MASTER IN ANALYTICAL CHEMISTRY

THESIS FOR THE NORWEGIAN PHARMACY DEGREE

CANDIDATUS PHARMACIAE

DEVELOPMENT OF AN ANALYTICAL METHOD FOR DETECTION AND QUANTIFICATION OF CANNABIS AND CANNABINOID ANALOGUES IN URINE

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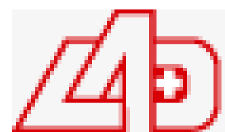
May 2009



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Preface

This master thesis was performed at the Swiss Laboratory for Doping Analysis (LAD) in Lausanne, Switzerland. The analytical work was performed in the period November 2009-May 2010.

Dr. Norbert Baume (LAD) has been the external supervisor, while Professor Einar Jensen (UiT) has been the internal supervisor.

Summary

Cannabis is a drug mainly consumed for its euphoric effects; users may become happy, careless and relaxed. The direct effects of cannabis in sports are performance decreasing, but cannabis can be used as a doping agent due to its relaxing properties. For these reasons, cannabinoids are prohibited in sports during competition by the World Anti-Doping Agency (WADA).

In the context of the fight against doping, urine is screened for the metabolite THC-COOH (11-nor-delta(9)-tetrahydrocannabinol-9-carboxylic acid or carboxy-THC) with a cut-off of 15 ng/mL as a reporting limit for adverse analytical finding. This criterion does not fully allow a differentiation of in- or out-of-competition abuse and might not distinguish between active and passive inhalation.

The presence of THC (Delta-9-tetrahydrocannabinol) and THC-OH (11-hydroxy-delta9-tetrahydrocannabinol) metabolite in urine has been proposed as indicators for recent cannabis use. Thus the need to develop and implement a method for detection and quantification of THC, THC-OH, and THC-COOH in urine appeared to be necessary in an anti doping laboratory. High sensitivity and specificity are required in such analytical method and for that purpose a GC-TSQ (Gas chromatography-triple quadrupole) was used.

A method for the detection of the compounds was developed regarding GC-TSQ parameters, sample extraction and conditions of hydrolysis. The method was validated for specificity, but, due to several problems, it was not possible to fully validate this new method. The preliminary results obtained during this project suggest that the method should be further validated.

Acknowledgments

I would like to thank my supervisor Dr. Norbert Baume and Dr. and PD. Martial Saugy, Technical Director of LAD for welcoming me as a master student, and giving me access to all necessary laboratory equipment and facilities.

I would also like to thank Dr. Norbert Baume and Einar Jensen for being my supervisors. I would like to thank Dr. Norbert Baume for being very helpful regarding the writing of my thesis and for spending a lot of time helping me with different problems in my project. I would like to thank Professor Einar Jensen for establishing the contact between Department of Pharmacy and Laboratoire Suisse D`Analyse du dopage, for reading through my thesis and giving me good advice.

I would like to thank everybody working at the LAD for welcoming me and helping me with the different problems that occurred during my stay. I want to thank some of you especially in the connection with my stay:

- Dr. Emmanuel Strahm and PhD student François Marclay for introducing me to the lab and the city, and driving me and my luggage around the first weeks in Lausanne. The help from Dr. Emmanuel Strahm has meant a great deal to me, he has introduced me to the GC-TSQ, being involved in my project and always answering my questions promptly and with great patience.
- The PhD students Flavia Badouid and François Marclay for all the discussions regarding my project, and for helping me out when everything seemed impossible. Flavia have been very helpful regarding practical problems during my stay.
- Carine Schweizer for good advice and helping me with different instruments on the lab.

Especially, I would like to thank my friend Ida Lange for coming with me to Switzerland, for being kind, impulsive and open minded – you have been a perfect exchange-partner!

At last I would like to thank my family and my friends for supporting me. Thanks also to my father for reading trough my report, and giving me advice during the writing process.

Lausanne, May 15th, 2010

Eirin Berge Steinshamn

1 Abbreviations

AcN: Acetonitrile

Bgluc: Beta-glucuronidase

BSTFA: O-bis(trimethlysilyl)acetamide

Carboxy-THC: THC-COOH, 11-nor-delta(9)-tetrahydrocannabinol-9-carboxylic acid

CB1: Cannabinoid 1

CB2: Cannabinoid 2

CIF: Cannabis influence factor

CP47,497: 5(1-dimetilheptil – 2 ((1R,3S) – 3 hydroxycyclohexyl)-phenol

CE: Collision energy

EI: Electron ionization

EPO: Erythropoetin

GC: Gas chromatography

GC-MS: Gas chromatography – Mass spectrometry

GC-TSQ: Gas chromatography – Triple quadrupole

h: hour

H. pomata: Helix Pomata

ICH: The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

IAAF: International Sport Federation

IOC: The International Olympic Committee

HCl: Hydrochloric acid

HP: Hewlett Packard

Hrs: Hours

HU-210: (6a*R*,10a*R*)- 9-(Hydroxymethyl)- 6,6-dimethyl- 3-(2-methyloctan-2-yl)- 6a,7,10,10a -tetrahydrobenzo [c]chromen- 1-ol

INT.STD: internal standards (THCd3, THC-OHd3, THC-COOHd3)

JWH-018: 1-pentil-3-(1-naftoil)indol

JWH-073:	Naphthalene-1-yl-(1-butylindol-3-yl)methanone
JWH-200:	2-(2-Methoxyphenyl)-1-(1-pentylindol-3-yl)ethanone)
JWH-250:	1-[2-(4-Morpholino)ethyl]-3-(1-naphthoyl)indole
K_2HPO_4:	Dipotassium hydrogen phosphate
KH_2PO_4:	Potassium dihydrogen phosphate
KOH:	Potassium hydroxide
LAD:	Laboratoire Suisse d`Analyse du Dopage
	Swiss Laboratory for Analysis of Doping
LC:	Liquid chromatography
LLE:	Liquid liquid extraction
LLOQ:	Lower limit of quantification
LOD:	Lowest level of detection
m/z:	Mass to charge ratio
MRM:	Multiple reaction monitoring
MS:	Mass spectrometry
MSTFA:	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
NaOH:	Sodium hydroxide
NL:	Intensity of a signal
RSD:	Relative standard deviation
$Si\text{-}CH_2CH_2CH_2NH_3^+$:	Protonized aminopropyl attached to silica.
SIM:	Single ion monitoring
S/N:	Signal to noise ratio
SPE:	Solid phase extraction
STD:	standards (THC, THC-OH, THC-COOH)
THC:	Delta-9-tetrahydrocannabinol
THCd3:	Delta9-tetrahydrocannabinol-d3
THC-COOH:	Carboxy-THC, 11-nor9-carboxy-delta-9-tetrahydrocannabinol
THC-COOHd3:	11-nor9-carboxy-delta9-tetrahydrocannabinol-d3
THC-COOHd9:	11-nor-9-carboxy-delta9-tetrahydrocannabinol-d9
THC-OH:	11-hydroxy-delta9-tetrahydrocannabinol
THC-OHd3:	11-hydroxy-delta9-tetrahydrocannabinol-d3
THC-TMS:	Delta-9-tetrahydrocannabinol-trimethylsilyl ether

THCd3-TMS: Delta9-tetrahydrocannabinol-d3-trimethylsilyl ether

THC-COOH-2TMS: Carboxy-THC, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol-2trimethylsilyl ether

THC-COOHd3-2TMS: 11-nor-9-carboxy-delta9-tetrahydrocannabinol-d3-2trimethylsilyl ether

THC-COOHd9-2TMS: 11-nor-9-carboxy-delta9-tetrahydrocannabinol-d9-2trimethylsilyl ether

THC-OH-2TMS: 11-hydroxy-delta9-tetrahydrocannabinol-2trimethylsilyl ether

THC-OHd3-2TMS: 11-hydroxy-delta9-tetrahydrocannabinol-d3-2trimethylsilyl ether

TMCS: Trimethylchlorosilane

TMS: Trimethylsilyl

TSQ: Triple stage quadrupole

µm: Microampere

eV: Electron volt

U: Units

UiT: University of Tromsø

ULOQ: Upper limit of quantification

WADA: World Anti-Doping Agency

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3 Aim of the thesis

This work has been done in the Swiss Laboratory for Doping Analyses (LAD) in Switzerland. In the context of anti doping analyses, several topics need constantly to be resolved. During the 8 months spent in the laboratory, I have focused on the cannabinoids detection in urine.

Presently, the detection of cannabis misuse in sports is based on the urinary detection of THC-COOH. A cut-off limit of 15 ng/mL is set by the WADA through the 2009 Code and the Prohibition List which is updated every year. This cut-off limit is highly debated as it is difficult to differentiate between passive and active intake of the drug and also to determine if cannabis has been used on purpose to increase the athletics performance or only as a “social drug”.

Numerous authors have already investigated the analysis of cannabinoids in different biological matrices and the quantification of THC and THC-OH in addition to THC-COOH could be helpful to answer the above questions.

Hence, the first aim of this work was to develop, validate and implement an analytical method based on GC-MS/MS (Gas chromatography technology to quantify THC, THC-OH and THC-COOH in urine. Sample preparation and analytical separation and detection have been tested in order to get the best quantification results and the lowest sensitivity of the method.

After the validation process, the objective will be to analyze “true” urine samples coming from doping control collection events. Negative and THC-COOH positive samples will be analyzed to quantify the 3 compounds of interest.

Recently, herbal blends containing synthetic cannabinoid analogues have been introduced on the drug market. These blends are illegally consumed as an alternative to cannabis. It is well known that the effects of these cannabinoids analogues are quite the same that the ones of THC and the abuse of such drugs would probably become a topic in the fight against doping. Thus, an analytical detection of herbal blends is now necessary for the anti doping laboratories.

In summary, the aims of this work will be:

1. Develop, validate and implement a new GC-MS/MS analytical approach to quantify THC and its metabolites in urine.
2. Quantify true samples that have already been found as adverse analytical finding with a THC-COOH concentration above the cut-off limit.
3. Develop and implement a detection method for the cannabinoids analogues in urine.

4 Introduction

4.1 General considerations about doping in sports

Doping in sport can be defined as

”*a practice that consists in the absorption of specific substances or the practice of specific medical acts in order to artificially enhance one’s physical or mental capacities*” [1].

The use of different substances to improve sport performance is an old phenomenon; even the Greeks in the antique period used different potions and diets to enhance performances. In 19th century, strychnine, caffeine, cocaine and alcohol were used to improve performance in sports. In 1928, doping was banned by the International Association of Athletics Federations (IAAF). Other federations did the same, but since there were no detection methods, the banning was not very effective. The first doping-tests were introduced in 1966, and drug-testing was introduced by most of the international sport federations by 1970. Unfortunately the tests were not always good enough; i.e. the use of anabolic steroids was forbidden in 1970, but a reliable test was not introduced until 1974. Another example is erythropoietin (EPO), which was introduced at the prohibited list inn in 1996, and a dependable test was not introduced until 2000 [2].

The fight against doping is coordinated by WADA. Their goal is:

“*to promote, coordinate and monitor the fight against doping in sport in all its forms*” [3].

One important achievement from WADA is the World Anti-Doping Code (Code). This is a framework of anti-doping rules, policies and regulations to harmonize the fight against doping. Included in the code is the prohibition list, which is a list over all the substances and methods, banned from sports in- and out of competition. The different compounds are divided in several classes which are forbidden either out-/and in-competition or only during competition (table 1).

	Out- / In-competition	In-competition
Classes	S1. Anabolic steroids S2. Peptide hormones S3. Beta-2 agonists S4. Hormones S5. Diuretics	S6. Stimulants S7. Narcotics S8. Cannabinoids S9. Glucocorticosteroids

Table 1: Forbidden substances out-/and in-competition

To be included on this list, a substance or a method needs to fulfill 2 of the following 3 criteria [2]:

a) Performance-enhancing

b) Possible health risk for the athlete

c) Damages the sports reputation.

As cannabis fulfills all these criteria, it is included on this list. First, cannabis can, but only indirectly, improve performance. When consuming cannabis, the user may become euphoric, and anxiety may be reduced and sociability can be increased with a person who is particularly nervous before a game. The relaxing effect of cannabis and the increased feeling of wellbeing, can help the athlete to sleep more easily [4]. The sleeping quality can also be improved because THC can reduce alertness and help athletes to escape from social pressure [5]. Second, use of cannabis can be a health risk for the athlete; the user could end up dysphoric and paranoid, or, if used regularly, the user can become chronically sedated and socially detached [4]. This kind of use is also associated with development of diseases as schizophrenia and depression [6]. Third, use of cannabis in a sport can damage the reputation of the sport; the association of cannabis consumption and sport will prevent the athletes to appear as healthy idols.

There are 35 anti-doping laboratories around the world, which daily screen for the presence of hundreds of drugs, metabolites and markers of prohibited substances in biological fluids. The analytical methods used in the anti-doping laboratories mainly rely on the use of chromatography combined with mass spectrometry (MS).

The anti doping laboratories have 3 main tasks that are

1. Widen the range of doping substances and doping methods that can be detected.
2. Prolong the interval of time between use of a doping substance/method and the time of possible detection.
3. Increase reproducibility and robustness of the analytical results [7].

4.2 Cannabis

4.2.1 Pharmacology and effects

The psychoactive substance of cannabis is Δ-9-tetrahydrocannabinol (THC). This compound binds to benzodiazepine, opioid and cannabinoid receptors. There are two types of cannabinoid receptors, cannabinoid 1 (CB1) and cannabinoid 2 (CB2) [5]. CB1 are found in the brain, especially in regions involved in memory, cognition, anxiety, reward, sensory perception, pain, endocrine function and motor coordination, and activation of CB1-receptors will then influence the properties controlled by these areas. CB2 receptors are present in the peripheral tissues [8], mainly in lymphoid tissues (i.e. tonsils, thymus and spleen). CB2-receptors can also be found in the immune system, which may explain the immune depressant effect of cannabis [9].

There is less knowledge with respect to the CB2-receptors than the CB1-receptor [9], thus the known effects of cannabis is mainly restricted to the effects in the brain.

The main reason for consumption of cannabis is the euphoric effect. The user may become careless, happy and relaxed, but could also end up dysphoric, anxious, psychotic, paranoid and in panic. Intake of cannabis can also be followed by depression or stress. If cannabis is consumed regularly, the user can become psychologically dependent, chronically sedated and social detached. Increasing the dose can give hallucinations and an alteration of the perception of reality [4, 8].

Cannabis possesses a generalized central nervous system depressant effect, which leads to drowsiness towards the end of intoxication. Intake of cannabis decreases reaction time and causes memory problems. Concentration, driving skills and the ability to process complex information are also impaired. These problems can last for several weeks, even if the user is not intoxicated [4, 8, 10].

4.2.2 Pharmacokinetics and detection of THC in blood

Cannabis is usually inhaled or ingested. Inhalation gives a faster absorption (plasma peak concentration is reached between 3 and 10 minutes) compared to ingestion (plasma peak concentration is reached after 1-2 hours). The amount absorbed is also reduced by a factor of 3 to 4 when ingested.

After entering the circulation, the cannabinoids are rapidly distributed throughout the body. Because of the high fat solubility, cannabinoids accumulate in fat tissues (i.e. liver, lung, spleen), and from there they are slowly released back into other body compartments [8] [11].

In blood 10 % of the cannabinoids are located in erythrocytes and the rest is found in plasma. Between 95 and 99 % of the drug is bound to plasma proteins, mainly lipoprotein and albumin.

Many authors have investigated the concentrations of cannabinoids in blood. A study has shown that the plasma concentration of THC decreases rapidly, after smoking cigarettes containing 16 and 34 mg cannabis. This caused average plasma concentrations of 84.3 ng/mL (range 50-129 ng/mL) and 162.2 ng/mL (range 76-267 ng/mL) for THC and THC-OH respectively. The plasma concentration decreased rapidly to reach a value below 5 ng/mL after 3-4 hrs. However, when the plasma concentrations of THC are decreasing, the amounts of metabolites (11-OH-THC and THC-COOH) were increasing. The peak concentration for THC, THC-OH and THC-COOH after start of smoking was observed after 8 min (range 6-10 min), 15 min (range 9-23 min) and 81 min (range 32-133 min) respectively [12].

6 hours after intravenous administration, pseudo equilibrium is reached between plasma and tissues, and THC is slowly released from tissues back into blood. At this point, the elimination starts to slow down, and the concentration in plasma of THC is < 2 ng/mL [13] [11].

Even though THC plasma concentration decreases rapidly, the terminal half-life is long, approximately 7 days. A complete elimination of a single dose may take up to 30 days [8]. The long terminal half-life is due to both accumulation of THC into lipophilic tissue, followed by a slow release back into blood and the binding of THC to plasma proteins [11, 14]. Thus the plasma half-life is dependent on the fat mass of the body but also to the frequency of use.

Different biological matrices (urine, blood i.e.) have been proposed for the detection of cannabis use. All have their limitations, and the choice of the appropriate sample is critical and depends on the context.

For interpretation of acute effects after cannabis use, blood analysis is preferred [11, 15]. However, there are some problems with detection of cannabis in blood. Several cannabinoids are present in blood after marijuana exposure and their relative concentrations change over time. Also, the concentration of THC decreases so rapidly that the samples need to be collected within 2 hours after intake. To avoid this limitation THC-COOH plasma concentration is often measured. Blood sampling is also invasive and requires trained personnel, which is not the case when analyzing urine.

Urine is still the preferred biological matrix for anti doping analyses even though blood is becoming more and more considered. Thus, cannabinoids have to be precisely detected and quantified in urine within anti doping laboratories.

Only small amounts of the THC are excreted in the urine because of extensive metabolism [5] and reabsorption of THC in the kidneys. The most abundant urinary metabolite of THC is 11-nor-Δ9-tetrahydrocannabinol (THC-COOH) [11]. THC-COOH is an inactive metabolite, and a result of the oxidation of the active metabolite 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH), which again is a result of oxidation of THC (Figure 1).

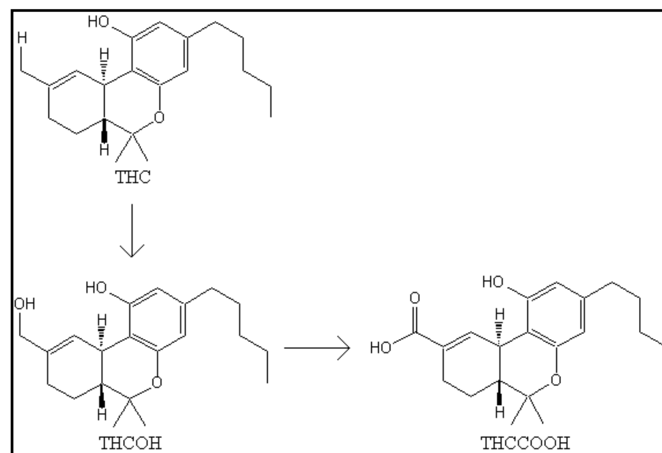


Figure 1: Metabolism of THC via THC-OH to THC-COOH

THC-COOH, THC-OH and THC are excreted in urine mainly as a glucuronic acid conjugate [16] (Figure 2). There are mainly two analytical techniques to analyze these compounds. The first is through liquid chromatography (LC) which requires a simple sample preparation as the glucuronised compounds could be detected as such [17]. The second analytical method is via gas chromatography and involves a more complex sample preparation including a hydrolysis.

THC-COOH is glucuronised by a ester-linkage, whereas an ether linkage attaches the glucuronide to THC and THC-OH (Figure 2) [18]. Thus THC-COOH can be submitted to basic hydrolysis, while THC and THC-OH must be hydrolyzed by enzymes. A study compared the yield of hydrolysis using β-glucuronidase produced from two different bacteria (*Helix Pomata* and *Escherichia coli*) and found that β-glucuronidase from *E.coli* gave the best yield for THC and THC-OH. However, there was no difference regarding THC-COOH [19].

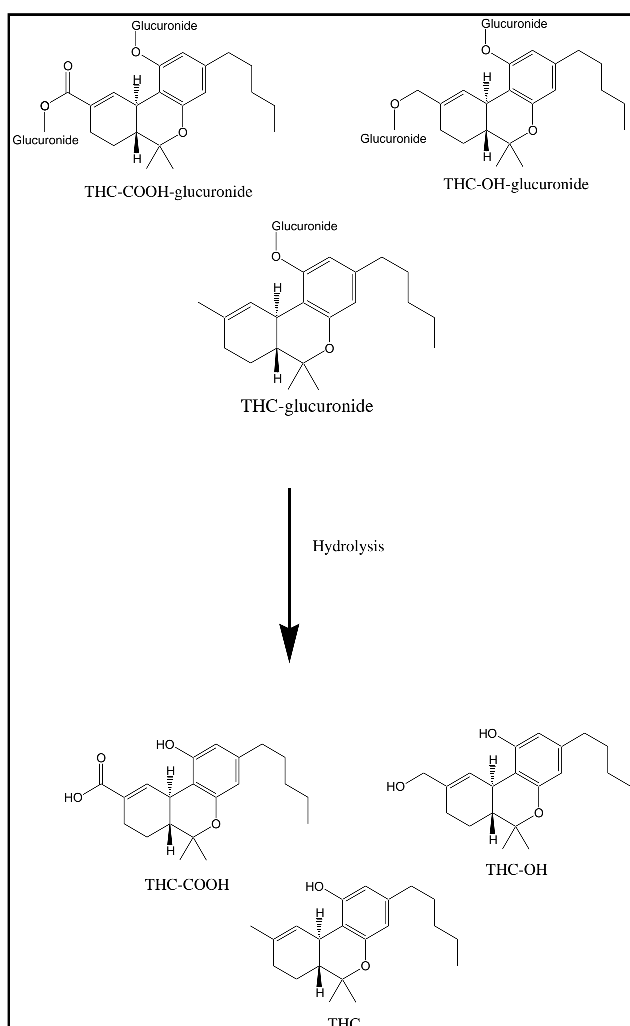


Figure 2: The glucuronide are linked to THC and THC-OH by an ether linkage and to THC-COOH by both an ester linkage and an ester linkage. The compounds are hydrolyzed to their non-conjugated forms and glucuronic acid. Inspired by [18, 20].

There is a conflict in reported half-life for excretion of metabolites; some have reported an average urinary half-life of THC-COOH of about 45 to 60 hours, whereas other groups have reported wider time ranges [14].

Different factors such as frequency of cannabis use, body fat mass and degree of urine dilution can affect the concentration of THC metabolites in urine. Considering these factors, it is problematic to determine the time of last consumption [11].

4.2.3 Passive versus active cannabis inhalation

Cannabis can be consumed either by inhalation or ingestion. Ingestion is usually done by eating muffins, cakes etc. containing cannabis. Inhalation is the preferred route, usually by smoking a cannabis cigarette (a “joint”). The smoke from this cigarette will not only be inhaled by the consumer, but also be distributed into the surrounding area exposing other people for inhalation of THC. Whether passive inhalation can give an adverse analytical finding or not is highly debated, and several people with a positive cannabis sample have claimed passive inhalation [21, 22].

Several studies have been performed to examine the detectability of passive inhalation [22-28], and in 2009, a meta analysis based on these studies (among others) concluded that it is unlikely to fail a doping-test due to unknown passive smoking of cannabis. The authors also indicated that urine samples positive for THC-COOH should be considered as an expression for active smoking [21]. However, the studies was performed several years ago (1977-1996) using a THC content in the cannabis cigarettes (5-95 mg [21]), that might not reflect the situation today.

In the 60`s and the 70`s, the normal THC content of cannabis was about 10 mg, but due to sophisticated breeding of cannabis plants, a THC-cigarette today could contain up to 150 mg, or 300 mg if laced with hashish oil, dependent on the sub specie of cannabis used [10]. This is confirmed by a report from USA, where the THC-content in cannabis increased from 4 to 10 % within the time period 1983 to 2007 [29]. The situation might be different in Europe as confiscated cigarettes in 1998 had a THC content of 10 mg THC [30]. Anyhow, these findings are suggesting that the THC-content in cannabis products varies with the time period and location and needs to be considered in future studies of passive inhalation.

As the urinary cannabinoid concentrations in urine are dependent on the amount inhaled, less abundant compounds such as THC and THC-OH might not be present in urine after pas-

sive inhalation. Thus the application of these compounds in urine analysis can give information about the consumption of cannabis; whether it is passive or active.

4.3 Cannabis and doping

4.3.1 Cannabis consumption in sport

The direct effect of cannabis on sporting performance is negative. It is an ergolytic drug, which means it impairs motor, psychomotor and exercise performance [4]. The reduced motor performance includes impaired measurement of tracking ability, body-sway, hand-eye-coordination, pursuit rotor performance, reaction time and physical strength among others [8]. Despite all these negative effects, the incidence of positive cases for cannabinoids is high [31] - it is actually on the top of the list of compounds detected by the anti-doping laboratories accredited by WADA. Usually, the consumption of cannabis happens outside sports facilities, and is thereby not controlled by team coaches and/or doctors [4].

4.3.2 The anti doping strategy for detection of cannabis

According to the IOC and WADA, urine samples are considered as adverse analytical findings if the total concentration of free and conjugated carboxy-THC in urine is above 15 ng/mL when determined by GC-MS [4, 5]. The limit of 15 ng/mL is supposed to distinguish between passive smokers and active use. It also decreases the risk of a false positive result because of intake of contaminated food products [5].

Unfortunately, there are some problems with this limit. Because of the long and diverse half-life of THC-COOH the strategy does not fully differentiate between use in-/and outside a competition, [4] [5, 13].

As several persons convicted for cannabis consumption claims that they have been passive exposed for cannabis [21, 22] and as the THC-content in cannabis have increased the last decades [10, 29], there is reason to believe that passive cannabis inhalation may give adverse analytical finding.

4.3.3 Approaches for determination of the time since cannabis consumption

Concentrations of THC, THC-OH in blood and urine have been examined by several studies to estimate the time of last cannabis consumption [11-13, 32-34].

In 1992, a study developed two models for interpreting THC and THC-COOH concentrations in blood to decide the time of cannabis consumption. The models managed to have a certain accuracy (90 % and 89 % for model I and model II respectively), but the confidence intervals allowed a duplication of the predicted time since consumption. Also, this models only took in account concentrations > 2 ng/mL, and only estimated consumption within the last 8 hours [12].

THC-concentration in urine has also been suggested as a biologic marker for recent cannabis use; urinary concentration above 2 ng/mL could be indicative of cannabis exposure within the last 5 h [13]. Also this study also only extended for 8 h after the drug administration.

In 2009, a study measured THC, THC-OH and THC-COOH concentrations in urine in light cannabis smokers, who consumed cannabis cigarettes containing 70 mg THC. THC was detected in concentrations of 0.1-1.3 ng/mL 2-8 hours after cannabis-consumption. The study also analyzed urine samples provided from LAD with a THC-COOH content >15 ng/mL, and found THC in concentrations up to 68.8 ng/mL. THC-OH was detectable in a somewhat higher concentrations, 0.1-14.4 ng/mL were detectable 2-72 hours after the consumption, and concentrations up to 213.6 ng/mL were detectable in urine samples provided from LAD [32]. Thus, both these compounds have potential as indicators for cannabis use in the context of doping.

THC concentrations in blood and THC and THC-OH concentrations in urine among chronic smokers have also been examined. Both these studies concluded that these compounds are not good indicators for recent consumption in chronic users. The first study measured urine concentrations of THC, THC-OH and THC-COOH, and as THC and THC-OH was detectable within 72 hours (>2.5 ng/mL) and 4 days (40 ng/mL) respectively [33]. In the other study THC was detected in blood 7 days after the start of the study in concentrations between THC between 0.3-0.7 ng/mL. Cannabis was consumed at least 48 hours before study start [34], which support the results from [35]. However, these two studies are not directly relevant for anti-doping, as chronic cannabis consumption is not compatible with sports.

Another method has interpreted concentrations of THC, THC-OH and THC-COOH for determining the time since cannabis use. This method is recommended for use in Germany for interpreting acute effects in cases of driving under influence [11]. However, as it is not likely that athletes are performing sports under the influence of cannabis, this method is neither applicable to doping.

The dilution level of urine could alter the determination of THC-COOH-concentration. A THC-COOH/creatinine ratio can adjust for some of the variability of measured THC-COOH, and this can be used to obtain a more accurate determination of THC-COOH in urine. In 1998, a study was performed to differentiate between recent and previous cannabis users based on calculations of THC-COOH/creatinine ratios, but the best prediction accuracy was 85.4 %, with false positive and a false negative rate of 5.6 % and 7.4 % respectively [36].

A dihydroxy metabolite (8β,11-dihydroxy-THC) that is eliminated within 24 h in urine has been suggested as a possible marker for recent marijuana use [37]. However, another group was only able to detect this metabolite in one of 8 cannabis users. This user was a heavy cannabis smoker until 1 week before the beginning of the study [13].

There are drawbacks with all these studies regarding the determination of the time of THC consumption. The determination of the THC/creatinine ratios gave a high positive rate, and there are problems with the detection of dihydroxy metabolite (8β,11-dihydroxy-THC). The interpretation of THC and/or THC-COOH and/or THC-OH concentrations is not applicable to chronic cannabis users, which however is of less relevance in sports.

4.3.4 Analytical methods for detection of cannabis

In screening of THC-COOH at LAD, THC-COOH-glucuronide is first hydrolyzed with β-glucuronidase from E.coli. Then, a liquid-liquid extraction is performed using an organic solvent. Finally, the extract is derivatized prior to analyses by GC-MS working in selective ion monitoring mode (SIM) [38].

For confirmation and quantification of THC-COOH at LAD, the urine sample is cleaned with a SPE prior to the injection on the GC-MS. THC-COOH is glucuronised in urine, so prior to the SPE the sample is subjected to basic hydrolysis with 11.8 N KOH at 60 °C in 20 minutes. Then the samples are mixed with acid, and extracted by a reversed phase solid phase extraction on a SPEC C18 column. The column is conditioned with organic solvent mixed with

an acid. After loading the sample, the column is washed with acid mixed with water. The analytes are eluted in a mix of organic solvents. Finally, the sample is evaporated and derivatized before injected into the GC-MS, which is in SIM-mode. The GC-MS is scanning for the ions 371, 473 and 488, which is characteristic for THC-COOH-2TMS (11-nor-9-carboxy-delta-9-tetrahydrocannabinol-2trimethylsilyl ether) and for the ions 380, 479 and 497, which is characteristic for THC-COOHd9-2TMS (11-nor-9-carboxy-delta9-tetrahydrocannabinol-d3-2trimethylsilyl ether), the internal standard [3]. For MS-spectra of these two compounds, see figure 3a and b.

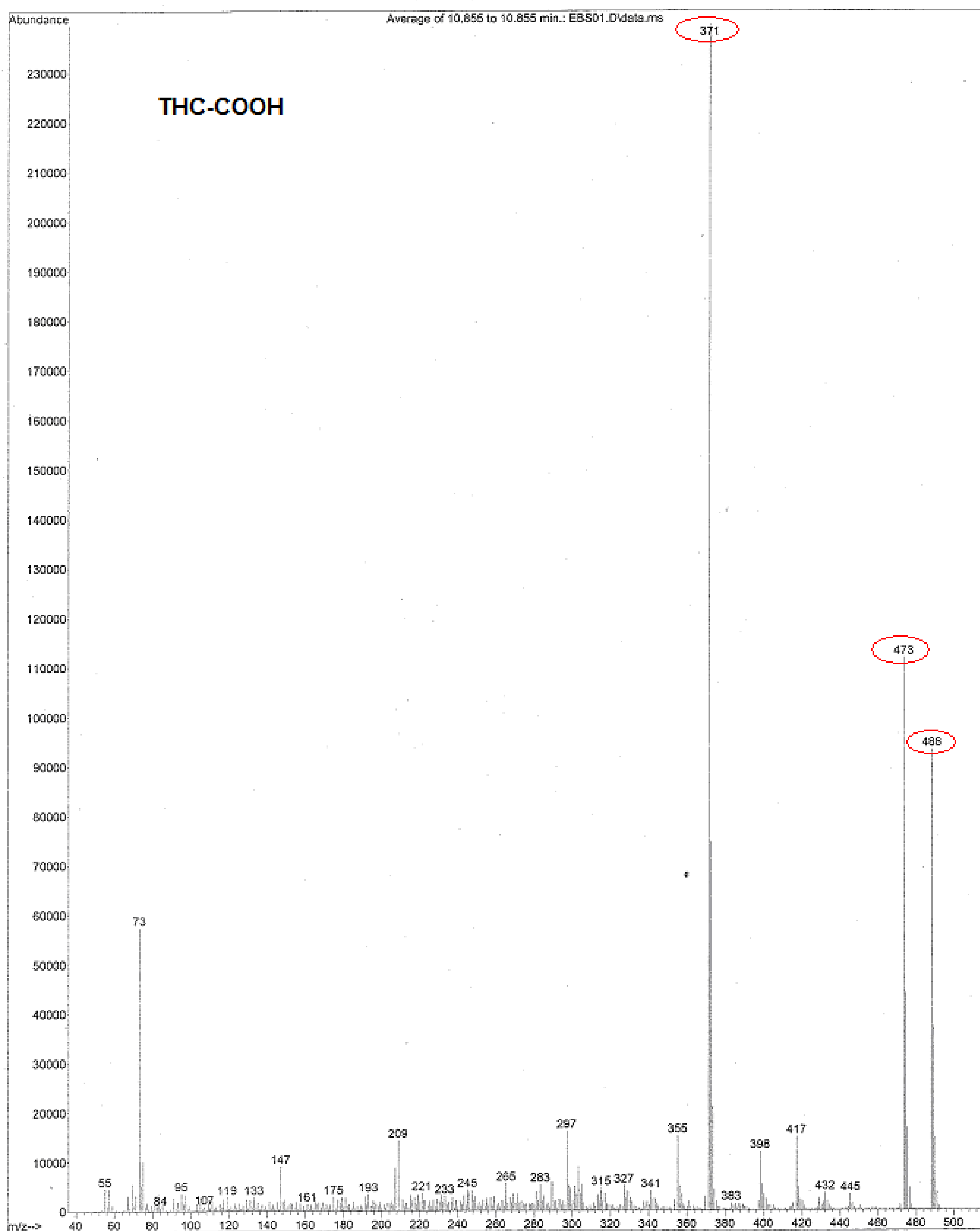


Figure 3a: MS-spectra of THC-COOH-2TMS.

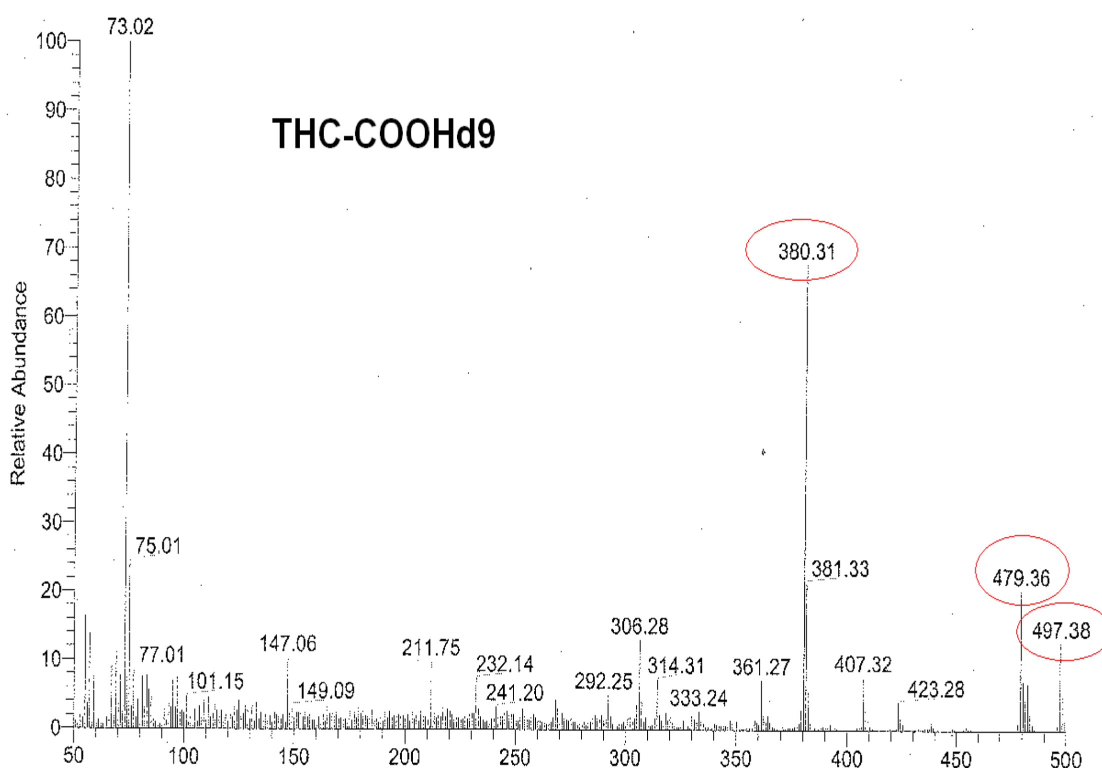


Figure 3b: MS-spectra of THC-COOHd9-2TMS.

Several analytical methods for detection of THC, THC-OH and THC-COOH in urine are published [13, 19, 32, 35, 37, 39]. These are different types of LLE extractions combined with GC-MS [13, 19, 37] or LC-MS/MS [39] or a mixed mode solid phase extraction combined with GC-MS [32, 35]. Because of the extensively metabolism of THC and THC-OH it is desirable to go as low in concentrations as possible. Thus the method obtained from [32] was suitable for this thesis because it provides the best LLOQ and LOD, and the best accuracy compared to the other methods used.

4.4 Sample preparation, detection and validation: theoretical points

4.4.1 Sample preparation

The sample preparations and detection methods used in this thesis will be discussed. Prior to analyzing a biological sample on i.e. a GC-MS, it is usually cleaned and concentrated [40].

Solid phase extraction

In solid-phase-extraction, the sample is solved a liquid and loaded on a column with a solid phase (sorbent). The analytes interacts with the sorbent and the liquid, and depending on the interactions, the analytes are either retained in the column or eluted with the liquid phase. The sorbent is usually made of silica, where functional groups are attached to optimize the interactions with the analytes. Different functional groups can be chosen, according to the properties of the analytes.

There are 4 different types of solid phase extractions:

1. *reversed phase extraction,* extract non-polar analytes from a water-based solution
2. *ion-exchange extraction,* extract ionic analytes from a water-based solution
3. *normal phase extraction,* extract polar analytes from a organic solution
4. *mixed-mode extraction,* extract analytes with both hydrophobic and ionic properties

The extraction procedure is common for all the types, and can be divided into four steps: conditioning, sample loading, washing and eluting (Figure 4).

The conditioning will activate and prepare the functional groups, to allow the interaction between the analytes and the solid-phase. Then, the sample is loaded on the column. The purpose of the washing procedure is to remove contaminants that have been retained on the column without removing the analytes. The last step should allow the elution of the analytes with the retention of the rest of the contaminants [40].

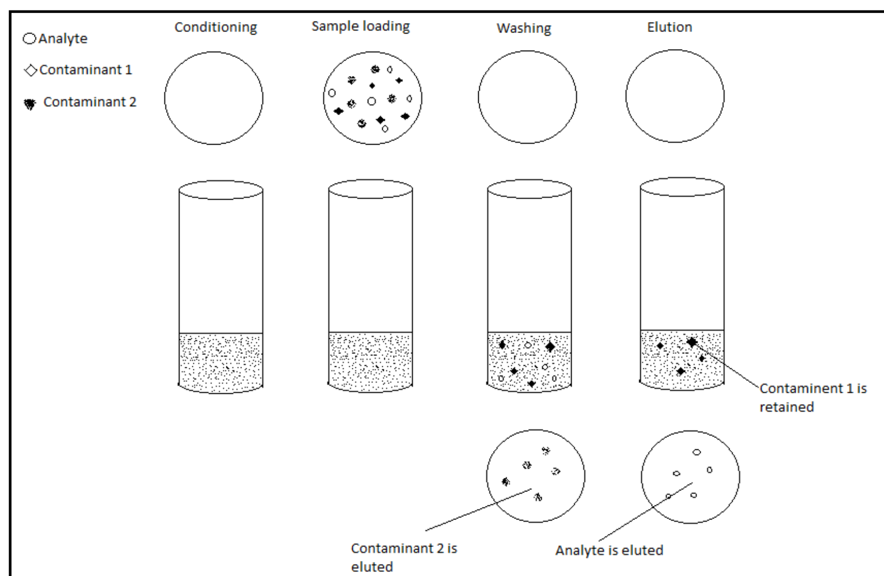


Figure 4: The extraction procedure in solid-phase extraction (inspired from [40]

Reversed phase SPE is used to clean up a non-polar analytes from aqueous solutions. The functional groups are hydrophobic, and so are the interactions between the analytes and the sorbent. The bigger the functional groups are, the more hydrophobic is the sorbent. Examples of sorbents are octadecyl (C18), octyl (C8), ethyl (C2) and cyclohexyl.

To promote the hydrophobic interactions between the analytes and the sorbent, the analytes are dissolved in a hydrophilic matrix, i.e. urine. The pH in the sample solution and the washing solution favor the non-ionic form of the analytes, with the purpose of increasing the interactions between analytes and sorbent. This will promote retention of the analytes while some contaminants are washed out. The elution solution is a organic solvent which will break the interactions between the analytes and the sorbent [40].

In mixed-mode extraction, the sorbent contains both hydrophobic and ionizable groups, which makes it possible to retain the analytes by hydrophobic interactions, ion exchange interactions and hydrophilic interactions. The column is usually conditioned with methanol and water. The pH can be adjusted in the conditioning step, the sample solution and the washing step, to promote the ionic interactions between analytes and sorbent. Since the analytes are retained by stronger interactions than in the reversed phase extraction, the sample can be washed more thoroughly, and the extract will usually be cleaner compared to a reversed phase SPE extract. The analytes is eluted with an elution solution that breaks all the interaction between the sorbent and the analytes [40].

Liquid-liquid extraction

In this type of extraction, the analytes are transferred from an aqueous phase to an organic liquid. The partition of the analytes between these two liquids is given by the partition coefficient, K, which should be high for the analytes and low for the contaminants.

$$K = [A]_{Organic}/[A]_{aq}$$

$[A]_{Organic}$ is the concentration of the compound in the organic solution, while

$[A]_{aq}$ is the concentration of the compound in the aquatic solution.

To get this partition coefficient as high as possible for the analyte, one have to consider the pH in the aqueous sample and the choice of the organic solvent. Regarding the pH in the aqueous sample, this should be adjusted favor the non-ionized form of the analyte to increase the solubility in the organic phase.

The organic solvent needs to be immiscible with water, and should have the same chemical properties as the analytes regarding polarity. If the analyte is an acid, the solvent should be proton accepting, and the opposite if the analytes is a base [41].

To increase the recovery of a LLE multiple extractions or increasing amounts of solvents could be used. If 83 % of an analyte is transferred to the organic solvent with one extraction, another extraction will increase the recovery to 97 %. The recovery can also be increased by increasing the amount of organic solvent, but these solvents are usually toxic and should be used in a small amount as possible.

Some contaminants will also be transferred to the organic phase after LLE. To increase the sample clean-up, a back-extraction can be performed with an aqueous solution having a pH allowing ionization of the analytes, which makes the analytes more soluble in the aqueous solution [40].

4.4.2 Gas chromatography

A GC separates the analytes in a sample extract and consists of an injector, a carrier gas, a reduction valve, a column and a detector.

The principle of GC is separation of the compounds in a sample due to interactions between a gaseous mobile phase and a stationary phase. The compounds are dissolved in an organic solvent and injected in the injector where it evaporates immediately. The most common injectors are split and split less injectors. In split less injections, the whole amount of injected sample are transmitted to the column, which makes it more suitable for anti doping analysis, as the concentrations of the doping agents in biologic matrices usually are low. The mobile phase, a carrier gas, will transport the sample from the injector into the column where the analytes are partitioned between a stationary phase and the gaseous mobile phase. Hence the compounds are retained at the stationary phase in different degrees and separated. The partition of the analytes between the two phases depends on the volatility of

the analyte, the column temperature, the properties of the stationary phase (polarity, thickness etc.), and on the speed of the carrier gas.

The analytes are derivatized before they are injected in the GC to make them more volatile and stable at high temperatures. In a derivatization process, these polar groups are remade to non-polar groups. The derivatization process used in this thesis was silylation, which is suited for compounds containing functional groups as OH, COOH, NH and NH_2. The hydrogen of these groups are replaced by a trimethylsilyl(TMS)-group [40].

4.4.3 Mass spectrometry

The mass-spectrometer is measuring masses. It cannot detect neutral molecules, only ions, thus it is necessary with an ion source which ionizes the compounds [42].

There are different ways of ionizing the analytes, but as only electron ionization (EI) was used, only this will be discussed here. EI is a common type of ionization in combination with GC-MS. In EI, the neutral molecules are guided into an ionization chamber in vacuum which contains an anode and a filament. The anode has a positive charge, and current is added to the filament. Between the filament and the anode there is a beam of electrons with energy of usually 70 eV, se Figure 5. When an electron with this energy impacts a molecule, an electron will be ejected from the molecule.

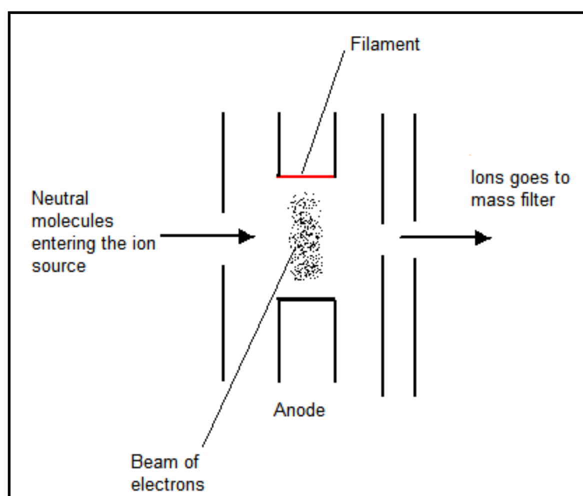
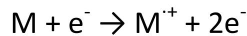


Figure 5: Sketch of ionization chamber in electron ionisation (Inspired by [42].

The molecule left will then have a positive charge and an unpaired electron, this is called the molecule ion ($M^{\cdot+}$). The mass will be the same, since the mass of the ejected electron is negligible compared to the remaining mass of protons plus neutrons.



$M + e^- \rightarrow M^{\cdot+} + 2e^-$

M = unionized molecule

$M^{\cdot+}$ = molecular ion

e^- = electron

The molecule ion is unstable, and most of the molecule ions will be fragmented into smaller pieces (fragment ions). The ionization process makes a fragmentation pattern which is typical for the molecule, and this pattern is often available in databases. These databases can be used for identification of the molecule [40, 43]. After they are ionized, the analytes are guided into a mass-separator.

The mass-analyzer is usually a quadrupole, which separates the ions according to their mass-to-charge-ratio (m/z). The quadrupole consists of 4 parallel rods, divided in two pairs, in a electrical field [40]. The pairs are charged, either positive or negative, and this charge changes from positive to negative (and back again) with a given frequency. The ions produced in the ion source travel along the longitudinal axis of the rods, and are attracted to the rods depending on their charge; positive charged ions are attracted to the negative charged rods, and opposite. A certain combination of charge and frequency allows preselected ions with a certain m/z ratio (SIM) or within a certain m/z-ratio range (scan) to travel through the filter [42]. These data will be transferred to a computer and treated electronically [40].

The different scan-opportunities are increased when using a mass-analyzer with three quadrupoles, a triple stage quadrupole (TSQ). The analysing quadrupoles are called Q_1 and Q_2, and they are separated by q, a collision cell (Figure 6). Q (the collision cell) fragments the ions escaping from Q1 before they enter Q2. The detector is placed after Q_2 [44].

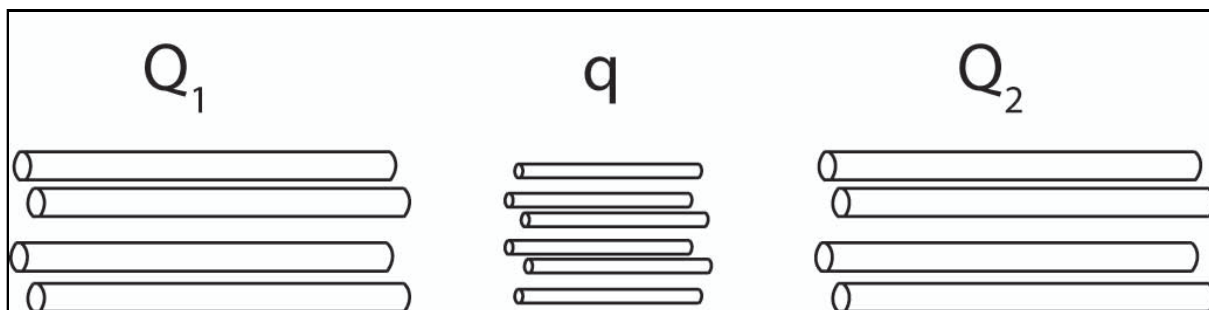


Figure 6: The quadrupoles in MS/MS: Q1 and Q2, separated by q. Reprinted with permission from Terje Vasskog.

Both Q1 and Q2 can be set in both SIM and Scan-mode [42]. This gives a wider range of scan-opportunities when compared to a regular MS-machine; product scan, parent scan and MRM (multiple reaction monitoring).

Product scan and MRM were used in this project. In a production scan (see Figure 7), the Q1 is set in SIM-mode at a selected ion and the Q2 is set in SCAN-mode. The selected ion is fragmented in q. The ions produced in q are further directed to Q2. Since Q2 is in Scan mode all ions produced in q are recorded. From this MS/MS spectrum abundant ions are candidates for analysis based on the use of MRM [42, 44]

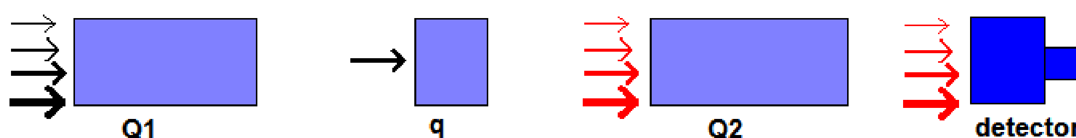


Figure 7: Product scan. All the product ions enter the Q1. Q1 choose one of these, which is fragmented in q. All the fragments from q pass the Q2, and are detected. This is one of the steps in setting up a MRM-method. The figure is inspired of [42].

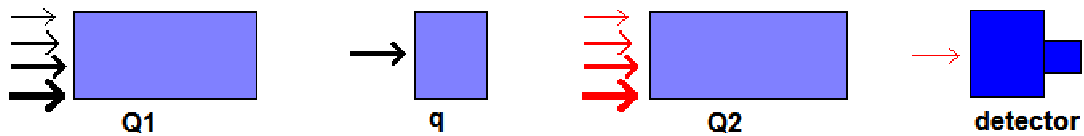


Figure 8: MRM-scan. All the ions fragmented in the ion source enter the Q1. In the first quadrupole, Q1, only the product ion is passing through and fragmented in q. The fragmented ions enter Q2, where only the selected fragment ions are passing through and detected. The figure is inspired of [42].

A MRM-method is more specific than a SIM-method. The risk for another compound having the same transition is reduced compared to having the same ion from the ion source.

The ability to detect small amounts is obtained, since the noise is reduced compared to a SIM-scan, and this give a higher signal to noise ratio [42].

As the urine concentrations of THC could be quite low (urine concentration >2ng/mL is indicative of cannabis exposure within the last 5 h [13]), there is need for a specific and sensitive detection method.

4.4.4 Validation procedure

Validation can be defined as:

"Establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes"[45]

The validation procedure documents that the method is fitted for its purpose.

The properties of the method are revealed by determining the following validation parameters:

- LOD
- LLOQ
- ULOQ
- Linearity
- Specificity

- Precision
- Accuracy
- Range
- Robustness

Lower limit of detection (LOD)

LOD is the lowest concentration where an analyte can be detected. It can be calculated by dividing the area of the signal on the area of the noise, and this ratio (signal to noise ratio, S/N) should be ≥ 3 [40]. The signal of the noise is the height of the baseline.

Lowest limit of quantification (LLOQ)

LLOQ is the lowest concentration where an analyte can be quantified. It can be calculated in the same way as LOD, but the S/N should be ≥ 10 [40].

If the signal to noise ratio is low, it is difficult to say how much of the signal that is due to the analyte, and how much that is due to the matrix, thus a reliable quantification would be difficult. There are no requirements for the LLOQ or LOD value in doping analysis, but the value should fit the purpose.

Upper limit of quantification (ULOQ)

ULOQ, upper limit of quantification, is the highest concentration where the analyte can be quantified, before having a saturated signal. At this point, the calibration curve will go from being linear to parabolic.

Linearity

Linearity is the ability of the method to give a linear calibration curve in a given concentration range. The ratio is given by the response of the analyte which is divided by the response of the internal standard, and allows a plot at different concentrations. A calibration curve is then obtained, and allows the calculation of urine of an unknown sample. The linearity of the equation is described by R, the regression coefficient [40]. R^2 should be as close to 1 as possible, but a value above 0.995 is satisfying.

Specificity

The specificity is the ability of the method to detect and quantify the analyte in presence of contaminations in the sample. The signal of the analyte should not be interfered by these. The specificity of the method can be tested by analyzing negative samples from 6 different subjects, and 3 samples per subject are analyzed. The signals in the negative urines are examined for interfering signals.

Range

This is the interval between the lower and the upper concentration where the method can quantify the analyte with a suitable accuracy, precision and linearity.

Precision

This parameter describes the diversity of the results, and are expressed by a relative standard-deviation (RSD), which is the standard deviation of the results divided by the mean value of the same results, and multiplied by 100. A low RSD indicates a good precision. 6 samples for 3 concentrations in the range are analyzed. The analyzed concentrations should reflect the concentration range.

Accuracy

Accuracy represents the closeness between the theoretical value and the calculated value. Hence, this parameter considers the uncertainty and the precision of the method. The uncertainty can be determined by calculation of the theoretical values in the sample by using a calibration curve. The calculated- and the theoretical value are plotted in a curve. The linearity and the slope of the curve demonstrate the correlationship between these values; a linear curve with a slope of 1 suggests a good correlation between these.

Robustness

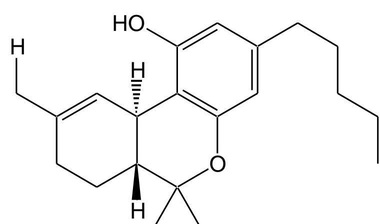
The robustness is an assessment on the ability of a method to stay unaffected by minor changes in the procedure, i.e. e. small variations in pH. This is to make sure that the analysis is not affected by variations that might occur in a sample preparation [40].

5 Material and methods

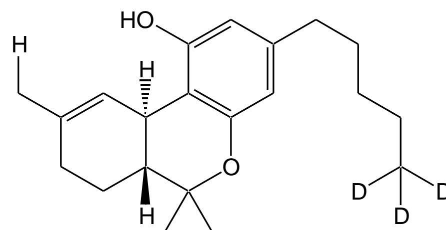
5.1 Chemical and reagents

THC(δ-9-tetrahydrocannabinol,*(−)-(6aR,10aR)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol*)1 mg/mL in ethanol, THCd3 (δ-9-tetrahydrocannabinol-d3, *(−)-(6aR,10aR)-6,6,9-trimethyl-3-([5`5`5`-trideuteropentyl])-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol*) 0,1 mg/mL in methanol, THC-OH(11-hydroxy-δ9-tetrahydrocannabinol, *6aR,10aR)-9-(Hydroxymethyl)-6,6-dimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H benzo[c]chromen-1-ol*) 0,1mg/mL in methanol,THC-OHd3(11-hydroxy-δ9-tetrahydrocannabinol-d3,*6aR,10aR)-9-(Hydroxymethyl)-6,6-dimethyl-3-([5`5`5`-trideuteropentyl])-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol-d3*) 0,1 mg/mL in methanol and THC-COOH (11-nor-delta(9)-tetrahydrocannabinol-9-carboxylic acid, *1-hydroxy-6,6-dimethyl-3-pentyl-6a,7,8,10a-tetrahydrobenzo[c]chromene-9-carboxylicacid*) 0,1 mg/mL were purchased from Lipomed (Arlesheim, Switzerland). THC-COOHd3 (11-nor-delta(9)-tetrahydrocannabinol-9-carboxylicacid-d3,*1-hydroxy-6,6-dimethyl-3-([5`5`5`-trideuteropentyl])-6a,7,8,10a-tetrahydrobenzo[c]chromene-9-carboxylic acid*) 0,1 mg/mL in methanol and THC-COOHd9 (11-nor-delta(9)-tetrahydrocannabinol-9-carboxylic acid-d9, *1-hydroxy-6,6-di[trideuterateromethy]-3-([5`5`5`-trideuteropentyl])-6a,7,8,10a-tetrahydrobenzo[c]chromene-9-carboxylic acid*) 0,1 mg/mL in methanol were purchased from Cerillant (Round Rock, TX, USA). Phosphoric acid 85 %, K_2HPO_2 (dipotassium hydrogen phosphate) (>98 %), β-glucuronidase type IX-A, 1`660`000 units/g solid from Escherichia coli and sodium chloride (HCl) 37 % fuming was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (99.9 %), KH_2PO_4 (potassium dihydrogen phosphate) (99.5-100.5 %) and acetic acid glacial (100 %) was purchased from Merck (Damstadt, Germany). BSTFA + TMCS 99:1, purchased from Sigma Aldrich (Buchs, Switzerland). β-glucuronidase type K12 140 U/mg was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Acetonitrile (AcN) (99.97%) was purchased from Biosolve (Valkenswaad Netherlands). Sodiumhydroxide (NaOH) pellets (>99 %) was purchased from VWR International (Leuven Belgium). Hexane (99.78 %) and potassium hydroxide (KOH) pellets > 85 %was purchased from Acros Organics (Geel, Belgium). Etyl acetate (99.8 %) was purchased from Panreac (Barcelona, Spain) and from VWR (Fontenay-sous-Bois, France). Ultrapure water was produced by a Milli-Q gradient

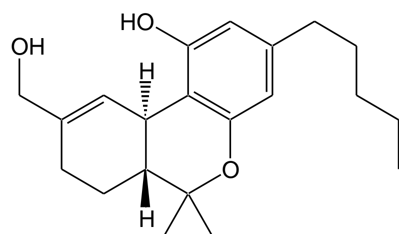
A10 water purification system with a Q-Gard 2 and a Quantum EX Ultrapure organix cartridge purchased by Milli-Q A-10 water purification system (Billerica, MA, USA).



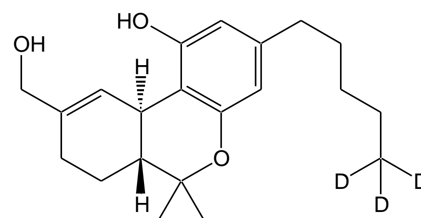
THC
Molecular weight: 314.5 g/mol
Monoisotopic mass: 314.2 u
pKa: 9.6
Average logP: 6.55 (±0.90)



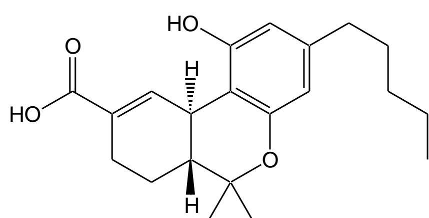
THCd3
Molecular weight: 317.5 g/mol
Monoisotopic mass: 371.2 u
pKa: 9.6
Average logP: 6.55 (±0.90)



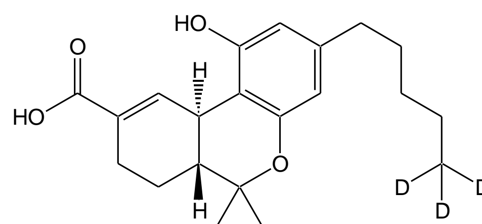
THC-OH
Molecular weight: 330.5g/mol
Monoisotopic mass: 330.2 u
pKa: 9.6
Average logP: 5.25 (±0.75)



THC-OHd3
Molecular weight: 333.5 g/mol
Monoisotopic mass: 333.2 u
pKa: 9.6
Average logP: 5.25 (±0.75)



THC-COOH
Molecular weight: 344.5g/mol
Monoisotopic mass: 344.2 u
pKa: 4.6
Average logP: 5.30 (±0.82)



THC-COOHd3
Molecular weight: 347.5g/mol
Monoisotopic mass: 347.2 u
pKa: 4.6
Average logP: 5.30 (±0.82)

Figure 9: Structure, molecular weights (MW) and pKa-values for the investigated compounds. The pKa-values and the logP are predicted values [46, 47]

5.2 Buffers and solutions

The solutions were made fresh every week, except β-glucuronidase solution and the stock solutions.

Stock solutions

THC was obtained in a concentration of 1 µg/mL in ethanol, while THC-d3, THC-OH, THC-OHd3, THC-COOH and THC-COOHd3 were obtained in a concentration of 1 µg/mL in methanol for the development of MRM-method.

For development of the sample preparation, a mix of THC, THC-OH and THC-COOH were obtained in a concentration of 1 µg/mL (standard solution), and a mix of THCd3, THC-OHd3 and THC-COOHd3 were obtained in the same concentration.

For the method validation mix of THC, THC-OH and THC-COOH were obtained in concentrations of 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL and 10 µg/mL.

THC-COOHd9 was obtained in a concentration of 1 µg/ml in methanol.

All the stock solutions were stored at -20°C.

Phosphate buffer, pH 6.8

4.33 g K_2HPO_4 and 3.42 g KH_2PO_4 were transferred to a beaker, and about 400 mL MilliQ water was added. The pH was adjusted to 6.8 with 0.1 M NaOH. The solution was transferred to a 500 mL cylinder and MilliQ-water was added up to 500 ml. The pH was measured, and adjusted to 6.8 with phosphoric acid 85%. The solution was stored at 5°C.

Acetic acid solution, 0.1 M

5.725 ml acetic acid pure was transferred to a 1000 mL flask, and MilliQ-water was added up to the mark. The solution was stored at 5°C.

Hydrochloric acid solution, 0.1 M

5 ml 37 % HCl fuming, was transferred to a 500 mL flask, and MilliQ water was added up to the mark. The solution was stored at 5°C.

β-glucuronidase solution, 10`000 UI/200 µg

603.7 mg β-glucuronidase from *E.coli*, type IX-A, 1`660`000 units/g solid, was transferred to a 20 mL flask, and dissolved in 20 mL phosphate buffer, pH 6.8. Aliquots of 1 ml were then in Eppendorf tubes transferred and stored at -20°C.

HCl 0.1 M/AcN 70/30

30 mL AcN was transferred to a 100 mL flask. The flask was filled with HCl 0.1 M up to the mark, and then shaked. Stored at 5°C.

Hexane/ethyl acetate 80/20

20 mL ethyl acetate was transferred to a 100 mL flask. The flask was filled with hexane up to the mark and shaked. Stored at 5°C.

5.3 Sample preparation

Spiking of urine

The final volumes used for spiking urine was: 1 mL urine samples, 200 µl phosphate buffer pH 6.8, 50 µl internal standards (1µg/ml) and 165 µl β-glucuronidase solution, type IX-A (10`000 U/200 µl).

Derivatization

After sample clean up the samples extract was evaporated under nitrogen, and resolved in 25 µl AcN mixed with 20 µl BSTFA:TMCS 99:1. The sample extract was derivatized at 85°C for 30 minutes, and 2 µl was injected in the GC-TSQ. When preparing samples for quantification in urine samples, the derivatization procedure was changed to 50 µl MSTFA, 90°C, 20 minutes.

Mixed mode SPE

Obtained from [32]

The extraction procedure for the mixed mode SPE can be found in table 2.

Condition	Load sample	Wash	Elute
Methanol: 3mL	1.4 mL	MilliQ water: 2 mL	Hexane:ethyl acetate, 80/20: 3 mL
MilliQ water 3 mL		HCl 0.1M/AcN: 2mL	
Acetic acid 0.1 M: 2 mL			

Table 2: The extraction procedure for the mixed mode SPE

Reversed phase SPE

Obtained from [38].

The extraction procedure for the reversed phase SPE can be found in table 3.

Condition	Load sample	Wash	Elute
Acetic acid 0.1 M in methanol: 200 µl	1.4 mL	Acetic acid 20 % in water: 1 mL	Hexane:ethylacetate 75/25: 2 × 500 µl
		Dry for 20 minutes	

Table 3: The extraction procedure for the reversed phase SPE.

LLE

Obtained from [48] and adjusted.

The sample was acidified with 1.5 mL acetic acid pure.

The LLE was performed with 2 × 3.5 mL hexane:ethyl acetate 90:10.

5.4 Instrumentation

Columns for solid phase extraction

UCT Clean Screen CSTHC203, octyl and aminopropyl, 200 mg/3mL solid phase extraction (SPE) columns were purchased by Laubscher Labs (Miecourt, Switzerland) and the Bakerbond C_{18} 30 mg/3mL SPE columns were purchased by Varian (Lake Forest, CA, USA).

GC-TSQ

The GC-TSQ system was composed of a Trace GC ultra Thermo Scientific coupled to a TSQ Quantum MS (Thermo) purchased from Brechbühler AG, (Schlieren, Switzerland) equipped with a Agilent 100-2000 (column material: HP-1; methyl polysiloxane, 17 x 0,2 mm I.D., 0.11

µm film thickness) from J&W Scientific (Böckten, Switzerland). 2 µl of the samples was injected in split mode, with a Triplus Autosampler Thermo Scientific purchased from Brechbühler AG (Schlieren, Switzerland). The injection was later changed to split less mode. The injector temperature was 260 °C. The temperature of the transfer line was 280 °C and the temperature of the source was 250°C. The carrier gas used was helium, with a flow of 1.5 mL/min in constant flow mode. The ramping of the oven temperature was as follow: 150°C at 1 minute to 280°C at 20°C/minute and held at this temperature for 280°C in 5 minutes. The ionization mode was in positive mode, and the ionization potential in the ionization source was 70 V, and the emission current was 250 µm. The dwell time was 20 ms.The collision gas used was argon.

Some experiments were also performed on the machine above, but with another column, Zebron 5MS column (colunmmaterial: 5 % polysilarylene/95 % Polydimethylsiloxane, 30 m x 0,25 mm I.D., 0,25 µ film thickness) from Brechbühler (Schlieren, Switzerland). The rest of the condition was held the same as above.

GC-MS

One GC-MS system was composed of Agilent 6890 Series GC-system from Agilent (Waldbronn, Germany) equipped with a Zebron 5 MS column (columnmaterial: 5% Polysilarylene/95 % Polydimethylsiloxane, 30 m x 0,25 mm I.D., 0,25 µ film thickness) from Brechbühler (Böckten, Switzerland). The GC was coupled to an Agilent 5973 detector (Waldbronn, Germany).

Helium was used as a carrier gas, with a flow of 1.1 mL/min in constant flow mode.

The ramping of the oven temperature was as follow: 150°C at 1 minute, to 280°C at 20°C/minute. 1 µl of the samples was injected in split less mode at 260°C with a Hewlett Packard 7683 series injector (Walbronn, Germany). The ionization potential was 70 eV and the emission current was 270 µm. The interphase temperature was 280°C, the source temperature was 200°C and the quadrupole temperature was 150°C. The dwell time was 50 ms. The temperature and column was adjusted during the project to improve the method. The ions chosen can be seen in table 4.

	THC-TMS	THC-OH-2TMS	THC-COOH-2TMS	THCd3-TMS	THC-OHd3-2TMS	THC-COOHD3-2TMS
Ion	303	371	371			
Ion	371	403	473	389	374	374
Ion	386	474	488			

Table 4: The ions used in GC-MS, SIM-mode. 3 ions per analyte were chosen in order to be able to have a quality control for the analysis.

5.5 Specificity

The specificity of the signal was tested by spiking 6 different male and 6 different female cannabis negative urines with β-glucuronidase, phosphate buffer and 50 µl methanolic standard of THC-COOHd9 (1 µg/mL), which were used as internal standard. 3 parallel urines per subject were spiked. One negative urine from one of these volunteers was spiked with 50 µl standards (1 µg/mL), 50 µl internal standards (1 µg/mL), phosphate buffer and β-glucuronidase.

The samples were hydrolyzed, extracted with the mixed mode SPE, evaporated, derivatized and injected. The signals in the negative urines were compared with the signal in urine spiked with analytes and internal standards.

The specificity test was first performed on GC-MS in SIM-mode. To improve the specificity, the temperature and the column ramping were adjusted. The experiment was later redone using the GC-TSQ in MRM-mode.

5.6 Linearity and detection limits

6 different urines, 3 males and 3 females, were spiked with different amount of the standards to a concentration in urine of 0.1, 0.5, 1, 3, 5, 7, 9, 10, 50, 100, 500 and 1000 ng/mL of THC, THC-OH and THC-COOH. To obtain the same amount of liquid in all the samples, the standard solutions were evaporated before adding urine, buffer, enzyme-solution and internal standards.

6 Results and discussion

During this thesis a GC-TSQ method has been developed to detect THC, THC-OH and THC-COOH. Specificity, selectivity and linearity have been tested as well as the sample preparation.

6.1 Method development

The aim was to develop and validate an analytical method for detection and quantification of THC, THC-OH and THC-COOH and one method for the cannabinoid analogues. Several technical problems hindered the progress of the project and therefore only sample preparation, specificity, selectivity and linearity for THC, THC-OH and THC-COOH have been studied.

6.1.1 Conditions of hydrolysis

THC, THC-OH, THC-COOH are all excreted mainly as glucuronide conjugates and should be hydrolyzed before they are injected in a GC [16]. Based on previous literature, only β-glucuronidase from *E.coli* was used [19]. A few parameters are important to optimize the conditions of hydrolysis. Three of them have been tested, which are the amount of enzyme, the time of hydrolysis and the temperature.

For investigation of the quantity of β-glucuronidase, a solution of β-glucuronidase type K12 of 140 U/mg (corresponding to at least 140 U/mL) was added in different quantities to 1 mL urine samples, previously quantified for THC-COOH (941 ng/mL, previously quantified at GC-MS). The quantities added were: 50 µl, 100 µl, 500 µl and 1 mL. Phosphate buffer internal standards were added to all the samples and the pH was between 6 and 7. Each sample was extracted in triplicate and hydrolyzed at 37°C for 16 hours.

As a reference, 1 mL urine previously quantified for THC-COOH (941 ng/ml, previously quantified GC-MS) was spiked with 125 µl KOH and internal standard, and subjected to basic hydrolysis at 60°C for 2 min [3]. The sample was neutralized with 450 µl acetic acid before the extraction.

All the samples were extracted with the reversed phase SPE, evaporated and derivatized.

2 µl of all the samples were injected in the GC-TSQ system in MRM-mode.

The ratios between the peak area of the transitions chosen for quantification of the analyte and the peak area of the transition chosen for quantification of the internal standard

were calculated for each quantity of β-glucuronidase solution (table 5). The chromatograms for the optimal quantity of β-glucuronidase and for the basic hydrolysis can be seen in figure 10.

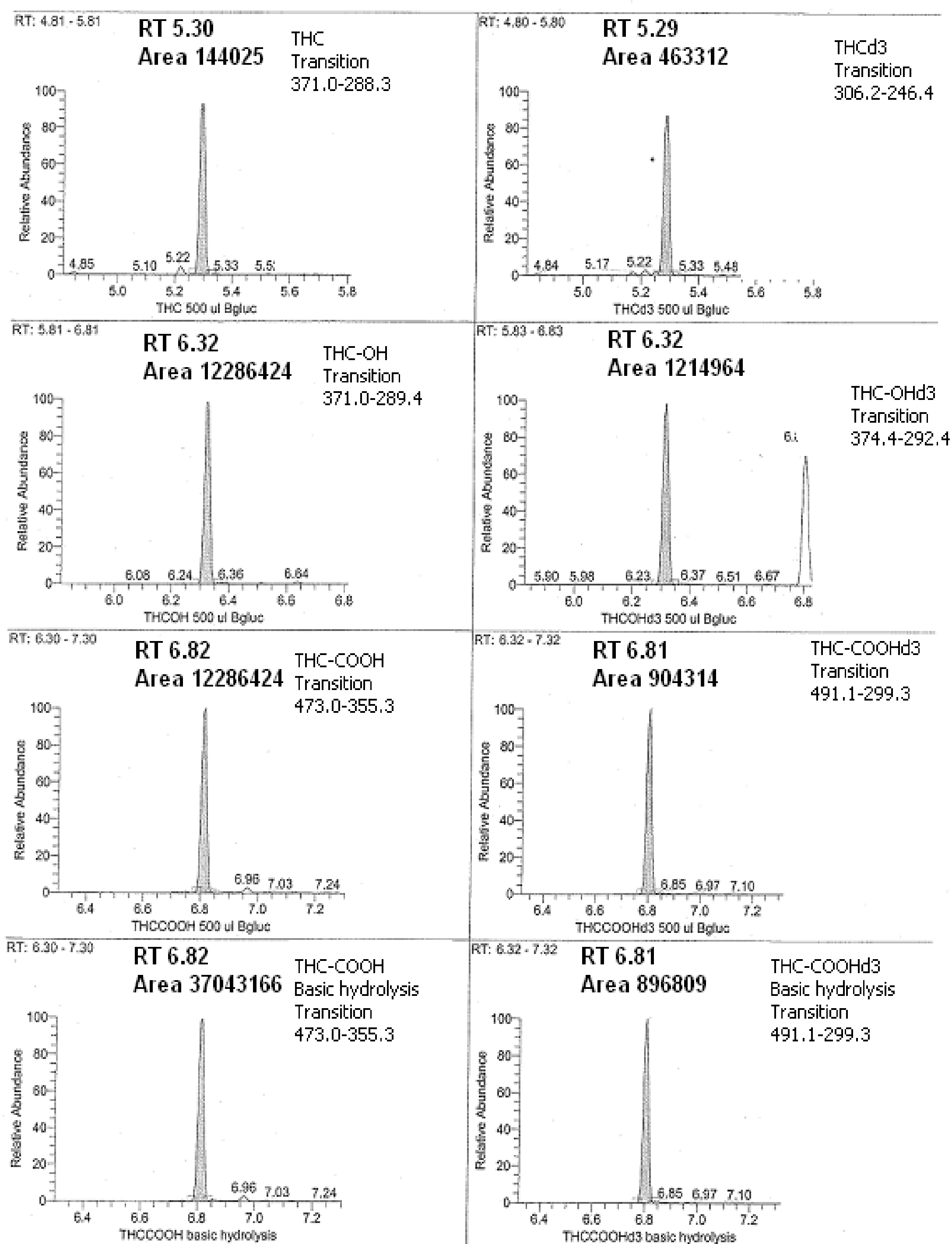


Figure 10: Chromatogram from GC-TSQ in MRM-mode, of the TMS-derivates of the compounds subjected to hydrolysis with 500 µl β-glucuronidase 140 ug/mL at 37°C for 16 hours (6 upper chromatograms) and of the TMS-derivates of the compounds subjected to basic hydrolysis (2 lower chromatograms).

		Quantity β-glucuronidase (µl)			
		50	100	500	100
Ratio standard/ internal standard	**THC-TMS/THCd3-TMS**	0.4	0.5	0.5	0.5
	THC-OH-2TMS/ THC-OHd3-2TMS	3.1	5.7	10.0	9.8
	THC-COOH-2TMS/ THC-COOHd3-2TMS	39.6	40.6	41.1	38.7

Table 5: The table shows the ratios of peak area for the TMS-derivates of the analytes/peak area for TMS-derivates of internal standard after addition of different quantities β-glucuronidase. The areas are obtained from figure 10.

A high ratio indicates an effective hydrolysis. Hence, the results in table 5 indicate that 500 µl is a suitable quantity of the enzyme solution as this is the best quantity for THC-OH-glucuronide and THC-COOH-glucuronide (the ratio is 10.0 and 41.1 respectively). For THC, the different quantities are almost not affecting the ratio.

The sample subjected to basic hydrolysis in this experiment confirmed this result: the peak area ratio of THC-COOH-2TMS/THC-COOHd3-2TMS in this sample was set to 100 %, and compared with the area ratio THC-COOH-2TMS/THC-COOHd3-2TMS from table 5. The highest percentage (127.3 %) indicates the most efficient hydrolysis regarding THC-COOH-glucuronide. The comparison can be seen in table 6.

		Confirmation procedure	50 µl β-gluc	100 µl β-gluc	500 µl β-gluc	1 ml β-gluc
Ratio anlyte /internal standard	**THC-COOH-2TMS/THC-COOHd3-2TMS**	32.3	39.6	40.6	21.1	38.7
	In percent	100.0	122.6	125.7	127.3	120.0

Table 6: Ratios of the peak area of THC-COOH-2TMS/peak area THC-COOHd3-2TMS for basic hydrolysis and for enzymatic hydrolysis when different quantites of β-glucuronidase were added. The ratios of THC-COOH-2TMS/THC-COOHd3-2TMS subjected to enzymatic hydrolysis are calculated as a percentage of the ratio THC-COOH-2TMS/THC-COOHd3-2TMS subjected to basic hydrolysis.

The results also indicate that THC-COOH-glucuronide is easier hydrolyzed compared to the two other analytes. THC-COOH-glucuronide can also be subjected to basic hydrolysis because of the ester linked glucuronide, but THC-glucuronide and THC-OH-glucuronide cannot undergo this type of hydrolysis, because of the glucuronide attached to those analytes are ether-linked [18].

Unfortunately, the costs would be too high if the optimal quantity (500 µl) of the enzyme solution of β-glucuronidase K-12 should be used in this project. Therefore, a new solution of β-glucuronidase type IX-A was made (10`000 U/200 µl). Different amounts, 50 µl, 100 µl, 150 µl, 200 µl and 300 µl of the latter enzyme solution were added to 1 mL negative urine with phosphate buffer and the internal standard. These samples were supposed to be extracted with the solid phase extraction, but this analysis was not performed because the enzyme solution clogged the reversed phase SPE column.

To spare some time in the project and based on previous publications [32, 35, 37], it was decided to use a volume of 165 µl (8250 U) per mL urine. Even if the usual amount of enzyme used is between 4000 and 5000 U/mL of urine a higher concentration would not affect the hydrolysis yield [19]

To determine the time of hydrolysis a urine sample previously quantified for THC-COOH (941 ng/mL) at GC-MS was used. β-glucuronidase, phosphate buffer and internal standard were added to the sample and different time and temperatures were tested for hydrolysis (1h at 50°C, 1.3h at 50°C, 2h at 50°C and 16h at 37°C). Three samples per condition were extracted with the mixed mode SPE, derivatized and 2 µl injected in the GC-TSQ. As the intensity of the signal decreased abnormally between 5 injections the results obtained from this analysis were not conclusive. The same samples were injected in a GC-MS.

In order to define the m/z ions for the SIM GC-MS method, the MS-spectra for the compounds were obtained by injecting methanolic standards of the analytes and the d3-internal standards in scan-mode (12a, b, c and 13a, b, c).

To evaluate the different hydrolysis conditions, the peak area of the TMS-derivate of the analytes was divided by the peak area of the TMS-derivate of the appropriate internal standard and the mean value of the ratio obtained from the three samples was calculated. The highest mean value indicates the most efficient hydrolysis (Table 7). For THC-glucuronide and THC-COOH-glucuronide the hydrolysis is barely more consistent at 50°C for 2h, followed by 37°C for 16h, the ratios are almost equal for these two conditions. Regarding the THC-OH-

glucuronide, the hydrolysis is clearly better at 37°C for 16 hours. Since the analytes should be quantified and not only detected, it is important to get the highest amount of compound analyzed, so the hydrolysis at 37°C for 16 hours was chosen.

	THC Area analyte-TMS/area internal standard-TMS		**THC-OH Area analyte-TMS/area internal standard-TMS**	**THC-COOH Area analyte-TMS/Area internal standard-TMS**		
Ion	**371**	**385**	**371**	**371**	**473**	**488**
Β-gluc 1h at 50°C	1.1	0.4	2.9	21.0	11.5	9.0
β-gluc 1.3h at 50°C	1.2	0.5	3.9	21.7	12.2	9.5
Β-gluc 2h at 50°C	1.2	0.5	4.4	22.0	12.4	9.8
Β-gluc 16h at 37°C	1.2	0.5	6.0	21.6	12.0	9.4

Table 7: The mean value of the ratio between the peak area for the TMS-derivates of the analytes and the peak area for TMS-derivates of the internal standards at different hydrolysis- times and temperatures.

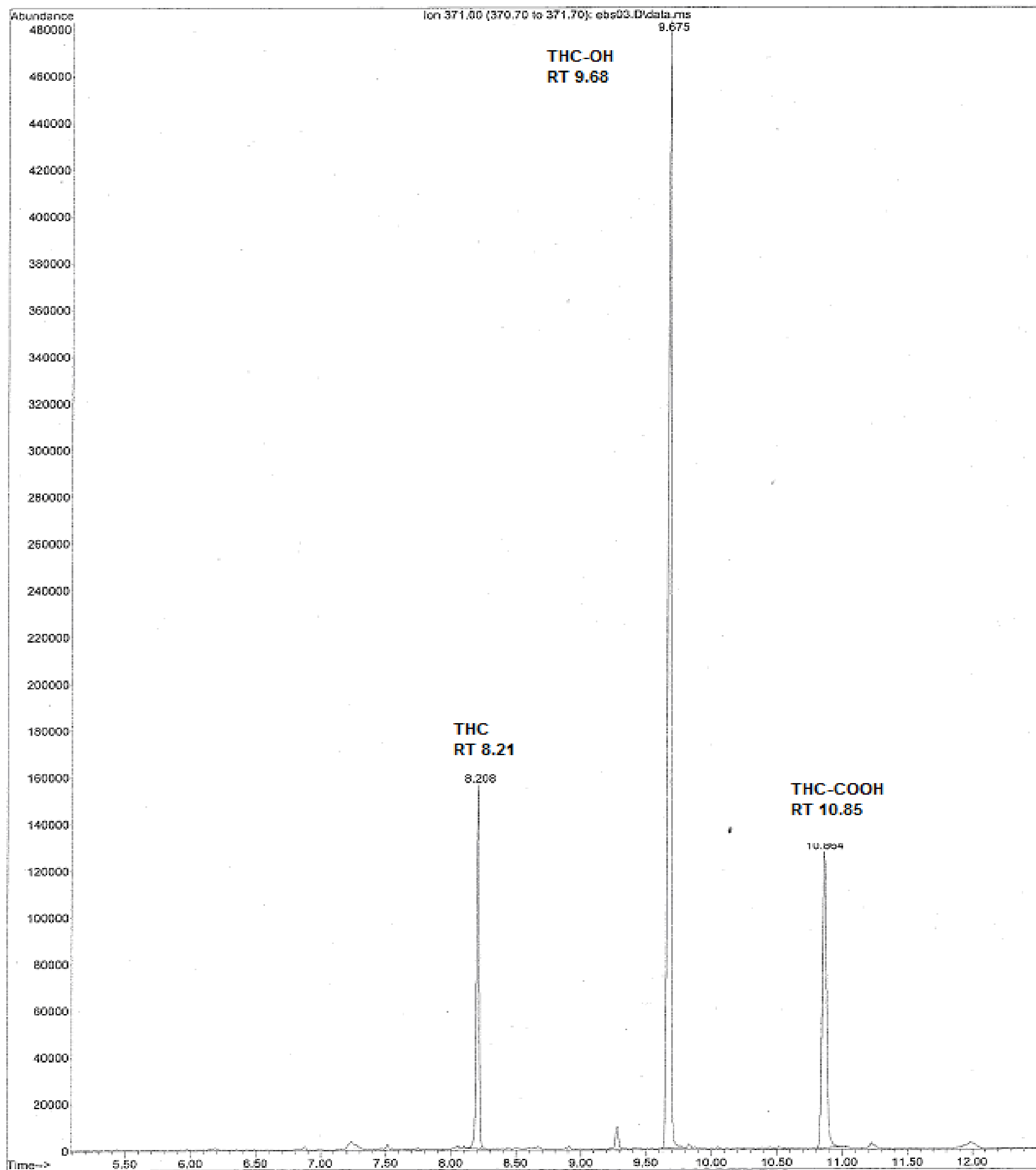


Figure 11: Chromatograms of the TMS-derivates of methanolic standards injected in GC-MS in full scan.

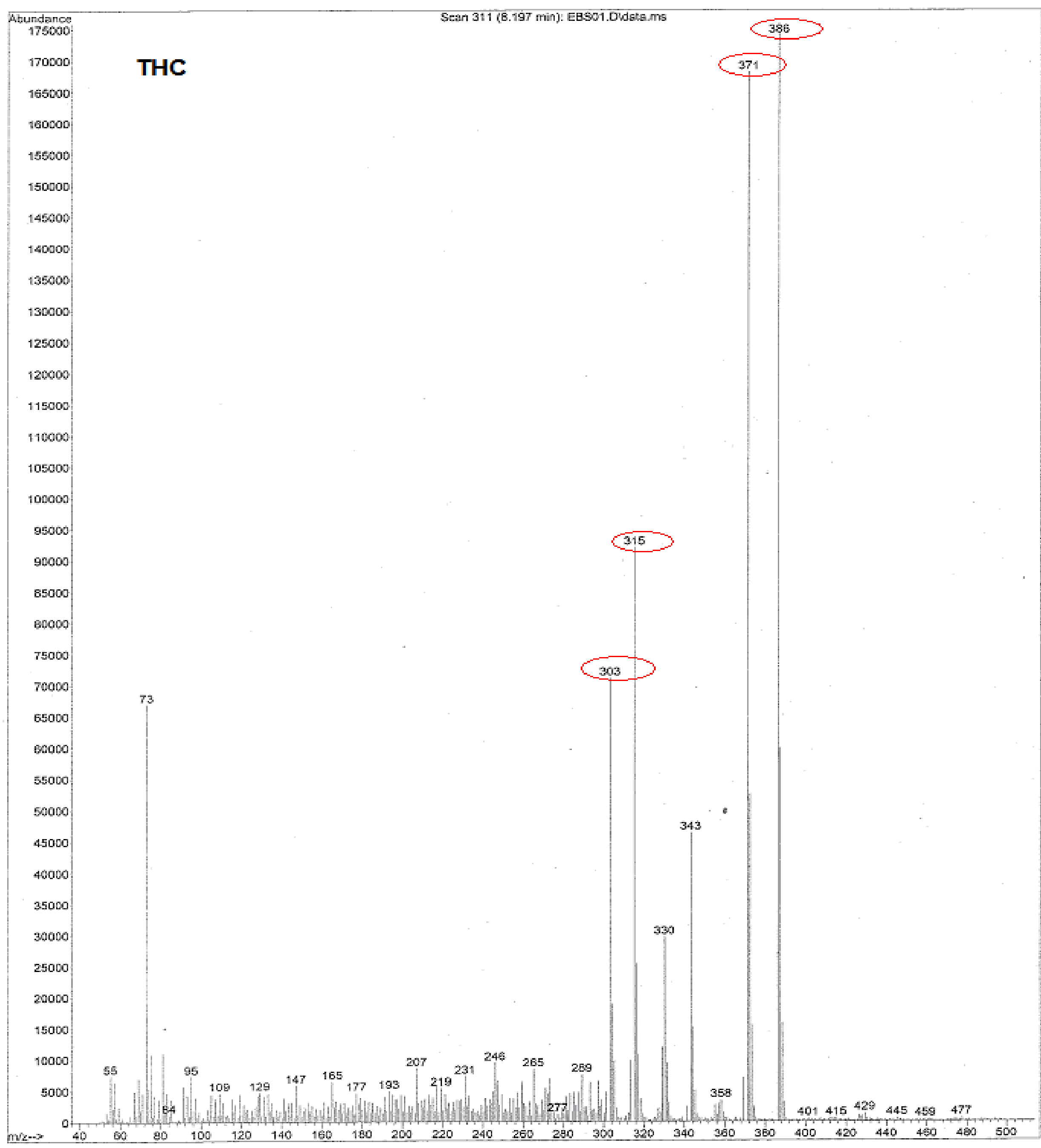


Figure 12a: MS-spectra of THC-TMS after injecting methanolic standards.

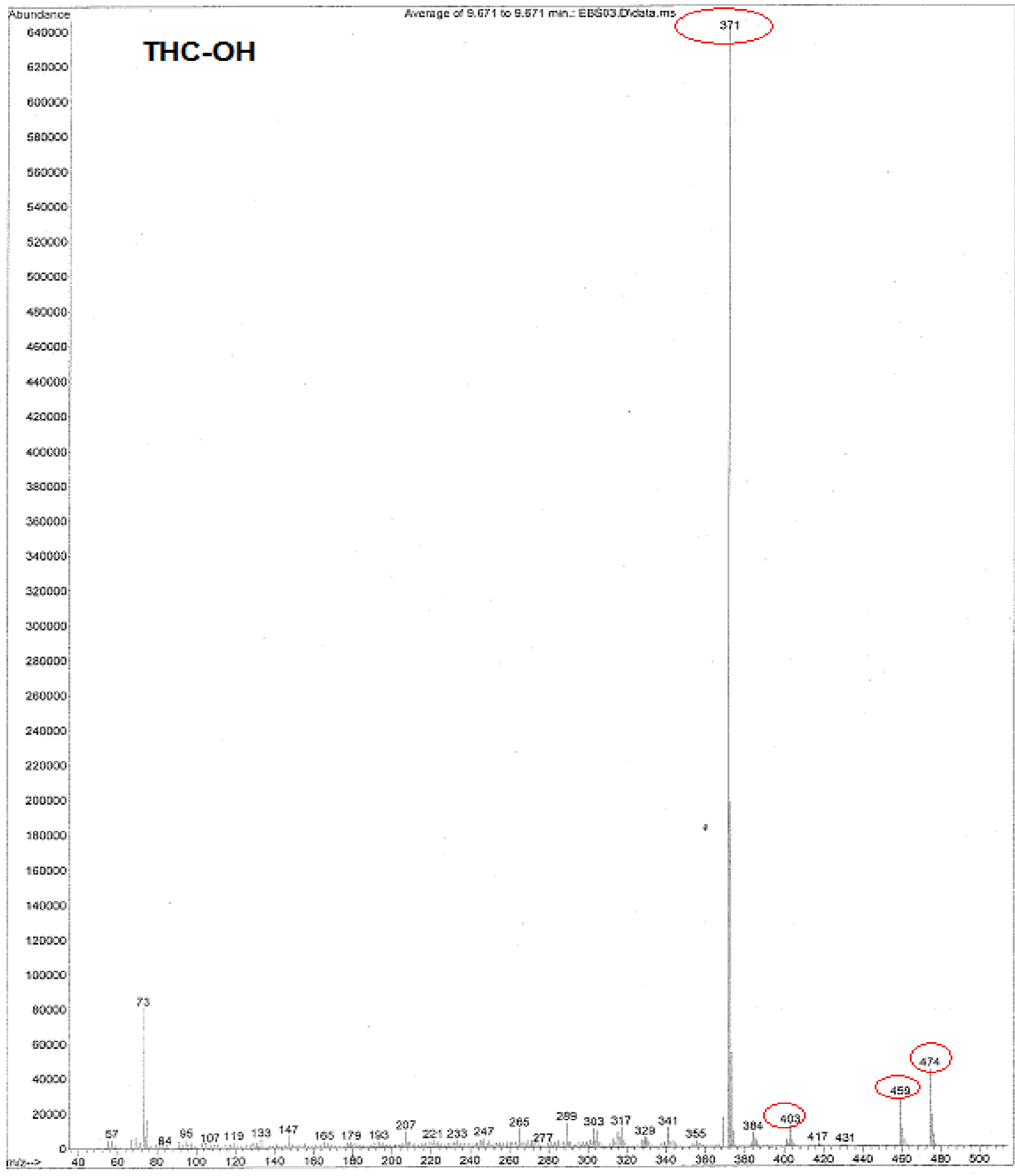


Figure 12b: MS-spectra of THC-OH-2TMS after injecting methanolic standards.

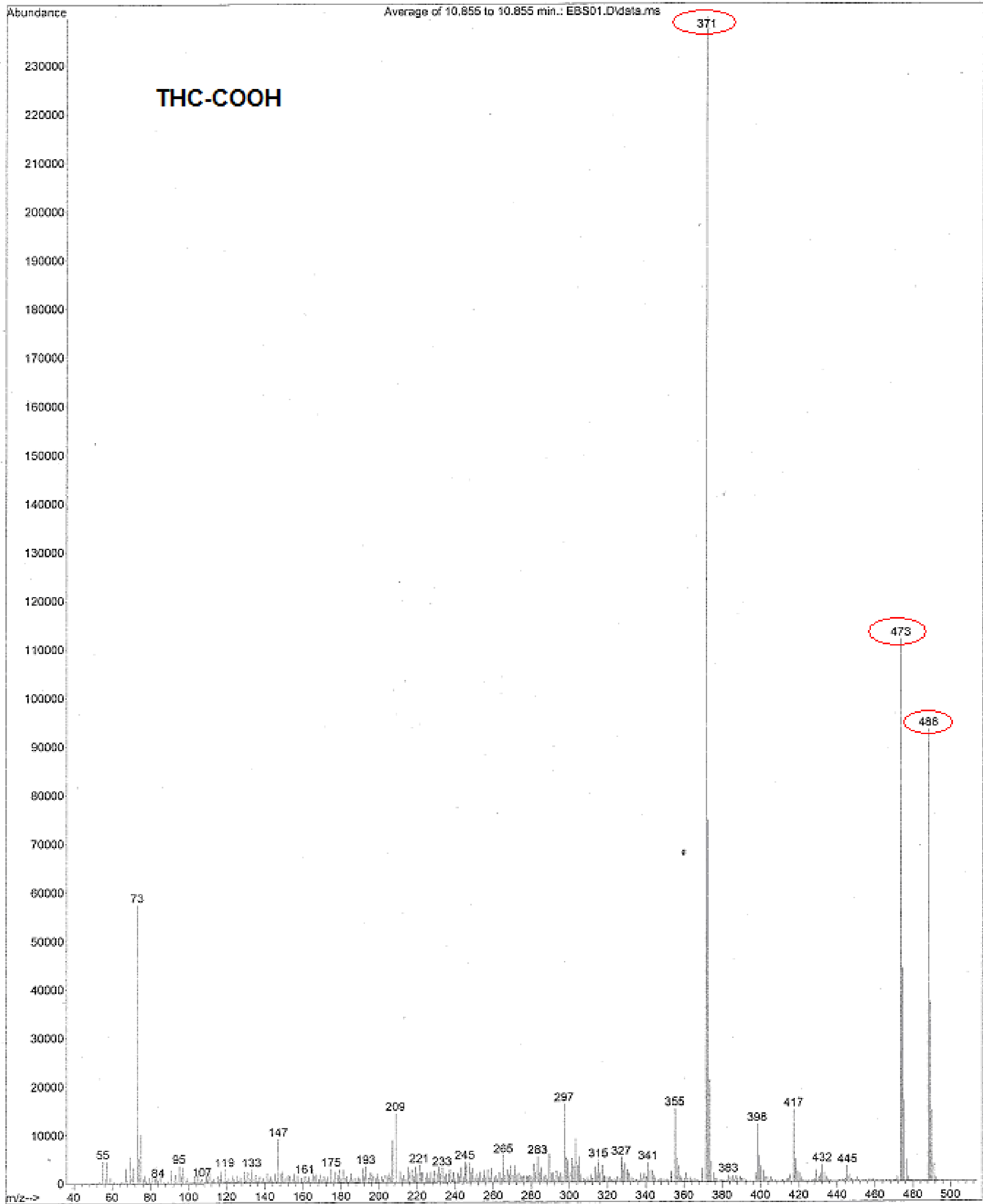


Figure 12c: MS-spectra of THC-COOH-2TMS after injecting methanolic standards.

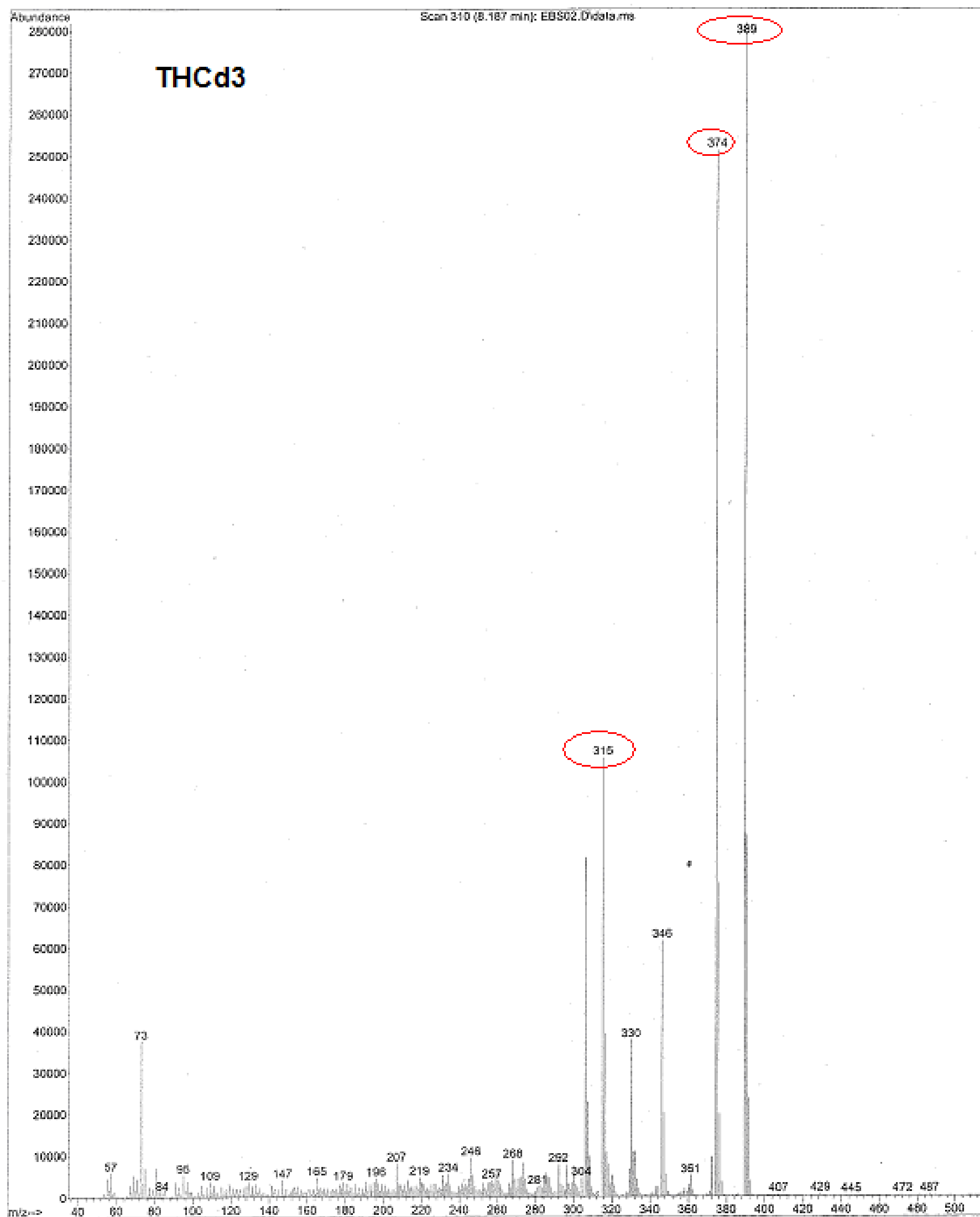


Figure 13a: MS-spectra of THCd3-TMS after injecting methanolic standards.

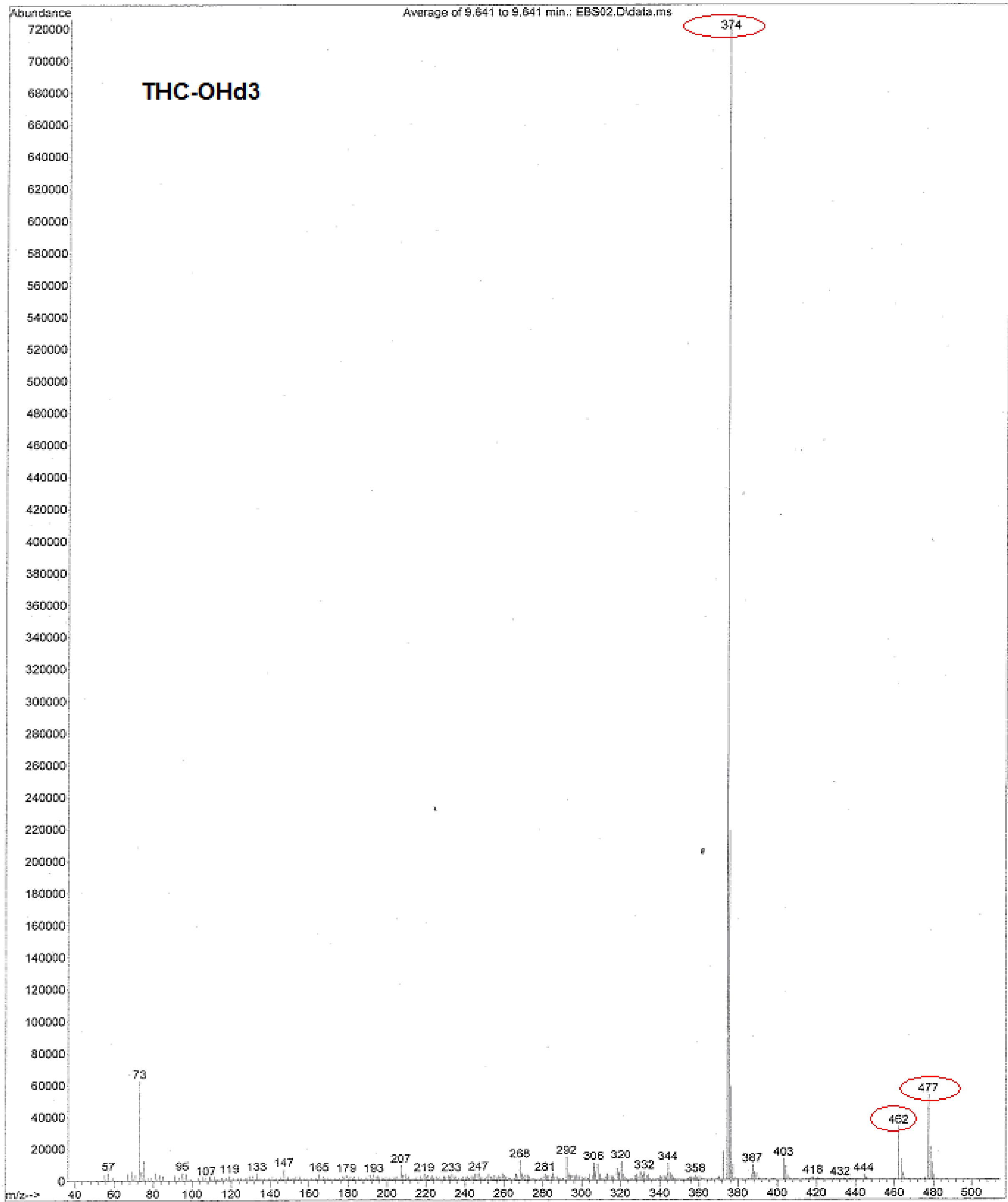


Figure 13b: MS-spectra of THC-OHd3-2TMS after injecting methanolic standards.

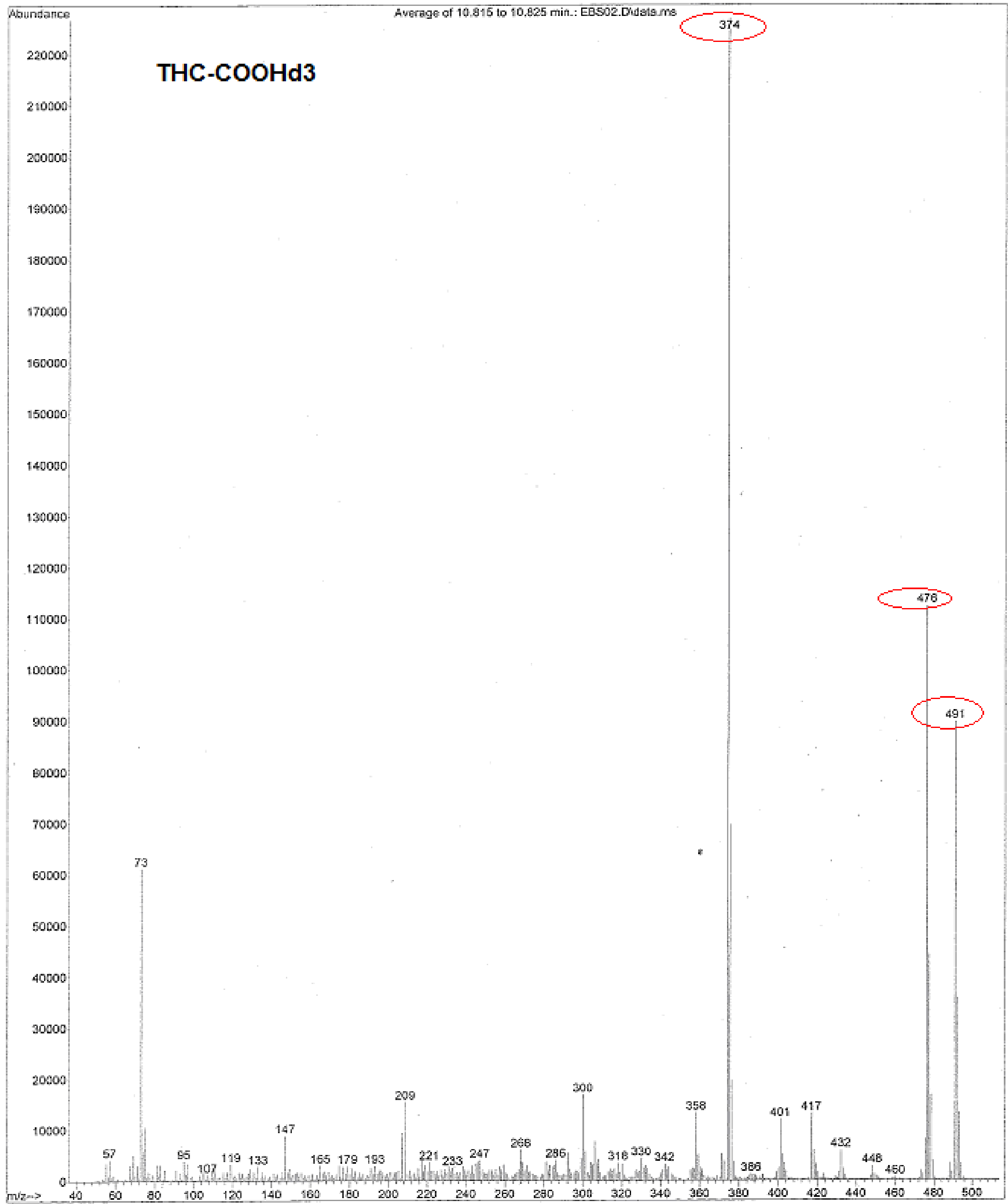


Figure 13c: MS-spectra of THC-COOHd3-2TMS after injecting methanolic standards.

6.1.2 Sample preparation

Three different sample preparations were tested, a mixed mode SPE, a LLE and a LLE combined with the reversed phase SPE. In the combined extraction the extracts from the LLE were dissolved in 1 mL of MilliQ water and acidified with 200 µl acetic acid pure. Two extractions were performed per sample preparation and the extractions were evaporated, derivatized and injected in GC-TSQ in MRM-mode.

Many of the experiments gave no results. The decision of sample preparation was based on noise in the chromatogram, practical reasons, literature review and analytical considerations. The LLE was excluded because of noise in the chromatogram (Figure 14), and the reversed phase SPE was excluded due to clogging of the column. The combination of these two was a complicated and time-consuming extraction; hence this is not a suited sample preparation for a screening procedure. As the mixed mode sample preparation had a low LOD, LLOQ and a good accuracy, it is well suited for the purpose of this thesis. The mixed mode sample preparation utilizes a number of interactions when retaining the analyte on the column, thus the sample are washed more thoroughly.

In this case, the analytes are very hydrophobic (logP are 5.30-6.55) with some polar, and in the case of THC-COOH ionizable, properties. The mixed mode extraction utilizes all these properties of the analytes; the octyl group will interact with the hydrophobic part of the molecule, while the aminopropyl group will interact with the hydrophilic and ionizable parts. Thus, the sample can be washed with both water and AcN. In LLE and reversed phase SPE the analytes are extracted or retained due to hydrophobic interaction, and the sample are not cleaned that thoroughly.

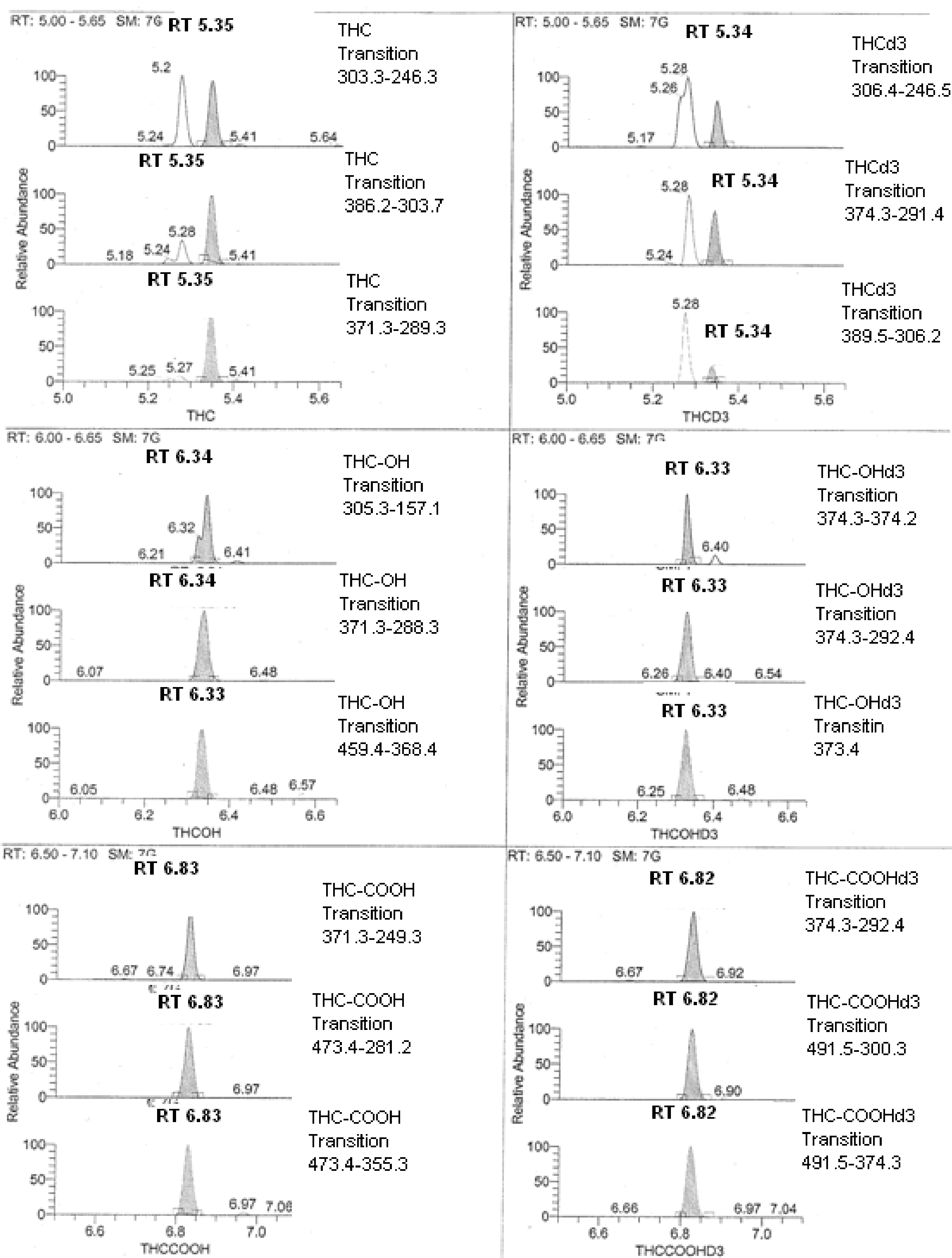


Figure 14: GC-TSQ chromatogram, MRM-mode, of urine samples spiked with standards and internal standards and extracted with LLE. In addition to noise in some of the chromatograms, there is an interfering signal for THC-OH-2TMS (305.3-157.1).

6.1.3 MRM method development

The retention time and the mass-spectra for all the TMS-derivates of analytes and internal standards were obtained, following injections of methanolic standards (2 µl injected from a solution of 4.5 ng/µL) in full scan (Figure 15). The same injection was performed for THC-COOHd9-2TMS (Figure 16).

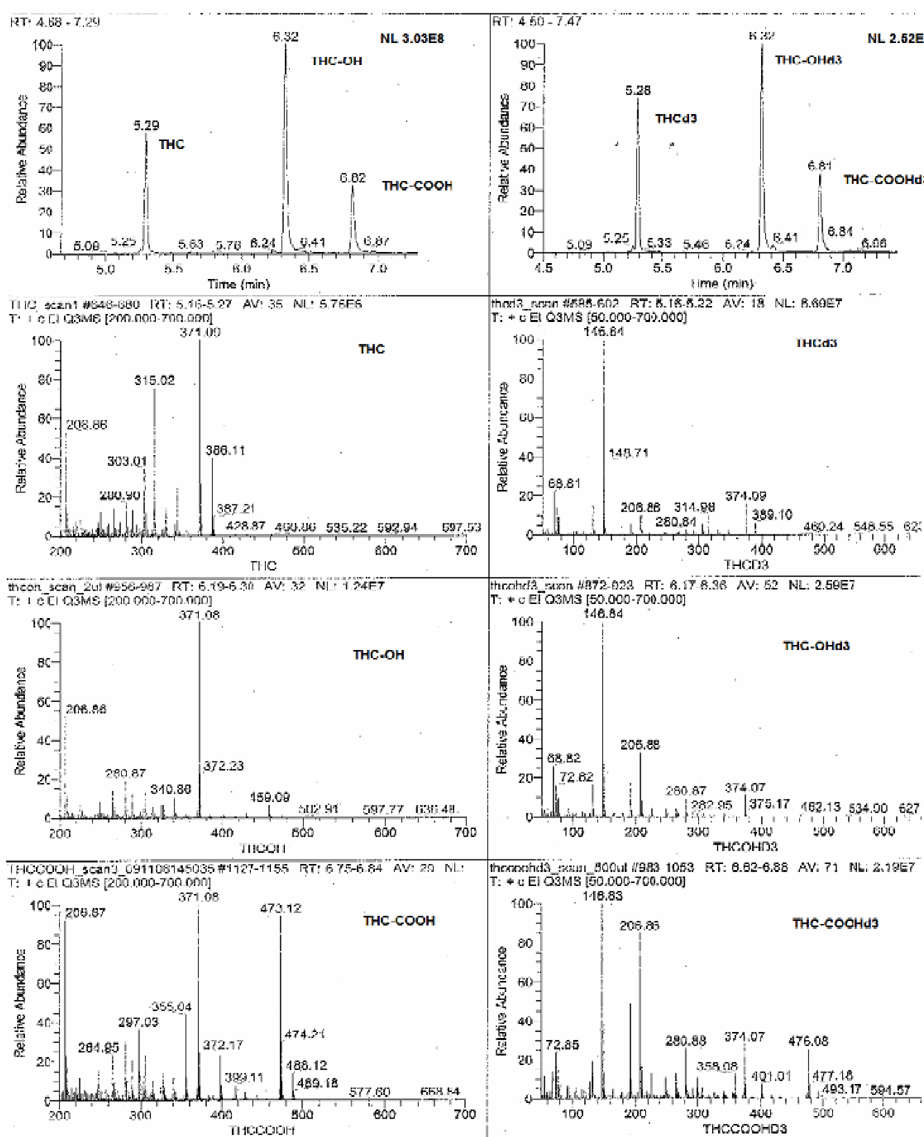
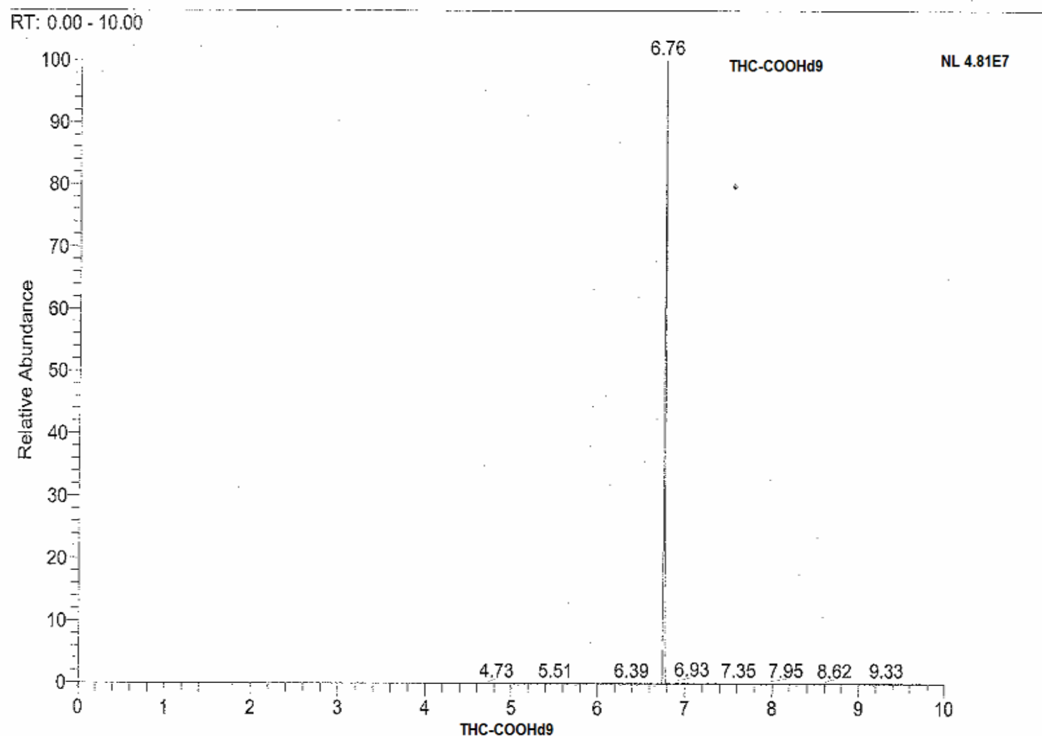


Figure 15: Chromatograms and MS-spectra for the TMS-derivates of the analytes and the TMS-derivates of the d3-analogues.



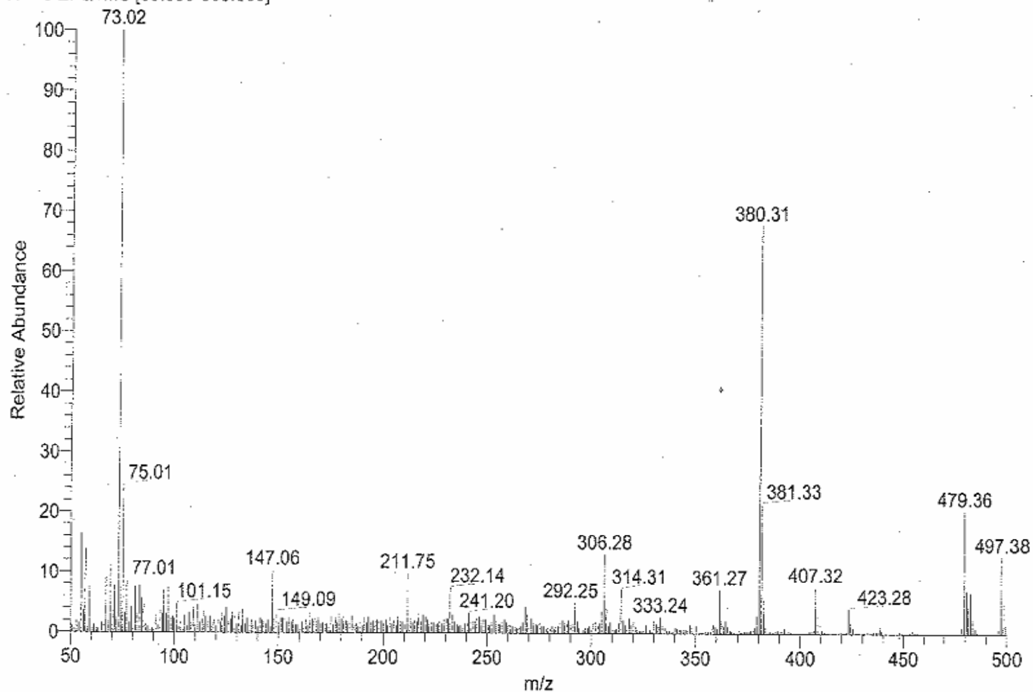


Figure 16: Chromatogram and MS-spectra for THC-COOHd9-2TMS.

Then, the most abundant product ions for each compound were chosen. The product scan for each ion was obtained at different collision energies. The 3 most abundant transitions were chosen at the collision energy which gave the optimal fragmentation. 3 transitions for each analytes were chosen in order to be able to have a quality control for the analysis. One quantification transition was chosen per compound, considering abundance in spiked urine and specificity. For chromatograms of THC-TMS, THC-OH-2TMS, THC-COOH-2TMS and the TMS-derivates of the d3-analogues, see figure 17.

Compound	**Parent ion ($M^{\cdot+}$)**	**MRM transitions (m/z)**	**Collision energy (eV)**	**Retention time (min)**
THC-TMS	428	**371.3-289.3**	25	5.30
		303.3-246.3	30	
		386.2-303.7	25	
THCd3-TMS	431	306.4-246.5	25	5.29
		374.3-292.4	20	
		389.5-306.8	25	
THC-OH-2TMS	474	**371.3-289.3**	20	6.32
		305.3-158.1	30	
		459.4-369.4	20	
THC-OHd3-2TMS	477	**374.3-292.4**	20	6.31
		477.5-374.4	20	
		374.3-274.2	30	
THC-COOH-2TMS	488	**473.4-355.3**	20	6.80
		371.3-249.3	30	
		473.4-281.2	40	
THC-COOHd32-TMS	491	491.5-300.3	30	6.80
		374.3-292.4	15	
		491.5-374.3	15	
THC-COOHd9-2TMS	497	380.2-292.35	20	6.76
		479.3-301.3	25	
		306.3-271.3	30	

Table 8: Parent ion, transitions, collision energy and the retention time chosen for the TMS-derivates of analytes and the internal standards. The quantification transitions are indicated in bold.

According to the instruments settings (column), the retention times could shift form one experiment to another.

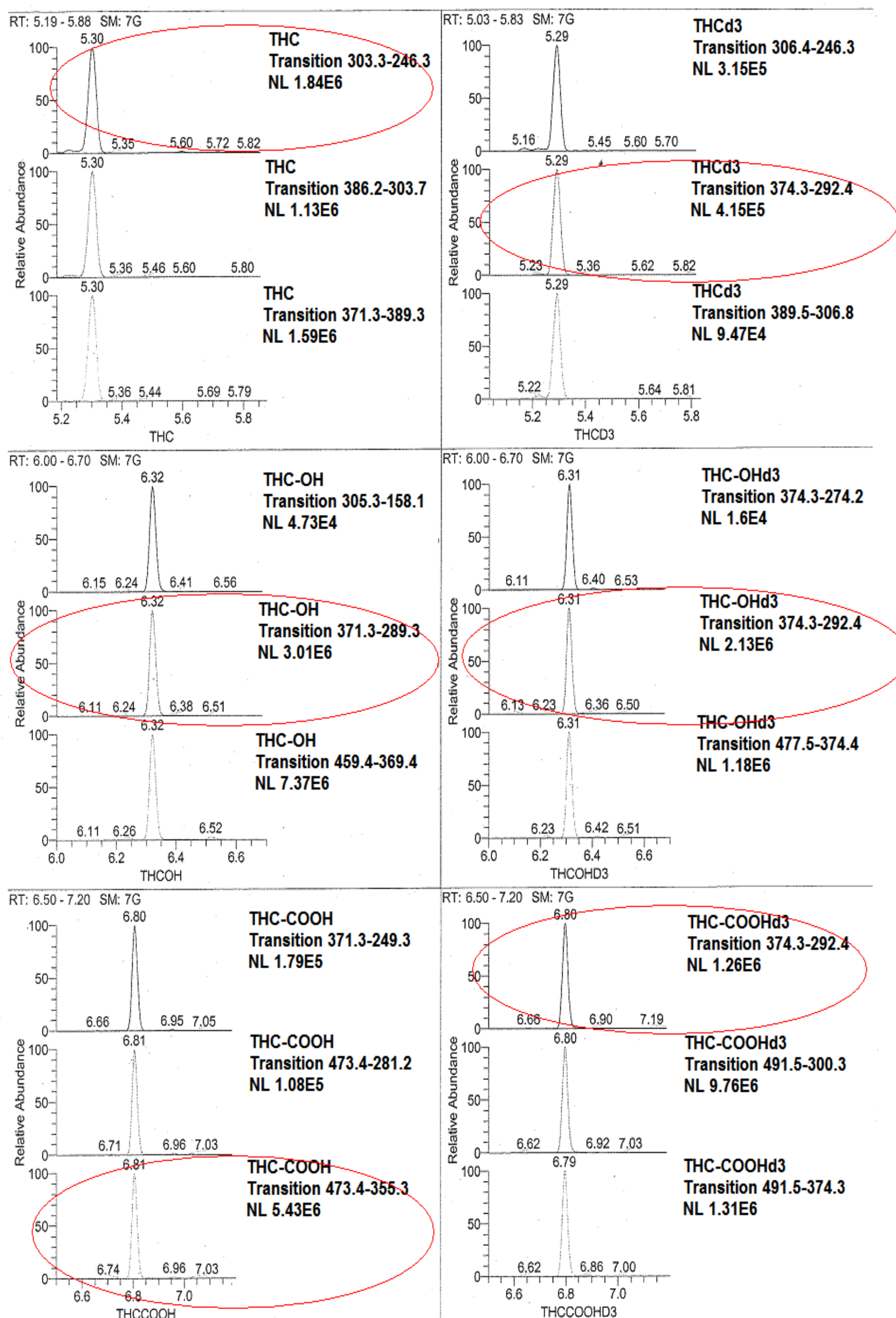


Figure 17: GC-TSQ chromatogram of the TMS-derivates of the analytes and the TMS-derivates of the internal standards. The quantification transitions are surrounded by a circle.

6.2 Specificity

In this experiment, three different GC-MS methods (changes in column type, pressure, oven ramping) were first used. Interference have been observed in each of the methods tested (Figure 18) and thus GC-MS appeared not to be optimal for detection of cannabinoids in urine. Hence, the GC-TSQ was further used in the project.

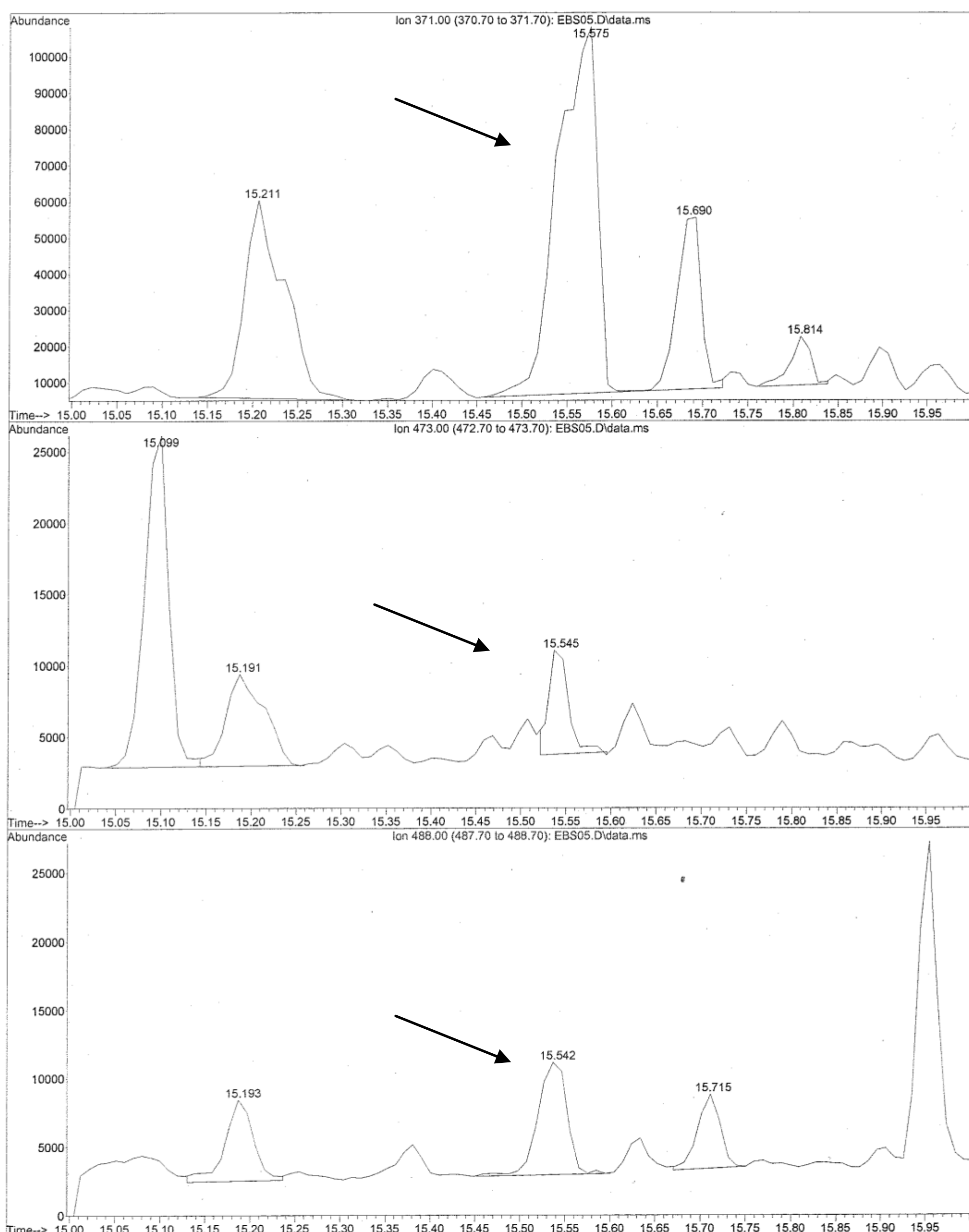


Figure 18: Negative urine injected in GC-MS. The arrows are indicating interfering signals for THC-COOH-2TMS.

The negative urines were compared to spiked urine (50 ng/mL of standards and internal standards) and examined for interfering signals (Figure 19). There were signals for THCd3-TMS (transition 374.3-292.4) and THC-OH-2TMS (transition 305.3-158.1 and 459.4-368.4), hence these transitions was used as qualifying transitions.

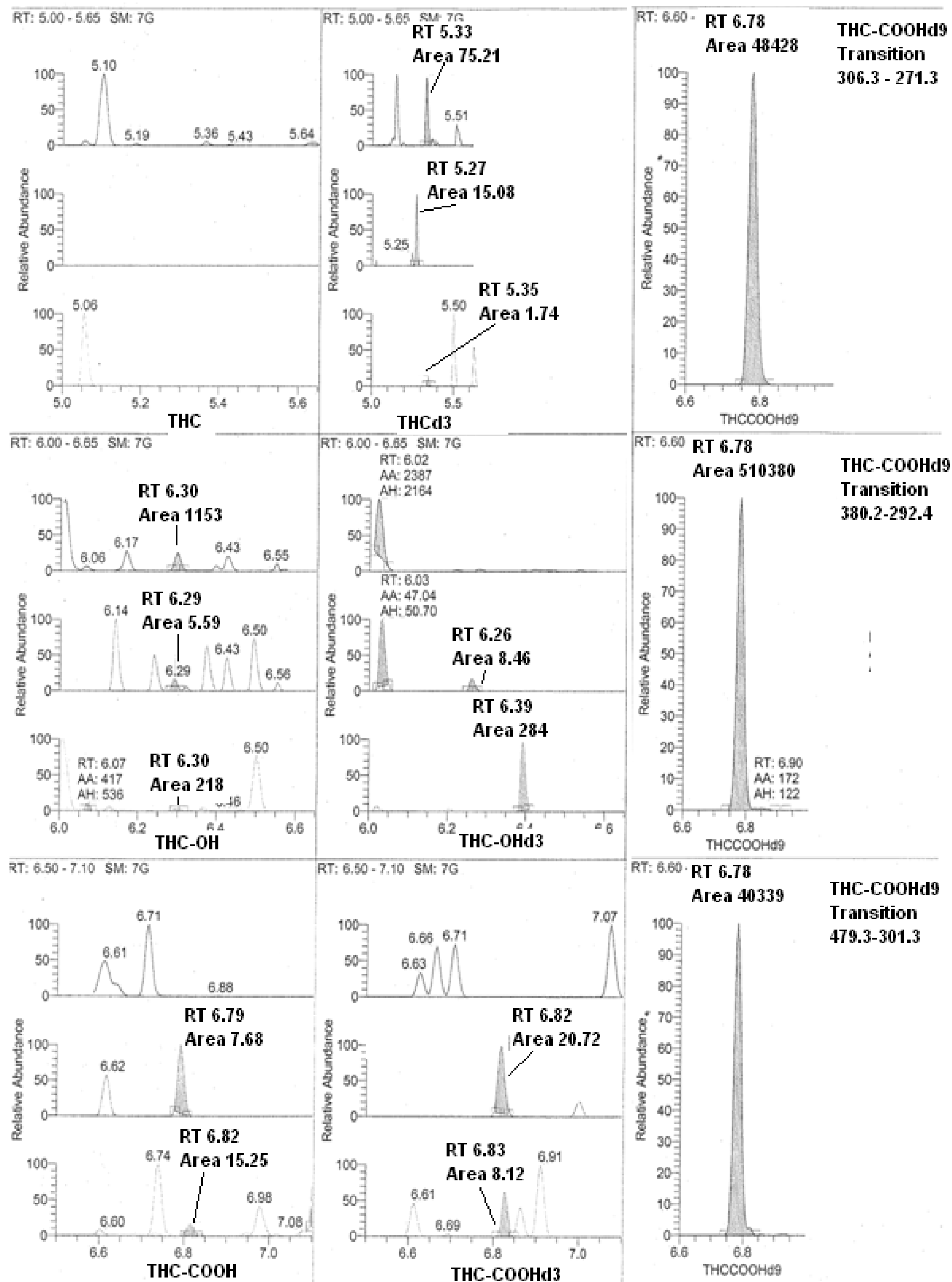


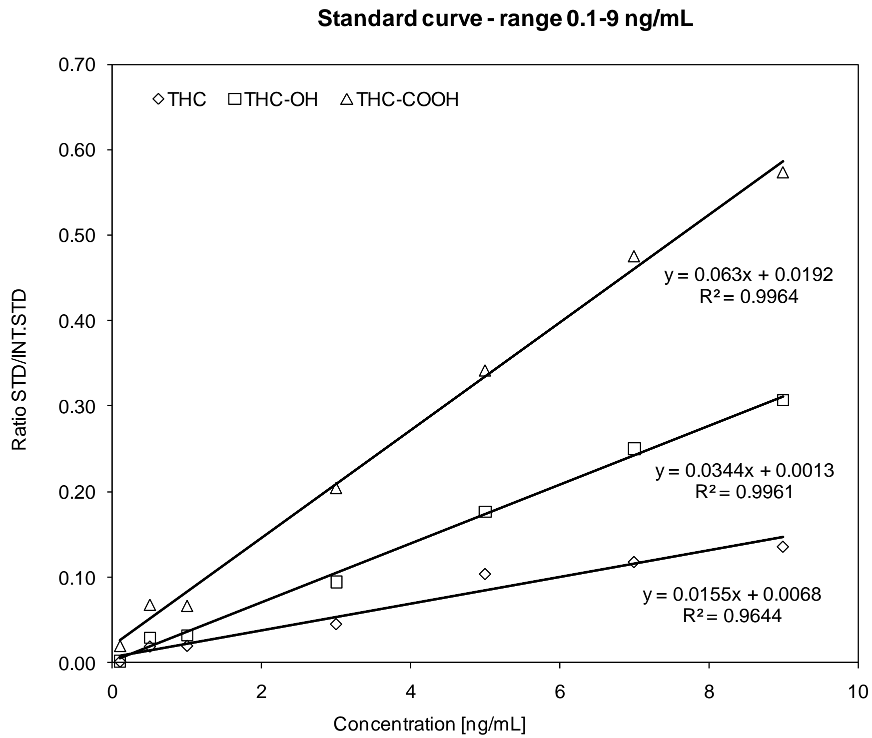
Figure 19: Negative urine from volunteer 2 injected in GC-TSQ. The chromatogram of THC-COOHd9-2TMS, internal standard used in this experiment, can be seen to the left in the chromatogram. The coelutions with a retention time close to the standards are shown.

6.3 Linearity and detection limits

Due to instrumental problems, only urine samples obtained from one person were analyzed in the concentration range 0.1 500 ng/mL. Hence, the estimates and further discussion of linearity, LOD, LLOQ and ULOQ are based on these data only.

The standard curves of THC, THC-OH and THC-COOH in the concentration range 0.1-9 ng/mL (further referred to as the lower concentrations) and 0.1-500 ng/mL were obtained (Figure 20). Considering the small concentration range, more samples have been extracted and analyzed to have a better estimation of LOD and LLOQ and also to avoid an analytical bias with higher concentrations. Indeed, when the concentrations of 100 and 500 ng/mL were introduced in the plot (Figure 20) the equation of the regression curve (slope and intercept) and the calculated concentrations changed (Table 10).

All the linearity coefficients are satisfying (R^2>0.995), except for THC at the lower concentration area. This suggest that the method is stable for THC-OH and THC-COOH, but not for THC, in this range.



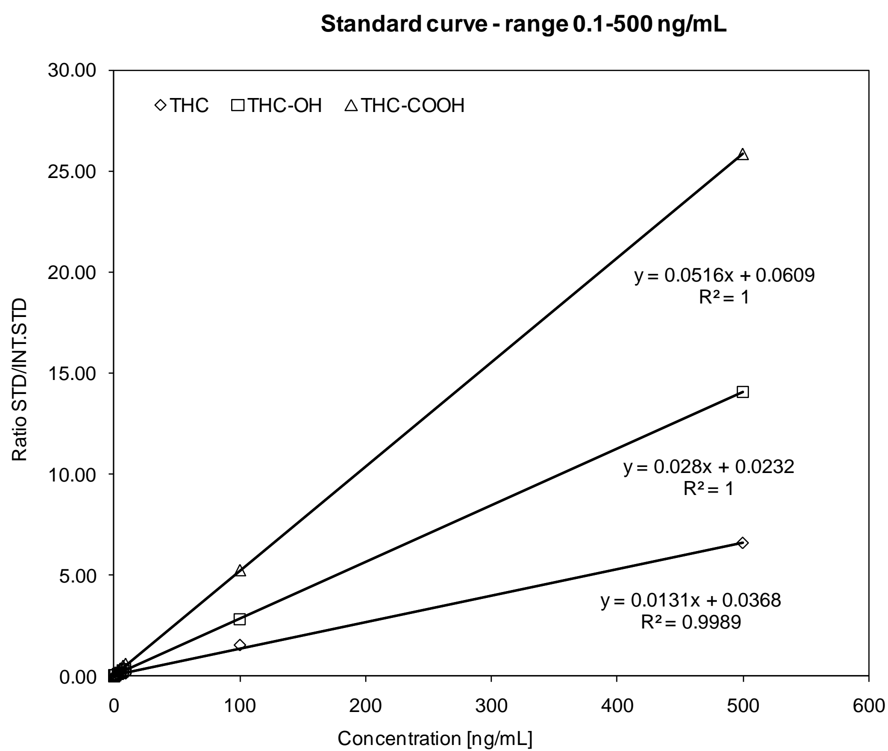
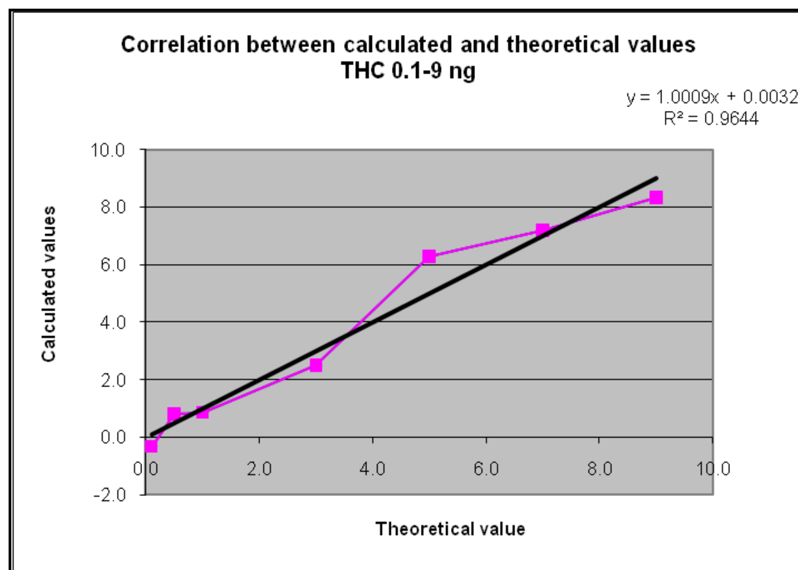


Figure 20: The standard curves for THC, THC-OH and THC-COOH in the concentration range 0.1-9 ng/mL (upper curve) and 0.1-500 ng/m L (lower curve).

The equations obtained from the standardcurves were used to calculate the concentrations (0.1, 0.5, 1, 3, 5, 7, 9, 100 and 500 ng/mL), and a 2 by 2 plot was done with the theoretical concentration (Figure 21- 23).

The correlation between the theoretical and calculated values is expressed by a function, and hence a curve, where *y* is the recalculated values and *x* is the theoretical values, and *a*, the slope, is describing the correlation between these values. A slope and a regression coefficient close to 1 indicate a good correlation. The curves can be seen in (Figure 21-23).



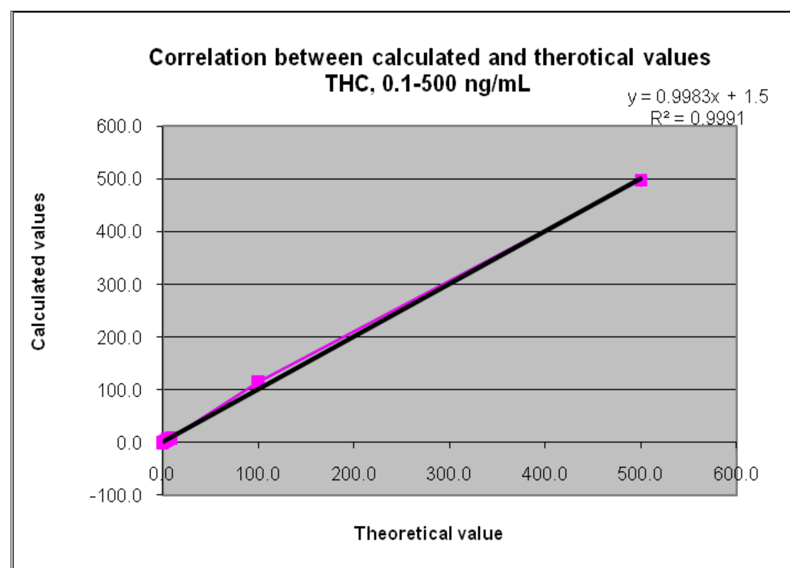
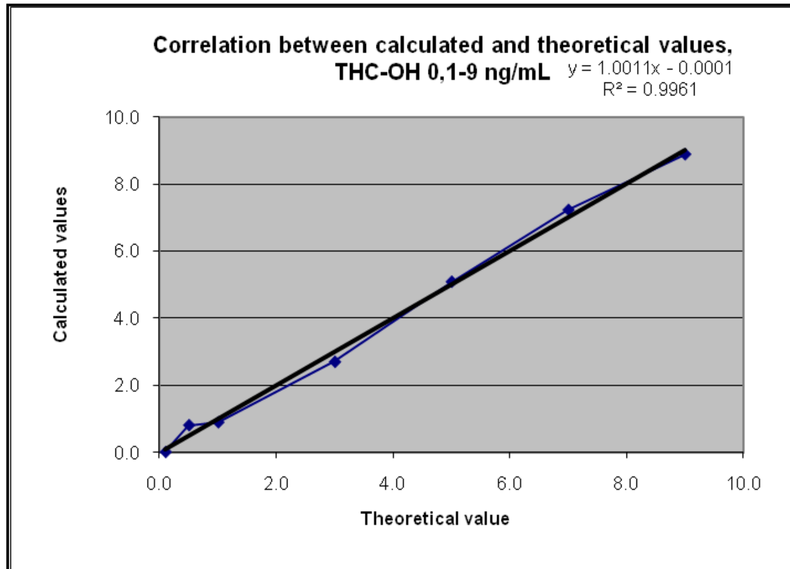


Figure 21: The curve shows the relationship between the theoretical and the calculated values for THC (concentrationrange 0.1-9 ng/mL and 0.1-500 ng/mL).



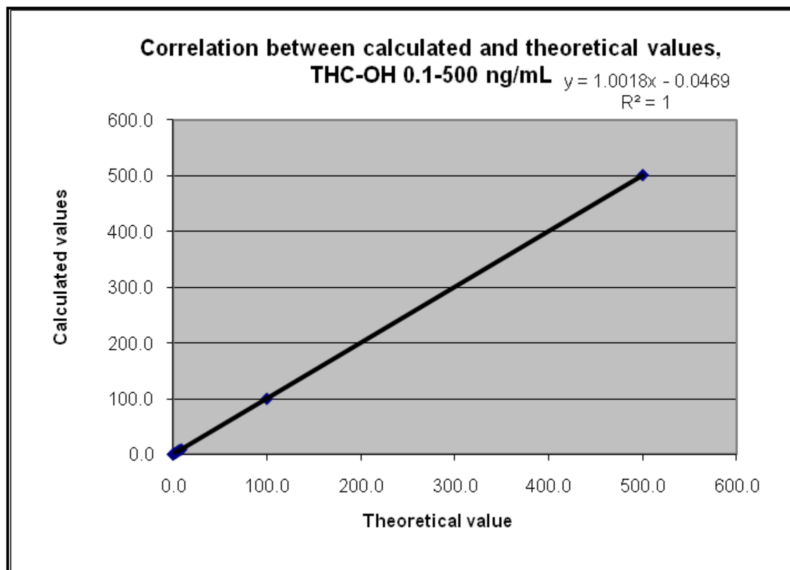
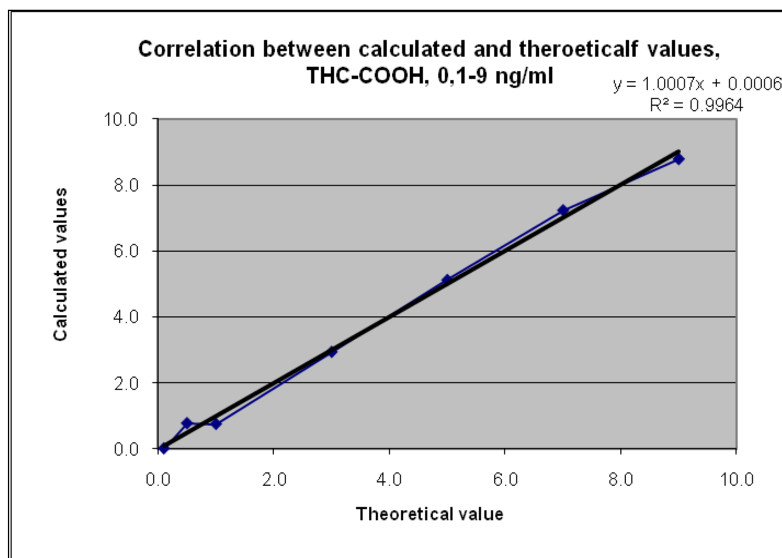


Figure 22: The curve shows the relationship between the theoretical and the calculated values for THC-OH (concentrationrange 0.1-9 ng/mL and 0.1-500 ng/mL).



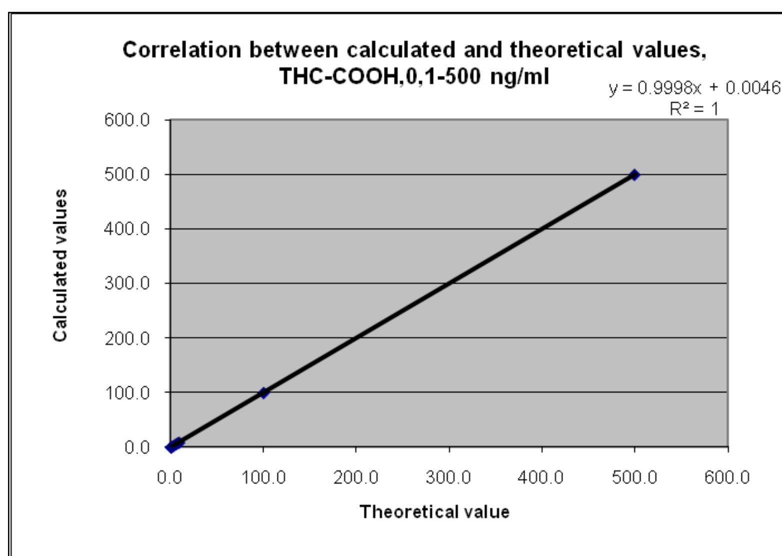


Figure 23: The curve shows the relationship between the theoretical and the calculated values for THC-COOH (concentrationrange 0.1-9 ng/mL and 0.1-500 ng/mL).

The point at 0.5 ng/mL deviates from the curve (Figure 20-23). This is probably due to an error when spiking the samples. Thus, the linearity and correlation may be improved if the experiment is done again.

The difference between the theoretical and calculated values can be expressed in percentage, and illustrates the uncertainty of the method. These values should not increase above a certain limit, which in this experiment was set to 17 %. This value was obtained from the validation for the quantification of THC-COOH on the Swiss Laboratory for Doping Analy-

sis[3]. For table over the theoretical and the calculated values, see table 9. For table over theoretical and the biased calculated values, see table 10.

The correlation is better for the higher concentrations, because the method is more stable in this range.

Theoretical values	Calculated values			Difference between values (%)		
	THC	THC-OH	THC-COOH	THC	THC-OH	THC-COOH
0.1 ng/ml	-0.3	0.0	0.0	424.2	88.3	92.7
0.5 ng/ml	0.8	0.8	0.8	-61.3	-62.3	-54.3
1 ng/ml	0.9	0.9	0.7	14.2	10.1	25.0
3 ng/ml	2.5	2.7	2.9	16.7	10.0	2.1
5 ng/ml	6.3	5.1	5.1	-25.6	-1.8	-2.4
7 ng/ml	7.2	7.2	7.2	-2.7	-3.3	-3.4
9 ng/ml	8.3	8.9	8.8	7.3	1.3	2.3
100 ng/ml	114.3	99.7	100.0	-14.3	0.3	0.0
500 ng/ml	498.2	500.9	499.9	0.4	-0.2	0.0

Table 9: The difference between the true and the calculated values

Theoretical values	Calculated values (biased)			Difference between values (%)		
	THC	THC-OH	THC-COOH	THC	THC-OH	THC-COOH
0.1 ng/ml	-2.7	-0.8	-0.8	2773.7	867.7	899.3
0.5 ng/ml	-1.3	0.2	0.1	367.2	57.0	73.2
1 ng/ml	-1.3	0.3	0.1	227.4	67.8	89.3
3 ng/ml	0.7	2.5	2.8	77.8	15.5	7.4
5 ng/ml	5.1	5.5	5.4	-2.8	-9.4	-8.9
7 ng/ml	6.2	8.1	8.0	11.3	-15.7	-14.7
9 ng/ml	7.6	10.1	9.9	15.8	-12.6	-10.4

Table 10: The difference between the theoretical and the calculated values (biased)

Regarding THC, the values in table 9 connfirmes the results obtained from the curve in Figure 21. For the lower concentrations, the percentage difference (table 10) is not sufficient below 7 ng/ml, which indicates that the method is unstable in the lower concentration range. This is confirmed qualitatively (upper curve, Figure 21) and by R^2, (Figure 20 and 21)

For THC-OH and THC-COOH, the linearity coefficient (Figure 22 and 23) indicates a stable method in the lower concentration range. This can also be confirmed qualitatively (Figure 22 and 23), and by the percentage differences in table 10. For THC-OH the percentage difference is sufficient at concentrations ≥1 ng/ml, and for THC-COOH, the percentage difference is sufficient at concentrations ≥ 3ng/ml.

Since THC and THC-OH are not implemented in screening and quantification of cannabis, no cut-off limit is set. The cut-off limit for THC-COOH is 15 ng/mL, all samples above this level are considered as adverse analytical findings.

Cannabis has doping potential when used the day before a competition, thus it will be useful if the analytes are detectable for 24 hours in urine.

The urinary concentration of THC and THC-OH of light cannabis smokers were detected in levels ≥ 0.1 ng/ml THC and ≥ 0.1 ng/ml THC-OH after 2-8 hours and 12-72 hours respectively [32]. In order to verify cannabis consumption the day before a competition these compounds should be detectable and quantifiable in these concentrations. As the limit from WADA regarding THC-COOH is 15 ng/mL, there is no need to detect this compound in lower concentration in this project.

The same study[32] analyzed urine samples obtained from the Swiss Laboratory for Doping Analysis, and quantified THC, THC-OH and THC-COOH in concentrations of 68 ng/mL, 213.6 ng/mL and 982.9 ng/mL respectively. Higher concentrations of these compounds in the samples may occur, thus the ULOQ in this experiment are suggested to be 100 ng/mL, 250 ng/mL and 1000 ng/mL for THC, THC-OH and THC-COOH respectively.

LLOQ are defined as signal to noise ratio ≥10, and LOD are defined as a signal to noise ratio ≥ 3. The mean values of the S/N in 6 different samples should be calculated to obtain these values.

Due to coeluting peaks in the chromatogram the noise was not obtained. Therefore, LOD and LLOQ were estimated qualitatively for THC, THC-OH and THC-COOH (table 11). The estimated values for THC and THC-OH are higher than the preferred concentrations, but the signal to noise ratio should be obtained to get a more accurate measurement before any conclusions are drawn.

Regarding ULOQ, the curve will change from linear to parabolic when the signal is saturated. The curve was still linear at 500 ng/mL for all the analytes; hence the ULOQ in this experiment was estimated to that value.

The estimated ULOQ is satisfactory regarding the quantification for THC and THC-OH, but should be higher for THC-COOH, see table 11.

	LOD	LLOQ	ULOQ
THC	5 ng/ml	5 ng/ml	500 ng/ml
THC-OH	5 ng/ml	5 ng/ml	500 ng/ml
THC-COOH	3 ng/ml	3 ng/ml	500 ng/ml

Table 11: LOD, LLOQ and ULOQ for THC, THC-OH and THC-COOH

LLOQ and LOD in this experiment are too high regarding THC and THC-OH and the regression coefficient indicates an unstable sample preparation for THC in the lower concentration area. Also the uncertainty is not satiyfying regarding the desired concentrations for THC and THC-OH.

The regression coefficient for THC-OH and THC-COOH are sufficient, and so are the LLOQ and LOD for THC-COOH. However, the detectionlimits were qualitatively determined and should be measured more precisely before any conclusions are drawn. Also, further measurement should be done regarding uncertainty for THC and THC-OH and regression coefficient and uncertainty for THC in the lower concentration range, to see if these values were due to errors when preparing the samples. Therefore, the method should be validated further.

6.4 Quantification of "true" samples

9 different urine samples from THC-consumers with a THC-COOH-concentration of 0.00, 15.22, 15.61, 15.72, 49.70, 100.10, 255.83, 502.56 and 947.84 ng/mL (previously quantified with GC-MS) was added internal standards, β-glucuronidase and phosphate buffer.

For the calibration curve, negative urine were spiked with different quantities of standard solution to contain the following concentrations of the standards; 0, 0.1, 0.5, 1, 5, 10, 50, 100, 500 and 1000 ng/mL. The standards were evaporated before adding urine, enzyme, phosphate buffer and internal standard.

All the samples were added β-glucuronidase, phosphate buffer and internal standards, extracted, evaporated and derivatized with (50 µl MSTFA, 20 minutes, 90°C).

Unfortunately, the retention time was changing during this experiment, see Figure 24, hence no results were obtained from this experiment.

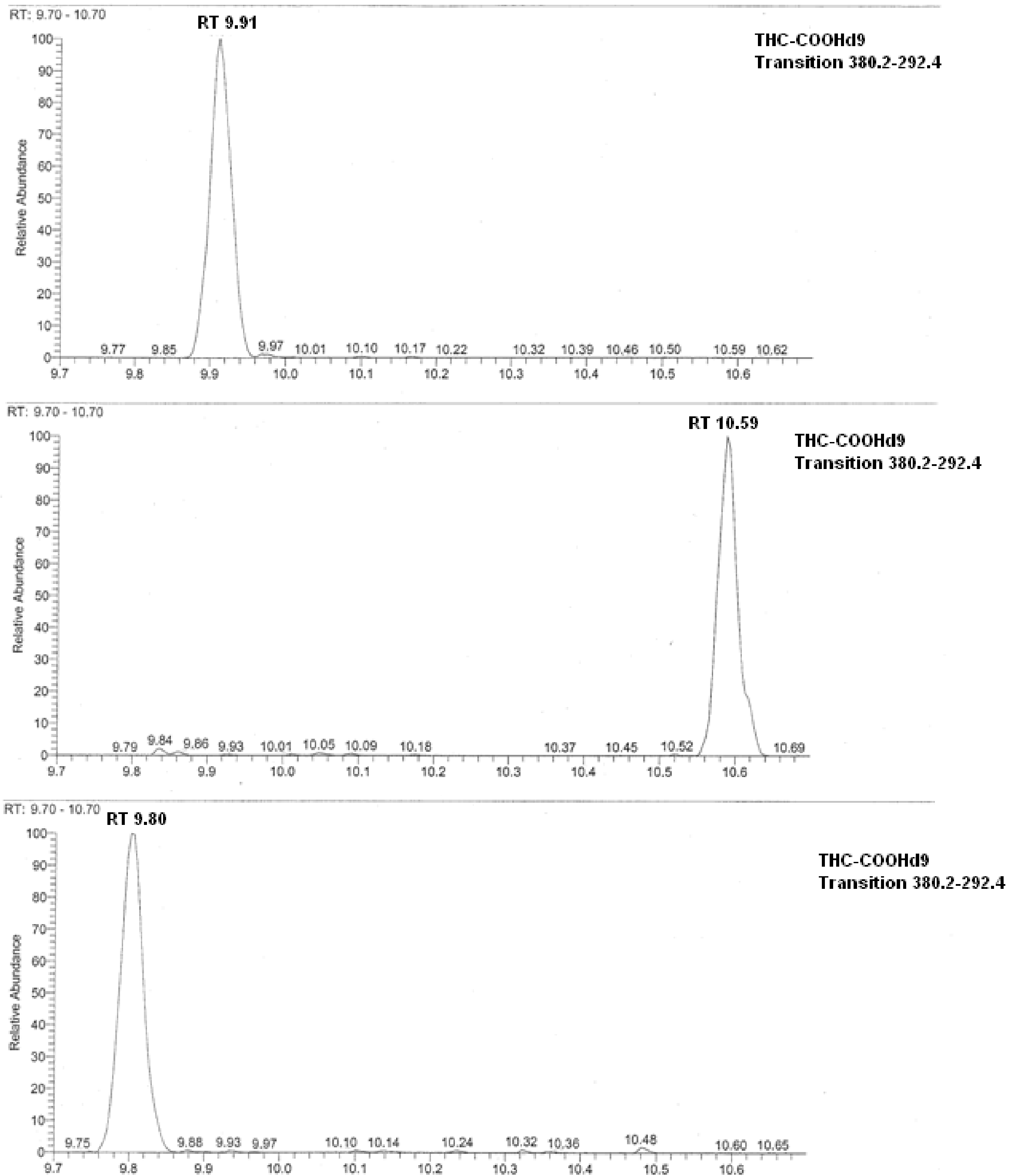


Figure 24: Negative urine from volunteer 2 injected in GC-TSQ, scan mode. The retentiontime for THC-COOHd9-2TMS is changing with over a half minute.

7 Trouble shootings

Several problems appeared during the accomplishment of this project. A lot of efforts have been made to resolve them at the time they emerged which is the main reason of the non achievement of some of the aims.

It is important to mention here few of these problems and to present the way that they have been treated.

- ✓ *Technical problems with the GC-MS/MS instrument*

  In most cases, analyses based on GC-MS/MS in the MRM mode are superior to the use of GC-MS in SIM-mode. However, a lot of technical problems with the instrument were experienced. Considerable time and effort was allocated to solve these problems, and this had great impact on the progress of the project.

- ✓ *Change in retention time*

  During the large injection sequences that have been done on the GC-TSQ, the retention times of the investigated compounds changed in a significant manner. One of the advantages of gas chromatography is its stable RT. Retention time is a key factor to evaluate different steps (specificity, LOD, LOQ,…) in the validation process of an analytical method. Therefore, these shifts in the RT had to be fixed before going further in the validation process. After many investigations, a leak in the gas system was discovered and then the RT was much more stable for a certain period of time which allowed the analyses of the specificity samples.

  Then the same trouble reappeared and it was thought that the problem was due to the derivatization agent since this was not adapted to the GC column installed (HP-1 MS). As acetonitrile was put in the final derivatization mixture, the polarity of this solvent could destroy rapidly the stationary phase inside the column. Another derviatization agent (MSTFA) has been tried as this has been demonstrated to be compatible with the GC column. Once again this solution did not resolve the troubles related to the RT.

- ✓ *Missing signals.*

  In some parts of the project, no signals (peaks) were obtained for the compounds, even not when methanolic standards were injected in full scan. This loss was probably related to a leak in the system and contamination of the ion source.

- ✓ *Missing scans*

  At some point of the project, scans coming from the acquisition system were missing. This was submitted again to the technician who had to come over in the laboratory to check all the electronics devices and fixed the problem.

- ✓ *Sample preparation*

  During the phase of sample preparation testing (LLE, reversed phase SPE and mixed mode SPE), a loss of sensitivity and even total loss of signal were observed for a long time. Many investigations were done on the machine for few days until the sample preparation itself was reconsidered. Indeed, buffers and solutions that were used in the sample preparations were more than a month old and therefore outdated. Fresh solutions were done and signal and sensitivity came back.

8 Conclusion and perspectives

This report reflects eight months of work and efforts. During this project, introductions to methods used in doping analyses have been given. The work has been instructive but also frustrating.

A GC-TSQ method and a sample preparation for detection of THC, THC-OH and THC-COOH in urine have been developed, validated for specificity and evaluated for linearity, LLOQ, ULOQ and LOD. Due to several problems during the project, the method was not fully validated. Even though problems prevented achievement of the initial aims, resolution of the trouble shootings linked to the instrument have been instructive and allowed acquirement of practical and theoretical aspects of working with a GC-TSQ.

Different sample preparations and conditions for hydrolysis were tested. The final extraction procedure includes an enzymatic hydrolysis with 8250 U/mL β-glucuronidase at 37°C for 16 hours followed by a mixed mode SPE sample preparation. Working with different types of instruments has improved the previous knowledge of the practical and theoretical aspects of GC-MS. The MRM transitions chosen during the method development revealed to be specific and adapted for the quantification of THC and its metabolites in urine matrix.

The results and discussion regarding the linearity and detection limits suggest that the method should be further validated.

Anyhow, the method is useful basis for further work.

Further, a MRM-method for the THC-analogues should be developed; using the same procedure as used for THC, THC-OH and THC-COOH, and a sample preparation should be developed, validated and implemented in the screening procedure in the Swiss Laboratory for Doping Analysis. First then, the relevance of these metabolites in urine should be investigated.

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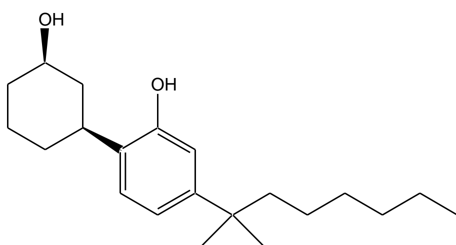
Appendix

9.1 Herbal blends

In 2004 herbal mixtures called 'Spice' was introduced on the marked, mainly via internet. They come in different versions, i.e. Spice Gold, Spice Silver, Spice Diamond, Spice Arctic Synergy, Tropical Spice and Spice Egypt. Other herbal blends, which say they have the same content as Spice, examples of these are Yuacatan Fire, Smoke, Sence and ChillX, are also available [49].

The herbal blends are declared incense and not for human consumption. Despite of this, they are smoked. Users have described cannabis-like effect after consuming these blends, but cannabis cannot be detected and the narcotic effects was suggested to be caused by added synthetic compounds or plant extracts [50]. Late in 2008 JWH-018 ((1-pentil-3-(1-naftoil)indol) [49]) was detected in one of the Spice products. Shortly after, CP47,497 (5(1-dimetilheptil – 2 ((1R,3S) – 3 hydroxycyclohexyl) [49] was also detected in a Spice product [49, 50]. Both these compounds are defined as synthetic cannabinoids [50]. After the detection in the mixtures, the Spice products was prohibited several countries of the EU [49]. In march 2009, another synthetic cannabinoid, HU-210 (6a*R*,10a*R*)- 9-(Hydroxymethyl)- 6,6-dimethyl- 3-(2-methyloctan-2-yl)- 6a,7,10,10a-tetrahydrobenzo [c]chromen- 1-ol), was detected in some herbal blends (Spice Gold, Spice Silver, Spice Diamond, Geni and Yuacatan Fire) by the Drug Enforcement Administration in USA [49, 51]. At the same time JWH-018 was replaced by the analogue JHW-073 ((Naphthalene-1-yl-(1-butylindol-3-yl)methanone [52]) [50].

The cannabinoid analogues act agonistically on the receptors CB1 and CB2 [53], and are classified into several groups. CP49,497 belongs to the non classic cannabinoids, which are bicyclic or tricyclic compounds [49]. The CP47,497 has 3-28 times greater potency compared with THC (depending on the applied model) [54] and the affinity for CB1 is 20 times greater than for THC [49]. For structural formula of CP47,497, see figure 25.

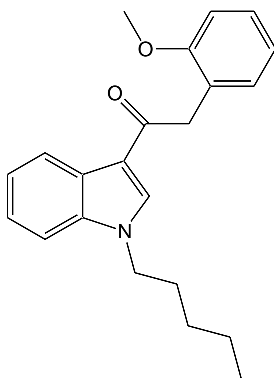


CP47,497
Molecular weight: 318.5g/mol
Monoisotopic mass: 318.3 u
pKa: 10.80
Average logP: 6.25 (±0.81)

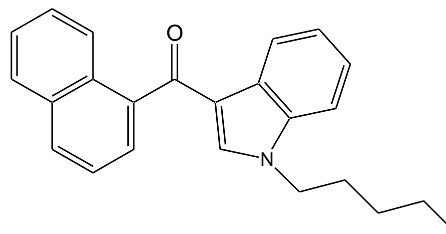
Figure 25: Structure, molecular weights (MW) and pKa-values for CP47, 497. The pKa-values and the logP are predicted values [46, 47]

The group aminoalkaloides includes JWH-018, JWH-073, JWH-200 and JWH-250. The compounds in this group have a chemistry structure different from the other groups. When compared to THC, JWH-018 has a 4 times greater potency for the CB1 receptor and 10 times greater potency for the CB2 receptor. In studies on rats, JWH-018 diminished locomotive activity, produced analgesia, hypothermia and catalepsy in a similar way that THC does. JWH-073 mainly affects CB1, but has a similar affinity of CB2 as that of THC. In the same way as JWH-018, the analogue JWH-073 will affect locomotive activity and produce analgesia and hypothermia similar to THC. There are no studies on catalepsy effects regarding this compound [49].
JWH 200 (1-[2-(4-Morpholino)ethyl]-3-(1-naphthoyl)indole) binds to CB1-receptor with a Ki 42 +- 5 nM [55]. The Ki-value is the inhibition constant for a drug; the concentration for a drug which would occupy 50 % of the receptors if no competing ligand was present. A study on mice have shown that JWH-200 have the same or superior effects in locomotor activity, tail-flick latency, hypothermia, and ring-immobility tests as THC[56].

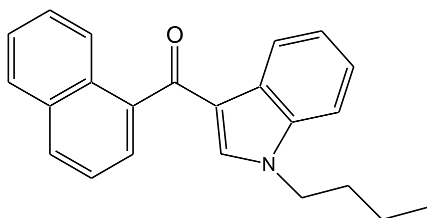
JWH-250 (2-(2-Methoxyphenyl)-1-(1-pentylindol-3-yl)ethanone) [52] was detected in May 2009 in spice products [52]. The compound has a high affinity for CB1 and CB2 receptors with a Ki = 11 nM and Ki = 33 nM, respectively. These values are a bit smaller than those for the THC (Ki = 40.7 nM and 36.4 nM respectively) [57, 58]. For structural formulas of JWH-018, JWH-073, JWH-250 and JWH-200, see figure 26.



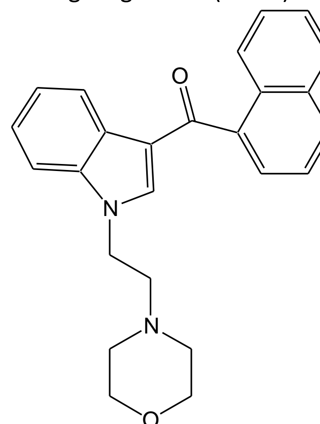
JWH-250
Molecular weight: 371.5 g/mol
Monoisotopic mass: 335.2 u
Average log P: 6.31 (±0.70)



JWH-018
Molecular weight: 341.5 g/mol
Monoisotopic mass: 341.8 u
Average logP: 6.36 (±0.60)



JWH-073
Molecular weight: 327.5 g/mol
Monoisotopic mass: 327.2 u
LogP: 5.91 (±0.50)



JWH-200
Molecular weight: 384.5 g/mol
Monoisotopic mass: 384.2 u
Average logP: 4.21
pKa: 6.60

Figure 26: Structure, molecular weights (MW) and pKa-values for JWH-250, JWH-018, JWH-073 and JWH-200. The pKa-values and the logP are predicted values [46, 47]

The last two groups of cannabinoids are classical cannabinoids (dibenzopyranoids) and endocannabinoids (eicosanoids). The first group includes THC and consists of a tricyclic structure. The compounds in the latter one are produced endogenously, and the majority of this group are derivates of arachidonic acid [49].

There are several problems with the human intake of these different blends. First of all, the only existing studies on the compounds (JWH-018, JWH-073, JWH-200, JWH-250 and CP47,497) are in test-tubes or with experimental animals. Thus, little is known about the safety and effects in humans [49]. Second, batch-to-batch-variation in the kind and differences in the amount of drug used can cause accidental overdosing [54]. And third, because

of the ease of synthesis of JWH-018 analogues, these could easily replace each other in legal replacement products [50]. It is also reason to be concerned about induction of psychic problems due to use of spice-products, since these compounds have cannabinoid-like effects. In a case report reactivation of psychotic symptoms after abuse of "Spice" was reported [59]

Because of the cannabinoid effects of Spice-products, there is a risk that these kinds of compounds could be used as performance-enhancing agents in sports, and thereby activate psychic problems among athletes. Thus, it is important to have a method for detection of these compounds in urine.

9.1.1 Current chromatographic methods for detection of cannabinoid analogues

Since these analogues are newly introduced into the marked, the literature on this field is limited. By start of this project, it was as far as I know, no published work regarding GC-MS/MS analysis of these compounds. However, in march 2010, a liquid-chromatography (LC) tandem MS method for quantification of JWH-018 in human serum was accepted in a journal [60]. In another accepted article, JWH-018, CP-47,497 and JWH-073 in herbal products were identified and analysed using GC-MS and LC-MS. The MS-spectra and a sample preparation of these herbal products were presented [61].

Two published articles have also managed to detect JWH-018 in herbal samples by GC-MS [50, 54], the same applies for JWH-073 [50]. CP49,497, JWH-018 and JWH-073 have also been detected by LC-MS [61]