DEVELOPMENT OF A LOW VOLUME DISPERSIVE LIQUID-LIQUID MICROEXTRACTION METHOD FOR THE SIMULTANEOUS DETERMINATION OF ILLICIT PSYCHOACTIVE DRUGS USED BY MOTORISTS IN THE KHOMAS REGION OF NAMIBIA

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ABSTRACT

In recent years, there has been an increase in fatal road accidents in Namibia claiming many lives each year. Traffic accidents are often related to the use of alcohol, illegal drugs or psychoactive medicines. A rapid low volume dispersive liquid-liquid microextraction (DLLME) method in combination with gas chromatography-mass spectrometry (GC-MS) detection was developed for the simultaneous determination of illicit psychoactive drugs in blood (those used most commonly in Namibia: cocaine, Δ^9- tetrahydrocannabinol, amphetamine and methamphetamine). Various DLLME parameters were optimised to improve the extraction efficiency of the analytes. The method was successfully applied for the simultaneous determination of the drugs from spiked blood in a single extraction with satisfactory sensitivity, accuracy, repeatability, linearity and recoveries. The limits of detection (LOD) for different drugs varied from 0.00001 to 0.055 µg/mL while the limits of quantification (LOQ) varied from 0.0001 to 0.1 µg/mL. The precision was in a range of 2-10 %. The method was linear in the ranges of 0.00001-10 µg/mL with the correlation coefficient (r^2) ranging between 0.998-0.999. The recoveries of the analytes were between 84 and 103 %. The validated method was subsequently successfully applied in the screening of drugs in the blood collected from motorists (samples which were originally collected for blood-alcohol determination). The developed method utilised very low volumes of dispersion and extraction solvents (about 6 and 4 µL respectively) and only 25 µL of blood. Since the DLLME procedure was performed in 300 µL micro-insert GC vials, it was easier to collect the sedimented volume (containing the drugs) formed at the

end of the extraction process with a GC injection needle without running the risk of mixing the separated layers. This solution was directly submitted to GC-MS analysis without any need for further treatment, e.g. evaporation and reconstitution, which significantly reduced the extraction time of the method. The method was also successfully automated with an Agilent Sample Prep Workbench 7696A. Due to these advantages, this method lends itself to be used in high throughput routine analysis of drugs and potentially other substances (e.g. of toxicological relevance) in trace amounts of blood.

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LIST OF ABBREVIATIONS AND SYMBOLS

Δ^9-THC	Delta-9-tetrahydrocannabinol
µL	Microliters
ACN	Acetonitrile
BAC	Blood Alcohol Concentrations
CapLC	Capillary Liquid Chromatography
CCl_4	Carbon tetrachloride
CE-UV	Capillary Electrophoresis with Ultraviolet detection
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
CI	Confidence Interval
DLLME	Dispersive Liquid-Liquid Microextraction
DLLME-SFO	Dispersive Liquid–Liquid Microextraction based on the Solidification of a Floating Organic Drop
DUI	Driving Under the Influence
DUID	Driving Under the Influence of Drugs
EI^+	Positive Electron Ionisation
EFs	Enrichment Factors
GC	Gas Chromatography
GC-FID	Gas Chromatography - Flame Ionization Detection
GC-MS	Gas Chromatography - Mass Spectrometry

HFLLLME	Hollow Fiber Liquid-Liquid-Liquid Microextraction
HPLC-UV	High Performance Liquid Chromatography with Ultraviolet detection
IL-UA-SE-ME	Ionic Liquid-based Ultrasound Assisted Surfactant-Emulsified Microextraction
IP-SA-DLLME	Ion Pair Based-Surfactant Assisted Dispersive Liquid–Liquid Microextraction
LC-MS/MS	Liquid Chromatography with tandem Mass Spectrometry
LC-UV/DAD	Liquid chromatography with Ultraviolet/ Diode Array detection
LLE	Liquid-liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave-assisted Extraction
MALDI-TOF-MS	Matrix-assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry
MDMA	3,4-Methylenedioxymethamphetamine
MOSS	Ministry of Safety and Security
MS	Mass Spectrometer
MVA	Motor Vehicle Accident
NaCl	Sodium Chloride
NFSI	Namibia Forensic Science Institute
OPPs	Organophosphorus Pesticides
PAHs	Polycyclic Aromatic Hydrocarbons

PCP	Phencyclidine
R^2	Regression Squares
RSD	Relative Standard Deviation
S/N	Signal to Noise
SDME	Single Drop Microextraction
SIR	Single Ion Reaction
SFE	Supercritical Fluid Extraction
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
UA-DLLME-SFO	Ultrasound-Assisted Dispersive Liquid–Liquid Microextraction b ased on Solidification of Floating Organic Droplet
UA-LDS-DLLE	Ultrsound-Assisted Low Density Solvent Dispersive Liquid-Liquid Extraction
LDS-SD-DLLME	Low-density solvent-based demulsification dispersive liquid-liquid microextraction
LSD	Lysergic Acid Diethylamide
UPLC-MS	Ultra Performance Liquid Chromatography - Mass Spectrometry
UPLC-PDA	Ultra Performance Liquid Chromatography - Photodiode Array Detection
USAEME	Ultrasound-assistsed emulsification microextraction

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DECLARATION

I Bronah Nawa Simasiku, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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CHAPTER 1

1. INTRODUCTION

1.1 Orientation of the study

In recent years, there has been an increase in fatal road accidents in Namibia claiming many lives each year. A report in the Namibian Sun newspaper by Smit (2013) indicated that road accidents which resulted in injuries or fatalities increased from 319 in 2009 to 381 in 2010. In 2011, the figure increased by 24% to 443 and the number further increased to 560 in 2012 (Smit, 2013). A recent statistics report by the Motor Vehicle Accident (MVA) fund shows that from 1st January 2013 to 05 August 2013, there have been about 2020 crashes, 338 fatalities and 3449 injuries on Namibian roads (Motor Vehicle, 2013). These reports are alarming considering the country's population size of about 2.2 million. MVA has identified road crashes as the third leading cause of death in Namibia after HIV/AIDS and malaria (Nakale, 2013).

The use of psychoactive drugs (licit and illicit) by motorists and their contribution to fatal road accidents in the country is often neglected as there are currently no specific reports on the prevalent use of these drugs by drivers. These drugs can affect the functions of the

brain, causing changes in behaviour, mood and consciousness. They can also impair the driving abilities and skills of a driver. According to the Road Traffic and Transport Act 22 of Namibia (1999) "[n]o person shall drive a vehicle on a public road while under the influence of intoxicating liquor or a drug having a narcotic effect, or with an excessive amount of alcohol in the blood (0.079 mg/ 100 mL of blood) or breath (0.37 mg/1000 mL)". The Khomas region has the highest motorists in the country and according to the Khomas Regional Governor, Laura McLeod-Katjirua, the Khomas region alone accounts for 51% of all crashes recorded every year in Namibia (Ikela, 2013). Standards analytical methods for analysis of drugs of abuse in blood include liquid-liquid extraction (LLE) and solid phase extraction (SPE). However, these techniques are complicated, time consuming, require larger amounts of sample and organic solvents and are difficult to automate with analytical instruments. In this research, a liquid-liquid microextraction (LLME) technique based on dispersive liquid-liquid microextraction (DLLME) with GC-MS was developed for rapid analysis of illicit drugs. This technique is rapid, inexpensive and offers a high enrichment factor for analytes with little use of solvents. In addition, conventional DLLME is arguably the most simple of all the LLME techniques. This method was used to investigate the presence of illicit drugs in the blood samples of traffic law offenders from the Khomas region.

The microextraction techniques received favourable responses and various modifications have been introduced, e.g., single-drop microextraction (SDME), continuous-flow microextraction (CFME) and hollow-fiber liquid-phase microextraction (HF-LPME). A

more recent technique which does not involve the use of either fiber or syringe has been termed DLLME. The LPME technique is also able to overcome some of the problems that are often encountered in solid phase microextraction (SPME) which include, but are not restricted to, the bending of syringe, leaching of fiber coating materials and the fragility of the fiber itself (Nuhu, Basheer, & Saad, 2011)

1.2 Statement of the problem

The National Forensic Science Institute (NFSI) laboratory under the Ministry of Safety and Security (MOSS) has observed that blood samples for most traffic law offenders including those involved in car accidents, are primarily requested for ethyl alcohol analysis and in rare cases for analyses of medicinal or drugs of abuse. Medicinal drugs and drugs of abuse like ethyl alcohol can also impair the driving capabilities of motorists. It is against this background that this research was carried out in order to investigate whether the blood samples that are primarily submitted for ethyl alcohol analysis to NFSI contain other drugs that could have contributed to the impairment of the driver's abilities. However, due to many drawbacks of the standard analytical methods for analysis of drugs of abuse, a convenient, rapid and inexpensive analytical method for extracting the investigated drugs of abuse from blood was to be developed prior to the investigation. It is for this reason that a DLLME method was developed which could offer these benefits as compared to conventional sample extraction techniques, LLE and SPE. The drugs

investigated were cocaine, delta-9-tetrahydrocannabinol (Δ^9-THC), amphetamine and methamphetamine as these are the most frequently confiscated and readily available drugs in the country (Haufiku, 2012). To the best of our knowledge, this research is the first to be carried out specifically on the simultaneous determination of the drugs under study using a low volume DLLME, and application of the method to study the prevalent use of psychoactive drugs by motorists in Namibia, as no such publications are yet available.

1.3 Objectives of the study

The main objective of this project was to develop a DLLME method that offer the rapid simultaneous determination of cocaine, Δ^9-THC, methamphetamine and amphetamine from blood specimens that can be used to investigate whether blood samples submitted for ethyl alcohol analysis at NFSI contains other psychoactive drugs that could have impaired the driving capabilities of the driver. The specific aims of the study were to develop and validate a DLLME method to be used for the screening of the drugs in blood and to analyse blood samples from NFSI (primarily submitted for blood alcohol analysis) for the illicit drugs under study.

1.4 Significance of the study

A low volume DLLME method for the simultaneous analysis of the drugs as compared to conventional DLLME, LLE and SPE might offer a reduction in extraction time, cost, as well as the use of toxic solvent. The overall outcome of this research might provide an insight on the current prevalent use of drugs by motorists in the Khomas region. This might be useful to the Ministry of Safety and Security (MOSS) and City police in improving the current strategies against driving under the influence of drugs (DUID) and implementation of strict laws. In addition, the results can be used to educate the Namibian public, especially motorists, on the risks of DUID in causing fatal accidents. Improvement in these areas might help to reduce the number of fatal road accidents related to DUID. Finally, the outcome of the research might motivate for further DUID prevalent research studies in other parts of the country.

1.5 Limitation of the study

In this research, only blood samples of motorists submitted to the NFSI for blood alcohol analysis during the year 2014 were to be analysed for the presence of illicit drugs. The research excludes the drivers who were tested for breath alcohol at the crime scene. However, all the samples collected for blood alcohol analysis by traffic officers are

submitted to the NFSI thereby giving a good representation of the general traffic offenders' population in the Khomas region.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Driving under the influence of drugs: an overview

Traffic accidents and the risk of severe injury are often related to the use of alcohol, illegal drugs or psychoactive medicines. In particular, the risk of traffic accidents has been found to increase with the use of illicit drugs (Gjerde, Normann, Christophersen, Samuelsen, & Morland, 2011; Hels, Lyckegaard, Simonsen, & Steentoft, 2013). In DUID studies, alcohol and drugs are frequently detected in injured drivers, more frequently than in the general driving population (Schulze, Schumacher, Urmeew, & Auerbach, 2006). The blood from drivers involved in accidents is primarily submitted for ethyl alcohol analysis in most road accidents because the relationship between alcohol and crash risk has been firmly established and accepted in many countries (Acar *et al*., 2013). Most drugs that affect the central nervous system may have the potential to impair driving ability and these include alcohol, drugs of abuse (opiates, amphetamines, cocaine and cannabis) and prescribed psychoactive drugs (Mura *et al*., 2003). In most studies where DUID cases were investigated, cocaine, cannabis and benzodiazepines were the most frequently detected illicit and medicinal drugs (Legrand *et al*., 2012; Stoduto, Mann, Ialomiteanu, Wickens & Brands, 2012; Vindenes *et al*., 2012; Steuer, Forss, Dally & Kraemer, 2014).

Cannabis and alcohol are the most popular drugs amongst recreational users, and most prevalent in injured and deceased drivers (Downey *et al.*, 2013).

2.2 DUID studies in various countries

The most common matrices analysed for drugs used by traffic offenders include oral fluid, blood and sometimes urine, if available. In this part of the review, the focus will be on DUID studies from different countries by various researchers showing the most common illicit and licit drug(s) used by motorists as well as DUID patterns among men and women, the summary of which is presented in Table 2-1.

A pilot study was conducted by Matzopoulos, Lasarow and Bowman (2013) in Gauteng and Western Cape provinces of South Africa aiming at testing the use of four substance use screening devices developed in Germany, under the local South African conditions and to assess their utility for detecting DUID as part of the standard roadblock operations of local law enforcement agencies. Out of the 269 cases studied, 28 % of motorists tested positive for breath alcohol, while 5 % of the drivers tested for both alcohol and drugs simultaneously and only 14% of the drivers tested positive for drugs only. After alcohol, amphetamine, methamphetamine and cocaine were the most common drugs of impairment detected. The results suggested that under normal enforcement procedures only 76% of drivers impaired by alcohol and other drugs would have been detected.

Davey, Armstrong, & Martin (2014) conducted an investigation on roadside drug detections after the introduction of oral fluid screening to detect the presence of illicit drugs in drivers of Queensland, Australia. This work was conducted with over 80,000 drivers over a 5 year period from December 2007 to June 2012. The most common drug types detected were methamphetamine (40.8%) followed by cannabis (29.8%) and methamphetamine/cannabis combination (22.5%). These results show that the usage of these illicit drugs especially methamphetamines and cannabis by motorists in Australia is relatively high. This pattern is similar to illicit drugs usage by motorists in Austria as shown in the study which was conducted over a period of 4 years during 2003–2007. In this study, the results from the EU-project Rosita in Austria as reported by Keller, Keller, Tutsch-Bauer, & Monticelli (2009) showed a wide variety of illicit drugs and medications in blood samples of the drivers. Of these, cannabis (50%), opiates (20%), amphetamines (18%), cocaine (15%) and benzodiazepines (20%) were the most prevalent.

The same trend was observed in a study conducted by Bezemer, Smink, van Maanen, Verschraagen, & de Gier (2014) on the prevalence of psychotropic medicines in drivers suspected of driving under the influence of medicinal and illicit drugs in the Netherlands. In total, 3038 blood samples of suspected impaired drivers in the Netherlands were analysed for the presence of medicinal and illicit drugs between January 2009 and December 2012. In 94% of the cases, medicinal and/or illicit drugs were detected.

Medicinal drugs were found in 33% of the blood samples, with the highest prevalence for anxiolytics. Illicit drugs were positive in 86% of the cases, with the highest prevalence for cannabis. Multiple drug use including medicinal and/or illicit drugs, were detected in at least 56% of the blood samples.

In 2012, a study was carried out with Brazilian drivers by Zancanaro *et al*. (2012) to investigate the prevalence of 32 psychoactive drugs. This study entailed the development and validation of an analytical method for analysing 32 prescription and illicit psychoactive drugs (amphetamines, benzodiazepines, cocaine, cannabis, opioids, ketamine and meta-Chlorophenylpiperazine) and metabolites in oral fluid samples. About 10% of the 2235 oral fluid samples collected from drivers on Brazilian Federal highways tested positive for at least one analyte investigated. Alone or in combination with other drugs, cocaine/metabolites were the analytes most detected in the samples (129; 5.8%), followed by amphetamines/metabolite (69; 3.1%), benzodiazepines (28; 1.2%), cannabinoids (23; 1.1%) and opioids (8; 0.4%). At least two psychoactive drugs from different classes accounted for 9.3% of the 236 positive samples. Cocaine was found in higher concentration in the samples with up to 1165 ng/mL recorded. The use of psychoactive drugs in this study was more prevalent as compared to the study conducted by Gjerde *et al*. (2014) which shows a significant decrease in DUI cases. These researchers compared the use of alcohol, illegal drugs and psychoactive medicinal drugs among random drivers in Brazil and Norway, two countries with the same legal limit for drunk driving, but with marked differences in legislation history, enforcement and penalties for DUI. Roadside

surveys were conducted on Fridays and Saturdays between noon and midnight. Samples of oral fluid were collected for analysis of drugs, whereas alcohol was determined by breath testing or by analysis of oral fluid. The weighted prevalence of driving with alcohol concentrations in breath or oral fluid equivalent to blood alcohol concentrations (BAC) above 0.2 g/L was 2.7% (95% confidence interval (CI) 2.2–3.3) in Brazil and 0.2% (95% CI 0.0–0.5) in Norway. Stimulants (amphetamines or cocaine) were found in samples at 1.0% (95% CI 0.7–1.4) of drivers in Brazil and 0.3% (95% CI 0.1–0.7) in Norway. The prevalence of amphetamines was highest among Brazilian truck drivers (3.6%; 95% CI 2.0–6.4). Tetrahydrocannabinol was found in samples at 0.5% (95% CI 0.3–0.8) of drivers in Brazil and 1.0% (95% CI 0.6–1.5) in Norway, whereas benzodiazepines or zopiclone were found in 1.0% (95% CI 0.7–1.4) and 1.7% (95% CI 1.2–2.4) of the samples from Brazil and Norway, respectively. Differences for drugs may be related to different patterns in the use of stimulants, cannabis and medicines.

Norway is one of the countries with the lowest DUID as shown in the above study, where the DUID patterns were compared with that of Brazil. Similar results were obtained in a case of a control study that was conducted by Bogstrand & Gjerde (2014) in Norway. In this study, blood samples from ‘cases’ and oral fluid samples from ‘controls’ were analysed for 15 drugs that have legislative concentration limits in Norway. The most prevalent illicit drug in the control group was tetrahydrocannabinol (THC; 0.58%), which was also commonly detected in samples of drivers arrested due to road crashes (15.6%) or arrested for other reasons (21.8%). Amphetamine/methamphetamine was most prevalent

among arrested drivers involved in crashes (30.6%) and drivers arrested for other reasons (56.9%), whereas only 0.18% of the control group was positive for amphetamine/methamphetamine.

In Spain, a random study was carried out by Go´mez-Talego´n, Fierro, Gonza´lez-Luque, Cola´s, Lo´pez-Rivadulla, & A´lvarez (2012), whereby roadside controls of 3302 representative samples of Spanish drivers' saliva were analysed for 24 psychoactive substances and alcohol breath tests. The results showed that about 17% of the drivers tested positive for the substances analysed, 6.6% of the drivers tested positive to alcohol (>0.05 mg/L in breath), 11% tested positive to any illicit drug, and 2% tested positive to one of the medicines analysed. Some drivers were positive in more than one substance. The most common illicit drugs among Spanish drivers were cannabis (7.7%) or cocaine (3.5%), either alone or in combination with other substances and the most prevalent medicines were benzodiazepines (1.6%).

In Sweden, an average trend is also observed as reported in a study by Jones, Holmgren, & Kugelberg (2008). This study was conducted over a period of 5 years (2000-2004) where they investigated cocaine and its major metabolite benzoylecgonine in blood samples from people arrested in Sweden for driving under the influence of drugs (DUID). Venous blood or urine was subjected to a broad toxicological screening for cannabis, cocaine metabolites, amphetamines, opiates and the major benzodiazepines. Over the study period,

26,567 blood samples were analysed and cocaine and/or BZE were detected in 795 cases (3%). In this study, motorists using cocaine were predominantly men (>96%) with an average age of 28.3 years. The concentrations of BZE were always higher than the parent drug; mean BZE/cocaine ratio was 14.2 (median 10.9), with a range between 1 and 55. Cocaine and BZE were the only psychoactive substances reported in 61 cases at mean (median) and highest concentrations of 0.095 mg/L (0.07) and 0.5 mg/L for cocaine and 1.01 (0.70) and 3.1 mg/L for BZE. Typical signs of drug influence noted by the arresting police officers included bloodshot and glossy eyes, agitation, difficulty in sitting still and incoherent speech.

In one of the recent control study case conducted in 2012, Hels *et al*. (2013) reported on the relative risks of severe driver injury when driving under the influence of ten substance groups (alcohol, amphetamines, benzoylecgonine, cocaine, cannabis, illicit opiates, benzodiazepines and Z-drugs, i.e. zolpidem and zopiclone, medicinal opioids, alcohol–drug combinations and drug–drug combinations). Data from six countries were included in the study: Belgium, Denmark, Finland, Italy, Lithuania and the Netherlands. Case samples (N = 2490) were collected from severely injured drivers of passenger cars or vans in selected hospitals in various regions of the countries. The highest risk of the drivers being severely injured was associated with drivers who tested positive with high concentrations of alcohol (≥0.8 g/L) alone or in combination with other psychoactive substances. For alcohol, risk increased exponentially with blood alcohol concentration (BAC). The second most risky category contained various drug–drug combinations,

amphetamines and medicinal opioids. Medium increased risk was associated with medium sized BACs (at or above 0.5 g/L and below 0.8 g/L) and benzoylecgonine. The least risky drug seemed to be cannabis, benzodiazepines, and Z-drugs. It was thus concluded that among psychoactive substances, alcohol still posed the largest problem in terms of driver risk of getting injured.

2.3 Psychoactive drug use patterns

The use of illicit and licit drugs or medicinal drugs varies among women and men at different seasons and times. Women tend to use more licit or medicinal drugs while men use more illicit drugs. This is evident from various studies on "driving under the influence of drugs" (DUID) carried out by various researchers. In South-East Hungary, a framework of the DUID Alcohol and Medicines EU-6 project was conducted by Institóris *et al*. (2013) to determine the incidence of alcohol use and the most frequent illicit and licit drug consumption in the general driving population. The results showed that illicit drug consumption was the highest among men of the ages 18–34, during the spring and on the weekend nights while, women over the age of 50 had the highest incidence of licit drugs, during the summer on week-days. All alcohol positive cases were men over the age of 35.

Similar results were obtained in a study by Davey *et al*. (2014) in a Queensland's 2007–2012 roadside drug testing program, which focused on the prevalence of three illicit drugs.

The data revealed a number of regional, age and gender patterns and variations of DUID across the state. The younger drivers were more likely to test positive for cannabis whilst older drivers were more likely to test positive for methamphetamine. The overall characteristics of drivers who tested positive for the presence of at least one of the target illicit drugs were male, aged 30–39 years, and driving a car on Friday, Saturday or Sunday between 6:00 pm and 6:00 am.

Another roadside study was conducted by Gómez-Talegón *et al*. (2012) in Spain to investigate the prevalence of psychoactive substances, alcohol, illicit drugs and medicines. The study showed higher figures for positive cases among males than females with statistically significant differences for alcohol, cannabis and cocaine. In addition to that, alcohol and cocaine positive cases were more frequently found among drivers on urban roads. There were differences in the prevalence of positive cases of alcohol, cannabis and cocaine, in relation to the period of the week.

2.4 Analytical methods in DUID studies

Liquid chromatography-mass spectrometry (LC-MS) and GC-MS are the most widely employed analytical techniques (Table 2-1) for qualitative and quantitative analysis of illicit drugs (Airado-Rodríguez, Cruces-Blanco, & García-Campa˜na, 2012). GC-MS is a generally reliable technique which offers high resolutions, good sensitivities and is

equipped with spectra libraries (Maurer, 2006). Matrix interference and an insufficient instrumental detection limit for traces of psychotropic drugs in biological samples, makes direct chromatographic separation and determination very difficult. Therefore, in order to obtain accurate, reliable and sensitive results, an extraction/pre-concentration method is required prior to chromatographic separation of psychotropic drugs (Shamsipur & Mirmohammadi, 2014).

In most roadside DUID studies, the screening of illicit drugs is primarily performed using immunoassay techniques (Table 2-1). However, these techniques are expensive and cannot be used for confirmation and quantification of drugs. Therefore, LLE and SPE are generally applied as sample preparation techniques in drug analysis and are compatible for use with analytical instruments. These techniques are however complicated, time-consuming; require large amounts of sample and organic solvents and difficult to automate with analytical instruments. The use of harmful chemicals and large amounts of solvents that can cause environmental pollution, present health hazards to laboratory personnel, not to mention extra operational costs for waste treatment.

Liquid-phase microextraction (LPME) is an emerging sample preparation technique that uses minimal amounts of solvent. It is rapid and inexpensive, offers a high degree of enrichment for the analytes in complex matrices with minimal exposure to toxic organic solvents as compared with the conventional methods (SPE and LLE). The LPME technique is also able to overcome some of the problems that are often encountered in

solid phase microextraction (SPME) which include, but are not restricted to, the bending of syringe, leaching of fiber coating materials and the fragility of the fiber itself (Nuhu, Basheer, & Saad, 2011). Most of the microextraction applications are employed in aqueous samples for the extraction of non-polar or moderately polar high molecular weight analytes (Cunha, Fernandes & Oliveira, 2011). LPME comprises four different techniques namely cloud point extraction (CPE), single-drop microextraction (SDME), DLLME and hollow-fiber LPME (HF-LPME). Conventional DLLME was the technique of choice for this study, since it is arguably the most simple of all the LPME techniques (Leong, Fuh, & Huang, 2014). This research aims at developing a rapid and affordable low volume DLLME technique for extraction of illicit drugs from blood obtained from motorists.

Table 2-1. A summary of DUID studies in various countries showing the most prevalent drug (s) as well as the analytical techniques used for analysis of the drugs

Country	**Most prevalent drug(s)**	**Population**	**Year**	**Sample preparation technique**	**Analysis method**	**Reference**
South Africa	Amphetamine, methamphetamine and cocaine	269	2008	-	LC-MS and GC-MS	Matzopoulos, Lasarow and Bowman (2013)
Queensland, Australia	Methamphetamine (40%), cannabis (29.8%), ecstasy (1.7%)	80000	2007-2012	-	Securetec Drug Wipe II Twin oral fluid device	Davey *et al*. (2014)
Austria	Cannabis (50%), opiates (20%), amphetamines (18%), cocaine (15%), benzodiazepines (20%)	1167	2003-2007	Immuno chemical analysis	HPLC, GC-MS	Keller *et al*. (2009)
Netherlands	Cannabis	3038	2009-2012	-	UPLC-MS/MS, GC-MS	Bezemer *et al*. (2014)
Brazil	Cocaine and metabolites (5.8%), amphetamines and metabolites (3.1%), benzodiazepines (1.2%), cannabinoids (1.1 %), opiods (0.4%)	2235	-	LLE	LC-MS/MS	Zancanaro *et al*. (2012)
Brazil	Alcohol (2.7%), amphetamines/cocaine (1%), tetrahydrocannabinol (0.5%), benzodiazepines/zopiclone (1%)	-	2008-2009	Quantisal oral fluid & Statsure saliva sampler	LC-MS/MS	Gjerde et al. (2014)
Norway	Alcohol (0.2%), amphetamines/cocaine (0.3%), tetrahydrocannabinol (1%), benzodiazepines/zipoclone (1.7%)					
Spain	Cannabis (7.7%), cocaine (3.5%), benzodiazepines (1.6%)	3302	2008-2009	Statsure saliva sampler	LC-MS/MS	Gómez-Talegón *et al*. (2012)
Sweden	Cocaine and metabolites (3%)	26567	2000-2004	SPE	GC-MS, LC-MS, GC-NPD	Jones *et al*. (2008)
Norway	Tetrahydrocannabinol, Amphetamine/methamphetamine	9375	2008-2009	-	GC-MS, LC-MS	Bogstrand & Gjerde (2014)

2.5 Dispersive liquid-liquid microextraction

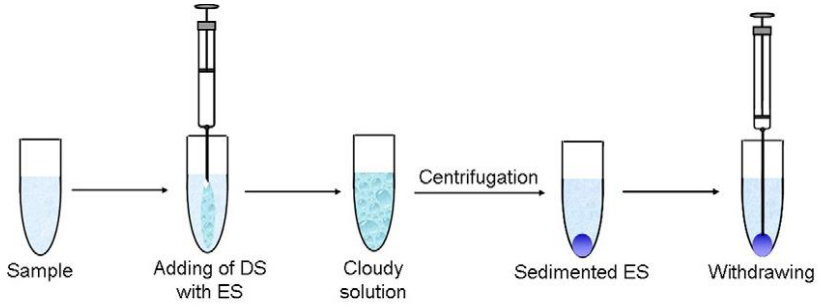
Sample preparation is considered the most time-consuming and error-prone step of the analytical process. It has always been a crucial step for the whole analytical procedure since complex matrices cannot be analysed directly even with advanced analytical instrumentation. This step is necessary to isolate the components of interest from the sample matrix (Pena-Pereira, Lavilla, & Bendicho, 2010). Extraction, clean-up as well as concentration of the target analytes are the main processes involved in sample preparation methods. In this respect, a large number of procedures have been developed so far using conventional sample preparation techniques (liquid-liquid extraction (LLE) and solid phase extraction (SPE).

In this research, a low volume DLLME technique for the determination of illicit drugs from blood is developed and validated. Therefore, this part of the review focuses on recent applications of DLLME on biological matrices, shortcomings and advances in DLLME as well as coupling and automation of DLLME.

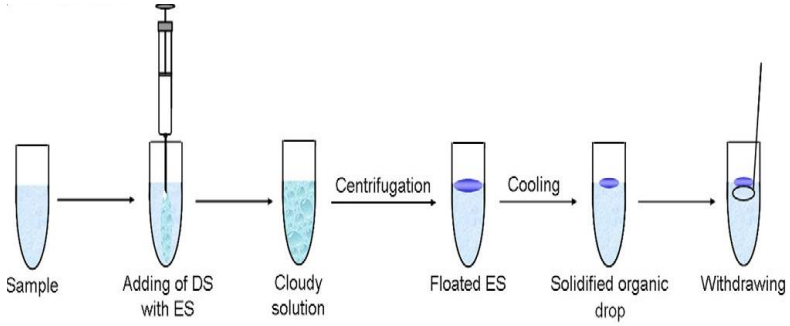
Dispersive liquid–liquid microextraction (DLLME) is an analytical technique that was introduced by Rezaee *et al*. (2006) for extraction and pre-concentration of organic compounds from water samples. The method offers simplicity of operation, rapidity, low cost, high recovery and enrichment factor; this is due to the large contact surface area of

the extraction solvent (Leong *et al*., 2014). In DLLME, the extracting solvent is mixed with a dispersing solvent that is miscible with both the former and with the aqueous sample. The mixture is rapidly injected into the sample with a syringe, producing high turbulence that leads to the formation of tiny droplets. Because of the large surface area between the extracting droplets and sample, the extraction time is drastically reduced. After centrifugation, the sedimented phase at the bottom of the tube is collected and either injected directly or evaporated to dryness before reconstitution and injection (Kohler, Schappler, Sierro , & Rudaz, 2013; Kocúrová, Balogh, Šandrejová, & Andruch, 2012).

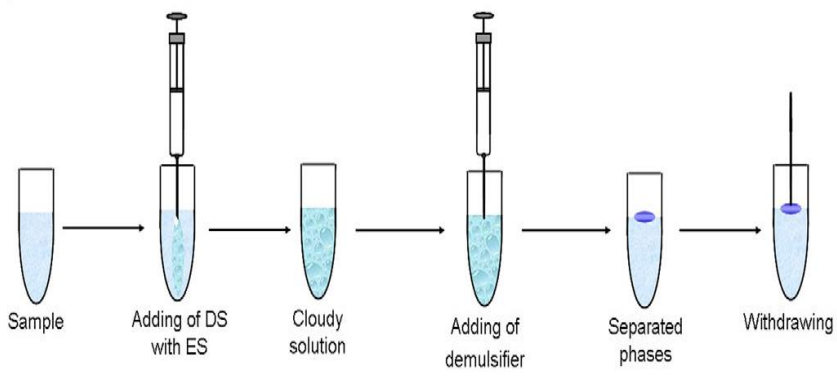
DLLME techniques are separated in two categories: DLLME with low-density extraction solvent and DLLME with high-density extraction solvent. These techniques have different operating modes such as manual shaking, air-assisted LPME (aspirating and injecting the extraction mixture by syringe), ultrasound-assisted emulsification, vortex-assisted emulsification, surfactant-assisted emulsification, and microwave-assisted emulsification (Figure 2-1). DLLME has been used in conjunction with other extraction techniques such as solid-phase extraction, stir bar sorptive extraction, molecularly imprinted matrix solid-phase dispersion and supercritical fluid extraction (Leong *et al*., 2014).


Sample
Adding of DS with ES
Cloudy solution
Centrifugation
Sedimented ES
Withdrawing

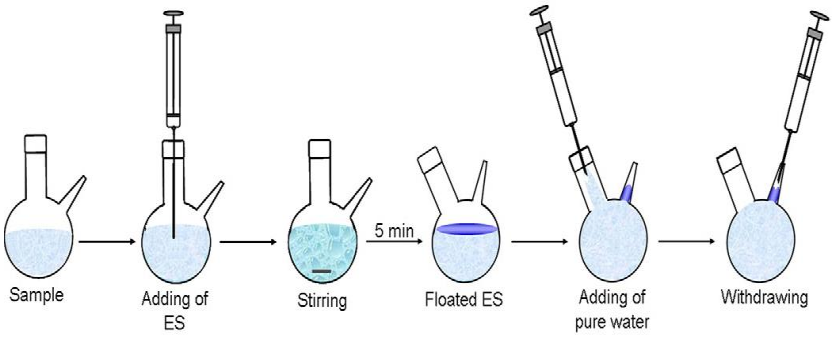
a)


Sample
Adding of DS with ES
Cloudy solution
Centrifugation
Floated ES
Cooling
Solidified organic drop
Withdrawing

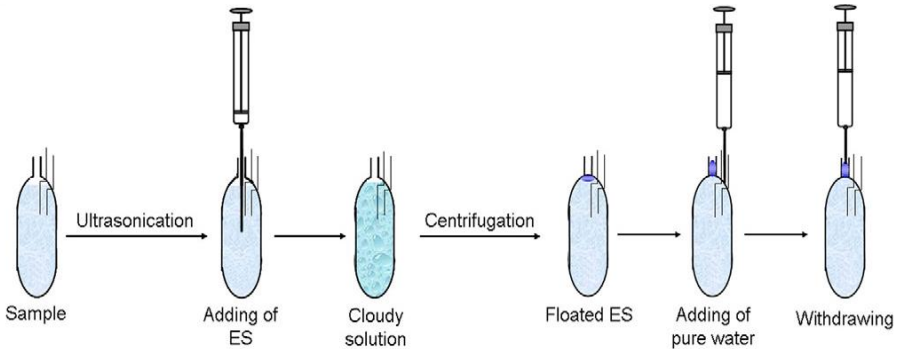
b)


Sample
Adding of DS with ES
Cloudy solution
Adding of demulsifier
Separated phases
Withdrawing

c)



d)



e)

Figure 2-1: Different DLLME techniques: (a) conventional DLLME; (b) dispersive liquid–liquid microextraction based on the solidification of a floating organic drop (DLLME-SFO); (c) low-density solvent-based solvent demulsification dispersive liquid–liquid microextraction (LDS-SD-DLLME); (d) magnetic stirring-assisted liquid phase microextraction; (e) ultrasound-assisted emulsification microextraction (USAEME) (Kocúrová et al., 2012).

2.5.1 Recent developments in DLLME with high density extraction solvents

High density extraction solvent also known as conventional DLLME, has in the very short time since its invention in 2006 grabbed the attention of researchers and has become very popular among analytical chemists (Kocúrová *et al*., 2012). This technique has found many applications in the separation and pre-concentration of various organic and inorganic analytes in various matrices such as biological matrices, environmental samples and food.

The applications of DLLME in the extraction of organic analytes from biological matrices are presented in Table 2-2. High extraction recoveries have been obtained in two different studies conducted by Vela-Soria and co-workers in 2013 and 2014. In their earlier work (2012), they introduced a new sample treatment method based on dispersive liquid–liquid microextraction for the extraction of the most commonly used parabens (methyl-, ethyl-, propyl-, and butylparaben) from human serum samples followed by separation and quantification using ultrahigh performance liquid chromatography– tandem mass spectrometry (UHPLC–MS/MS). In this work, the extraction recoveries were in the ranges of 96-106 %, with the limit of detection (LOD) between 0.1-0.2 µg/L. However, the method had a high relative standard deviation (RSD) of up to 11% (Vela-Soria *et al*., 2013). Their work in 2014, was an extension of previous work and entailed the development of a DLLME for the extraction of six parabens (methyl-, ethyl-, isopropyl-, propyl-, isobutyl and butyl paraben), six benzophenones (benzophenone-1, benzophenone-

2, benzophenone-3, benzophenone-6, benzophenone-8 and 4-hydroxybenzophenone) and two bisphenols (bisphenol A and bisphenol S) in human urine samples, followed by GC–MS/MS analysis. This method was satisfactorily applied for the determination of target compounds in human urine samples from 20 randomly selected individuals (Vela-Soria, Ballesteros, Zafra-Gómez, Ballesteros & Navalón, 2014). In this work, the LOD was lower and ranged between 0.06-0.2 μg/L compared to the previous work. The differences in these experiments could have been influenced by the matrix effect of the samples; in the 2013 work, the analytes were extracted from human plasma and in 2014 from urine.

In 2011, Saraji, Boroujeni & Bidgoli applied DLLME and hollow fiber liquid–liquid–liquid microextraction (HFLLLME) combined with HPLC–DAD for the determination of three narcotic drugs (alfentanil, fentanyl, and sufentanil) in biological samples (human plasma and urine). The results showed that both methods exhibited good linearity, precision, enrichment factor and detection limit. Under optimal condition, the limits of detection ranged from 0.4 to 1.9 μg/L for DLLME and from 1.1 to 2.33 μg/L for HFLLLME, respectively. The enrichment factors (EF) were from 275 to 325 and 190 to 237 for DLLME and HFLLLME, respectively.

In 2014, Shamsipur & Mirmohammadi used DLLME coupled with high performance liquid chromatography with ultraviolet detection (HPLC–UV) as a fast and inexpensive technique for the determination of imipramine and trimipramine in urine samples. Under

optimised experimental conditions, the enrichment factors and extraction recoveries were between 161.7–186.7 and 97–112%, respectively. The linear range and LOD for both analytes were found to be 5–100 ng/mL and 0.6 ng/mL, respectively. The RSD for 5 ng/mL of the drugs in urine samples were in the range of 5.1–6.1% (n = 5). The developed method was successfully applied to real urine sample analyses. A similar method was developed by Airado-Rodríguez *et al*. (2012) for the analysis of 3,4-methylenedioxymethamphetamine (MDMA), lysergic acid diethylamide (LSD) and phencyclidine (PCP) in human urine. The linear responses for MDMA, PCP and LSD in the urine matrix were between 10.0 and 100 ng/mL approximately, and LODs of 1.00, 4.50 and 4.40 ng/mL were recorded for MDMA, PCP and LSD, respectively. This method was successfully applied for the analysis of the three drugs of interest in human urine with satisfactory recovery percentages.

A simple, rapid and sensitive method termed DLLME combined with high-performance liquid chromatography-ultraviolet detection (HPLC-UV) has been proposed by Xiong, Ruan, Cai & Tang (2009) for the determination of three psychotropic drugs (amitryptiline, clomipramine and thioridazine) in urine samples. Under optimal DLLME conditions, the absolute recoveries of amitryptiline, clomipramine and thioridazine from the urine samples were 96, 97 and 101%, respectively. The LOD and limit of quantification (LOQ) of the proposed approach were 3 and 10 ng/mL for amitryptiline, 7 and 21 ng/mL for clomipramine, as well as 8 and 25 ng/mL for thioridazine, respectively. The RSDs for nine replicate determinations at 0.100 µg/mL level of target drugs were less than 4.8%. Good

linear behaviours over the investigated concentration ranges were obtained with the values of $R^2 > 0.998$ for the target drugs. This proposed method was successfully applied to the real urine samples from two female patients under amitryptiline and clomipramine treatment, respectively.

DLLME has been widely used in environmental samples as well as food applications, however only a few publication in such fields was reviewed as the focus of this research is on biological matrices (blood). In 2014, Zhang, Zhang, & Jiao developed a quick, easy, cheap, effective, rugged and safe (QuEChERS) method combined with DLLME for the analysis of ten pyrethroids in various fruit juices using gas chromatography-electron capture detection (GC–ECD). This QuEChERS–DLLME method has found its widespread applications to all the fruit juices including those samples with more complex matrices (orange, lemon, kiwi and mango), while DLLME was confined to fruit juices with simpler matrices (apple, pear, grape and peach). These methods provided acceptable recoveries and repeatability. In addition, the applicability of two methods were demonstrated with the real samples and further confirmed by gas chromatography–mass spectrometry (GC–MS).

Like in other matrices, DLLME has also found many applications for the extraction of analytes from water samples. To mention a few, the technique was used to develop a

simple and sensitive method for the determination of trace levels of inorganic species in environmental water samples by Jafarvand & Shemirani (2011).

Table 2-2: A comparison of various DLLME methods for the determination of narcotic drugs in biological samples

Method	**Analyte (s)**	**Sample**	**Enrichment factor**	**Recovery (%)**	**LOD (µg/mL)**	**SD (%)**	**Linearity (µg/mL)**	**Reference**
DLLME	Parabens	Serum	-	96-106	0.1-0.2	3.1-11	Up to 100	Vela-Soria *et al*. (2013)
DLLME	Parabens, benzophenone, bisphenols	Urine	-	94-105	0.06-0.2	2.0-14.9	Up to 300	Vela-Soria *et al.* (2014)
DLLME and HFLLLME	Narcotic drugs (alfentanil, fentanil, sufentanil)	Plasma and urine	275-325	11-49	0.4-1.9	-	5-1000	Saraji, Boroujeni, & Bigoli (2011)
DLLME	Imipramine, trimipramine	Urine	161.7-186.7	97-112	0.6-1	5.1-6.1	5-100	Shamsipur & Mirmohammadi (2014)
DLLME	MDMA, LSD, PCD	Urine	3.5-5.7	81.7-91.1	1.0-4.5	1.4-121	10-100	Airado-Rodriguez *et al*. (2012)
DLLME	Psychotropic drugs (amitryptiline, clomipramine, thioridazine)	Urine	23.5-24.1	96-101	3.8	4.8	0.02-6.0	Xiong *et al*. (2009)

2.5.2 Other high density solvents DLLME techniques

There are various DLLME techniques that have been recently developed by various researchers. These include; microwave assisted extraction with DLLME, dispersed solvent-assisted emulsion DLLME and assisted vortex surfactant enhanced emulsion DLLME which are discussed below.

2.5.2.1 Microwave assisted extraction with DLLME

A microwave-assisted extraction (MAE)- DLLME procedure coupled with gas chromatography–mass spectrometry (GC–MS) was developed by Ghasemzadeh-Mohammadi, Mohammadi, Hashemi, Khaksar, & Haratian (2012) for the extraction and quantification of 16 polycyclic aromatic hydrocarbons (PAHs) in smoked fish. The MAE–DLLME method coupled with GC–MS provided excellent enrichment factors (in the range of 244–373 for 16 PAHs) and good repeatability (with the RSD of 2.8 and 9%) for spiked smoked fish. The calibration graphs were linear in the range of 1–200 ngg^{-1}, with the square of the correlation coefficient (R^2) > 0.981 and detection limits between 0.11 and 0.43 ngg^{-1}. The extraction recoveries of those compounds in smoked fish were from 82.1% to 105.5%. A comparison of this method with previous methods demonstrated that the proposed method was accurate, rapid, reliable and gives very good enrichment factors and detection limits for extracting and determining PAHs from smoked fish.

Tsai, Chen, & Feng (2013) explored DLLME for extraction and concentration of lipoic acid in human urine. To improve the detection of lipoic acid by both capillary liquid chromatography (CapLC) with UV detection and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), microwave-assisted derivatisation with 4-bromomethyl-6, 7-dimethoxycoumarin was performed to render lipoic acid chromophores for UV detection and also high ionisation efficiency in MALDI.

All parameters that affected lipoic acid extraction and derivatisation from urine were investigated and optimised. In the analyses of human urine samples, the two methods had a linear range of 0.1–20 μM with a correlation coefficient of 0.999. The detection limits of CapLC–UV and MALDI-TOF MS were 0.03 and 0.02 μM (S/N ≥3), respectively.

In 2012, Pizarro, Sáenz-González, Pérez-del-Notario & González-Sáiz proposed a microwave assisted extraction (MAE) in combination with DLLME method. This was a new approach for the sensitive determination of cork taint responsible compounds in cork stoppers and oak barrel sawdust. The optimised method showed satisfactory linearity (correlation coefficients over 0.991), repeatability (below 10.4%) and inter-day precision (below 11.2%). The results obtained proved the suitability of the combination of MAE with DLLME as a sensitive sample preparation methodology for the analysis of haloanisoles and halophenols in solid enological matrices.

An analytical procedure based on microwave-assisted dispersive liquid–liquid microextraction (MA-DLLME) and spectrophotometric coupled with chemometrics methods to determine uranium was proposed by Niazi, Khorshidi, & Ghaemmaghami (2015). Under the optimum conditions, the calibration graphs were linear in the range of 20.0–350.0 ng/mL with a detection limit of 6.7 ng/mL and the enrichment factor of this method for uranium reached at 135. The RSD was 1.64%. The partial least squares modelling was used for multivariate calibration of the spectrophotometric data. This

procedure allowed for the determination of uranium synthesis and real samples such as wastewater with good reliability of the determination.

2.5.2.2 Vortex assisted DLLME

Yang, Wang, Zhao, Zhou, & Liu (2013) developed a novel vortex-assisted surfactant-enhanced-emulsification liquid–liquid microextraction using a low density solvent for the determination of eight organophosphorus pesticides (OPPs) in water samples. The key point of this method was the application of a special home-made extraction device. The influence parameters relevant to this method were systemically investigated and the optimum conditions were as follow: 35 μL of toluene was used as extraction solvent and 0.2 mmol/L Triton X-100 was chosen as the surfactant to enhance the emulsification. The developed method has been successfully applied to the determination of eight OPPs pesticides in river, reservoir and well water samples with recoveries between 82.1 and 98.7%.

Behbahani, Najafi, Bagheri, Bojdi, Salarian & Bagheri (2013) developed a simple, rapid and efficient method, based on surfactant assisted dispersive liquid–liquid microextraction (SA-DLLME), followed by high performance liquid chromatography (HPLC) for simultaneous pre-concentration and trace detection of zonisamide and carbamazepine in biological samples. Under optimum extraction conditions, the limits of detections (LODs)

were 2.1 and 1.5 µg/L for urine samples, and 2.3 and 1.6 µg/L for plasma samples. Linear dynamic ranges of 5–300 and 5–200 µg L^{-1} were obtained for zonisamide and carbamazepine respectively in all samples. The applicability of the proposed method was evaluated by extraction and determination of the drugs in urine and plasma samples.

Yousefi & Shemirani (2013) evaluated a simple ion pair based-surfactant assisted dispersive liquid–liquid microextraction (IP-SA-DLLME) method for extraction and pre-concentration of Cr(VI) and Cr(III) in aqueous samples. Under the optimised conditions and pre-concentration of 10 mL of sample, an enrichment factor of 159 and a detection limit of 0.05 µg/L were obtained. Validation of the method was performed by a spiking-recovery method and comparison of results with those obtained by electrothermal atomic absorption spectrometry (ET-AAS) method.

2.5.2.3 Supercritical fluid extraction DLLME

A supercritical fluid extraction (SFE) requires a lower quantity of organic solvents, has a short extraction time, and allows for the extraction of thermally labile compounds under mild conditions. SFE method combined with DLLME followed by gas chromatography with flame ionisation detection (GC-FID) was developed by Jowkarderis & Raofie (2012). This method was applied for the extraction and determination of 4-nitrotoluene and 3-nitrotoluene in soil samples and satisfactory results were obtained (RSDs < 6.5%).

2.5.2.4 Capillary electrophoresis - DLLME

Meng, Wang, Luo, Shen, Wang & Guo (2011) were the first to develop new method for pre-concentration, chiral separation and determination of multiple illicit drugs on forensic samples using DLLME and capillary electrophoresis (CE) with ultraviolet (UV) detection. Under optimum conditions, linearity of the method was 0.15–6500 mg/L for all target analytes. The LODs (S/N = 3) were 0.05–0.20 mg/L. Excellent repeatability (RSD around 4.4%) was achieved. The feasibility of this method was demonstrated by analysing spiked forensic samples.

2.5.2.5 Low density solvent DLLME

One of the shortcomings of DLLME is the use of chlorinated solvents. Therefore, in recent years, interest in DLLME has been focusing on the use of low-toxicity solvents and more conveniently practical procedures (Leong *et* al., 2014). Low density solvent DLLME involves the use of extraction solvents that are lighter than water. Researchers have recently attempted to use solvents with densities lower than that of water and to perform the extraction without using a disperser solvent and without the need for centrifugation (Kocúrová *et al*., 2012).

For the first time, Guo & Lee (2011) developed a low-density solvent-based solvent demulsification dispersive liquid–liquid microextraction technique for fast, simple, and efficient determination of 16 priority polycyclic aromatic hydrocarbons (PAHs) in environmental samples followed by GC–MS analysis. The method was simple and easy to use, and some additional steps, usually required in conventional DLLME or similar techniques, such as centrifugation, ultrasonication or agitation of the sample solution, or refrigeration of the extraction solvent, were not necessary. Under the optimised conditions, this method provided a good linearity in the range of 0.05–50 μg/L, low limits of detection (3.7–39.1 ng/L), and good repeatability of the extractions (RSDs below 11%, n = 5). The proposed method was successfully applied to the extraction of PAHs in rain water samples, and was demonstrated to be fast, efficient, and convenient.

Zhong, Li, Zhong, Luo & Zhu (2013) developed a simple and effective one-step sample extraction technique termed ultrasound-assisted low-density solvent dispersive liquid–liquid extraction (UA-LDS-DLLE) coupled with ion chromatography (IC) for the determination of three alkanolamines and two alkylamines in complex samples. Linearity ranges of 0.3-50 mg/Land limits of detection varying from 0.072 to 0.12 mg/L were achieved. The recoveries ranged from 86.9–108.5% with the RSDs of 1.2–6.2%. The developed method provided an attractive alternative to the analysis of trace amounts of target analytes in a large number of cosmetics.

Li, Xue, Chen & Li (2013) developed a three-phase microextraction technique by combining low-density solvent-based, DLLME and single-drop microextraction (SDME) for the first time, which was a new format for the determination of chlorophenols in environmental water samples. The extraction procedure included a 2 min DLLME pre-extraction and a 10 min SDME back-extraction. A portion of low-density solvent (toluene) was used as organic phase and injected into the aqueous sample (donor phase) with methanol as disperser. Under optimal conditions, the method showed a low detection limit (0.016-0.084 µg/L) for the five chlorophenols, good linearity (from 0.2-250 µg/L, depending on the analytes) and repeatability (RSD below 8.2%, n = 5). The efficient features of the method which included simplicity and rapidity were demonstrated by the analysis of chlorophenols in environmental water samples.

2.5.2.6 Solidification of floating organic drop

Dispersive liquid–liquid microextraction with solidification of floating organic drop (DLLME-SFO) is one of the most interesting sample preparation techniques developed in recent years (Vera-Avila, Rojo-Portillo, Covarrubias-Herrera, & Peña-Alvarez, 2013). It is a simple, rapid and sensitive DLLME technique involving the use of low-toxicity solvents.

In 2014, Ahmadi-Jouibaria, Fattahi & Shamsipur developed a DLLME-SFO technique combined with high-performance liquid chromatography–ultraviolet detection (HPLC–

UV) for the determination of amphetamine and methamphetamine in urine samples. The results obtained showed that DLLME-SFO combined with HPLC–UV is a fast and simple method for the determination of these drugs in urine.

This technique was also used by Vera-Avila *et al.* (2013) for the determination of pollutants from different chemical families. The compounds studied included: 10 polycyclic aromatic hydrocarbons, 5 pesticides (chlorophenoxy herbicides and DDT), 8 phenols and 6 sulfonamides, thus, covering a large range of polarity and hydrophobicity. After optimisation of extraction conditions, using 1-dodecanol as extractant, the procedure was applied for extraction of each family from 10-mL spiked water samples. The absolute recoveries as well as the recovery values for pollutants were >70% within this group.

In the same year, Suh *et al*. (2013) developed a DLLME-SFO technique for the determination of duloxetine in human plasma samples by high performance liquid chromatography with fluorescence detection (HPLC-FLD). In this technique, the disperser was redundant because the small amount of remaining acetonitrile, which acts as a protein precipitating reagent, was also employed as a disperser; therefore, organic solvent consumption was reduced as much as possible. This method was successfully applied to the pharmacokinetic study of duloxetine in human plasma and was shown to be an alternative green approach compared with the conventional solid-phase microextraction (SPME) and dispersive liquid–liquid microextraction techniques.

A novel ultrasound-assisted dispersive liquid–liquid microextraction based on solidification of the floating organic droplet method (UA-DLLME-SFO) combined with gas chromatography (GC) for the determination of eight pyrethroid pesticides in tea was developed by Houa, Zheng, Zhang, Ma, Ling, & Zhao (2014). The developed method was considered to be simple, rapid and precise to satisfy the requirements of the residual analysis of pyrethroid pesticides.

2.5.2.7 Use of ionic liquids in DLLME

Other attempts to avoid the use of chlorinated solvents, is the recent use of ionic liquids (ILs) in DLLME. A review by Trujillo-Rodríguez, Rocío-Bautista, Pino, & Afonso (2013), summarised the most recent analytical developments employing ILs in DLLME. They distinguished four main operation modes (1) conventional IL-DLLME; (2) temperature-controlled IL-DLLME; (3a) ultrasound-assisted, (3b) microwave-assisted or (3c) vortex-assisted IL-DLLME and (4) *in-situ* IL-DLLME. In these modes, the dispersive solvent can be an organic solvent, a surfactant or a hydrophilic IL. In some cases, a dispersive solvent is not even necessary. They also discussed the practical applications of IL-DLLME to determine metals and organic compounds in a variety of samples.

Lai, Ruan, Liu, & Liu (2014) presented a novel and rapid IL-DLLME method combined with liquid chromatography and a fluorescence detector for the analysis of ochratoxin A

(OTA) in rice wines. Under the optimised experimental conditions, good linearity was obtained with a correlation coefficient of 0.9998 and LOD of 0.04l g/L. The recoveries ranged from 75.9% to 82.1% with an RSD below 10.4%. This method was successfully applied for the analysis of OTA samples from several rice wine brands collected in Guangdong province, China.

Another DLLME technique involving the use of ionic liquids namely vortex-assisted ionic liquid DLLME (VA-IL-DLLME) was developed by Gure, Lara, García-Campaña, & Megersa (2015). This technique was combined with capillary liquid chromatography for the determination of four sulfonyl urea herbicides: flazasulfuron, prosulfuron, primisulfuron-methyl and triflusulfuron-methyl in wine samples. The ionic liquid (IL) 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6MIM]$ $[PF_6]$) was used as an extraction solvent while methanol was the dispersion solvent, assisted by a vortex mixer. Under the optimum conditions, the limits of detection and quantification for this method were in the ranges of 3.2–6.6 g/kg and 10.8–22.0l g/kg, respectively; lower than the maximum residue limits set by the EU for these matrices. This method was successfully applied to different wine samples and satisfactory recoveries were obtained.

An ionic liquid-dispersive liquid–liquid microextraction combined with micro-solid phase extraction (IL-DLLME-SPE), and high-performance liquid chromatography (HPLC) for the determination of tricyclic antidepressants in water samples was developed by Ge &

Lee (2013). The optimised IL-DLLME-SPE method was suitable for the determination of tricyclic antidepressants in water samples.

Xu *et al*. (2011) proposed an ionic liquid-based microwave-assisted dispersive liquid–liquid microextraction (IL-based MADLLME) method followed by derivatisation for the pre-treatment of six sulfonamides (SAs) prior to their determination by HPLC. The proposed method was applied to the analysis of river water, honey, milk, and pig plasma samples, and the recoveries of analytes obtained were in the range of 95.0–110.8, 95.4–106.3, 95.0–108.3, and 95.7–107.7, respectively. The RSDs varied between 1.5 and 7.3% (n = 5). The results showed that the proposed method was a rapid, convenient and feasible method for the determination of SAs in liquid samples.

Similarly, Mesa, Padró, & Reta (2013) used a combination of microwave-assisted solvent extraction and dispersive liquid–liquid microextraction with an ionic liquid generated *in situ* to determine six heterocyclic aromatic amines (3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, 3-amino-1-methyl-5H-pyrido[4,3-b] indole, 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine, 2-amino-9H-pyrido-[2,3-b] indole, 2-amino-3-methyl-9H-pyrido-[2,3-b] indole and 2-amino-1,6-dimethylimidazo [4,5-b]-pyridine) in cooked beef burgers. The optimized microwave extraction procedure showed a repeatability (RSD%) between 5.4 and 10.9%, enrichment factors between 19 and 30, limits of detection between 0.35 and 2.4 ng/mL and recoveries between 69 and 100% were achieved.

For the first time, Rajabi, Ghanbari, Barfi, Asghari & Haji-Esfandiari (2014) developed a novel, efficient and environmentally friendly method, named ionic liquid-based ultrasound assisted surfactant-emulsified microextraction (IL-UA-SE-ME) in which they compared with normal-, reversed-, surfactant assisted-dispersive liquid–liquid microextraction and ultrasound-assisted surfactant-based emulsification microextraction methods for the analysis of three bioactive and flavouring compounds (*para*-anisaldehyde, *trans*-anethole and its isomer estragole) in some fresh plant (fennel and basil) extracts and urine samples. The results showed that IL-UA-SE-ME is a much more effective method and under the optimum conditions the limits of detection, limits of quantification, linear ranges, recoveries, and enrichment factors were in the range of 16-22 ng/mL, 49-67 ng/mL, 0.04-90 μg/mL, 94.3-101.1%, and 118-127%, respectively.

Wu *et al*. (2013) developed a novel, simple and rapid shaking-based method of ionic liquid dispersive liquid phase microextraction for the determination of six synthetic food colourants (Tartrazine, Amaranth, Sunset Yellow, Allura Red, Ponceau 4R, and Erythrosine) in soft drinks, sugar- and gelatin-based confectionery. High-performance liquid chromatography coupled with an ultraviolet detector was used for the determinations. The extraction procedure did not require a dispersive solvent, heat, ultrasonication, or additional chemical reagents. 1-Octyl-3-methylimidazolium tetrafluoroborate ([C_8MIM] [BF_4]) was dispersed in an aqueous sample solution as fine droplets by manual shaking, enabling the easier migration of analytes into the ionic liquid phase. Under the optimum experimental conditions, the proposed method showed

excellent detection sensitivity with the LOD within 0.015–0.32 ng/mL and good spiked recoveries from 95.8–104.5% were obtained.

2.5.2.8 Automation of DLLME

Another limitation to the further development of DLLME is the lack of approaches to its automation. Recently, Maya, Horstkotte, Estela, & Cerdà (2014) reviewed and discussed applications of a novel approach to perform fully automated in-syringe DLLME based on the use of computer-controlled bi-directional syringe pumps. The in-syringe-DLLME technique enables precise flow control over the extractant and the concomitant detection of the analytes "in-syringe", within a peripheral flow network, or by introduction into coupled detectors.

Falkova, Alexovič, Pushina, Bulatov, Moskvin, & Andruch (2014) presented two easily performed automated DLLME procedures; one sequential injection and the other, stepwise injection –based on on-line ultrasound-assisted surfactant-mediated extraction followed by spectrophotometric determination of total anthraquinones in medicinal plants. The suggested procedures were validated according to reference methods and the sampling frequencies were 12 h^{-1} for sequential injection and 6 h^{-1} for stepwise injection, respectively.

As cited in most of these reviews, DLLME has been used in combination with different analytical techniques such as capillary liquid chromatography, liquid chromatography with fluorescence detection/UV/DAD, GC-MS, capillary electrophoresis with UV, or UPLC tandem mass spectrometry for pre-concentration and determination of analytes in different types of samples.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents were analytical grade. Distilled water was used in all sample preparation procedures. Stock solutions of cocaine, Δ^9-tetrahydrocannabinol (1000 µg/mL) in methanol, amphetamine-HCl and methamphetamine-HCl standards in purge and trap (P&T) methanol (1000 µg/mL) were purchased from Restek. Working standards at suitable concentrations were prepared in acetonitrile and stored at 4^{o}C. Chloroform, dichloromethane, carbon tetrachloride, methanol, acetone and acetonitrile were purchased from Merck (Germany).

3.2 Instrumentation

Analyses were performed on a Perkin Elmer Clarus 680 GC coupled to a Clarus turbomass 600T single quadrupole mass spectrometer. A Perkin Elmer Elite-5 MS (30 m × 250 mm × 0.25 µm) was used for chromatographic separation of the target analytes. The mass spectrometer (MS) was operated in positive electron ionisation mode (EI^+) with the

transfer line temperature of 190^{o}C and the ion source maintained at 200^{o}C. The ionisation energy was 70 eV. The solvent delay was from 0-2 min. The carrier gas, ultra-pure helium gas (99.999%) purchased from Afrox (Windhoek, Namibia) was passed through the Perkin Elmer triple air filter before entering the instrument. NIST MS search 2.0 (2011) was used for identification of the compounds. Litmus papers purchased from Merck, as well as a Crison 2000 digital pH meter (Crison instruments S.A, Barcelona, Spain) combined glass-Ag/AgCl (KCl 3M) electrode, calibrated with certified solutions before use were used for pH measurements. GC micro-insert vials were purchased from La-Pha-Pack. A vortex mixer VM-300 and GS-6 centrifuge (Beckman) were also used during sample preparation.

3.3 Sample collection and storage

The blood used in this research was obtained from blood samples that were submitted by the Namibian police to NFSI for blood alcohol analysis during the year 2014. These blood samples were drawn from suspects by qualified physicians or nurses and stored into venoject tubes (10 mL), containing sodium fluoride (100 mg) and potassium oxalate (22.50 mg) as chemical preservatives and anticoagulants, respectively. When the blood samples are submitted to NFSI, they are kept at -20 ^{o}C (for up to 2 months depending at the time of analysis) and then transferred to 4 ^{o}C directly prior to analysis. Blank blood samples used in this research were obtained from these blood samples after performing a

routine general screen for drugs under study (cocaine, Δ^9-tetrahydrocannabinol, amphetamine and methamphetamine). To confirm that these drugs were not present, blank blood samples were used as negative controls during method development.

3.4 Preparation of calibration standards

A series of working standards were prepared by dilution of the stock solution using acetonitrile. The working standards were analysed and the data was used to generate calibration curves prior to optimisation of each DLLME parameter. The following concentrations were used to generate the calibration curves; 1 µg/mL, 2.5 µg/mL, 5 µg/mL, 7.5 µg/mL, 10 µg/mL, 15 µg/mL and 20 µg/mL.

3.5 Mass spectrometer parameters

The selected ion reaction (SIR) mode parameters used for analysis are summarised in Table 3-1. When the MS was operated in full scan mode, the scan range was between 44 and 315, with a scan time of 0.5 sec and an interscan delay of 0.05 sec.

Table 3-1. The MS parameters in SIR mode that were used in the analyses

Compound	Monitored Ions	Time (Min)
Amphetamine	44, 91, 120, 134	3-4
Methamphetamine	58, 91, 134, 149	3-4
Cocaine	82, 182, 272, 303	6-8
Δ^9-tetrahydrocannabinol	231, 271, 299, 314	7-11

3.6 Optimisation of gas chromatography method

Prior to DLLME method optimisation, the gas chromatograph (GC) method was optimised. The optimised GC parameters included an oven temperature programme, split ratio and carrier gas flow rate. Different oven temperature programmes in the ranges of 40-280 ^{o}C, at different ramping speeds were investigated for the effectiveness in separating the analytes. The enhancement of the split ratio in separating the analytes was studied by varying the split ratios between 5 and 50. Finally, the flow of helium was also optimised by investigating flow rates between 1.0 and 2.0 mL/min. The injection port was held at 250 ^{o}C throughout method optimisation. The GC method was optimised using the working standards of the drugs under study. To evaluate the sensitivity of the GC method, blank blood samples and spiked blood samples were analysed with the optimised method.

3.7 Screening for blank samples

Since there were no blank blood samples available, blood samples submitted to NFSI for ethyl alcohol analysis were randomly selected and screened for cocaine, Δ^9-tetrahydrocannabinol, amphetamine and methamphetamine using the NFSI standard laboratory procedure for drugs of abuse. This involved protein precipitation followed by LLE as well as sample drying and reconstitution of analytes in GC compatible solvents for analysis. The blood samples in which none of these drugs were detected, were used as sample blanks for the development of the DLLME method.

3.8 Sample pre-treatment

To obtain a concentration of 1.20 µg/mL of each drug under study, 25 µL of blank blood was spiked with 1 µL of standard solution in acetonitrile containing 30.0 µg/mL of each analyte, i.e. cocaine, amphetamine, methamphetamine, and Δ^9-tetrahydrocannabinol. The dilution effect was not taken into consideration since the spiked volume was very little. This was followed by the addition of 25 µL of acetone to the spiked blood sample to precipitate out proteins. The mixture was vortexed for about 5 minutes followed by centrifugation at 4000 rpm for 5 min. The resultant 25 µL supernatant was transferred to a 300 µL GC micro-insert vial and diluted 10 times with 0.5 M NaOH at a desired pH. For multiple analyses, 1 mL of blank blood was spiked at the same concentration and

respective procedures were followed in order to have the same final concentration prior to DLLME.

3.9 DLLME procedure overview

After sample pre-treatment, the resultant 250 µL sample solution was transferred to a 300 µL GC micro insert crimp vial. A specified volume of a solvent mixture containing a dispersion and extraction solvent was rapidly injected into the sample solution. The vial was capped and then gently shaken for a specified time (The solvent mixture volumes and shaking times are reported in the relevant results sections). This led to the formation of a cloudy solution. The sample was centrifuged and 1 µL of the sedimented volume was collected using a 10 µL GC syringe and directly injected into the GC-MS for analysis. Throughout method development and validation, all samples were prepared and analysed in triplicate. The type and volume of dispersion and extraction solvents, sample pH, sample ionic strength, extraction time and centrifugation time were optimised. The effect of the sample matrix on the extraction efficiency of the analytes was also investigated.

3.9.1 Selection of extraction and dispersion solvents

Acetonitrile, methanol and acetone were used as dispersion solvents while dichloromethane, chloroform and carbon tetrachloride were used as extraction solvents. Each solvent mixture was prepared by mixing a volume of 870 µL dispersion solvent and 130 µL extraction solvent (based on literature). In order to yield a constant volume for each experiment, the use of various volumes of each solvent mixture were evaluated in the ranges of 10-40 µL. The dispersion and extraction solvent combination that gave optimum results was selected to be used in subsequent method development steps.

3.9.2 Optimisation of sample pH

Different solutions of 0.5 M NaOH were prepared at various pH levels and used for sample dilutions. Solutions of HCl (0.1 N) and NaOH (1 M) were also prepared and used for final pH adjustments of samples. Litmus papers were used for preliminary pH measurements and the final pHs of the solutions were confirmed using a pH meter. The effect of pH on DLLME procedure was evaluated at sample pH levels of 9, 10, 11 and 12. The pH level at which optimum results were obtained was selected for use in further optimisation steps.

3.9.3 Optimisation of dispersion solvent volume

To investigate the effect of the dispersion volume on the extraction efficiency of the analytes, different dispersion: extraction solvent mixtures with varying ACN contents were investigated. The following dispersion:extraction solvent combinations were prepared: (i) 130 µL:130 µL, (ii) 390 µL:130 µL, (iii) 570 µL:130 µL, (iv) 770 µL:130 µL, (v) 870 µL:130 µL, (vi) 930 µL:130 µL. A specified volume from each combination was injected in the sample(s) while ensuring that the sedimented phase remained below 10 µL for all preparations. The volume of chloroform was constant at 130 µL, during the preparation of solvent combinations. The dispersion volume that gave optimum recoveries was selected and used in further DLLME optimisation.

3.9.4 Optimisation of extraction solvent volume

The extraction efficiency of different solvent mixtures with varying chloroform content was investigated for the drugs under study. The following extraction:dispersion solvent combinations were prepared; (i) 20 µL:130 µL, (ii) 40 µL:130 µL, (iii) 70 µL:130 µL, (iv) 100 µL:130 µL, (v) 130 µL:130 µL. The optimised dispersion volume was kept constant. A specified volume of each combination was injected in the sample(s) while ensuring that the sedimented phase remained almost the same for all preparations. The extraction

volume that gave the best recoveries of the analytes was kept constant in conjunction with the dispersion volume for further method development.

3.9.5 Optimisation of ionic strength

Different mass concentrations of NaCl from 0-0.1 g/mL were prepared directly by adding specified amounts of the salt to the NaOH solution at the optimum pH. The following mass concentrations of NaCl (g/mL) were added to the solutions: 0.00, 0.01, 0.05, 0.1 and 0.05. A specified amount was used to dilute the sample and adjust the pH before DLLME. The ionic strength condition that gave optimum results for the extraction efficiency of the analytes was selected.

3.9.6 Optimisation of extraction time

To optimise the extraction time, DLLME was performed at different times. Timing began after the injection of the dispersion and extraction solvents into the samples and capping of vials. The samples were gently shaken for a specified time and then centrifuged prior to analysis. The samples were prepared in triplicates and extracted at 0.5, 1, 2.5, 5 and 10 min. The extraction time that gave optimum results for the extraction of the analytes was selected.

3.9.7 Optimisation of centrifugation time

To optimise the centrifugation time, samples were centrifuged at different times after extraction. The following times were investigated: 2.5, 5, 10 and 15 min. For each time interval, samples were prepared and analysed in triplicates. The centrifugation time at which the best extraction recoveries of the analytes were obtained was selected as the optimum condition for this method.

3.9.8 Effect of sample matrix

Two separate blood samples (25 µL each) were spiked at 1.20 µg/mL with the analytes under study, followed by protein precipitation. To sample one (diluted sample), a tenfold dilution was performed with 0.5 M NaOH at pH 12 prior to DLLME procedure, while sample two (undiluted sample) was not diluted. A 1 M NaOH solution was used to adjust the sample pH to 12. A standard solution (in ACN) was also prepared at a concentration of 1.20 µg/mL equivalent to the concentration level of the two samples. Samples were prepared in triplicate. The optimised DLLME procedure was performed on the samples and the samples were analysed directly with GC-MS.

3.9.9 Method validation

Once all the necessary parameters that affect the extraction efficiency of the method were optimised, it was necessary to validate the method first in terms of its linearity. This was performed by preparing a series of spiked samples with final concentrations in the range of 0.000001-1 µg/mL. These concentrations correspond to the concentration range 0.00001-10 µg/mL in undiluted blood. The linear calibration curve for each drug was used to determine the limit of quantification (LOQ) as well as the limit of detection (LOD).

- The enrichment factor (*EF*) was defined as the analyte concentration in the sedimented phase (C_{sed}) in relation to the initial concentration of the analyte (C_0) in the original sample.

$$EF = C_{sed}/C_o \qquad (1)$$

- The extraction recovery (ER %) was defined as the percentage of the total analyte which was extracted in the sedimented phase.

$$ER\ \% = EF \times (V_{sed}/V_{aq}) \times 100 \qquad (2)$$

Where V_{sed} is the total sedimented volume and V_{aq} is the total sample volume

- Matrix effect (*ME%*) was defined as a percentage of the peak area of sample spiked prior to extraction (*A*) over the peak of the standard solution (*B*)

$$ME\ (\%) = (A/B) \times 100 \qquad (3)$$

- Relative standard deviation (%*RSD*) was defined as the percentage of the standard deviation divided by the mean.

$$\%RSD = (Standard\ deviation/Mean) \times 100 \qquad (4)$$

3.9.10 Application of the method

Thirty blood samples were randomly selected from the NFSI laboratory. These samples were primarily submitted to NFSI in 2014 for blood alcohol analysis. These samples were collected from motorists suspected of driving under the influence of alcohol in Khomas region, Namibia. The samples were initially analysed for blood alcohol before subjection to the screening of the drugs of abuse under study using the current optimised and validated method.

CHAPTER 4

4 RESULTS

4.1 Optimisation of the GC-MS method

A dilute solution containing a mixture of each drug under study was first analysed and the identity of the compounds was confirmed using the library spectra. To obtain a shorter analysis time, the GC method was optimised by varying oven temperature programmes, split ratios as well as carrier gas flow rate. For the optimised GC method, the GC was operated at a split ratio of 10:1, the initial oven temperature was 90^{o}C which was ramped at 30 ^{o}C min^{-1} to 300 ^{o}C and held for 4 min. The carrier gas was held constant at 1.33 mL/min throughout the analysis. The total run time of the optimised GC method was 12 min.

The sensitivity of the developed method was evaluated and the results are shown in Figures 4-1 and 4-2. The total ion chromatogram acquired in full scan mode showed that all analytes were resolved from each other and acceptably resolved from any excipient peaks in the blood. The optimised GC method showed a good separation of the analytes, therefore the method was sensitive. The retention time of each analytes under the optimised GC method are shown in Table 4-1.

Table 4-1 The retention times of the analytes under optimised GC method

Analyte	Retention time
Amphetamine	3.24 ± 0.05
Methamphetamine	3.52 ± 0.05
Cocaine	7.52 ± 0.05
Δ^9-THC	8.29 ± 0.05

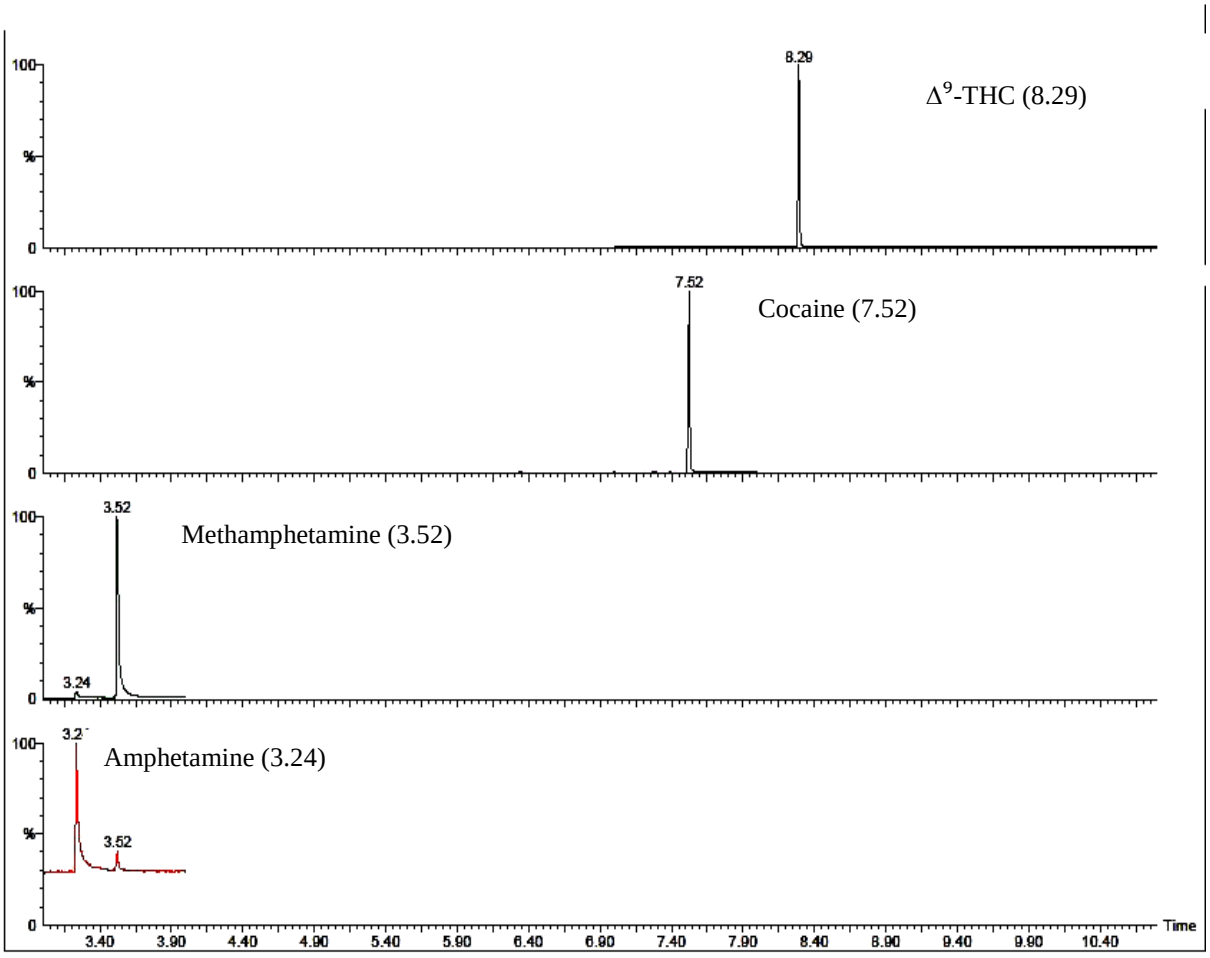


Figure 4-1: A SIR scan of total ion chromatograms of a spiked blood sample (1.20 µg/mL) analysed

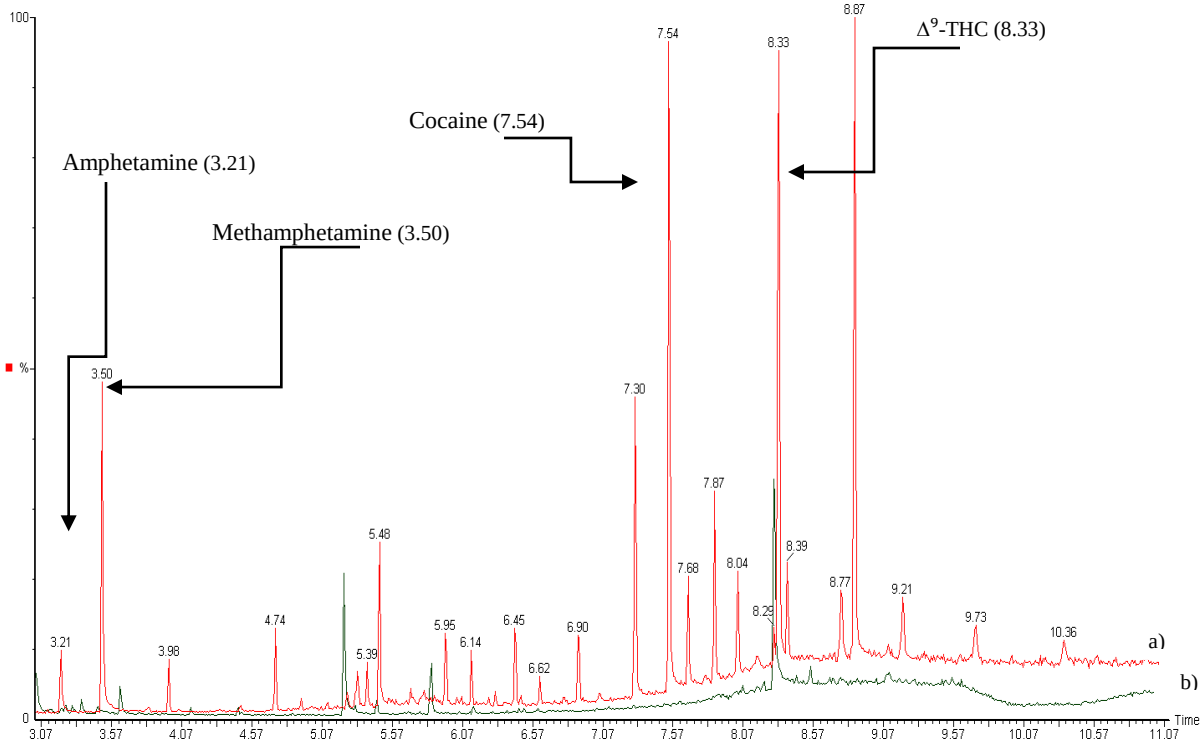


Figure 4-2: An overlay of the full scan total ion chromatograms of the spiked blood sample (a) (1.20 µg/mL) versus blank blood sample (b) obtained using the optimised GC-MS parameters.

4.2 Screening for blank samples

After extraction of the samples using the standard laboratory method, samples were analysed using the optimised GC-MS method. Figure 4-2 above shows a chromatogram obtained for one of the blank blood samples. From the chromatogram, it can be observed that no peaks eluted at the expected retention times of the analytes of interest.

4.3 Selection of dispersion and extraction solvents

The influence of the dispersion and extraction solvents on the extraction efficiency of the analytes under study was evaluated. Different combinations of dispersion:extraction solvents studied are shown in Table 4-2. Various volumes (10-35 µL) of solvent mixture were injected into the sample solution to obtain a sedimented phase of approximately 4 µL for each solvent combination. A two-phase system was not observed and no sedimentation formed with any of the dispersive solvents when dichloromethane was used as an extraction solvent (solvent combinations 7 – 9). Therefore, only two extraction solvents (chloroform and carbon tetrachloride) were studied in conjunction with the dispersion solvents and the results obtained are shown in Figure 4-4.

Fairly good results were obtained with solvent combination 1, as compared to other solvent combinations. Variations in the extraction recoveries were observed for different solvent combinations especially for combinations 2, 3 and 6. While poor recoveries of the analytes were obtained with combinations 4 and 5. On average, solvent combination 1 gave optimum recoveries for all analytes under study, therefore, acetonitrile and chloroform were selected as the best dispersion and extraction solvents for extracting these analytes.

Table 4-2: Different solvent combinations used during the selection of dispersion and extraction solvents.

Solvent combination	Dispersion solvent	Extraction solvent
1	Acetonitrile	Chloroform
2	Acetonitrile	Carbon tetrachloride
3	Methanol	Chloroform
4	Methanol	Carbon tetrachloride
5	Acetone	Chloroform
6	Acetone	Carbon tetrachloride
7	Acetonitrile	Dichloromethane
8	Methanol	Dichloromethane
9	Acetone	Dichloromethane

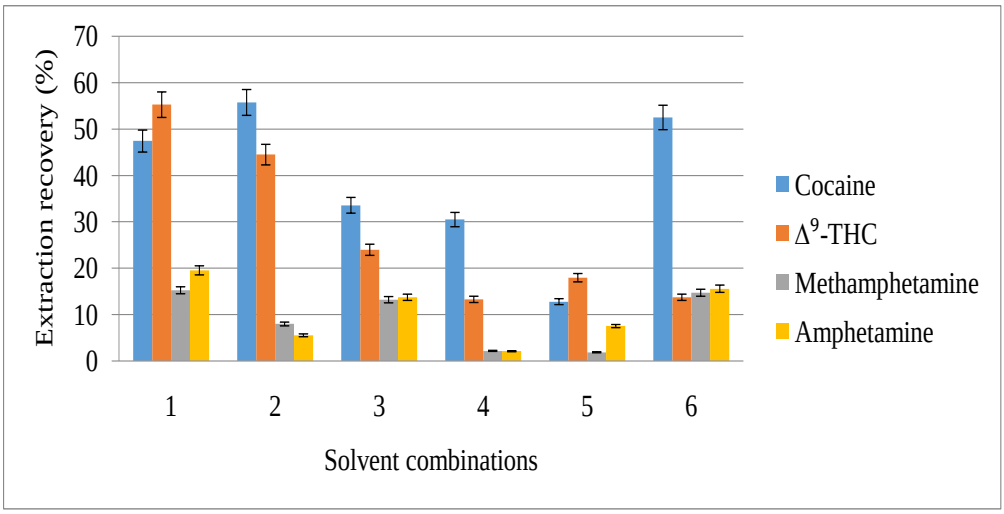


Figure 4-3: The effect of dispersion and extraction solvent combinations on the extraction efficiency of the analytes under study.

4.4 Optimisation of pH

During the selection of dispersion and extraction solvents, the pH of the sample solutions was maintained at pH 9 (most of the drugs are basic). After this, the effect of pH on the extraction efficiency of the analytes from the aqueous phase was investigated and the results are presented in Figure 4-5. A significant increase in the extraction recoveries of the analytes was observed with an increase in the sample pH. At pH 9, the recoveries of the two amphetamines was extremely low as compared to cocaine and Δ^9-THC, however, this significantly improved at higher pH levels, reaching peak recoveries at pH 12. Based on these observations, pH 12 was chosen as the optimum pH for extraction of all the drugs as the recoveries were above 50 % for all the analytes.

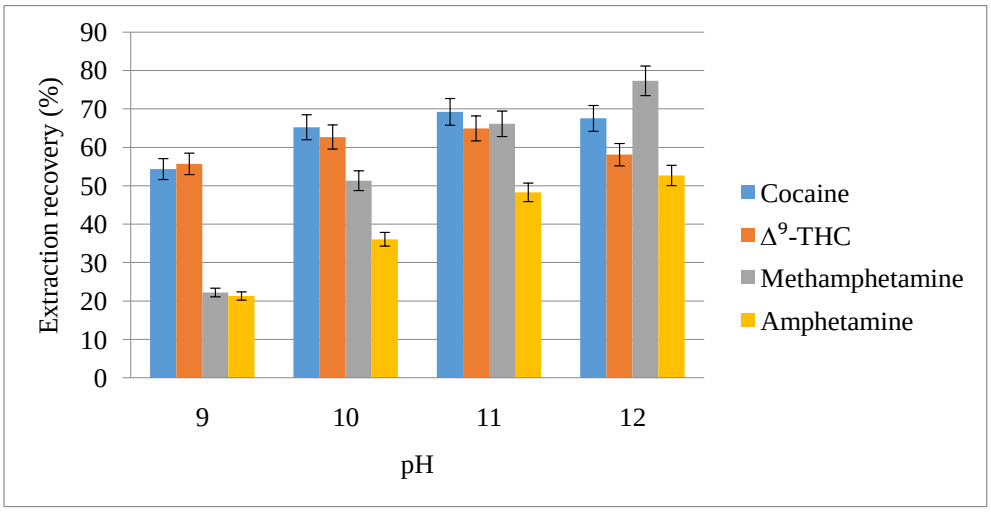


Figure 4-4: The effect of sample pH on the extraction efficiency of the analytes under study.

4.5 Optimisation of dispersion solvent volume

An increase in the sedimented volume was observed with a decrease in the dispersion solvent volume and vice versa. Sedimented volumes varied greatly, ranging between 2 and 20 µL for different solvent combinations when a constant volume of 35 µL was injected from all solvent combinations. It was for this reason that, different injection volumes were tried in order to get the sedimented volume between 3 and 10 µL. Acetonitrile volumes above 930 µL could not be used as no cloudy solution formed upon injection and thus no sedimentation was observed after centrifugation. Similarly, the acetonitrile volume below 130 µL could not be used due to higher sedimented volumes obtained. The results

obtained after optimisation of the injection volumes are depicted in Figure 4-6. Fairly good recoveries were obtained from a combination of 130 µL acetonitrile in conjunction with 130 µL chloroform. Variations in the extraction recoveries of the analytes were observed for other volumes of acetonitrile with fixed chloroform volume. Based on these results, the 130 µL of ACN was chosen as the optimum volume since it gave the best recoveries for all the drugs. This volume was thus kept constant throughout the remaining method development procedures. The sedimented volume for this combination was approximately 6 µL.

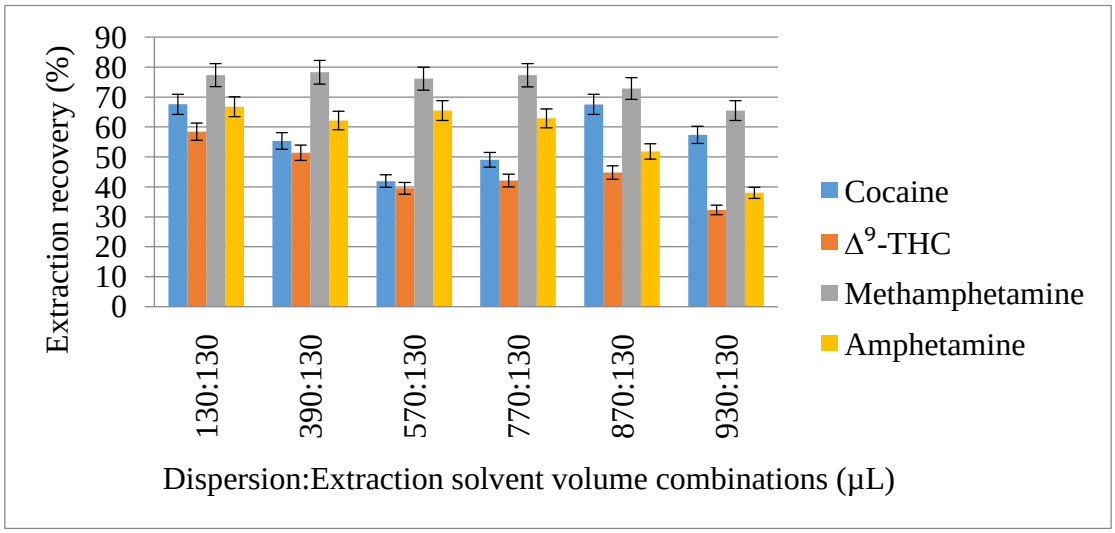


Figure 4-5: The effect of different volumes of acetonitrile (dispersion solvent) on the extraction efficiency of the drugs under study.

4.6 Optimisation of extraction solvent volume

As with the optimisation of the dispersion volume, the extraction volume also needed to be optimised as it played the most significant role in the extraction of the analytes from the samples. From the results, it was observed that the sedimented phase decreased with the decrease in extraction volume, while the increase in extraction volume showed opposite results. Similarly, to the optimisation of dispersion volume, sedimented volumes varied greatly between different solvent combinations and therefore the injection volume was optimised first in order to obtain sedimented volumes between 3 µL and 10 µL. When the extraction volume below 20 µL was used, no cloudy solution formed upon injection and thus no sedimentation formed after centrifugation, therefore 20 µL was the minimum injection volume optimised. Similarly, the extraction volume above 130 µL could not be optimised due to the increase in sedimented phase.

The results in figure 4.7 show the extraction recoveries obtained after optimisation of the extraction volumes. From the results, it was observed that, the combination 130:100 µL gave the highest recoveries for all drugs as compared to other combinations. Based on this, combination 130:100 µL was selected as the optimum combination for the extraction of the analytes under study. The sedimented volume for this combination was approximately 6 µL.

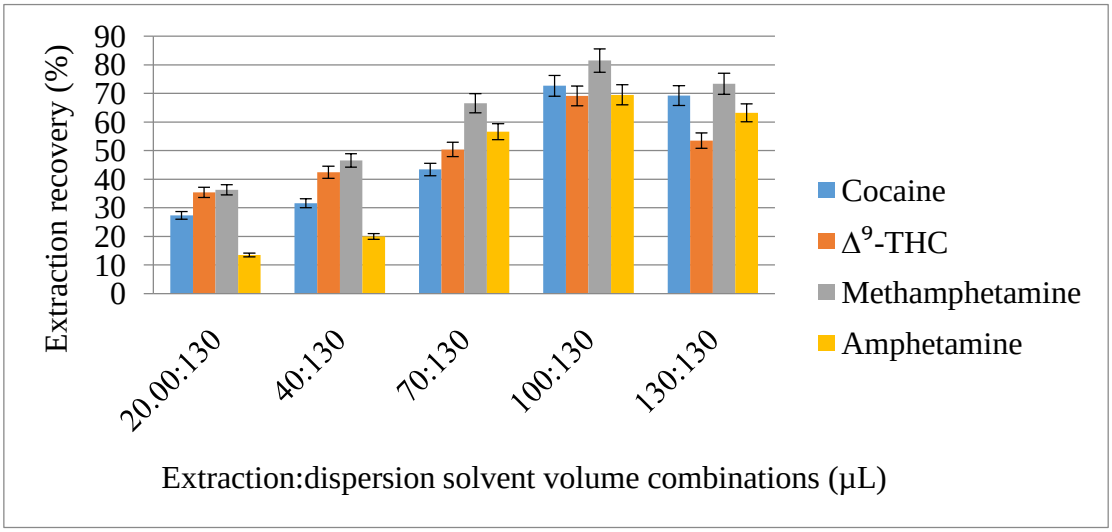


Figure 4-6: The effect of different volumes of chloroform (extraction solvent) on the extraction efficiency of the drugs under study

4.7 Optimisation of ionic strength

To study the effect of the ionic strength of the sample on the extraction efficiency of the analytes, different amounts of NaCl were added to the samples. From the results obtained, it was observed that addition of NaCl did not have much effect on the sedimented volume which remained constant at about 6 µL. However, from the results as shown in Figure 4-8, the extraction recoveries significantly dropped with the addition of salt compared to the recoveries obtained when no salt was added to the samples. Based on these observations, no salt was added further during method development as it did not enhance the extraction efficiency of the analytes.

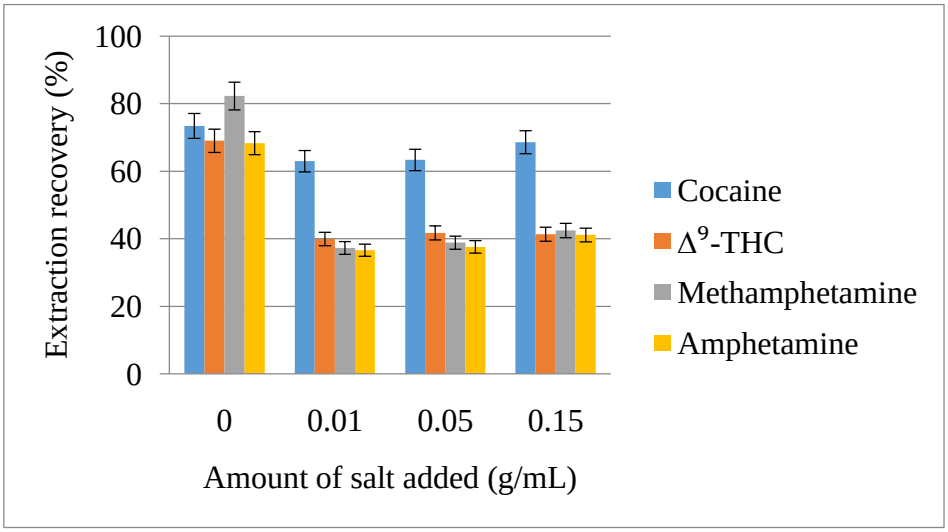


Figure 4-7: The effect of salt (NaCl) addition on the extraction efficiency of the drugs under study.

4.8 Optimisation of extraction time

The extraction time is one of the most crucial elements in the extraction procedure; therefore, it was of great importance to optimise it. During the optimisation, it was observed that the sedimented volume remained constant at about 6 µL throughout all analyses. The extraction recoveries of the analytes are shown in Figure 4-9. High recoveries above 60 % of all the analytes were achieved within 30 min, peaking at about 2.5 min. Based on these results, an extraction time of 2.5 min was considered as the optimum DLLME time for the analytes under study.

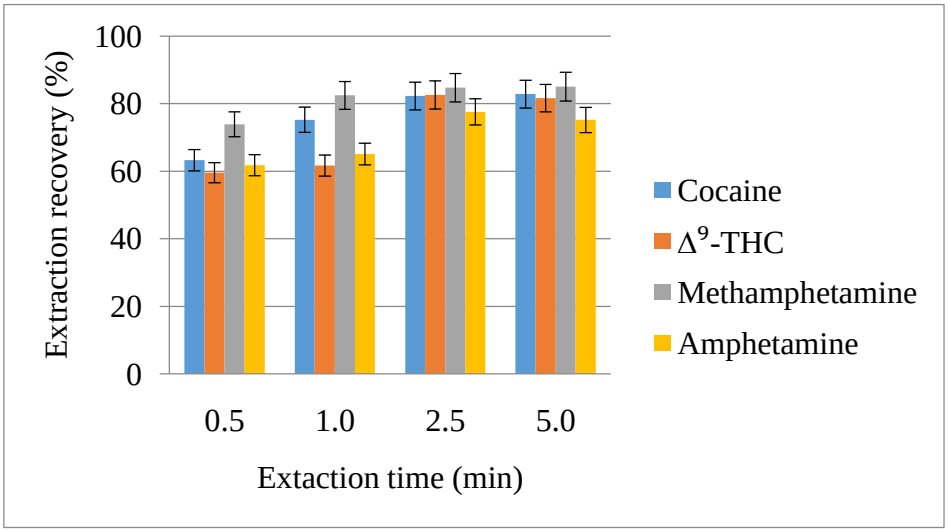


Figure 4-8: The effect of extraction time on the extraction efficiency of the drugs under study.

4.9 Optimization of centrifugation time

The last DLLME parameter optimised before the validation of the method was the centrifugation time. This parameter had no effect on the sedimented volume as it remained constant at about 6 µL throughout all analyses. The extraction recoveries of the analytes obtained during the optimisation process are shown in Figure 4-10. From the results, it was observed that the optimum extraction recovery was reached immediately at 2.5 min and almost remained constant thereafter. Based on these results, the centrifugation time of 2.5 min was used as the optimum time for this developed DLLME method.

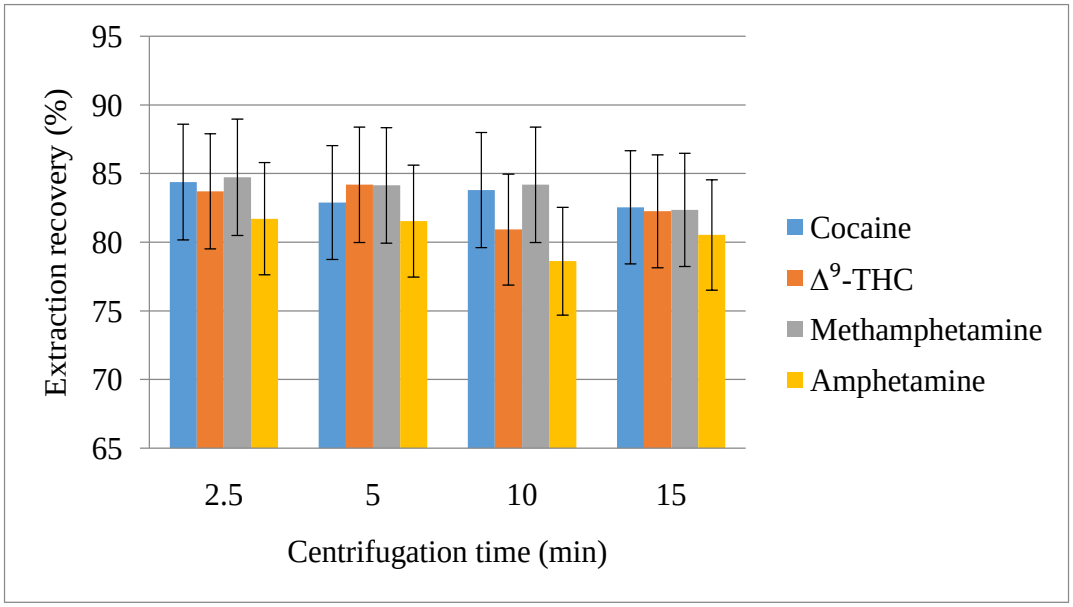


Figure 4-9: The effect of centrifugation time on the extraction efficiency of the drugs under study.

4.10 Matrix effect

During method development, samples (after protein removal) were diluted ten folds prior to DLLME to minimise blank blood usage. After the development and optimisation of the DLLME method, it was of great importance to study the matrix effect under optimised conditions. This was achieved by a comparison of sedimented phases, extraction recoveries and signal-to-noise ratios of the two samples (diluted and undiluted samples). A reduction in the sedimented volume was observed with the undiluted sample as compared to the diluted sample under the same optimised DLLME conditions. A significant reduction in the extraction recoveries of the analytes under study was observed with

undiluted blood, especially for cocaine and the two amphetamines as depicted in Figure 4-11. The signal-to-noise (S/N) ratios as depicted in Figure 4-12 and 4-13 were higher for the sample diluted prior to DLLME as compared to the undiluted samples. The recoveries of the two amphetamines were drastically affected when samples were not diluted compared to the diluted sample (Table 4- 3).

Based on these results, it was therefore necessary to dilute the samples prior to DLLME as it reduced the matrix effect, thus significantly increasing the extraction recoveries and improving the signal-to-noise ratios.

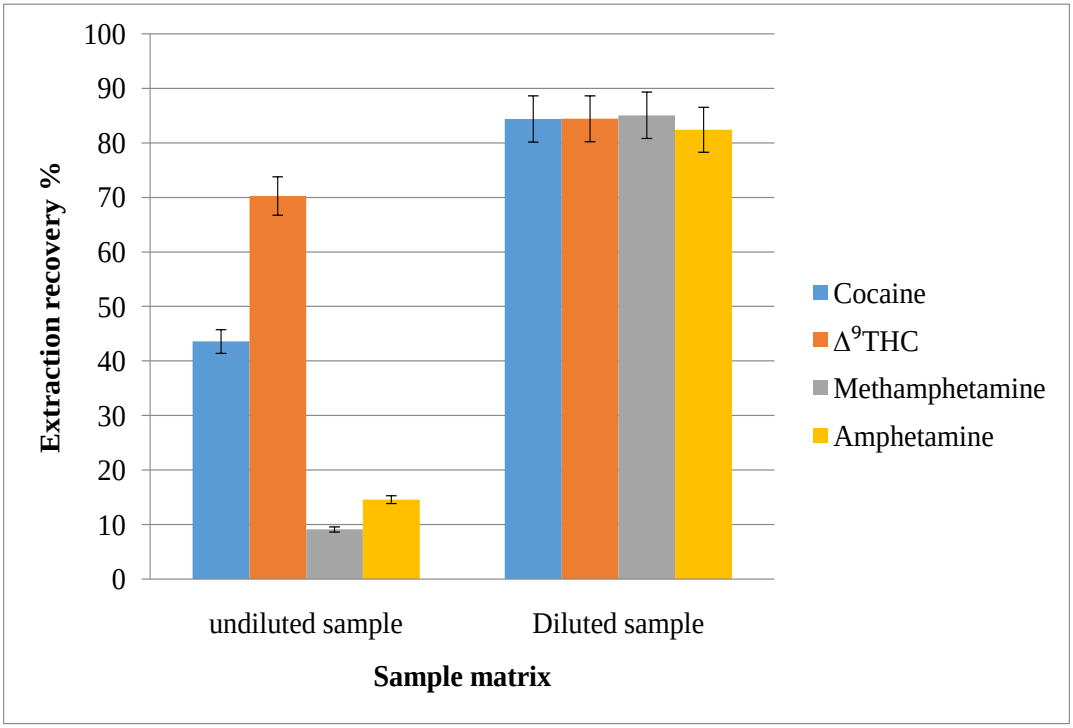


Figure 4-10: A comparison of the extraction recoveries of the analytes obtained from the undiluted and diluted samples prior to DLLME.

Table 4-3: A summary of the matrix effects for undiluted blood versus diluted blood.

	Cocaine	**Δ^9-THC**	**Methamphetamine**	**Amphetamine**
Undiluted sample (peak area)	5576	2705	1440	870
Diluted sample (peak area)	5402	2651	7906	3378
Standard (peak area)	5840	2784	8539	4378
Matrix effect undiluted (%)	95	97	17	20
Matrix effect diluted (%)	92	95	93	77

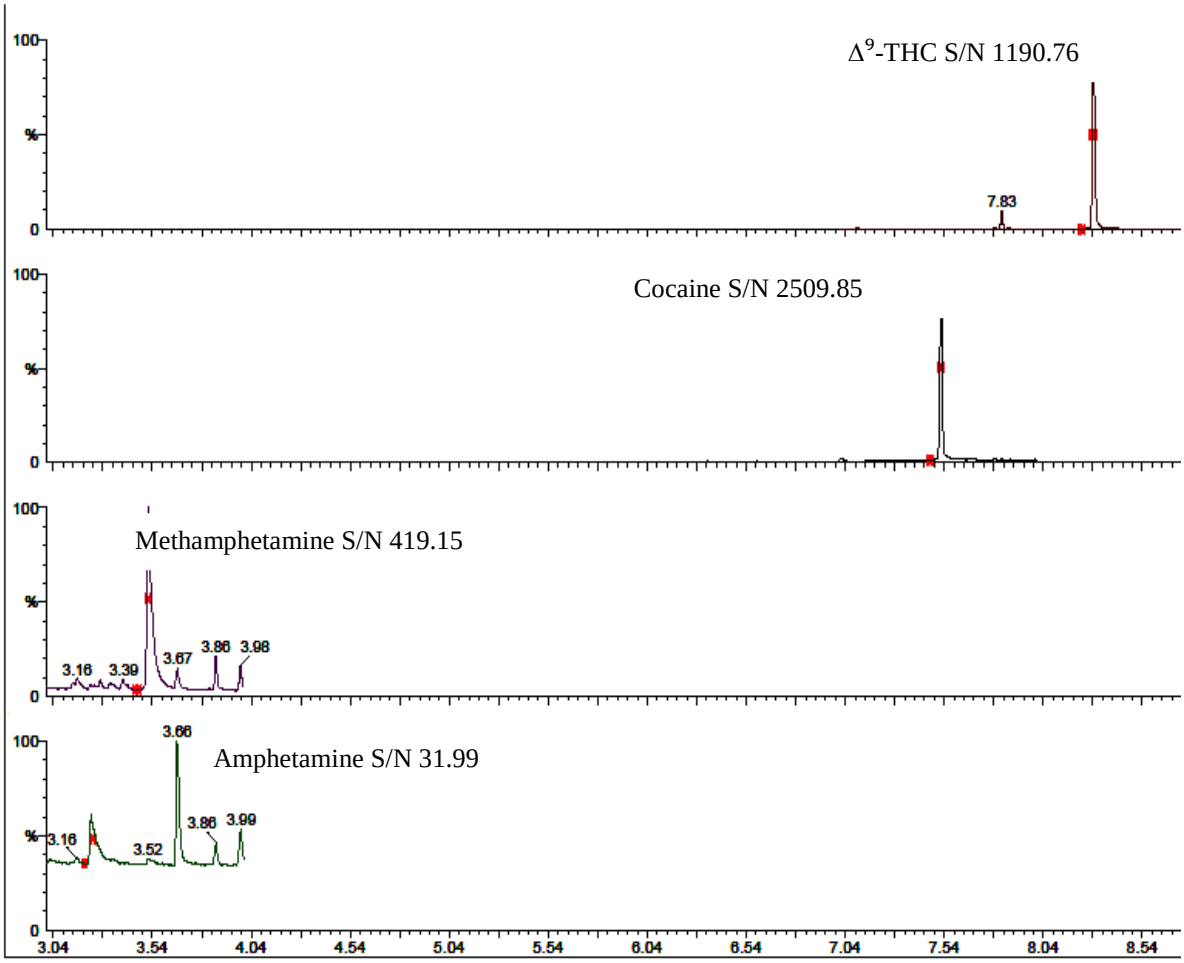


Figure 4-11: Signal-to-noise ratio obtained from the analysis in SIR mode of undiluted blood sample under optimised DLLME conditions, blood spiked at 1.20 µg/mL.

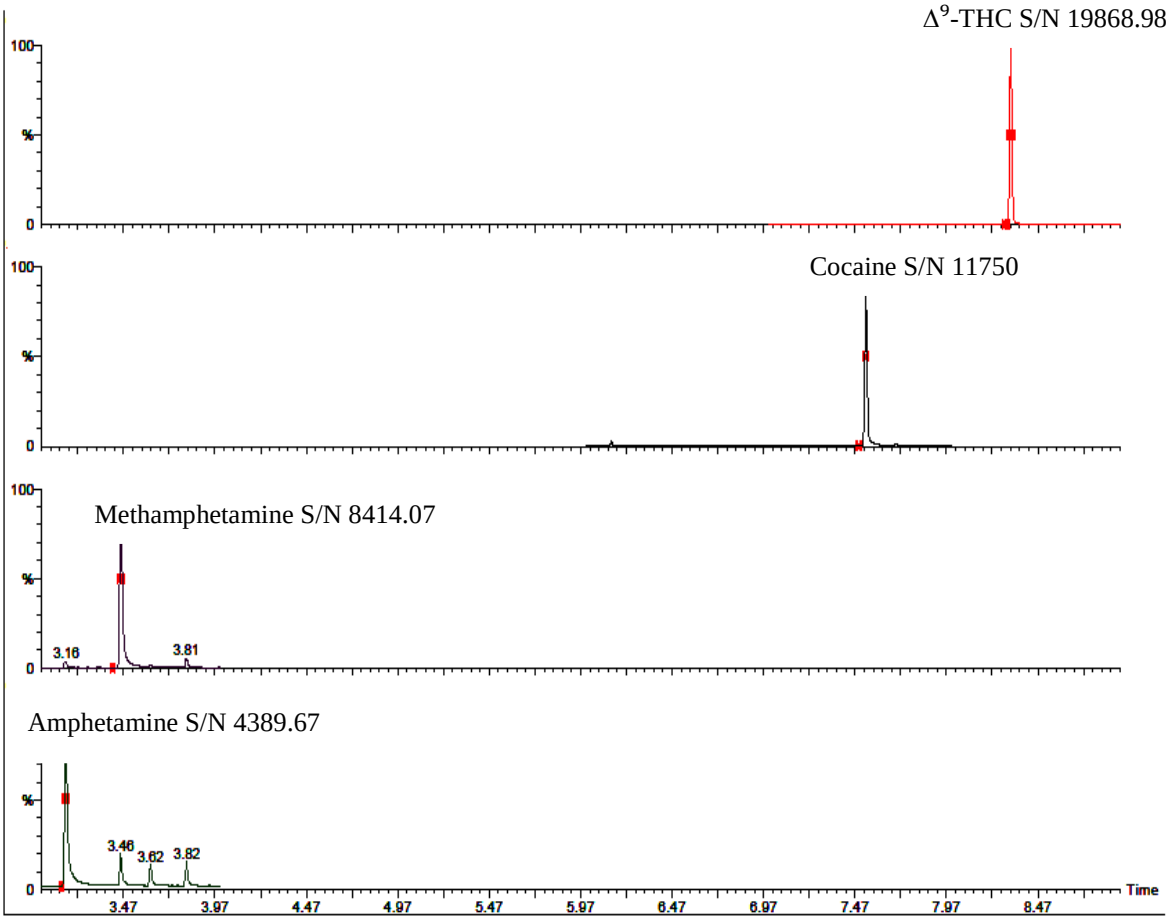


Figure 4-12: The S/N ratios of drugs under study obtained from the analysis of diluted blood under optimised DLLME conditions. Blood spiked at 1.20 µg/mL before protein removal and then diluted to 0.12 µg/mL prior to DLLME.

4.11 Extraction trend of the analytes

A general overview of the extraction recovery trends obtained for each analyte under optimised DLLME parameter during method development are summarised below.

4.11.1 Effects of optimised parameters on the extraction of cocaine

The extraction trend of cocaine under various optimised parameters is shown in figure 4-14. From the graph, it can be observed that the extraction recovery of cocaine significantly improved after pH optimisation. Optimisation of the dispersion solvent volume did not improve the recovery of cocaine. However, a slight improvement was observed after the optimisation of the extraction solvent volume. Since salt addition affected the recoveries of all analytes, no salt was added in further optimisation. Moreover, a significant increase in the recovery was obtained when the extraction and centrifugation times were optimised.

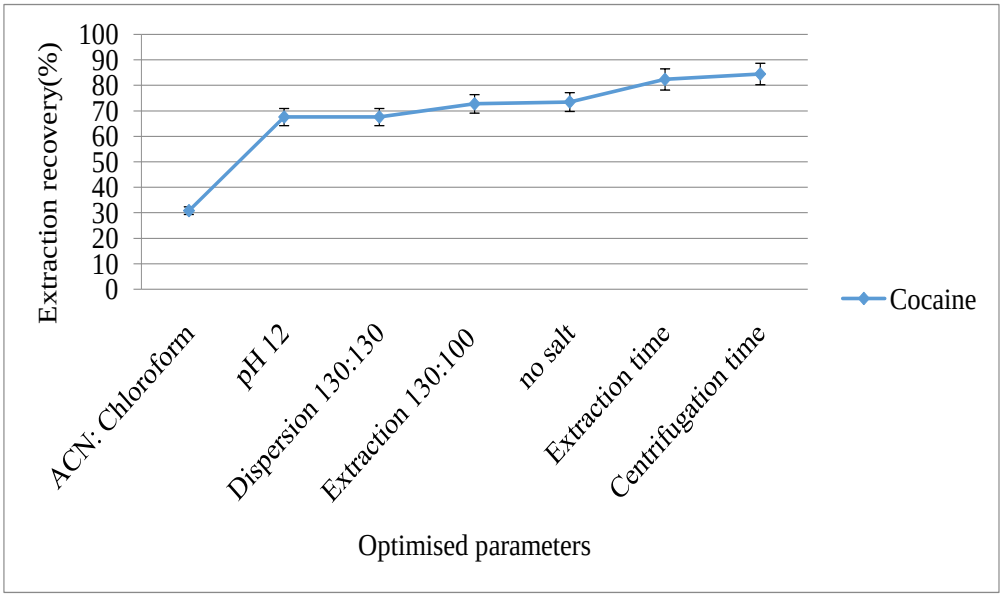


Figure 4-13: The effects of optimised parameters on the extraction efficiency of cocaine.

4.11.2 Effects of optimised parameters on the extraction of Δ^9-THC

The extraction trend of Δ^9-THC under various optimised parameters is shown in Figure 4-15. From the graph, it can be noted that the extraction recovery of Δ^9-THC significantly improved after pH optimisation. No change was observed in the extraction recovery after the volume of the dispersion solvent was optimised. However, a significant improvement was observed after the optimisation of the extraction solvent volume. Finally, a significant increase in the recovery was obtained when the extraction and centrifugation times were optimised.

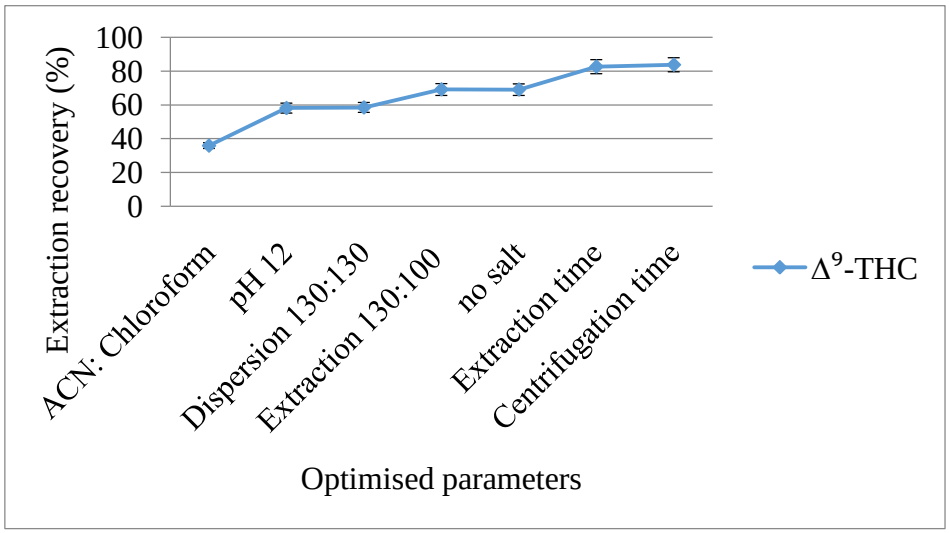


Figure 4-14: The effects of optimised parameters on the extraction efficiency of Δ^9-THC.

4.11.3 Effects of optimised parameters on the extraction of methamphetamine

The extraction trend of methamphetamine under various optimised parameters is shown in Figure 4-16. From the trend, it can be observed that the extraction recovery of methamphetamine significantly improved after pH optimisation. No change was observed in the extraction recovery after the volume of the dispersion solvent was optimised. However, a slight improvement was observed after the optimisation of the extraction solvent volume. Moreover, a significant increase in the recovery was obtained when the extraction and centrifugation times were optimised.

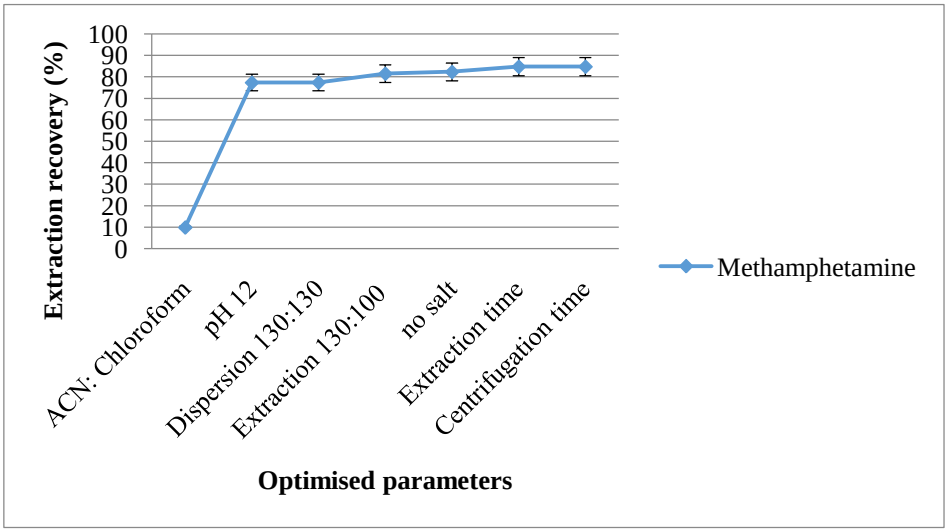


Figure 4-15: The effects of optimised parameters on the extraction efficiency of methamphetamine.

4.11.4 Effects of optimised parameters on the extraction of amphetamine

The extraction trend of amphetamine under various optimised parameters is shown in Figure 4-17. The extraction recovery of amphetamine significantly improved after pH optimisation. Unlike other analytes, optimisation of the dispersion volume significantly improved the extraction recovery of amphetamine, with a slight improvement observed after the optimisation of the extraction solvent volume. Finally, a significant increase in the recovery was obtained when the extraction and centrifugation times were optimised.

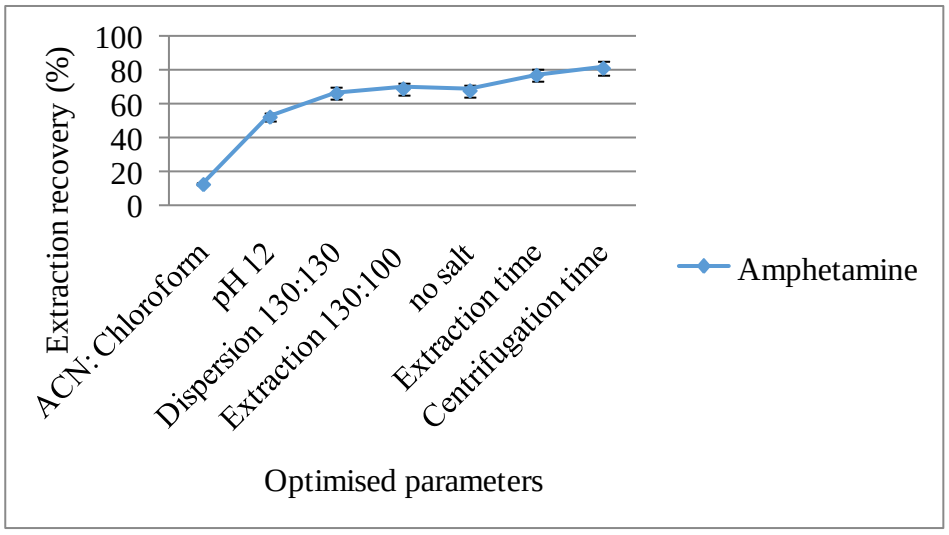


Figure 4-16: The effects of optimised parameters on the extraction efficiency of amphetamine.

4.12 Method validation

The following parameters were validated under optimised DLLME conditions; linearity of the method, LOD, LOQ, sensitivity, % RSD, enrichment factor and extraction recovery and the results are summarised in Table 4-4 and Table 4-5.

4.12.1 Linear calibration range

The linearity of the developed method was studied for each of the analytes using different concentrations of spiked diluted blood. The results are reported to the corresponding concentrations in undiluted blood in the ranges of LOQ to 10 µg/mL. All analyses were performed in triplicate for each concentration.

4.12.1.1 Calibration curve for cocaine

Figure 4-18 shows the calibration curve obtained for cocaine. The method was linear in the range of 0.01-10 µg/mL with $r^2 = 0.9982$. The LOD based on the concentration value giving three times signal-to-noise ratio for three analyses was observed at 0.001 µg/mL, while the LOQ based on the concentration value giving ten times signal to noise ratio for three analyses was observed at 0.01 µg/mL.

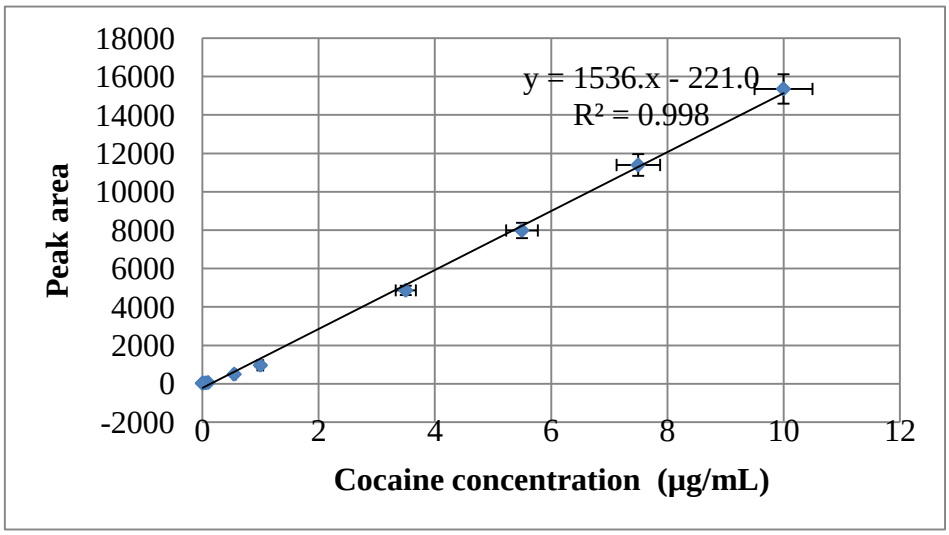


Figure 4-17: The calibration curve obtained for cocaine after the analysis of spiked blood samples in the concentration range of 0.01-10 µg/mL.

4.12.1.2 Calibration curve for Δ^9-THC

Figure 4-19 shows the calibration curve for Δ^9-THC. The method was linear in the ranges of 0.0001-10 µg/mL with r^2 = 0.9985. The LOD based on the concentration value giving three times signal to noise ratio for three analyses was observed at 0.00001 µg/mL, while the LOQ based on the concentration value giving ten times signal to noise ratio was estimated at 0.0001 µg/mL.

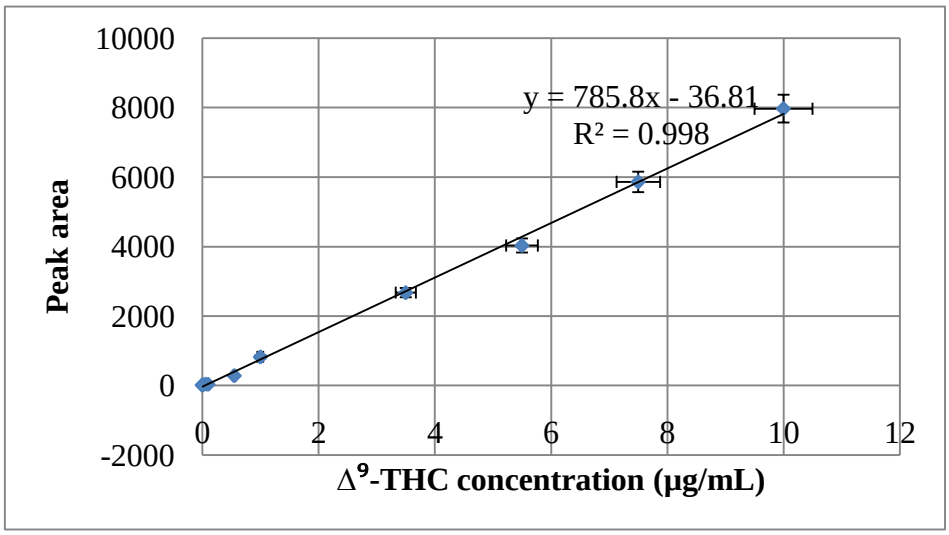


Figure 4-18: The calibration curve obtained for Δ^9-THC after the analysis of spiked blood samples in the concentration range of 0.0001-10 µg/mL.

4.12.1.3 Calibration curve for methamphetamine

Figure 4-20 shows the calibration curve obtained for methamphetamine. The method was linear in the ranges of 0.01-10 µg/mL with r^2 = 0.9971. The LOD based on the concentration value giving three times signal to noise ratio for three analyses was observed at 0.001 µg/mL, while the LOQ based on the concentration value giving ten times signal to noise ratio for three analyses was observed at 0.01 µg/mL.

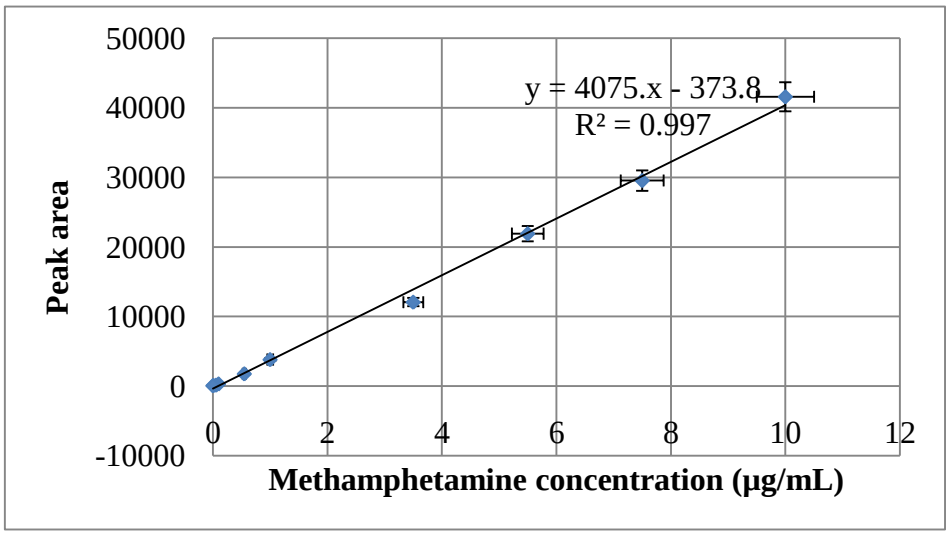


Figure 4-19: The calibration curve obtained for methamphetamine after the analysis of spiked blood samples in the concentration range of 0.01-10 µg/mL.

4.12.1.4 Calibration curve for amphetamine

The calibration curve obtained for amphetamine is shown in Figure 4-21.The method was linear in the ranges of 0.1-10 µg/mL with the regression square (r^2) = 0.9976. The LOD based on the concentration value giving three times signal to noise ratio for three analyses was observed at 0.055 µg/mL while the LOQ based on the concentration value giving ten times signal to noise ratio for three analyses was observed at 0.1 µg/mL.

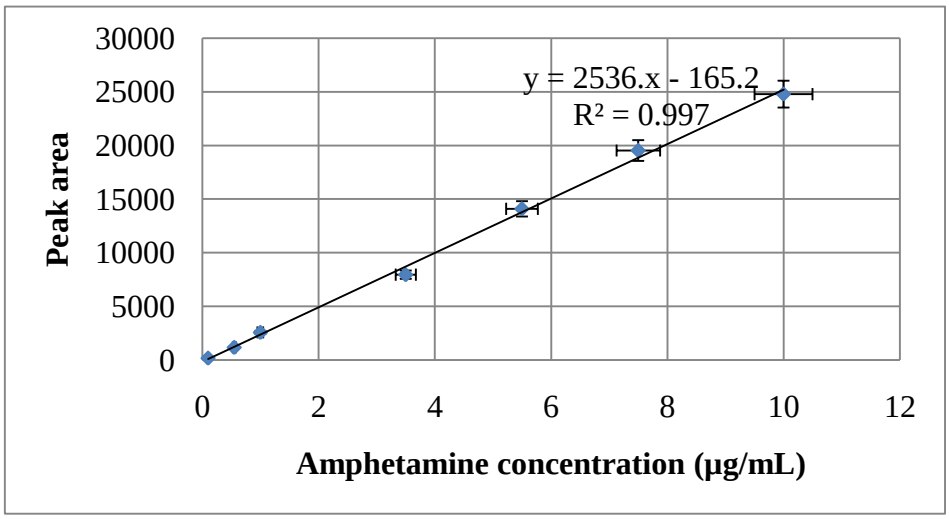


Figure 4-20: The calibration curve obtained for amphetamine after the analysis of spiked blood samples in the concentration range of 0.1-10 µg/mL.

Table 4-4: A summary of calibration curve equations, calibration range, regression square, LODs and LOQs obtained after method validation.

Drug	**Calibration curve**	**R^2**	**Linear range (µg/mL)**	**LOD (µg/mL)**	**LOQ (µg/mL)**
Amphetamine	y=25369x-165.2	0.997	0.1-10	0.055	0.1
Methamphetamine	y=40753x-373.8	0.997	0.01-10	0.001	0.01
Cocaine	y=15365x-221	0.998	0.01-10	0.001	0.01
Δ^9-THC	y=7858x-36.81	0.998	0.0001-10	0.00001	0.0001

4.12.2 Enrichment Factor

The enrichment factor was determined for each analyte based on equation (1) as described in the methodology section. The enrichment factors were calculated from the data used to construct the calibration curves for each drug. The obtained enrichment factors were 3.6, 3.6, 3.6 and 3.5 for amphetamine, methamphetamine, cocaine and Δ^9-THC, respectively (Table 4-5).

4.12.3 Selectivity

The ability of this analytical method to differentiate the analytes in the presence of other components in the sample matrix, as well the ability of the method to quantify the analytes was evaluated by analysing different blank blood samples in triplicate. No interfering peaks were found at the retention times of the drugs under study. See Figure 4-3.

4.12.4 Precision

The precision of the developed method describes the closeness of individual measurements of the analytes in triplicate was expressed as %RSD. The %RSDs were calculated from the data obtained for three different concentrations of spiked blood

extracted in triplicates under optimum conditions as presented in Table 4.5. The obtained %RSDs for the optimised method were between 5 and 10 % for amphetamine, 2 and 5 % for methamphetamine, 3 and 9% for cocaine and 4 and 7 % for Δ^9-THC.

4.12.5 Extraction Recovery

The extraction recovery was determined for 3 samples spiked at different levels and analysed in triplicate under optimum DLLME conditions as presented in table 4-5. The obtained extraction recoveries for were between 96-102 % for amphetamine, 95- 103 % for methamphetamine, 84-101 % for cocaine and 74-102 % for Δ^9-THC.

Table 4-5: A summary of the EFs, %RSDs and extraction recoveries obtained during method validation.

		0.5 µg/mL		**5 µg/mL**		**10 µg/mL**	
Drug	**EF**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**
Amphetamine	3.6	7	96	5	102	10	98
Methamphetamine	3.6	5	95	2	99	3	103
Cocaine	3.6	3	84	5	97	9	101
Δ^9-THC	3.5	7	74	4	94	7	102

4.13 Method application

In order to have an insight as to whether blood samples primarily submitted to NFSI for ethyl alcohol analysis contained other drugs of abuse, the current optimised DLLME method was used to screen about 30 blood samples. Of the 30 samples screened, 12 tested positive for ethyl alcohol with levels exceeding the country's legal limit of 0.79 mg/100 mL of blood. However, none of the drugs under study were detected in any of the tested samples.

CHAPTER 5

5. DISCUSSION

The main aim of extraction is to transfer the target analyte from the aqueous phase to the organic phase. In order to obtain high extraction efficiencies, it is necessary to investigate the effects of all parameters that can probably influence the performance of extraction. In developing the current DLLME method, the parameters that were optimised included the type and the volume of the extraction and the dispersion solvents, sample pH, sample ionic strength, extraction and centrifugation times.

5.1 Selection of extraction and dispersion solvents

The selection of an appropriate extraction and dispersion solvent is one of the most important factors in DLLME in order to effectively extract the analytes of interest from aqueous sample. According to the governing principles of DLLME, the extraction solvent should demonstrate a high extraction capability of the compounds of interest, low solubility in water, miscibility with the dispersion solvent should form tiny droplets in the presence of a dispersion solvent, should have a higher density than water as well as good chromatographic behaviour (Farajzadeh, Nouri, & Nabil, 2013). Three solvents that were readily available and met these requirements are carbon tetrachloride (CCl_4) (density, 1.59

g/mL and boiling point, 76.8 ^{o}C), dichloromethane (CH_2Cl_2) (density, 1.33 g/mL and boiling point, 39.8 ^{o}C) and chloroform ($CHCl_3$) (density, 1.48 g/mL and boiling point, 61.2 ^{o}C). An appropriate dispersion solvent for DLLME is required to achieve a rapid extraction of analytes into the extraction solvent. Therefore, a dispersion solvent should be miscible with both the aqueous phase (sample solution) and the extraction solvent, it should also be capable of dispersing the extraction solvent to form very fine droplets in aqueous phase. Acetonitrile, methanol and acetone are some of the commonly available dispersion solvents meeting these requirements and therefore their efficiency in DLLME in conjunction with the extraction solvents were investigated to extract the analytes under study from blood.

Even though CH_2Cl_2 met the requirements for DLLME procedures, it did not form a two phase system and sedimentation in this study. Similar results were also obtained in other studies (Xiong *et al.*, 2009). This may be due to the high solubility of CH_2Cl_2 in water (Saraji *et al.*, 2011). However, there are some studies in which CH_2Cl_2 was effectively used in DLLME. One such study was conducted by Cabarcos *et al*. (2013) in which a mixture of 630 µL acetone and 230 µL dichloromethane were used as dispersion and extraction solvents respectively, for the extraction of phosphatidylethanol in blood. However, the volume of dichloromethane (230 µL) used in their study was more than the volume used in this study of which only 35 µL (a mixture of 30 µL dispersion solvent and 5 µL dichloromethane) was injected into the sample. From this, it can be perceived that, sedimentation could have formed with dichloromethane if larger amounts of solvents were

used. However, this would not be feasible for this study as it aimed at using low amounts of solvents and sample.

From the six solvent combinations that were studied, the solvent combination 1 is the one that met the entire DLLME requirement and gave optimum recoveries of the drugs. The poor extraction recoveries of the analytes by other solvent combinations demonstrate that the solvents were not very effective or compatible with the extraction of the analytes under study from the aqueous solution. However, these solvents were successfully used by other researchers in DLLME procedures to extract other analytes from various matrices. Some of the works include that of the determination of benzophenone-3 and its metabolites in human serum by Tarazona, Chisvert & Salvado (2013) in which they used 70 µL acetone and 30 µL chloroform under optimised conditions as their dispersion and extraction solvents respectively. Another study where acetone and chloroform were effectively used in DLLME was that of Vela-Soria *et al*. (2013) where they developed a new treatment by DLLME for the determination of parabens in human serum samples. Research where methanol and CCl_4 were used as dispersion and extraction solvents respectively was the one conducted by Ito *et al*. (2011) where they developed a DLLME for analysing tricyclic antidepressants in urine. From these studies, it can be concluded that the inability of other solvent combinations to effectively extract the analytes under study in this research does not limit their efficiency in extracting other analytes from biological matrices.

5.2 Optimisation of sample pH

pH affects the ionisation states of analytes and hence the extraction efficiency of the analytes. Analytes can only be effectively extracted into organic phase once they are uncharged and this is achieved by adjusting the sample pH. All the drugs investigated in this study were basic except for Δ^9-THC which is a weakly acidic drug; therefore, to convert them to a neutral form and to enhance their affinity for the extraction solvent, the pH of the aqueous solution should be higher than the pKa of the basic analytes. In practical terms, a neutral deprotonated base exists in the solution of pH > pKa + 2 therefore in order to effectively neutralise the analytes; the pH of the solution should be adjusted sufficiently high so that additional selectivity can be achieved through adequate control of pH (Jouyban, Sorouraddin, Farajzadeh, Somi & Fazeli-Bakhtiyari, 2015). Amphetamine has a functional group of a primary amine, methamphetamine, a secondary amine, cocaine, a tertiary amine and Δ^9-THC, a phenol. The pKa values of these drugs are: cocaine (~8.6), methamphetamine (~10), and amphetamine (~10). Since the pKa values of these drugs are above 8, it was necessary to investigate their extraction recoveries at pH >8 in order for these analytes to be neutralised. However, since Δ^9-THC is a weak acid with a pKa value of ~10.6, at pH higher than 10.6, it is deprotonated and thus ionised.

The significant role of sample pH was observed from the trend of the results whereby the extraction recoveries of the drugs increased significantly with an increase in pH, peaking

at pH 12. The low recoveries of the amphetamines at low pH as well as the drop in the recovery of Δ^9-THC at high pH is consistent with expected protonation of the basic drugs at lower pH and ionisation of Δ^9-THC at high pH (Jouyban *et al*., *2*015). However, pH levels did not drastically affect the extraction recoveries of cocaine which was ranging between 50 and 60% recovery.

5.3 Optimisation of the dispersion solvent volume

The volume of the dispersion solvent plays a significant role in the extraction efficiency of the analytes from aqueous phase. When a higher volume of a dispersion solvent is used, the analyte tend to be more soluble in the aqueous phase than in the organic phase (Ghambari & Hadjmohammadi, 2012). The formation of a cloudy solution diminishes with the increasing volume of dispersion solvent thereby lowering the extraction recoveries of analytes (Mudiam *et al*., 2012). During the optimisation of the dispersion volume, an inversely proportional relationship was observed between the sedimented volume and the dispersion volume. Increases in the dispersion solvent volume led to a decrease in the sedimented volume and vice versa. Since the volume of the extraction solvent (chloroform) was kept constant at 130 µL, it significantly influenced this trend. The decrease in the sedimented volume at higher acetonitrile volume might have been due to the increasing solubility of the extraction and dispersion solvents in the aqueous phase (Xiong *et al.,* 2009). The volume of dispersive solvent affects the formation of

water/dispersive solvent/extractant solvent emulsion system, thereby affecting the dispersion of target analytes between dispersive and extractant solvents, and ultimately the extraction efficiency (Xiao, Tang, Yin, Xiang, & Xu, 2013). The sedimented volume also plays a significant role in DLLME as it affects the enrichment factor as well as the extraction recoveries of analytes. Since DLLME was performed in GC micro-insert vials (300 µL) with the sample size of 250 µL, it was very crucial to maintain the sedimented volume to at least 6 µL in order to have high enrichment factors and in return, higher extraction recoveries for the analytes under study. Optimisation of the dispersion solvent volume significantly improved the extraction recoveries of amphetamine while it had a limited effect on the recoveries of cocaine, methamphetamine and Δ^9-THC (Figure 4-14 to Figure 4-17).

5.4 Optimisation of the extraction solvent volume

The volume of the extraction solvent just like the dispersion solvent plays a significant role in the extraction of the analytes from the aqueous phase. Increasing the extraction volume leads to a higher sedimented volume which lowers the enrichment factor. In return, this affects the extraction recoveries of the analytes. This can be attributed to the dilution effect of the analyte as the sedimented volume increases (Saraji *et al.,* 2011; Ghambari & Hadjmohammadi, 2012).

To study the effect of the extraction solvent on the extraction efficiency of the analytes the optimised dispersion volume of 130 µL was fixed and only the volume of the extraction solvent was varied as shown in Figure 4-8. As with the dispersion volume, the sedimented volume increased with an increase in the extraction volume and this has the same effect on the extraction of the analytes. At low extraction solvent volume, no sedimentation was observed and this was contrary to the effect of the dispersion solvent volume whereby the reverse was observed. This might be that the extraction solvent was more soluble in the aqueous phase than with the dispersion volume and this increases the affinity of the analytes to the aqueous phase.

5.5 Optimisation of the ionic strength

The sample ionic strength effect on the extraction efficiency of the analytes under study was of great importance as NaCl can influence analytes to move from aqueous phase to organic phase. This is because the addition of salt improves the extraction efficiency in liquid–liquid extraction due to the salting-out effect (Peng *et al*., 2015). For analytes that are relatively soluble in water, a salting-out agent is used in order to improve the recoveries in liquid–liquid extraction, as it generally makes the analytes less soluble in water (known as the salting-out effect) (Jouyban *et al.,* 2015). On the other hand, the presence of a salt in the extraction system promotes phase separation of the two layers after centrifugation (Jouyban *et al.,* 2015). This effect of ionic strength was studied by

adding different NaCl concentrations (ranging from 0.01- 0.1 % w/v) to the samples, resulting in a reduction of the extraction recoveries of the analytes as compared to the sample where no salt was added. The addition of salt to the sample could have affected the solubility of the extraction solvent in the aqueous phase by altering the physical properties of the extraction film and therefore the diffusion of the analyte from the aqueous phase to the organic solvent phase was reduced (Cheng, Zhou, Zuo, Dai, & Guo, 2010; Peng *et al*., 2015). It was therefore concluded that the addition of salt to the sample did not enhance salting out but it caused a suppression of analytes in the aqueous phase. Therefore, no salt was added in further optimisation.

5.6 Optimisation of the extraction time

The extraction time is one of the most important variables involved in the extraction process and in DLLME, it is defined as the time interval between the injection of the binary mixture of disperser and extraction solvents and centrifugation (Tarazona *et al.*, 2013). Sometimes this parameter is not considered as a very crucial parameter in DLLME, because the equilibrium state is quickly achieved due to the large surface area produced during dispersion of the solvent into the sample (Suh *et al*., 2013; Kohler *et al*., 2013). A fast equilibration state was observed in the current method, as recoveries above 60 % were attained within 0.5 min of extraction, with the maximum recoveries obtained within 2.5 min of extraction. This short extraction time is one of the greatest advantages of DLLME

and it indicates a fast mass transfer of analytes from the sample solution to the extraction solvent due to an infinitely large surface area (Ghambari & Hadjmohammadi, 2012).

5.7 Optimisation of the centrifugation time

Centrifugation is a mandatory process and it plays a significant role in accelerating the collection of the extractant droplet which settles at the bottom of the conical tube (Fernandez *et al*., 2013; Farajzadeh, Nouri, & Nabil, 2013). In this research, it was observed that longer centrifugation times did not enhance the extraction recoveries of the analytes as the optimum recoveries were obtained within 2.5 min. This time was very short as compared to other DLLME methods whereby centrifugation times were around 5 min (Saraji *et al.*, 2011; Fernandez *et al.*, 2013). A shorter centrifugation time could have been due to the size and shape of the GC micro-insert vials (300 µL) that were used during the DLLME procedure which could have enhanced the organic layer to rapidly settle at the bottom.

5.8 Matrix effect

Matrix effect have been known to either increase or decrease the MS signal because of interfering substances specific to the sample that co-migrate with targeted analytes and affect their ionisation (Oldekop, Herodes, & Rebane, 2014). Matrix effect is known to

cause a compound's response to differ when analysed in a biological matrix compared to a standard solution (Eeckhaut, Lanckmans, Sarre, Smolders, & Michotte, 2009). Therefore, sample pre-treatment such as LLE or SPE greatly reduces the presence of endogenous compounds; however, a small amount can remain in the sample, which can lead to matrix effect (Kohler *et al*., 2013). In order to eliminate matrix effect, proteins were precipitated out of the blood followed by a ten times dilution with 0.5 M NaOH at specified pH prior to DLLME. From the results, it was observed that sample dilution significantly reduced the matrix effect especially for the two amphetamines compared to the undiluted sample. Sample dilution is one of the measures taken to reduce or assess the matrix effect, and dilution of the final extracts reduces matrix effects and therefore helps to achieve lower quantitation limits (Oldekop *et al.*, 2014). Small dilutions do not have a great impact on the reduction of matrix effect, however, total elimination of matrix effects by dilution of the sample may be limited by the sensitivity of the instrument (Oldekop *et al.*, 2014).

5.9 Method Validation

Table 5-1 shows a comparison of the performance of the developed and validated method in this study with other methods reported in literature for the analysis of amphetamine, methamphetamine, Δ^9-THC and cocaine. Lowest LODs and LOQs for cocaine, amphetamine and methamphetamine are reported by Fisichella, Odoardi, & Strano-Rossi (2015) and Meng *et al*. (2011). The differences of which can be attributed to the analysis

method used. The lowest LODs and LOQs were obtained when LC/MS/MS and CE-UV were used for the analysis of samples prepared by DLLME, while the highest LODs and LOQs were obtained when GC-MS and HPLC were used (Table 5.1). Other validation parameters (Table 5.1) of the current methods were comparable with the reported methods in literature. No publications on DLLME for Δ^9-THC were available, therefore it was assumed that the current method was the first to be developed for the extraction of Δ^9-THC from blood using DLLME.

Table 5-1: A comparison of the linear calibration ranges, regression coefficients (R^2), LODs, LOQs, EFs, %RSDs and extraction recoveries of amphetamine, methamphetamine and cocaine.

Analyte	**Linear range (µg/mL)**	**R^2**	**LOD (µg/mL)**	**LOQ (µg/mL)**	**EF**	**RSD (%)**	**Recovery (%)**	**Analysis method**	**References**
Amphetamine	0.055-10	0.997	0.055	0.1	3.6	5-10	96-102	GC-MS	Current method
Methamphetamine	0.001-10	0.997	0.001	0.01	3.6	2-5	95-103		
Cocaine	0.001-10	0.998	0.001	0.01	3.6	3-9	84-101		
Δ^9-THC	0.00001-10	0.998	0.00001	0.0001	3.5	4-7	74-102		
Amphetamine	-	-	0.0005	0.002	-	5-10	90-119	LC-MS/MS	Fisichella *et al*. (2015)
Methamphetamine	-	-	0.0005	0.002	-	0.4-5	99-124		
Cocaine	-	-	0.0005	0.002	-	1-12	87-106		
Amphetamine	0.0060-10	0.9987	0.002	-	-	4.5-5.7	83.5-88.3	GC-MS	Meng *et al*. (2015)
Methamphetamine	0.0060-10	0.9981	0.002	-	-	3.6-4.7	79.3-90.4		

D-Methamphetamine	0.0006-6	0.9992	0.0002	-	545	3.2	85.9-87.9	CE-UV	Meng *et a.*, (2011)
L-methamphetamine	0.0006-6	0.9994	0.0002	-	552	3.3	86.3-88.9		
Amphetamine	0.03-3	0.995	0.008	-	17	7.8	56.5	HPLC	Ahmadi-Jouibari *et al.* (2014)
Methamphetamine	0.01-2	0.997	0.002	-	25	6.2	62.4		
Amphetamine	-	-	-	-	-	-	76	CE-UV	Kohler *et al.* (2013)
Methamphetamine	-	-	-	-	70	-	75		
Cocaine	-	-	-	-		-	-		

5.10 Advantages of the developed method

A comparison of the current method with other DLLME methods reported in literature was made and the results are shown in table 5.1, 5.2 and 5.3. Several benefits of the current method in comparison with reported methods are briefly discussed below.

The current developed and validated DLLME method utilizes about 10 µL of the solvent mixture combination (about 6 µL of acetonitrile and 4 µL chloroform). These volumes are very low as compared to volumes used in previously reported DLLME methods and so far, the lowest volumes ever reported in literature. This significantly reduced the usage of toxic solvents.

The DLLME procedure was performed in 300 µL micro-insert GC vials, which made it easier to collect the sedimented volume with a GC injection needle without running a risk of mixing the aqueous and organic layers. Therefore, evaporating the extraction solvent and reconstituting analytes in fresh solvent prior to GC analysis, as performed in previously reported DLLME methods, was not necessary as samples were analysed directly. This had two main advantages: (i) no toxic solvents were released into the environment by evaporating the solvents, and the sample preparation time was reduced as no extra steps were required after DLLME prior to GC-MS analysis.

However, when the procedure is performed in centrifuge tubes (as it was in most cases reported in literature Tables 5-2 and 5-3), it requires more sample and more solvents in order to effectively extract the analytes from the sample. A higher sedimented volume is also required in order to make it easier for extracting the sedimented phase without mixing the aqueous and organic layers. Higher sedimented volumes calls for larger amounts of solvents and this has a dilution effect which significantly reduces the extraction recovery. Therefore in order to obtain good recoveries, the organic layer should be evaporated to dryness and sample reconstituted in little solvent volumes. Another disadvantage of performing the extraction in centrifuge tubes is the high risk of drawing some of the aqueous layer together with the organic layer. The aqueous solution is detrimental especially when analyses are performed with GC-MS.

The total sample amount subjected to DLLME per analysis was about 250 µL of the diluted sample. Since samples were diluted ten folds prior to DLLME analyses, only 25 µL of blood sample was needed for each analysis. This amount of sample is very little as compared other developed DLLME methods and it allows for multiple analyses from one sample. This is very economical especially in forensics where samples are normally limited while requiring multiple analyses.

The DLLME process excluding sample pre-treatment was performed under 10 minutes, while the entire process from sample pre-treatment to sample analysis was performed under 30 min. This significantly reduced on the time spend on sample preparation especially in conventional LLE where the process may take longer than 30 min. This, and other advantages mentioned above, make it suitable for use in fields for screening drugs of abuse from motorists when portable GC-MS equipments are available.

The entire DLLME process was successfully automated using an Agilent Sample Prep Workbench, 7696A. The automation of the method significantly reduced on the amount of manual labour required to prepare each sample and it also allowed for sample preparation overnight. This is a great advantage as it allows for high output of sample analysis in back logged laboratories.

Table 5-2: A comparison of various experimental parameters of the current developed method with other DLLME methods used for the analysis of similar drugs of abuse.

Reference	**Analyte (s)**	**Matrices**	**Dispersion & extraction solvents mixture**	**Centrifuge tube size**	**Sample amount per analysis**	**pH**
Current method	Amphetamine, methamphetamine, cocaine & Δ^9-THC	Blood	5.6 µL acetonitrile and 4.4 chloroform	300 µL GC micro-insert vial	25 µL	12
Fisichella *et al*, (2015)	Drugs of abuse, benzodiazepines, psychotropic medications	Blood	250 µL methanol and 100 µL chloroform	15 mL	500 µL	9
Meng *et al*. (2015)	Methamphetamine, amphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine, methcathinone, ketamine, meperidine, and methadone)	Blood & urine	100 µL toluene	2 mL	1 mL before dilution	13
Meng *et al*. (2011)	Heroin, DL-methamphetamine, DL-3, 4-methylenedioxymethamphetamine and DL-ketamine	Bank notes, craft paper, plastic bag & silver paper	500 µL Isopropyl alcohol and 41 µL chloroform	10 mL	5 mL solution	9
Ahmadi-Jouibari *et al*. (2014)	Amphetamines	Urine	300 µL acetonitrile and 30 µL 1-undecanol	10 mL	2 mL	10.2
Kohler *et al*. (2013)	Amphetamines and their derivatives, opiates, cocaine and its metabolites and pharmaceuticals	Urine	1.4 mL isopropyl alcohol and 600 µL dichloromethane	15 mL	4 mL	≥11.5

Table 5-3: A comparison of various experimental parameters of DLLME methods in general to those of the current developed method.

Reference	**Analyte (s)**	**Matrices**	**Dispersion & extraction solvent mixtures**	**Analysis Method**	**Centrifuge tube size**	**Sample amount per analysis**	**pH**
Vela-Soria *et a*l. (2013)	Parabens	Serum	500 µL acetone and 750 µL chloroform	UPLC-MS	15 mL	1 mL before dilution, 10 mL after dilution	2
Suh *et al*. (2013)	Duloxetine	Plasma	900 µL acetonitrile and 500 µL 1-undecanol	HPLC-UV	15 mL	1 mL before dilution, 4 mL after dilution	
Jouyban *et al*. (2015)	Antiarrhymic drugs	Plasma	1340 µL acetonitrile and 100 µL dichloromethane	HPLC-UV	-	650 µL before dilution, 5 mL after dilution	11.5
Cabarcos *et al*. (2013)	Phosphatidylethanol	Blood	630 µL acetone and 230 µL dichloromethane	LC-MS	-	200 µL before dilution, 1.6 mL after dilution	-
Fernandez *et al*. (2013)	Benzodiazepines	Plasma	2 mL methanol and 250 µL	UPLC-PDA	15	500 mL before dilution, 5 mL after dilution	9
Farajzadeh, Nouri, & Nabil (2013)	Amantadine	Urine & Blood	750 µL methanol and 10 µL dibromomethane	GC-FID	10	1 mL before dilution, 5 mL after dilution	10
Tarazona *et al*. (2013)	Benzophenone-3 and metabolites	Serum	70 µL acetone and 30 µL chloroform	LC-MS	1.5 mL eppendorf tube	800 µL	-

Barfi *et al*. (2015)	Anti-inflammatory drugs	Urine	350 µL acetone and 55 µL n-octanol	GC-FID	10 mL	5 mL	3
Ghambari & Hadjmohammadi (2012)	Warfarin	Plasma	150 µL methanol and 150 µL 1-octanol	HPLC-UV	Special extraction cell	11	2.3
Xiao *et al*. (2013)	Sildenafil, Verdenafil, Aildenafil	Plasma	20 µL methanol and 20 µL $[C_8MIM][PF_6]$	HPLC-UV	1.5 mL polyethylene tube	960 µL	7.2
Carrasco-Correa *et al*. (2015)	Parabens	Blood & Urine	500 µL acetone and 750 µL chloroform	Capillary LC-UV	15 mL	1 mL before dilution, 10 mL after dilution	2
Shamsipur & Mirmohammadi (2014)	Imipramine & trimipramine	Urine	800 µL acetonitrile and 50 µL chloroform	HPLC-UV	10 mL	8.5 mL	8.5
Vela-Soria *et al*. (2014)	Benzophenone-UV	Serum	3.5 mL acetone and 500 µL chloroform	LC-MS/MS	15 mL	1 mL before dilution, 10 mL after dilution	2

5.11 Method application

The absence of cocaine, Δ^9-THC, amphetamine and methamphetamine from the screened samples does not rule out the possibility that some motorists in the Khomas region of

Namibia drive under the influence of other narcotic drugs other than ethyl alcohol. The sample size used in screening for these drugs of abuse was small (about 30 blood samples) which was not representative of the motorists population that are suspected to be driving under the influence of narcotic drugs. Screening of more cases could have given a better insight on the usage of narcotic drugs by motorists in Khomas region. From this investigation, a conclusion on the frequency distribution of psychoactive drug use in Khomas region as well as the identification of the most frequently used psychoactive drugs by drivers in Khomas region could not be firmly established. Therefore, it is recommended that more cases be screened and analysed to get a representative population of motorists using narcotic drugs in Khomas region.

CHAPTER 6

6. CONCLUSIONS

A short GC-MS method under 12 min that was sensitive to the analytes under study was successfully developed and optimised prior to the development of a DLLME method. Various DLLME parameters were successfully optimised to improve the extraction efficiency of the method. Hence, a fast, simple, convenient and environmental friendly DLLME method was successfully developed while utilising minimum amounts of sample and solvents. The method was successfully optimised for the determination of amphetamine, methamphetamine, cocaine and Δ^9-THC. The developed method utilised low volumes of dispersion and extraction solvent of about 6 and 4 µL, respectively. The sedimented volume at the bottom of the micro-insert vial was very little, about 6 µL and this was easily collected with a GC syringe due to the conical shape of the micro-insert vial. In addition, there was no need to evaporate the solvent, so the samples were directly analysed and this significantly reduced the overall sample preparation time. Low volumes of sample (25 µL) were required per analysis, hence the method is suitable for trace analysis in very small samples of blood, e.g. taken from crime scenes. In addition, the method was successfully automated using an Agilent Sample Prep Workbench, 7696A. The validated method provided good recoveries of the analytes under study and it was successfully applied to screen the blood samples from traffic law offenders for the presence of these drugs. Due to the many advantages of this method as compared to other

DLLME methods reported in literature, it is suitable for field screening of drugs of abuse from motorists when portable GC-MS are available.

RECOMMENDATIONS

One of the shortcomings of the current method is the need for manual injection. This is because the sedimented volume (6 µL) collected at the bottom of the GC micro-insert vials, is below the needle-depth level of the GC-MS instrument used for the analysis. This can be overcome by increasing the volume of the extraction solvent to at least 40 µL in order to obtain a higher sedimented phase volume. However, this will require a compromise on the sample amount as well as sensitivity of the method. Hence, an instrument with the capability to adjust the injection needle to the required depth will be the best solution to this problem.

The current method can be improved and it has potential for wider applications in forensic and clinical studies. This could be achieved by optimising and validating the current method also for other drugs (medicinal or illicit) of interest. This would significantly improve the costs of screening for these drugs especially in cases where each drug is screened separately. Since drugs of abuse are not limited to blood, the method can further be extended to other specimens such as urine and saliva, or it can be extended to post mortem drug analysis. The method can be employed for further DUID studies in future, as it is convenient, fast, cheap and environmentally friendly. Obtaining conclusive DUID patterns for our country might help in developing new strategies for combating the issue, as more lives are continuously being lost on the Namibian roads at alarming rates.

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