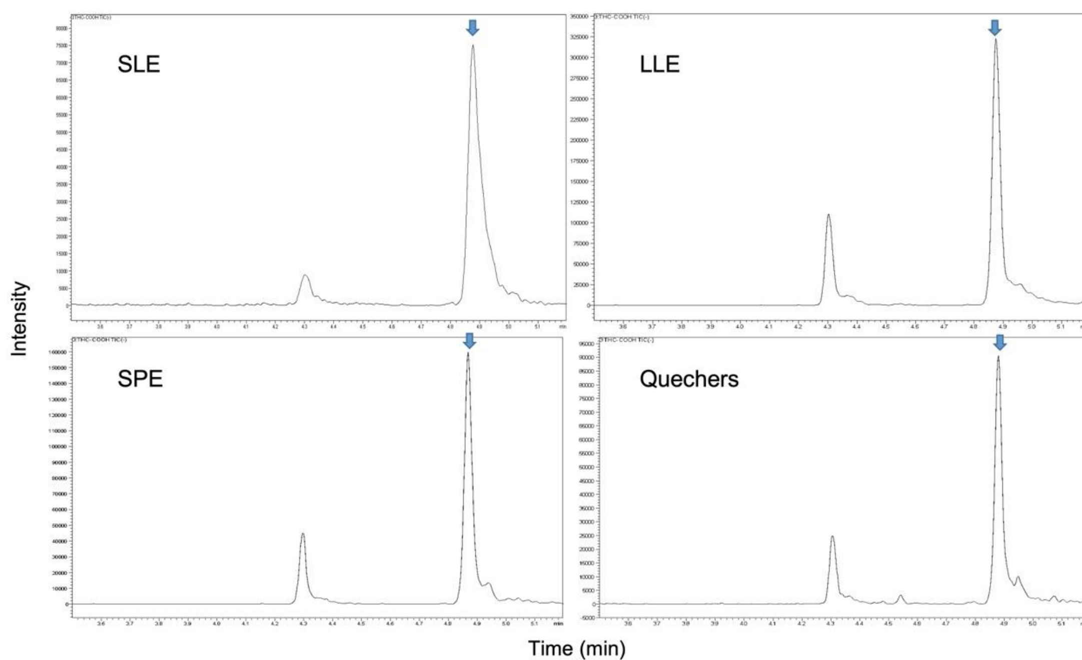

Figure 6. Total Ion MRM Chromatogram (TIC) for THC-COOH in blood at 10 ng/mL after undergoing SLE, LLE, SPE and QuEChERS extraction procedures.

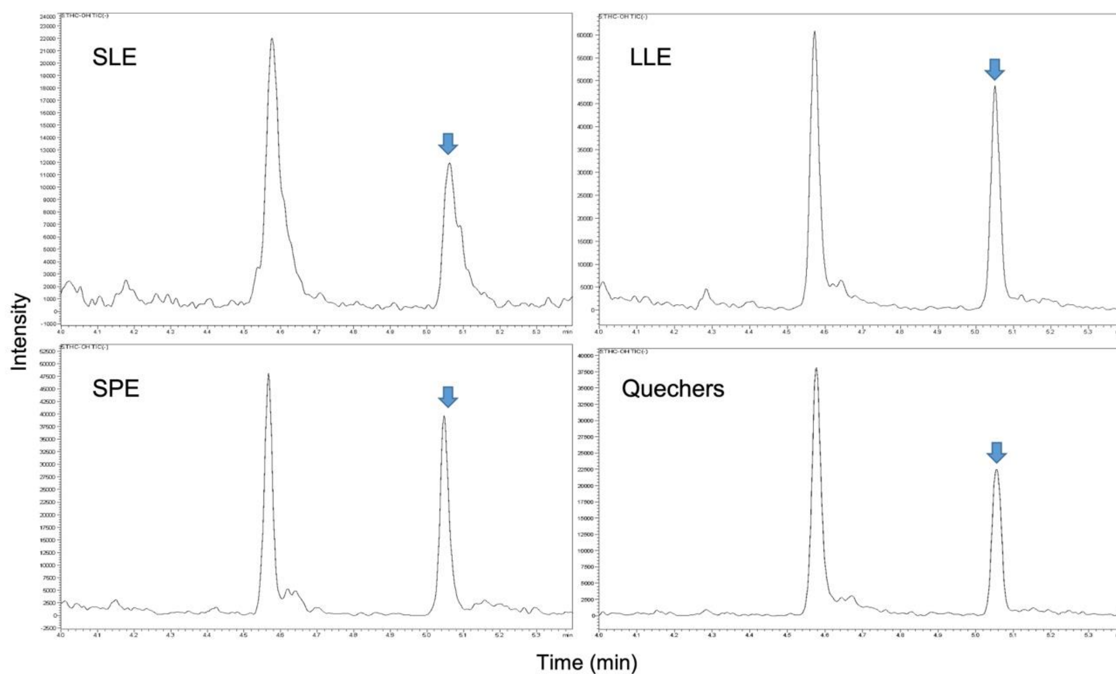

Figure 7. Total Ion MRM Chromatogram (TIC) for THC-OH in blood at 10 ng/mL after undergoing SLE, LLE, SPE and QuEChERS extraction procedures.

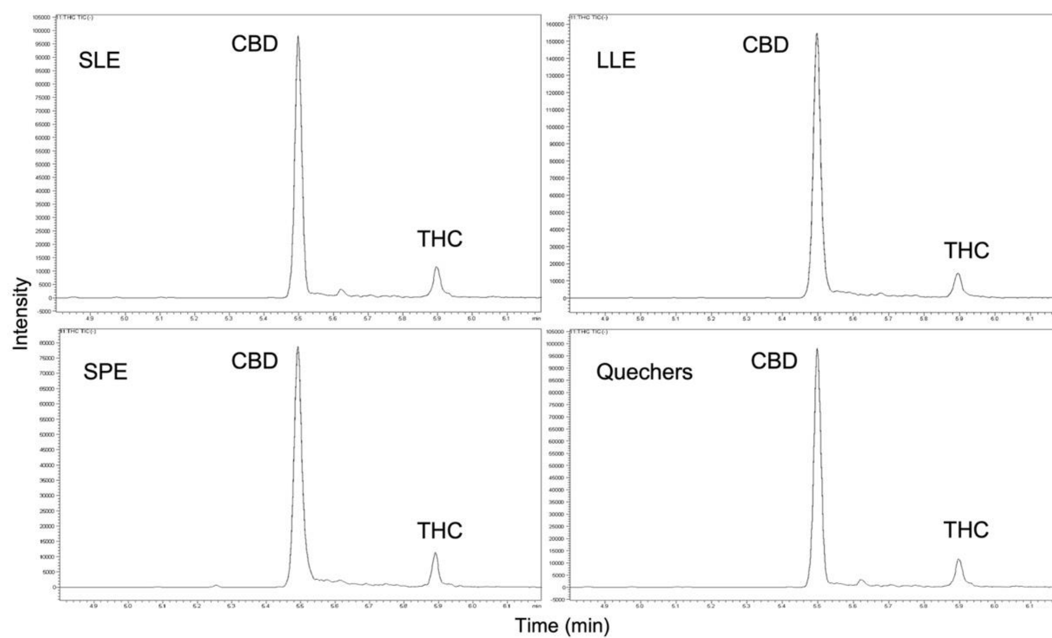

Figure 8. Total Ion MRM Chromatogram (TIC) for CBD and THC in blood at 10 ng/mL after undergoing SLE, LLE, SPE and QuEChERS extraction procedures.

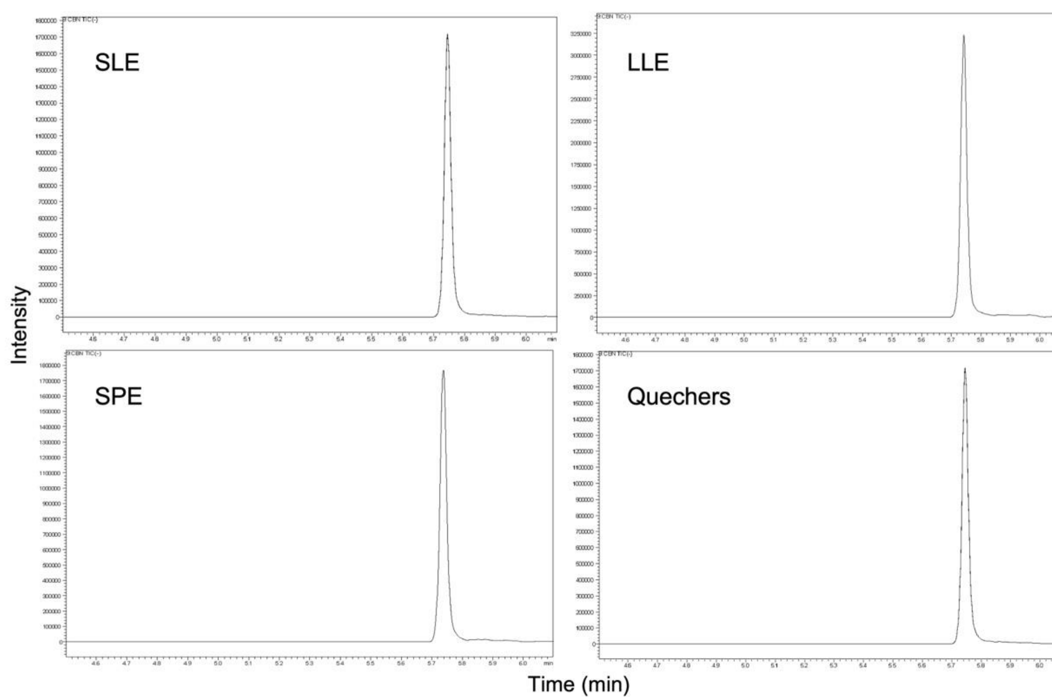

Figure 9. Total Ion MRM Chromatogram (TIC) for CBN in blood at 10 ng/mL after undergoing SLE, LLE, SPE and QuEChERS extraction procedures.

Extraction efficiency, matrix effect and process efficiency were evaluated at 10 and 100 ng/mL for the different extraction procedures (Table 8). Among the different extraction techniques, LLE yielded the best results overall for extraction efficiency (50.9-100.8%), followed by SPE (14.5-89.4%), SLE (8.4-31.6%) and QuEChERS (17.4-26.1%). Focusing on the different analytes, SPE was the extraction procedure that yielded the best extraction efficiency for CBD-COOH (86.8-89.4%), and LLE for the rest of analytes, CBD-OH (50.9-55.4%), THC-COOH (76.3-83.2%), THC-OH (67.4-69.7%), CBD (72.8-77.6%), CBN (90.6-100.8%) and THC (84.5-96%).

Table 8. Extraction efficiency results (%) for each analyte at Low QC (10 ng/mL) and Hig QC (100 ng/mL) for LLE, SLE, SPE and QuEChERS extration procedures.

Analyte	LLE		SLE		SPE		QuEChERS	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
CBD-COOH	55.3	58.6	24.5	8.4	86.8	89.4	22.0	22.8
CBD-OH	55.4	50.9	30.8	27.0	36.2	43.6	24.9	26.1
THC-COOH	83.2	76.3	29.1	26.4	66.0	70.6	20.4	23.2
THC-OH	69.7	67.4	25.5	24.4	65.1	62.8	21.4	23.4
CBD	77.6	72.8	25.8	22.4	21.6	14.5	22.1	24.7
CBN	100.8	90.6	31.6	28.3	31.6	34.8	18.8	23.0
THC	96.0	84.5	23.8	26.1	21.5	19.7	17.4	17.4

Regarding matrix effect, the summary of the results is shown in Table 9. In LLE, ion enhancement was present for CBD-COOH and CBD-OH (up to 119.3%), and ion suppression for CBD, CBN and THC (up to -66.3%). In SLE, ion enhancement was present for CBD-OH (28.9%) and ion suppression was present for CBD and THC (between -37.4 and -52.3%). In SPE

ion suppression was present for almost all analytes, CBD-OH, THC-COOH, THC-OH, CBD, THC, with values up to -65.5%. In QuEChERS, only two analytes, CBD and THC, showed ion suppression (-45 to -58 %). Most of the procedures showed a variation among the different sources (CV) less than 20%, except SPE when analyzing the parent drugs, CBD, CBN and THC.

Table 9. Matrix effect (%) and CV (%) results for each analyte at Low QC (10 ng/mL) and Hig QC (100 ng/mL) for LLE, SLE, SPE and QuEChERS extration procedures.

Analyte	LLE		SLE		SPE		QuEChERS	
	Low QC (CV)	High QC (CV)	Low QC (CV)	High QC (CV)	Low QC (CV)	High QC (CV)	Low QC (CV)	High QC (CV)
CBD-COOH	119.3 (9.4)	67.4 (7.5)	-3.4 (16)	1.5 (13.2)	16.2 (13.1)	-20.2 (15.5)	9.3 (5.5)	-16.5 (4.2)
CBD-OH	49.3 (3.5)	8.6 (5.1)	24.6 (17.1)	28.9 (16.7)	5.0 (13.9)	-30.6 (7.4)	10.1 (3.3)	-4.5 (10.2)
THC-COOH	5.2 (6.8)	-3.9 (6.3)	-7.1 (7.4)	-15.4 (2.3)	-62.1 (10.1)	-68.1 (7.2)	-12.0 (5.5)	-19.7 (5.6)
THC-OH	13.8 (10.1)	-17.9 (12.3)	2.9 (10.7)	-16.0 (10.1)	-55.7 (13.5)	-65.5 (21)	-1.7 (9.5)	-7.5 (12.8)
CBD	-14.0 (6.0)	-34.7 (7.3)	-37.4 (9.5)	-20.6 (5.0)	-74.4 (38.9)	-67.7 (25.6)	-24.7 (5.5)	-45.1 (6.0)
CBN	-22.2 (9.1)	-37.1 (9.1)	-1.9 (6.9)	18.9 (3.2)	-14.1 (22.1)	-13.9 (11)	16.7 (5.7)	-13.6 (2.5)
THC	-59.6 (9.3)	-66.3 (6.6)	-47.5 (19.6)	-52.3 (8.4)	-49.3 (50.9)	-47.8 (24.1)	-12.1 (13.8)	-58.1 (6.2)

The process that yielded the best process efficiencies for all the compounds was LLE (Table 10). In this procedure, the process efficiency was >50% for all compounds, except for THC (28.5-38.8%). In SPE, the process efficiency was >50% only for CBD-COOH, and low (4.7-5.5%) for CBN. For the rest of the compounds, the process efficiency ranged from 10.3 to 38%. In SLE and QuEChERS, all compounds showed process efficiencies <50%, between 12.4 and 38.4% and between 7.3 and 27.5%, respectively.

Table 10. Process efficiency results (%) for each analyte at Low QC (10 ng/mL) and High QC (100 ng/mL) for LLE, SLE, SPE and QuEChERS extration procedures.

Analyte	LLE		SLE		SPE		QuEChERS	
	Low QC	High QC	Low QC	High QC	Low QC	High QC	Low QC	High QC
CBD-COOH	121.2	98.1	23.7	15.8	100.9	71.4	24.0	19.0
CBD-OH	82.7	55.3	38.4	34.8	38.0	30.3	27.5	25.0
THC-COOH	87.5	73.2	27.0	22.4	25.0	22.5	17.9	18.6
THC-OH	79.3	55.3	26.2	20.5	28.9	21.7	21.0	21.7
CBD	66.7	47.5	16.1	17.8	5.5	4.7	16.7	13.6
CBN	78.4	57.0	31.0	33.6	27.2	30.0	21.9	19.8
THC	38.8	28.5	12.5	12.4	10.9	10.3	15.3	7.3

The sensitivity of the four most optimal extraction procedures was evaluated through a calibration curve using the calibrators 0.5, 1, 5, 10, 50 and 100 ng/mL. The estimated LOQ was similar for all the compounds in all the procedures, except THC. A LOQ of 0.5 ng/mL was observed for CBD-COOH, CBD-OH, THC-COOH, CBD and CBN. THC-OH had a LOQ of 5 ng/mL, and THC showed a LOQ of 5 ng/mL for all the procedures, except SPE (1 ng/mL).

3.4 Comparison with Previously Published Procedures

Many studies have been published on the identification and quantification of THC and its metabolites, THC-COOH and THC-OH, in multiple biological matrices such as whole blood, plasma, and serum (Koenig et al., 2011; Kim et al., 2017; Teixeira et al., 2007; Tiscione et al., 2016; Fernandez et al., 2008; Fabritius et al., 2013; Scheidweiler et al., 2016; Schwope et al., 2011; Frei et al., 2022; Gray et al., 2010; Dziadosz et al., 2017; Sanchez-Gonzalez et al., 2016; Jamwal et al., 2017; Haedener et al., 2016; Palazzoli et al., 2018; Dybowski & Dawidowicz, 2018) A few of these studies have focused on developing a method to identify and quantify THC and its metabolites using LLE as an extraction method (Fernandez et al., 2008; Tiscione et al.,

2016). Fernandez et al were able to recover THC, THC-OH and THC-COOH at rates of >100% utilizing hexane:ethyl acetate at 90:10 ratio as the organic phase (Fernandez et al., 2008). These results differ from our preliminary results during method optimization. In our experiments, hexane:ethyl acetate at 90:10 ratio produced poor process efficiency rates (< 53%) for THC and its metabolites. However, utilizing a different ratio of hexane:ethyl acetate (70:30) produced process efficiency >55% for the metabolites, except for THC (<30%). Regarding the sensitivity of the LLE procedure, another study was able to detect THC and THC-OH at 1 ng/mL, with an LLE procedure that used hexane:ethyl acetate at 80:20 ratio (Tiscione et al., 2016). Whereas, utilizing our method for LLE, the LOQ for THC and THC-OH was 5 ng/mL.

Other studies have been published on the identification and quantification of THC and its metabolites using SPE as an extraction method (Scheidweiler et al., 2016; Koenig et al., 2011; Schwope et al., 2011; Teixeira et al., 2007; Frei et al., 2022). The most common SPE type was cartridges was reversed phase (Ferreiros et al., 2013; Schwope et al., 2011; Teixeira et al., 2007; Koenig et al., 2011; Frei et al., 2022), but also mixed mode anion exchange (Gasse et al., 2016) and cation exchange (Concheiro et al., 2021) have been used. In terms of sensitivity, our method was able to produce better LOQs for THC-COOH then other publications (Teixeira et al., 2007; Schwope et al., 2011; Sorensen & Hasselstrom, 2017; Koenig et al; 2011; Frei et al., 2022). In Teixeira et al. (2007) they obtained a LOQ of 2 ng/mL for THC and THC-COOH and a LOQ of 20 ng/mL for THC-OH in blood. In Frei et al. (2022) an LOQ of 3 ng/mL was obtained for THC-COOH in blood. In Schwope et al. (2011), an LOQ of 1.0 ng/mL was obtained for THC-COOH. Utilizing our method with SPE, the LOQ for THC-COOH was 0.5 ng/mL, 1 ng/mL for THC and 5 ng/mL for THC-OH.

QuEChERS were originally developed for the determination and identification of pesticides in food and plants. However, some publications have attempted to use this novel method for the determination of THC and its metabolites in blood samples (Dybowski & Dawidowicz., 2018). Although Dybowski & Dawidowicz (2018) were able to obtain recovery rates that exceeded 55% for THC and its metabolites, in our method the extraction efficiency rates were lower (17.4-26.1%). The Dybowski & Dawidowicz (2018) study did not look at QuEChERS ability to determine CBD and its metabolites.

Compared to THC, fewer studies have been published on the identification and quantification of CBD and its metabolites, CBD-COOH and CBD-OH (Malaca et al., 2021; Pichini et al., 2021; Kevin et al., 2018; Sempio et al., 2022). Among these studies, only two analyzed THC, CBD, and their metabolites at the same time (Kevin et al., 2018; Sempio et al., 2022). Kevin et al. (2018) was able to obtain extraction efficiencies that exceeded 75% for all CBD and THC metabolites and over 60% for their parent drugs utilizing SLE, compared to our SLE method that produced <30% extraction efficiency for all analytes. This difference between Kevin et al. and our procedure could be due to the fact Kevin et al. (2018) conducted a protein precipitation using cold acetonitrile before the SLE, because it increased recovery rates. In our procedure no protein precipitation was performed before the SLE. In terms of sensitivity, our method was able to produce a better LOQ for CBD-OH (0.5 ng/mL) than their method (1 ng/mL).

Sempio et al. (2022) obtained LOQ ranging from 0.74-4 ng/mL for CBN, CBD, THC, and their metabolites. Our method proved to be more sensitive as we were able to obtain LOQs ranging from 0.5-1 ng/mL for CBN, CBD, CBD-COOH, CBD-OH, THC-COOH, and THC. Malaca et al. (2021) obtained LOQ of 1 ng/mL for CBD and CBD-COOH and a LOQ of 20

ng/mL for CBD-OH. Our method proved to be more sensitive as we were able to obtain LOQs of 0.5 ng/mL for CBD and its metabolites.

Some differences observed between our methods and previous publications could be due to the different types of biological samples (plasma and serum vs. whole blood). Plasma and serum samples are considered analytically cleaner than blood, and therefore, a better sensitivity may be expected (Sempio et al., 2021; Dziadosz et al., 2017; Kevin et al., 2018; Malaca et al., 2021; Pichini et al., 2021). Another aspect to take into consideration are the types of tubes employed in the sample preparation. Many of these publications chose to use glass tubes instead of polypropylene tubes (Kevin et al., 2018; Fernandez et al., 2008; Tiscione et al., 2016). Studies have found that using glass tubes instead of polypropylene tubes may increase recovery rates. This is due to the high affinity cannabinoids have toward materials such as propylene, resulting in them sticking to the tube and reducing recovery rates.

4. Conclusion

Investigating different analytical procedures allowed us to determine the best method to identify and quantify THC, CBD, and their metabolites in whole blood. In LC-MSMS, most cannabinoids work better in negative mode, except THC that yielded higher intensity in positive mode. Regarding the extraction procedures, although the methods evaluated showed different matrix effect, extraction efficiency and process efficiency, all of them were able to achieve low LOQs between 0.5 and 5 ng/mL. The procedure that yielded the best process efficiency was LLE, and the worst was QuEChERS. Compared to current published literature, the present methods were more sensitive in identifying THC-COOH and CBD metabolites in blood with LOQs of 0.5 ng/mL.

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