

National Student Research Conference

ÁKOS LEMLE

**Method development for the
simultaneous identification of acidic and basic
drugs of abuse in blood**

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List of abbreviations

3-CMC	3-chloromethcathinone
BSTFA	<i>N,O</i> -bis(trimethylsilyl)trifluoroacetamide
GC	gas chromatography
LOD	limit of detection
LOQ	limit of quantification
<i>m/z</i>	mass / charge
MBTFA	<i>N</i> -Methyl-bis(trifluoroacetamide)
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MS	mass spectrometry
MSTFA	<i>N</i> -methyl- <i>N</i> -(trimethylsilyl)trifluoroacetamide
MTBSTFA	<i>N</i> -(tertbutyldimethylsilyl)- <i>N</i> -methyl-trifluoroacetamide
NENP	<i>N</i> -ethyl-norpentedrone
SIC	single ion chromatogram
SIM	single ion monitoring
SPE	solid phase extraction
TBAH	tetrabutylammonium hydroxide
THC	delta-9-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-delta-9-tetrahydrocannabinol
THC-OH	11-hydroxy-delta-9-tetrahydrocannabinol
TIC	total ion chromatogram
TMCS	trimethylchlorosilane

1 Introduction

Illicit drugs have always been a way for humans to escape mental pain and excessive stress. While in the short term, it seemingly alleviates the problems, but then, these substances introduce even more problems. Drugs have many acute and chronic side effects, and some substances can even induce aggression from the user. Therefore, it is important to make people rather face their problems than to use illicit drugs.

In order to confirm that a person used a kind of illicit substance, instrumental analysis is required. These are long procedures that involve a sample preparation step, and the target compounds are generally detected using a mass spectrometer coupled with a gas or liquid chromatograph.

In my research, I developed a rapid analytical method with the use of a derivatization agent that can simultaneously derivatize different functional groups in aqueous environments without the need for long and expensive solid phase extraction (SPE).

Most components can only be measured after a derivatization step when using gas chromatography. Most of the derivatization agents do not work in aqueous environments, so the compounds need to be extracted beforehand. However, the underivatized molecules are generally hydrophilic therefore, the extraction can be long and tedious. Moreover, most derivatization reactions take a long time and require high temperature.

On the other hand, chloroformates can be used to derivatize most functional groups with active hydrogen. Unlike the majority of the derivatization agents, these reactants work in aqueous environments, so the extraction can be performed more rapidly and efficiently after the derivatization. This way, acidic and basic compounds can be derivatized and extracted simultaneously. Additionally, the whole reaction can be performed under one minute at room temperature.

During my research I developed an analytical method for the identification and quantification of the most prevalent narcotics in blood samples. I used isobutyl chloroformate to simultaneously derivatize the acidic and basic compounds and I performed the measurements on a mass spectrometer coupled with a gas chromatograph.

2 Literature overview

2.1 Illicit drugs

Humans always endeavored to escape discomfort. While the most straightforward way of easing mental pain is to face and solve the aligning problems, some people have always sought seemingly easier methods. The date when the first narcotic was used is unknown.¹

However, most drugs not only have a desired effect, but can also cause potentially unwanted symptoms and the user may experience addiction. Addiction is defined as a chronic desire of repeated drug consumption and the inability to reduce the dosage of the agent. Illicit substances cause these effects by altering the biological systems in the brain that are responsible for the rewarding and reinforcing effects. These alterations are often persistent, causing the desire of re-administration.²

As a result of drug administration, the system adapts to the changes and develops mechanisms to neutralize the effect of the substance. These neutralizing mechanisms remain even when the user stops the administration, causing withdrawal effects such as negative mood and elevated stress.^{2,3}

In Hungary, the most prevalent illicit drugs are cannabinoids, amphetamine and its derivatives, and cathinone derivative stimulants. In the following sections, some of these compounds are discussed in more detail.⁴

2.1.1 Marijuana

Marijuana has been known to humans for thousands of years. Its main active ingredient is delta-9-tetrahydrocannabinol (THC), and after consumption, it causes a long-lasting euphoric state. Marijuana is typically smoked in cigarette but can be administered orally along with food or can be drunk in the form of a tea.^{5,6}

In addition, marijuana along with other cannabinoids can be used for medical purposes.⁷ For example, cannabinoids can be used to help patients with chronic pains, asthma and eye disorders.⁸ They are found to be beneficial for patients that were admitted to chemotherapy.⁹ However, whether cannabis should be used for medical purposes or not, is disputed. The studies that were conducted on this matter often contradict each other.⁸

Chronic use of marijuana can result in excess weight gain and reduced background EEG activity. It can also cause respiratory problems and nausea.¹⁰ Some studies found that THC also has neurotoxic properties.¹¹

When a drug molecule enters the system, it inevitably takes part in biochemical reactions, and as a result, the structure of the drug changes. The resulting molecules are called metabolites and this whole biochemical process is called metabolism.¹²

The two main metabolites of THC are 11-Hydroxy-delta-9-tetrahydrocannabinol (THC-OH) and 11-Nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) (Figure 1). In order to establish drug abuse in the case of a suspect, the metabolites must also be screened alongside the parent molecule.¹³ The time of the last cannabis use can be estimated based on the concentration ratio of THC and THC-COOH.^{14,15}

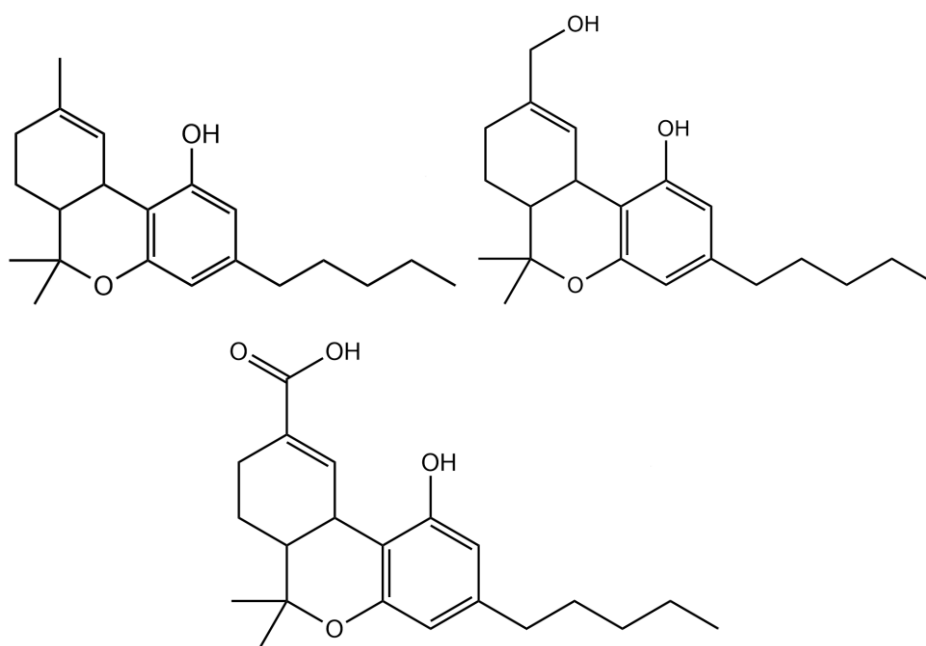


Figure 1 The structural formula of THC (top left), THC-OH (top right) and THC-COOH (bottom)

2.1.2 Amphetamine, a synthetic stimulant

Stimulants increase the amount of available dopamine in the brain which causes euphoria and joy. Chronic administration changes some of the brain functions by altering the neurons. High doses of stimulant consumption have harmful effects on the neuron synapses. Some studies imply that chronic usage can cause permanent neurological

problems. By repeated administration, the system develops tolerance to the stimulants, making the consumer elevate the dosage to reach the desired effect.¹⁶

Amphetamine (Figure 2) is a stimulant that is often used to treat children with ADHD. While it has proven to reduce the symptoms¹⁷, some studies imply that amphetamine administration reduces the rate of weight and height growth of children.¹⁸

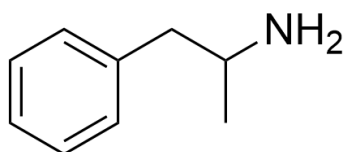


Figure 2 The structural formula of amphetamine

Amphetamine was given to soldiers during the World War II to increase their alertness and performance and during the sixties, it was also used to increase the performance of professional sporters. Hence, the positive effects of amphetamine were widely known.¹⁹

On the other hand, amphetamine can cause many acute intoxication symptoms such as vomiting, increased heart rate and nausea. There are also chronic adverse effects such as insomnia, anorexia, weight loss and cardio-vascular diseases.²⁰ Studies suggest that aggressive behavior can also be a response to the drug.²¹

2.1.3 Cathinone derivatives

Cathinone (Figure 3) is also a kind of stimulant. It naturally occurs in the plant *Catha edulis* (khat). Its molecular structure and effects are very similar to those of the amphetamine. Khat originates from East Africa, and its leaves were chewed for centuries by local residents for their stimulant effects. Khat leaves are best consumed soon after harvest, because cathinone decomposes rapidly.²²

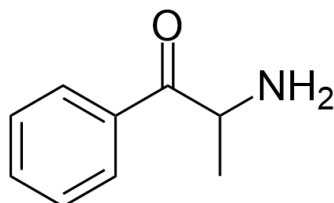


Figure 3 The structural formula of cathinone

Prior to the first isolation of cathinone, its two derivatives had already been synthesized from ephedrine, namely methcathinone (ephedrone) and 4-methylmethcathinone (mephedrone).^{23,24} Ephedrine naturally occurs in the plant ephedra which is a shrub native to China. During the sixteenth century, ephedra was used to treat circulatory diseases and cough.²⁵ Ephedrine is still used as a medicine and it is available in Hungary. For example, the Coderit N medicine aimed to cure dry cough, and it is available in local pharmacies.²⁶ Ephedrine is also used as a medicine for e.g. asthma and hypotension in the United States.²⁷

It had already been on the US market before the introduction of stricter medicine approval protocols, and despite the fact that studies imply that it has stimulant and adverse effects, similar to the amphetamine, it still has not been banned yet.²⁸

Shortly after the identification of cathinone, more synthetic derivatives were created. These synthetic derivatives were intended to cure medical issues. Nowadays, synthetic cathinones are highly demanded on the black market due to their amphetamine-like stimulant effects, prevalence and affordable price.²²

N-ethyl-norpentedrone (NENP), also known as N-ethyl-pentadone (Figure 4), is a new psychoactive component that first appeared on the black market after 2010, and it was identified in Europe in 2014.²⁹ It is also a synthetic cathinone derivative. Its effects only last for a couple of hours, therefore, often re-administered after the end of its effects. NENP has social behavior related side-effects like reduced social exploration, and after repeated administration, it can induce aggression.³⁰

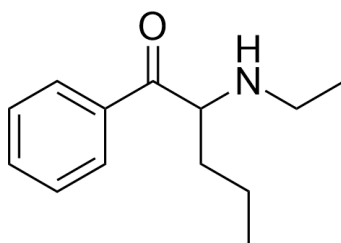


Figure 4 The structural formula of N-ethyl-norpentedrone (NENP)

The main metabolite of NENP is N-ethyl-norpentedrol where the keto group is converted to hydroxylic group by reduction.³¹

2.2 Methods of illicit drug identification

Illicit drugs can be screened for most rapidly using enzymatic immunoassays (EIAs). Such tests are convenient to use and can provide a result very quickly, but they cannot substitute a thorough laboratory assay. EIAs are often not sensitive or specific enough, therefore their positive results need to be confirmed.^{32,33}

Immunoassays use an antibody that can bind to the unique 3-dimensional structure of a target or to a class of targets. However, these antibodies do not have the required specificity to only bind to the designated target, therefore e.g. medicines that share a similar structure can yield false positive results. Moreover, an antibody can only detect very similar structures and not all the illicit compounds. To mitigate these problems outlined, samples that were tested positive are sent to laboratories to confirm the immunoassay test result.³³

During such confirmatory analyses, HPLC-MS and GC-MS based methods are used to identify and quantify the illicit drugs in biological matrices. For GC-MS analysis, the compounds are extracted from the matrix using liquid-liquid extraction or solid phase extraction (SPE), then derivatized to reduce their hydrophilicity. The different sample preparation methods for illicit drug measurement in blood using GC-MS are shown in Table 1.

While SPE can be used to extract the target compounds with high efficiency, such a procedure can be long and tedious. The first step of a SPE is the sorbent conditioning that increases the active surface area. Then the matrix, containing the target compounds, is passed through the cartridge. After that, the contaminants can be rinsed using a polar solution or water. Then, an organic solvent is used to elute the target compounds.³⁴

To accelerate the liquids passing through the cartridge, vacuum can be used,³⁵ but even then, this whole procedure can be tedious and time consuming, furthermore it requires one-time use cartridges and high volume of solvents which makes it expensive. So, if the required limit of detection and accuracy can be achieved without using SPE, it is recommended to use liquid-liquid extraction instead.

Table 1 Different GC-MS sample preparation methods for illicit drugs

V_{blood} / mL	Extraction	Derivatization agent	LOD / (ng/mL)	LOQ / (ng/mL)	Target samples	Citation
2	liquid-liquid	iodomethane	0.2-0.4	-	THC, THC-COOH	³⁶
0.5	SPE	BSTFA with 1% TMCS	0.29-0.33	0.87-0.99	THC, THC-OH, THC-COOH	³⁷
0.2	liquid-liquid	trifluoroacetic anhydride (TFAA)	0.22-0.81	0.65-2.4	amphetamine-like stimulants	³⁸
2	liquid-liquid	hexyl-chloroformate	1-50	5-50	cathinones, amphetamine-like stimulants	³⁹
1	SPE	MSTFA	5-10	10-25	cocaine, amphetamines, cannabinoids	⁴⁰
1	liquid-liquid	propyl-chloroformate	0.2-0.9	0.5-2.4	THC, THC-OH, THC-COOH	⁴¹
1	SPE	iodomethane	0.3-3	-	THC, THC-COOH	⁴²

Abbreviations: LOD: limit of detection, LOQ: limit of quantitation, SPE: solid phase extraction, BSTFA: *N,O*-bis(trimethylsilyl)trifluoroacetamide, MSTFA: *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide

2.3 Derivatization methods

Most components are only measurable with GC-MS, if they are derivatized. Derivatization reduces the polarity of the compounds of interest, which enables the detection of symmetric and narrow peaks.⁴³ However, most of the time derivatization is performed following the extraction step, which makes the latter less efficient and more time consuming. Moreover, basic compounds can only be extracted to the apolar phase if the aqueous phase is alkalinized, and acidic compounds can only be extracted from an acidic aqueous phase.

2.3.1 Silylation agents

The most prevalent derivatizing agents are silylation agents, and they react with the active hydrogen containing functional groups (Figure 5). The most widespread silylation agents are *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-(tertbutyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA). Trimethylchlorosilane (TMCS) is often used as catalyst to help the reaction.⁴³ These derivatization agents require high temperature and long reaction times, which makes the sample preparation more time consuming.⁴⁴ It is also

true that silylation agents are sensitive to water, therefore the components need to be extracted from the aqueous phase and only then can be derivatized.⁴⁵

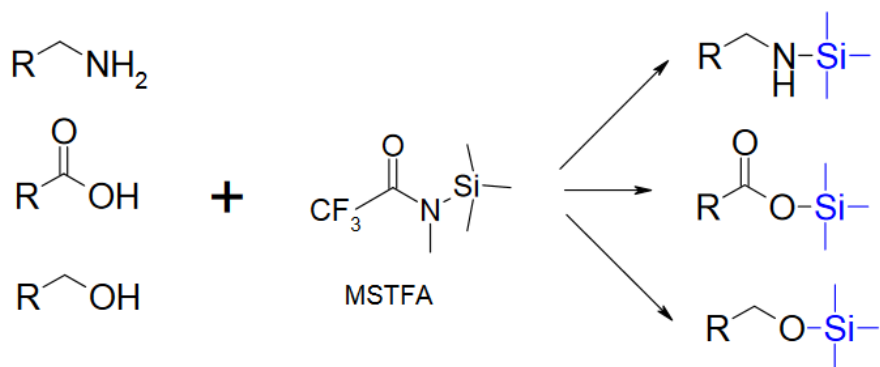


Figure 5 Silylation using MSTFA⁴⁶

2.3.2 Alkylation

Another widespread derivatization method is alkylation. For example, iodomethane in the presence of tetrabutylammonium hydroxide (TBAH), can be used to derivatize hydroxyl and carboxylic groups. The main advantage of this method compared to silylation is the shorter reaction time, and the ability to perform it at room temperature.⁴⁷

2.3.3 Anhydrides

Hydroxyl groups can be acylated using acid anhydrides (Figure 6). This method can be applied even more rapidly however, it requires higher temperature.⁴⁸ The excess derivatization agent must be evaporated following the reactions, which reduces the time-efficiency of the procedure. However, this step is crucial, because organic anhydrides can cause damage to the analytical column.⁴⁹

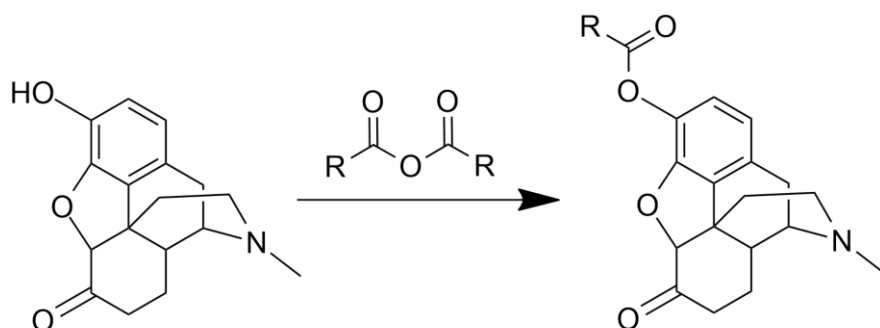


Figure 6 Derivatization using anhydrides

2.3.4 Chloroformates

So far, all the above-mentioned derivatization methods require a long reaction time and can only be performed in the organic phase, following the extraction. However, it is not the case with chloroformates. Chloroformates can react with the active hydrogen containing function groups (Figure 7) in aqueous solutions, which makes it possible to simultaneously derivatize basic and acidic components, and following the reaction, the extraction can be performed more rapidly and efficiently. The whole procedure can be done in less than a minute, at room temperature, which makes the sample preparation less time-consuming. These derivatization agents can even work in biological matrices such as urine and blood. In the case of blood samples, protein precipitation is necessary prior to derivatization.^{39,41}

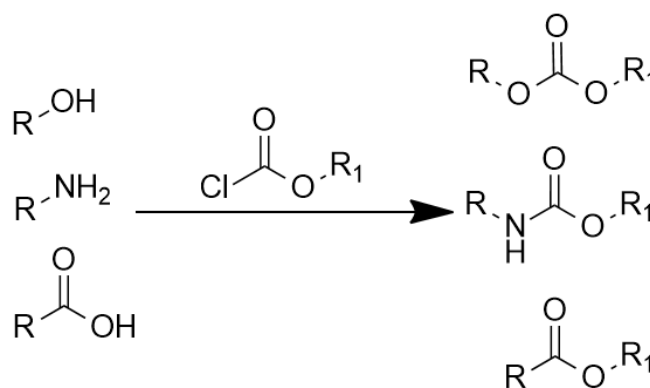


Figure 7 Derivatization using chloroformates

2.4 The chemical properties of chloroformates

Chloroformates are known to react with water and form hydrogen chloride and carbon dioxide. Depending on the reacting chloroformate, the analogous symmetric carbonate can also be formed, and in the case of ethyl chloroformate, ethylene is one of the products. The rate of hydrolysis depends on the analogous alcohol. It is the slowest for those derived from primary alcohols, faster for secondary alcohol analogues and those that can be derived from tertiary alcohols have the highest rate of hydrolysis. The reaction mechanism is $\text{S}_{\text{N}}1$ for the latter group and $\text{S}_{\text{N}}2$ for the other two groups. The reaction can be catalyzed by bases.⁵⁰

When reacted with aliphatic alcohols at room temperature, carbonates and hydrogen chloride are formed. This reaction is facilitated by alkali metals and their hydroxides and

tertiary amines, making the reaction more rapid and resulting in a higher yield. The reason behind the catalytic effect is the reaction with the forming hydrogen chloride.⁵⁰ However according to some studies, this reaction only occurs in aqueous environments if the hydroxyl group is adjacent to the carboxylic group.^{51,52} On the other hand, some studies found that chloroformates can be used to derivatize aliphatic alcohols in water.^{53,54}

Phenols do not react with chloroformates at room temperature, and even at higher temperatures, only small yield can be achieved. However, in the presence of bases, the reaction proceeds readily with high yield. Pyridine and amines are excellent catalysts for the reaction.^{50,51}

Chloroformates can also react with carboxylic acid, resulting in esters, hydrogen chloride and carbon dioxide. During the reaction, a mixed anhydride intermediate is formed. Depending on the structure of the reactants and the catalyst, it is also possible that the reaction stops when the anhydride is formed (Figure 8). In earlier studies, mixed anhydrides were prepared using chloroformates, carboxylic acid and pyridine as a catalyst to absorb the resulting hydrogen chloride.⁵⁰ Amines can also react with chloroformates, resulting in analogous amides.⁵⁵

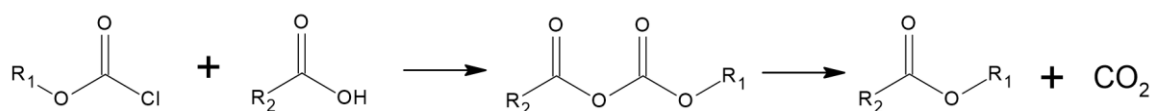


Figure 8 The reaction of chloroformates with carboxylic acids

While chloroformates are hydrolyzed in water, the rate constant of hydrolysis is smaller by several magnitudes compared to the rate constant of reaction with amines. The hydrolysis rate constant of ethyl chloroformate in 0.2 M sodium perchlorate is $5.6 \cdot 10^{-4} \text{ s}^{-1}$. When ethyl chloroformate is reacted with ethylamine or aniline, the rate constants of those reactions are $390 \text{ L mol}^{-1} \text{ s}^{-1}$ at pH 5.5 and $22.7 \text{ L mol}^{-1} \text{ s}^{-1}$ at pH 2.38 respectively.⁵⁶

3 Research goals

During a sample preparation for GC-MS measurement, the analytes are derivatized to reduce their polarity and make the separation more efficient. The derivatization method is generally performed after the extraction, which makes the latter less efficient and, in this way, acidic and basic compounds can only be extracted separately. Moreover, in some cases solid phase extraction (SPE) is necessary for the extraction, making the preparation more time consuming and tedious. It is also true that most derivatization reactions require long time and high temperature.

The goal of my research was to develop an analytical method that can identify some of the most common illicit drugs and new psychoactive compounds of Hungary in blood samples.⁴ The target compounds are THC and its main metabolites (THC-OH and THC-COOH) and the most prevalent stimulants (N-ethyl-norpentedrone, N-ethyl-norpentedrol, amphetamine, methamphetamine, MDA, MDMA, mephedrone, 3-CMC) simultaneously in blood samples. My further goal was to construct a calibration curve for the quantification of the target samples.

Because THC-COOH is acidic, and the stimulants have basic amino groups, I found chloroformate derivatization the most suitable for my goal. I planned to use isobutyl chloroformate to rapidly derivatize all the components in the aqueous phase at room temperature. My further goal was to avoid the use of SPE.

4 Experimental section

4.1 Determining the retention times and specific fragments

First, target components were added to 0.50 mL of urine. Each sample contained only one target component. A blank sample was also prepared in the same way as the others.

The pH was set using 150 μ L of 1 M NaOH. After that, 15 μ L of pyridine catalyst and 15 μ L of isobutyl chloroformate were added. The samples were shaken by hand three times and kept idle for approximately one minute for the reaction to complete.

For the extraction, 1.00 mL of isooctane was added to each sample, and they were shaken using an automatic shaker for 20 min at 1200 rpm at room temperature. Then, 800 μ L of supernatant was separated from each sample and evaporated in nitrogen gas flow. After that, they were reconstituted in 150 μ L of isooctane, vortexed for 10 s, and 100 μ L of each sample was added to a GC-MS measuring vial.

4.2 Optimizing the consecutive steps of sample pretreatment

Different approaches were tested to optimize chromatographic efficiency and improve the sensitivity of target compound determination.

4.2.1 Optimizing the derivatization reaction

In the first attempt, the circumstances that influence the derivatization reaction were optimized. NENP was added to 0.50 mL of urine in 10 μ g/mL concentration, along with 150 μ L of 1 M NaOH, 15 μ L of pyridine and 15 μ L of isobutyl chloroformate.

Then, the following approaches were tested. A different sample was prepared for each.

1. Shaking three times by hand and waiting 1 min
2. 10 s vortex
3. 30 s vortex
4. 60 s vortex
5. 1 min of automatic shaking for 1 min at 1200 rpm
6. 3 min of automatic shaking for 1 min at 1200 rpm
7. 5 min of automatic shaking for 1 min at 1200 rpm
8. Shaking three times by hand and waiting 10 min

The extraction was performed according to the procedure described in chapter 4.1.

4.2.2 Optimizing the amount and type of the catalyst

THC, THC-COOH, THC-OH, NENP, N-ethyl-norpentedrol and amphetamine were added to 0.50 mL of blood at 1 µg/mL concentration. Then pyridine and triethylamine were used as catalysts in different amounts while keeping the other aspects of the sample preparation constant.

The following amounts of pyridine were tested:

1. 7.50 µL
2. 15.0 µL
3. 30.0 µL

Triethylamine was also tested in the following amounts:

1. 3.00 µL
2. 15.0 µL

4.2.3 Testing different approaches for the protein precipitation

Unlike urine, blood contains plenty of proteins that have different functional groups. These can react with the derivatization agent, lowering the sensitivity for the target compounds. Therefore, it is crucial to precipitate the proteins from the blood samples.

For my first experiment, THC, THC-COOH, THC-OH, NENP, N-ethyl-norpentedrol and amphetamine were added to 1.00 mL of whole blood with anticoagulants, at 1 µg/mL concentration, and the following approaches were tested:

1. Adding 1.50 mL of acetonitrile
2. Adding 1.00 mL of methanol

In both cases, the samples were vortexed for 10 s and centrifuged for 5 min at 3500 rpm. 0.50 mL supernatant was separated from the sample of each approach.

In the next experiment, the same target compounds were added in the same concentration to 0.50 mL of blood plasma that did not contain anticoagulants and to anticoagulant containing whole blood. For the precipitation, different amounts of acetonitrile were used:

1. 1.40 mL
2. 0.50 mL
3. 0.25 mL

The precipitation was performed using Eppendorf tubes. This time the samples were vortexed for 10 s and centrifuged for 5 min at 11000 rpm.

From those samples that were precipitated using 1.40 mL acetonitrile, 1.40 mL of supernatant was separated. In this case, the supernatants were evaporated to the final volume of 0.50 mL. In case of 0.50 mL of acetonitrile addition, 0.25 mL of supernatant was separated, and in case of 0.25 mL acetonitrile addition, 0.20 mL of supernatant was separated.

4.2.4 Optimizing the amount of derivatization agent, the type of alkalization agent and catalyst

In this experiment, following the protein precipitation the derivatization was performed using twice the amount of the NaOH, pyridine and isobutyl chloroformate.

4.2.5 Two-step derivatization by amidation

The two step derivatization of the carboxylic group of the THC-COOH was tested to form an amide group. To achieve that, following the chloroformate derivatization, 10 μ L of diethylamine was added to the sample and it was kept idle for 5 min.

4.2.6 Further optimization of alkalization of the medium

During the experiments, the pH of the reaction mixture was always adjusted. 150 μ L of different solutions were added to achieve a basic medium.

The following solutions were tested:

1. 1 M NaOH
2. 1 M NaOH with 10 mg/mL NaCl
3. Solution of 5 M KOH and saturated NaHCO_3 in 1 : 1 ratio

4.2.7 Optimizing the extraction conditions

In this experiment, successive extractions were used, 1 mL of isooctane was added to a sample, and it was automatically shaken for 10 min at 1200 rpm. Then, 800 μ L of supernatant was isolated and 800 μ L of isooctane was re-added. The sample was once

again shaken for additional 10 min, and 800 μL of supernatant was isolated again and pooled with the first batch. A control sample was also prepared that was extracted in the way described in chapter 4.1.

4.3 Determination of analytical performance characteristics

To make the method suitable for quantitative analysis, cannabiol was selected as internal standard for the THC, THC-OH and THC-COOH, and for the other compounds, the internal standard was methoxyphenamine. The retention times and the specific fragments of the internal standards were determined by adding them to 0.50 mL of urine in 1 $\mu\text{g}/\text{mL}$ concentration.

Then, the matrix matched calibration solution series for all the compounds was constructed. The target compounds were added to 0.50 mL of blood plasma in the range of 1-100 and 1-1500/(3000) ng/mL , respectively (Table 2). Internal standards were added at the beginning of the sample preparation.

For the sample preparation, 1.40 mL acetonitrile was used to precipitate the proteins, then the samples were vortexed, and centrifuged for 5 min at 11000 rpm. 1.40 mL supernatant was separated and evaporated in nitrogen stream to the final volume of approximately 0.5 mL. Then, the samples were vortexed for 10 s. Protein precipitation was not performed for urine samples.

The rest of the sample preparation was performed according to chapter 4.1, except that instead of 15 μL pyridine, 3 μL triethylamine was used as catalyst and after the final evaporation, the samples were reconstituted in 110 μL isooctane instead of 150 μL .

The isobutyl chloroformate was always used in great excess. In the highest calibration point, the total concentration of the target compounds and internal standards was $7.60 \cdot 10^{-8} \text{ mol}/\text{mL}$ and the concentration of the derivatization agent was $2.31 \cdot 10^{-4} \text{ mol}/\text{mL}$.

Table 2 The concentrations of the calibration points

Component	c / ng·mL ⁻¹						
Serial number	1	2	3	4	5	6	7
THC	1	5	20	40	70	100	-
THC-COOH	1	5	20	40	70	100	-
THC-OH	1	5	20	40	70	100	-
N-ethyl-norpentedrone	1	10	30	100	300	1000	1500
N-ethyl-norpentedrol	1	10	30	100	300	1000	3000
amphetamine	1	10	30	100	300	1000	1500
methamphetamine	1	10	30	100	300	1000	1500
MDA	1	10	30	100	300	1000	1500
MDMA	1	10	30	100	300	1000	1500
mephedrone	1	10	30	100	300	1000	1500
3-CMC	1	10	30	100	300	1000	1500

Additionally, a blank sample was also prepared. The internal standards were spiked in a 100 ng/mL concentration to all the calibration solutions and to the blank. All the prepared samples had the exact same volume following the addition of the standard compounds. The standards were initially dissolved in methanol, so the blank contained the same volume of methanol as the total volume of the standard solutions in all the other samples.

The instrument was operated in single ion monitoring mode.

4.4 Determination of the reproducibility and stability

To test the stability of the prepared samples, three parallels of the fourth calibration point were prepared according to chapter 4.3, and were measured right after the sample preparation, 24 hours later and 48 hours later. After 48 h, all the parallels were measured three times successively.

For the reproducibility test, three parallels of the fourth calibration point were prepared in the same way as described in chapter 4.3 and were measured three times in total. Of the nine chromatograms, the reproducibility of the method was tested.

The N-ethyl-norpentedrol was excluded from both studies because of the lack of available reference standard for the experiment.

5 Blood samples of suspected people

To evaluate the practical suitability of my method, blood samples of suspects with different sampling times were selected and prepared (Table 3). All these samples were stored at 2-8 °C.

To obtain the official results presented in the expert's report, the stimulants were analyzed using GC-MS after derivatization (acylation) with *N*-Methyl-bis(trifluoroacetamide) (MBTFA).

The THC and its metabolites were determined using GC-MS after MSTFA derivatization for sample 1-5, and using a UHPLC-MS/MS screening method for sample 6-15. For the latter, the official confirmation analyses have not been conducted yet.

Table 3 The blood samples of alleged drug users

Serial number	Sampling time	Date of analysis		Identified compounds according to the official results / ng·mL ⁻¹
		Stimulants	THC compounds	
1	2024.04.30.	2024.06.25.	2024.06.17.	amphetamine: 406 NENP: < 10 THC: 1.34 THC-COOH: 16.4
2	2024.05.15.	2024.07.04.	2024.07.03.-04.	amphetamine: < 10 THC-COOH: 4.23
3	2024.05.19.	2024.07.04.	2024.07.03.-04.	mephedrone: 45.0 THC-COOH: 7.90
4	2024.05.22.	2024.07.09.	-	MDA: < 10 MDMA: 54.4 NENP: < 10 N-ethyl-norpentadrol: +
5	2024.05.21.	2024.07.09.	2024.07.03.-04.	NENP: < 10 THC-COOH: 6.09
6	2024.07.14.	2024.10.17.		amphetamine: 60.4 MDA: 23.3 MDMA: 298 methamphetamine: 12.8 THC: + (screening)
7	2024.07.28.	2024.10.17.		NENP: 73.6 THC-COOH: + (screening)
8	2024.08.01.	2024.10.17.		amphetamine: 56.8 methamphetamine: 419
9	2024.08.11.	2024.10.17.		THC-COOH: + (screening)
10	2024.08.11.	2024.10.17.		methamphetamine: 63.6 NENP: 43.3 N-ethyl-norpentadrol: + THC-COOH: + (screening)
11	2024.08.14.	2024.10.17.		amphetamine: 266 THC-COOH: + (screening)
12	2024.08.18.	2024.10.17.		NENP: 11.2 THC-COOH: + (screening)
13	2024.08.26.	2024.10.17.		NENP: 18.7 N-ethyl-norpentadrol: + THC: + (screening)
14	unknown	2024.10.17.		amphetamine: 20.4 methamphetamine: 17.2 THC: + (screening)
15	2024.08.29.	2024.10.17.		MDA: 20.8 MDMA: 271 NENP: 158 N-ethyl-norpentadrol: + THC: + (screening)

Quantitative: GC-MS measurement, MBTFA derivatization for the stimulants and MSTFA derivatization for the THC compounds; Screening: UHPLC-MS/MS measurement

6 Instrumental parameters

GC-MS instrument: Shimadzu GCMS-QP2010S

Physical properties of the column:

- Column: ZB-5HT INFERNO
- Thickness: 0.18 μm
- Diameter: 0.18 mm
- Length: 20 m
- Carrier gas: He

Ion source: electron ionization

Analyzer: quadrupole

Settings:

- Column temperature: 90 $^{\circ}\text{C}$
- Injection temperature: 270 $^{\circ}\text{C}$
- Injection: Splitless (1 min splitless time)
- Inlet pressure (initial): 116.6 kPa (constant linear velocity)
- Column flow: 0.66 mL/min
- Split ratio: 22.9
- Ion source temperature: 260 $^{\circ}\text{C}$
- Interface temperature: 290 $^{\circ}\text{C}$

The temperature gradient program of the GC oven is given in Table 4.

Table 4 The temperature gradient program

Rate / $^{\circ}\text{C}\cdot\text{min}^{-1}$	Final temperature / $^{\circ}\text{C}$	Hold time / min
-	90	1
20	290	15

Total chromatogram time: 26 min

The single ion monitoring (SIM) parameters are given in Table 5. The solvent delay time was 4 min.

Table 5 The SIM parameters

Start time	End time	Event time / s	Monitored m/z values						
7.21	7.74	0.10	144	91	162	158	176	-	-
8.25	9.52	0.10	144	158	200	102	136	-	-
12.08	13.00	0.10	297	313	271	395	295	238	312
16.08	16.48	0.10	426	385	-	-	-	-	-

Full scan determination mode parameters:

- Event time: 0.5 s
- Scan speed: 1428
- m/z range: 40 – 700

GCMS Solution (Shimadzu) software was used to control the instrument and for the qualitative and quantitative evaluations, and ChemDraw Pro was used to determine the tentative structures of the identified fragments throughout the research.

7 Results

7.1 The retention times and specific fragments

The retention times and the representative fragments of each compound were determined (Table 6). The THC-COOH can be derivatized in two ways, either an ester or an anhydride can be formed (Table 6, THC-COOH 1-2).⁵⁰ This phenomenon will be discussed in more details later.

Table 6 The retention times, the theoretical molecular ions and the representative fragments of the derivatized target compounds.

Target component	t_R / min	Theoretical [M^+] m/z	Representative fragments (m/z)
THC	12.275	414.28	297; 313
THC-COOH (1)	14.550	500.31	426
THC-COOH (2)	16.275	544.30	426
THC-OH	12.800	412.26	312
N-ethyl-norpentedrone	8.883	305.20	200; 144
N-ethyl-norpentedrol	9.283	407.27	200
amphetamine	7.408	235.16	144; 91; 162
methamphetamine	7.542	249.17	158; 176
MDA	9.243	279.15	144
MDMA	9.317	293.16	158
mephedrone	8.633	277.17	158
3-CMC	8.825	297.11	158
Internal standard	t_R / min	Theoretical [M^+] m/z	Representative fragments (m/z)
cannabinol	12.567	410.25	395; 295; 238
methoxyphenamine	8.450	297.18	158; 102

Based on the m/z values of the fragments and on the literature, the tentative structure of each specific fragment was determined (Figure 9-11 and Figure A1-A8).

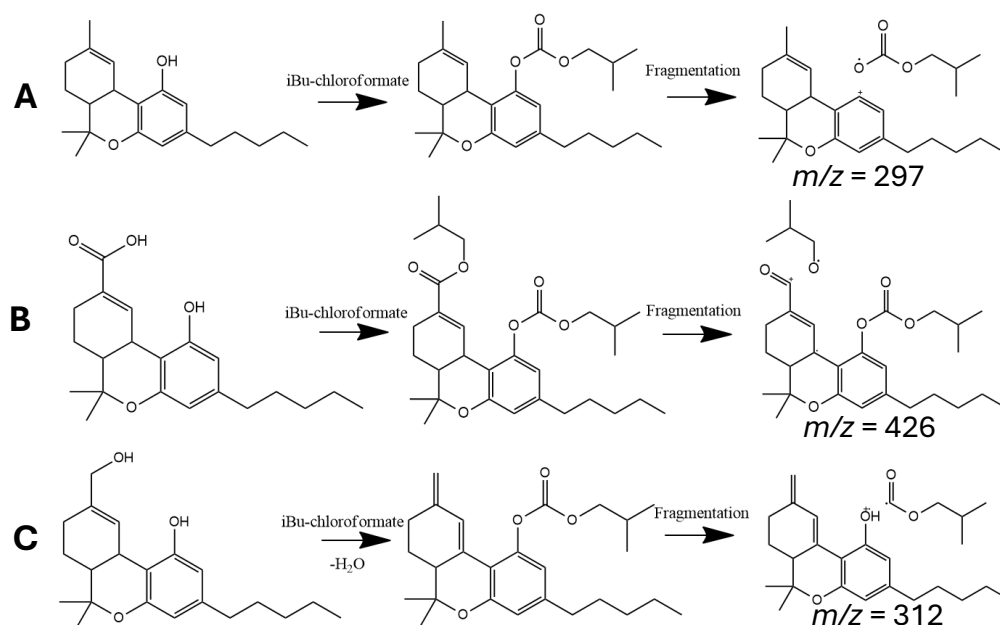


Figure 9 Derivatization and fragmentation of THC (A), THC-COOH (B) and THC-OH (C)

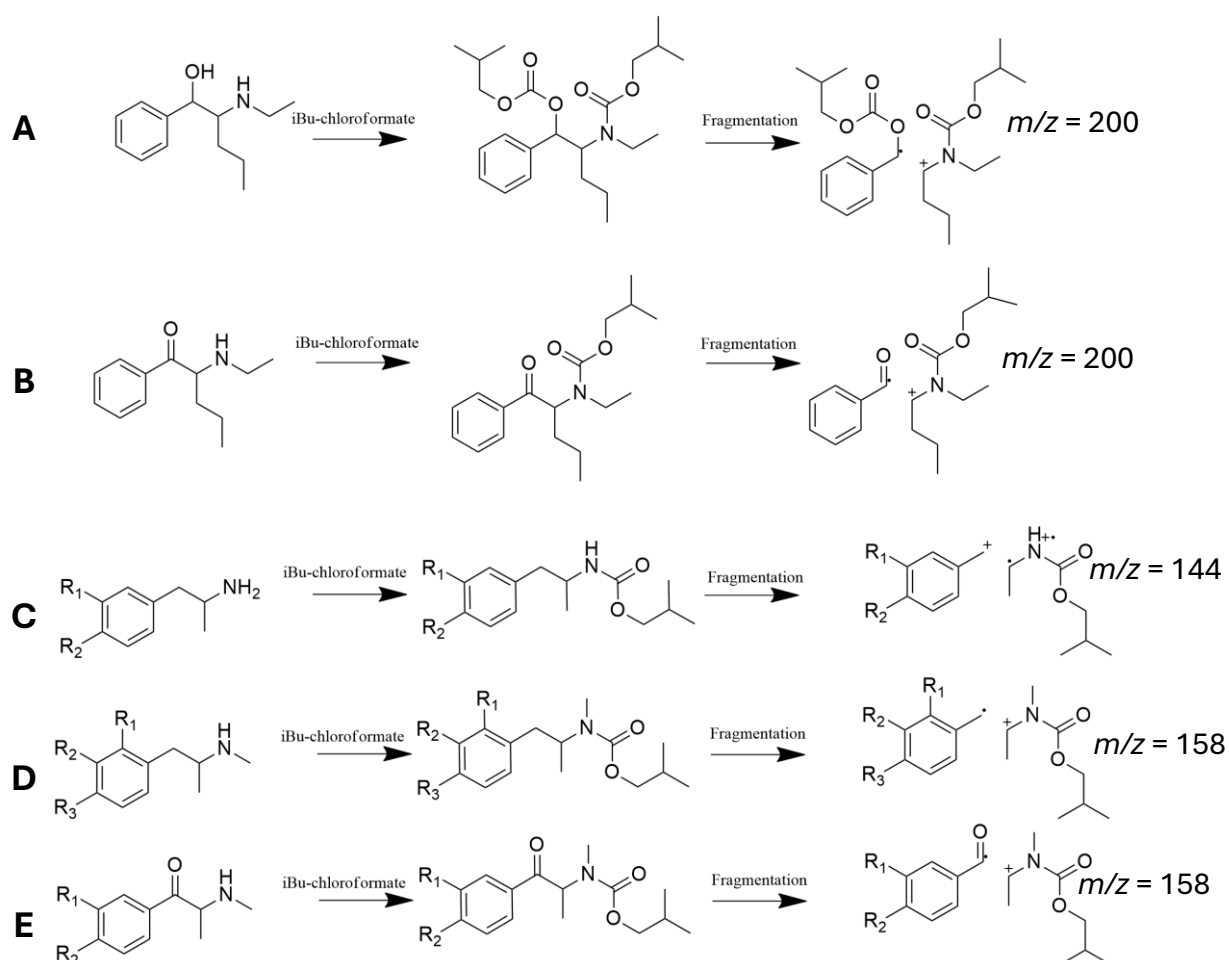


Figure 10 Derivatization and tentative fragmentation of N-ethyl-norpentadol (A), N-ethyl-norpentedrone (B), amphetamine and its derivatives (C), methamphetamine and its derivatives (D) and the miscellaneous cathinone derivatives (E)

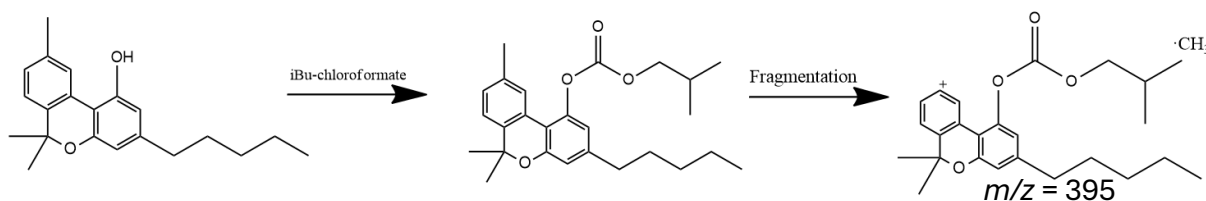


Figure 11 Derivatization and tentative fragmentation of cannabinol

The m/z values and the structures of the THC and its two main metabolites correlate with the results of Stefanelli et. al. In that research, propyl chloroformate was used to derivatize those three compounds, and for the THC and THC-OH, the same m/z values were observed. For the THC-COOH, m/z value 412 was observed, which only differs from the value 426, by 12 which is attributed to the usage of a different chloroformate.⁴¹

For the stimulants and for the internal standards, I derived the possible tentative fragment structures using ChemDraw.

7.2 The optimal circumstances of the derivatization reaction

The optimization was performed on the NENP. As it is shown in figure 12 (black line), method 1 (shaking three times by hand) resulted in the highest sensitivity, therefore the research was continued using that approach. On the total ion chromatogram (TIC) the peak of the underivatized NENP was not present, since the derivatization reaction was complete.

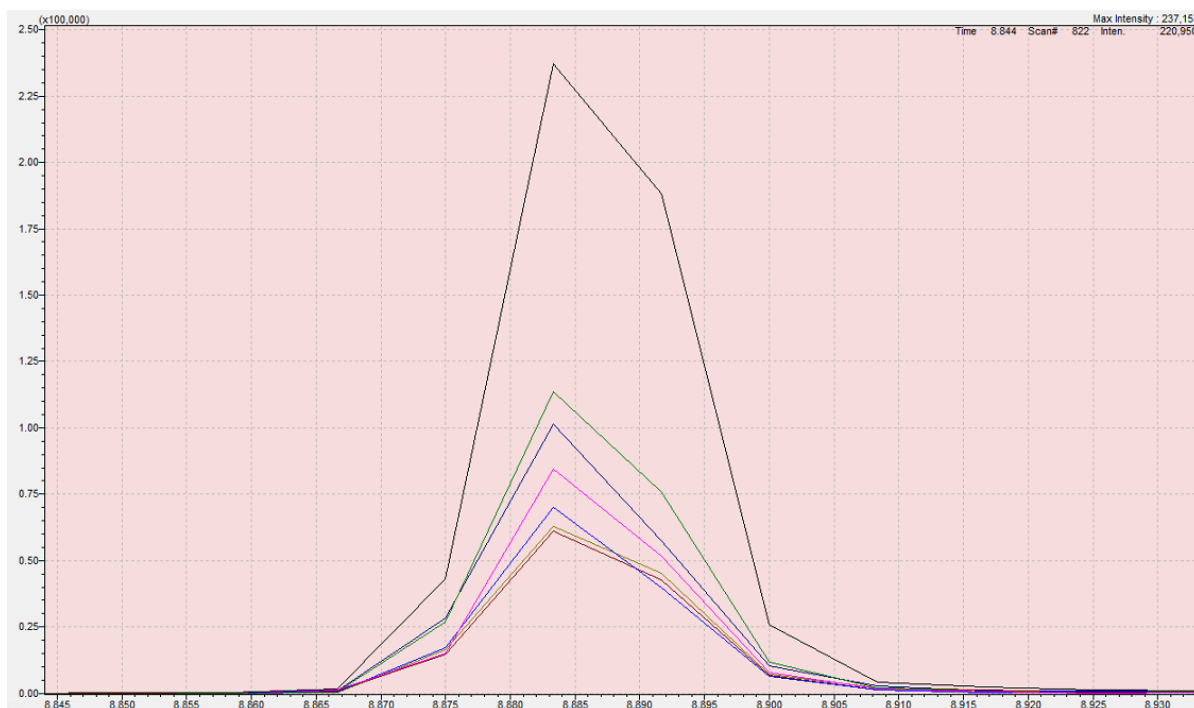


Figure 12 7 overlaid single ion chromatograms (SIC) of NENP peak (m/z 200) under different circumstances. Black: method 1; Pink: method 2; Blue: method 3; Brown: method 4; Green: method 5; Dark blue: method 6; Grey: method 7. Where the method numbers represent the circumstances listed at chapter 4.2.1.

It was also interesting to observe that, in contrast with the method where the waiting time was one minute, waiting ten minutes for the derivatization did not significantly improve the sensitivity.

7.3 Selection of the optimal catalyst and the result of the two-step derivatization by amidation

When pyridine and triethylamine were tested in different amounts, it was not possible to select a catalyst with a certain volume that is the best for every compound. The usage of 7.5 μL of pyridine instead of 15 μL resulted in a sensitivity increase for some of the components but caused a huge loss of peak area for the THC-COOH. For this component, using 15 μL triethylamine increased the sensitivity by more than 300%. The other components reacted differently to the different catalysts, but those differences were not as high as I observed in the case of the THC-COOH.

When only 3 μL of triethylamine was used as a catalyst, the intensity of all the components increased even further. Therefore 3 μL of triethylamine was considered optimal for most of the compounds.

7.3.1 Two-step derivatization by amidation and the catalyst dependence of THC-COOH

Because only the THC-COOH has a carboxylic group among the target compounds, amide derivatization was only assessed on that compound. The reaction was successful, and it increased the sensitivity for that component, but not significantly. It is also interesting that the use of different catalysts influenced the retention time of the non-amidated THC-COOH (Figure 13).

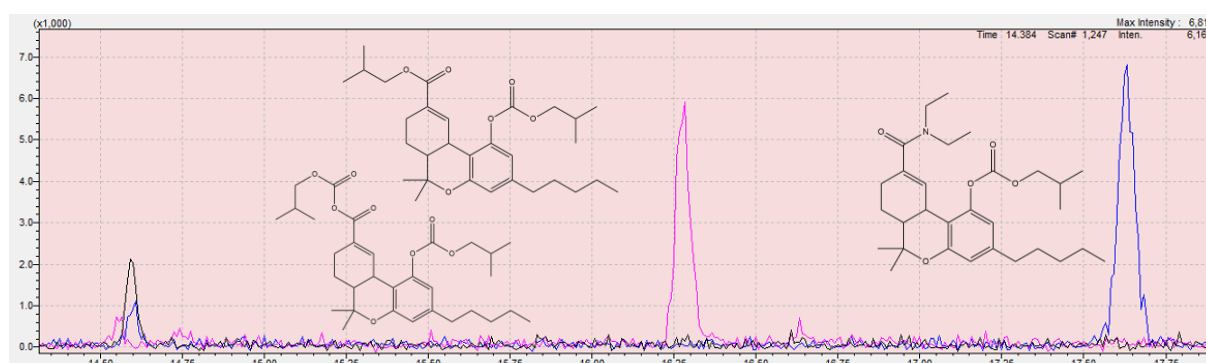


Figure 13 The 3 overlaid SICs ($m/z = 426$) of THC-COOH samples. Black: prepared using pyridine without amidation; Pink: triethylamine catalyst without amidation; Blue: triethylamine catalyst with amidation

The altered retention time can be explained by the fact that a carboxylic group can be derivatized by chloroformates in two possible ways. One, in which an ester is formed, and one, in which a mixed anhydride is formed (Figure 14).⁵⁰ Both cases, the same representative fragment is formed in the ion source.

According to the results, whether anhydride or ether is formed during the derivatization reaction depends on the catalyst. This phenomenon can be explained by the different size and basicity (pK_b of pyridine = 8.77; pK_b of triethylamine = 3.25) of the two catalysts. In previous synthetic organic chemistry studies, chloroformates were used along with pyridine to synthesize and isolate mixed anhydrides.⁵⁰

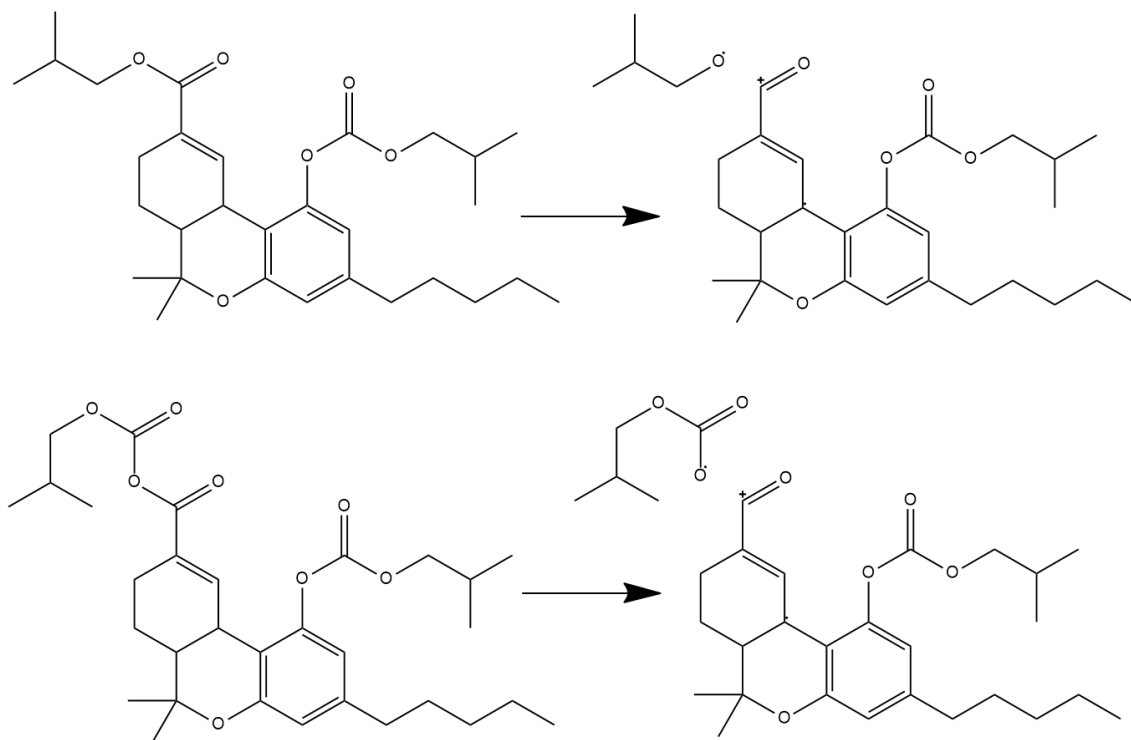


Figure 14 The two possible ways of derivatization of the carboxylic group and its fragmentation.

When the carboxylic group is transformed into an amide, the fragmentation also results in the exact same fragment (Figure 15).

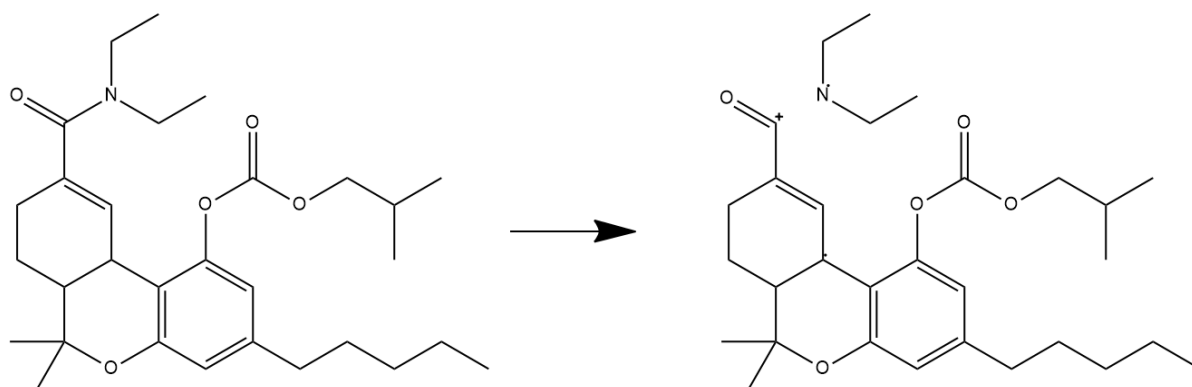


Figure 15 The fragmentation of the amidated THC-COOH

7.4 The optimal alkalization of the medium

The pH not only depends on the inorganic base solution that is added during the sample preparation, but also on the catalyst. Therefore, during the method development, these two factors were often changed together.

When 15 μ L of triethylamine was used along with 1 M NaOH, the sensitivity was higher for all compounds when the base solution contained 10 mg/mL salt.

By using a solution of 5 M KOH and saturated NaHCO_3 in 1 : 1 ratio instead of NaOH with salt, along with triethylamine catalyst, the sensitivity was slightly lower for the THC-COOH, but it was detected in only one peak instead of two (Figure 16).

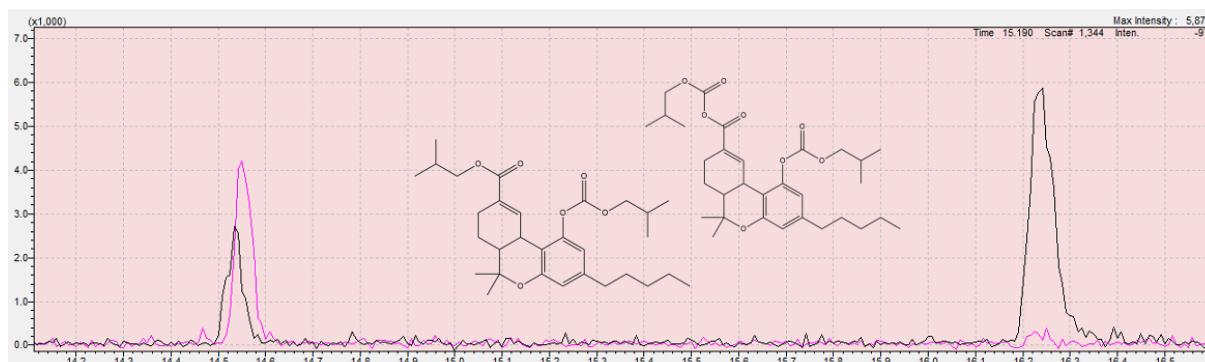


Figure 16 The SICs THC-COOH ($m/z = 426$). Black: when the sample was treated with NaOH with salt. Pink: when it was treated with KOH : $\text{NaHCO}_3 = 1 : 1$ solution.

7.5 The optimal protein precipitation

When 1.50 mL of acetonitrile or 1.00 mL of methanol was used, because of the high volume of the samples and the precipitation agents, test tubes were required instead of Eppendorf tubes. However, with test tubes, the centrifuge can be operated on up to 3500 rpm instead of 11000 rpm.

Therefore, in that case, the sedimentation was not complete, which presumably resulted in sensitivity loss. For the identification of THC and N-ethyl-norpentedrol, the method using methanol precipitation was the optimal, but for the THC-COOH, NENP and amphetamine, method of acetonitrile precipitation was the most suitable.

Overall, the precipitation method was the one where 1.40 mL of acetonitrile was used to precipitate the proteins. The intensities of the target compounds were approximately the same for blood plasma and for anticoagulant containing whole blood, but from the latter matrix, a significantly higher volume of proteins was precipitated.

7.6 The optimal extraction conditions

Extraction in two times did not increase the sensitivity significantly, therefore it can be concluded that the regular extraction is already optimal.

7.7 Derivatization with twice the amount of NaOH, pyridine and isobutyl chloroformate

When twice the amount of NaOH, pyridine and isobutyl chloroformate was used, the sensitivity remained almost the same for all compounds (Figure 17).

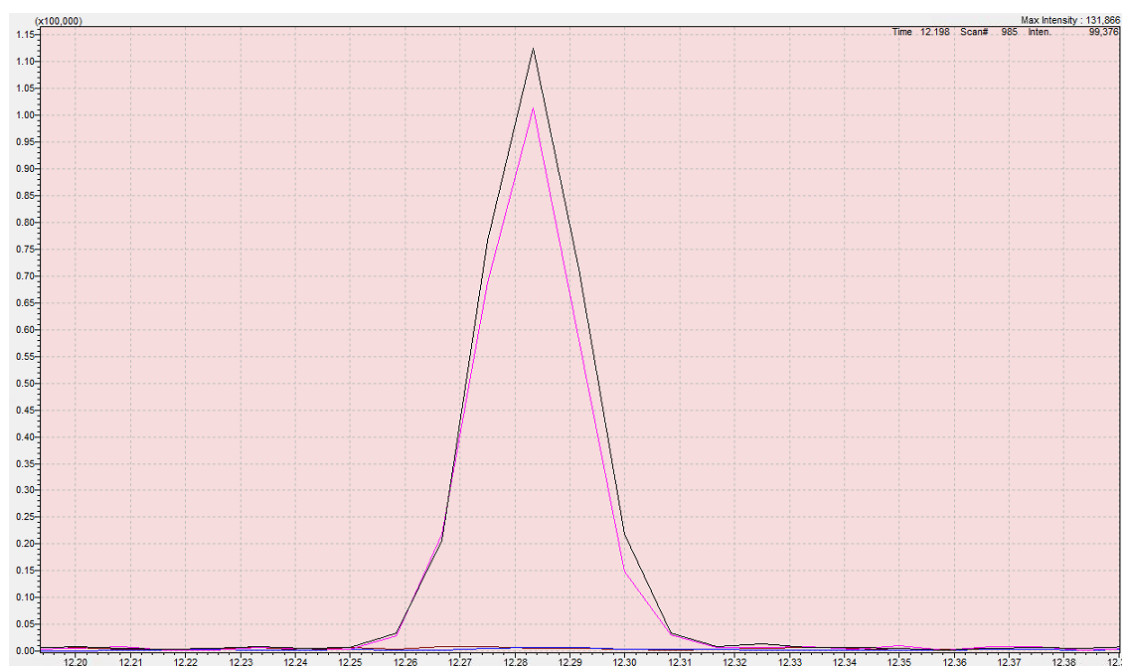


Figure 17 The SIC of THC ($m/z = 297$) under different circumstances. Black: Control; Pink: twice the amount of the mentioned agents; Blue and brown: blanks for both cases

7.8 Quantitative results

After the results were obtained, all the component-peaks were integrated using the instrument manufacturer's software, Shimadzu GCMS Solution Postrun Analysis. Then, the calibration curves were constructed with the use of the same software (Table 8). The outlier points were excluded for each compound (Table 7). Each point was weighted by $\frac{1}{A^2}$, where A is the peak area.

Table 7 The included final calibration points

Target component	Used points / ng·mL ⁻¹
THC	1; 5; 20; 40; 70; 100
THC-COOH	5; 40; 70; 100
THC-OH	20; 40; 100
N-ethyl-norpentedrone	10; 100; 300; 1000; 1500
N-ethyl-norpentedrol	100; 300; 1000; 3000
amphetamine	10; 30; 100; 300; 1000; 1500
methamphetamine	10; 30; 100; 300; 1000; 1500
MDA	10; 100; 300; 1500
MDMA	30; 100; 300; 1000; 1500
mephedrone	100; 300; 1000; 1500
3-CMC	10; 100; 300; 1000; 1500

Table 8 The calibration curve parameters for each compound. *I* is the peak area and *c* is the concentration

Target component	Equation of the calibration curve	Correlation coefficient	Mean response factor	Response factor RSD%
THC	$I = 1.222 \cdot 10^{-2}C + 2.245 \cdot 10^{-3}$	0.9972	$1.281 \cdot 10^{-2}$	9.1
THC-COOH	$I = 3.694 \cdot 10^{-3}C - 7.128 \cdot 10^{-3}$	0.9975	$3.275 \cdot 10^{-3}$	22
THC-OH	$I = 5.058 \cdot 10^{-4}C + 6.205 \cdot 10^{-4}$	0.9997	$5.235 \cdot 10^{-4}$	2.81
N-ethyl-norpentedrone	$I = 4.920 \cdot 10^{-3}C + 5.481 \cdot 10^{-3}$	0.9978	$5.079 \cdot 10^{-3}$	7.6
N-ethyl-norpentedrol	$I = 7.365 \cdot 10^{-5}C + 1.877 \cdot 10^{-3}$	1.000	$8.054 \cdot 10^{-5}$	10
amphetamine	$I = 8.216 \cdot 10^{-3}C - 9.812 \cdot 10^{-3}$	0.9979	$8.020 \cdot 10^{-3}$	7.7
methamphetamine	$I = 7.462 \cdot 10^{-3}C - 7.316 \cdot 10^{-4}$	0.9983	$7.479 \cdot 10^{-3}$	5.2
MDA	$I = 2.505 \cdot 10^{-3}C - 3.538 \cdot 10^{-3}$	0.9950	$2.439 \cdot 10^{-3}$	12
MDMA	$I = 4.824 \cdot 10^{-3}C + 7.212 \cdot 10^{-2}$	0.9956	$5.571 \cdot 10^{-3}$	18
mephedrone	$I = 6.631 \cdot 10^{-3}C + 0.2034$	0.9983	$7.415 \cdot 10^{-3}$	12
3-CMC	$I = 3.049 \cdot 10^{-3}C - 5.245 \cdot 10^{-3}$	0.9983	$2.944 \cdot 10^{-3}$	10

Mean response factor and RSD%: The average factors and the corresponding RSD%-s of the calibration points.

The limit of detection (LOD) and limit of quantification (LOQ) for each compound was also determined (Table 9). LOD was calculated as the concentration of a peak that is three times higher than the peak or noise measured in the blank sample. LOQ values were calculated by multiplying the LODs by 3.3. The lowest points of the calibration curve were selected in a way that the RSD% of the response factors of the calibration curve should not exceed 25%.

Table 9 Limits of detection and quantification

Target component	Limit of detection (LOD) / ng·mL ⁻¹	Limit of quantification (LOQ) / ng·mL ⁻¹
THC	0.174	0.574
THC-COOH	0.018	0.0594
THC-OH	0.132	0.436
N-ethyl-norpentedrone	1.29	4.25
N-ethyl-norpentedrol	49.8	164
amphetamine	0.771	2.54
methamphetamine	1.44	4.75
MDA	2.29	7.54
MDMA	7.23	23.8
mephedrone	1.74	5.75
3-CMC	0.294	0.970

7.9 Recoveries

The recoveries were calculated for all the calibration points within the calibration range (Table10).

Table 10 The recoveries of the calibration points above the LOQ

Component	Point 1	Point 2	Point 3	Point 4	Point 5	Point 6	Point 7
THC	99.9	99.6	110	89.2	103	103	
THC-COOH		100	59.5	92.2	107	103	
THC-OH			99.3	102	152	98.9	
N-ethyl-norpentedrone		99.2	76.2	106	108	96.6	93.0
N-ethyl-norpentedrol				99.8	100	99.9	99.8
amphetamine		99.5	101	104	108	91.0	100
methamphetamine		101	94.6	106	105	93.8	102
MDA		99.4	80.8	106	111	80.9	89.2
MDMA		139	94.7	112	109	90.9	99.1
mephedrone				97.3	108	98.9	96.9
3-CMC		100	76.5	92.4	108	101	101

7.10 Reproducibility of the retention times

The mean RSD% of the retention times in the calibration curve was calculated (Table 11).

Table 11 The average and the RSD%s of the retention times in the calibration points

Component	Mean t_R / min	RSD% t_R / min
methoxyphenamine	8.453	0.00966
cannabinol	12.570	0.0150
THC	12.275	0.0227
THC-COOH	16.248	0.0549
THC-OH	12.803	0.0274
N-ethyl-norpentadron	8.888	0.0172
N-ethyl-norpentadrol	9.281	0.0264
amphetamine	7.413	0.0464
methamphetamine	7.540	0.0438
MDA	9.242	0.0159
MDMA	9.323	0.0204
mephedrone	8.644	0.0215
3-CMC	8.825	0.0178

7.11 Result of the stability test

The peak area ratios of the analytes and the internal standards were calculated and plotted (Figure 19). The prepared samples can be measured up to 48 hours after preparation without losing accuracy. The peak area ratio wasn't shown to be declining over time, according to the data.

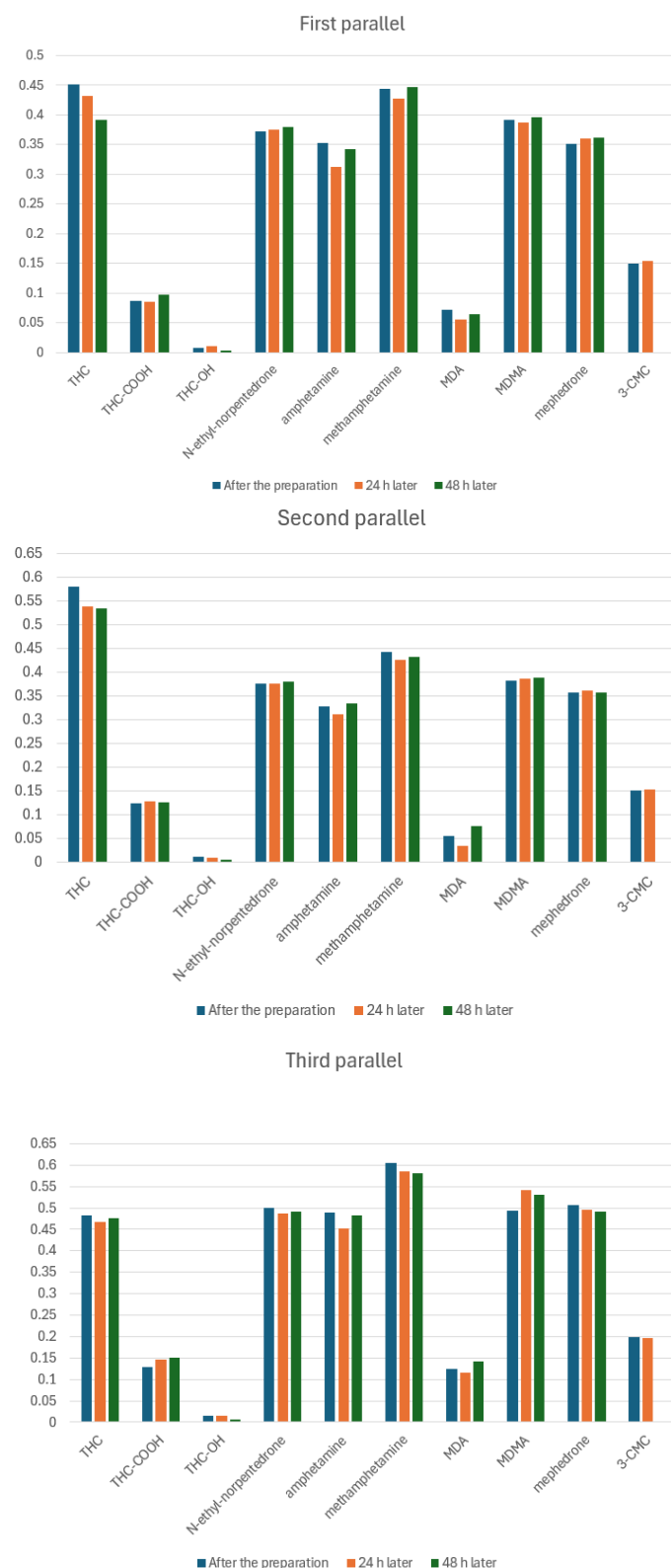


Figure 19 The area ratios of the analytes and the internal standards in each sample after the preparation, 24 h later and 48 h later.

7.12 Result of the repeatability determination

Three types of repeatability were tested:

1. Three parallel injections (48 h samples, Table 12);
2. Three parallel injections from the same GC vial after different storage times (0 min, 24 and 48 h, Table 13);
3. Three different samples (Table 14)

Table 12 The mean and RSD% of each parallel between three successive injections, 48 h after the sample preparation.

Component	First sample (n = 3)		Second sample (n = 3)		Third sample (n = 3)		RSD% range
	mean	RSD%	mean	RSD%	mean	RSD%	
THC	0.391	5.90	0.533	4.30	0.476	2.06	2.06-5.90
THC-COOH	0.097	5.81	0.127	2.55	0.151	7.86	2.55-7.86
THC-OH	0.004	33.1	0.005	26.3	0.006	17.5	17.5-33.1
NENP	0.380	0.44	0.379	1.25	0.490	0.79	0.44-1.25
amphetamine	0.342	8.68	0.333	5.48	0.483	7.95	5.48-8.68
methamphetamine	0.446	2.56	0.432	0.69	0.580	2.29	0.69-2.56
MDA	0.065	18.2	0.076	44.5	0.141	3.59	3.59-44.5
MDMA	0.395	0.67	0.388	0.90	0.531	1.51	0.67-1.51
mephedrone	0.362	2.03	0.357	1.71	0.492	2.52	1.71-2.52
3-CMC	0.169	1.09	0.158	1.73	0.193	3.14	1.09-3.14

Table 13 The mean and RSD% of each parallel sample (0 min, 24 h and 48 h).

Component	First sample (n = 3)		Second sample (n = 3)		Third sample (n = 3)		RSD% range
	mean	RSD%	mean	RSD%	mean	RSD%	
THC	0.331	2.10	0.360	3.01	0.316	1.41	1.41-3.01
THC-COOH	0.031	11.0	0.042	10.3	0.049	7.20	7.20-11.0
THC-OH	0.003	19.3	0.003	41.0	0.003	13.4	13.4-41.0
NENP	0.512	2.03	0.425	0.56	0.536	2.71	0.56-2.71
amphetamine	0.574	1.57	0.442	1.91	0.594	1.58	1.57-1.91
methamphetamine	0.621	3.10	0.511	2.83	0.650	2.42	2.42-3.10
MDA	0.164	18.1	0.139	2.42	0.205	0.99	0.99-18.1
MDMA	0.522	3.23	0.458	2.60	0.529	1.69	1.69-3.23
mephedrone	0.620	3.01	0.520	3.51	0.641	2.72	2.72-3.51
3-CMC	0.146	0.42	0.126	0.33	0.145	2.69	0.33-2.69

Table 14 The mean area ratio and RSD% of the three parallel samples.

Component	First sample (n = 3)	Second sample (n = 3)	Third sample (n = 3)	Mean	RSD%
THC	0.391	0.533	0.476	0.467	15.2
THC-COOH	0.097	0.127	0.151	0.125	21.8
THC-OH	0.004	0.005	0.006	0.005	23.6
NENP	0.380	0.379	0.490	0.417	15.3
amphetamine	0.342	0.333	0.483	0.386	21.8
methamphetamine	0.446	0.432	0.580	0.486	16.8
MDA	0.065	0.076	0.141	0.094	43.7
MDMA	0.395	0.388	0.531	0.438	18.4
mephedrone	0.362	0.357	0.492	0.404	19.0
3-CMC	0.169	0.158	0.193	0.173	10.5

The difference of the RSD% between the successive measurements (3 injections from the same vial) and the 3 injections from the same sample in different time-intervals (0 min, 24 and 48h) was not significant for all the compounds except for the THC-OH and the MDA (Table 12 and 13, range of RSD%). Overall, the prepared samples can be measured multiple times with high repeatability and the difference between parallels is also acceptable for most of the target compounds.

Higher standard deviation between different biological samples is a natural phenomenon,⁵⁷ but in the future, more experiments must be conducted to reduce it, especially in the case of the THC-OH and the MDA. Furthermore, the low sensitivity for the forementioned two compounds must also be addressed.

8 Results of the blood samples of suspected people

With the use of the calibration curve, the target compounds were identified, and the concentrations were calculated in the blood samples of suspected people (Table 16). The date of the sample preparation was 2024.10.14.

Table 16 The concentration of the identified components in the blood samples

Serial number	Sampling time	Identified compounds according to my measurement
1	2024.04.30.	THC-COOH: 7.94 ng/mL Amphetamine: 224 ng/mL
2	2024.05.15.	THC-COOH: 4.22 ng/mL
3	2024.05.19.	THC-COOH: 5.04 mephedrone: 25.7 ng/mL
4	2024.05.22.	NENP: 57.5 ng/mL MDA: 63.7 ng/mL
5	2024.05.21.	THC-COOH: 5.79 ng/mL
6	2024.07.14.	THC-COOH: 8.49 ng/mL amphetamine: 32.7 ng/mL methamphetamine: 6.47 ng/mL MDMA: 181 ng/mL
7	2024.07.28.	THC-COOH: 15.9 ng/mL NENP: 91.6 ng/mL
8	2024.08.01.	amphetamine: 36.7 ng/mL methamphetamine: 287 ng/mL
9	2024.08.11.	THC-COOH: 44.8 ng/mL
10	2024.08.11.	THC-COOH: 38.1 ng/mL NENP: 53.4 ng/mL methamphetamine: 53.0 ng/mL
11	2024.08.14.	THC-COOH: 153 ng/mL amphetamine: 105 ng/mL
12	2024.08.18.	NENP: 48.6 ng/mL
13	2024.08.26.	THC-COOH: 4.54 ng/mL NENP: 21.4 ng/mL
14	unknown	THC-COOH: 5.19 ng/mL NENP: LOD < NENP < LOQ amphetamine: 38.2 ng/mL methamphetamine: 23.2 ng/mL
15	2024.08.29.	THC: 4.10 ng/mL THC-COOH: 67.3 ng/mL NENP: 154 ng/mL MDMA: 183 ng/mL

The quantitative results obtained by this method were compared with the official data presented in the expert's report. The N-ethyl-norpentedrol is not quantified in the official analysis and all stimulant results below 10 ng/mL are considered negative. The percentage of difference between the result of this method and the official results were plotted (Figure 20).

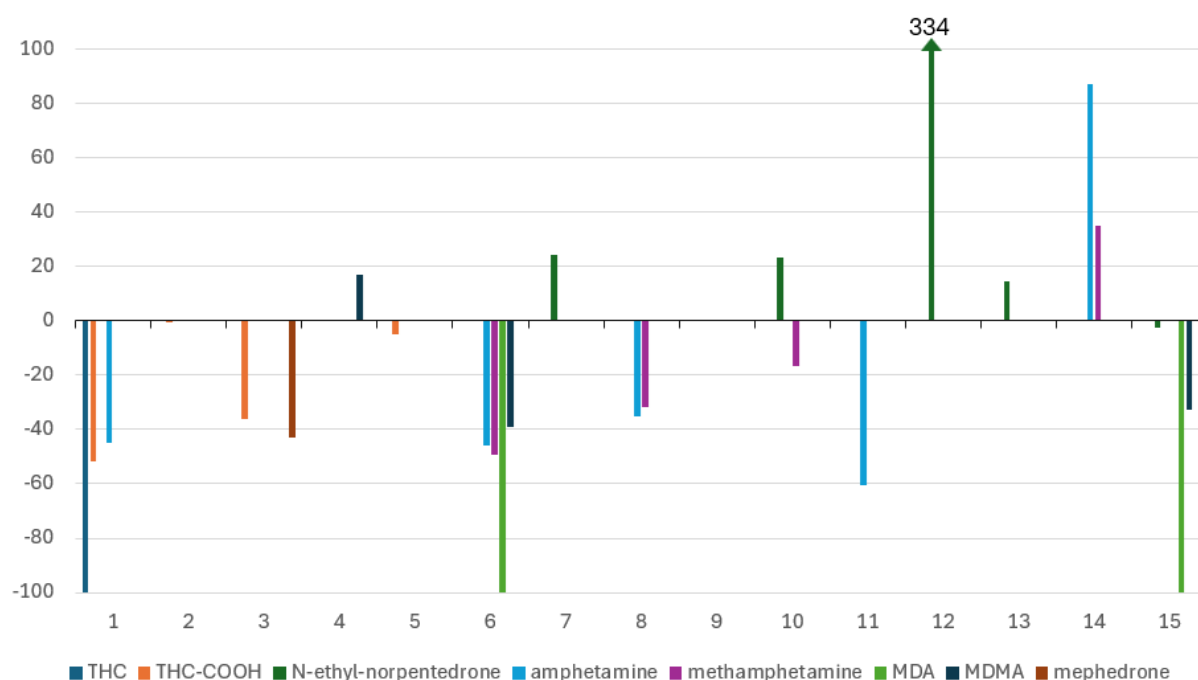


Figure 20 The difference between the result of this method and the official results in percentages.

Since analytes can decompose over time in blood samples⁵⁸, the significantly lower concentrations measured in this study can be attributed to the time elapsed between the sample preparation for official analysis and our method development.

In most cases, parallel biological samples can only be measured with an accuracy of 30%⁵⁹, and the error range of two different samples can overlap, so a maximum error difference of 60% between the two methods is considered acceptable.

9 Evaluation of the requirements for validation

In the Department of Forensic Toxicology, an analytical method must fulfill certain requirements for validation and this method fulfills some of the requirements. The RSD%s of the retention times of the analyte peaks are below 2% for all the components and the calibration curve has a squared correlation coefficient above 0.99 with less than three

outlier points for each target compound. The LOD is < 1 ng/mL for the THC components, and it is ≤ 10 ng/mL for all the stimulants except for the N-ethyl-norpentadrol. The recoveries deviate from 100% by less than 15% with no more than 2 outliers for each compound.

However, for validation proceedings more experiments are necessary. For example, stability and reproducibility tests must be conducted on more than 6 parallel samples, and the RSD% of those should be below 15% for all the compounds.

10 Conclusions

In my research, I developed a rapid analytical method that can simultaneously identify acidic and basic drugs of abuse in blood samples. For derivatization, I used isobutyl chloroformate that can react with the analytes in aqueous solution. Then, the derivatized compounds can be extracted efficiently, so SPE is not necessary during the sample preparation.

The method was tested on THC, THC-COOH, THC-OH, NENP, N-ethyl-norpentadrol, amphetamine, methamphetamine, MDA, MDMA, mephedrone and 3-CMC. For quantitative analysis, cannabinol is used as an internal standard for the THC and its metabolites, and methoxyphenamine is used for the rest of the compounds. Numerous experiments were conducted to optimize the sample preparation procedure and as a result, the final protocol was established:

The sample preparation was performed on 0.50 mL of blood samples. Internal standards (cannabinol and methoxyphenamine in 100 ng/mL concentration each) were added at the beginning of the procedure. 1.40 mL acetonitrile was used to precipitate the proteins, then the samples were vortexed, and centrifuged for 5 min at 11000 rpm. 1.40 mL supernatant was separated and evaporated in nitrogen stream to the final volume of approximately 0.5 mL. Then, the samples were vortexed for 10 s.

The solution was alkalized using 150 μ L of 1 M NaOH. Then, 3.00 μ L of triethylamine catalyst and 15 μ L of isobutyl chloroformate were added. The samples were shaken by hand three times and kept idle for approximately one minute for the reaction to complete.

For the extraction, 1.00 mL of isooctane was added to each sample, and they were shaken using an automatic shaker for 20 min on 1200 rpm at room temperature. Then, 800 µL of supernatant was separated from each sample and evaporated in nitrogen gas flow. After that, they were reconstituted in 110 µL of isooctane, vortexed for 10 s, and 100 µL of each sample was added to a GC-MS vial.

It was proven that depending on the catalyst, THC-COOH can be derivatized in two ways, either anhydride or ester can be formed as a result. By two-step derivatization, the carboxylic group can be transformed into an amide group.

According to the stability test, a prepared sample can be measured up to 48 h after the preparation without significant loss of accuracy. The method has also proven to be sufficiently reproducible for all the compounds, except the THC-OH and the MDA.

The method fulfills many of the requirements for validation. For example, the calibration curve has the required correlation coefficient with less than three outlier points and the recoveries are also sufficient. The required LOD values are achieved for all the compounds except for the N-ethyl-norpentadrol.

In the future, the repeatability of parallel samples must be enhanced, and samples must be simultaneously prepared using both the official and this method to obtain comparable results.

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Appendix

Miscellaneous fragment structures

The possible structures of the fragments other than the most intensive ones are given in figures F1-F5. The fragment with $m/z = 295$ of the cannabinol can be the result of fragmentation upon electron impact and chemical ionization subsequently.

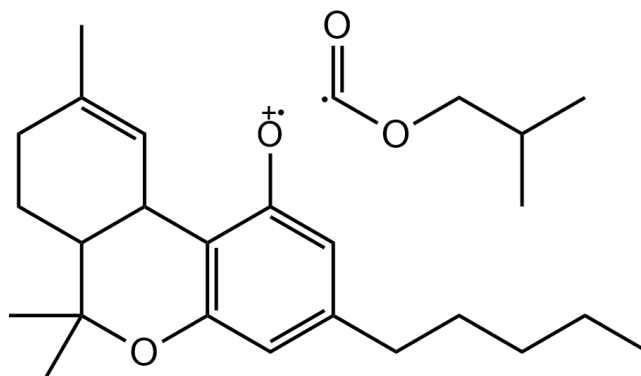


Figure A1 The THC fragment with $m/z = 313$

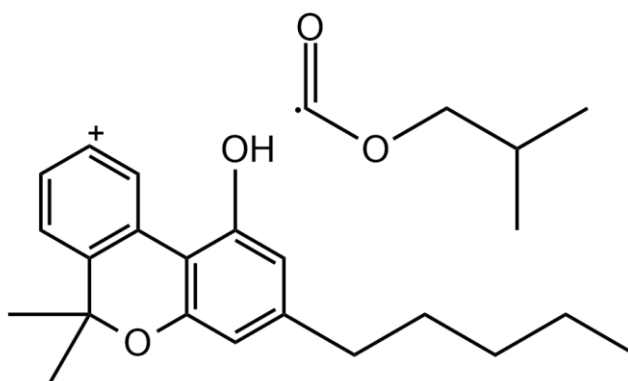


Figure A2 The cannabinol fragment with $m/z = 295$

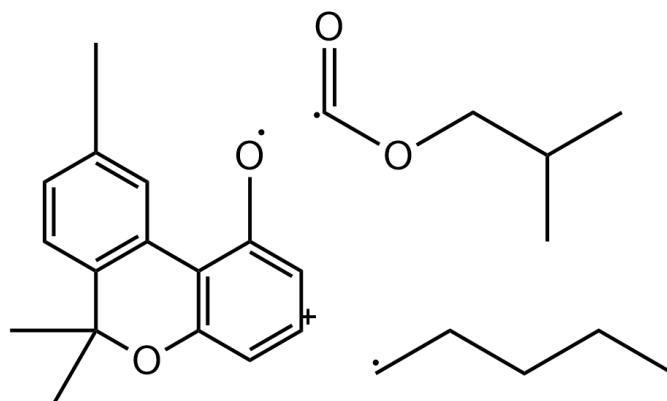


Figure A3 The cannabinol fragment with $m/z = 238$

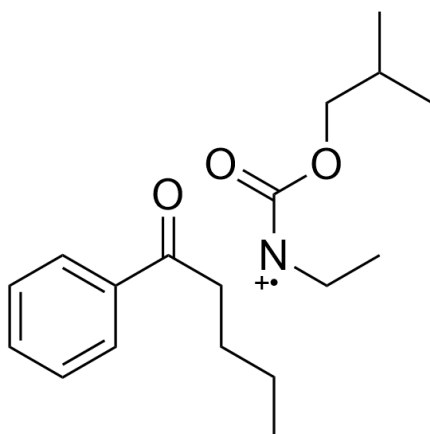


Figure A4 The N-ethyl-norpentedrone fragment with $m/z = 144$

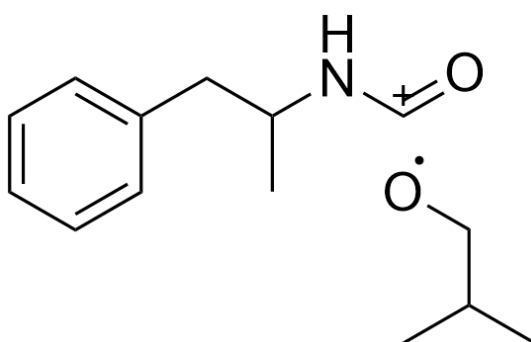


Figure A5 The amphetamine fragment with $m/z = 162$

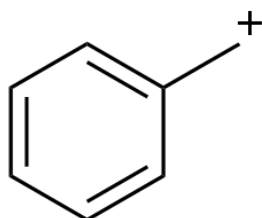


Figure A6 The amphetamine fragment with $m/z = 91$

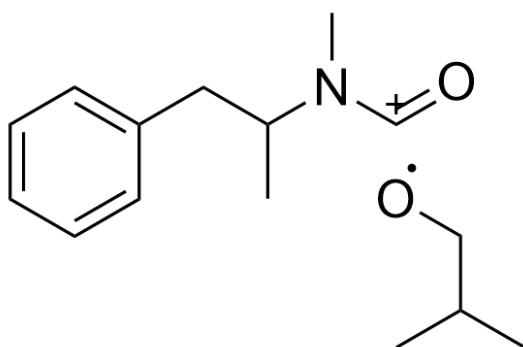


Figure A7 The methamphetamine fragment with $m/z = 176$

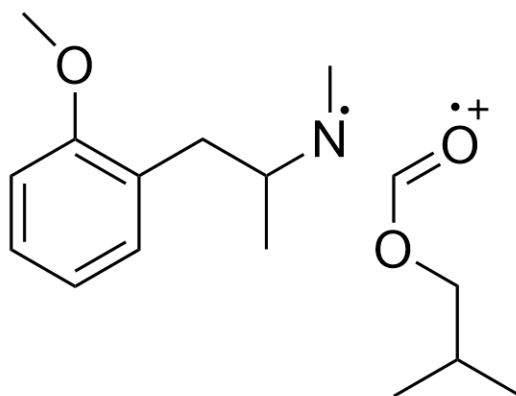


Figure A8 The methoxyphenamine fragment with $m/z = 102$

Mass spectra of the target compounds

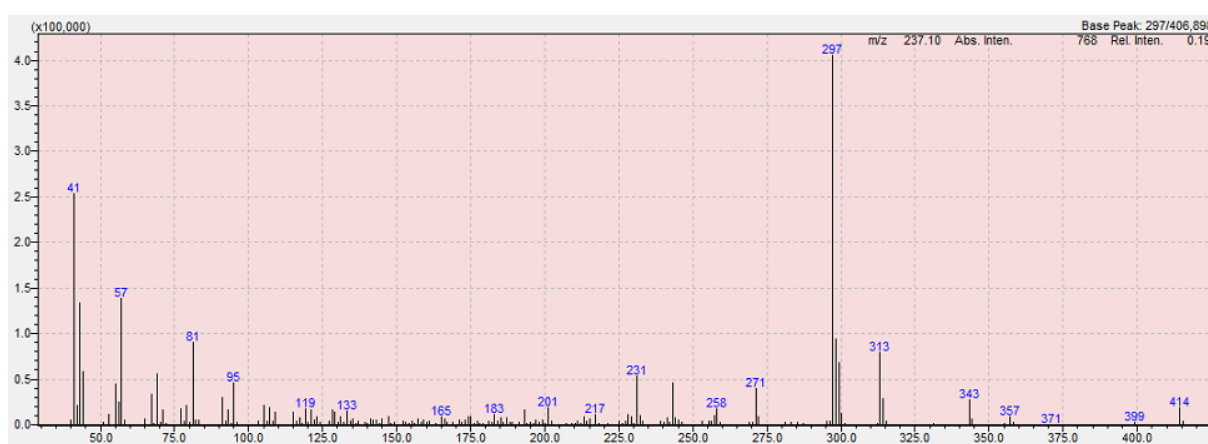


Figure A9 The mass spectrum of the derivatized THC

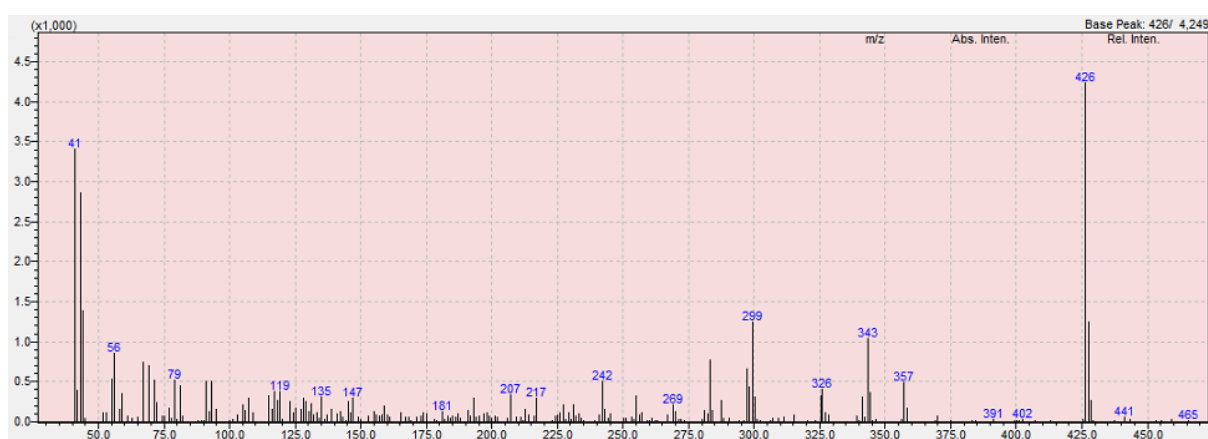


Figure A10 The mass spectrum of the derivatized THC-COOH with $t_R = 14.550$ min in ester form

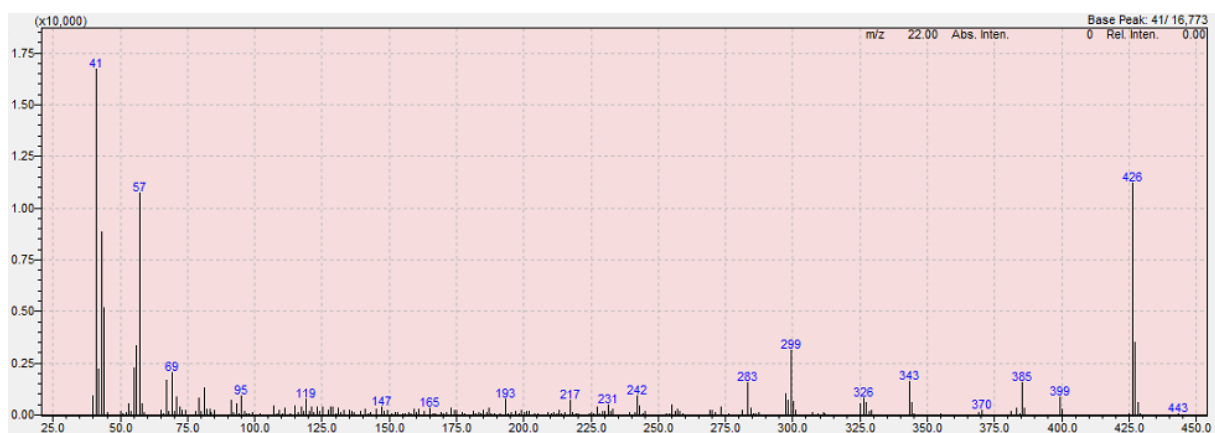


Figure A11 The mass spectrum of the derivatized THC-COOH with $t_R = 16.275$ min in anhydride form

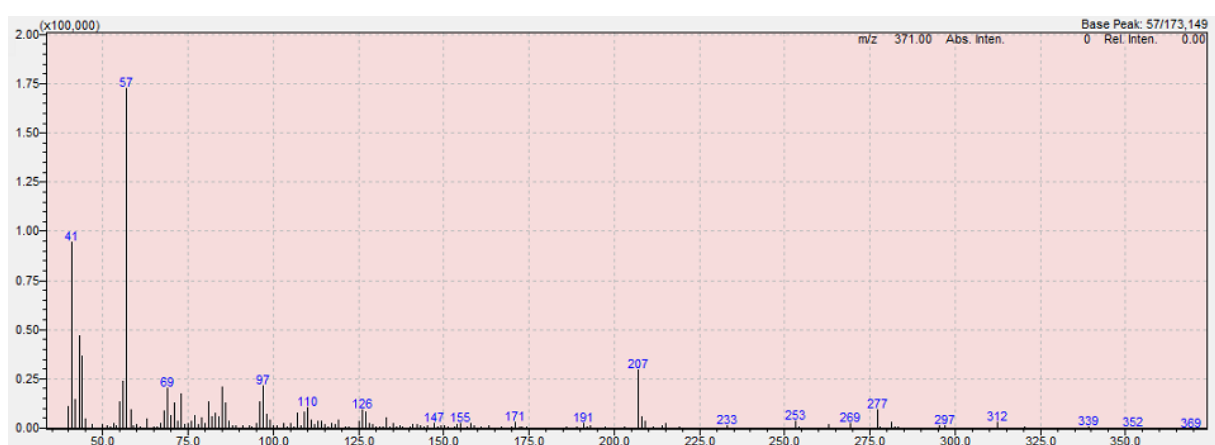


Figure A12 The mass spectrum of the derivatized THC-OH

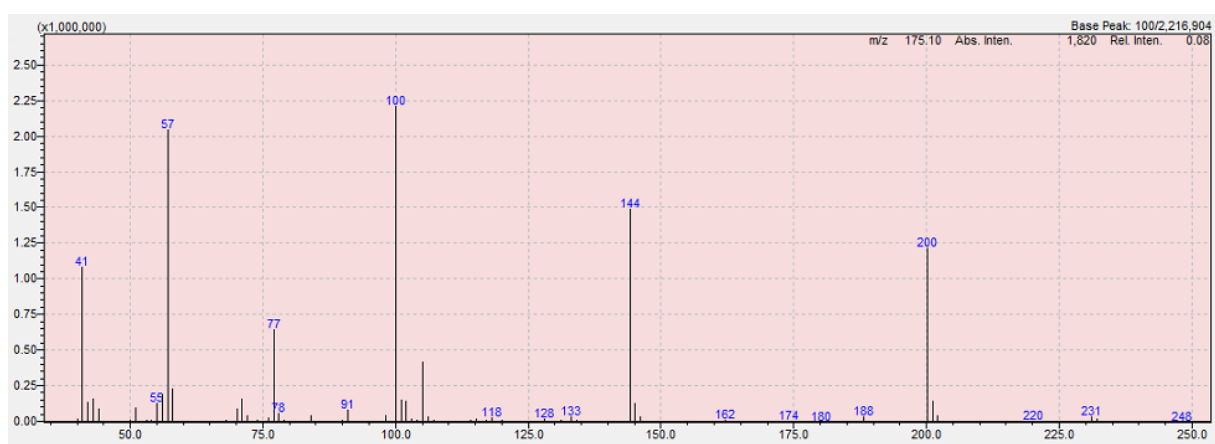


Figure A13 The mass spectrum of the derivatized NENP

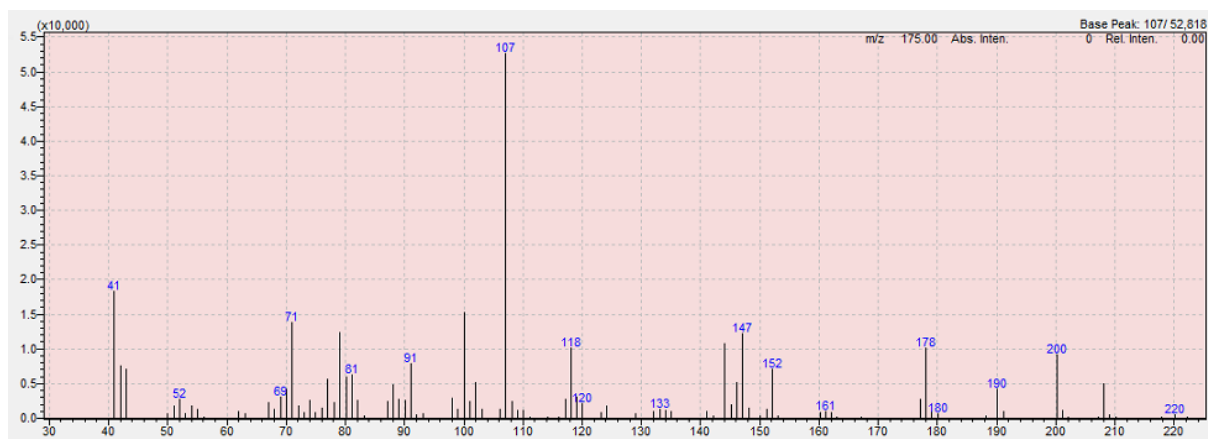


Figure A14 The mass spectrum of the derivatized N-ethyl-norpentadrol

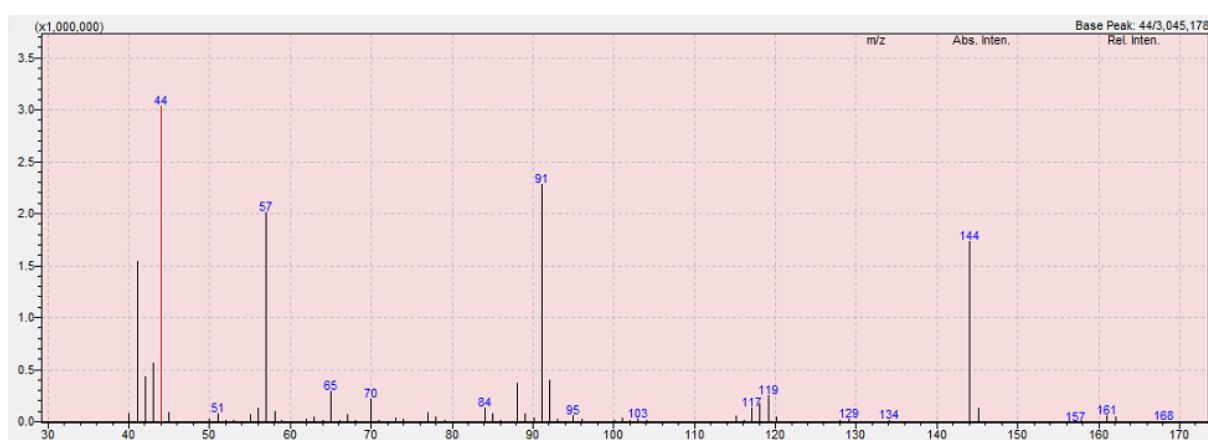


Figure A15 The mass spectrum of the derivatized amphetamine

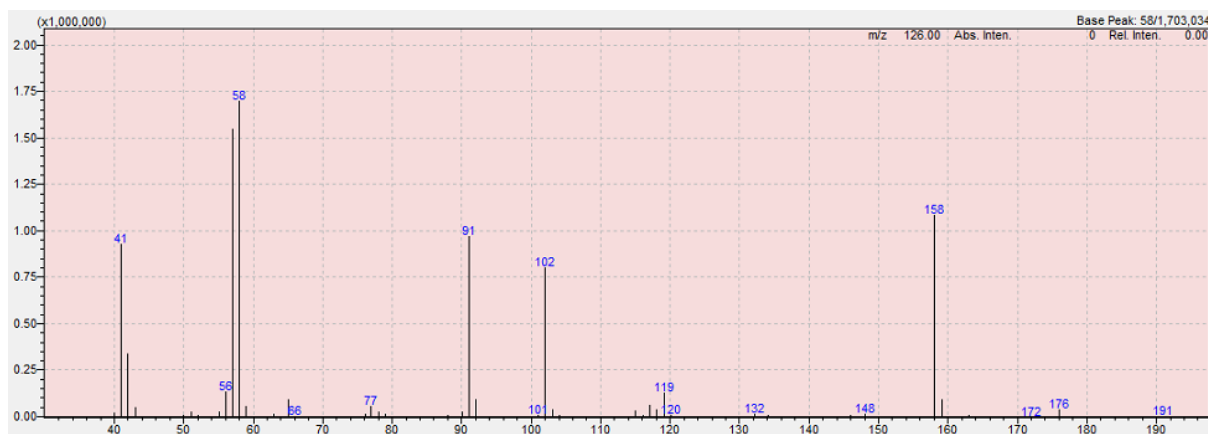


Figure A16 The mass spectrum of the derivatized methamphetamine

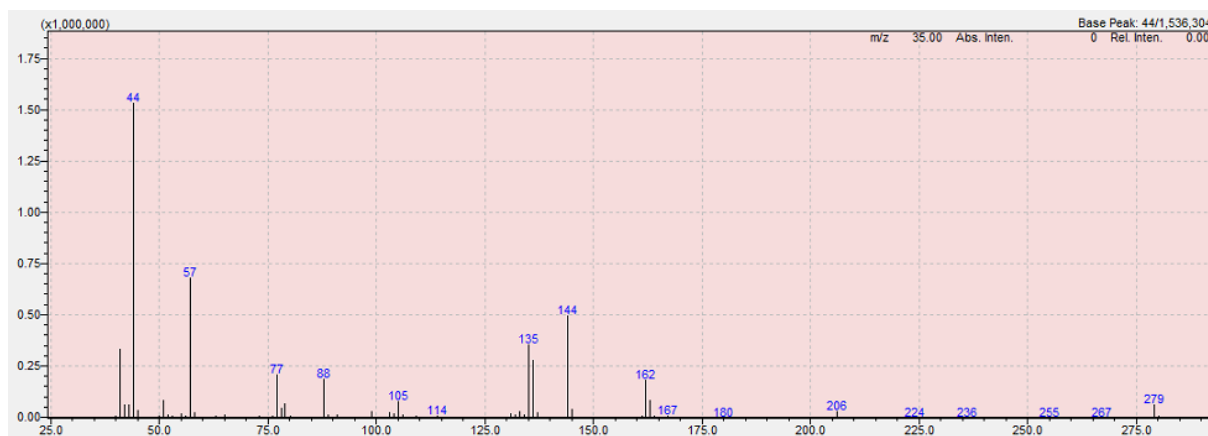


Figure A17 The mass spectrum of the derivatized MDA

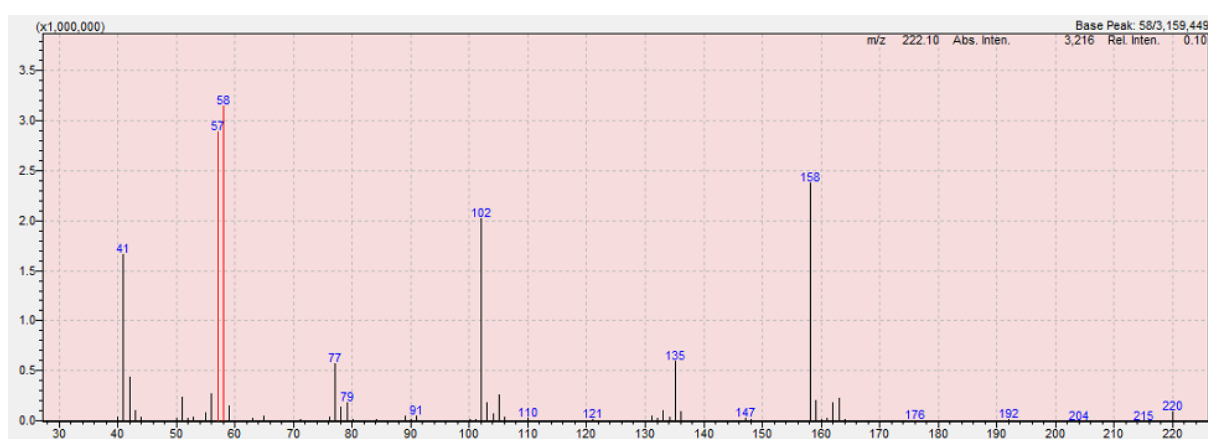


Figure A18 The mass spectrum of the derivatized MDMA

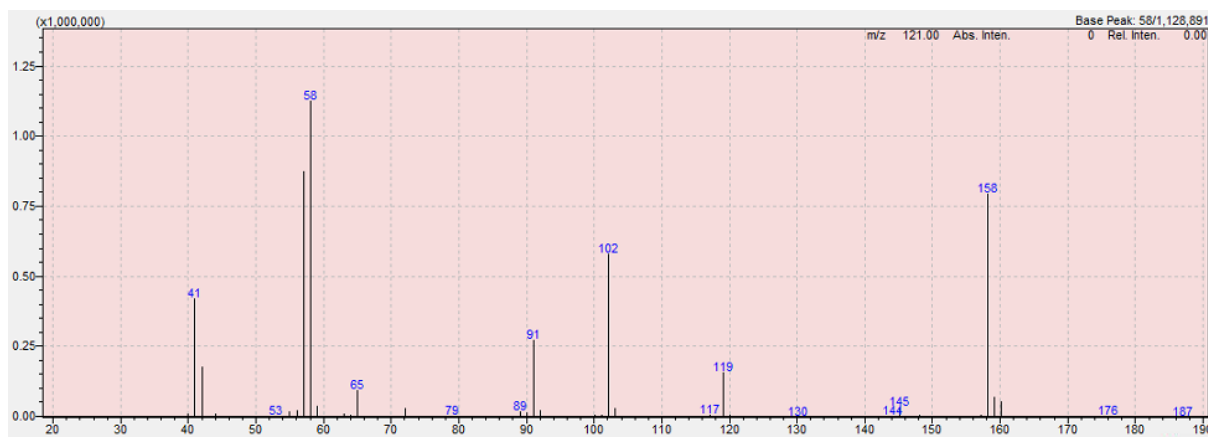


Figure A19 The mass spectrum of the derivatized mephedrone

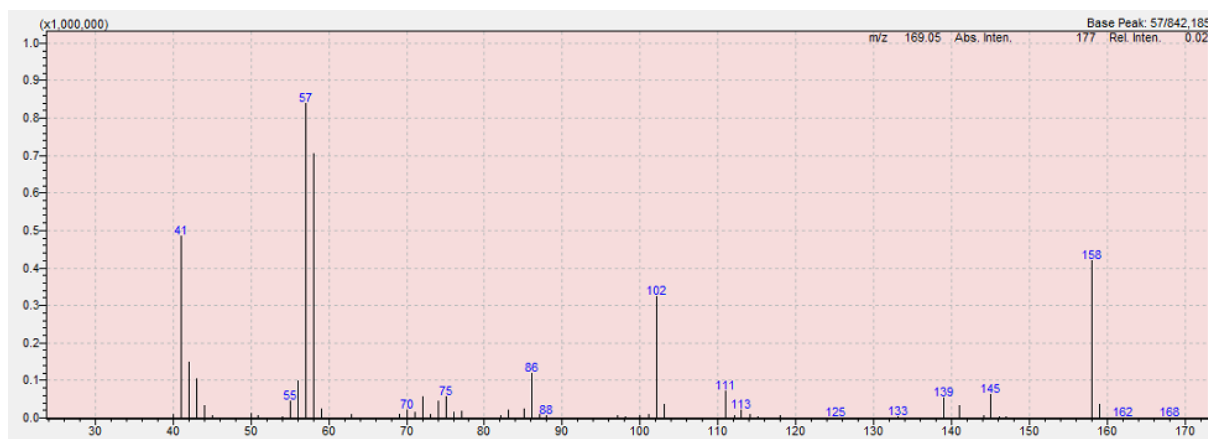


Figure A20 The mass spectrum of the derivatized 3-CMC