Fall 12-2021

Impact of Blood Collection Tubes in Cannabinoid Quantitative Analysis

Tega E. Obruche-Akponah
CUNY John Jay College, tegategs32@gmail.com

Impact of Blood Collection Tubes in Cannabinoid Quantitative Analysis

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

Tega Obruche-Akponah

December 2021

Impact of Blood Collection Tubes in Cannabinoid Quantitative Analysis

Tega Obruche-Akponah

December 2021

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

Thesis Committee:

Thesis Advisor: Dr. Marta Concheiro-Guisan

Second Reader: Teeshavi Acosta, M.S.

Third Reader: Sabra Botch-Jones, M.S., M.A., D-ABFT-FT

Acknowledgements

Firstly, I would like to thank the Almighty God for making it possible to be part of this program and for a successful completion of my thesis. Being an international student comes with its restrictions and challenges, but I was able to make it this far and I have the Lord to thank for all that.

My deepest appreciation goes out to Professor Marta Concheiro-Guisan. I want to thank you for all the knowledge and skills you have instilled all through this research process. Working closely with you has made me not only a better scientist but a better individual. You made the stressful times bearable, and I appreciate your patience and guidance through all the ups and downs throughout this process and all through my academic program.

More special thanks to Teeshavi Acosta who has always been very supportive and understanding. Thank you for all your words of encouragement and support throughout my research process and for never hesitating to provide both academic and moral assistance in any way you could.

I also want to thank Professor Sabra Botch-Jones for taking out time out of your schedule to review my research and for your curiosity and engagement in my research. I appreciate your interest and contribution to my research.

Great appreciation to my family, especially my father who has supported my whole academic journey. I would have hardly come this far without my family support, and I am deeply grateful for this.

I also thank my classmates who have always been sources of encouragement through the good and tough times. I am truly proud of how far we have come.

Lastly, my sincere appreciation goes out to all members of faculty and staff of the forensic science department who have contributed to my knowledge and experience throughout my program.

Abstract

In forensic toxicological cases, sample stability and storage are pivotal for obtaining adequate analytical results. Currently, limited information is available about the impact of different preservatives and anticoagulants present in the blood tubes used in the collection of blood samples containing cannabinoids. This study investigated the impact of sodium fluoride (gray top) and sodium citrate (blue top) blood collection tubes on the concentrations of tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) and 5 THC metabolites (THC-OH, THCCOOH, THC-diOH, THC-glucuronide, THCCOOH-glucuronide) in whole blood samples, stored at room temperature, 4ºC and -20ºC for up to 3.5 months. The samples were extracted using QuEChERS extraction technique. LC-MSMS quantification was performed in positive electrospray ionization mode, and two transitions were monitored using multiple reaction monitoring mode. In this method, the limits of quantification ranged from 0.5 – 5 ng/mL with a linearity range up to 100 ng/mL. Bias ranged from -4.9 to 25.2%, and all compounds also showed an acceptable imprecision, except THC-glucuronide and THC-diOH. Both extraction and process efficiency ranged from about 2.4% to 41.4%, and most of the analytes showed ion suppression at low concentrations (up to -61.4%) and no matrix effect at high. With respect to the stability studies, THC, THCCOOH, CBD and CBND were mostly stable, although stability issues occurred at low concentrations stored at -20°C after 3.5 months of storage. THCCOOH-glucuronide, THC-glucuronide and THC-OH showed some stability issues at room temperature, and THC-diOH was the most unstable analyte. Overall, better stability was achieved using sodium citrate collection tubes.

Table of Contents

1. Introduction

Over past decades, marijuana has been the most prevalent federally illicit drug used in the United States (Substance Abuse and Mental Health Services Administration [SAMHSA], 2020). According to the Center of Disease Control (CDC), as of 2019, about 48.2 million Americans reportedly used marijuana (Center for Disease Control and Prevention (CDC), 2019), increasing from about 25.8 million in 2002 (Substance Abuse and Mental Health Services Administration, 2020). According to a SAMHSA survey, marijuana remained the most used illicit drug from 2002 to 2019, with the highest use recorded among people aged between 18 and 25 years (Substance Abuse and Mental Health Services Administration, 2020).

Delta-9-tetrahydrocannabinol (THC) is the main psychoactive ingredient in marijuana, and it produces wide range of effects in the body (Sharma et al., 2012). THC produces its psychoactive effects by acting on the CB1 and CB2 cannabinoid receptors (Sharma et al., 2012), triggering behavioral and cognitive impairment. During metabolism in the body, THC is hydroxylated at the carbon-11 to form 11-hydroxy-delta 9-tetrahydrocannabinol (OH–THC), which in turn undergoes oxidation to produce 11-nor-carboxy-delta 9-tetrahydrocannabinol (THCCOOH) (Lacroix & Saussereau, 2012). Glucuronides of THC and THCCOOH are also formed in phase II metabolism reactions. THC is highly lipophilic, and therefore, it shows a high tendency to be stored in fatty tissues and has a large volume of distribution (Wolff & Johnson, 2014). Due its accumulation in fat, THC may be steadily released back into the blood for a longer period of time after ingestion (Compton, 2017).

From a federal standpoint and according to the Drug Enforcement Agency (DEA), cannabis remains a schedule I controlled substance in the United States (Compton, 2017). However, several states have adopted laws that decriminalize marijuana, allow it for medical purposes and/or permit

its recreational use (Governors Highway Safety Association [GHSA], 2021). Cannabis is available for medical use in 36 States and for recreational use in 17 States and the District of Columbia (World Drug Report, 2019). These changes in the legal status are expected to have an impact in the prevalence of this drug, increasing its use.

Cannabis (marijuana) is the most predominant drug in most cases of driving under the influence of drugs (DUID) (Coulter et al., 2008; Brady & Li, 2014; O'Malley & Johnston, 2013). Majority of drivers in cases of marijuana driving/crashes have reportedly been young adults or teenagers (National Institute of Drug Abuse [NIDA], 2020; O'Malley & Johnston, 2013; Vindenes et al., 2012), and most cases were found to be common among nighttime drivers (Brady & Li, 2014; National Highway Traffic Safety Administration [NHTSA], 2007). In these cases where drugs of abuse are being investigated in drivers, establishing the suitable biological matrix and cut-off concentrations is crucial for interpretation purposes (Vindenes et al., 2012). As such, blood is an ideal matrix for DUID cases because of its correlation between concentrations of drugs and impairment. But there are limitations when using blood to interpret marijuana impairment while driving because of how rapidly this drug is eliminated from the body and detection of concentrations that confound interpretations (Compton, 2017). After cessation of smoking, THC levels drop rapidly within the first few minutes and slowly declines as lower concentrations are reached (Compton, 2017). Studies have reported THC peak concentrations ranging between 15 ng/mL to 33 ng/mL within 15 – 30 minutes after smoking,and declining to less than 2 ng/mL after 22 hours (Schwope et al., 2011). This could be challenging due to the time lag between exposure to this drug, time of driving/accident crash and time for law enforcement officials to obtain a sample for analysis. On the other hand, THC has a long half-life (about 7 days), making the drug detectable in the system long after impairment has worn off, especially in chronic users (Bédard

et al., 2007; Karschner et al., 2009; Peterman, 2019). This lack of correlation between THC concentrations and impairment could confound the establishment of a relationship between cannabis and impaired driving (Bédard et al., 2007).

The investigation of additional cannabinoids, such as cannabinol (CBN) and cannabidiol (CBD), THC metabolites and the metabolite-to-parent ratios in a blood sample may help to improve the interpretation of this biological sample. Scheidweiler et al. (2013) propounded that 11-nor-carboxy-delta 9-tetrahydrocannabinol (THCCOOH concentrations increase more rapidly than THCCOOH-glucuronide immediately after smoking, making the ratios of THCCOOH-glucuronide/THCCOOH useful in indicating recent exposure to cannabis. However, within about 1-2 hours after smoking the ratio returns to baseline and becomes constant. Samples obtained approximately 2-3 hours after smoking may not be efficacious in indicating recent use from these ratios because they would have returned to baseline (Bédard et al., 2007). In a study carried out on 25 participants, THCCOOH was reportedly present in all participants up to 7 days after cessation of smoking, with a concentration range of 2.8 – 91.7 ng/mL on day 1 to 0.4 – 36.5 ng/mL on day 7 (Karschner et al., 2009). Low concentrations of THC-OH were also detected in some participants over the 7-day period, with an average range of 0.1 – 0.5 ng/mL (Karschner et al., 2009). CBD, CBN and THC-glucuronide could be feasible markers for recent cannabis exposure as they were detectable for the first 2 - 4 hours after cessation of smoking (Desrosiers et al., 2014; Schwope et al., 2011).

These interpretative challenges have impelled a few states in the United States to adopt “per se laws” regarding driving under the influence of marijuana, some other states use “zero tolerance” laws (Wong et al., 2014). Zero tolerance laws make it a criminal offence for an individual to drive with any measurable amount of THC and/or its metabolites in their

system/biological fluid (Arkell et al., 2021). Currently, several states have adopted zero tolerance laws against THC and/or THC-COOH; 9 of these states (Arizona, Delaware, Georgia, Indiana, Oklahoma, Pennsylvania, Rhode Island, South Dakota, and Utah) have zero tolerance against both THC and its metabolites and only 3 states (Iowa, Michigan, and Wisconsin) have zero tolerance exclusively against THC (Compton, 2017). Additionally, 7 states have adopted per se laws stating legal limits for marijuana in blood and other biological samples while driving. Per se laws make it a criminal offense to drive with a specific concentration of THC and/or its metabolite in the blood or other biological fluids (Arkell et al., 2021). Colorado, Montana, Illinois, and Washington have legal limits of 5 ng/mL in blood for only THC (Bédard et al., 2007). Nevada and Ohio both have the same legal cut off 2 ng/mL and 50 ng/mL for THC and THCCOOH in blood, respectively (Bédard et al., 2007).

In forensic toxicological cases, especially those involved with DUID, sample stability and storage are pivotal for obtaining adequate analytical results. Considering the usual delay between sample collection and analysis, and possible back log of cases in laboratories because of varying prioritization, depending on how urgent the results of the cases are needed for court, most samples could spend weeks to months in forensic laboratories prior to analysis. Several publications have investigated the stability of THC and some of its metabolites in blood under different storage conditions (time and temperature) (Scheidweiler et al., 2013; Sorensen & Hasselstrom, 2018; Stout et al., 2000), but limited information is available about the impact of different preservatives and anticoagulants present in the blood tubes used in the collection of the samples (Sorensen & Hasselstrom, 2018; Toennes & Kauert, 2001). Also, most of the studies investigate the stability of mainly THC, THC-OH and THC-COOH (Koenig et al., 2011; Meneses & Mata, 2020; Salimiasl et al., 2017; Sorensen & Hasselstrom, 2018; Wiedfeld et al., 2018) and only a few investigated the

stability of other metabolites, including the glucuronides (Scheidweiler et al., 2013, 2016)

Studies carried out on the blood samples stored with sodium fluoride containing vacutainers (gray top tubes) have reported a decrease in concentrations of THC, THC-OH and THC-COOH (Sorensen & Hasselstrom, 2018), including CBD and CBN (Wiedfeld et al., 2018). Sorensen and Hasselstrom (2018) has also found greater stability in gray top tubes compared to cannabinoids stored in tubes containing a sodium citrate mixture additive. Other studies which have analyzed THC-COOH and THC-COOH glucuronide determined THC-COOH concentrations to be lower in serum samples preserved in gray top vacutainers (Toennes & Kauert, 2001). Gray top tubes contain sodium fluoride potassium oxalate additives (Toennes & Kauert, 2001). The sodium fluoride additive improves the stability of the phase II metabolite, THC-COOH glucuronide (Sorensen & Hasselstrom, 2018). It prevents its degradation by inhibiting phosphatase and esterase activity preventing hydrolysis of the ester linkage between THCCOOH and glucuronide (Asbridge, 2014; Scheidweiler et al., 2013). This hydrolysis can cause increase in free THCCOOH concentrations, altering accurate quantification and interpretation of these two metabolites. These studies are summarized in Table 1.

Table 1. Studies of cannabinoid stability in blood. RT: room temperature.

Study	Target Analytes	Collection Tube/Additive	Stability
Toennes, 2001	THC-COOH, THCCOOH-glucuronide	Gray Top tubes	All decreased
Wiedfeld, 2019	THC, THC-OH, THC-COOH	Gray top tubes	All decreased
Scheidweiler, 2016	THC, THC-OH, THC-COOH, THCCOOH-glucuronide	Gray top tubes	All stable at RT, 4°C and -20°C for 1 week, 1 week and 3 months respectively, THCCOOH and THCCOOH-glucuronide unstable

Sorensen, 2017	THC, THC-OH, THC-COOH, CBD, CBN	Gray top tubes & sodium citrate mixture additive	All decreased at -20°C More decrease in sodium citrate samples
Scheidweiler, 2013	THC, THC-OH, THC-COOH, THC-glucuronide, THCCOOH-glucuronide, CBD, CBN	Green top tubes (Sodium heparin)	All stable for 3 months at -20°C except THCCOOH-glucuronide

THC and metabolites are highly lipophilic, so they could stick to sample containers during storage. However, the tendency for THC and metabolites to adhere to sample containers made of plastic or rubber may be different depending on the type of the biological sample. Stout et al. (2000) reported a rapid loss in THCCOOH at 4°C in urine samples preserved in polyethylene and polypropylene containers. Another study reported that blood may prevent the adherence of cannabinoids to polypropylene tubes at 4°C (Johnson et al., 1984).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a widely accepted and applied analytical technique in quantification of cannabinoids and their metabolites, using electrospray in either positive (Bédard et al., 2007; Coulter et al., 2008; Lacroix & Saussereau, 2012; Peterman, 2019) or negative mode (Brady & Li, 2014; Scheidweiler et al., 2016). Gas chromatography mass spectrometry (GC-MS) is also accepted and extensively utilized in quantification of cannabinoids (Johnson et al., 1984; Toennes & Kauert, 2001), but LC-MSMS has reportedly been preferred by some researchers as a more sensitive method that is less time consuming (Asbridge, 2014; Lacroix & Saussereau, 2012). Regarding extraction procedures, the most common clean-up of blood samples for cannabinoids is solid phase extraction (SPE) and it has been extensively utilized by various studies (Brady & Li, 2014; Coulter et al., 2008; Koenig et al., 2011; Raikos, 2014; Scheidweiler et al., 2016, Simões et al., 2011). Liquid-liquid extraction has also been employed in the past (Purschke et al., 2016). Currently, there is very limited research

on the use of QuEChERS (QUick, Easy, CHeap, Effective, Rugged, and Safe) extraction in cannabinoid quantification in blood. QuEChERS extraction involves a two-step cleanup. In the first step, the analyte is extracted from the sample using extraction salts and in the second step, the supernatant from the step one undergoes cleanup in dispersive SPE (dSPE) tubes. Compared to other extraction techniques like SPE, QuEChERS is a much easier and faster extraction technique (Salimiasl et al., 2017). It is also less cost effective, as it requires less use of organic solvents or chemicals during sample cleanup (Salimiasl et al., 2017). QuEChERs has been utilized in the quantification of THC, THC-OH and THCCOOH in blood using GC-MSMS (Dybowski & Dawidowicz, 2018).

A comprehensive understanding of the stability of THC and its metabolites in blood in different types of collection tubes is important for proposing the right collection and storage conditions. The objective of the proposed research is to evaluate the impact of Vacutainer tube selection in quantitative analysis of THC, THC-OH, THC-COOH, THC-glucuronide, THC-COOH glucuronide, CBD and CBN, in whole blood samples. The blood collection tubes investigated were BD Vacutainer Fluoride plastic tubes (gray-top) and BD Vacutainer Citrate plastic tubes (blue top) at three temperatures (room temperature, 4ºC and -20ºC) for up to 3.5 months. Blood samples were extracted using QuEChERS method and analyzed by LC-MS/MS.

2. Materials and Methods

2.1. Reagents, Standards, and Supplies

THC, THC-OH, THCCOOH, THCCOOH-glucuronide, CBD, CBN, THC-d_3, THCCOOH-glucuronide-d_3, THC-OH-d_3, THCCOOH-d_3, THCCOOH-glucuronide-d_3, CBD-d_3, and CBN-d_3 were purchased from Cerilliant (Round Rock, TX) at 100 µg/mL or 1 mg/mL in methanol. THC-diOH and THC-diOH-d_6 at 100 µg/mL and THC-glucuronide at 10 µg/mL in methanol were from

ElSohly Laboratories (Oxford, MS). Methanol (LC-MS grade), acetonitrile (HPLC grade, LC-MS grade), and formic acid (LC-MS grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Ten mL Sarstedt tubes were from Fisher Scientific. QuEChERS 800 mg $MgSO_4$/200 mg Sodium Chloride 15 mL tubes and QuEChERS 150 mg $MgSO_4$/50 mg CEC 18 2 mL tubes (dSPE) were purchased from United Chemical Technologies (Bristol, PA).

2.2. Blood Samples and Collection Tubes

Sheep blood, Alsever, pooled, was purchased from Carolina Biological Supply Company (Burlington, NC). The following human whole blood pools were purchased from BioIVT (Westbury, NY): Human whole blood sodium heparin, human whole blood K_2EDTA (pool 1), human whole blood K2EDTA (pool 2), human whole blood 3.2% sodium citrate and human whole blood KOx-sodium fluoride. Buffered sodium citrate (9NC) (2.7mL, 13 x 75mm) and sodium fluoride 10 mg potassium oxalate 8 mg (FX) (4.0 mL, 13 x 75 mm) blood collection tubes were purchased from Becton, Dickson, and Company (Franklin Lakes, NJ).

2.3. Preparation of Working Solutions

Cannabinoid working solutions at 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL were prepared from 10 μg/mL stock solutions of THC, THCCOOH, THC-OH, THC-diOH, THC-glucuronide, THCCOOH-glucuronide, CBD, and CBN in methanol. THC-glucuronide standard was present at the 0.01 and 0.1 μg/mL mixture, but not at 1 μg/mL due to limited availability (individual stock solution at 1 μg/mL).

2.4. Preparation of Calibrators and Quality Controls

A calibration curve was prepared with calibrators at 0.5, 1, 5, 10, 50 and 100 ng/mL. Quality controls (QC) were prepared at 5 and 50 ng/mL. Calibrators and QCs were prepared in Sarstedt tubes using 0.5 mL of sheep blood. See Table 2 for preparation of calibrators and QCs.

Table 2. Volume of the corresponding working solution for calibrators and QCs fortification.

Working Solutions	0.5 ng/mL	1 ng/mL	5 ng/mL	10 ng/mL	50 ng/mL	100 ng/mL
0.01 μg/mL	25 μL	50 μL	-	-	-	-
0.1 μg/mL	-	-	25 μL	50 μL	-	-
1 μg/mL	-	-	-	-	25 μL	50 μL

2.5. Preparation and Storage Conditions of Stability Samples

For the low concentration stability samples at 5 ng/mL, eight tubes containing 3 mL of human blood each (preserved with sodium fluoride or sodium citrate) were fortified with 25 μL of 0.1 μg/mL cannabinoid standard solution. For high concentration stability samples at 50 ng/mL, eight tubes containing 3 mL of human blood each (preserved with sodium fluoride or sodium citrate) were fortified with 25 μL of 1 μg/mL cannabinoid standard solution. A total of sixteen 3mL aliquots were prepared in the corresponding Vacutainer tube containing sodium fluoride or sodium citrate as preservatives. After fortification with the standards, the 3 mL pools were vortexed for 30 s and mixed in a rotor for 30 min. After mixing, 0.5 mL duplicates were taken from each aliquot to measure the time zero (t_0) prior storage; the rest (2 mL) were stored at room temperature, 4°C

or -20°C. Room temperature samples were stored and extracted after 3 days and 1 week. 4°C samples were stored and extracted after 1 week, and -20°C samples were stored and extracted after 3.5 months. At each time point and storage condition, the stability samples were analyzed in duplicate (see Fig. 1).

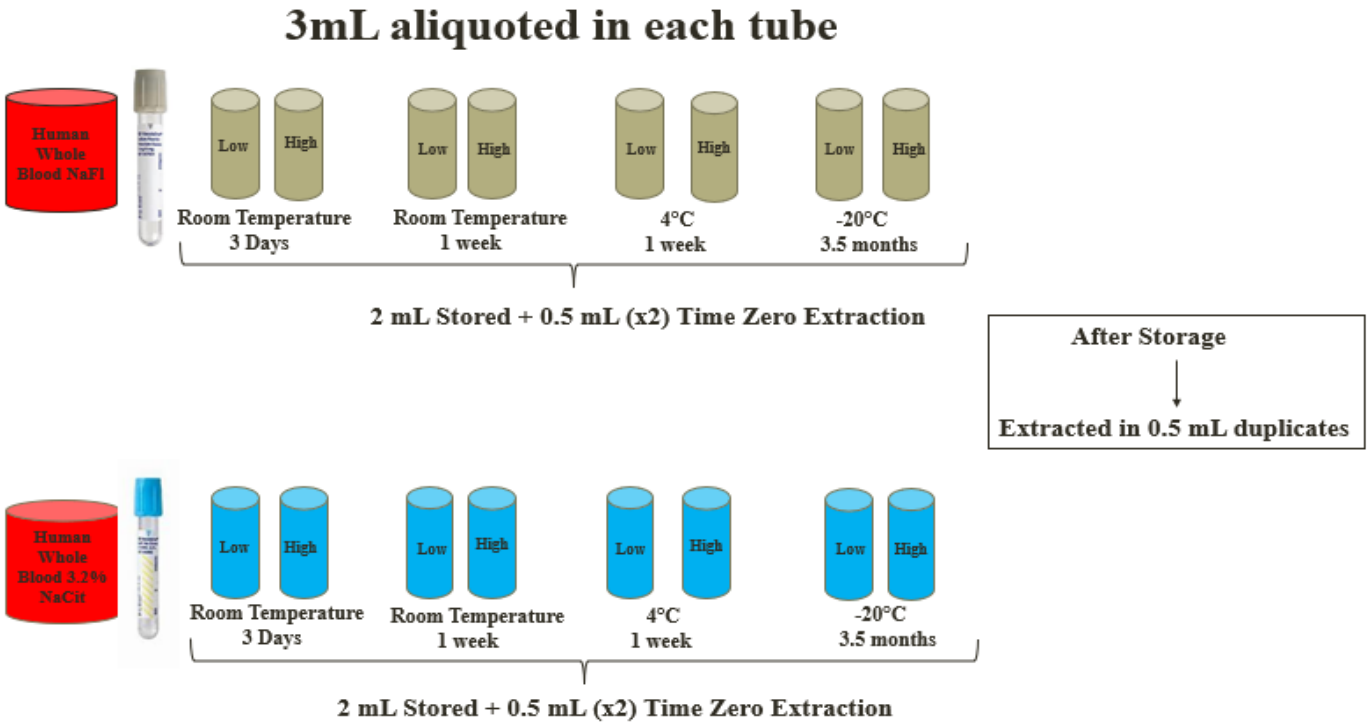

Fig 1. Preparation of stability samples in sodium fluoride and sodium citrate tubes at different storage conditions.

2.6. Blood Sample Extraction

One hundred µL of 0.1 µg/mL internal standard solution were added to 0.5 mL blood sample in a Sarstedt tube. The tube was capped, vortexed for 30 s, and mixed for 10 min in the rotor. One and a half mL of water was added to each sample and the tubes were vortexed for 30 s. Two mL acetonitrile was added to 15 mL-QuEChERS tube, the samples were transferred to these tubes, vortexed and mixed for 10 min in a rotor. After centrifugation at 7000 rcf for 10 min at 10°C, 1

mL of the supernatant was transferred to dSPE 2 mL tubes. Samples were vortexed for 1 min and centrifuged at 12000 rcf for 5 min at room temperature. Purified samples were transferred to clean Sarstedt tubes and evaporated with nitrogen in a TurboVap™ (Biotage, Charlotte, NC) at 50°C. Reconstitution was performed by adding 200 µL of initial conditions mobile phase (0.1% formic acid in water:0.1% formic acid acetonitrile, 70:30, v/v). Reconstituted samples were transferred to appropriately labeled LC-MSMS vials.

2.7. LC-MSMS

Samples were analyzed on an LC-MSMS 8050 instrumentation 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). A Kinetex F5, 2.6 µm, 2.1 x 100 mm, 100 Å pore size chromatographic column from Phenomenex (Torrance, CA) was employed for this analysis. 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.

The triple quadrupole mass spectrometer was operated with an electrospray ionization (ESI) source in positive mode. The following parameters were employed for the source: nebulizing gas flow at 2 L/min, heating gas flow at 10 L/min, drying gas flowed at 10 L/min, interface temperature at

300°C, and heat block temperature at 400°C. Ions were monitored by multiple reaction monitoring (MRM). See Table 3 for MSMS optimization parameters for all target cannabinoids.

Table 3. MRM transitions, entry (Q1) and exit (Q3) quadrupole voltages and collision energy (CE) for each target analyte.

Compound	MRM Quantifier Transition	Q1 (V)	CE (V)	Q3 (V)	MRM Qualifier Transition	Q1 (V)	CE (V)	Q3 (V)
THC	315.2 > 193.0	-24	-26	-19	315.0 > 123.3	-20	-34	-20
THC-d_3	318.2 > 193.2	-11	-21	-20	318.2 > 123.2	-22	-37	-12
THC-COOH	345.0 > 327.4	-23	-18	-21	345.0 > 299.3	-17	-21	-30
THC-COOH-d_3	348.0 > 330.4	-23	-18	-11	348.0 > 302.3	-30	-20	-20
THC-OH	331.0 > 193.4	-30	-25	-29	331.0 > 200.9	-22	-25	-20
THC-diOH-d_3	334.0 > 201.2	-25	-23	-29	334.0 > 196.2	-13	-28	-21
THC-diOH	347.0 > 311.3	-10	-17	-19	347.0 > 329.3	-25	-10	-21
THCCOOH-glucuronide	521.0 > 345.4	-36	-21	-22	521.0 > 327.3	-24	-24	-22
THCCOOH-glucuronide-d_3	524.0 > 348.3	-40	-16	-16	524.0 > 330.3	-26	-29	-23
THC-glucuronide	491.0 > 315.4	-25	-20	-15	491.0 > 193.3	-30	-34	-30
CBD	315.2 > 193.2	-30	-23	-28	315.2 > 123.0	-11	-30	-21
CBD-d_3	318.2 > 196.2	-12	-24	-13	318.2 > 123.2	-16	-37	-24
CBN	311.3 > 194.9	-24	-30	-21	311.3 > 222.9	-14	-23	-23
CBN-d_3	314.2 > 223.1	-13	-23	-10	314.2 > 208.1	-25	-34	-13

2.8. Method Validation

Method validation was performed according to the set standards by ANSI/ASB Standard 036, 1st Ed. 2019 (LeBeau, 2020). The following parameters were analyzed: linearity, bias, precision,

limit of detection (LOD), limit of quantification (LOQ), matrix effect, extraction efficiency and process efficiency. Data acquisition and processing was performed by Lab Solutions software (Shimadzu) and calculations were performed using Microsoft Excel.

2.8.1. Linearity and Limit of Quantification (LOQ)

Samples were analyzed within a 4-day period. On each day, calibrators within the concentration range 0.5-100 ng/ml were prepared and analyzed. Residuals fell between ± 20% of the target concentration for linearity to be considered acceptable. The LOQ was the lowest calibrator with bias within ±20% and imprecision below 20%.

2.8.2. Bias and Imprecision

Low and high QCs at 5 ng/mL and 50 ng/mL, were prepared in triplicates (n = 3) following each calibration curve on days 1- 4 (n=12). Bias was determined by measuring the percent error of QC samples and was determined to be acceptable if results were within ± 20% of the target concentration. Imprecision was assessed by measuring the coefficient of variation (CV) and was considered acceptable if below 20%.

2.8.3. Matrix Effect (ME), Extraction Efficiency (EE) and Process Efficiency (PE).

ME, EE, and PE were evaluated at 5 and 50 ng/mL preparing 3 sets of samples. Set 1 (“neats”) were neat standards at 5 or 50 ng/mL (n=3). Set 2 (“before”) were blood samples from six different sources (one sheep blood and five human blood pools) fortified with the standards and internal standards before the extraction. And set 3 (“after”) were blood samples from six different sources (one sheep blood and five human blood pools) fortified with the standards and internal standards after the extraction, just before the evaporation. Calculations for ME, EE and PE were performed using Microsoft Excel with the formulas highlighted in Table 4. Matrix effect results

are considered acceptable if they are within ± 25% of the target concentration, and if the CV among the different sources do not exceed 20%.

Table 4. Formulas employed for matrix effect, extraction efficiency and process efficiency determination.

Parameter (%)	Formula
Matrix Effect	$(Mean_{After} - Mean_{Neat} / Mean_{Neat}) \times 100$
Extraction Efficiency	$(Mean_{Before} / Mean_{After}) \times 100$
Process Efficiency	$(Mean_{Before} / Mean_{Neat}) \times 100$

2.9. Criteria for Confirmation of Cannabinoid Analytes

For an analyte to be confirmed as present within the samples, its retention time had to be within 2% of the average retention time of the calibrators, the 2 MRM transitions had to be detected (one quantifier and one qualifier transition) and show the correct ion ratio within 20% of average ion ratio of the calibrators.

2.10. Data Analysis of Stability Samples

Stability samples were considered stable if they quantified within ±20% of their corresponding time zero sample. For each concentration at each storage condition, a trendline was built by Google Sheets.

3. Results and Discussion

3.1. Linearity and Limits

This method achieved a linearity range of 0.5 ng/mL - 100 ng/mL, 1 ng/mL – 100 ng/mL and 5 ng/mL – 100 ng/mL depending on the analyte (see Table 5). Method calibration was accomplished

via a linear model, non-forced and $1/x^2$ weighting for all analytes. The LOD was equal to the LOQ, which was the lowest calibrator (Table 5). See Table 6 for mean r^2, slope and intercept values.

Table 5. Limit of quantification **(**LOQ) for all target analytes.

Analyte	LOQ
THC-glucuronide	0.5 ng/mL
THC THCCOOH-glucuronide CBD CBN	1 ng/mL
THC-OH THCCOOH THC-diOH	5ng/mL

Table 6. Calibration curve parameters for each target cannabinoid (n=4).

Analyte	r^2 ± SD	Slope ± SD	Intercept ± SD
THC-OH	0.9312 ± 0.092	0.8722 ± 0.098	0.0046 ± 0.044
THC-diOH	0.9449 ± 0.047	1.8561 ± 0.495	0.1810 ± 0.136
THC-Glucuronide	0.9472 ± 0.032	16.1326 ± 4.037	0.2210 ± 0.159
THCCOOH-Glucuronide	0.9870 ± 0.010	1.4054 ± 0.185	0.0141 ± 0.019
THCCOOH	0.9918 ± 0.007	0.4771 ± 0.054	0.0038 ± 0.008
THC	0.9920 ± 0.004	0.8208 ± 0.095	0.0037 ± 0.005
CBD	0.9911 ± 0.004	0.8388 ± 0.070	0.0084 ± 0.004
CBN	0.9912 ± 0.006	0.8624 ± 0.143	0.0146 ± 0.005

3.2. Imprecision and Bias

Bias and imprecision results of 3 replicates over a period of 4 days (n=12) was monitored at 5 ng/mL (low QC) and 50 ng/mL (high QC) for each analyte. Bias ranged from -2.3 – 25.2% for low QC samples and from -4.9 – 2.7% for high QC samples. Overall, target analytes all showed acceptable bias, within ± 20% of the target concentration, except THC-glucuronide with a bias of 25.2% at low QC (See Table 7). Imprecision ranged from 5.7% - 45.4 % at low QCs and from

5.5% - 31.8% at high QCs. All compounds showed an acceptable imprecision (<20%), except THC-glucuronide (25.4-35%) and THC-diOH (28.7-45.4%). See Table 8.

Table 7. Bias results at 5 ng/mL (low QC) and 50ng/mL (high QC) for quantitative transitions n blood (n= 12).

Analyte	Low QC	High QC
	% Bias	% Bias
THC-OH	6.2	1.1
THC-diOH	16.9	6.1
THC-glucuronide	25.2	-
THCCOOH-glucuronide	5.2	-5.9
THC-COOH	-2.3	-2.7
THC	-1.5	-4.9
CBD	-0.5	2.7
CBN	2.7	0.2

Table 8. Imprecision (CV, %) results at 5 ng/mL (low QC) and 50 ng/mL (high QC) for quantitative transitions in blood (n= 12).

Analyte	Low QC		High QC	
	Intraday Imprecision	Interday Imprecision	Intraday Imprecision	Interday Imprecision
THC-OH	13.8	21.0	6.5	10.7
THC-diOH	45.4	30.6	28.7	31.80
THC-glucuronide	35.0	25.4	-	-
THCCOOH-glucuronide	13.9	10.9	9.3	11.3
THC-COOH	28.9	17.5	4.9	7.5
THC	5.7	5.8	-	8.7
CBD	13.5	22.9	6.2	11.7
CBN	8.6	7.5	5.5	8.7

3.3. Matrix Effect, Extraction Efficiency and Process Efficiency

Matrix Effect, Extraction Efficiency and Process Efficiency were calculated at 5 ng/mL and 50 ng/mL using 6 whole blood pools, sheep blood and 5 human blood pools containing the following additives: sodium heparin, K_2EDTA (pool 1), K_2EDTA (pool 2), sodium citrate and sodium fluoride.

For extraction efficiency at 5 ng/mL, 4 cannabinoids were above 20%, 2 were below 20% and 2 were below 10%. At 50 ng/mL, 5 cannabinoids were below 20%, one was below 10% and only one was above 20%. For process efficiency at 5 ng/mL, 5 cannabinoids were between 10% and 15%, 2 were below 10% and one was above 20% (See Table 9).

Table 9. Extraction efficiency and process efficiency of cannabinoids at 5 ng/ml (low QC) and 50 ng/mL (high QC).

Analytes	Extraction Efficiency (%, n=6)		Process Efficiency (%, n=6)	
	Low QC	High QC	Low QC	High QC
THC-OH	25.2	15.5	15.4	15.5
THC-diOH	3.5	24.8	34.2	41.4
THC-glucuronide	13.7	-	7.9	-
THCCOOH-glucuronide	3.7	2.7	3.0	2.4
THCCOOH	18.4	11.9	12.1	9.6
THC	32.7	16.5	14.0	11.9
CBD	26.3	17.3	11.7	17.6
CBN	27.3	17.0	11.1	12.9

For matrix effect at low QC, all but 2 cannabinoids showed ion suppression varying between -37.8 and -61.4% with coefficient of variations (CV) between 7.5 and 34.7%. At high QC, THC-OH, THCCOOH-glucuronide, CBD and CBN showed no matrix effect (from -8.4 to -25.7%); THC-diOH showed ion enhancement (66.3%) and THC ion suppression (-30.8%). The CV among the 6 different sources ranged from 6.5 to 24.8% (see table 10).

Table 10. Matrix Effect and %CV results of cannabinoids at 5 ng/ml (Low QC) and 50 ng/mL (High QC) (n=6)

Analyte	Low QC		High QC	
	Matrix Effect %	%CV	Matrix Effect %	%CV
THC-OH	-37.8	24.2	2.7	14.7
THC-diOH	13.1	10.9	66.3	10.9
THC-glucuronide	-42.8	7.5	-	-
THCCOOH-glucuronide	90.1	7.5	-8.4	6.5
THCCOOH	-61.4	21.8	-21.3	19.5
THC	-61.1	37.3	-30.8	24.8
CBD	-59.5	32.4	0.3	21.7
CBN	-60.4	34.7	-25.7	23.2

3.4. Comparison with Other Analytical Methods

Cannabinoids have been extracted from blood samples by different techniques, including solid phase extraction and liquid-liquid extractions (Brady & Li, 2014; Coulter et al., 2008; Purschke et al., 2016). Both techniques are efficient and commonly used in toxicology, but they are time consuming and/or expensive. QuEChERS is a QUick, Easy, CHeap, Effective, Rugged, and Safe procedure that has been routinely used for the extraction of pesticides in food, but with limited applications in forensic toxicology. Dybowski and Dawidowicz, (2018) developed a QuEChERS method for the analysis of THC, THC-OH and THC-COOH in whole blood by GC-MSMS. We expanded the scope of analytes and developed a method that also included THC-diOH, THC-glucuronide, THCCOOH-glucuronide, CBD and CBN.

3.5. Stability Studies

For THC, all samples stored in the different storage conditions were considered stable except when stored in sodium fluoride tubes at 3.5 months at -20°C at both low and high QCs (Fig. 2).

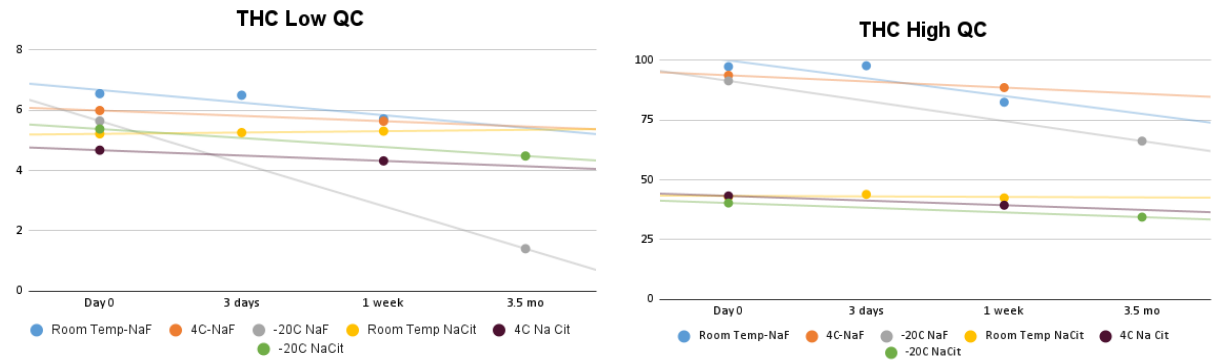

Fig. 2. Trend lines of THC in whole blood samples fortified at low and high concentrations in sodium fluoride and sodium citrate collection tubes at different storage conditions.

For THC-COOH, most of the samples were stable except the low QC samples that were stored at -20°C for 3.5 months, in both sodium fluoride and sodium citrate storage tubes (Fig. 3).

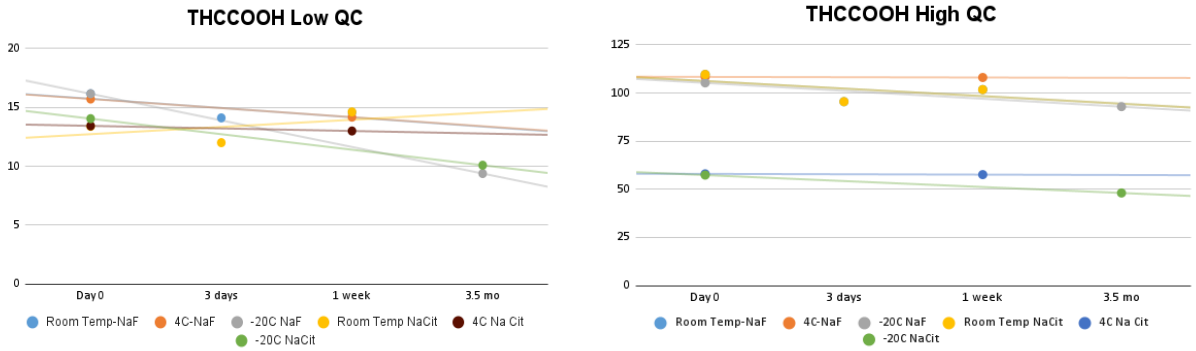

Fig. 3. Trend lines of THCCOOH in whole blood samples fortified at low and high concentrations in sodium fluoride and sodium citrate collection tubes at different storage conditions.

THCCOOH-glucuronide was stable at all storage conditions except at room temperature, in the sodium fluoride tubes (Fig. 4).

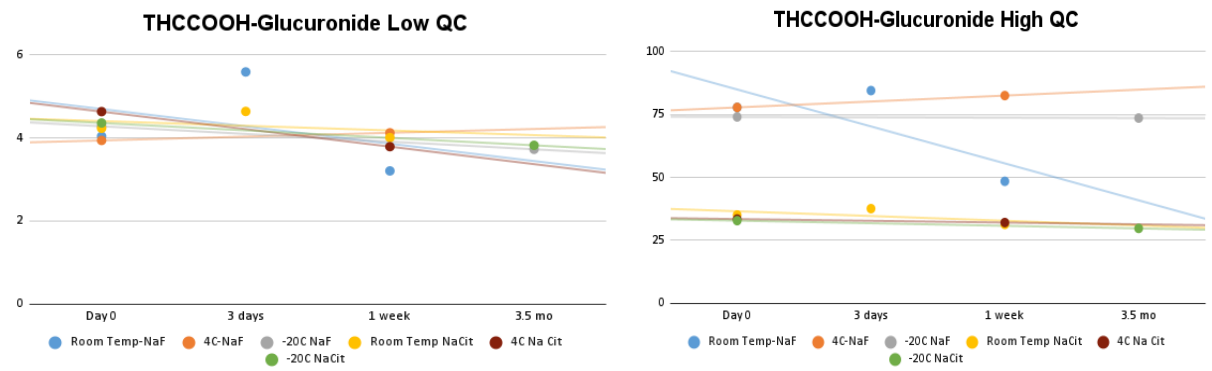

Fig. 4. Trend lines of THCCOOH-glucuronide in whole blood samples fortified at low and high concentrations in sodium fluoride and sodium citrate collection tubes at different storage conditions.

THC-OH was generally stable at all conditions in the sodium citrate tubes but stability issues occurred in at room temperature and -20°C for 3.5 months in sodium fluoride tubes (Fig. 5).

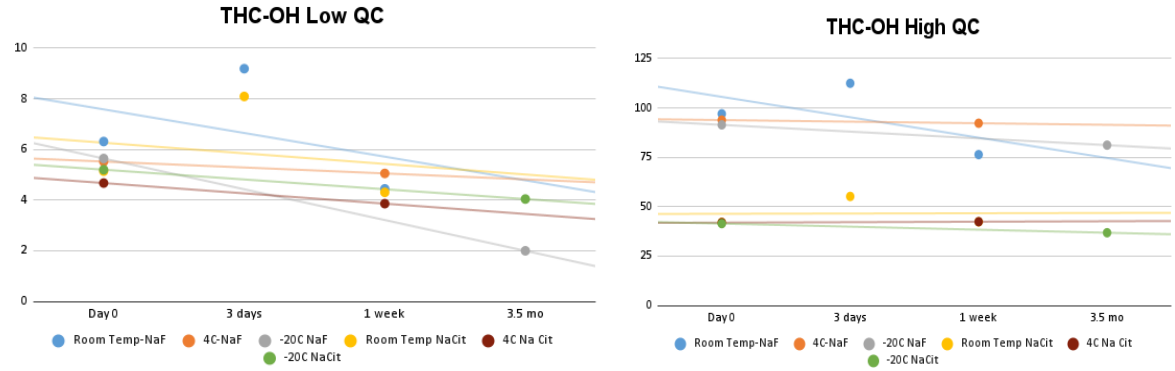

Fig. 5. Trend lines of THC-OH in whole blood samples fortified at low and high concentrations in sodium fluoride and sodium citrate collection tubes at different storage conditions.

Stability issues were observed for THC-diOH in sodium fluoride tubes at all the storage conditions and in sodium citrate tubes at room temperature and 4°C (Fig. 6).

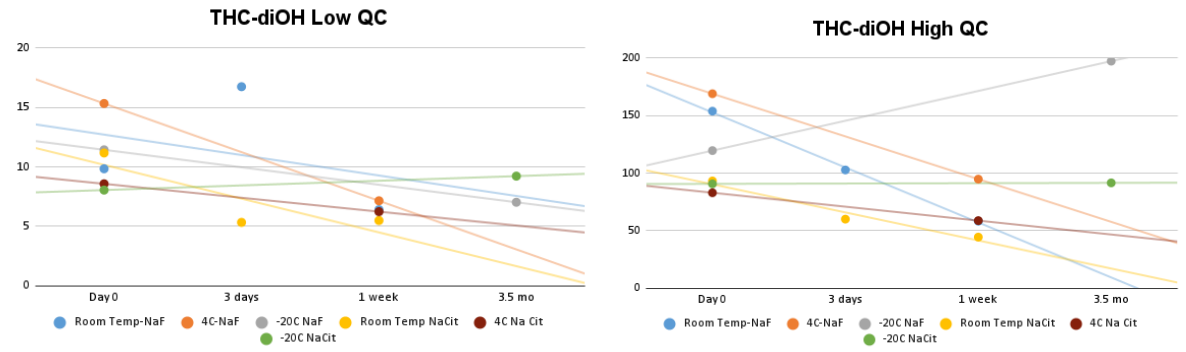

Fig. 6. Trend lines of THC-diOH in whole blood samples fortified at low and high concentrations in sodium fluoride and sodium citrate collection tubes at different storage conditions.

For THC-glucuronide, stability issues occurred at room temperature and 4°C in sodium citrate tubes and at 4°C in sodium fluoride tubes (Fig. 7).

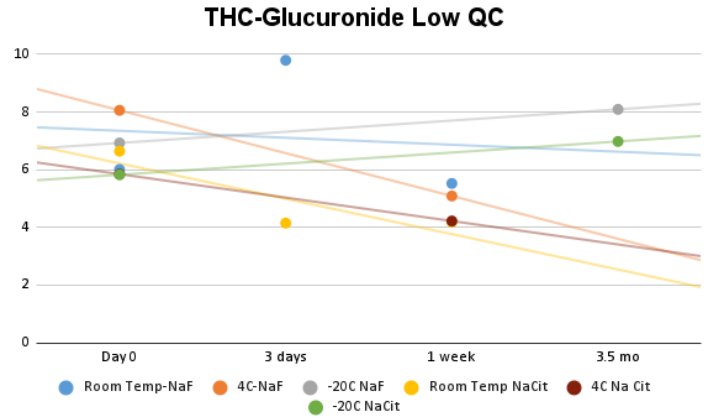

Fig. 7. Trend lines of THC-glucuronide in whole blood samples fortified at low concentrations in sodium fluoride and sodium citrate collection tubes at different storage conditions.

CBD and CBN were mostly stable except when stored in sodium fluoride tubes at -20°C in low QC samples (Fig. 8 for CBD; Fig.9 for CBN).

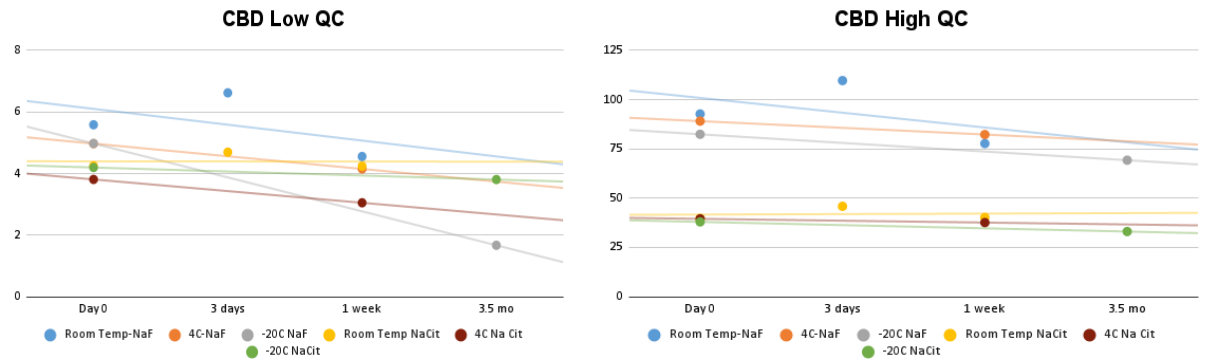

Fig.8. Trend lines of CBD in whole blood samples fortified at low and high concentrations in sodium fluoride and sodium citrate collection tubes at different storage conditions.

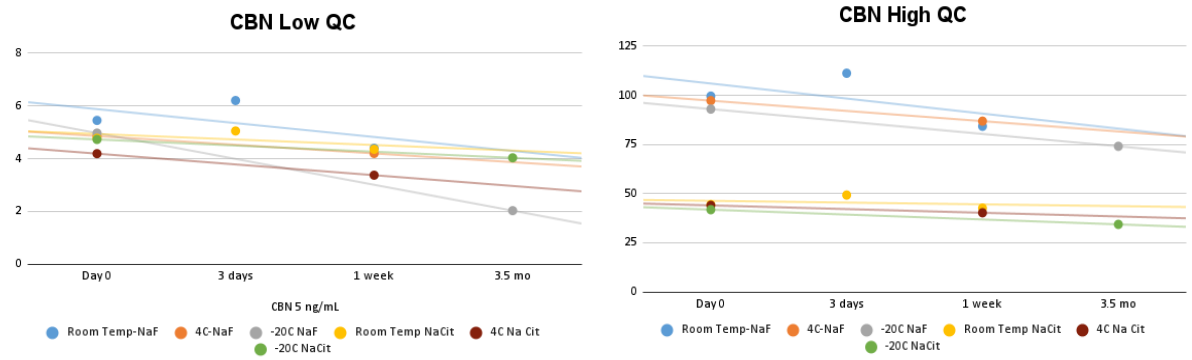

Fig.9. Trend lines of CBN in whole blood samples fortified at low and high concentrations in sodium fluoride and sodium citrate collection tubes at different storage conditions.

In this study, each storage condition had a time zero sample, to enable a more accurate monitoring of changes in the concentration over time. From our findings, THC and THCCOOH were both stable for one week at room temperature and 4°C in sodium fluoride and sodium citrate tubes. These results correlate with a similar study carried out on gray top tubes, which found THC to be stable for one week at room temperature and for up to 6 months at 4°C (Scheidweiler et al., 2016), and THCCOOH to be stable for up to 4 weeks at 4°C (Scheidweiler et al., 2016). However, the same group reported a decrease in THC concentrations when stored in sodium heparin (green top) tubes for one week at room temperature (Scheidweiler et al., 2013). Eibel et al. (2019) also

found THC and THC-COOH to be unstable for one week when stored in sodium heparin tubes. Regarding long-term storage, THC and THC-COOH have been stable for up to 4 and 3 months respectively, at -20°C in both gray top tubes and green top tubes (Scheidweiler et al., 2013, 2016), but our study observed a decrease in both THC and THC-COOH concentrations, specially at low concentrations, after 3.5 months at the same storage temperature in sodium fluoride tubes.

In our study, THCCOOH-glucuronide and THC-OH were unstable when stored for 1 week at room temperature, but stable at 4°C for one week in sodium fluoride tubes. This correlates with a similar finding where both metabolites decreased in sodium fluoride tubes after storage for one week at room temperature and remained stable at 4°C (Scheidweiler et al., 2016).

Currently, there are limited studies on the stability of THC-glucuronide and THC-diOH, but our study assertained that THC-diOH showed the least stability, as it was only stable at -20°C in sodium citrate tubes. THC-glucuronide showed stability issues in sodium fluoride (one week at 4°C) and in sodium citrate tubes (3 days and one week at room temperature, and one week at 4°C). CBD and CBN showed very similar stability patterns as they both only decreased at-20°C after 3.5 months at low concentrations.

In forensic toxicological cases, the gray top tubes containing sodium fluoride and potassium oxalate additive are the recommended tubes for blood collection (Toennes & Kauert, 2001). It is also ideal for samples to be stored frozen, approximately at -20°C for effective long-term storage (Sorensen & Hasselstrom, 2018). However in this study, contrary findings were observed upon storage of blood samples in sodium fluoride and sodium citrate tubes at different storage conditions. Analytes were mostly unstable in blood samples stored at -20°C when stored in sodium fluoride tubes for a 3.5 month period. Instead, samples were the most stable in the sodium citrate tubes. Thus, further research is recommended for the determination of the ideal

blood collection tube and storage conditions to be used in forensic investigations involving cannabinoid quantification in blood.

4. Conclusion

A simple, cheap, and sensitive method was developed for the quantification of CBD, CBN, THC and 5 metabolites in blood using LC-MSMS, with a LOQ from 0.5 to 5 ng/mL. Among all the target cannabinoids, THC-diOH was the most unstable analyte. Overall, the analytes had the most stability issues at low concentrations at -20°C after 3.5 months of storage, and greater stability was achieved when the samples were stored in sodium citrate collection tubes.

References

Arkell, T.R., Spindle, T. R. Kevin, R.C., Vandrey, R., & McGregor, I.S. (2021). The failings of perse limits to detect cannabis-induced driving impairment: Results from a simulated driving study. *Traffic Injury Prevention*, 22(2), 102-107, DOI:10.1080/15389588.2020.1851685

Asbridge, M. (2014). Driving After Marijuana Use: The Changing Face of "Impaired" Driving. *JAMA Pediatrics*, 168(7), 602-604.

Bédard, M., Dubois, S., & Weaver, B. (2007). The Impact of Cannabis on Driving. *Canadian Journal of Public Health, 98*(1), 6-11.

Brady, J.E., & Li, G. (2014). Trends in Alcohol and Other Drugs Detected in Fatally Injured Drivers in the United States, 1999-2010. *American Journal of Epidemiology*, 179(6), 692–699. https://doi.org/10.1093/aje/kwt327

Centers for Disease Control and Prevention (CDC) (2019). Marijuana and Public Health. Retrieved from https://www.cdc.gov/marijuana/index.htm on July 22, 2020

Compton, R. (2017, July). Marijuana-Impaired Driving - A Report to Congress. (DOT HS 812 440). Washington, DC: National Highway Traffic Safety Administration.

Coulter, C., Miller, E., Crompton, K., & Moore, C. (2008). Tetrahydrocannabinol and Two of its Metabolites in Whole Blood Using Liquid Chromatography-Tandem Mass Spectrometry. *Journal of Analytical Toxicology, 32*(8), 653-658.

Desrosiers, N., Himes, S. K., Scheidweiler, K. B., Concheiro-Guisan, M., Gorelick, D. A., & Huestis, M. A. (2014). Phase I and II cannabinoid disposition in blood and plasma of occasional and frequent smokers following controlled smoked cannabis. *Clinical Chemistry*, 60(4), 631–643. https://doi.org/10.1373/clinchem.2013.216507

Dybowski, & Dawidowicz, A. L. (2018). Application of the QuEChERS procedure for analysis of Δ9-tetrahydrocannabinol and its metabolites in authentic whole blood samples by GC–MS/MS. *Forensic Toxicology, 36(*2), 415–423.

Eibel, Ameline, A., Raul, J.-S., & Kintz, P. (2019). Stability of Δ9-THC, 11-OH-THC and THC-COOH in Whole Blood in Presence of Formalin Solution. *Journal of Analytical Toxicology*, 43(6), e1–e3. https://doi.org/10.1093/jat/bkz037

Governors Highway Safety Association (GHSA) (2021) Marijuana Drug Impaired Driving Laws. DOI: https://www.ghsa.org/statelaws/issues/drug%20impaired%20driving

Johnson, J. R., Jennison, T. A., Peat, M. A., & Foltz, R. L. (1984). Stability of delta 9-tetrahydrocannabinol (THC), 11-hydroxy-THC, and 11-nor-9-carboxy-THC in blood and plasma. *Journal of analytical toxicology*, *8*(5), 202–204. https://doi.org/10.1093/jat/8.5.202

Karschner, Schwilke, E. W., Lowe, R. H., Darwin, W. D., Pope, H. G., Herning, R., Cadet, J. L., & Huestis, M. A. (2009). Do 9-tetrahydrocannabinol concentrations indicate recent use in chronic cannabis users? *Addiction*, 104(12), 2041–2048. https://doi.org/10.1111/j.1360-0443.2009.02705.x

Kim, J., Castro, A., Lendoiro, E., Cruz-Landeira, A., López-Rivadulla, M., & Concheiro, M. (2018). Detection of in utero cannabis exposure by umbilical cord analysis. *Drug Testing and Analysis*, 10(4), 636–643. https://doi.org/10.1002/dta.2307

König, S., Aebi, B., Lanz, S., Gasser, M., & Weinmann, W. (2011). On-line SPE LC-MS/MS for the quantification of [DELTA] 9-tetrahydrocannabinol and its two major metabolites in human peripheral blood by liquid chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 400(1), 9. https://doi.org/10.1007/s00216-011-4708-x

Lacroix, C., & Saussereau, E. (2012). Fast liquid chromatography/tandem mass spectrometry determination of cannabinoids in micro volume blood samples after dabsyl derivatization. *Journal of Chromatography, 905*, 85-95.

LeBeau, M. (2020). ANSI/ASB Standard 036 for Method Validation in Forensic Toxicology Has Replaced SWGTOX's Version. *Journal of Analytical Toxicology*, 44(4), 414–414. https://doi.org/10.1093/jat/bkz115

Meneses, V., & Mata, D. (2020). Cannabinoid Stability in Antemortem and Postmortem Blood. *Journal of Analytical Toxicology*, 44(2), 126–132. https://doi.org/10.1093/jat/bkz073

National Highway Traffic Safety Administration. (2007). National Highway Traffic Safety Administration (NHTSA) Notes. *Annals of Emergency Medicine, 49*(2), 218-219.

National Institute on Drug Abuse (NIDA). (2020) What is the scope of marijuana use in the United States? Retrieved from https://www.drugabuse.gov/publications/research-reports/marijuana/what-scope-marijuana-use-in-united-states, on July 22, 2020.

O'Malley, P., & Johnston, L. (2013). Driving after drug or alcohol use by US high school seniors, 2001-2011. *American Journal of Public Health, 103*(11), 2027-2034.

Peterman, D. R. (2019). Marijuana use and highway safety. Congressional Research Service.

Purschke, K., Heinl, S., Lerch, O., Erdmann, F., & Veit, F. (2016). Development and validation of an automated liquid-liquid extraction GC/MS method for the determination of THC, 11-OH-THC, and free THC-carboxylic acid (THC-COOH) from blood serum. *Analytical and Bioanalytical Chemistry*, 408(16), 4379–4388. https://doi.org/10.1007/s00216-016-9537-5

Raikos, N., Schmid, H., Nussbaumer, S., Ambach, L., Lanz, S., Längin, A., König, S., Roth, N., Auwärter, V., & Weinmann, W. (2014). Determination of Δ9-tetrahydrocannabinolic acid A (Δ9-THCA-A) in whole blood and plasma by LC–MS/MS and application in authentic samples from drivers suspected of driving under the influence of cannabis. *Forensic Science International*, 243, 130–136. https://doi.org/10.1016/j.forsciint.2014.07.026

Salimiasl, S.M., Khodayar, M.j., Mousavi, Z., & Akhgari, M.. (2017). Comparison of the Modified QuEChERS Method and the Conventional Method of Extraction in Forensic

Medicine to Detect Methadone in Post-Mortem Urine by GCMS. *Asia Pacific Journal of Medical Toxicology*, 6(3), 79–85. https://doi.org/10.22038/apjmt.2017.9402

Scheidweiler, K. B., Schwope, D. M., Karschner, E. L., Desrosiers, N. A., Gorelick, D. A., &Huestis, M. A. (2013). In vitro stability of free and glucuronidated cannabinoids in blood and plasma following controlled smoked cannabis. *Clinical Chemistry*, *59*(7), 1108+.

Scheidweiler, K.B., Newmeyer, M., Barnes, A., &Huestis, M. (2016). Quantification of cannabinoids and their free and glucuronide metabolites in whole blood by disposable pipette extraction and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 1453, 34-42.

Schwope, D. M., Karschner, E. L., Gorelick, D. A., & Huestis, M. A. (2011). Identification of recent cannabis use: Whole-blood and plasma free and glucuronidated cannabinoid pharmacokinetics following controlled smoked cannabis administration. *Clinical Chemistry, 57*(10), 1406-14014.

Sharma, P., Murthy, P., & Bharath, M. M. S. (2012). Chemistry, metabolism, and toxicology of cannabis: clinical implications. *Iranian Journal of Psychiatry*, 7(4), 149–156.

Simões, S.S., Ajenjo, A. C., & Dias, M. J. (2011). Qualitative and quantitative analysis of THC, 11-hydroxy-THC and 11-nor-9-carboxy-THC in whole blood by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid*

Communications in Mass Spectrometry, 25(18), 2603–2610. https://doi.org/10.1002/rcm.5165

Sørensen, L., & Hasselstrøm, J. (2018). The effect of antioxidants on the long-term stability of THC and related cannabinoids in sampled whole blood. *Drug Testing and Analysis*, 10(2), 301-309.

Stout, P., Horn, C., & Lesser, D. (2000). Loss of THCCOOH from urine specimens stored in polypropylene and polyethylene containers at different temperatures. *Journal of Analytical Toxicology, 24*(7), 567-571.

Substance Abuse and Mental Health Services Administration. (2020). Key substance use and mental health indicators in the United States: Results from the 2019 National Survey on Drug Use and Health (HHS Publication No. PEP20-07-01-001, NSDUH Series H-55). Retrieved from https://www.samhsa.gov/data/

Toennes, S., & Kauert, G. (2001). Importance of Vacutainer Selection in Forensic Toxicological Analysis of Drugs of Abuse. *Journal of Analytical Toxicology, 25*(5), 339-343.

Vindenes, V., Lund, H., Andresen, W., Gjerde, H., Ikdahl, S., Christophersen, A., & Øiestad, E. (2012). Detection of drugs of abuse in simultaneously collected oral fluid, urine and blood from Norwegian drug drivers. *Forensic Science International, 219*(1-3), 165-171.

Wiedfeld, C., Krueger, J., Skopp, G., & Musshoff, F. (2018). Comparison of concentrations of drugs between blood samples with and without fluoride additive—important findings for Δ9-tetrahydrocannabinol and amphetamine. *International Journal of Legal Medicine*, 133(1), 109–116. https://doi.org/10.1007/s00414-018-1797-5

Wolff, K., & Johnston, A. (2014). Cannabis use: A perspective in relation to the proposed UK drug-driving legislation. *Drug Testing and Analysis, 6*(1-2), 143-154.

Wong, K., Brady, J., & Li, E. (2014). Establishing legal limits for driving under the influence of marijuana. *Injury Epidemiology,* 1(1), 1-8.

World Drug Report (2019) Cannabis and hallucinogens (United Nations publication, Sales No. E.19.XI.8). Retrieved from https://wdr.unodc.org/wdr2019/prelaunch/WDR19_Booklet_5_CANNABIS_HALLUCINOGENS.pdf