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THE SYNTHESIS, ANALYSIS AND CHARACTERISATION OF PIPERAZINE BASED DRUGS

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A Thesis in partial fulfilment of the requirements of Anglia Ruskin University for the degree of PhD in Analytical Chemistry

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ABSTRACT

FACULTY OF SCIENCE AND TECHNOLOGY

DOCTOR OF PHILOSOPHY

The Synthesis, Analysis and Characterisation
of Piperazine Based Drugs

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This study developed a GC-MS method for the simultaneous detection of piperazines and congeners in street samples of amphetamine type stimulants. This research investigated the clandestine routes of synthesis and chemical profiles of phenylpiperazines, represented by 1-(4-fluorophenyl)piperazine (4-FPP) and 1-(3-trifluoromethylphenyl)piperazine (3-TFMPP). These drugs are part of the increasingly prevalent illicit new psychoactive substances. The presence of (2, 3, 4) FPP and (2, 3, 4) TFMPP positional isomers has been identified by other researchers as a limitation due to their similar chemical profiles.

The method was optimized and confirmed as compliant with the International Conference on Harmonisation and the Center for Drug Evaluation and Research guidelines on validation. 4-FPP and 3-TFMPP were synthesised using potential routes for clandestine laboratories. Simple extraction and analysis of 11 street samples was conducted using the method developed. Furthermore, the stability of 22 drugs during analysis was investigated.

Limits of detection were in the range 5 – 1.95ng/mL free base on column. The synthesised samples were identified as 4-FPP and 3-TFMPP. Several impurities were observed in the synthesised samples, which were identified and categorised as residual reactants, isomers of 4-FPP and of 3-TFMPP and by-products of synthesis. The percentage yields of the synthesised samples obtained were up to 82.4% 4-FPP and 78.7% 3-TFMPP.
The street samples were found to contain MDMA, 3-TFMPP, BZP, caffeine, ephedrine and other impurities.

The analytical method simultaneously separates 19 of the most common drugs found in piperazine samples and achieves for the first time the GC-MS separation of (i) 2-FPP, 3-FPP and 4-FPP and (ii) 2-TFMPP, 3-TFMPP and 4-TFMPP at the same time from a sample matrix containing all the 19 compounds. This method provides operational laboratories with a more effective method for the chemical characterisation of street samples of piperazines and also provides novel stability data.

Keywords: piperazines, method, charaterisation, street drugs, stability.

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ABBREVIATIONS

ABBREVIATIONS FOR DRUGS

2-FPP	1-(2-fluorophenyl)piperazine
3-FPP	1-(3-fluorophenyl)piperazine
4-FPP	1-(4-fluorophenyl)piperazine
2-TFMPP	1-(2-trifluoromethylphenyl)piperazine
3-TFMPP	1-(3-trifluoromethylphenyl)piperazine
4-TFMPP	1-(4-trifluoromethylphenyl)piperazine
3-CPP	1-(3-chlorophenyl)piperazine
2-MeOPP	1-(2-Methoxyphenyl)piperazine
4-MeOPP	1-(4-Methoxyphenyl)piperazine
4-MePP	1-(4-methylphenyl)piperazine
Amp	(+)-Amphetamine SO_4
BEH	Benzoylecgonine hydrate
BZP	1-benzylpiperazine
Dap	Dapoxetine
Dex	Dextromethorphan
DBZP	1,4-dibenzylpiperazine
EME	Ecgoninemethylester
MBZP	1-(4-methylbenzyll)piperazine
MDMA	3,4-Methylenedioxymethamphetamine
Meth	(+)-Methamphetamine hydrochloride
Nic	Nicitinamide

OTHER ABBREVIATIONS

AMC	Analytical methods committee for the Royal society of chemistry
ACMD	Advisory council on the misuse of drugs, UK
AOAC	Association of analytical chemists
DEA	Drug enforcement agency for the US
ECDD	Expert committee on drug dependence for WHO
EMCDDA	European monitoring centre for drugs and drug addiction
EURACHEM	a focus for analytical chemistry in Europe

EUROPOL	European Union law enforcement agency
FDA	US Food and drug adminstration
FTIR	Fourier transform infrared spectrometry
GC-MS	Gas chromatograph - Mass Spectrometry
HETP	Height equivalent of a theoretical plate
HPLC	High performance liquid chromatography
ICH	Internation conference on harmonisation
IUPAC	International union of pure and applied chemists
LTG	London toxicology group
m/z	Mass to charge ratio
RI	Retention Index
RRT	Relative retention time
R_s	Resolution
RSC	Royal Society of Chemistry
RSD	Relative standard deviation
RT	Retention time
TIC	Total ion chromatogram
UNODC	United Nations Office on Drugs and Crime
USP	United States Pharmacopeia
UV-Vis	Ultraviolet visible spectrometry
WHO	World Health Organisation

NOTATIONS

α	Selectivity
σ	Standard deviation
σ^2	Variance
K	Partition coefficient
k	Capacity factor
N	Plate number

LIST OF APPENDICES

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THE SYNTHESIS, ANALYSIS AND CHARACTERISATION OF PIPERAZINE BASED DRUGS

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CHAPTER 1

INTRODUCTION

1.1 AN OVERVIEW OF DRUGS OF ABUSE

Drugs of abuse regardless of their origin have a complex profile containing not only the active substances but also impurities such as potential by-products of synthesis, solvents and degradation products (UN, 2001; Inoue et al., 2008). In addition, cutting agents and adulterants are added along the distribution chain mainly so as to increase the volume so that more doses can be sold, e.g. starch, flour, sugar, caffeine (Andreasen et al., 2009). This effectively increases the profits for the drug seller. An adulterant unlike a cutting agent is pharmacologically active; it is stimulating and as such it normally enhances the psychoactive effects of the drug (adulterants are further discussed in section 1.2.2.1). The type and amount of adulterant or cutting agent depends on the illicit drug, the market and the desired effects (UN, 2001; Cole et al., 2011; Elliot, 2011). In some cases more than one is added, thereby further increasing the complexity of the drug profile.

Theoretically, investigation of all the components of a sample provides a complete "history" of the sample and is of use in characterising samples. According to the United Nations (UN, 2001) studies on characterisation/impurity profiling of seized drugs can provide insight into diverse law enforcement investigative issues, ranging from dealer-user relationships, drug source, distribution networks, and trafficking routes to manufacturing methods and precursors used. Such information may also be used to identify and control precursors and other chemicals by regulatory authorities. To evaluate the profile of a drug through analytical investigation involves identification, quantification, chemical characterisation and profiling (Bartos and Gorog, 2008; Inoue et al., 2008). These are therefore further discussed in section 1.8.

According to the UN (2001) and DEA (2011) drugs of abuse can be classified by origin and pharmacological effects. Categorisation by origin is useful in gaining insight into how the drug arises and evaluation of the similarities of their origin. This is of use in characterisation and profiling of a substance (UN, 2001; Aalberg et al., 2005a; Bartos and Gorog, 2008). Whilst categorisation by pharmacological effects gives an understanding of

the psychoactive effects of the substance, both sought by the user and the associated adverse effects. These can have a socio-economic impact, e.g. health risks. Consequently, these categories are discussed further. Categorisation by pharmacological effects is variable however they are mainly classified into depressants, stimulants and hallucinogens (DEA, 2011; Dargan and Wood, 2013). This is further discussed in the section 1.5 on pharmacological effects.

Table 1.1 gives an over view of the classes by origin. As shown in the table illicit drugs can be classified by origin into natural, semi-synthetic and synthetic substances. The natural group has been in existence the longest, e.g. cocaine is reported to have been in use since circa 470 – 370 B.C (Brick and Erickson, 2013; Seymour and Smith, 2011). The semi-synthetics arose in the late 1800s when Bayer Laboratories discovered a new pain reliever, diacetylmorphine (heroin). A compound derived from morphine by the addition of two acetyl groups. The drug had a high potential for abuse as it was 2 to 3 times more potent than morphine and was a stronger psychoactive substance. The emergence of the synthetic group started with amphetamine which was intended as a medical drug in the late 1920s (Dargan and Wood, 2013). Its "success" as an illict drug saw the development of its derivatives and other synthetic drugs (Shulgin and Shulgin, 1991).

Table 1.1 Classification/categories of drugs by origin (Arbo et al., 2012; Brick and Erickson, 2013; Dargan and Wood, 2013; DEA, 2011; King, 2009; Seymour and Smith, 2011; Shulgin and Shulgin, 1991; Winstock and Wilkins, 2011).

Drug category	**Definition**	**Origin**	**Examples**
Natural	Substances that are only produced naturally without chemical alteration.	Plants Fungi	• Cocaine: derived from the coca plant, *Erythroxylum coca and Erythrxylum novogranatense.* • Cannabis: derived from the leaves of various species of *Cannabis*. • Natural opiates e.g. morphine, codeine. • Khat (Catha edulis) and kratom. Magic mushrooms: various mushroom species of *Psilocybe*.
Semi-synthetic	These are drugs that are derived by synthesis from a natural psychoactive drug.	Made by derivation from natural opiates such as morphine, codeine and thebaine. These substances were initially derived for use as pharmaceuticals.	Heroin Oxycodone
Synthetic	These are a relatively new generation of drugs, completely man made and are derived wholly from chemical synthesis.	They are mainly made by modification of another drug molecule, commonly a pharmaceutical drug or a controlled psychoactive drug with the aim of circumventing legal controls.	• Amphetamines e.g amphetamine, methamphetamine, MDMA. • Piperazines (BZP, 3-TFMPP, 4-FPP). • Tryptamines (LSD).

A general review of the trends in the history of drugs of abuse (King, 2009; King and Kicman, 2011; Dargan and Wood, 2013; UNODC, 2013a) confirmed that in the early years the natural class accounted for all the abused substances. However, from the 1940s the drug scene changed due to the emergence of synthetic and semi-synthetic drugs. This can be seen in the trends in substances under legal control (Fig 1.1).

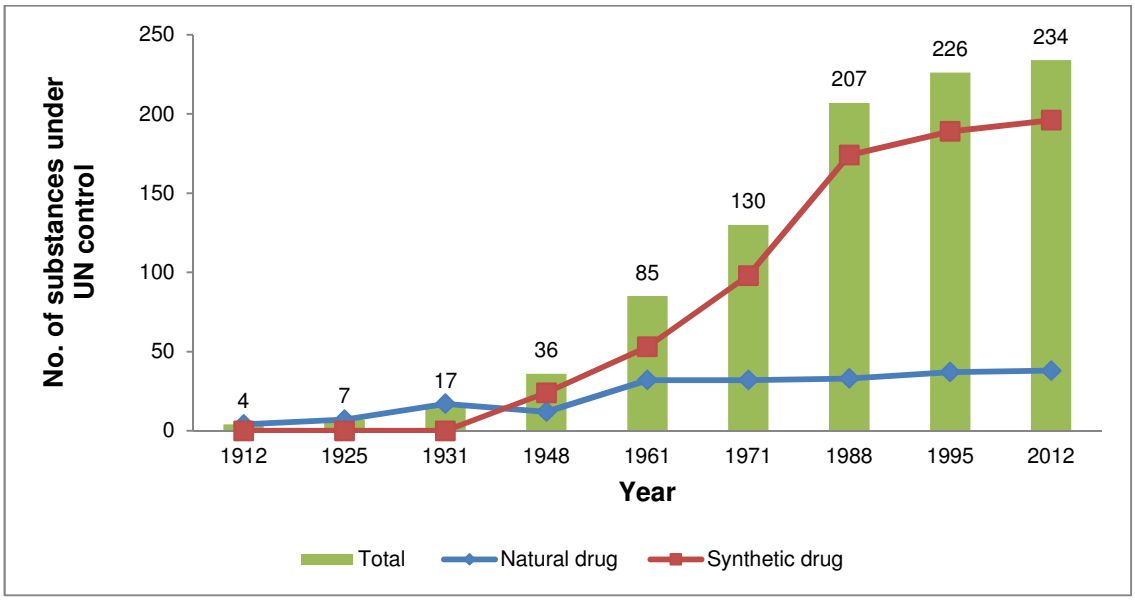


Figure 1.1 Trend in substances controlled by the UN, showing changes in the type and number of substances (data from UNODC, 2013a).

Figure 1.1 shows that globally there has been an increase over the years in the number of drugs of abuse controlled by the UN in the period 1912 - 2012. With this increase synthetic drugs have increased exponentially since the 1940s (UNODC, 2013a) by 2012 accounting for 84% (196 substances) of all the substances controlled by the UN. In comparison the natural class accounted for 16% (UNODC, 2013a). The marked increase in 1948 can be attributed to the emergence of phenethylamines (Shulgin and Shulgin, 1991; Dargan and Wood, 2013). The increasingly large discrepancy in the drug types with time is due to the emergence of a larger variety in the types of synthetic relative to the natural drugs.

It has been recently reported by the UNODC (2013a) that for the first time the international drug control system is floundering under the speed, creativity and unfailing regularity of appearance of new psychoactive substances (NPS). Furthermore, for the first time the number of NPS actually exceeds the total number of substances under international control

(234). Currently 250 NPS are listed (UNODC, 2013b; EMCDDA-Europol; 2012). Furthermore, it was reported in the UNODC Global Smart program (UNODC, 2014) that synthetic drugs pose a threat and a significant drug problem globally. The report stated that "after cannabis, amphetamine-type stimulants (ATS) are the second most widely used drugs across the globe". Further to this is the exponential increase highlighted above. This shows that there is need to keep up with investigation of these newly developing substances as a way of gaining information, increasing regulations, reducing risk, potential health issues and prevalence. This study will investigate a new psychoactive substances; phenylpiperazines with a focus on 1-(4-fluorophenyl)piperazine (4-FPP) and 1-(3-trifluoromethylphenyl)piperazine (3-TFMPP).

1.2 PROPERTIES OF 4-FPP AND 3-TFMPP

The properties of a class of synthetic drugs of abuse, i.e., piperazines will be reviewed so as to gain an insight into their characteristics.

1.2.1 PHYSICAL AND CHEMICAL PROPERTIES

4-FPP and 3-TFMPP are isomeric, exhibiting (2, 3 and 4) positional isomers. The structures, CAS numbers (chemical identity numbers), molecular mass, boiling points and characteristic mass spectral ions are shown in Figure 1 and Table 1.2. These properties are characteristic of these compounds and as such are of use in their identification (de Boer et al., 2001; Takahashi et al., 2009; UNODC, 2013b).

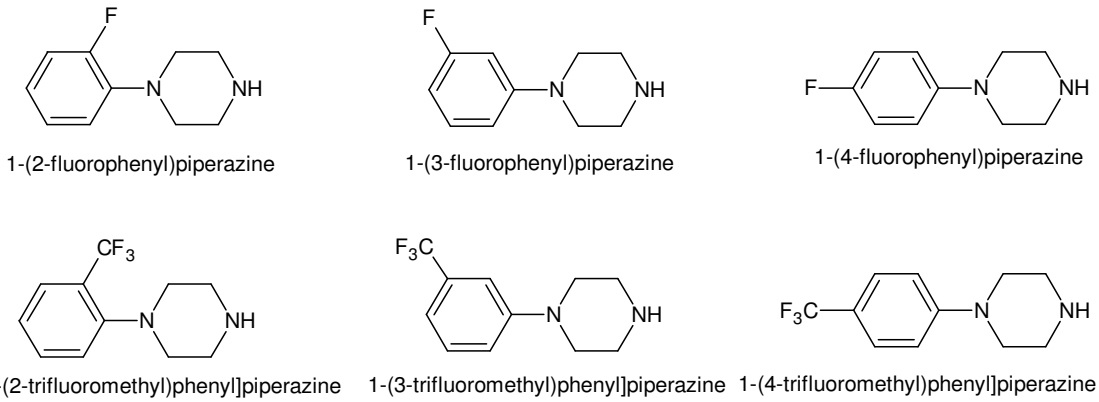


Figure 1.2 Chemical structures of selected piperazine based psychoactive drugs; the main drugs of focus for this research.

Table 1.2 Properties of FPP and TFMPP compounds (UNODC 2013c; NIST, 2014; Takahashi et al., 2009; de Boer et al., 2001; RSC Chemspider, 2013)

Compound	**Common name**	**CAS No.**	**Molecular mass (g/mol)**	**Boiling point (^{o}C)[1]**	**Characteristic mass spectral ions m/z[2]**
1-(2-fluorophenyl)piperazine	2-FPP	1011-15-0	180.22	398.35 –401.84	138(100), 180(M^+), 122, 56
1-(3-fluorophenyl)piperazine	3-FPP	3801-89-6	180.22	358.33 –372.97	138(100), 180(M^+), 122, 56
1-(4-fluorophenyl)piperazine	4-FPP	2252-63-3	180.22	440.24	138(100), 180(M^+), 122, 56
1-(2-trifluoromethylphenyl)piperazine	2-TFMPP	63854-31-9	230.23	344.27 – 350.91	188(100), 230(M^+), 172, 145
1-(3-trifluoromethylphenyl)piperazine	3-TFMPP	15532-75-9	230.23	190.92 – 198.95	188(100), 230(M^+), 172, 145
1-(4-trifluoromethylphenyl)piperazine	4-TFMPP	30459-17-7	230.23	309.10	188(100), 230(M^+), 172, 145

[1] At (^{o}C/760mmHg). [2] A discussion of the ions and mass spectra is conducted Chapter 7 (Validation).

1.2.2 PHYSICAL PROPERTIES OF PIPERAZINE (4-FPP AND 3-TFMPP) STREET DRUGS ON THE MARKET

In street drugs, 4-FPP and 3-TFMPP exist in the free base or salt form, such as the hydrochloride salt (Nikolova and Danchev, 2008; Kenyon et al., 2010; Kelleher et al., 2011). These drugs are sold as powders, liquids, tablets and capusules (Vorce et al., 2008; Nikolva and Danchev, 2008), however they have been reported mostly as tablets and capsules (Nikolva and Danchev, 2008; DEA, 2009a, b; Kenyon et al., 2010; Kuleya et al., 2014; Yuk, 2010). Piperazine drugs are easily available in pubs, clubs and herbal shops. The internet has also been widely reported to be the main source of new psychoactive substances (ACMD, 2011; Arbo et al., 2012; Baron et al., 2011; EMCDA – Europol, 2013). Figure 1.3 shows images of some of the different clandestine drugs containing 4-FPP and 3-TFMPP encountered in illegal drugs marketed under a variety of names.

(I)


(II)

(III)


(IV)

(V)


(VI)

(VII)

Figure 1.3 Images of some the different clandestine drugs containing 4-FPP and 3-TFMPP; Majik tablets (I), Exotic tablets (II, III), 3-TFMPP powder (IV, VI), Obama logo tablets (VI) and Playboy bunny tablets (VII) (DEA, 2009a; Yuk, 2010; EMCDDA, 2011; UNODC, 2013b).

According to EMCDDA (2011) and DEA (2011) they often carry logos similar to those seen on ecstasy (Figure 1.3). For example, 4-FPP was reported in seized Playboy bunny shaped ‘ecstasy’ tablets. This is likely so as to mislead the user into thinking they are a

form of ecstasy. Table 1.3 gives examples of some of the street drugs that have been reported as containing piperazines.

Table 1.3 Examples of steet drugs containing piperazines (DEA, 2010; Yeap et al., 2010; Yuk, 2010; Kelleher et al., 2011; Arbo et al., 2012; ECDD, 2012)

Street name	**Composition**
Exotic, A2, Charge, Nemesis, Legal X	BZP 50 - 200mg 3-TFMPP 5 - 225mg
Altitude	BZP 45mg 3-TFMPP 15mg Vitamins, minerals
Super E[1]	4-FPP 3-TFMPP Caffeine
E-XTC	"Not for human consumption" dicalcium phosphate, ketones and magnesium stearate but was actually found to contain TFMPP
"ecstasy"[1]	4-FPP

[1] Dosages not given

Piperazine drugs have been reported to exist as drug cocktails and that they are rarely found as the only active ingredient present (Davies et al, 2010; Kenyon et al., 2010; Yeap et al., 2010; Kelleher et al., 2011; Winstock and Wilkins, 2011). This is evidenced by the composition of the street samples given in Table 1.3. The most common combination was found to be BZP and TFMPP (DEA, 2009a, 2010; Kenyon et al., 2010; Yuk, 2010; Kelleher et al., 2011). According to a DEA report (2009a, 2009b) caffeine and nicotinamide are routinely found as adulterants in most piperazine drug cocktails. Furthermore, other drugs such as dapoxetine, dextromethorphan, diazepam and cocaine have also been reported to exist with these drugs (Staack, 2007; Nikolova and Danchev, 2008; DEA, 2009 a, b). Table 1.4 shows the list of congeners found in street samples containing 4-FPP and 3-TFMPP and Figure 1.4 shows their chemical structures. Congeners can be defined as other chemical components which together with the main drug aid in give it its distinctive character or physiological effects.

Table 1.4 Congeners found with 4-FPP and 3-TFMPP drugs of abuse on the street (Staack, 2007; Nikolova and Danchev, 2008; Vorce et al., 2008; Kenyon et al., 2010; Kelleher et al., 2011; UNODC, 2013a).

Substance (chemical name)	**Common name**
1-benzylpiperazine	BZP
1,4-dibenzylpiperazine	DBZP
1-(4-methylbenzyl)piperazine	MBZP
1-(3-chlorophenyl)piperazine	CPP
1-(4-methylphenyl)piperazine	MePP
(2S)-1-phenylpropan-2-amine	(+)-Amphetamine
(2S)-N-methyl-1-phenylpropan-2-amine	(+)-Methamphetamine
methyl (3S,4R)-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylate	Cocaine
1S)-N,N-dimethyl-3-naphthalen-1-yloxy-1-phenylpropan-1-amine	Dapoxetine
7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one	Diazepam
Dextromethorphan: d-methorphan	Dextromethorphan
(1R,2S)-2-(methylamino)-1-phenylpropan-1-ol	Ephedrine
1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione	Caffeine
7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one	Nicotinamide

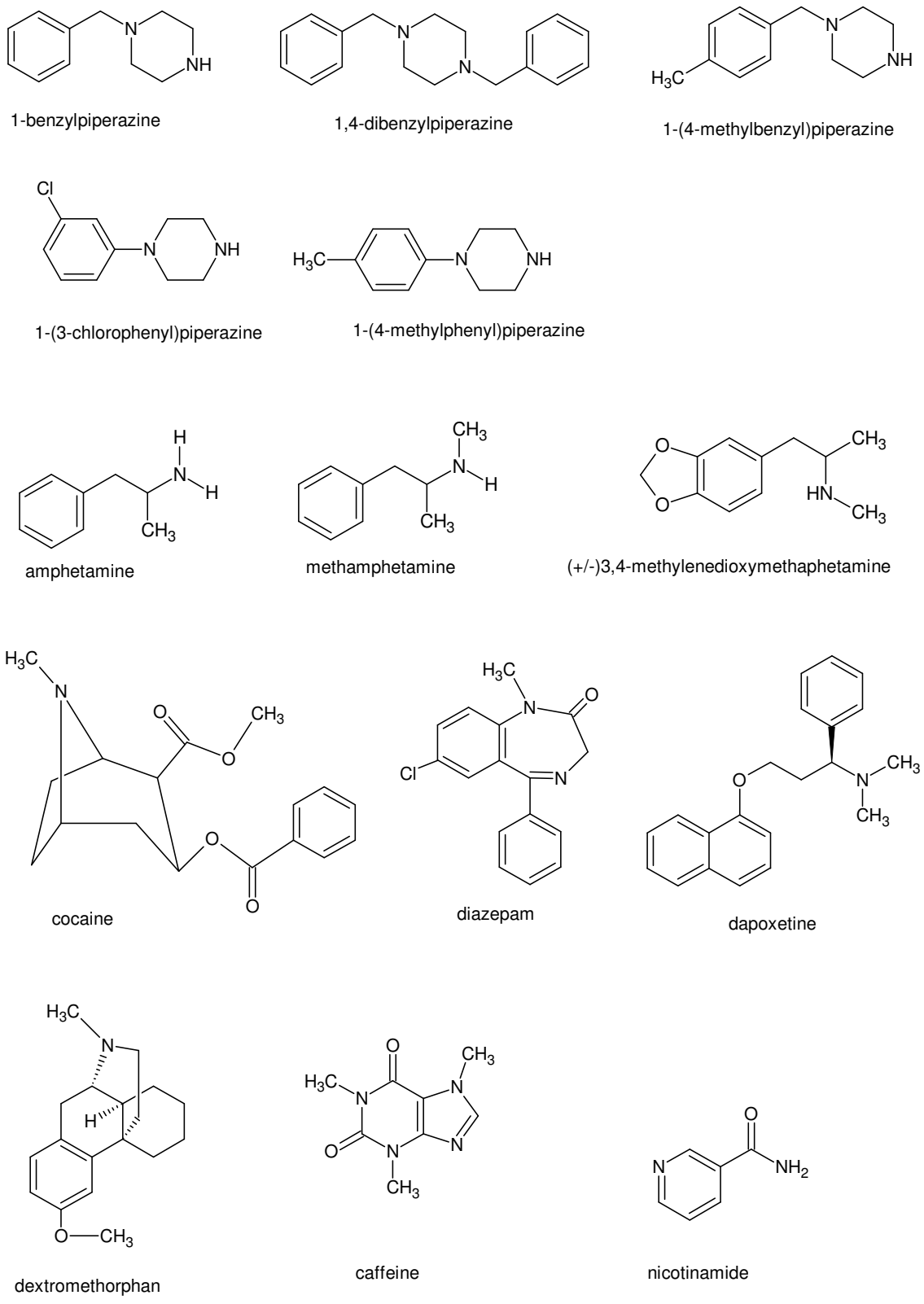


Figure 1.4 Drugs and other substances found in street samples containing 4-FPP and 3-TFMPP.

The dosages for the drugs vary and are often not reported on the label (Kelleher et al., 2011). Doses for BZP, 3-TFMPP, CPP and caffeine have been routinely reported by many researchers (LTG, 2006; Kenyon et al., 2010; Kelleher et al, 2011; Yeap et al., 2010; EMCDDA, 2011). The stated doses ranged between 50 - 200mg BZP and 5 - 75mg TFMPP and 90 - 110mg 3-CPP (EMCDDA, 2011; Kenyon et al., 2010; Kelleher et al., 2011). However, dosages for 4-FPP or any of the other drugs have not yet been reported. This could be due to the fact that research on 4-FPP is very limited in comparison to the previously stated piperazines. This can be attributed to the fact that it is relatively newer on the market as a drug of abuse than either BZP or 3-TFMPP (section 1.3).

Furthermore, the drugs are often not labelled with safety data or ingredients (Yuk, 2010; Elliot, 2011). Where this is present it is often misleading, e.g. the drugs are labelled as containing research chemicals (ACMD, 2011; Baron et al., 2011). In some cases tablets said to be piperazines were found to contain caffeine and in some cases 'ecstasy' tablets actually contained piperazines (Davies et al, 2010; DEA, 2009a). The implication is that the composition, dose and purity and potential adverse effects of the drug are unknown to the user. This puts users at risk of potential health problems. Consequently, this study investigates and provides information on the characterisation of piperazine street samples. This will beuseful to other researchers and might also be of use to law enforcement agencies

1.2.2.1 Why adulterate?

It is evident from section 1.2.2 that piperazine drugs of abuse are highly adulterated. A considerable number of congeners were identified (Table 1.2) as present in 4-FPP and 3-TFMPP street samples. The aims of adding adulterants are to add bulk, to enhance or mimic and to facilitate administration of the drug (Cole et al., 2011). Adulterants are also used to reduce the effect of adverse reactions. Consequently, this gives rise to the presence of the congeners in 4-FPP and 3-TFMPP street samples. The adulterants have been categorised according as;

1.2.2.1.1 True adulterants (such as caffeine, nicotine, nicotinamide)

These are non-psychoactive but pharmacologically active drugs. Their purpose is mainly to add bulk and enhance the effect of the psychoactive substances as a result of their own pharmacological properties (DEA, 2011; Barceloux, 2012) These substances have been

widely reported in drugs of abuse (Davies et al., 2010; Kenyon et al., 2010; Kelleher et al., 2011). Caffeine is one of the most common adulterants found in cocaine, amphetamine, methamphetamine, ecstasy and piperazine street drugs (LTG, 2006; Davies et al., 2010; Cole et al., 2011). The stimulant properties of caffeine can create similar, although usually milder effects to the primary drug (Davies et al., 2012). Nicotinamide also known as niacinamide is a water-soluble B-complex vitamin (B3) used in the treatment of vitamin B3 deficiency and is also commonly used as an adulterant (DEA, 2011). In some cases they are also used due to the fact that they facilitate drug administration (Cole et al., 2011; Davies et al., 2012) such as caffeine when is smoked with heroin. Huizer (1987) showed that due to its physiochemical effects, caffeine lowers the sublimation temperature of heroin, resulting in it vaporising at a lower temperature. This slightly increases its recovery and decreases its pyrolitic decomposition. Consequently, when heroin is smoked with caffeine there is a slightly increased efficiency (Andreasen et al., 2009).

1.2.2.1.2 Use of other drugs of abuse as adulterants

Piperazines such as BZP, DBZP, MBZP, MePP and CPP are psychoactive illicit drugs used to adulterate each other with the purpose of mimicking ecstasy. In combination the drugs have a synergistic effect and it has been reported that the BZP and 3-TMPPP combination is synergistic such that the effect is similar to ecstasy (Elliot, 2011). Piperazines are also reported as adulterants in other non-piperazine based illicit substances such amphetamine, methamphetamine, MDMA and cocaine (Arbo et al., 2012). The use of 4-FPP and 3-TFMPP as adulterants is mainly due to the following: a) their psychoactive effects can mask the fact that a reduced amount of the drug is present, b) they are cheaper (section1.4.2) and, c) they are more easily available as the legal controls on them are less extensive (Davies et al., 2012; Cole et al., 2011). Consequently, besides 4-FPP and 3-TFMPP other piperazine drugs are increasingly reported as adulterants (Yeap et al., 2010; Kelleher et al., 2010, Dargan et al., 2013).

1.2.2.1.3 The use of pharmaceuticals with anti-depressant or pain relieving properties

The use of these substances as adulterants has been widely reported (Cole et al., 2011; DEA, 2011; Arbo et al., 2012). The adulterants are mainly common pharmaceuticals such as diazepam, paracetamol and lidocaine. Their prevalent use as adulterants can be attributed to their physiological effects. This type of adulterants have sedative and analgesic effects and as such they appear to enhance the psychoactive effect of the drug, as they give

feelings of relaxation, for example diazepam. They have been found in illicit drugs such as piperazines, amphetamines, cocaine, heroin (Nikolova and Danchev, 2008; DEA, 2009a; King and Kicman, 2011). Andreasen et al. (2009) reported paracetamol as being widely present in amphetamine street samples. Furthermore, the UNODC (2013b) stated that their use has been "commonly observed among users of heroin who use benzodiazepines to enhance its effects". In addition, it has been reported that they also facilitate administration of the drug for example procaine relieves pain in cocaine intake (Cole et al., 2011).

1.3 HISTORY OF PIPERAZINE DRUGS OF ABUSE

Piperazines are derived by taking advantage of, or remodelling of piperazine based pharmacological drugs (Nikolova and Danchev, 2008; Lecompte et al., 2008; Yeap et al., 2010; Arbo et al., 2012; Dargan and Wood, 2013). The skeleton structure of all these drugs is the piperazine moiety (Figure 1.5).

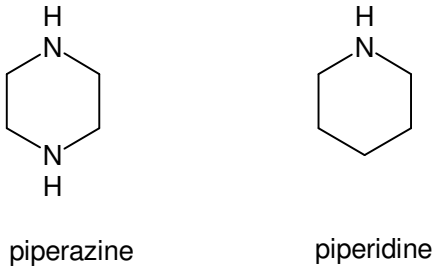


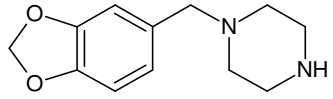
Figure 1.5 Chemical structures of piperazine and piperidine

The presence of two reactive amine groups on the piperazine ring makes it amenable to chemical derivatisation (for example as shown in Figure 1.16 for the synthesis of 3-TFMPP). The synthesised drugs structurally are 1-arylpiperazines and benzylpiperazines and as such are psychoactive, the degree of which is dependent on the substituent (Staack, 2007; Kenyon et al., 2010; Yeap et al., 2010). According to King (2009) and UNODC (2013a) there are at least 12 substituted piperazines drugs on the clandestine drug market (Table 1.5). In addition to these, thienylmethylpiperazines have been reported to a lesser extent (Arbo et al., 2012).

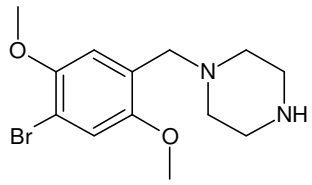
Table 1.5 Piperazine based psychoactive drugs currently found on market (UNODC, 2013a).

Common name	**Abbreviation**
1-Benzylpiperazine	BZP
1-Benzyl-4-methylpiperazine	MBZP
1,4-Dibenzylpiperazine	DBZP
3,4-Methylenedioxy-1-benzylpiperazine	MDBZP
1-(4-Bromo-2,5-dimethoxybenzyl)piperazine	2C-B BZP
1-Phenylpiperazine	N/A
1-(3-Chlorophenyl)piperazine	3-CPP
1-(3-Chlorophenyl)-4-(3-chloropropyl)piperazine	3-CPCPP
1-(4-Fluorophenyl)piperazine	4-FPP
4-Methylphenylpiperazine	4-MePP
1-(4-Methoxyphenyl)piperazine	4-MeOPP
1-(3-Trifluoromethylphenyl)piperazine	3-TFMPP

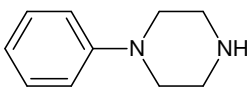
The structures of piperazine drugs not previously shown in Figures 1.2 and 1.3 are given in Figure 1.6



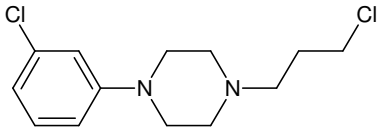
3,4-methylenedioxy-1-benzylpiperazine



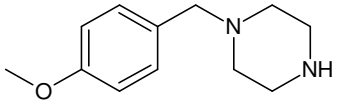
1-(4-bromo-2,5-dimethoxybenzyl)piperazine



1-Phenylpiperazine



1-(3-chlorophenyl)-4-(3-chloropropyl)piperazine



1-(4-methoxyphenyl)piperazine

Figure 1.6 Structures of illicit piperazine drugs MDBZP, 2C-B BZP, phenylpiperazine, 3-CPCPP and 4-MeOPP.

In 1943 the Wellcome Research Laboratories introduced BZP as an antihelmintic agent (Staack and Maurer, 2005; Elliot and Smith, 2008; King and Kicman, 2011; Barceloux,

2012). This was followed by research into its pharmacological use as an anti-depressant (Staack, 2007) and in the 1970s indications were that it induced psychoactive behaviour and had addictive properties similar to amphetamines (Staack and Maurer, 2005). This led to a discontinuation of the study trials due to perceived potential for abuse (Yeap et al., 2010). It can therefore be extrapolated that drug users took advantage of this information and furthermore that abuse of piperazine drugs started with 1-benzylpiperazine (BZP). The 1990s saw the use of BZP for recreational purposes as a rave drug marketed extensively over the internet. The drug was sold as a "herbal high", a term coined due to its similarity to piperidine (Figure 1.5) in black pepper (King, 2009; Dargan and Wood, 2013).

According to Arbo et al., (2012) the first documented abuse of BZP was in 1996 in the USA. In 2002 BZP was marketed as a legal alternative to methylamphetamine in New Zealand (Winstock and Ramsey, 2010) and its use became widespread. With the success of BZP as an illicit substance, a variety of other benzylpiperazines also came onto the illicit drug market such as 3,4-methylenedioxy-1-benzylpiperazine. The 2000s saw the emergence of phenylpiperazines (Staack, 2007; ACMD, 2011; King and Kicman, 2011). The most commonly used were reported as 3-CPP and 3-TFMPP. In 2004, 3-CPP was reported to be used in 26 member states of the EU (King 2009; Arbo et al., 2012).

3-TFMPP is a drug normally used as a pharmacological probe in animal drug trials (Elliot, 2011; Staack et al., 2003). Abuse of 3-TFMPP together with BZP was first reported in the late 1990s in the USA and Scandinavia (ECDD, 2012). In the 2000s its use became widespread in New Zealand, Australia, Europe, Japan and Bulgaria (Inoue et al., 2008; Nikolova and Danchev, 2008; Yuk, 2010; ACMD, 2011). Second to BZP, 3-TFMPP is the most abused piperazine drug (Staack et al., 2003; Staack, 2007; Yeap et al., 2010).

4-FPP was discovered as a metabolite of the pharmaceutical drug Niaprazine (Figure 1.7), an antihistamine with psychoactive properties (Kelleher et al., 2011). This could be the reason it has found use as a drug of abuse. 4-FPP was initially encountered in New Zealand during 1982 in party pills and its use increased in 2003 (Inoue et al., 2008; Vorce et al., 2008; Dargan and Wood, 2013). Comparatively, use of 3-TFMPP is more widespread than 4-FPP. This could be attributed to the fact that 3-TFMPP is found commonly in drugs containing BZP and it has been reported that BZP is one of the two most commonly used

piperazines. In addition, 4-FPP emerged relatively later on the market; as such the users are not as familiar with the drug.

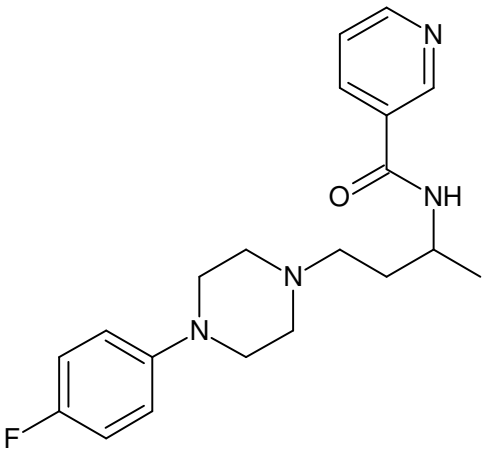


Figure 1.7 Structure of niaprazine

Currently, use of piperazines is reported to be spreading globally and increasing in many countries. (Takahashi et al., 2009; Kenyon et al. 2010; Yeap et al., 2010; Arbo et al., 2012; UNODC, 2013b; EMCDDA, 2014). As such, to gain an insight into the prevalence of piperazines, a review of the trends in abuse of these drugs is presented.

1.4 TRENDS IN USE AND ABUSE OF PHENYLPIPERAZINES

1.4.1 GLOBAL PERSPECTIVES

The use of new psychoactive substances has been widely reported globally including Australia, New Zealand, EU, and the USA. To obtain a more global perspective, in 2012 the UNODC carried out a survey on the global spread of NPS, in 80 countries in Europe, Asia, the Americas, Africa and Oceania. The results indicated that all the NPSs were widespread with 70 countries reporting the presence of NPSs. Europe had the highest rate of prevalence with 31 countries in the EU reporting the presence of an NPS (UNODC, 2013a). The global distribution of NPS by region is shown in Figure 1.8.

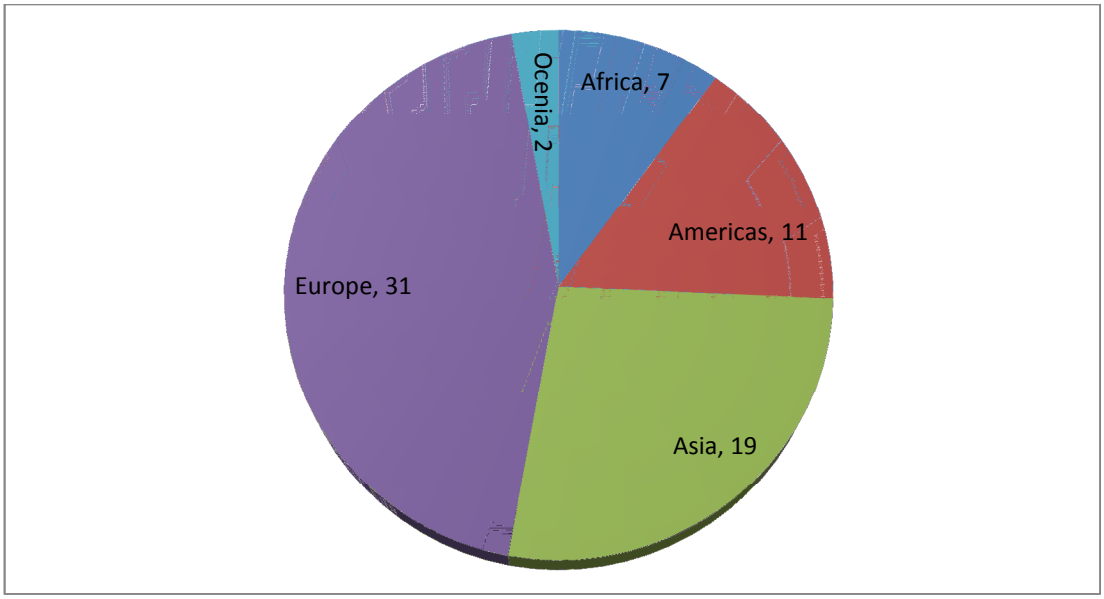


Figure 1.8 Global emergences of NPS by region, showing the number of countries reporting the presence of NPS by 2012, a total 70 countries (UNODC, 2013a).

Trends in drugs of abuse can be attributed to any of the following;

a) Easy availability to the end user, due to the internet and clubs.
b) Legislative controls
c) Cost of the street drug
d) Knowledge of the drug and/or its perception
e) Technology
f) Production
g) History of illicit drug use

Consequently, these are contributing factors to the global distribution observed in Figure 1.8. Historically, Europe has a high rate of illicit drug use (UNODC, 2013a; EMCDDA, 2013a; EMCDDA-Europol, 2012). whilst most developing countries, e.g. in Africa are emerging as drug users. Cost limitations and low technology could all be contributors. Furthermore, lack of information regarding use of illicit drugs in countries such as India, China and in Africa might mean the data is not exact. However, it has been reported that generally rates of drug abuse (consumption and production) are generally higher in Europe than in these countries, which confirms the trend in NPS in Figure 1.8. All the different types of NPSs were reported in all the regions except Africa (which at the present time had

no record of synthetic cannabinoids and phenethylamines). The global prevalence by the type of drug is shown in Figure 1.9.

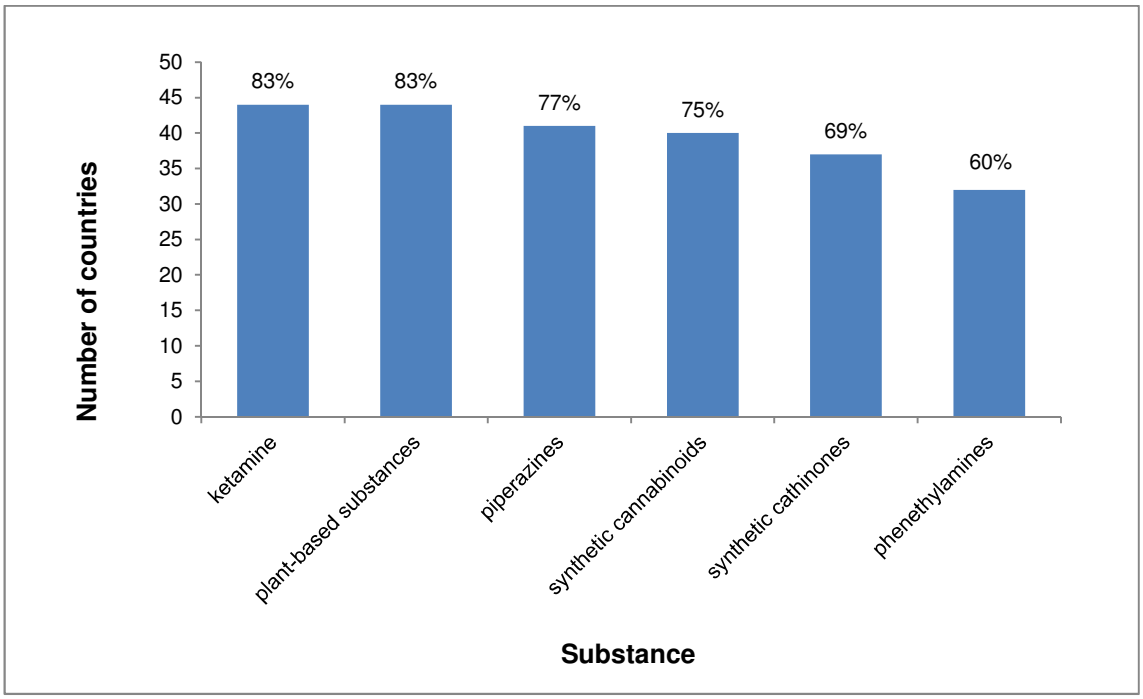


Figure 1.9 Global emergence of new psychoactive substance by type, showing the number of countries reporting the substance to UNODC up to 2012, also shown as a percentage of the countries studied (UNODC, 2013a).

Ketamine and plant-based substances were found to have the highest prevalence, each being found in 44 countries, (83% of the countries studied). The most common were kratom, khat and *Salvia divinorum*. Piperazines accounted for 77%, synthetic cannabinoids 75% and phenethylamines had the lowest at 60%. The implication of the results is that all the groups of NPS have become globally widespread. Furthermore, they confirm earlier reports that use and abuse of piperazines and other NPS has spread to several countries (Nikolav and Danchev, 2008; Takahashi et al., 2009; Elliot, 2011; EMCDDA, 2011). It is therefore suggested that the high spread of NPSs is largely attributed to their easy availability over the internet (Davies et al., 2010; King and Kicman, 2011) and also the aggressive and often misleading marketing of these substances. They are often marketed as legal and herbal highs. Furthermore, currently there is lack of adequate information on their adverse effects.

The lower prevalence of phenethylaminess can be attributed to the fact that legislation on MDMA and other amphetamines is more extensive. Amphetamine, methamphetamine and MDMA are listed in Schedule I of the United Nations 1971 Convention on Psychotropic Substance. Whilst cannabis and THC are controlled, none of the synthetic cannabinoids are under international control (Coulson and Caulkins, 2012; UNODC, 2014). This is likely the reason for their high prevalence. Furthermore, for the tryptamines only magic mushrooms; psilocin, psilocybin, DET, DMT, and etryptamine are under international control (UNODC, 2013c). None of the piperazines are under international control (legislation of piperazines will further be discussed in section 1.6). In, addition national controls on amphetamine and MDMA are more widespread than for the other drugs (Dargan and Wood, 2013; UNODC, 2013b). It has been reported that users view plant based substances as being herbal and therefore harmless (Dargan and Wood, 2013). Such perceptions are likely to have an impact on prevalence of these substances. Synthetic cathinones, cannabinoids and plant-based substances are also the latest in emerging NPS, consequently regulations and other pertinent information such as health risks and adverse reactions are still very scarce.

From previous discussions it can be seen that piperazines constitute a relatively significant share of the global NPS market. The question therefore arises as to which of the piperazines are mostly abused and whether 3-TFMPP and 4-FPP the drugs of focus in this study are a pertinent problem. Most regions reported the emergence of piperazines before 2012 (UNODC, 2013a). Figure 1.10 shows the global distribution of the top 5 most commonly reported piperazines in drugs of abuse seizures reported to the UNODC as per the 2012 survey (UNODC, 2013a).

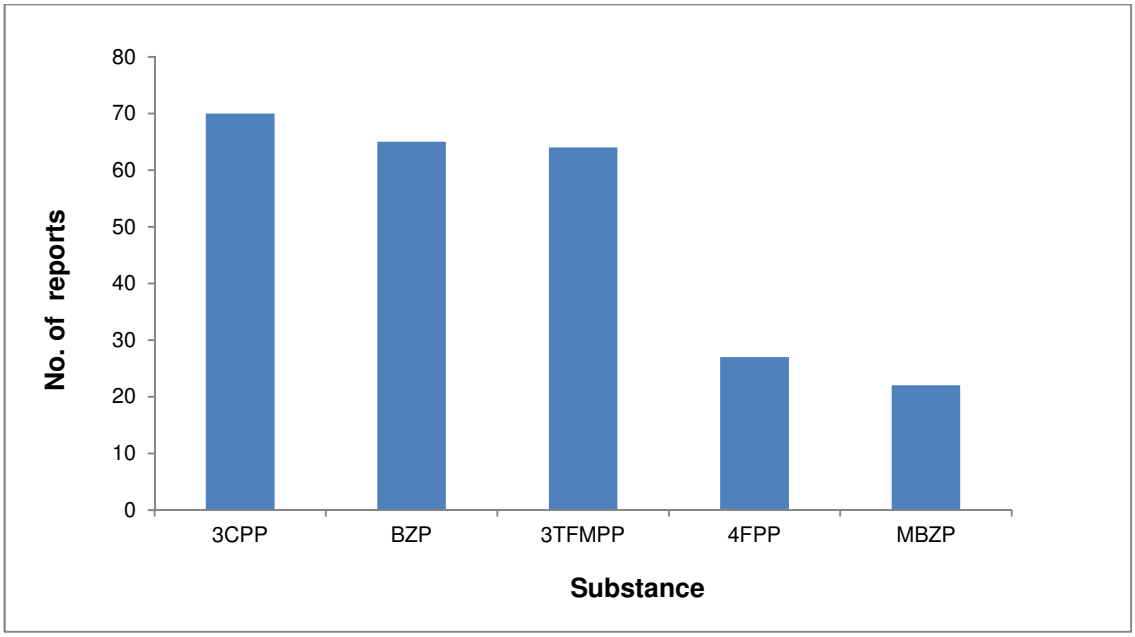


Figure 1.10 The top five piperazines reported to the UNODC up to 2012 (UNODC, 2013a).

3-CPP was the most commonly reported piperazine. It has been reported that by 2006 almost 10% of illicit pills sold in the European Union contained 3-CPP to mimic illicit ecstasy (Staack, 2007; King, 2009; EMCDDA, 2011). The data in Figure 1.10 is consistent with this view. The graph also shows that 3-TFMPP and 4-FPP the main drugs of focus for this research are a significant part of the piperazine based illicit drug market.

1.4.2 EU PERSPECTIVES

In the previous discussion, Europe was identified as having the highest prevalence of NPS globally. It has been reported that the United Kingdom is the country that identified the most NPS in the European Union accounting for 30 per cent of the total during the 2005 - 2010 period (EMCDDA-Europol, 2012; UNODC, 2013a). The situation in the UK is further shown Figure 1.11 for the number of substances by seizures analysed in the UK for phenethylamines (MDMA) and piperazines.

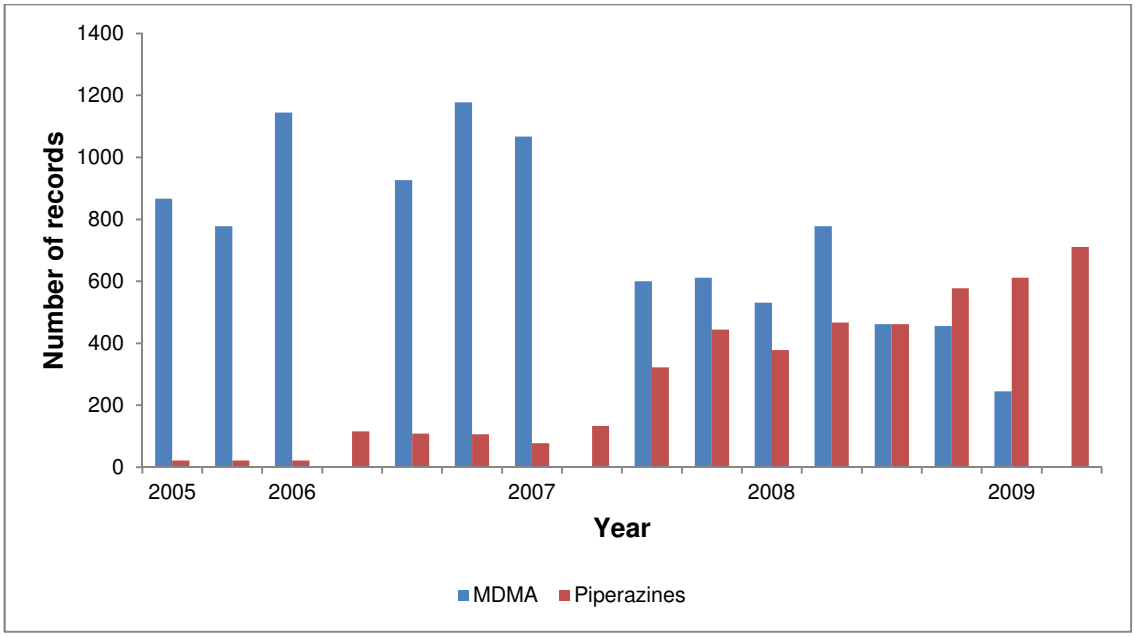


Figure 1.11 Number of seizure records for Forensic Science Services (Seizure date July 2005 - March 2010) (data from UK Focal point, 2012).

Figure 1.11 shows there was a general decrease in MDMA seizures from 2007 to 2009, comparatively piperazines increased from 2007 to 2009 then decreased thereafter. Data thereafter was not available. However reports for 2012 and 2013 have shown a re-emergence of piperazines; most countries such as UK, Belgium and Denmark reported ≥ 20% piperazines in the tablets they analysed (UK Focal Point, 2012, 2013; EMCDDA, 2014). Furthermore, the UK Focal point (2012) reported there was a resurgence of MDMA in 2011 with the decrease in other NPS. The trends shown in Figure 1.11, are also linked to the trends in other NPS such as cathinones. Whilst not shown in Figure 1.11 according to the EMCDDA (2011), prior to 2009 cathinone seizures were not significant but increased from 2009 such that by 2010 cathinone seizures were higher than those of piperazines and MDMA. The observed trends can mainly be attributed to legal controls on MDMA having resulted in the upsurge in piperazines. Thereafter, legal controls on piperazines in December 2009 (King and Kicman 2011), resulted in a drop in their prevalence. This created a market for other NPS as evidenced by the rise in cathinones and the resurgence of MDMA.

It has been discussed that prevalence of use or distribution is linked to street prices of the drugs (UK Focal Point, 2013). To try and evaluate whether the trends observed above are

linked to cost of the drugs, the street prices of new psychoactive substances in the UK are shown in Table 1.6.

Table 1.6 Prices of new psychoactive substances in the UK (2010 – 2013).

Drug	**Price per capsule or tablet**	**Source**
Amphetamines	£10 per gram	UK Focal point (2013)
Ecstasy	£4.00 per tablet £36 per gram (in 2010)	UK Focal point (2013)
Ketamine	£21 per gram	UK Focal point (2013)
Cathinones	£5.50 per tablet	Davies et al. (2010)
Piperazines	£3.30 – £5.85 per tablet	Davies et al. (2010)
Caffeine/ephedrine (no psychoactive drug present)	£2.50 – £3.40 per tablet	Davies et al. (2010)

The table shows that piperazines are cheaper than the other psychoactive drugs available. Considering they are marketed as ecstasy, this could be a contributing factor to their rise in use in the face of a decline of ecstasy. Cannabinoids and cathinones are relatively more expensive, however this could be because they are relatively newer on the market hence their high price and high prevalence.

The discussions above show that the use of piperazines in the UK is prevalent and growing. Hence, there is a need to keep up with not only their analytical investigation but also to gain an insight into their psychoactive properties due to their pharmacological effects.

1.5 PHARMACOLOGICAL EFFECTS OF PIPERAZINE DRUGS OF ABUSE

The perceived biological and bio-behavioural action of a drug determines how a drug is defined. Its pharmacological effects are the physiological and biochemical changes that it produces in the body. Psychoactive drugs are those drugs that modify our physiological traits such as behaviour and cognition (Brick and Erickson, 2013). Psychoactive substances are taken for their perceived stimulating effect; because they promote a sense of well-being and alertness among others. For example, amphetamine is associated with ‘feelings of increased confidence, sociability and energy (Barceloux, 2012).

The site of action of psychoactive substances is the Central Nervous System (CNS) and Peripheral Nervous System (PNS) in the brain, mainly the medial forebrain bundle known as the mesolimbic dopamine system (MDS) in the CNS (Elliot, 2011; Barceloux, 2012; Gee and Schep, 2013). Neurochemicals known as neurotransmitters naturally found in the brain cells (neurons) located in the MDS are stimulated and/or released during metabolism of drugs. These are released or inhibited as a natural part of the nervous system, to regulate the various bodily functions such as sleep, feeling and movement. Psychoactive drugs produce their effect by altering the activity of neurotransmitters in the brain. It is then the presence of the neurochemicals that promote feelings of well-being, craving, alertness etc. perceived with the drug (Barceloux, 2012; Brick and Erickson, 2013).

1.5.1 MECHANISM OF ACTION

In the pharmacology of drugs of abuse the most common neurotransmitters are dopamine, serotonin, norepinephrine (noradrenaline) and Gamma-aminobutyric acid (GABA). Dopamine is the neurotransmitter most affected by psychoactive drugs (Arbo et al., 2012; Elliot, 2011; Staack, 2007). Neurotransmitters are either inhibitory (-) (decrease), or excitatory (+) (increase) the probability of an action potential in the neuron. A drug may influence the activity of a neurotransmitter through the following mechanisms; (Staack and Maurer, 2005; Barceloux, 2012; Dargan and Wood, 2013);

- increase the release of a neurotransmitter.
- directly activate or block the neurotransmitter receptors.
- inhibit reuptake (transport) of the transmitter into the neuron, consequently keeping. it in the synapse where it is free to interact with receptors again.
- inhibit enzymes in, or near the neuron that would breakdown the neurotransmitter.

Drugs that directly activate receptors are agonists and those that block are antagonists. Figure 1.12 below shows a general mechanism of action of a neuron in response to a psychoactive drug.

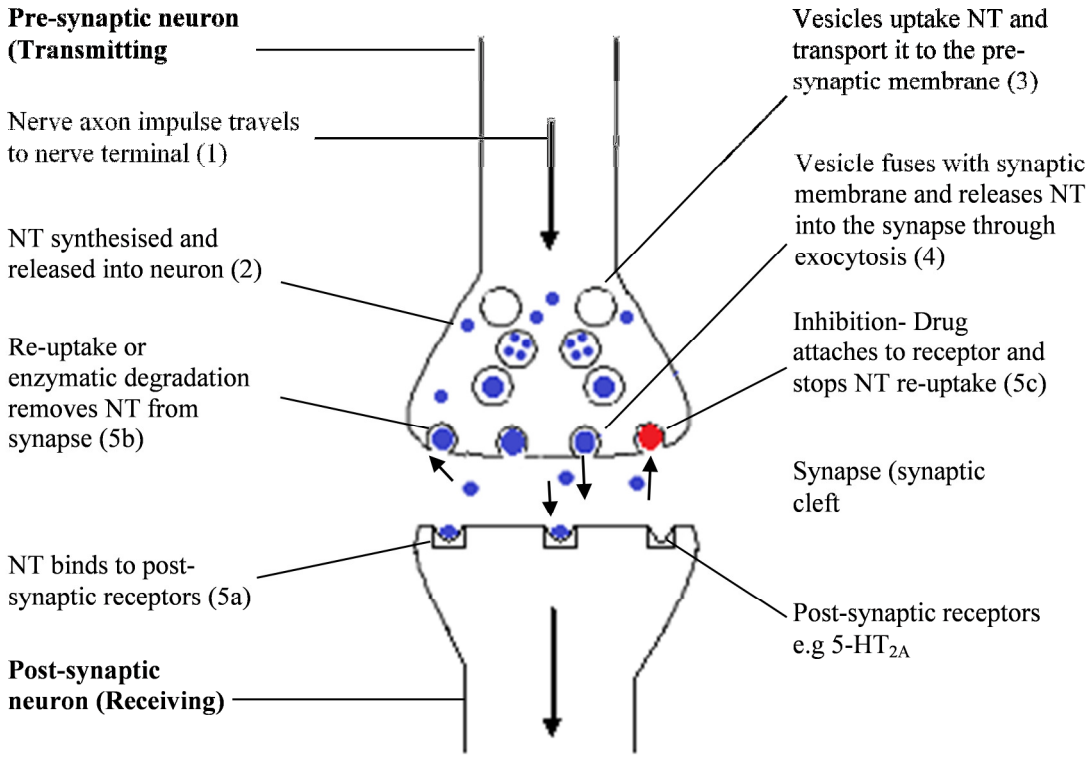


Figure 1.12 Schematic diagram of neurotransmitter mechanism in response to a psychoactive drug. The processes occur sequentially as numbered (1 – 5).

The concentration of the neurotransmitter in the synapse influences the degree of effects felt on the body's functions. Psychoactive drugs increase the concentration of neurotransmitters in the synapse, thereby deriving the associated functional effects of that neurotransmitter, such as increased alertness for noradrenaline. As shown in Figure 1.12; in the pre-synaptic neuron synthesis of the neurotransmitter (NT), e.g. dopamine, serotonin occurs. This is followed by uptake of the neurotransmitters into vesicles for transportation to the membrane. The vesicles fuse with the pre-synaptic membrane and release the neurotransmitters into the synapse. In the synapse, the neurotransmitter then a) diffuses to the post-synaptic membrane and binds to the post-synaptic receptors (e.g. 5-HT_{2A} for serotonin). This activates the receptors and consequently modulates the body's functions regulated by that neurotransmitter, resulting in the perceived psychoactive effects, such as an increased feeling of happiness b) the neurotransmitters in the synapse diffuse back to the pre-synaptic membrane for re-uptake or enzymatic degradation. This process is inhibited by antagonist drugs as they attach to the receptors, thereby blocking access to the neurotransmitter and consequently resulting in a build-up of neurotransmitters in the synapse (Dargan and Wood, 2013; Gee and Schep, 2013).

Therefore, different drugs of abuse produce different psychological effects, depending on the neurotransmitter they act upon. Drugs that act on adrenaline are adrenergic and those that work on serotonin are serotogernic, whilst dopaminergic drugs act on the dopamine pathway. However, they are drugs which due to their structural similarity to neurotransmitters mimic the actions of the neurotransmitter as they are able to bind to the neurotransmitter's receptors. Such drugs are sympathomimietic (Gee et al, 2005, DEA, 2011). Hence, drugs can be categorised (section 1.1) according to their pharmacological effects into stimulants, hallucinogens and depressants, e.g. piperazines, LSD and diazepam, respectively (Dargan and Wood, 2013; DEA, 2011; EMCDDA, 2013a). The pharmacological actions of selected drugs will be discussed below.

1.5.2 PHARMACOLOGICAL ACTIVITY OF SELECTED DRUGS

The pharmacological effects of selected psychoactive drugs of abuse and the responsible neurotransmitter functional activity are described in Table 1.7. Piperazines, amphetamines, cocaine and generally most of the drugs under study exhibit dopanergic, serotogernic and adregernic effects (Elliot, 2011; Dargan and Wood, 2013).

Table 1.7 Pharmacological effects of selected psychoactive drugs showing drug categories, the responsible neurotransmitter functional activity and drug effects (Arbo et al., 2012; Brick and Erickson, 2013; Elliot, 2011; Dargan and Wood, 2013).

Category/ definition	**Examples of selected drugs**[1]	**Main neurotransmitter/ Functions Affected**	**Drug Effects (sought by users)**
Stimulants Substances that increase the activity of the brain CNS and body processes	Most psychoactive drugs e.g. Amphetamine Methamphetamine MDMA Cocaine Benzylpiperazines e.g. BZP	*Dopamine (+) or (-)* Reward pathways, cognition (Mainly dopamine, lesser extent norepinephrine and serotonin). *Norepinephrine(-)* Fight or flight response, sensory processing, movement, sleep, mood, memory	Well-being, pleasure, euphoria, forgetfulness, stimulation, mental alertness, loss of reality, weight loss. Alertness, energy rush, hyperactivity, anxiety
	Most psychoactive drugs e.g. phenylpiperazines e.g 4-FPP	*Serotonin(±)* Mood regulation, sleep, sexual desire, appetite	Suppressed appetite, euphoria, arousal
Hallucinogens Substances that cause distortions in perceptions of reality	MDMA LSD Ketamine	Communication in the neural system is disrupted on various neurotransmitters that affect any of the body functions, e.g. sensory perception, sleep, hunger and muscle control	Distortion of the senses often with visual images, disorientation, intense mood swings
Depressants Substances that slow down brain activity, anxiety, memory, anesthesia	Benzodiazepines e.g. diazepam Barbiturates Opiods e.g. morphine	*Gamma-aminobutyric acid (GABA), (-)* Brain neural activity is slowed down	Relaxation, drowsiness, lethargy, pain relief, sedative effects

[1] Drugs that are part of/or impact on this research.

Amphetamines are mainly dopaminergic and are sympathomimetic; in addition they also potentiate the release of small amounts of norepinephrine and serotonin. Dopamine acts to regulate the body's reward pathways, cognition, movement, attention, memory (Brick and Erickson, 2013). An increase in dopamine is often associated with intense feelings of pleasure (Barceloux, 2012; Gee and Schep, 2013). Amphetamine is less potent than methamphetamine, but in uncontrolled situations the effects are almost indistinguishable (Barceloux, 2012; Brick and Erickson, 2013; Elliot, 2011). The reason why amphetamines are sympathomimetic is shown in Figure 1.13.

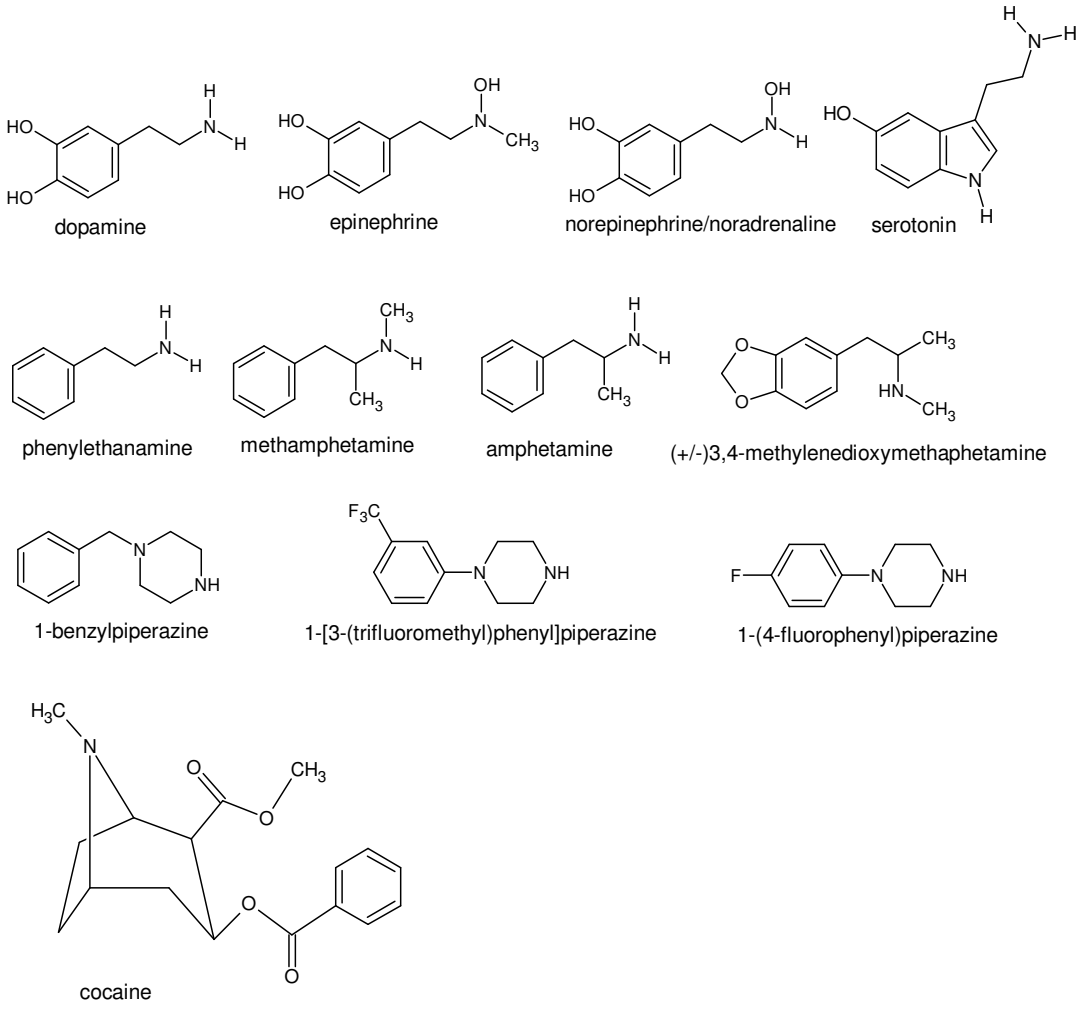


Figure 1.13 Structures of selected neurotransmitters comparative to some of the psychoactive drugs to determine similaties in structure to a neurotransmitter resulting in sympathomimetic traits (adapted from Gee et al., 2005; Barceloux, 2012).

It can be seen from the structures in Figure 1.13 that amphetamines, e.g. amphetamine, methamphetamine and MDMA contain in their structure the phenylethanamine moiety, which is present in catecholamines such as dopamine, epinephrine and noradrenaline.

Hence, they are structurally similar. However, cocaine acts in a different manner; it inhibits the re-uptake of dopamine. The mechanism of inhibition was shown Figure 1.12). This leads to an increase in dopamine. Since the dopamine remains in the synapse, it continues to exert its effect. The difference in its action in comparison with amphetamines may be due to the difference in structural similarity in comparison to the neurotransmitters mentioned above. Whilst cocaine does not contain the phenyethanamine moiety, it contains the benzyl group which is also present in these neurotransmitters. Consequently, these drugs are mainly dopanergic, they mimic the actions of nervous system that runs on the neurotransmitter epinephrine (Gee et al., 2005; Staack and Maurer 2005; Arbo et al., 2012) and the drug users experience the effects they seek (Table 1.7), e.g. increased alertness.

In comparison, piperazines are predominantly serotoninergic agonists (Elliot, 2011; Staack and Maurer, 2005; Gee and Schep, 2013). The use of piperazines as recreational drugs arises from their chemical properties as 1-arylpiperazines which give them the ability to bind to serotonin receptors in the human nervous system (Staack et al, 2003; Kenyon et al., 2010; Yeap et al., 2010; Gee and Schep, 2013). Serotonin (5-hydroxytryptamine) is also known as 5-HT. It has both inhibitory and excitatory (±) effects. Agonistic psychoactive substances such as piperazines and tryptamines potentiate the release of serotonin as per the mechanism described in Fig 1.12. The effects of the increase in serotonin (Table 1.7) are a distortion of the senses, arousal, disorientation, suppressed appetite, euphoria (Arbo et al., 2012; Barceloux, 2012). Piperazines to a slighter extent also increase dopamine thereby creating stimulating feelings of euphoria, alertness and social activeness. These properties give piperazines status as party pills (Yeap et al., 2010). However, they exhibit less potent properties than amphetamine (Elliot, 2011).

Animal studies have demonstrated that BZP unlike most piperazines is mainly sympathomimetic and dopaminergic. It stimulates the release and inhibits the reuptake of dopamine. However, to a lesser extent it also potentiates the release of serotonin and noradrenaline (Staack and Maurer, 2005; Elliot, 2011). BZP has 10% the potency of amphetamine. Active doses of BZP are 50 - 100mg (Nikolova and Danchev, 2008). Higher doses of BZP were found to increase pulse rate, blood pressure (systolic and diastolic) and pupillary dilation (Elliot, 2011; Arbo et al., 2012; Gee and Schep, 2013).

3-TFMPP it is a non-selective agonist at the 5-HT_{2A}, 5-HT_{1B}, and 5- HT_{2C} serotonin receptors in the brain (Elliot and Smith, 2008). Consequently, it potentiates an increase in serotonin levels. On its own it produces mild hallucinogenic and stimulus effects (Vorce et al., 2008, Staak, 2007, Barceloux, 2012). It has no dopaminergic effects and this might be the reason it exerts milder psychoactive effects when compared to BZP and amphetamines.

The psychoactive effects of 4-FPP are derived from its ability to raise serotonin and dopamine levels. In vitro studies (Nikolova and Danchev, 2008) have indicated that 4-FPP acts mainly as a 5-HT_{1A} serotonin receptor agonist. In addition, it also inhibits the reuptake of serotonin and norepinephrine. As a drug of abuse it produces very mild psychoactive effects. It is slightly stimulating, producing mildly hallucinogenic and euphoric effects. This may explain why it is commonly used in combination with other drugs, such as 'ecstasy'. The active 4-FPP doses are between 20mg - 150mg, higher doses cause a range of side effects including migraine headaches, muscle aches, anxiety, nausea and vomiting (Nikolova and Danchev, 2008, Elliot and Smith, 2008).

Caffeine an adulterant exerts sympathomimetic effects, however unlike amphetamines or piperazines it acts by antagonizing adenosine phosphate receptors in the brain, and may also inhibit the enzyme that breaks down cyclic Adenosine monophosphate (cAMP), consequently increasing cAMP and thereby phosphorylation of proteins and subsequent membrane activity of the neuron. Caffeine increases energy metabolism through the brain, hence the perceived alertness with caffeine use. However, it also decreases cerebral blood flow inducing brain hypo-perfusion, which may result in its analgesic effects. Nicotine stimulates 2 subtypes of the receptor for the neurotransmitter acetylcholine (Seymour et al., 2011; Barceloux, 2012; Brick and Erickson, 2013).

The effects of 3-CPP and MDMA are somewhat comparable, but unlike MDMA and BZP, 3-CPP has little effect on the dopaminergic system (EMCDDA, 2009). It can therefore be suggested that the ability of BZP to raise dopamine unlike the phenylpiperazines 3-TFMPP and 4-FPP is linked to its structural similarity to phenylethylamine (Figure 1.13), a structural moiety found in dopamine as is the case with amphetamines and cocaine. It can be seen in Figure 1.13 that BZP like cocaine contains the benzyl group which is absent in phenypiperazines. The benzyl group is present in the neurotransmitters dopamine,

serotonin and noradrenaline. It is therefore suggested that this structural similarity results in its dopaminergic properties.

It has been discussed that clandestine drugs are commonly found in combination in street samples as is the case with 4-FPP and 3-TFMPP (Section 1.2.2 above). As such, there is possibility of pharmacological drug-drug interactions. Drug-drug interactions can be (Elliot, 2011; Arbo et al., 2012; Dargan and Wood, 2013) a) Additive- the drug combination exhibits an effect equal to the sum effect of each drug, b) Synergistic (potentiation) - drug combination exhibits an effect greater to the sum effect of each drug. For example, the combination of BZP and 3-TFMPP is synergistic, giving it its enhanced effects not felt with either drug individually. Hence, the combination is perceived to mimic the psychoactive effects of 'ecstasy'. This is due to the fact that BZP is dopaminergic whereas 3-TFMPP is mainly serotonergic, therefore in combination there is an increase in serotonin and dopamine levels. According to Vorce et al., (2008), clandestine laboratories have exploited this synergy in such a way that both 3-TFMPP and 4-FPP are now commonly encountered in illicit drugs combined with other psychoactive substances such as BZP or cocaine, and c) antagonistic- drug combination exhibits an effect less than the sum effect or individual effect of each drug; one drug competitively blocks another by occupying the receptors.

1.5.3 METABOLISM OF PIPERAZINE DRUGS OF ABUSE

Metabolism of piperazines is by cytochrome P450 (CYP) and its isoenzymes which results in glucuronidation and/or sulfation (Elliot, 2011, Barceloux, 2012). The metabolism of 3-TFMPP was studied by Staack, et al. (2003) and showed that TFMPP was extensively metabolized mostly by CYP2D6 and to a minor extent by the isoenzymes CYP1A2 and CYP3A4 (Figure 1.14). This is through hydroxylation of the aromatic ring and degradation of the piperazine moiety to mostly the metabolites N-(hydroxy-3-trifluoromethylphenyl)ethylenediamine (IV), N-(3-trifluoromethylphenyl)ethylenediamine (III), 3-trifluoromethylaniline (V), and hydroxy-3-trifluoromethylaniline (VI). Similar studies on BZP (Arbo et al., 2012; Staack et al., 2003) identified that BZP was metabolised by cytochrome P450 and subsequent catechol-O-methyltransferase catalysed methylation to N-4-hydroxy-3-methoxy-BZP. Subsequent degradation follows to produce the metabolites 4-hydroxy-BZP, 3-hydroxy-BZP, piperazine, benzylamine and N-benzylethylenediamine

occurs. Comparatively 3-TFMPP is almost exclusively metabolised prior to renal excretion (98 – 99%), whilst BZP is excreted mostly un-metabolised. This may have an effect on the toxicity of the drug metabolites and also on analytical investigation, since the detection of 3-TFMPP in subjects is conducted by testing for its metabolites whereas for BZP this is conducted through the BZP molecule itself (Elliot, 2011; Gee and Schep, 2013). The metabolism of phenylpiperazines is shown in Figure 1.14 for 3-TFMPP as an exemplar (Staack and Maurer, 2003; and Barceloux, 2012). The metabolism of 4-FPP has not yet been reported. It is therefore proposed that 4-FPP is similarly metabolised to TFMPP since studies on BZP and 3-TFMPP (Staack et al., 2003; Staack and Maurer, 2005; Arbo et al., 2012; Barceloux, 2012) have shown similar routes of metabolism. Furthermore effects of the drug whilst mild were similar (section 1.5.2).

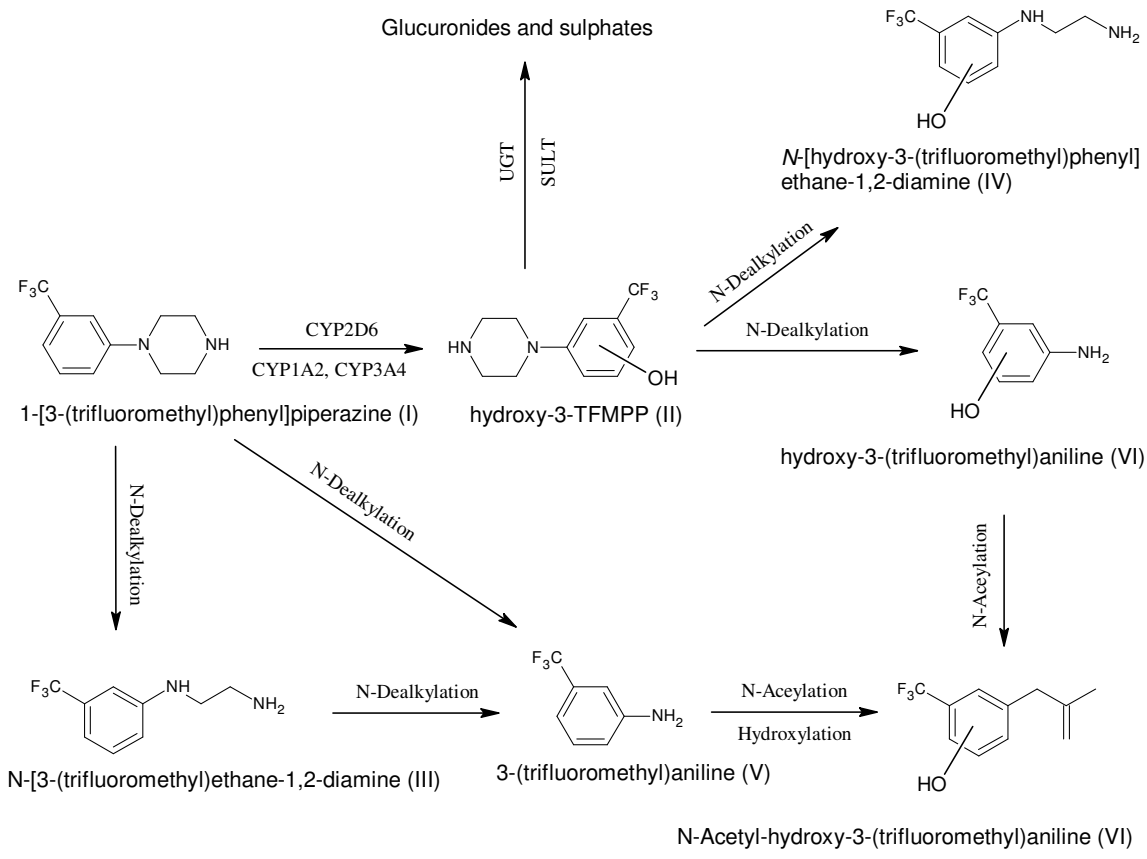


Figure 1.14 Metabolism of 3-TFMPP (Adapted from Staack and Maurer (2005) and Barceloux (2012).

1.5.4 EFFECT OF FLUORINATION ON PHARMACOLOGICAL ACTIVITY

The presence of fluorine in a compound alters the binding affinity to receptors and the metabolism of the compound. Fluorine has been shown to improve target receptor selectivity and or binding properties, metabolic stability, body distribution and excretion of phenethylamines (Trachsel, 2012). According to Filler and Saha (2009) fluorine has the advantage that it increases lipid solubility, thereby enhancing rates of absorption and transport of drugs *in vivo*. In addition the C-F bonds are stronger than C-H bonds which imparts stronger oxidative and thermal stability. It has been reported by Schifano et al. (2015) that fluorination of synthetic cannabimimetic compounds may increase the lipophilicity of the compound, which enhances their absorption through biological membranes/blood brain barrier. This is due to the strongly negative inductive effects of fluorine which influences their acidity/basicity. Fluorine substitution increases the acidity of drugs which are organic acids and decreases those which are basic such as amines including amphetamines and piperazines (Jones 1982, Park et al., 2001). However this is also affected by other substituents on the compound. 3-TFMPP unlike 4-FPP contains a trifluoromethyl group, which decreases its basicity due to the electron withdrawing properties of the methyl group. This affects their binding affinities to the 5-HT_{2A} receptors. This could be the reason why 3-TFMPP has stronger psychoactive effects than 4-FPP. Trachsel (2012) reported that studies showed indications that $5HT_{2A}$ receptor affinities might be enhanced by the introduction of the methyl group. Furthermore, Filler and Saha (2009) reported that the trifluoromethyl group is more lipophilic than the methyl or chloro group and this significantly increased its pharmacological activity

1.5.5 ADVERSE EFFECTS OF PIPERAZINE DRUGS OF ABUSE

Adverse effects of drugs of abuse drugs as amphetamines and BZP is a topic that has been studied by many researchers (Elliot, 2011; Gee and Schep, 2013; Staack, 2007; Staack and Maurer, 2005; Yeap et al., 2010). However, studies pertaining to 3-TFMPP and 4-FPP are limited, as these are relatively new psychoactive substances. Furthermore, according to Arbo et al. (2012) although in the market piperazine designer drugs have the reputation of being safe, several experimental and epidemiological studies indicate risks for humans. Adverse reactions to piperazines include vomiting, headache, palpitations, anxiety, insomnia, confusion, irritability and tremors (Davies et al., 2010; Yeap et al., 2010; Elliot,

2011). They exhibit effects typical of a serotonin syndrome; anxiety, dizziness, confusion, shivering, sensitivity to light and noise, fear of losing control, migraine and panic attacks. Amphetamine leads to insomnia, users may feel irritable, restless, anxious, depressed and lethargic. It has been reported that chronic use of amphetamines may cause such adverse toxic effects as psychosis and schizophrenia (Brick and Erickson, 2013). Since piperazines have similar pharmacological activity to amphetamines it can be assumed similar chronic adverse effects are also realised with their prolonged use.

'Drug-drug' interactions (section 1.5.3) can generate adverse effects. The BZP/TFMPP combination has been reported to cause a range of side effects including dehydration, seizures, jaw-clench, mild to severe nausea, vomiting, toxic psychosis (panic and extreme paranoia), high blood pressure, persistent headache, flu-like symptoms, stiff neck, post-trip exhaustion, impotence, anxiety, migraine muscle aches, as well as a come-down syndrome' (Nikolova and Danchev, 2008; Elliot, 2011). Adulterants such as caffeine and nicotinamide may also cause adverse effects. It has been reported that caffeine causes nervousness, diuresis, and insomnia among others. Nicotine results in craving, drowsiness, bad dreams, headaches, depression, increased appetite among others (Seymour et al., 2011; Barceloux, 2012; Brick and Erickson, 2013). Davies et al., (2012) in their study based on the risks associated with caffeine in "illegal highs" found there was a risk of significant caffeine toxicity related to the high caffeine content of some novel psychoactive substances. The authors reported caffeine content in street samples of up to 940mg per tablet. Furthermore, it was indicated that doses >200mg can lead to adverse effects such as anxiety and agitation.

However, these drugs are marketed as alternatives to MDMA and LSD and are misleadingly considered safe, being described as 'drug harm minimisation solution' (Yeap et al., 2010; Davies et al., 2010). Contrary to this, there have been a reports of deaths where have piperazines been implicated in Europe (Elliott and Smith, 2008; EMCDDA, 2009); Elliott, 2011).

1.6 LEGISLATIVE CONTROL OF 3-TFMPP AND 4-FPP

Drugs listed under the UN Convention are subject to global controls whilst those not listed under any international legislation can be subject to the national controls existing in

different countries. These results in different classification and legislative control of drugs, hence a drug might be illegal in one country whilst legal in another and as such can be easily sourced from these countries. This therefore can have a negative effect on controlling the substances.

Currently, there are no international controls for any piperazine based drugs of abuse. None of the piperazines are listed in the United Nations 1971 Convention on Psychotropic Substances (DEA, 2011; Coulson and Caulkins, 2012; UNODC, 2013a). However, several (BZP, TFMPP, 3-CPP, MDBP) were pre-reviewed by the WHO Expert Committee on Drug Dependence in 2012 (UNODC, 2013a). This might in the future lead to stricter controls internationally.

Not all countries currently control piperazine based drugs. This is potentially a contributing factor to the popularity and widespread use of these drugs. As a result of the increasing trend in the use of piperazine drugs, risk assessments were conducted, for example in the EU by the EMCDDA in 2008; as a consequence piperazines came under EU-wide control after 2008 (UNODC, 2013). BZP is now controlled in the European Union and several other countries such as New Zealand and Japan. In New Zealand as of October 2008; BZP, TFMPP, 4-FPP, MBZP MeOPP are all Class C controlled drugs (King, 2009; Arbo et al., 2012). Control of 4-FPP and 3-TFMPP is not widespread (Nikolova and Danchev, 2008; ECDD, 2012). However, in most EU countries, 3-TFMPP is controlled including Belgium, Denmark (December 2005), Greece, Estonia, Italy, Lithuania, Malta and Sweden (March 2006). Outside of the EU 3-TFMPP is controlled in Japan (October 2003), Bulgaria, Australia and (Nikolova and Danchev, 2008; ACMD, 2011; Coulson and Caulkins, 2012; ECDD, 2012; UNODC, 2013a). Legal controls on 4-FPP are even less prevalent than for 3-TFMPP.

With reference to the UK, in December 2009 piperazines drugs became listed under the Misuse of Drugs Act 1971 (Amendment) Order 2009 (Acts of Parliament, 2009; EMCDDA, 2013a); BZP and the all other psychoactive piperazine derivatives were listed in Part 3 of the Schedule as Class C drugs (Acts of Parliament, 2009; King 2009; ACMD 2011). In the UK, controlled substances are listed in Schedule 2 of the Misuse of Drugs Act and are categorised into three classes according to the severity of perceived potential health risks to humans due to the use of the drug. The drugs in class A are those that are high risk,

such as cocaine and heroine and class C contains the low risk group. The scheduling of NPS in the UK is shown in Table 1.8.

Table 1.8 UK controls of new psychoactive substances, NPS (Acts of Parliament, 2009; Dargan and Wood, 2013).

Substance	**Year controlled**	**Class**
Cannabinoid agonists	2009	B
Cannabinols	1971	B
Cathinones	2010	B
Fentanyls	1986	A
Phenethylamines	1977	A
Phenyl and benzylpiperazine	2009	C
Tryptamines	1977	A

It can be seen that all piperazine based drugs of abuse are in class C; this includes BZP, 3-TFMPP and 4-FPP. However, amphetamines are in class B (Acts of Parliament, 2009; ACMD, 2011). It was highlighted in section 1.5 that the whole purpose of manufacture of piperazine based drugs is that they mimic the psychoactive effects of amphetamine type drugs. In addition, drugs such as 3-TFMPP and 4-FPP are not used alone but in combination with other drugs. As such, the following implications can be drawn; the lack of more comprehensive controls imparts the sense that the drugs are harmless (Davies et al., 2010; Yeap et al., 2010). Furthermore, scheduling piperazine based drugs in lower class than amphetamines gives the sense that these are safer than MDMA and other amphetamines. This gives the potential for a growth in use and clandestine synthesis of piperazine drugs, especially in view of the increasing market for NPS (section 14) and the fact that the precursors and chemicals (section 1.7) used in the manufacture of these drugs are not controlled. Precursors for the synthesis of amphetamine; 1-phenyl-2-propanone, norephedrine and norpseudoephedrine are controlled, listed in Table I of the United Nations 1988 Convention Against Illicit Traffic in Narcotic Drugs and Psychotropic Substances (UNODC, 2013). The corresponding EU legislation is set out in Council Regulation (EEC) No 3677/90 (as later amended), which governs trade between the EU and developing countries. This could have been a major contributing factor in the decrease of

phenethylamines on the illicit market. Such controls can also have a similar impact in curbing abuse of piperazines if instigated.

The selling, marketing and easy availability of piperazines and other new psychoactive substances such as cathinones and tryptamines openly on the internet, is a challenge to legislation, as these substances can be sourced from off-shore sources were they might be legally available. They may also be purchased from non-UK internet sites and then supplied from overseas, both EU and non EU (ACMD, 2011). The likely reason for the lack of more comprehensive controls on piperazines globally is because they are relatively new substances on the market (section 1.3). As such, there is lack of adequate information pertaining to their analytical properties including their safety. This can be seen by the case of 3-TFMPP in the USA where it was scheduled in 2002 and then later unscheduled in 2004 due to inadequate foreseen risk to users, such that it is currently uncontrolled in the USA (Coulson and Caulkins, 2012).

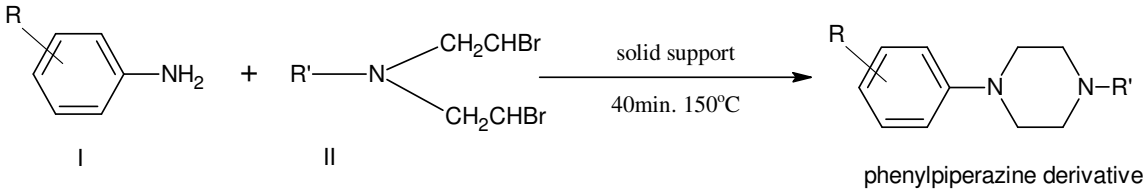
1.7 SYNTHESIS OF 4-FPP AND 3-TFMPP DRUGS OF ABUSE

It is discussed in sections 1.1 and 1.8.3 that information on routes of synthesis, precursors and impurities can provide an insight into links between illicit drug samples. This has been identified as pertinent to characterisation of street drugs (UN, 2001). Since this research will characterise street samples, it is therefore imperative to identify potentially viable routes for clandestine synthesis of phenylpiperazine drugs of abuse using 4-FPP and 3-TFMPP as their representatives. Sources of literature on clandestine methods of synthesis were found to be both scientific (peer reviewed literature) and the informal (the internet websites not peer reviewed). According to the UNODC (2013c) and EMCDDA (2009) no clandestine synthesis of piperazines has been reported. A review of literature on piperazines confirmed this observation. However, according to ACMD (2011) chemists responsible for the development of new illicit drugs have a sophisticated knowledge of the chemical/pharmacological scientific literature. As such both the scientific and informal literature were reviewed as potential sources of methods for the illicit manufacture of 4-FPP and 3-TFMPP.

Early studies (Baltzly et al., 1944) on synthesis of piperazines concentrated on BZP and its derivatives. The study detailed the preparation of mono-substitued piperazines in moderate

yields by reaction of piperazine with acylating or alkylating agents and has become a foundation for current research on other piperazines such as that published by Cymerman and Young (1962) on the synthesis of BZP from piperazine and benzoyl chloride. In addition, some of the routes of clandestine synthesis of piperazines were found to be similar to those of Baltzly et al., (1944). For example, the EMCDDA (2009) and UNODC (2013c) reported that clandestine synthesis of BZP involved the reaction of piperazine monohydrochloride with benzyl chloride. In addition, the reaction also formed another psychoactive substance 1,4-dibenzylpiperazine (DBZP) as a side-product. The precursors were readily available, or could be easily manufactured from commercially available chemicals. In the same reports another piperazine, 3-CPP is also stated as being manufactured from piperazine with m-dichlorobenzene. These routes of synthesis can be extrapolated for the synthesis of other benzyl and phenylpiperazine drugs.

Several studies have been reported on the synthesis of phenylpiperazines, the focus of which was on synthesis of the drugs as pharmaceutical intermediates (Pollard and Wicker, 1954; Kiritsy et al., 1978; Mishani et al., 1996; Liu and Robichaud, 2005). These methods mainly included acylation, alkylation, and cyclisation and were found to vary both in the complexity of reaction methods and reagents used. Common precursors were identified as piperazine, anilines, benzene and haloamines (Figures 1.15 – 1.17). It is worth noting that of the few published scientific methods for the preparation of 3-TFMPP and 4-FPP, most of these were for 3-TFMPP (Kiritsy et al.; 1978; Liu and Robichaud; 2005; Mishani et al., 1996). Taking into consideration the complexity of these methods, availability and cost of the precursors it can be deduced that it is most likely some of these methods of synthesis can be extrapolated for clandestine synthesis. Below is a further review of these methods.
Mishani et al., (1996) described the synthesis of the phenylpiperazine ring by the reaction of aniline derivatives with (2-bromoethyl)-N-(alkyl) amine on an aluminium solid support. The reaction is shown in Figure 1.15.



I = a fluoroaniline compound

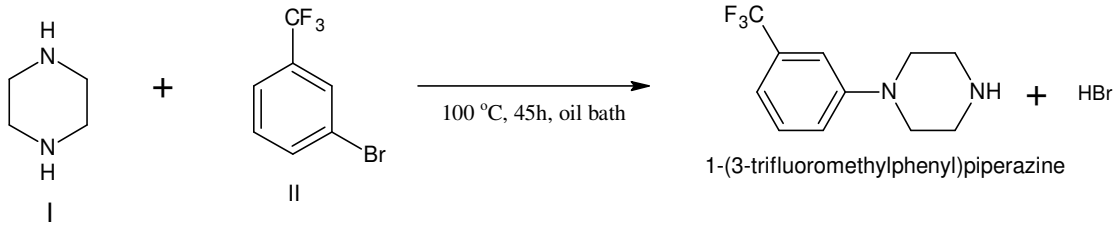
II = 2-bromoethyl)-N-(alkyl)amine

for 3-TFMPP: R = CF_3, R' = H

Figure 1.15 Scheme for the synthesis of 1-(3- trifluoromethylphenyl)piperazine (Mishani et al., 1996).

The method is described as rapid and the yield high. The authors stated 80% yield of 3-TFMPP. Whilst the reaction scheme is shown for 3-TFMPP the authors reported that the route of synthesis can be used for any phenylpiperazines. As such, it can be used to manufacture other psychoactive drugs such as 3-CPP, 4-MePP and 4-FPP by changing the substituent on the aniline (I) in Figure 1.15.

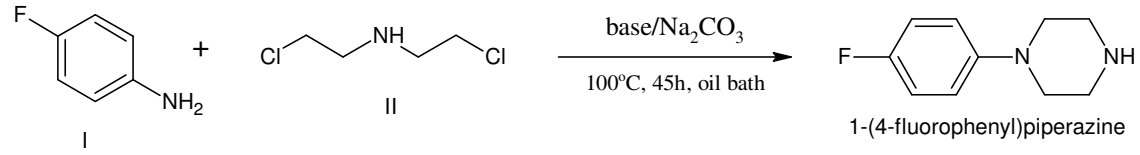
A study by Kiritsy et al. (1978) prepared 3-TFMPP as intermediates during the synthesis of pharmaceutical substances using piperazine and a benzene derivative, with comparable yields. The authors stated a yield of 81.2% of 3-TFMPP. The same authors also investigated the synthesis of 4-FPP from fluoroaniline and bis-2-chloroethylamine. However, this method gave low yields and was reported to yield 30% 4-FPP. The reactions are shown in Figure 1.16a and 1.17b for 3-TFMPP and 4-FPP respectively.



I = piperazine

II = 3-bromo-1-(trifluoromethyl)benzene

Figure 1.16a: Scheme for the syntheses of 1-(3-trifluoromethylphenyl)piperazine (Kiritsy et al., 1978; Shaman Australis Botanic 2003).

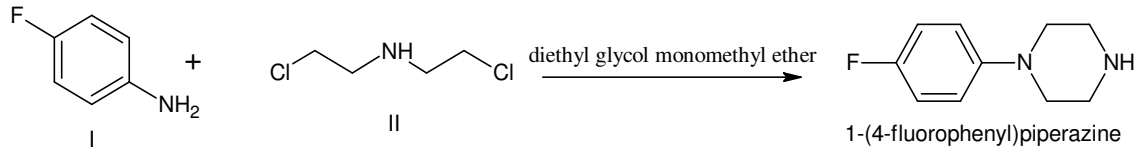


I = 4-fluoroaniline

II = Bis(2-chloroethy)lamine

Figure 1.16b: Scheme for the syntheses of 1-(4-fluorophenyl)piperazine (Kiritsy et al., 1978; Shaman Australis Botanic, 2003; ECDD, 2012).

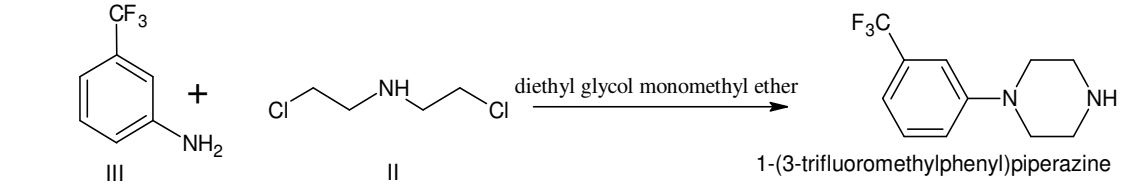
In 2005, a general method for the synthesis of N-aryl piperazines from anilines was developed by Liu and Robichaud (2005); the reaction is shown in Figure 1.17a and 1.17b for 4-FPP and 3-TFMPP respectively below. According to the authors the method gave yields of 87% 4-FPP and 67% for 3-TFMPP.



I = 4-Fluoroaniline

II = Bis(2-chloroethyl)amine

Figure 1.17a: Scheme for the synthesis of 1-(4-fluorophenyl)piperazine piperazine (Liu and Robichaud, 2005; EMCDDA, 2009).



III = 3-trifluoromethylaniline

Figure1.17b: Scheme for the synthesis of 1-(3- trifluoromethylphenyl) piperazine (Liu and Robichaud, 2005; EMCDDA, 2009).

Comparison of the methods reveals that the studies by Kiritsy et al. (1978) and Liu and Robichaud (2005) give high yields. The methodology is relatively easier and cheaper compared to that of Mishani et al., (1996) which requires a solid support. As such, these are the most likely methods to be used in clandestine synthesis of these drugs. However, the

route of synthesis for 4-FPP by Kiritsy et al. (1978), Figure 1.16b is unlikely to be of use due to the low yields (30%) generated. The highest yield encountered by any of the methods was 81.2%. This implies that impurities are present in the final product. These could be residual precursors and by-products. Consequently, if no further purification of the product is conducted it implies that the drug on the street will have impurities in it. This could have health implications and as such it is important that these are identified and be of use in characterising and profling street drugs.

This review on synthesis also highlights the prevalence of informal sources of literature (encountered on the internet) on manufacture of these drugs such as Erowid, Shaman Australis and many other drug forums/chartrooms. According to these sources, the drugs can be manufactured through several routes; a) the reaction between diethanolamine with 3-chloroaniline b) the reaction between 3-chloroaniline with bis(2-chloroethyl)amine c) the reaction between piperazine with 3-dichlorobenzene (EMCDDA, 2009). According to Shaman Australis (2003) all known psychoactive piperazines can generally be derived from piperazine and benzyl chloride or a substituted form of benzyl chloride. The precursors were identified to be the same as those in the methods published by Kiritsy et al. (1978) and Liu and Robichaud (2005), as was highlighted in Figures 1.17a and 1.18 respectively.

The methods of synthesis reported by the EMCDDA (2009) and UNODC (2013c) are a distinct possibility in illicit laboratory synthesis, as the materials are easily available and the methods are not complex. The EMCDDA further suggests that currently there have been no reports of clandestine synthesis of 3-TFMPP and 4-FPP, as currently these drugs are available from commercial sources such as chemical suppliers (ECDD, 2012). It was found that whilst the UNODC acknowledges that the routes of synthesis are simple and product yields high. It shares the same view as the EMCDDA as according to the UNODC it is unlikely BZP, 3-CPP or 3-TFMPP found in illicit products have been synthesised clandestinely. However, this approach fails to acknowledge the significance of the internet, where methods for the preparation are freely available. Furthermore, the impacts of the recent regulations controlling these drugs may drive the distributors and users into clandestine manufacture. This view is supported by Haroz and Greenberg (2006), who in their study state that, ‘‘*information accessibility through the internet has facilitated accessibility, synthesis and production of substances by individuals*’’. In addition, most of the designer drugs on the market exist because of synthetic approaches published in the

open scientific and medical literature and the exploitation of this research by clandestine manufacture of drugs for illicit purposes is drawn (King and Kicman, 2011).

The ECDD (2012) reported a single existence of clandestine laboratory for the manufacture of 3-TFMPP. However, no details were available as to the origin of this claim, precursors or route of synthesis. The ECDD further indicates that whilst specific information is not available, chemical synthesis is likely to involve piperazine. This was found to be similar to BZP synthesis (Cymerman and Young, 1962). In addition it indicated that TFMPP is available from retail chemical suppliers (ECDD, 2012). Consequently, if the method does use the precursors stated then it is similar to that of Kiritsy et al. (1978) (Figure 1.16a).

As such the methods by Kiritsy et al. (1978) (Figure 1.16a) and Liu and Robichaud (2005) (Figure 1.17a, b) were identified as potential routes of illicit synthesis. Hence these methods were adapted for use in this research.

1.8 REVIEW OF ANALYSIS METHODS AND TECHNIQUES

The analysis drug of abuse is carried out for the purpose of identification, quantitation, characterisation and impurity profiling. However in all the cases specific identification of the drug is paramount before further testing such as quantification can be carried out. A variety of benzylpiperazines and phenylpiperazines have been reported in street samples (King, 2009). In addition, isomers may also exist for example the (2, 3, 4) positional isomers of CPP, FPP and TFMPP (Takahashi, 2009; UNODC, 2013a; Gee and Schep, 2013). This results in a complex sample matrix and this poses a challenge to the identification and accurate quantification of these substances. As such, there is need for the method of analysis employed to be highly specific so as to selectively analyse for the target compound.

Currently, there exists a variety of methods, techniques and tests for the analysis of drugs of abuse. However, studies pertaining to the analysis of 3-TFMPP and 4-FPP are currently limited. This is likely to be due to the substances being relatively new on the market as previously discussed (section 1.3). Most of the analytical methods published were found to be on screening, detection and quantitation of piperazines, based on metabolic and toxicological studies of urine and blood samples and not on the drug in its original form

(Staack et al., 2003; Elliot and Smith, 2008; Vorce et al., 2008; Elliot, 2011). This further implies the need to develop methods not for biological samples but for the analysis of the drug as is in street samples.

1.8.1 IDENTIFICATION METHODS

The process of chemical identification often involves the application of two categories of qualitative techniques, i.e., preliminary screening tests and confirmatory tests. American systems apply two or more independent tests depending on the test used, i.e., use of only one confirmatory test would require one or more less conclusive techniques (DEA, 2011). Given the diversity of drugs and methods screening tests provide a means of narrowing the scope and focusing the direction of the analysis. Hence, further confirmatory tests for the unknown substance are more easily identified. According to the UNODC (2013c) a screening test is defined as a preliminary test which is used to indicate or eliminate a class or group of drugs. For this reason screening tests have become common in routine analysis of unknown drugs (Baker and Phillips, 1983; UN, 1994) and have been applied to piperazines (Takahashi, 2009; UNODC, 2013b). They entail a) physical tests such as appearance, melting and boiling point, b) colour tests and c) thin layer chromatography (TLC).

1.8.1.1 Presumptive tests

Colour tests are used as part of presumptive tests for drugs to distinguish between different chemical classes of compounds. They have good sensitivity, with limits of detection in the range 1 – 50µg (Takahashi et al., 2009; O'Neal et al., 2010). As such, they provide a guide to the identity of a drug. Consequently, these tests will be sensitive enough for use in this study as the analytes have been identified to exist in relatively high doses (mg/tablet range) in the street samples (Davies et al., 2010; Kenyon et al., 2010). However, they cannot specifically confirm the identity of a particular substance and as such are used in conjunction with more confirmatory tests such as Gas chromatograph (GC) or Fourier Transform Infrared Spectrometry (FTIR) techniques (section 1.8.1.2).

Even though presumptive analysis is common in analysis of drugs of abuse, it was found that studies involving presumptive analysis of piperazines are very few. Of the studies found the tests applied were the Marquis, Simon's reagent and Dragendorff tests. Inoue et

al., (2004) in their study applied the Marquis, Simon's reagent and Dragendorff tests for the identification of piperazines. This study set the ground work for presumptive analysis of piperazines. It is likely the basis of the presumptive tests recommended by UNODC (2013c) for the analysis of piperazine drugs. In the study these tests were able to discriminate between amphetamines and piperazines. The UNODC recommends the use of Marquis, Simon's reagent and Dragendorff tests for piperazines. Philip et al., (2013) developed a novel presumptive test for piperazines using sodium 1,2-napthoquinone-4-sulphonate (NQS). The authors reported that the test can distinguish BZP from its analogues and piperazines from most of the analytes tested including amphetamines. This gives it an advantage comparative to the other tests. Currently there is no presumptive test specific to piperazines and none so highly selective for BZP. However, since the test is novel it has not yet had wide application. The concepts and reactions behind the presumptive tests that will be applied in this research will be further discussed in the chapter for theoretical concepts (Chapter 2 section 2.8.2).

In contrast to piperazines, preliminary screening of MDMA, other amphetamine drugs and cocaine has been more extensive (Cole, 2003; Baker and Phillips, 1983; Dargan and Wood, 2013). Consequently, since these substances will potentially be present with 4-FPP and 3-TFMPP the screening tests for amphetamines will thus provide useful comparative data and methods for this research.

1.8.1.2 Identification confirmatory tests

Further confirmatory identification made use of more selective techniques. The techniques routinely used were identified as TLC or instrumental techniques such as infra-red spectrometry (IR), Ultraviolet–Visible spectrometry (UV-Vis) and chromatographic methods, such as high performance liquid chromatography (HPLC) and gas chromatography (GC) (Baker and Phillips, 1983; de Boer et al., 2011; Dargan and Wood, 2013; UNODC, 2013c). In addition, for chemical characterisation and impurity profiling where drugs may require synthesis it was found that structural elucidation and identification of unknown substances was mainly by infra-red spectrometric methods, FTIR and also nuclear magnetic resonance spectroscopy (UN, 2001; Aalberg et al., 2005a; Bartos and Gorog, 2008; Inoue et al., 2008). This is due to the techniques providing more highly specific structural data. However, for routine analysis tandem methods with mass spectrometry (MS) were common such as liquid chromatography-mass spectrometry (LC-

MS and GC-MS) (Anderson et al., 2007a; Lecompte et al., 2008; Takahashi et al., 2009; Schurenkamp, 2010). This can be attributed to the advantage of the mass spectral library. Arbo et al. (2012) in a review of piperazine compounds as drugs of abuse identified similar analytical approaches for the identification of these drugs. Consequently, in this study confirmatory identification will be by UV-Vis, FTIR and GC-MS.

1.8.2 QUANTITATIVE METHODS

In the analysis of drugs of abuse, quantitative analysis is almost exclusively instrumental. Common techniques were similar to those for identification, i.e., GC-MS, LC-MS and in addition capillary electrophoresis (Baker and Phillips, 1983; Vorce et al., 2008; Kelleher et al., 2011; UNODC, 2013c). It has been discussed (section1.2.2) that 4-FPP, 3-TFMPP or other piperazine drugs are often found as a combination of drugs. In addition, impurities from synthesis are often present in street samples (UN, 2001; Bartos and Gorog, 2008). Hence, consideration is given to issues of selectivity and sensitivity. GC-MS is one such technique, as it has the required high selectivity and sensitivity (ng range) (Barwick, 1999; Khopkar, 2012). Furthermore, it has been successfully applied in other studies (de Boer et al., 2001; LTG, 2006; Maher et al., 2009; Takahashi et al, 2009; Kelleher et al., 2011). Of the instrumental techniques, GC and HPLC techniques have found wide application for the analysis of drugs of abuse and have been in use since 1970s. The applicability of these techniques can be seen by the UNODC recommended methods for the identification and analysis of piperazines in seized materials; TLC, GC-FID, GC-MS, GC-IRD, HPLC, capillary electrophoresis and FTIR (UNODC, 2013. The use of LC-MS as an alternative to GC-MS simplifies sample preparation and eliminates derivatization procedures and in addition can be applied to less volatile substances. However, GC-MS has the advantage of a wider application (Bowers et al., 2002; Kronstrand and Jones, 2000) and it has been used more extensively for drugs of abuse as such provides more extensive referral material.

Whilst the GC-MS technique has found a wide application in the analysis of drugs of abuse in the analysis of piperazine drugs, most of the studies have been on bioanalysis, such as toxicological studies not on analysis of street drugs (Staack, and Maurer, 2005; Elliot and Smith, 2008). Furthermore, shortcomings were identified in the methods reviewed for piperazines drugs and street samples. In a study by Inoue et al. (2004) to determine BZP, MeOPP, TFMPP, FPP and their isomers, a variety of techniques were applied ; colour tests,

TLC, IR, GC-MS and LC-MS. However, a limitation of the methods was the inability to separate the FPP isomers. These limitations were also encountered in a similar study to create a psychoactive drugs database by Takahashi et al. (2009). Isomers possess similar chemical and physical properties, resulting in similar analytical profiles and making it difficult to distinguish between, for example their chromatographic and spectrometric behaviour. Consequently it is often difficult to distinguish between the isomers. In their, studies Elliott and Smith (2008) and Vorce et al. (2008) indicated that 3-TFMPP and 4-TFMPP appear to have identical retention time and identical fragmentation spectra. The authors reported that they both exhibited similar principal ions (m/z 230 (M^+), 188(100), 122 and 56. As such, it was impossible to identify the specific isomer by GC/MS. They further reported that the resolution of these compounds was achieved by using LC-MS. In these studies it was observed that the researchers highlighted the need for HPLC for the separation of isomers, consequently such techniques as LC-MS will likely be appropriate.

Studies have been reported where isomers have been successfully separated, e.g. Maher et al. (2009) in a study on regiosomers of TFMPP successfully separated the isomers. However, the study whilst successful was qualitative and as such was not applied to street samples. A study by (UNOD, 2013c) developed three GC-MS methods for the analysis of materials containing piperazine drugs (BZP, MeOPP, FPP, TFMPP). The investigation was successful in separating the isomers. The methods by UNODC similarly to that of Maher et al. (2009) may face limitations when applied to actual street samples, as they do not take into account the congeners present in a street sample. In addition, for purposes of characterisation and profiling they may face a further challenge due to the solvent used. Whilst the results of drug stability studies with the solvent were not indicated, methanol can cause hydrolysis and esterification reactions (sections 2.7.2 and 2.7.3).

Among the instrumental techniques reviewed, GC-MS was chosen for use in this study. This was mainly due to its sensitivity, selectivity and diverse application to the psychoactive drug investigation (de Boer et al., 2001; Anderson et al, 2007a; Elliot and Smith; 2008) and in addition availability. Furthermore, in forensic investigations identifying the exact isomer maybe critical and may have legal implications, e.g., it is the meta- isomer of TFMPP which is the controlled psychoactive substance in some countries such as the USA (Coulson and Caulkins, 2012). In addition, it is the 4-FPP and 3-TFMPP isomers that have been reported as pharmacologically active drugs of abuse (Staack, 2007;

DEA, 2011; Arbo et al., 2012; Dargan and Wood, 2013), hence it is necessary to identify the exact isomer. Consequently, for purposes of conclusive identification more than one identifying variable is applied, such as the use of retention indices and retention times in addition to the mass spectra and often more than one technique is considered. GC-MS has the advantage that it offers all three. It therefore follows that a GC-MS method will be developed to overcome the limitations highlighted by the discussions above.

1.8.3 CHEMICAL CHARACTERISATION AND IMPURITY PROFILILNG OF STREET DRUGS CONTAINING 4-FPP AND 3-TFMPP

In clandestine production of drugs the main processes are a) synthesis of the active substance (or procurement from commercial sources), b) addition of cutting agents, adulterants, lubricants and binders c) mixing of the powders and d) compression of this mixture into tablets using tableting machine with specific settings (UN, 2001; Milliet et al., 2009). These processes define the physical and chemical characteristics of the finished drug and can therefore be used to check for links between samples. Identification of routes of synthesis and characterization/impurity profiling of seized drugs studies can provide an insight into diverse law enforcement investigative issues; ranging from dealer-user relationships, drug source, distribution networks, and trafficking routes to manufacturing methods. Drug profiling is made up of characterisation (physical and chemical) and impurity profiling (UN, 2001).

Physical characterisation involves a visual inspection of the) physical properties such as whether it is a tablet, powder, its colour, dimensions, i.e., generally its appearance and b) packaging. Chemical characterisation is the process of establishing a characteristic chemical signature of major, minor and trace components of a drug (UN, 2001, Bartos and Gorog, 2008). This involves detailed chemical analysis to determine their identity and relative concentrations using an appropriate method. In addition, it aids in the identification of possible routes of their clandestine synthesis. All this gives a complex chemical profile of each drug sample. Comparison of chemical profiles of different drug substances can then be carried out. This can be used to establish a) whether or not there is a link between one or more drugs, b) links on relationships between, for example distribution networks and, c) methods of clandestine synthesis including chemicals used in the manufacture.

Impurity profiling is the determination of minor "trace" components in the sample. According to King (2009) and EMCDDA, (2013a) impurity profiling is defined as the characterisation of naturally occurring or synthetic by-products in a drug to form a 'fingerprint' that maybe characteristic of its origin or manufacture. The aim of which is the detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities as well as residual solvents (Bartos and Gorog 2008; Verweij, 1992). The knowledge of the impurity profile of drugs of abuse in forensic analysis is of great importance since the impurities can be characteristic of the synthetic route and starting material and hence the origin of the drug (UN, 2001). Verweij (1992) in their study on MDA and MDMA reported that it was an established fact that street samples of these substances were clandestinely synthesised. The author concluded that establishing the presence of contaminations derived from different origins in MDA or MDMA preparations can assist in establishing the route of synthesis adopted by the individuals illegally producing these amphetamines. As such, a direct comparison of the profiles can provide links between different samples seized and also conclusions as to the methods of synthesis used. This together with chemical characterisation provides a more holistic picture of the drug. This study carried out physical and chemical characterisation; hence the concepts behind these processes are further discussed in Chapter 2 (Theoretical concepts).

No chemical characterisation and impurity profiling of 4-FPP and 3-TFMPP has been carried out to date. Hence, of studies on other drugs of abuse were reviewed so as to gain an insight into the process of characterisation and profiling. A study by Inoue et al. (2008) on characterisation and profiling of methamphetamine in seized drugs reviewed methods for characterisation and profiling. Both liquid-liquid extraction (LLE) and solid phase extraction (SPE) techniques utilising solid phase micro-extraction were found to be effective sample treatment techniques for the extraction and isolation of impurities. The methods of analysis involved mainly gas chromatography with a flame ionisation detector (GC/FID), GC/Thermal desorption, GC-MS and NMR. Other studies showed a similar approach. Andersson et al. (2007b) similarly investigated sample preparation techniques as part of their studies on profiling amphetamines. The study concluded that LLE was better suited to the extraction of amphetamine impurities than SPE due to lack of information on the long-term impact of SPE columns on the stability of the analytes. In a related study Andersson et al. (2007a) developed optimised and validated GC-FID and GC-MS methods and in addition carried out comprehensive profiling of amphetamine drugs. A study by

Dayrit and Dumlao (2004) profiled impurities in seized methamphetamine samples by GC–MS and GC/FID. These studies confirm the use of GC-MS for this study and that both LLE ans SPE can be employed during profiling. However, in this study consideration will be given to LLE due to the findings by Andersson et al. (2007b) stated above. Furthermore, these studies also highlight that impurities are present in street drugs. As such this confirms the fact that 4-FPP and 3-TFMPP drugs synthesised illicitly are most likely to have impurities.

Kelleher et al. (2011) in a research for the National Advisory Committee on Drugs of Dublin analysed new psychoactive substances by GC-MS. In the study, street samples containing suspected new psychoactive substances were analysed. The results showed that street samples do not necessarily contain the substances indicated on the label e.g. 'E-XTC' was labelled as "not for human consumption" but contained dicalcium phosphate, ketones and magnesium stearate and no TFMPP. This is likely for the purpose of circumventing legal controls. Also, a variety of drugs are present in street samples (Table 1.3). Baron et al. (2011) and the London toxicology group (LTG), (2006) reported similar findings. The LTG in their study on analytical profiles of piperazines analysed samples (26) consisting of capsules and tablets. These were found to contain mostly a combination of piperazine drugs with the most common being BZP and 3-TFMPP. The dosages were in line with the results of other researchers and were diverse. Reported dosages were 60 - 200 mg BZP, 4 - 72mg 3-TFMPP. (The effective dose and physiological effects was in discussed section 1.5). Dosages were not stated for the other piperazines found present; 4-FPP, 3-CPP, MePP, MBZP and MeOPP. These findings confirm the discussion in section 1.2 where it was highlighted that a number of drugs were likely to be present in street samples containing 3-FPP, 3-TFMPP or any piperazine drug of abuse.

It is evident from the discussion above that the composition and purity of street samples is often unclear. Furthermore, it has been highlighted there is lack of information on characterisation of piperazine based drugs of abuse, consequently there is need to characterise piperazine street drugs. The approach and techniques highlighted in the review for other drugs were extrapolated in the characterisation of piperazines in this study.

1.9 A REVIEW OF METHOD DEVELOPMENT, OPTIMISATION AND VALIDATION

It has been identified in previous discussions (section 1.8) that there are limitations in the existing methods for the analysis of piperazines; 4-FPP and 3-TFMPP. Thus this gives rise to the need to develop the relevant method(s) for their investigation. A method that will therefore be applied for the qualitative and quantitative analysis of street samples containing 4-FPP and 3-TFMPP. Further application of the same method will be used for the chemical characterisation and impurity profiling of the street samples. In addition the method should be able to selectively analyse for 4-FPP and 3-TFMPP in a sample with a complex matrix of various drug combinations, impurities and isomers of 4-FPP and 3-TFMPP.

Several studies on development of methods for drugs of abuse have been reported (Staack et al., 2003; Inoue et al; 2004; Andersson et al., 2007a; Elie et al., 2013; Kelleher et al., 2011; UNODC, 2013c). The review on method development below focused on GC-MS as this is the technique to be used. In studies where GC-MS methods were developed, the selection of stationary and instrumental parameters, such as flow rate of the mobile phase and sensitivity of the detector were identified as paramount to development of the preliminary method (Andersson, 2007a; de Boer et al., 2001; Elie et al., 2013; Maher et al., 2009; Takahashi et al., 2009). In addition, it was also observed that solvents, sample preparation techniques and stability of the analytes are also an inherent part of development. Furthermore, the method was then review optimised and validated before application.

According to ICH guidelines (2005), optimisation ensures that all the variables in the method are those that give the best results and validation gives an indication that the method is fit for purpose. This process involves manipulating method variables. It was found that chromatographic concepts such as Plate theory, van Deemeter equation are often employed in determining which variables can be varied so as to optimise chromatographic data (Chan et al., 2010; Dijkistra et al., 1996; Horacio et al., 2008; McNair and Miller, 2009; Khopkar, 2012). Validation is the sequence of events carried out to ensure that the method is suitable for its purpose, i.e., it achieves the desired outcome (Eurachem, 1998;

ICH, 2005; Horacio et al., 2008; Lavanya et al., 2013). The concepts behind optimisation and validation will be discussed in detail in Chapter 2.

In studies where GC-MS methods were developed, it was observed that the following method variables were optimised; instrumental parameters such as the detector, mobile phase flow rate, injection temperature, oven temperature, ionisation energy and in addition sample preparation techniques such as extraction techniques, solvent selection, derivatisation methods and stability of samples (Andersson, et al., 2007a; 2007b, Lock at al., 2007; Inoue et al., 2008). Different approaches to method validation were found to exist showing a wide range of parameters to be validated (Eurachem, 1998; Horacio et al., 2008; ICH, 2005; Lavanya et al., 2013; Thompson et al., 2002). The extent of validation was also found to depend on use of the method. For example, qualitative methods did not require determination of limit of quantitation or linearity (Appendix 4). Lavanya et al. (2013) in a review on analytical method validation had similar findings. In addition, the author reported the following method variables were basically validated; selectivity/specificity, linearity, range, accuracy, precision, method detection limits and robustness.

Byrska et al., (2010) developed and validated a GC-MS method for the determination of 6 piperazine drugs; 3-MePP, BZP, 3-TFMPP, MBZP, MeOPP and 3-CPP. The approach used involved evaluation of different columns and several temperature programs. The column selected was HP-5MS comprising of 5%-Phenyl-95%-Dimethylpolysiloxane stationary phases. This type of column has been found to be widely used in analysis of drugs of abuse.The UNODC (2013c) in its recommended methods for piperazines; Inoue et al. (2004) in analysis of benzylpiperazine like substances; Takahashi et al. (2009) in a study on creation of a psychoactive library and Andersson et al. (2007a) in developing a GC-MS method for the profiling of amphetamines all selected this type of stationary phase for use. This implies that this particular stationary phase might be the best suited to analysis of the drugs in this study which also contain piperazines and amphetamines (section 1.2.2).

It has been stated above that optimisation of instrument variables can enhance chromatographic results. Santali et al., (2011) in their study on developing a method for mephedrone and Inoue et al., (2008) in a study on developing a method for profiling methamphetamine using GC-MS observed that both efficiency and tailing were temperature dependent. Furthermore, in optimising their methods variation of both injector

and column temperatures similarly improved efficiency and tailing. It therefore follows that in this research optimisation of instrument variables will be investigated so as to enhance performance of the preliminary method. In addition, validation will also be conducted so as to ascertain suitability of the method for use. The validation will determine the parameters selectivity/specificity, linearity, accuracy, precision, method detection limits and robustness.

1.10 INVESTIGATION OF THE STABILITY OF DRUGS AND EFFECT OF SOLVENTS DURING ANALYSIS

The stability of a drug substance can be defined as its ability to retain its properties (e.g. chemical and physical properties) within specified limits throughout its time of usage or storage (ICH Q1A(R2), 2003; FDA, 2008). Loss of stability can occur through degradation during any of the different stages of a drugs life cycle, such as during formation, distribution, storage of the finished product and laboratory investigations. Degradation leads to a loss in concentration of the drug and the possibility of degradation products (or artefacts) which can suddenly appear in the sample matrix (Yoshioka and Stella, 2000; ICH Q1A(R2), 2003). These impact on the quality of analysis results for example degradation products in the sample matrix can interfere with the analytes during analysis and as such require investigation of stability.

Stability studies on drugs of abuse during laboratory investigations and storage have been reported (Aalberg et al., 2005b; Gunnar et al., 2004; Karinen et al., 2011; Moody et al., 1999; Nowatzke and Woolf, 2007). Karinen et al., (2011) investigated the storage of drugs in the refrigerator, freezer and at ambient conditions involving an extensive range of drugs in the solvents used methanol, acetonitrile, or a mixture of the two. Loss in concentration (> 20%) was reported for some of the drugs indicating a loss of stability.

Aalberg et al. (2005b) in a study on profiling of amphetamines investigated the stability of impurities of amphetaminmes in organic solvents as a function of time and temperature. This study found the impurities were most stable in the solvents iso-octane, toluene and ethanol. However, whilst the temeperature effects were not determined, it is a well-known fact that temperature affects stability (Yoshioka and Stella, 2000). In other studies; Gunnar et al. (2004), Moody et al. (1999) and Nowatzke and Woolf (2007) also similarly found that

degradation of drug substances occurs with time. It therefore follows there is need to establish the limits during which the drug is stable to enable accurate analytical results.

A review of literature on analytical investigations of drugs of abuse showed that a wide variety of solvents are used during analysis. Methanol is the most commonly used solvent during drug analysis (de Boer et al., 2001; Staack and Maurer, 2005; Anderson et al., 2007b; Elliot and Smith, 2008; Takahashi et al., 2009), other solvents commonly used are ethyl acetate, ether, ethanol, propanol dichloromethane, chloroform, DMSO and toluene. Their use can be attributed to the fact that most drugs of abuse are polar and consequently methanol and ethyl acetate are good solvents for such drugs. Both are polar and volatile enough for use on the GC-MS. In addition methanol is miscible with aqueous solutions hence can be used on the HPLC. Furthermore, ethyl acetate is amenable to use during sample derivatisation commonly used in GC-MS analysis. Currently there is very limited use of 2-methylpropan-2-ol in analysis of drugs of abuse (de Boer et al., 2001; Inoue et al., 2004; Staack and Maurer, 2005; Andersson et al., 2007a; Staack 2007; Elliot and Smith, 2008; Takahashi et al., 2009; Kenyon et al., 2010). However, it has potential for use as analytical solvent in analysis of drugs of abuse. It has similar properties to methanol and is less reactive as it is a tertiary alcohol (Table 2.3 and section 5.7.1); as such it can be used as an alternative solvent in cases where the other solvents prove unsuitable. Its reactivity makes it suited to characterisation and profiling.

According to the FDA (2008) it is necessary to establish chemical stability of the drug analytes in solution or in the proposed matrix and how stability maybe maintained. Consequently, selected solvents will be reviewd further in Chapter 2, so as to evaluate their potential reactivity and effect on the stability of the drugs used in this research (Table 4.1).

No literature was found on thermal and chemical stability of 4-FPP and 3-TFMPP or any of the piperazines in various solvents either as part of method development or as independent investigations. Furthermore, stability studies have focused on storage and none were encountered on stability during analysis on the instrument's autosampler. Analytes can spend prolonged periods on the autosampler awaiting the sample to run, in addition the temperature on the autosampler maybe above ambient due to the presence of the injector and oven. These may influence the stability of the analyte. In addition, literature pertaining to metabolic degradation of 3-TFMPP and 4-FPP was evaluated. Studies by Staack (2007)

showed that 3-TFMPP is extensively metabolised by hydroxylation at the aromatic ring and also by degradation of the piperazine moiety. These results have implication on the stability of 3-TFMPP in alcoholic solvents due to the possibility of hydroxylation. It is therefore evident there is a lack of adequate information on stability and stability limits to be applied for routine laboratory analysis of these drugs, hence this will be investigated in this research.

1.11 AIMS AND OBJECTIVES OF THE STUDY

Research on phenylpiperazines drugs is as yet not exhaustive. From the literature review, it was identified that there is need to address the lack of research for both the synthesis and analysis of 4-FPP and 3-TFMPP for purposes of chemical characterisation and profiling of these drugs. In addition, no literature on profiling of piperazines was found. Very few analytical investigations have been conducted on 4-FPP, even though it has been identified as prevalent in drugs of abuse (UNODC, 2013a). Furthermore, no studies were encountered on drug stability of these substances during analytical laboratory conditions and limited studies on the other drugs investigated in this study.

Furthermore, it was also identified that research on isomers for psychoactive substances is very limited which poses a challenge both on analysis and related toxicity issues. To date separation of the positional isomers for TFMPP and FPP has not been achieved in a street sample containing other drugs. It has been discussed that the determination of the exact isomers is necessary in case work and may have legal implications with regards to scheduling status of these drugs (sections 1.6 and 1.8). This study addresses these issues and provides novel information.

Currently, there is a lack of information on the stability of piperazines during analysis. In addition, no stability studies of the drug whilst on the GC-MS autosampler were encountered. The temperature effects of the injector and column oven in close proximity to autosamplers and also the duration of stay of the analyte on the autosampler were highlighted in section 1.10 as potential causes of degradation thereby raising the need for establishing their stability.

It has been identified in previous discussions (section 1.4) that new psychoactive substances are an emerging and increasing global problem. In addition the complexity of the drugs poses health risks (section1.5). The drugs have been reported to exist as combinations of two or more drugs and commonly with cutting agents and adulterants (Davies, 2010; Kelleher et al., 2011; UNODC, 2013b). It can therefore be concluded that if not investigated to provide scientific data this problem cannot be minimized or halted. This is evidenced by the UNODC (2013b) which states that "The increasing number of NPS appearing on the market has also become a major public health concern, not only because of increasing use but also because of the lack of scientific research and understanding of their adverse effects". Consequently, this research will investigate one group of the NPS, i.e., piperazines and develop methods of analysis and also provide research data. As such the following aims arise;

a) To develop a method, optimise and validate a GC-MS method for the simultaneous analysis of 4-FPP, 3-TFMPP, their positional isomers and their congeners in street samples.
b) To carry out stability studies and establish stability limits for piperazine drugs of abuse and their congeners during laboratory analysis and also determine the solvent in which these compounds are most stable during analysis and storage
c) To synthesise the substituted phenylpiperazine derivatives (3-TFMPP and 4-FPP) and thereby determine the viability of the synthesis routes for potential illicit use.
d) To analyse the synthesised 3-TFMPP and 4-FPP and chemical impurities arising from the synthesis (by-products, positional isomers, residual solvents and precursors) using the developed GC-MS method, FTIR and UV-Vis to provide analytical data.
e) To carry out the chemical characterisation and profiling of 3-TFMPP and 4-FPP drugs of abuse using the data derived from the synthesis and the developed GC-MS method.

CHAPTER 2

THEORETICAL CONSIDERATIONS UNDERLYING CONCEPTS APPLIED IN THE RESEARCH

2.1 INTRODUCTION TO THE STUDY

This chapter investigates the theoretical concepts underpinning the experimental work for this research so as to gain insight into the processes involved. Principles of chromatographic processes impacting on this research were discussed (sections 2.1 and 2.2). In addition, the concepts underpinning optimisation and validation of methods, sample treatment and stability studies, including the factors influencing them were reviewed (sections 2.3 – 2.5). The aspects behind synthesis, characterisation and profiling of street drugs of abuse were also discussed (section 2.8).

2.2 BASIC CONCEPTS OF GC-MS INSTRUMENTATION AND ANALYSIS

Two important aspects arise in the analysis of samples containing more than one analyte or an analyte in a sample matrix with detectable elements; a) ability of the system to selectively separate the analyte from other components in the sample matrix and b) the emission of a signal characteristic of the analyte and its concentration, thereby enabling its detection, identification and quantitation. The ability to separate different substances in the chromatographic column during analysis lies at the heart of many separation techniques such as GC-MS and HPLC. The basic concepts of GC-MS instrumentation are highlighted in Figure 2.1.

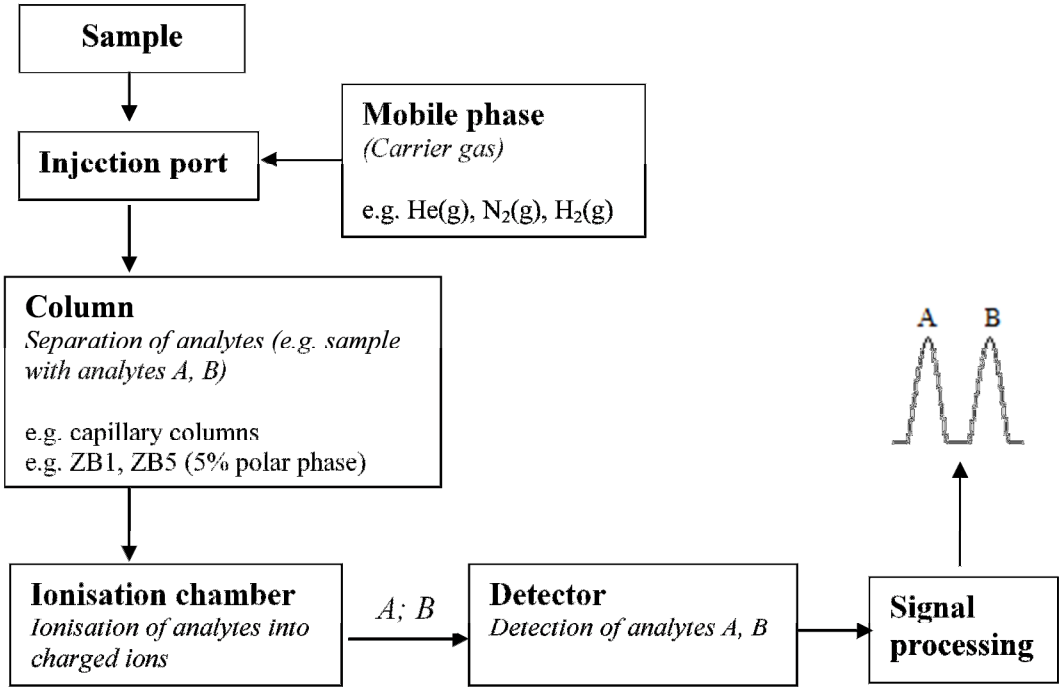


Figure 2.1 Schematic block diagram of a GC-MS instrument.

The types of injection techniques for capillary GC-MS are split, splitless, direct on column, thermal desorption and pyrolysis (Grob, 1994). Split and splitless injection were found to be common for analysis of street samples of drugs (de Boer et al., 2001; Inoue, 2004; Takahashi, 2009; Dargan and Wood; 2013; UNODC, 2013c), will be discussed further. In the split mode an aliquot of the sample is passed into the column whilst the rest is vented. The amount depends on the split ratio. This has the advantage of avoiding overloading the column. However, the limitation is that high sample concentrations are required (>0.01% mass/vol) (Barwick, 1999; Grob, 1994; Kaur, 2010). In addition, the disadvantage is that the carrier gas is continuously vented into the laboratory. In splitless mode, the injection vent is closed. The sample is first pre-concentrated in the solvent at the top of the column before entry into the column and hence is advantageous for trace analysis. The limitation is that the boiling points of the analytes have to be 393 - 493°K above the boiling point of the solvent so as to facilitate the solvent pre-concentrating the sample (Grob, 1994; Kaur, 2010; Miller, 2005). Both injection modes were found applicable to the study, however, the split mode was chosen as doses of drugs in street samples are high in the mg/tablet range (Arbo et al., 2012; Davies et al., 2010; Kenyon et al., 2010; Kelleher et al., 2011).

Mobile phases for GC analysis are inert gases under pressure continuously pumped throughout the system, mainly He, H_2, N_2 and Ar (Kaur; 2010; Kronstrand and Jones, 2000). The carrier gas moves the analytes through the system thereby facilitating elution from the column after injection.

2.2.1 SEPARATION IN THE COLUMN

The sample is injected into the injection chamber (Figure 2.1). Continuous movement of the mobile phase carries the sample through the column. The resulting process of separation is schematically shown in Figure 2.2.

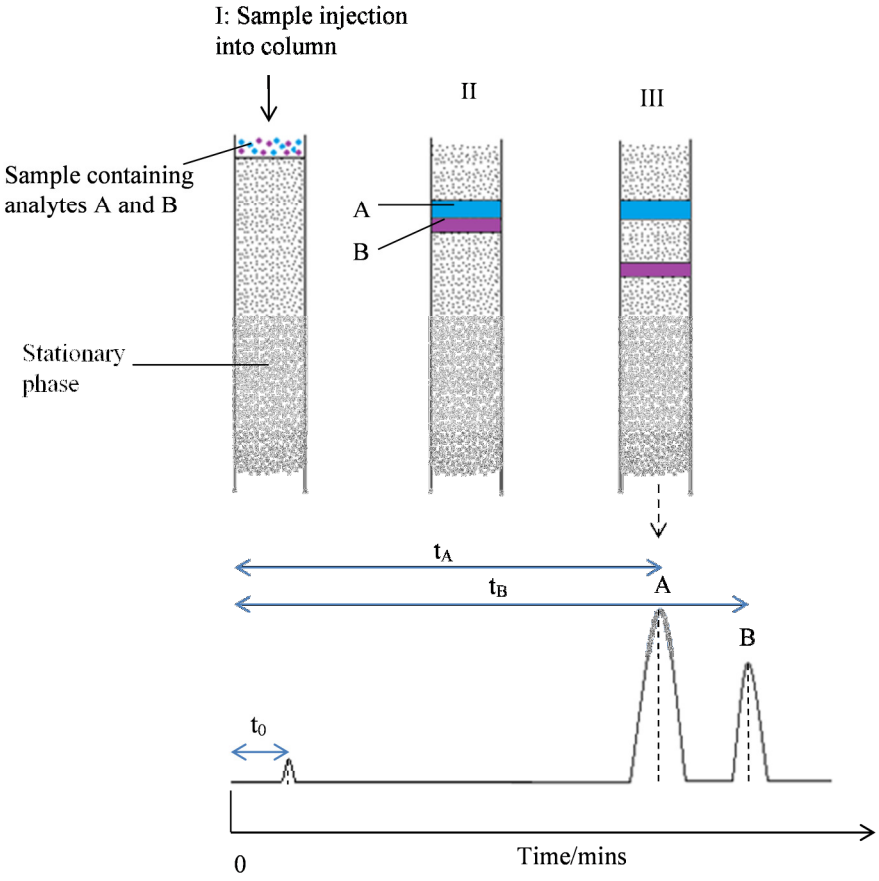


Figure 2.2 Separation of a sample containing analytes A and B in the column, depicting the progression of events sequentially from I – III. The time taken for the analyte to elute is shown as t_A and t_B.

In the column, partition of the analytes between the mobile phase and the stationary phase occurs. Compounds show differences in partitioning behaviour depending on their chemical properties. Substances with a greater affinity for the stationary phase bond more strongly

with the stationary phase and are consequently retained longer in the column. This results in separation of the analytes into bands (II). Movement of the mobile phase not only moves the analytes bands down the column but further separates them. Consequently they elute at different rates from the column and are thus separated (III) (Skoog et al., 2007).

It therefore follows that during method development, selection of column is critical to obtaining good results. Furthermore in method development a stationary phase should be selected which is suitable to the type of drugs under investigation. It has been found by many reseachers that like separates like (Kaur, 2010, Maher et al., 2009). Thus, it follows that as the drugs in this study are polar, polar stationary phases were considered so as achieve good resolution. This is in line with the other studies conducted on similar drugs, such as cocaine, amphetamines and piperazine. In their investigations Andersson et al. (2007a), de Boer et al. (2001), Inoue et al. (2004) and UNODC (2013c) employed polar columns (mainly 5%-Phenyl-95%-dimethylpolysiloxane). Kaur (2010) suggested the following rules of thumb, these will be considered in this research so as to maximise the quality of the chromatographic results and are discussed under effect of operational variables (section 2.4);

- A long column gives a longer elution time but high resolution.
- Small internal diameter gives high efficiency.
- Film thickness should be thin; about 1µm. thicker films become unstable giving rise to pooling and excessive column bleed.

2.2.2 ANALYTE DETECTION

On elution from the column, the mobile phase carries the analytes into the detector. Where the GC instrument is coupled to an MS detector, after elution from the column the sample first enters the ionisation chamber, where either electron impact ionisation (EI) or chemical ionisation (CI) occurs (Khopkar, 2012). This generates charged ions which enter the detector and are separated on the basis of mass to charge ratio. The detector determines the time of arrival of the analyte into the detector; this is relative to its retention time, t_r. The earlier separation of analytes A, B (Figure 2.2) in the column results in analytes eluting from the column and subsequently arriving at the detector at different times. Those that are least retained elute first and subsequently have the shortest retention time as shown in

Figure 2.2 retention times t_A and t_B for analytes A and B respectively. Equation 2.1 shows calculation of the retention time (Grob and Barry, 2004; Skoog et al., 2007).

Retention time of analyte A = uncorrected retention time – retention time of un-retained compound.

$$t'_A = t_A - t_0 \qquad [2.1]$$

where: t_0 is the retention time of the un-retained compound (minutes)

t_A is the retention time for analyte A (minutes)

t_B is the retention time for analyte B (minutes)

t'_A is the corrected retention time for analyte A (minutes)

In addition, the detector signal is processed and generates a chromatogram with a peak for each analyte. Further data processing produces a mass spectrum. The mass spectra and retention times can "fingerprint" a compound. Hence, these are useful qualitative tools in identification of substances. In the chromatograms generated on analysis the area under a peak is relative to the concentration of that analyte. The height of the peak can also be used in place of concentration (Kaur, 2010; Khopkar, 2012). This imparts quantitative aspects to the technique.

2.3 FACTORS INFLUENCING THE QUALITY OF CHROMATOGRAPHIC RESULTS

A good chromatographic peak profile is one with narrow, sharp, symmetrical peaks, i.e., the peaks a show a normal distribution pattern, thus are Gaussian shaped. In addition, a "clean" baseline is prerequisite. Where more than one analyte is present, the peaks are well separated (equation 2.11) with a resolution, $R_s > 2$ (Huber 1996; ICH, 2005). The quality of the chromatogram is influenced by several factors such as the partition coefficient, retention (capacity) factor, column efficiency and selectivity. Investigation of these parameters aids in determining the performance of the chromatographic system. In this study, column efficiency (plate number), tailing, selectivity and resolution is evaluated as measures of the quality of the chromatographic peak profiles in order to determine the impact of optimisation (Chapter 6). Consequently, these parameters and others which

influence them are described below (CDER, 2004; Eurachem, 1998; Thompson et al., 2002; ICH, 2005; Kaur, 2010; Khopkar, 2012).

The *partition coefficient*, K is an equilibrium constant which refers to the spatial distribution of the analyte between the stationary phase and the mobile phase. K is given by the molar concentration of the analyte in the stationary phase relative to the mobile phase at equilibrium (equation 2.2). The rate and degree of partition is governed by the analyte's vapour pressure, which is determined by its boiling point, degree of volatility and column oven temperature. The partition coefficient is also dependent on the chemical affinity of the analyte for stationary phase. It therefore follows that analytes with a larger partition coefficients are retained longer in the column and consequently have longer retention times. The partition coefficient can be calculated as shown in equation 2.2 (Huber 1996; ICH, 2005).

$$K = C_s/C_m \qquad [2.2]$$

where: C_s = concentration of analyte in stationary phase (mol/dm^{-3})

C_m = concentration of analyte in mobile phase (mol/dm^{-3})

The *capacity factor* (retention factor), k describes the migration rate of an analyte in a column. This can be calculated by equation 2.3 (CDER, 2004; Skoog et al, 2007).

$$k = \frac{t_r - t_0}{t_0} = t_r' \qquad [2.3]$$

where: t_0 is the retention time of the un-retained compound (minutes)

t_r is the gross retention time of the analyte = unadjusted retention time (minutes)

$t_{r'} = t_r - t_0$ and is the retention time of the analyte (minutes)

When an analyte's capacity factor is less than one, it implies the analyte is not highly retained in the column and the elution is very fast. High capacity factors (greater than 20) mean that elution takes a very long time. An ideal capacity factor for an analyte is between one and five (IUPAC, 2014). The process of separation is also dependent on other factors, mainly the type of stationary phase, flow rate and temperature (Barwick, 1999; Khopkar, 2012; Maher et al., 2009).

Plate Column efficiency is measured as plate count (also known as plate number) and plate height. *Plate number*, N is a measure of the ability of a column to produce narrow sharp peaks. It shows the resolving power to separate analytes. According to the Plate theory (Kaur, 2010; Khopkar, 2012) a chromatographic column is made up of a large number of separate layers, called theoretical plates. These plates govern the shapes of the resulting peaks in the chromatogram which is also influenced by the flow rate. Complete, separate equilibration of the sample between the stationary and mobile phase occur in each of these "plates" (equation 2.9). The movement of the mobile phase down the column results in the transfer of the equilibrated mobile phase from one plate to the next and with it the analyte. The higher the number of theoreticals in a column, generally the sharper (narrower) and more intense (higher) the resulting peaks (Figure 2.3). This results in the better the separation from adjacent peaks (equation 2.11). This concept assumes a peak is symmetrical, as such shows a normal distribution pattern, i.e., it is Guassian shaped. The peak-widths in such a peak are related to the standard deviation (σ) of the peak according to Figure 2.3 and the equations 2.4 – 2.8 (Skoog et al, 2007; CDER, 2004; IUPAC, 2014).

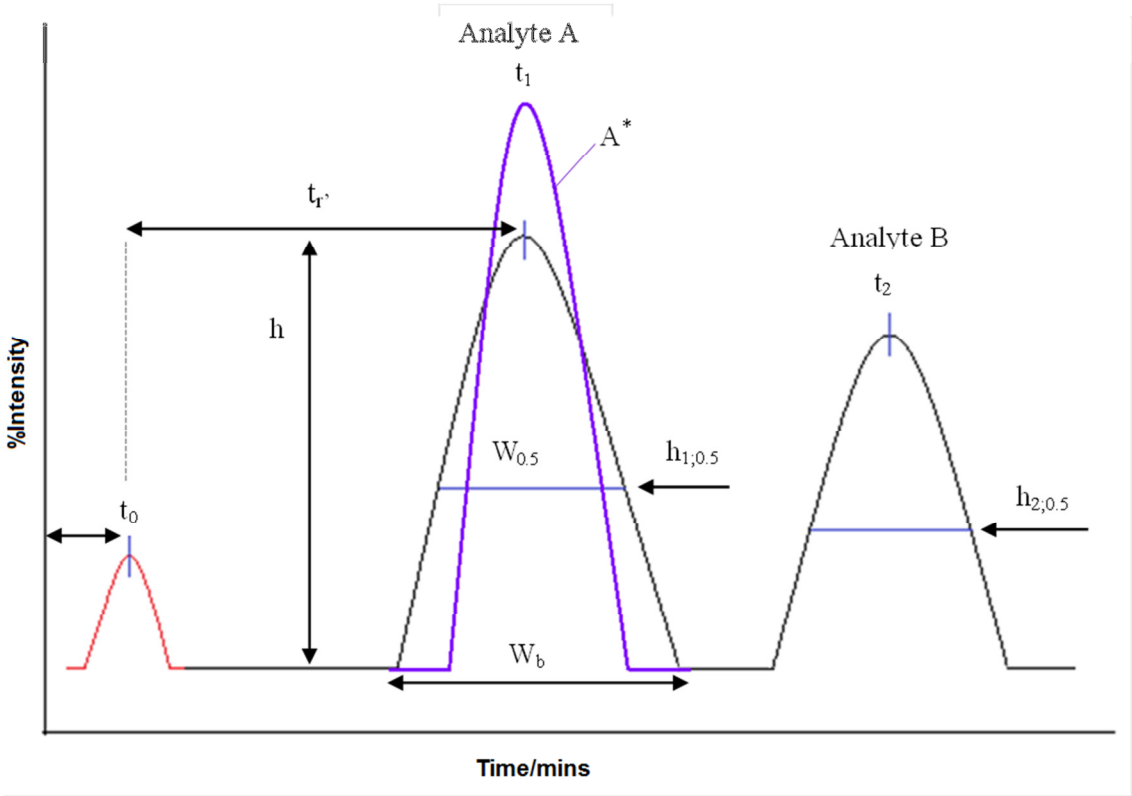


Figure 2.3 Schematic representations of determination of plate number and the effect of peak shape on it. Showing the effect of an increase in plate number on Analyte A (peak A*)

where: $W_{0.5}$ is peak width at half height of the peak (seconds)

W_b is peak width at base of the peak (seconds)

A* is the analyte peak with a higher value of plate number

In a Gaussian peak 96% of the area is under $\pm 2\sigma$ and the base peak width is 4σ hence,

$$W_b = 4\sigma \quad [2.4]$$

$$W_{0.5} = 2\sigma 2\sqrt{2\ln 2} = 2.355\sigma \quad [2.5]$$

$$N = \left(\frac{t_{r\prime}}{\sigma}\right)^2 \quad [2.6]$$

Deriving σ from equation 2.5 gives 2.6

$$\sigma = \left(\frac{W_{0.5}}{2.355}\right) \quad [2.7]$$

substituting for σ into equation 2.7 gives 2.8, the formula for calculating N

$$N = 5.545\left(\frac{t_{r\prime}}{W_{0.5}}\right)^2 \quad [2.8]$$

Plate height, H is defined as the height equivalent of the theoretical plate (HETP) determined by plate number (refer to discussion of column efficiency). It is a measure of the length of the column needed for the equilibrium process to proceed. As such it is an alternative measure to plate number, N for column efficiency. H is given by equation 2.9 (IUPAC, 2014).

$$H = HETP = \frac{\sigma^2}{N} = \frac{L}{N} \quad [2.9]$$

where: H is the height equivalent of a theoretical plate (μm)

L length of column (μm)

N is plate number

Selectivity, α is a measure of the separating power of a column. It describes the separation of analyte 1 and 2 on the column. A selectivity of one or less means compounds cannot be separated as they are more or less retained to the same extent (Horacio et al., 2008). If

analyte 2 elutes faster than analyte 1, selectivity is greater than 1. This shows that the peaks are separated. It can be determined from equation 2.10 (CDER, 2004; IUPAC, 2014).

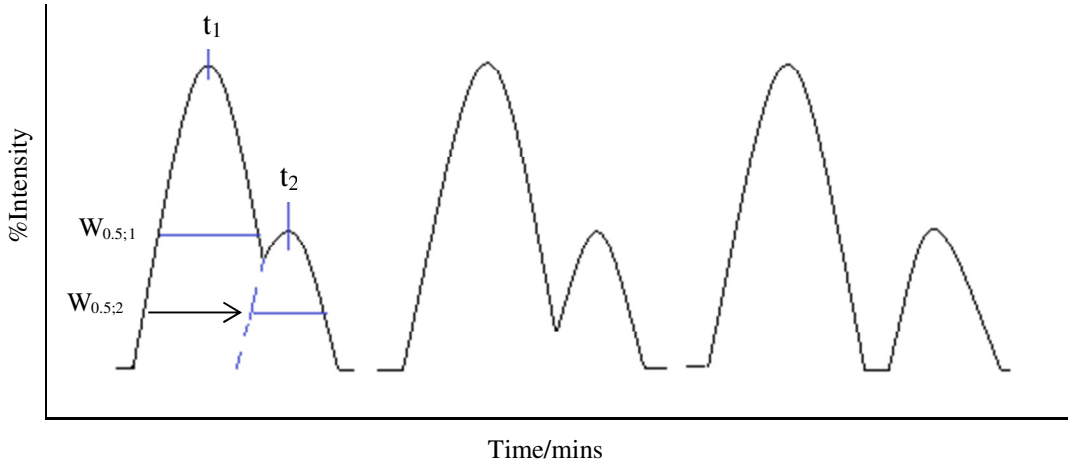
$$\alpha = \frac{k_1}{k_2} \qquad [2.10]$$

where: α is selectivity

k_1 is capacity factor of the less retained peak (eluting first)

k_2 is capacity factor of the more retained peak (eluting second)

Resolution, R_s is a measure of peak overlap defined by the relative number of peak widths between peaks. It aids choice of column and optimisation (CDER, 2004; Horacio et al., 2008). Figure 2.4 below shows a schematic diagram of resolution of analytes and equation 2.11 its calculation.



$R_s = 1.0$ $R_s = 1.5$ $R_s = 2.0$

Figure 2.4 Schematic diagram for the determination of resolution of analytes (Adapted from CDER, (2004).

where: t_2 is the retention time of the more retained peak

t_1 is the retention time of the more retained peak

$$R_S = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{k+1}\right)\sqrt{N} \qquad [2.11]$$

It can be seen that the equation for resolution, equation 2.11 consists of three terms; (i) plate number, ii) selectivity and (iii) capacity factor. Selectivity and capacity factor are a measure of retention. Therefore these parameters describe the separation between analytes. Consequently, these parameters influence resolution. Hence, increases in plate number and selectivity increases resolution whilst an increase in capacity factor reduces resolution. It follows that to obtain high resolution, the three terms (efficiency, selectivity and capacity factor) must be optimised (the concept is discussed in section 2.4).

Peak broadness and symmetry define the general shape of the peaks. These terms are affected by band broadening which in turn is dependent on kinetic variables (these are also discussed in section 2.4.

Peak broadness refers to the width of a peak. Good peaks are narrow. This facilitates for more accurate triangulation of peak area as such accurate quantification. Also, narrow peaks reduce peak overlapping and increases peak separation. In addition, consistent retention times and peak areas are obtained (Bowers et al., 2002; Gonzalez and Herrador, 2007).

Peak asymmetry describes peak shape, as previously highlighted theoretically a peak should be Gaussian shaped. However, tailing and fronting can occur when the peak shape becomes distorted as shown in Figure 2.5. Peak symmetry is dependent on the adsorption isotherm for the distribution of the solute between solid phase and the gas phase. When this varies with concentration peak asymmetry occurs. Fronting normally results when the amount of sample introduced into the sample is too large. Other factors such use of a column with poorly packed stationary phase (inconsistent packing causes voids, which affect equilibration of the analyte in the phases).With increase in peak asymmetry and tailing, the resolution between peaks decreases (Skoog et al., 2007).

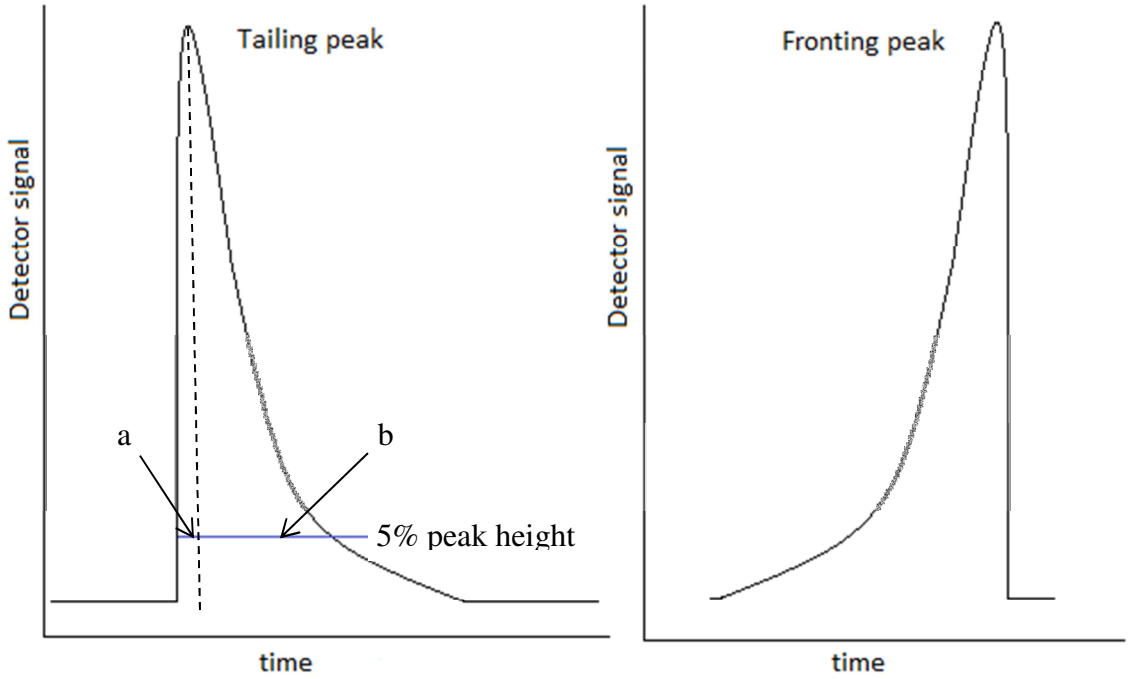


Figure 2.5 Schematic diagram showing peak asymmetry.

Peak asymmetry can be used as a measure of peak tailing. A value of < 1 indicates fronting whilst > 1 imples peak tailing (IUPAC, 2014; McNair and Miller, 2009). Tailing can be calculated from equation 2.12;

$$T = \frac{a + b}{2a} \qquad [2.12]$$

where: T is the tailing factor

b is the front half of peak width at 5% height of the peak

a is the back half of peak width at 5% height of the peak

The parameters that were discussed plate number, selectivity, resolution, peak asymmetry and capacity factor are dependent on method operational parameters such as injection temperature. In addition, other analytical aspects such as the degree of fragmentation observed in mass spectra of an analyte are similarly affected. As such, method operational variables can be varied so as to obtain the best chromatographic results. This gives rise to the concept of optimising the method (section 2.4 and chapter 6).

2.4 EFFECT OF OPERATIONAL VARIABLES AND OPTIMISATION

Operational variables are those aspects of a method that can manipulated, such as instrumental parameters, column physical properties, physical and chemical properties of the stationary phase, mobile phase, flow rate, detector sensitivity, oven temperatures, injector temperature and ionisation energy (where an MS detector is used). Consequently, variables which negatively affect peak profiles need to be minimised and those which improve performance optimised (Andersson et al., 2007a; Hibbert, 2007; Inoue et al., 2008). Consequently, these are manipulated so as to;

a) Generally, an improvement in chromatographic peak profile in terms of peak shape, broadness, intensity;
b) Increase column efficiency measured as plate number, N.
c) Increase selectivity, α, > 1.0
d) Increase peak resolution, $R > 1.5$
e) Decrease peak tailing or peak asymmetry, $T < 1.5$
f) Maintain or decrease retention time

The Plate and Rate theories explain the effects of chromatographic variables. They are useful tools in deriving variables that can be improved. Consequently, they have found wide application in determination of method performance and optimising methods (Moody, 1982; Kaur, 2010; Khopkar, 2012; IUPAC, 2014). The Rate theory describes the shapes and breadths of elution bands. According to the Rate theory, the resulting band shape of a chromatographic peak is affected by kinetic variables, i.e., the rate of elution and the different paths available to analyte molecules, as they travel between particles of stationary phase. As such, this theory can be used to determine the variables that can improve column efficiency.

The van Deemeter equation arises from the Rate theory. It describes some of the relationships and effects of the experimental variables that affect band broadening.
Band broadening is due to 3 main processes in the column; a) multiple path of an analyte through the column packing, b) molecular diffusion and c) effect of mass transfer between phases. These processes are influenced by variables such as effect of mobile phase flow rate. Below is a list of the influencing factors (Hibbert, 2007; Skoog et al., 2007);

- μ: the linear velocity of the mobile phase (cms^{-1})
- d_p: the diameter of stationary phase particle size (cm)
- D_m: diffusion coefficient of analyte in the mobile phase (cm^2s^{-1})
- D_s: the diffusion coefficient of analyte in the stationary phase (cm^2s^{-1})
- λ: packing factor related to particle size
- γ: obstruction factor due to the diffusion restriction by the column
- d_f: effective film thickness of liquid phase coating
- k: capacity factor

It is evident that these factors are related to the column and analyte properties. Hence, they can be manipulated to optimise performance. This is reason it has found wide application in determination of optimum values such as flow rate and temperature during optimisation. The Van Deemter equation is given by equation 2.13 (Moody, 1982; CDER, 2004; Horacio et al., 2008; Kaur, 2010; Khopkar, 2012; IUPAC, 2014).

$$HETP = H = A + B/\mu + C\mu \qquad [2.13]$$

where: A refers to Eddy diffusion

B refers to Longitudinal diffusion

C refers to Resistance to mass transfer

Eddy diffusion

$$A = 2\lambda d_p \qquad [2.14]$$

This term describes movement of the analyte through the column. The analyte moves randomly following different paths through the stationary phase particles. This causes band broadening because of differences in paths in lengths. Consequently, the peak shape is broadens. As shown in equation 2.14 Eddy diffusion is dependent on particle size. Larger particles increase the distance the analyte has to travel and as such increase dispersion effects. It is also influenced by particle shape and tightness of packing.

Longitudinal diffusion

$$B/\mu = \frac{2\gamma D_m}{\mu} \qquad [2.15]$$

In the column separation occurs in bands, and the concentration of analyte distribution is not homogenous; it is less at the edges of the band than at the centre. Consequently, diffusion of analyte molecules from the centre to the edges occurs. This results in band broadening. However, if the velocity of the mobile phase is high then the analyte spends less time in the column, which decreases the effects of longitudinal diffusion. Molecular diffusion in the liquid phase is much lower than that in the gas phase hence this term is negligible in HPLC.

It can be seen from equation 2.15 that longitudinal diffusion is dependent on the mobile properties and flow rate. It is also affected by temperature and molecular mass of the analyte.

Resistance to mass transfer

$$C = \frac{8}{\pi} \times \frac{k d_p^2 \mu}{(1+k) Ds} \qquad [2.16]$$

The time analyte taken for the analyte to equilibrate between the stationary and mobile phase is dependent on the velocity of the mobile phase. At high flow rates, the mobile phase moves ahead of an analyte that has a strong affinity for the stationary phase. This results in band broadening. For such analytes band broadening worsens with increasing velocity of the mobile phase. Equation 2.16 shows that the C-term decreases with increase in velocity whilst it increases with increase in particle size or particle porosity. It is also affected by temperature, retention factor. A graphical representation of equation 2.13 is shown in (Figure 2.6).

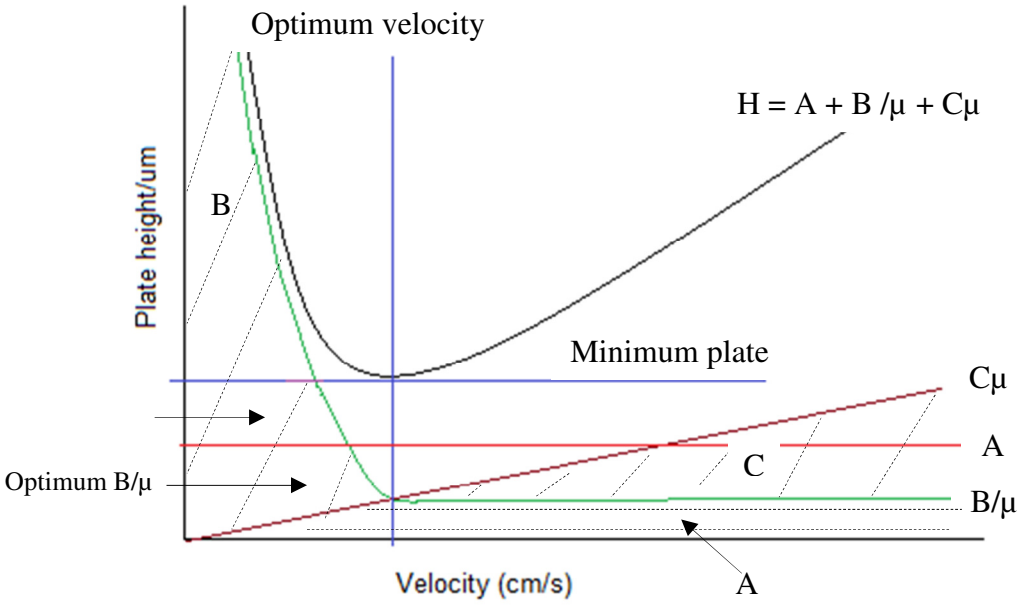


Figure 2.6 Van Deemer plot for the determination of optimum conditions (adapted from Khopkar, 2012; Moody, 1982; Kaur, 2010)

Consequently, from the discussion above it follows that reducing eddy diffusion, longitudinal diffusion and resistance to mass transfer will reduce HETP (equation 2.13). Thus, according to equation 2.9 reducing HETP effectively increases plate number, which is desirable so as to obtain optimal separations; sharp sand symmetrical chromatographic peaks must be obtained. From the discussion above it has been identified that this can this can be achieved through the following;

a) Column selection
   - Column length (L) - the shorter the column, the higher the efficiency.
   - Particle size- the smaller the particle size the lower the HETP hence the higher the value of N.
   - Packing quality- tightly packed column reduces the A term in equation 2.13, minimises eddy diffusion. As a result band broadening is minimised. For GC analysis capillary columns are therefore more advantageous. As such these will be applied to this study.

b) Mobile phase- increasing the flow rate, μ reduces the B-term in equation 2.13; however it also increases the C term. Consequently, there exists an optimum for flow rate which is a compromise of the two terms. This can be deduced by graphical van Deemeter plots of HETP versus velocity such as Figure 2.6.

c) Temperature- in accordance with kinetics, increases in temperature reduces resistance to mass transfer. This leads to a decrease in HETP as such an increase in efficiency.

Operational variables therefore have a profound effect on the quality of the results generated. Furthermore, it is possible to manipulate these variables and thereby influence the underlying chromatographic processes. This can then result in an improvement in the results in terms of plate number, selectivity, resolution, peak asymmetry, capacity factor, retention time and mass spectra. Consequently the column, injector port and oven temperatures, carrier gas flow rate, MS scan rate and MS ionisation energy were be investigated in this research (Chapter 7) so as to optimise the methods required to separate piperazine compounds from mixtures such as in street drugs.

2.5 CONCEPTS TO METHOD VALIDATION

The need for validation is highlighted in section 1.9. The parameters commonly investigated during method validation were identified as selectivity, specificity, linearity, linear, range, accuracy, precision limits of detection and quantitation (Eurachem, 1998; Thompson et al., 2002; ICH, 2005; Thompson, 2005; Rambla-Alegre et al., 2012; Lavanya et al., 2013). These are be investigated in this study and consequently discussed.

2.5.1 FACTORS CHARACTERISTIC OF METHOD VALIDATION

2.5.1.1 Linearity

2.5.1.1.1 Calibration curves

Linearity can be defined as the relationship that occurs when detector response (area or peak height) is directly proportional to concentration. As such a plot of detector response versus concentration gives a linear plot defined by equation 2.17 (similar to equation 3.16).

$$y = x + c + \varepsilon \qquad [2.17]$$

where: y = dependent variable (detector response)

x = independent variable (concentration)

m = slope

c = intercept

ε = random error

In validation studies, such plots (calibration plots) are used to investigate linearity and other linearity dependent parameters (limits of detection and quantitation). This involves the use of statistical analysis such as regression analysis (section 3.11). In this study, calibration curves were established by plotting peak area ratio versus concentration. Peak area ratio is calculated according to the equation 2.18;

$$\text{Peak area ratio} = \frac{\text{peak area of analyte standard}}{\text{peak area of internal standard}} \quad [2.18]$$

2.5.1.1.2 Testing for linearity

The linearity of the calibration plot needs to be established so as to ensure accuracy in quantitative determinations. Linearity can be determined by several methods among them; a) visual inspection of the calibration line plots, b) regression analysis and determination of correlation coefficients, c) use of residual plots (Thompson, 2005; Lavanya et al., 2013), d) use of other statistical tests such as Fischer-Snedecor test, Lack-of-fit and Mandel's fitting test (Eurachem, 1998; Gonzalez and Herrador, 2007; Rambla-Alegre et al., 2012).

The use of correlation coefficients in establishing linearity is a routine procedure commonly used by researchers. If linearity is achieved the linearity plots are visually linear with a regression coefficient, $R^2 > 0.99$ and RSD < 2% for repeated injections of the calibration solutions (Chan et al., 2010). However, it has been established that use of correlation coefficients on their own are inadequate as even a non-linear graph can give a high correlation coefficient (Miller, 1991; Rambla-Alegre et al., 2012; Thompson, 2005; Van Loco et al., 2002) Consequently linearity should further be established by other statistical tests for lack of fit or and or its significance. Analysis of residuals of the regression plot was identified as a common test (Thompson, 2005) in evaluating linearity, hence it will be applied in this research to confirm wether or not the calibration data (Chapter 7) follows a linear trend.

Analyses of the residuals of the regression analysis show the degree of deviations from linearity (section 3.2.11 equation 3.20). If there is no lack of fit (linear), deviations are not statistically significant, hence a plot of the residuals shows a random distribution with a mean of zero and no defined shape as shown in the comparator graph Figure 2.7 (Miller, 1991; Thompson, 2005).

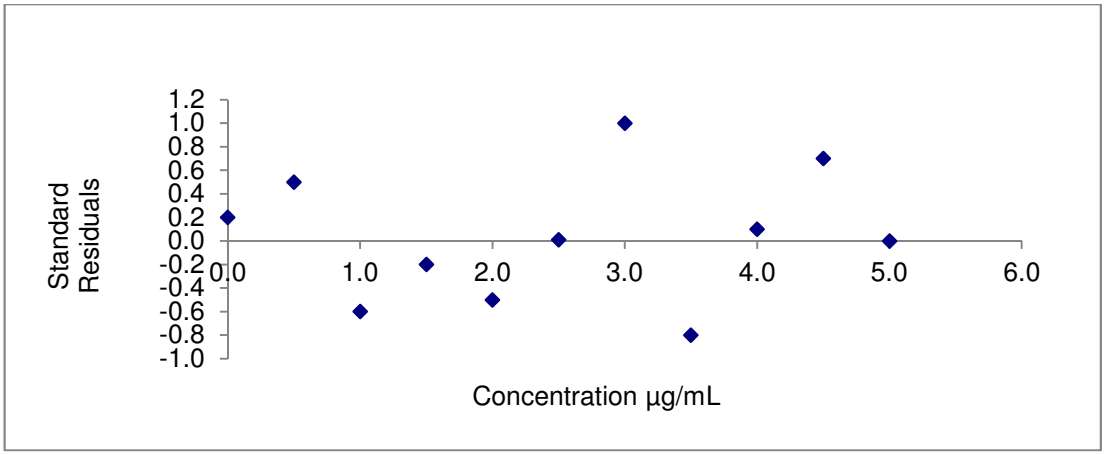


Figure 2.7 Expected shape of standard residual plot for a linear function showing no definite shape, i.e., constant variance (adapted from Miller, 1991; Thompson, 2005).

If there is lack of fit (non-linearity), a plot of the residuals shows a defined pattern (non-random distribution), as shown in the comparator graph Figure 2.8 (Thompson, 2005). Furthermore, the standard deviation of the replicates of each point on the plot is reasonably less than the systematic deviation of the residuals and hence statistically significant (Rambla-Alegre et al., 2012). In addition, as confirmation the randomness of the residuals can be statistically evaluated. The Runs test (section 3.2.13) is reported to be applicable to such a determination (Sprent and Smeeton, 2007; Chatterjee and Simonoff, 2012).

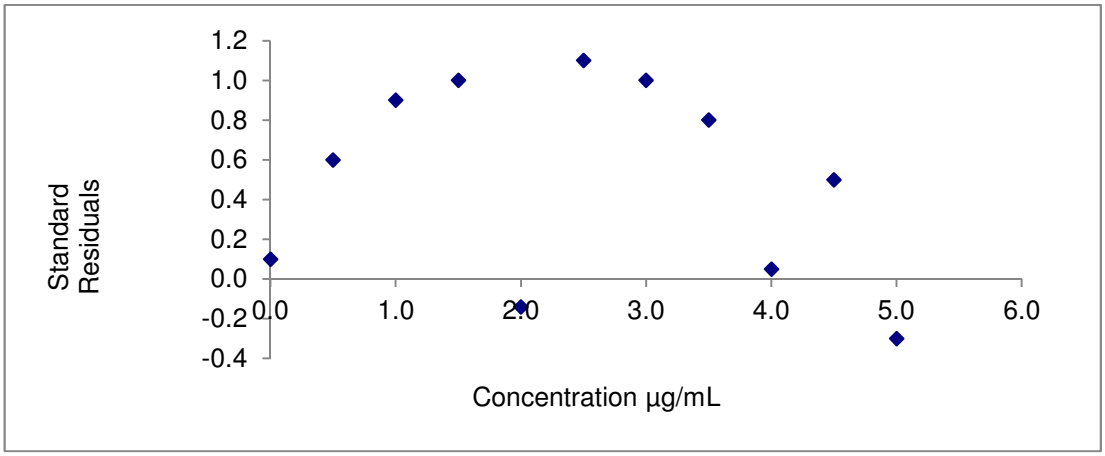


Figure 2.8 Expected shape of standard residual plot for a non-linear function showing a bow trend (adapted from Miller, 1991; Thompson, 2005).

Since the detector response is directly proportional to concentration and is employed to calculate the concentration of unknown (Crockett, 1986), therefore it follows that linearity is desirable, as it makes for accurate determination of unknown substances.

2.5.1.2 Range

For most analytes linearity is observed for a specific concentration range, giving rise to the linearity range and working range. The linearity range is the concentration range covering the area where the method was confirmed as having acceptable linearity, accuracy and precision. The working range defines the target concentration range for analysis. It is narrower and encompasses or is above the limit of quantitation (ICH, 2005), (see section 3.2.11).

2.5.1.3 Limit of detection (LOD)

According to ICH guidelines, the limit of detection is the minimum level at which the analyte can be detected with accuracy and precision. It is dependent on the analytical method and instrument sensitivity. The calibration curve of the analyte can be utilised to evaluate the LOD, through use of the residual standard deviation of the response and the slope. The calculation is given by equation 2.19 (1CH, 2005; IUPAC, 2014).

$$LOD = \frac{3.3\sigma}{S} \qquad [2.19]$$

2.5.1.4 Limit of quantitation (LOQ)

This is the minimum level at which the analyte can be quantified with acceptable accuracy and precision and instrument sensitivity. Similarly to the LOD the LOQ is given by equation 2.20 (1CH, 2005; IUPAC, 2014).

$$LOQ = \frac{10\sigma}{S} \qquad [2.20]$$

where: σ is the standard deviation of the response (based on standard deviation of the y-intercepts of the regression line).

S is the slope of the calibration curve

2.5.1.5 Accuracy

Accuracy refers to the "the closeness of test results obtained by that method to the true value. The criterion for good accuracy is 98 – 102% of the expected amount (Gonzalez and Herrador, 2007; Van Loco et al., 2002). Accuracy can be determined by determining analysing a standard of known concentration and calculating the actual amount determined

by the method under investigation relative to the known concentration as recovery (%). This is given by equation 2.21;

$$\% \text{ recovery} = \frac{\text{amount of substance determined by method}}{\text{actual known amount}} \times 100 \qquad [2.21]$$

2.5.1.6 Precision

Precision of an analytical method refers to the degree of variability in the results obtained between a series of measurements from the same homogenous sample using the same method. There are three different levels of precision: 1) repeatability refers to precision determined by multiple analyses of the same sample over a short interval of time, 2) intermediate precision refers to variability determined over a longer time period, conditions may vary such as different analytes or sample, and 3) reproducibility refers to variability using different laboratories. Precision is normally expressed as standard deviation or relative standard deviation (%RSD) and must be less than 2% for most methods (Eurachem, 1998, ICH, 2005; Gonzalez and Herrador, 2007).

2.5.1.7 Specificity/Selectivity

Specificity and selectivity are an expression of the sensitivity of the method to the analytes present in a sample. Specificity is the ability of the method to selectively, unequivocally determine the analyte in the presence of other substances in the sample matrix, such as impurities and degradants. This is determined through calculating selectivity value and or resolution, the higher the resolution between peaks the greater the specificity. Ideally selectivity should be > 1 (CDER 2004, Horacio et al., 2008). It is possible for lack of specificity to occur, however according to Lavanya et al. (2013) this may be compensated by other supporting analytical procedure(s) such as use of selected ion monitoring in GC-MS.

2.5.1.8 Robustness

The final part of validation is testing the method for robustness. Robustness refers to the ability of the method to withstand small changes in operating conditions (ICH, 2005), such as using different instruments, analyst or a similar column but from a different supplier. Robustness provides a measure of the reliability of operating the method under normal day to day conditions.

2.5.2 QUALITY CONTROL

Quality control refers to the processes or checks and standards that are routinely conducted in order to ensure that all the work generated is maintained at the required standard of quality. Corrective actions to problems are then taken before it affects the quality of the work. In laboratory analysis this can be conducted by setting up control charts, such as Shewhart charts (ICH, 2005) to monitor, for example, instrument performance and temperature. Such approaches have been widely used by researchers (Andersson et al., 2007a; Hibbert, 2007). System suitability testing has been described as a way of monitoring instrument performance (ICH, 2005). Repeated measures are carried out. The standard deviation is then determined (Horacio et al., 2008). This can provide a measure of consistency of detector performance. This approach is utilised in this study, control charts will also be applied as they have good practical application. Peak area ratios will be used as a measure of detector response.

It has been highlighted that columns affect the quality of chromatographic results. It is therefore critical to monitor their performance so as to check whether the column has decomposed. The general background in a chromatogram might show the presence of distinct peaks or an abundance of small peaks. This is a result of contamination due to column bleed, hydrocarbons, and phthalate plasticizers (McMaster, 2007; McNair and Miller, 2009). The background signal can interfere with analysis scan and decrease the sensitivity level for detecting target compounds. Furthermore, specific ions in the background can interfere with a single-ion or extracted ion chromatogram.

Columns bleed is the vapourisation of stationary phase coating resulting in the presence of peaks in the chromatogram due to the stationary phase. Generally, stationary phases are thermally stable, however, due to age or high temperature degradation can occur (McNair and Miller, 2009). This needs to be minimised so as to prevent interference with the analyte peaks, maintain chromatographic baseline stability, prolong the life of the column and prevent any fouling of detector (Barwick, 1999; Kaur, 2010). Contaminant mass ions due to column bleed of polysiloxane stationary phases have been reported at m/z 73, 207, 281 and 327 with the 207 ion being the most abundant followed by 281 (Miller 2005; McMaster, 2007). Furthermore, it has been identified section 1.8 that polysiloxane columns are

routinely applied in analysis of drugs of abuse. Consequently, as this study uses a polysiloxane based column the ion at m/z 207 will be monitored.

2.6 SAMPLE TREATMENT: DERIVATISATION

Derivatisation is generally performed to alter reactivity or change a physical property such as solubility, boiling point, melting point, thermal stability. It enhances volatility, thereby improving resolution and improves peak shape for quantitative analysis (Inuoe et al., 2004; Telepchak, 2004; UNODC, 2013c). The process involves altering the functional groups. The analyte is tagged with a detector-oriented compound (a compound with high detector sensitivity such as pentafluoropropionic anhydride (PFPA), molecular weight 310.05Da, boiling point 69 - 70^{o}C and N,O Bis(trimethylsilyl)trifluoroacetamide (BSTFA), molecular weight 257.40Da, boiling point 45 - 55^{o}C with trimethylchlorosilane (TMCS) as a catalyst (Quirke, 1994; Inoue et al., 2007; Takahashi et al., 2009). The structures of PFPA, BSFTA and TMCS are shown in Figure 2.9.

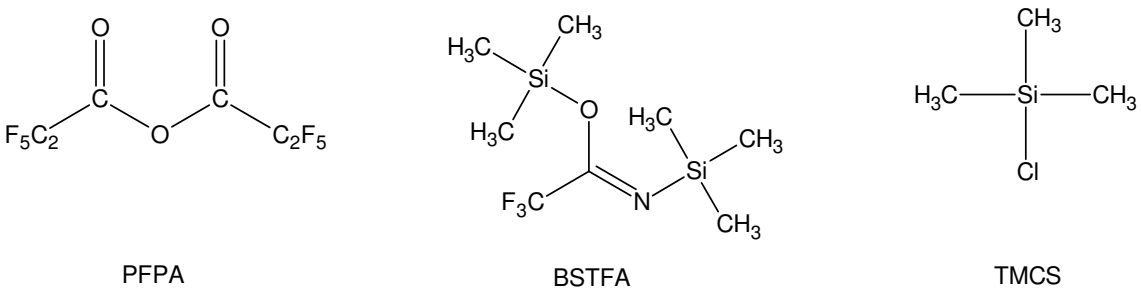
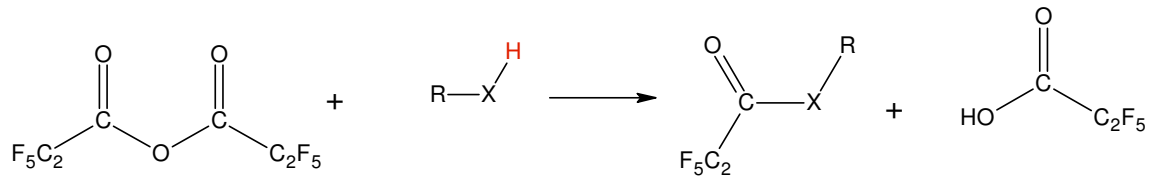


Figure 2.9 Structures of derivatising agents.

BSTFA is a silylation agent, unlike PFPA which acylates. It introduces trimethylsilyl (TMS) -Si(CH_3)$_3$ or DMS -Si(CH_3)$_2$ or N,O-bis(trimethylsilyl) acetamide in place of the functional group i.e., substitution of reactive H in polar groups such as -COOH, -OH, - NH and -SH to obtain their derivatives. As can be seen from their structures, these compound are very reactive and therefore can be used on a wide range of drugs, they also have very low boiling points hence are highly volatile hence one of their major functions is to impart volatility. These agents can derivatives such compounds amides, secondary amines, alcohols and as such will be applicable to the drugs under study (amphetamines, piperazines). In this study 4-FPPA will be investigated as an example to determine the effect of derivatisation on the analytes under investigation.

The generalised derivatisation reaction schemes for compounds containing the functional groups NH (e.g. piperazines, tryptamines, MDMA), COO (e.g. cocaine and heroin) and SH (e.g. thienylmethylpiperazines) functional groups is shown in Figure 2.10 PFPA.The reaction for derivatisation of 4-FPP is also proposed (Figure 2.11).



X = O, NH, NR', COO

R, R' = Alkyl, Aryl group

Figure 2.10 Generalised PFPA derivatisation reaction (adapted from Knapp, 1979; Telepchak, 2004; Miller, 2005).

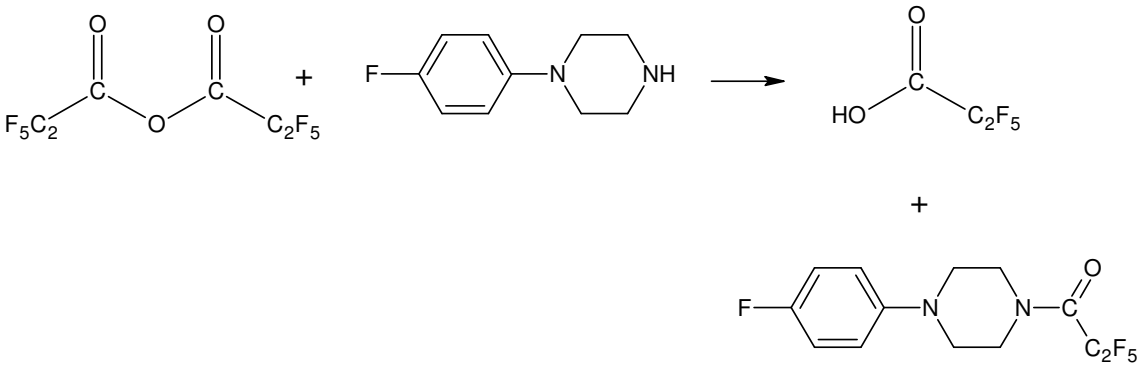


Figure 2.11 Proposed PFPA derivatisation reaction for 4-FPP.

2.7 ASPECTS OF SOLVENTS AND STABILITY STUDIES

The presence of degradation in a sample can be demonstrated by variables such as potency (concentration of the drug) and degradation products (or artefacts), changes in colour, pH and other physical properties (Yoshioka and Stella, 2000; ICH Q1A(R2), 2003). Any of these variables can be measured as a test for stability. In studies on drugs of abuse concentration is used as a measure of degradation and the presence of degradation products also confirms of degradation (Aalberg et al., 2005b; Karinen et al., 2011). Furthermore,

degradation products can also give an indication of the degradation pathway that occurred. Hence, these will be monitored in this research.

2.7.1 FACTORS INFLUENCING STABILITY

Factors affecting stability can be divided into a) intrinsic factors, such as the molecular structure of the drug itself and b) environmental factors- temperature, air, light, humidity, solvent effects, additives and excipients (cutting agents). In the molecular structure the reaction centres and substituents around the reaction centre affect its degradation, i.e., degradation pathway, kinetics and reactivity. For example, steric hinderance or electron withdrawing groups next to the reactive centre can make it less reactive thereby imparting a greater degree of stability. In solid state properties, such as melting point, crystallinity and hygroscopisity are critical and in addition, mechanical forces such as pressure and grinding - physical state of the drug may affect solid state and chemical degradation (Yoshioka and Stella, 2000; FDA, 2008). Therefore the stability of substances under analysis is affected by the prevailing conditions in the laboratory (environmental and analytical). Often these factors can act in combination. Degradation can occur both in the solid-state and more commonly in solution. In-solution, factors such as solvent choice critically arise.

2.7.2 EFFECT OF SOLVENTS ON STABILITY

The properties of a solvent, such as boiling point, viscosity, UV absorption, refractive index, density, and polarity determine its selection for analytical use. It was reported in section 1.10 that solvents influence the stability of drugs. It therefore follows that a good solvent is one which not only dissolves the reactants and reagents but whose properties do not interfere with the analysis. Hence, it does not affect the outcome or accuracy of the results. A good solvent therefore;

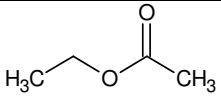
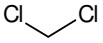
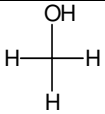
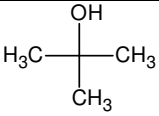
- Should have an appropriate boiling point. The boiling point of the solvent should be lower than that of the analytes. It is well known that in gas chromatography separation is based on their volatility (Khopkar, 2012). Hence, if the solvent and analyte have similar boling points the solvent peak will co-elute with the analyte.

- Does not contain ions which will interfere with the detector. In gas chromatography some detectors are element specific, e.g. acetonitrile will be a poor choice of solvent where Nitrogen phosphorous detector (NPD) is used (Barwick, 1999; Kaur, 2010).
- Should be inert to the reaction conditions so as to prevent degradation of the analytes. For example aldehydes or ketones such as acetone are rarely used because they may undergo chemical reactions with the analytes. In addition, alcohols such as methanol and ethanol may also react with the analytes (Yoshioka and Stella, 2000).
- Should be thermal stable under routine laboratory conditions.

In this study the solvent selected for use will be applied to all the analytical work in the research, identification, quantitative determination of street samples, characterisation and profiling. Consequently, the solvent required should not react with the drugs or any substances in the street samples as this might generate artefacts which can mask analytes.

This research investigated the solvents shown in Table 2.1. Their use in analysis of drugs of abuse was identified in section 1.10. The properties of the solvents (Gokel, 2004), are also given in the table. The potential for solvent-drug reactions, which can result in degradation were reviewed (Yoshioka and Stella, 2000; Lawrence, 2004; Jones, 1982) so as to gain an insight into their influence on chemical stability of the drugs for this research.

Table 2.1 Chemical properties of the solvents investigated (Gokel, 2004).

	Ethyl acetate	**Dichloromethane**	**Methanol**	**2-methylpropan-2-ol**
Structure				
Dipole moment (μ)	1.84	1.60	1.68	1.67
Dielectric constant (ε)	6.11	9.14	32.62	10.9

The solvents methanol and 2-methyl-propan-2-ol are alcohols and can undergo reactions with analytes characteristic of alcohols depending on the chemical functional groups present in the drug analyte, such reactions as esterification with drugs with organic acids properties can arise. Oxidation of the solvent can occur, resulting in products which are

potentially reactive; oxidation of methanol produces formaldehyde and in excess oxidising agent, the formaldehyde is further oxidised to formic acid and then to carbon dioxide and water. However, the reaction is very minimal and requires a catalyst. Methylene chloride in its pure dry state is a very stable compound. However, as a solvent methylene chloride has potential to decompose producing chemicals which can act as degradants (Tanabe and Matsuda, 1961). Dichloromethane can undergo atmospheric degradation; on exposure to air methylene chloride slowly reacts with photochemically generated hydroxyl radicals. It is known that in the presence of moisture methylene chloride has a propensity to undergo slow hydrolysis to give formaldehyde and hydrogen chloride and in alkaline solution the formaldehyde formed undergoes further reaction with hydroxide ion to give methyl alcohol and formic acid. The rate of reaction whilst minimal varies greatly with changes in temperature and pH and commercial reagents used for laboratory analysis are stabilised to minimise its hydrolysis. Reactions for the hydrolysis are shown in Figure 2.12 below.

$CH_2Cl_2 + OH^-$ (or H_2O) $\rightarrow CH_2ClOH + Cl^-$ (or HCl)

$CH_2ClOH + H_2O \rightarrow CH_2(OH)_2 + HCl$

$\rightarrow CH_2O + H_2O,$

in alkaline solution

$2CH_2O + OH^- \rightarrow HCO_2^- + CH_2OH$

Figure 2.12 Hydrolysis of dichloromethane (Tanabe and Matsuda, 1961)

Both formaldehyde and formic acid are reactive species which can affect the stability of analytes present in the solution

2.7.3 POTENTIAL CHEMICAL DEGRADATION REACTION PATHWAYS

During analytical investigation drug analytes can undergo different types of chemical degradation pathways, such as hydrolysis, oxidation, photochemical degradation, esterification and isomerisation. The type of reaction and its likelihood depend on the functional group present in the drug and also on the adjacent functional groups which influence its reactivity. The compounds (structures in Figure 1.4) under investigation in the research are heterocyclic polar compounds, exhibiting a variety of functional groups. They can generally be categorised as;

- 1-arylpiperazines, benzylpiperazines and amphetamines. These contain the amine and fluorine as reaction centres (for fluorinated piperazines such as FPP, CPP and TFMPP).
- Compounds containing ester bonds; mainly the non–piperazines alkaloid cocaine, benzylecgonine, ecgonine methyl ester and dextromethopharn.
- Compounds containing amides bonds; caffeine (an alkaloid) and diazepam (a benzodiazopinone)
- Other combinations such as dapoxetine and nicotinamide (heteromonocyclic pyridine)

Due to the presence of moisture in the solvents and atmosphere, hydrolysis is the most common degradation pathway for most drug compounds. Hydrolysis is often the major degradation pathway for substances having ester and amide functional groups due to nucleophilic attack of the hydroxide ion or water at the ester (Yoshioka and Stella, 2000; Lawrence, 2004) consequently cocaine and diazepam are likely to be subject to hydrolysis. Accordingly the degradation of cocaine is well known (Cole, 2003; Yoshioka and Stella, 2000; Staack and Maurer, 2005), the degradation generally follows the reaction scheme in Figure 2.13; shown for the formation of a carboxylic acid ester.

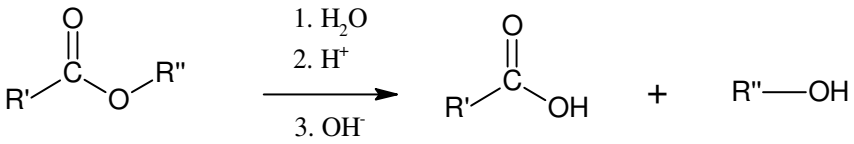


Fig. 2.13 General hydrolysis of the drugs containing ester bonds.

Drugs with a reactive nitrogen or amide bonds can also undergo hydrolysis, e.g. diazepam can undergo ring opening due to reversible hydrolysis of the amide and azomethine bonds. The reaction is generalised in Figure 2.14.

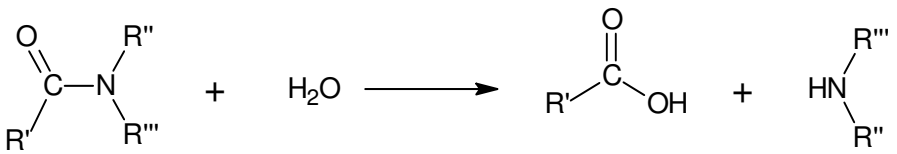
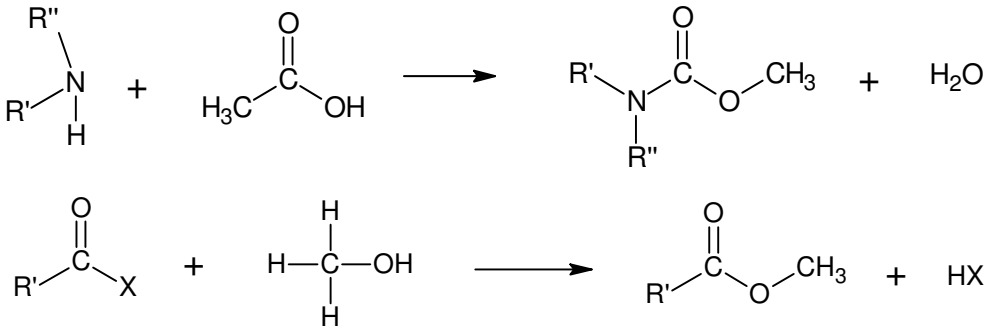


Figure 2.14 General hydrolysis of the drugs containing amide bonds.

Oxidation reactions are also a potential degradation pathway; in the presence of reactive oxygen or other oxidants in the sample matrix or atmosphere (dissolved atmospheric oxygen in the sample or solvent) can result in degradation of the drug analytes e.g. amines (piperazines) can be oxidised to the respective amine oxide (Jones, 1982). Such a reaction usually requires catalysis and elevated temperatures and as such under routine analysis is minimal. Esterification of drugs containing carbonyl bond or amine bonds can occur in the presence of organic acids, consequently use of ethyl acetate and methanol as solvents gives potential to such reactions with the piperazines, cocaine, diazepam and caffeine, such reactions generally occurs as shown below in Figure 2.15.



(X = halide, -OR', OH^-)

Figure 2.15 General esterification reactions of the drugs containing amine bonds.

Furthermore, considering that piperazine drugs of abuse exist as combinations in street drugs (Yeap et al., 2010) the likelihood of drug-drug interactions or drug-excipient interaction occurring during chemical analysis exists. This has implications of potential degradation and hence raises the need to investigate stability during analysis.

2.8 CONCEPTS TO CHARACTERISATION OF STREET SAMPLES

In Chapter 1 (section 1.8.3) physical and chemical characterisation were identified as inherent components of drug profiling. In addition, presumptive testing was identified as part of chemical characterisation. Hence, the concepts behind physical characterisation and the presumptive tests that will be applied in this research are discussed below. The general concept behind chemical characterisation was discussed in section 1.8.4.

2.8.1 PHYSICAL CHARACTERISATION

Physical features are those features that are present on the tablet after manufacture, i.e., "tableting". These are mainly the shape, logo, colour, dimensions or any other markings (Cheng et al., 2003; Makino et al., 2003; Milliet et al., 2009) e.g. Figure 1.3. According to Milliet et al. (2009) most physical features (except the logo and colour) are observed to be quite persistent in time. As such they can be utilised as a tool to provide links between different samples of tablets. For example, tablets produced from the same machine have similar features due to the characteristic traits of the tableting machines. A defective tablet die machine may even produce a characteristic mark, such as a chipped surface, this can aid in identification of its source. It therefore follows that such samples can be linked irrespective of when they were produced. As such, post-tableting links maybe useful intelligence tools for law investigation purposes.

In a study by Milliet et al. (2009) on profiling of MDMA, the authors found that generally organic impurities confirmed the links highlighted by physical profiling in about half of the cases studied (11 out of 19). In other study, also profiling of MDMA by Makino et al. (2003) and Zingg (2005) also investigated similar physical characteristic of tablets and similarly concluded that physical characteristics produce useful links. It can be extrapolated that similar samples from the same source will therefore contain similar chemical profiles, thereby confirming the links between them. However, this is not always the case. Cheng et al. (2003) in a study also on profiling of several amphetamines that reported that some tablets with similar physical appearances had different chemical compositions. Furthermore, some manufacturers used several colour dyes and metal dies with different shapes, logos, or letters, e.g., rectangular CC, circular 88, and AP were found at a local ecstasy tablet-manufacturing scene. The purpose of doing this is most likely to mislead law enforcement agents. As such, physical characterisation is not used on its own but in complement to chemical analysis.

2.8.2 PRESUMPTIVE TESTS

The concept behind the test is the production of a specific colour change due to a chemical reaction between the analyte and the reagent. Hence, these tests provide a visual means of identification. Table 2.2, highlights the compounds that give a positive reaction to the

presumptive tests used in this research (Marquis and Simon's test) and the expected colour changes.

Table 2.2 Reactions of colour tests used in this investigation (Cole, 2003; Kovar and Laudszun, 1989; UNODC, 1994; 2013c)

Substance	**Expected colour**	
	Marquis reagent	**Simon's reagent**
Amphetamine	Orange colour changing to brown	NR[1]
Other amphetamine derivatives e.g. 2,5-dimethoxyamphetamine (DMA)	Yellow to yellowish brown	NR[1]
Methamphetamine	Orange colour changing to brown	Blue
MDMA	Black	Blue
Other methamphetamine derivatives e.g. 2,5-dimethoxymethamphetamine (DMMA) and N-substituted derivatives such ethylamphetamine	Blue	Blue
Piperazines	NR[1]	Blue
Piperidine	NR[1]	Deep blue

[1] NR is no reaction observed.

The Marquis test is a qualitative test for aromatic compounds. The test is based on the reaction of the substance with the Marquis reagent, i.e., a solution of formaldehyde in the presence of concentrated sulphuric acid (section 8.2.4.2.1) The reagent can distinguish between unsubstituted phenethylamines (amphetamine, methamphetamine) and their ring-substituted analogues such as MDMA (Kovar and Laudszun, 1989; UNODC, 1994). It can be seen in Table 2.2 that the Marquis reagent produces an orange colour with amphetamine and methamphetamine whilst a dark blue/black colour is obtained with MDMA. Hence, these compounds can visually be distinguished. For this reason it is widely used as a presumptive test for 'ecstasy' (Cole, 2003; UNODC, 2013c). Piperazines have been reported to give no reaction or a faint colouration (UNODC, 2013c). Similarly, the London toxicology group (LTG) found that of the piperazines only 2-MeOPP gave a faint pink colour with Marquis reagent (LTG, 2006). It can also be applied to other drugs such as heroin and codeine. However, it also produces colour changes with a large number of heterocyclic compounds and this is a limitation as it would give ambiguous results if used

on sample containg more than one such compound. Figure 2.16 shows the general reaction to the Marquis test.

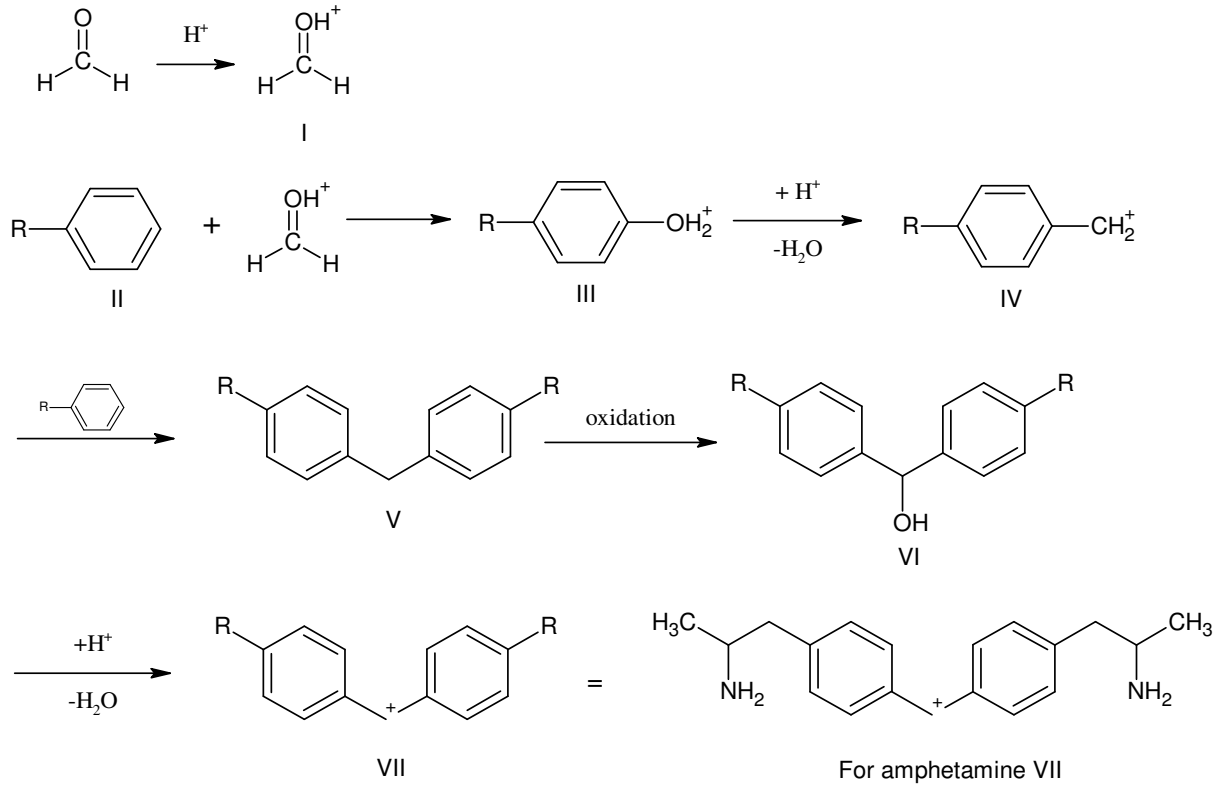


Figure 2.16 Reaction of aromatic compounds with Marquis reagent (adapted from Kovar and Laudszun, 1989).

In Figure 2.16, formaldehyde in the presence of an acid forms a carbonium ion (I). This reacts with the aromatic ring (II) in the presence of sulphuric acid to form a carbenium ion (IV). To stabilise the carbenium ion a further reaction with a second aromatic ring occurs (V). Oxidation occurs due to traces of heavy metals such as iron in sulphuric acid to give (VI). This undergoes hydrolysis in the presence of sulphuric acid to give a carbenium ion (VII). This carbenium ion is responsible for the pink colour observed with aromatic compounds, however the final colour is dependent on the type of drug. The different substituents for R in reaction implies different carbenium ions are formed at the end of the reaction resulting in the colours observed (Table 2.2).

Simon's reagent is comprised of solutions of sodium nitroprusside, acetaldehyde and sodium carbonate (UNODC, 2006; Takahashi et al., 2009). Simon's reagent is used to test

for secondary amines on the basis of formation of a blue Simon-Awe complex (Kovar and Laudszun, 1989). It can be seen it Table 2.2 that no colour is produced with primary amines (e.g. amphetamine) whislt secondary amines produce a blue colour with methamphetamine derivatives). In comparison, it has been reported to be less sensitive to piperazine drugs (IV) than secondary amines such as methamphetamine or MDMA (UNDOC, 2013c). As such, this could prove a limitation to use of the test on its own. The reaction mechanism is shown in Figure 2.17.

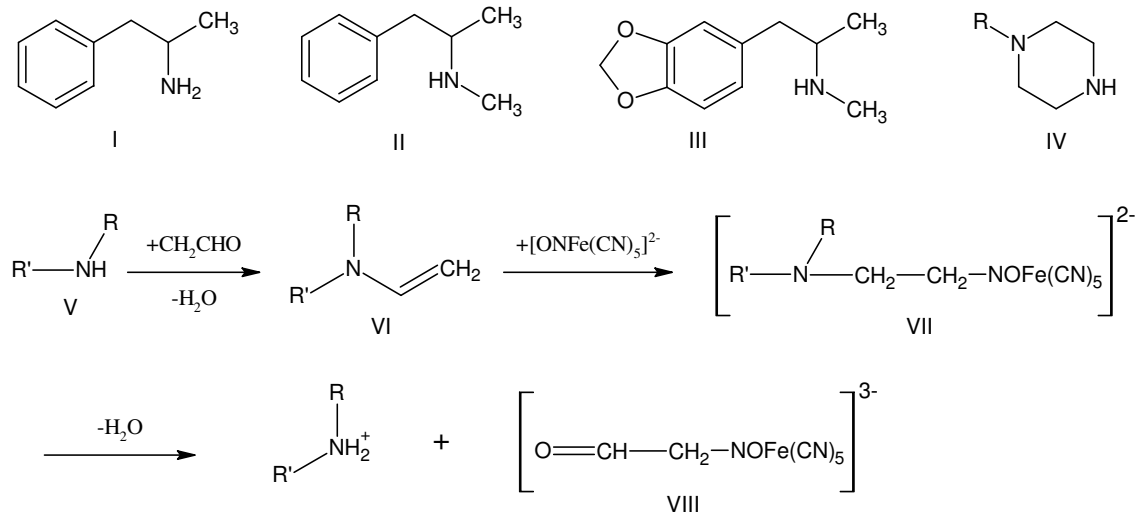


Figure 2.17 Reaction of secondary amine compounds with Simon's reagent (adapted from Kovar and Laudszun, 1989).

Reaction of the secondary amine with formaldehyde results in the formation of an enamine intermediate product (VI). This reacts with the sodium nitroprusside anion ($[ONFe(CN)_5]^{2-}$) to give the immonium ion (VII) and is subsequently hydrolysed to the Simon-Awe complex (VIII), this is responsible for the blue colour change. This is a ferro cyano complex in aqueous solution and is characteristically blue. Simon's test distinguishes between primary and secondary amines. Secondary amines give a blue a positive reaction (blue colour) with Simon's reagent (Table 2.2). Hence, the Marquis and Simon's test will be useful in this study as street samples are expected to contain amphetamines such as MDMA and other aromatic compounds (such as BZP and other piperazine derivatives, cocaine, diazepam, caffeine).

Another test that has been reported in the analysis of piperazines is the Dragendorff reagent (UNODC, 2013c). The reagent tests for the presence of an alkaloidal base, however tertiary amines often show a strong positive result. Piperazines may also give a mildly positive result. Whilst reports on presumptive tests are limited, the use of Dragendorff is less than that of Marquis and Simon's reagents. In the UNODC (2013c) study the results for the piperazines and amphetamines were orange to orange-red and not easily distinguishable. As such, this reagent was not considered for use in this research.

It was identified (section 1.2), that piperazines are often marketed as 'ecstasy' and are found in combination with other drugs such as methamphetamine, MDMA and adulterants. The Marquis test will therefore be a valuable tool in this study in detecting the presence of amphetamines. The limitation of presumptive tests is that the reactions are generally specific to a class of drugs and not the actual drug substance. For example, it would be difficult to specifically identify amphetamine in the presence of MDMA by Marquis reagent or methamphetamine if piperidine is present in the sample (Table 2.2). The actual colour observed depends on many factors, such as the concentration of the drug and whether the drug is a salt or free base form (O'Neal et al., 2000). In addition, the presence of impurities in the sample matrix may interfere and mask the colour change (Baker and Phillips; 1983; O'Neal et al., 2000). Consequently, more than one colour test is used, this gives more discriminating results. Hence, in this study for the analysis of street samples, preliminary screenings were carried out using the Marquis and Simon's tests.

2.8.3 SYNTHESIS (ORGANIC)

Synthesis is the process of chemically reacting compounds so as to produce a desired substance. The initial starting materials to a reaction are defined as precursors. The purpose of synthesising a drug substance is to obtain the product in the highest yield possible and at acceptable purity. This is influenced by several factors. Typically, this is dependent on temperature, catalysts, concentration of precursors, reaction time and reaction route and furthermore, on the skill of the manufacturer. For example, use of the wrong temperature might mean the reaction will not have enough activation energy for the precursors to react. If the reaction time is too short the reaction is likely not have had enough time to reach completion. If the amount of precursors used are not the reaction's stoichiometric ratios one or more will be in excess. In all these cases the result will be a) poor product yield and b)

residual precursors will be present giving poor product quality. Side reactions can occur resulting in by-products such the formation of the 2 and 3) positional isomers of 4-FPP and (2 and 4) positional isomers of- 3-TFMPP (the reactions would follow a similar mechanism to that shown in Figures 1.17a; b for the synthesis of phenylpiperazines.

In addition, Liu and Robichaud (2005) reported the possibility of a competitive side reaction in the reaction of anilines (I) with bis(2-chloroethylamine). The product of the reaction 4-FPP or 3-TFMPP and the reactant bis(2-chloroethylamine) occurs the presence of a base. The reaction scheme in Figure 2.18 is proposed. However, in the method used the author also reported that the use of diethyl glycol monomethyl ether as a solvent minimises the competitive side reaction (Liu and Robichaud, 2005). Hence, its occurrence is reduced since this was the solvent used. Assuming that the side reaction minimally occurs, products (II) and III) could be produced as impurities. This not only gives a poor quality product but results in low yields of the product.

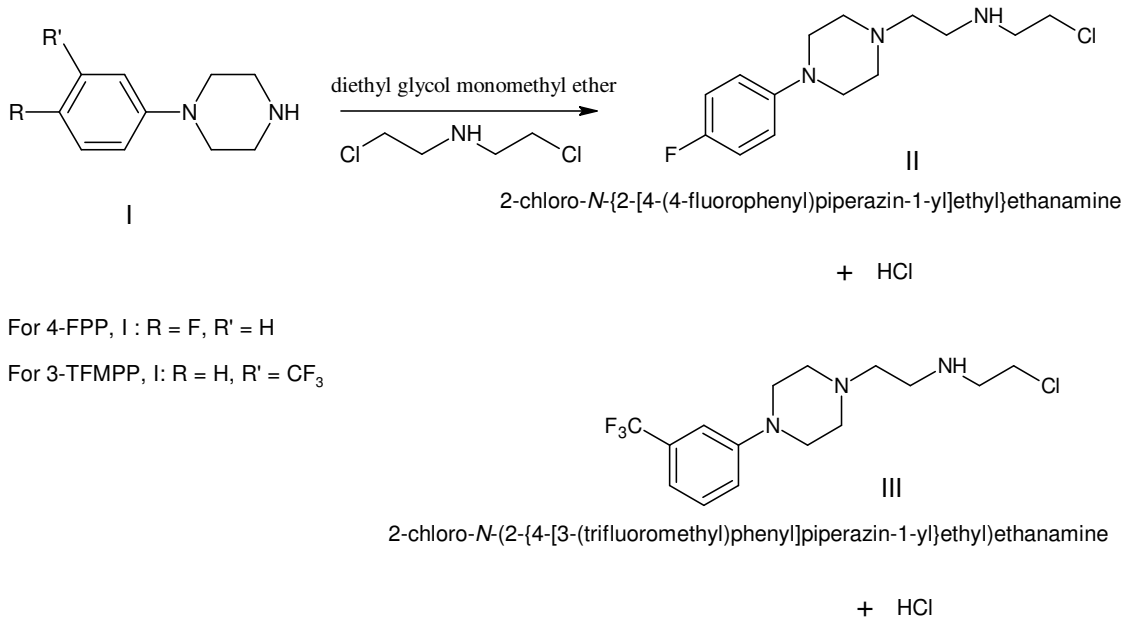


Figure 2.18 Impurities in the synthesis of phenylpiperazines from bis(2-chloroethy1)amine hydrochloride and substituted anilines.

In the current study, 4-FPP and 3-TFMPP were synthesized from fluoroanilines according to previously published procedures (Liu and Robichaud, 2005) and an adaptation of the method for the isomer 4-FMPP for the synthesis of the 3-TFMPP isomer (Kiritsy et al., 1978; Shaman Australis Botanic 2003). It was recorded the method by Kiritsy et al. for 4-FPP was similar in reagents to that of Liu and Robichaud, 2005. The schematic diagram for

the syntheses reactions were shown in Chapter 1, Figures 1.17a,b (Liu and Robichau, 2005) and Figures 1.6a,b (Kiritsy et al., 1978; Shaman Australis Botanic 2003). The mechanisms for the reactions involved are shown in Figures 2.19 and 2.20 below. Due to 4-FPP having electron-donating substituents the mechanism (Figure 2.21) involved ring-closure via reaction of bis(2-chloroethy1)amine hydrochloride and the appropriate substituted anilines (Kiritsy et al., 1978). For 3-TFMPP the mechanism involved fusion of anhydrous piperazine and the corresponding substituted halobenzenes due to the electron-withdrawing properties of the (-methyl group) substituent. The intermediate carbocation formed is stabilised through resonance (structures IV- VIII). Whilst the main product is 3-TFMPP resonance can result in the presence of 2 and 4 (TFMPP) isomers. The procedure can be applied to any other N-(4-substituted pheny1)piperazines.

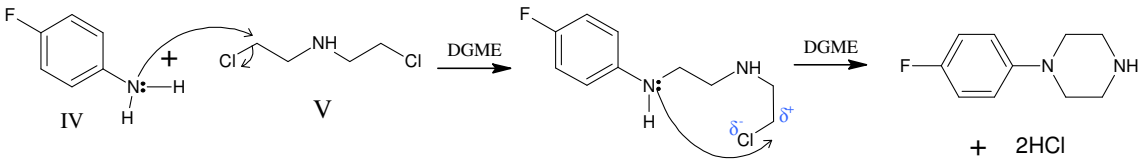


Figure 2.19 Mechanism for the synthesis of phenylpiperazines from bis(2-chloroethy1)amine hydrochloride and substituted anilines.

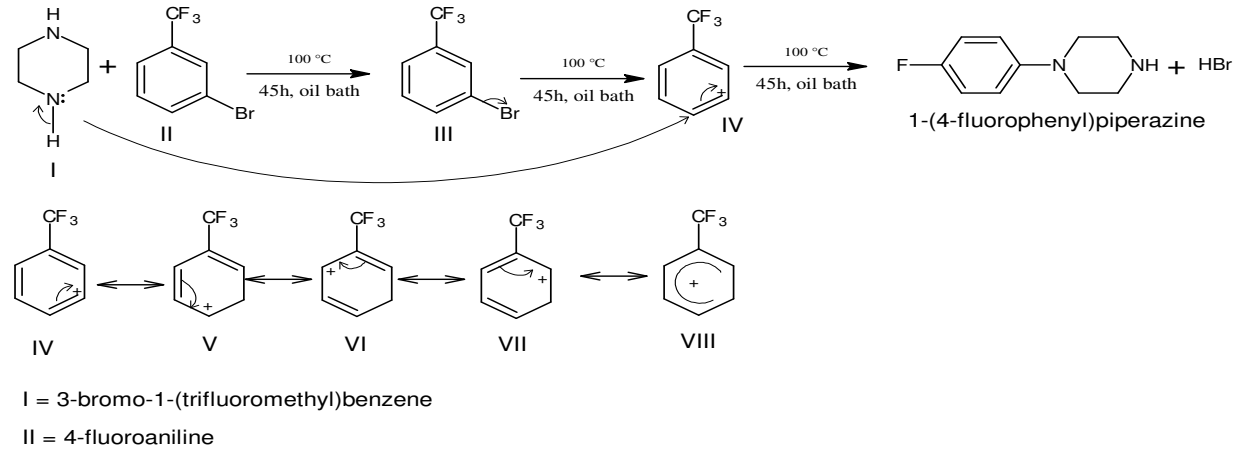


Figure 2.20 Mechanism for the synthesis of phenylpiperazines from anhydrous piperazine and substituted halobenzenes.

CHAPTER 3

STATISTICAL DETERMINATIONS AND OTHER CALCULATIONS

3.1 INTRODUCTION TO STATISTICAL EVALUATIONS AND CALCULATIONS

In this chapter the tests applied in testing the validity of the results generated during this research are discussed. The mean, standard deviation, relative standard deviation and variance are common and useful statistical tests applied in research work and have been applied in analysis of drugs of abuse such as Andersson et al. (2007a), Davies et al. (2010) and Kelleher et al. (2011). The tests were for descriptive statistical evaluation of research data such as the degree of variation and error. Consequently, they were applied in this study for similar evaluation. These tests are described in sections 3.2.1 - 3.2.3. Other statistical tests used were Pearson's correlation coefficient; t-test, ANOVA, Chi square, Friedman test, Wilcoxon signed-rank test, Gain and loss, linear regression, Mann- Kendal test and Runs test for randomness (sections 3.2.4 - 3.2.13). In addition, the calculation of identification parameters (relative retention time and relative retention index) and quantitative determinations for analytes are described (sections 3.2.14 and 3.2.15).

3.2 TESTS APPLIED

3.2.1 MEAN

The mean or average of a sample $\bar{x}$ is given by the sum divided by the sample size. For a sample of size n where i = 1, 2, ….,n (Corder and Foreman, 2009).

$$\bar{\mathrm{x}} = \frac{1}{\mathrm{n}} \sqrt{\sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{x}_i} \qquad [3.1]$$

3.2.2 STANDARD DEVIATION RELATIVE STANDARD DEVIATION AND VARIANCE

Standard deviation, σ and variance, σ^2 are measures of the variation of the data from the mean. Standard deviation is given by the square root of the variance. These parameters were determined according to equations 3.2 and 3.3 (Sprent and Smeeton, 2007);

$$\sigma = \sqrt{\sum_{i=1}^{n} \frac{(x_i - \bar{x})^2}{n-1}} \qquad [3.2]$$

$$\text{Variance} = \sigma^2 \qquad [3.3]$$

The relative standard deviation, %RSD is defined by the equation 3.4 (Sprent and Smeeton, 2007; Corder and Foreman, 2009).

$$\%\text{RSD} = \frac{\sigma * 100\%}{\bar{x}} \qquad [3.4]$$

3.2.3 CONFIDENCE INTERVALS

The interval is a range of values for which there is a specified probability, 100(1-α)% that the population mean or parameter lies within it (Corder and Foreman, 2009). For example it can be said with 95% confidence (α = 1- 0.95 = 0.05 or 5%) that the mean is defined by this value. As such it provides a description of the accuracy of an estimated mean value. The interval can be computed using either the normal or t-distribution. In this study, computations applied the t-distribution since it is the one applicable to small populations (n < 30) and is given by (Corder and Foreman, 2009; Gopal, 2006);

$$\bar{x} \pm \left(\frac{\sigma}{\sqrt{n}}\right) t \qquad [3.5]$$

Where σ is equivalent to the estimated standard deviation of the sample, t is the t-statistic for the t-distribution at 1-α level of confidence (or $1 - {}^{\alpha}/_{2}$ for a two tailed distribution) and n-1 degrees of freedom. It is further discussed in section 3.2.5.

3.2.4 PEARSON'S CORRELATION

It is a measure of whether there is a significant relationship, i.e., correlation between two variables. Pearson's correlation coefficient is given by r, where -1< r < +1, the closer to one the greater the similarity. A value of 0 indicates there is no relationship, +1 indicates a perfect positive correlation and -1 indicates a perfect negative correlation, however it is only applicable to continuous data (Gopal, 2006; Sprent and Smeeton, 2007; Corder and Foreman, 2009). Pearson's correlation is given by the equation 3.6 (de Souza and Junqueira, 2005; Taverniers et al., 2004; Corder and Foreman, 2009);

Let x and y be the measured variables with sample of size n where i = 1, 2, ….,n

$$r = \frac{\sum_{i=1}^{n}[(x_i - \bar{x})(y_i - \bar{y})]}{\{\sum_{i=1}^{n}(x_i - \bar{x})^2][\sum_{i=1}^{n}(y_i - \bar{y})^2]\}^{\frac{1}{2}}} \qquad [3.6]$$

As such in this study this test was applied to compare the data derived from the different solvents used in the stability studies. The objective was to statistically determine if different solvents produce similar stability profile for the drugs (Chapter 5 on stability studies). In addition it was also applied to evaluate if there was correlation between synthesised drugs samples and reference standards in the chapter for characterisation of street samples (Chapter 8).

3.2.5 T- TEST

T-tests are a basic inferential test used for evaluating the significance of the relationship between two sample means (μ_0 and μ_1), with respect to the variation in the data. This is achieved by testing the hypothesis H_o: $\mu_o = \mu_1$, i.e., there are no differences between the two samples hence their means are equal. The principle behind significance testing is the calculation of the test statistic and its comparison to tabulated critical values. In addition, the determination of the probability of occurrence (p) by comparison of the p value to the level of significance used (α). If $p < \alpha$, it implies that the means are not equal, hence H_o is rejected (Huber, 1996; Sprent and Smeeton, 2007). There are several types of statistical tests that utilises a t-distribution. These are (Gopal, 2006);

a) One-sample t-test (uni-variate test): this is a measure of whether a sample mean is different from a hypothesized value. This by investigating the value of a single sample mean and its comparison of the sample mean with a theoretical value such as a known population mean or some other fixed value.
b) Independent samples t-test : compares two means from different groups
c) Paired samples t-test: compares two means that are repeated measures of the same subject

The principle assumptions are the population data from which the sample data are drawn are normally distributed and that the variances of the populations to be compared are equal (Sprent and Smeeton, 2007). In a normally distributed population, the population standard deviation, σ is known and the difference between each value of the variable x and the population mean, μ is defined by the standard variable, *Z* where;

$$Z = \frac{\mathrm{x} - \mu}{\sigma} \qquad [3.7]$$

Consequently for the sample mean, $\bar{x}$

$$Z = \frac{\bar{\mathrm{x}} - \mu}{\sigma_{\mathrm{x}}} \qquad [3.8]$$

Where σ_x is the standard error of the mean, $\sigma_{\mathrm{x}} = \sigma/\sqrt{\mathrm{n}}$. Substituting for σ_x into equation 3.8 gives;

$$Z = \frac{\bar{\mathrm{x}} - \mu}{\sigma/\sqrt{n}} \qquad [3.9]$$

Equation 3.9 has more practical applications than 3.7 and was applied in the calculation of the z-statistic in significance tests and in this research. It has been reported that when the sample size is small (< 30) and the standard deviation is unknown the sample distribution no longer follows a normal distribution pattern, instead it widens and follows a t-distribution where the t-statistic is defined by the equation 3.10 (Gopal, 2006; Corder and Foreman, 2009);

$$t = \frac{\bar{x} - \mu}{s/\sqrt{n}} \qquad [3.10]$$

where s is the estimated standard deviation.

Consequently, in this research the following types of t-tests were applied when the following conditions existed; for the comparison of two samples (drug A and drug B) subjected to the same measure, the independent t-test was be applied. For comparison of the drugs in optimisation studies (Chapter 6) such as checking for effects of before and after subjection to changes in temperature the paired t-test was applied.

3.2.6 ANALYSIS OF VARIANCE (ANOVA)

ANOVA is a statistical test for heterogeneity of means by analysis of group variances and is used to compare the means of two or more groups of data (Huber, 1996). The types of ANOVA are a) ANOVA One-way; this is applied where there is only one factor (one treatment type, one response) and b) two- way which is applied when two factors are investigated (2 treatment types or 2 responses). The concept behind ANOVA is the calculation of the mean observation and variance within each group. This is followed by comparison of the variances among the means to the average variance within each group. The hypothesis test is that the observations in the different groups have the same mean, i.e., Ho: $\mu_1 = \mu_2, \ldots\ldots, \mu_n$. As such the weighted and group variance will be same as the within group variance. However, the variance among the means increases if at least one of the means is not equal. The significance of the difference between the means is indicated by the test statistic, F, where F is the ratio of variance among the means to the average variance within the groups (Huber, 1996; Corder and Foreman, 2009). The F statistic has a known distribution as such its probability of occurrence can be calculated.

Assumptions of ANOVA are similar to the t-test and the tests are similar. For more than two samples it would mean carrying out multiple t-tests since the t-test is for comparison of two samples. This would lead to increased errors in the analysis (Sprent and Smeeton, 2007; Corder and Foreman, 2009), hence the use of ANOVA. As such in this research for comparison of more than two samples, ANOVA is one of the tests that were applied. The test statistic, F is given by equation 3.11 (Corder and Foreman, 2009) and is determined as follows;

Let y be the response observed, where y is a random variable with equal variances, independent errors and a normal distribution. Let k be the number of groups investigated and n the number of observations. For the y_{ij} observation; i = 1, 2,…., k and j = 1, 2,…., n. The total sum of the squares (SST) is given by sum of the squares when subjected to treat (SSA) and the error of the sum of the squares (SSE), i.e.,

SST = SSA + SSE

$$\text{SST} = \sum_{i=1}^{k}\sum_{i=1}^{n}(y_{ij} - \bar{\bar{y}})^2 = \sum_{i=1}^{k}\sum_{i=1}^{n}(y_{ij})^2 - \frac{\sum_{i=1}^{k}\sum_{i=1}^{n}(y_{ij})^2}{kn}$$

$$\text{SSA} = \frac{1}{n}\sum_{i=1}^{k}\sum_{i=1}^{n}(y_{ij}) - \frac{1}{kn}\sum_{i=1}^{k}\sum_{i=1}^{n}(y_{ij})^2$$

SSE = SST – SSA

$$F = \frac{\text{MSA}}{\text{MSE}} \qquad [3.11]$$

where: $\bar{\bar{y}}$ is the group mean (mean of means)

MSA is the mean sum of squares for the treatment

MSE is the mean error sum of squares

If $p < \alpha$ reject the null hypothesis, as the deduction is that at least one of the means is not same as the others.

3.2.7 CHI SQUARE

Chi square is a goodness-of-fit test used to measure of the deviation of a sample from an expected value. It is used to determine whether a sample data are consistent with a hypothesised distribution (Gopal, 2006). In stability studies the objective was to determine whether the concentration of the analyte remained the same for a period of time i.e., H_o: $x_1 = x_2 =, …., = x_n$ where x is the concentration of the analyte and N is the sample size (or number of determinations). Therefore in this research the chi-square test was one of the

tests used in analysis of stability results (Chapter 5). The chi square distribution is the distribution of the sum squares of a set of normally distributed random variables. The chi square distribution is the distribution of the sum squares of a set of normally distributed random variables. The test statistic, chi square, χ^2 is given by equation 3.12 (Gopal, 2006; Corder and Foreman, 2009). Let the probabilities of various classes in a distribution be p_1, p_2, …, p_k

$$\chi^2 = \sum_{i=1}^{k} \frac{(m_i - Np_i)^2}{Np_i} \qquad [3.12]$$

Critical values of chi square were used for comparison (Laurencelle and Frangois, 2002).

3.2.8 FRIEDMAN TEST

This is a non-parametric test used to compare more than two related samples. The null hypothesis of the test is that when subjects are subjected to different treatments, the treatments produce identical effects. The alternative hypothesis is that at least one of the treatments tends to yield larger values than the other treatments (Friedman, 1937; Corder and Foreman, 2009; Best et al., 2009). The test does not make assumptions that the data is normally distributed and uses ranks of data. The objective of the test is it provides a means of identifying differences in the distribution under k number of treatments.

Martin et al. (1993) in their study on Friedman tables and Best et al. (2009) in their study on non-parametric rank tests reported that the test is an alternative to the F-test for two-way analysis of variance when there is reason to believe that the assumptions underlying the classical ANOVA are not satisfied by the data. For this reason this test was deemed appropriate to use in the study as the data under investigation cannot be assumed to follow a normal distribution. In addition, it involves ranking, which was especially useful as the data under consideration was very diverse for the different subjects (drugs) which can result in swamping of the smaller values. Consequently, ranking gives a clearer picture. The descriptive statistics derived from the ranking would also be of use in interpretating the pattern between the results, such as the medians and rank sums. Furthermore, Martin et al. (1993) reported that it is applicable in situations where multiple correlated measures are

obtained on the same subjects. In this research such a situation existed where several different instrumental variables, i.e., different column and oven temperatures were applied on the subjects. In addition different parameters, i.e., retention time, plate number, resolution, selectivity and retention were then determined on the same subjects. As such the test was considered ideal for use and was applied in evaluation of optimisation (Chapter 6) and validation data (Chapter 7).

The Friedman Test statistic, F_r is calculated by equation 3.13 (Friedman, 1937; Martin et al., 1993; Best et al., 2009; Corder and Foreman, 2009).

$$F_r = \left(\frac{12}{nk(k+1)} \sum_{i=0}^{n} {R_i}^2 \right) - 3n(k+1) \qquad [3.13]$$

$$\text{Corr} = \sum_{i=1}^{n} \sum_{j=1}^{k} ({t^3}_{ij} - t_{ij}) / (nk(k^2 - 1)$$

where: F_r is the Friedman test statistic

n is the number of subjects or cases (= rows)

k is the number of conditions (= ranks or columns)

R_i is the sum of the ranks for ranks i

df is the degrees of freedom = k-1

Corr is the correction factor for ties

If ties exist on ranking the correction factor, Corr is applied and

$F_{rcorr} = F_r / \text{Corr}$

3.2.9 WILCOXON SIGNED-RANK TEST

The Wilcoxon signed-rank test (Wilcoxon, 1945) a nonparametric test equivalent to the t-test and similarly is used for the analysis of two samples. It determines the magnitude of departures from a hypothetical mean ranks the data and calculates the test statistic (Toutenburg, 2002). The assumptions made are that a) the dependent variable is measured at the ordinal or interval/ratio level, b) the independent variable consists of two categorical, matched pairs, i.e., the same subjects are present in both groups and, c) assumption of

population symmetry. It does not assume normality of data hence can be used when this assumption has been violated rendering use of the dependent t-test is inappropriate. When participants are subjected to 2 different treatments at different time intervals violation of normality assumption can occur (Corder and Foreman, 2009). As such this test was deemed appropriate for this study were the drugs were subjected to two different oven temperatures and the responses measured through a number of variables (Chapter 6). The Friedman test was used for evaluation when more than two temperatures were investigated (Phase 1 of the optimisation).

The test is based on the concept that if a true mean or median (θ) exists then there should be an equal number of positive and negative ranks. For a data set consisting of N paired observations (x_1, y_1), (x_2, y_2),...., (x_N, y_N), where the X and Y random variables are correlated. The differences are defined as (Kang and Kvam, 2007);
$D_i = x_i - y_i$, $i = 1, 2, ..., N$

The following assumptions are made about the distribution of the random variables: a) the distribution of D_i each is symmetric b) the D_i's are mutually independent and c) the D_i's have the same median. As such, the hypothesis tested is H_o: $\theta_1 = \theta_2$ hence $\theta_1 - \theta_2 = 0$ meaning; H_o: There is no difference between the two treatments. H_1: There is a difference between the two treatments. The Wilcoxon signed-rank test statistic is given by T, equation 3.14 (Kang and Kvam, 2007; Sprent and Smeeton, 2007; Toutenburg, 2002).

$$T = \min\left[\sum_{i=0}^{n} R^2{}_i(D_i > 0), \sum_{i=0}^{n} R^2(D_i < 0)\right] \qquad [3.14]$$

$$\text{Mean} = E(t) = \sum_{i=0}^{n} r_i/2$$

$$\text{Variance} = \sigma^2 \sum_{i=0}^{n} r_i/4$$

The standardised test statistic, $Z = \frac{T - E(T)}{\sigma}$

where: σ is the standard deviation

r_i is the ranks for ranks i

R_i is the sum of the ranks for ranks i

The criteria for the test are that for sample sizes, $n \leq 20$ by comparison to literature Wilcoxon tables. For sample sizes, $n > 20$ the normal distribution Z score tables are used.

3.2.10 GAIN AND LOSS ANALYSIS

Gain and loss graphs are one of the many types of existing control charts. They are used in change analysis. They are used to monitor deviations or variability from a target value. They are useful for detection, correction and reduction of process that cause undesirable change. According to Cheung et al. (2012) control charts allow for visual distinction between meaningful and random change by distribution pattern. As such, they were found useful in this study to enable determination of the changes in the chromatographic profile of an analyte caused by variation of experimental variables, such as oven temperature. Hence, parameters which caused the highest variations were identified. This facilitated selection of optimum parameters during the optimisation process (Chapter 6). In addition they provided easier evaluation of the large experimental data generated. In the study % gain or loss in plate number and tailing was evaluated using this method. With the aim of determining which experimental variable (different injector and oven temperatures) caused the most increase in N and decrease in T. The % Gain or loss (or % change) is given by equation 3.15;

$$\% \text{ G or L} = \frac{\text{Parameter experimental value - parameter value in control} \times 100\%}{\text{parameter value in control}} \quad [3.15]$$

Where % G or L is % gain or loss respectively, this is then plotted to give a graphical depiction of the changes (Bersimis and Parakis, 2007; Kang and Kvam, 2007; Cheung et al., 2012).

3.2.11 SIMPLE LINEAR REGRESSION ANALYSIS

Regression analysis is a method for investigating the relationship between two or more variables. Various types of regression analysis exist; however for routine analysis ordinary

least squares (OLS) regression analysis is applied (de Souza and Junqueira, 2005; Miller, 1991; Tavernier et al., 2004). OLS investigates the relationship between one independent variable (x) and one dependent variable (y). The OLS model is typically given by equation 3.1 (Taverniers et al., 2004), for n measurements, i = 1, 2,……., n.

$$y_i = \beta_0 + \beta_1 x_i + \varepsilon_i \qquad [3.16]$$

where: y_i is the detector response

x_i is the concentration

β_1 is the slope slope

β_0 is the intercept

ε_i is the random error (or residual)

OLS works on the principle of finding the best-fit straight line ($\hat{y}$) for the given data. This is also known as the predicted or expected line. This it does by determining estimates of the intercept (β_0) and the slope (β_1) such that the predicted (or expected) line plot minimises the sum of the squared differences between the actual line plot (y) and the predicted plot ($\hat{y}$). The concept behind this is that there is a variance between the observed y values and the predicted y values due to random errors. Residuals (ε) are the differences between the observed value of y and the expected or predicted y value. This is schematically shown in Figure 3.1.

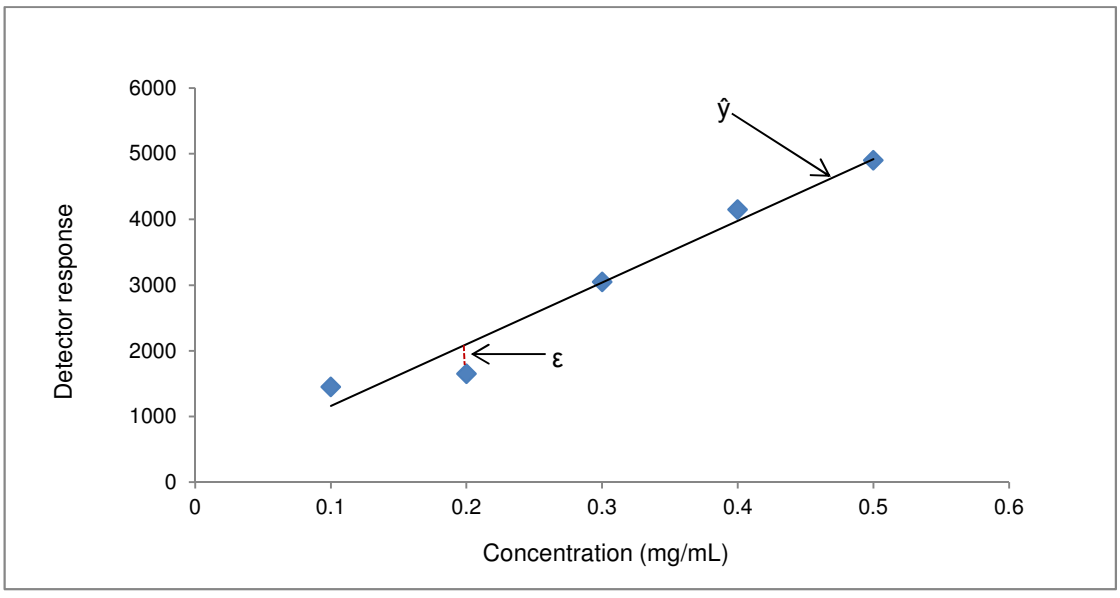


Figure 3.1 Schematic diagram of ordinary least squares linear regression plot

The predicted linear regression model is given by equation 3.17, for n measurements, i = 1, 2,……., n..

$$\hat{y}_i = \hat{\beta}_1 x_i + \hat{\beta}_0 \qquad [3.17]$$

where: $\hat{y}$ is the expected or predicted y value

x_i is the concentration

$\hat{\beta}_1$ is the slope of the predicted plot

$\hat{\beta}_0$ is the intercept of the predicted plot

The values for the slope and intercept are estimated using equations 3.18 and 3.19.

$$\beta_0 = \bar{y} - m\bar{x} \qquad [3.18]$$

$$\beta_1 = \frac{\sum_{i=1}^{n}[(x_i - \bar{x})(y_i - \bar{y})]}{\sum_{i=1}^{n}[(x_i - \bar{x})^2} = \frac{S_{xy}}{S_{xx}} \qquad [3.19]$$

The residual or random error is given by equation 3.20;

$$\varepsilon_i = y_i - \hat{y}_i \qquad [3.20]$$

The residual sum squares, SSres has n - 2 degrees of dreedom and can be broken down into two terms, that due to pure error which has n-1 degrees of freedom and is given by SSε and that due to lack of fit, SS_{lof}. Hence,

SSres = SS_{lof} + SSε

SSres = $\sum_{i=1}^{n}(y_i - \hat{y}_i)^2$

Given that SSε = $\sum_{i=1}^{n}(yi_i - \bar{y})^2$

where $\bar{y} = \frac{1}{n}\sum_{i=1}^{n} y_i$. Therefore,

SS_{lof} = SSres - SSε

To test for lack of fit the mean squares are calculated and the ratio of mean squares due to lack of fit relative to pure error determined. This gives the variance ratio, F.

$$\text{MS}\varepsilon = \frac{SS\varepsilon}{df} = \frac{SSe}{n-1}$$

$$\text{MSlof} = \frac{SSlof}{df} = \frac{SSlof}{n-2}$$

The variance ratio F is given as (de Souza and Junqueira, 2005; Miller, 1991);

$$F = \frac{MSlof}{MS\varepsilon} \qquad [3.21]$$

The significance of F can be determined at the desired level of confidence, α and from stastical tables of critical values. H_o: there is a lack of fit or non-linearity. H_1 there is linearity. If calculated $p > \alpha$ then H_o is rejected (Miller, 1991).

To test for linearity or how good a fit are the actual data points to the regression line the coefficient of determination R^2 is used (Gopal, 2006). It is a measure of the total variance in y that is explained by the regression equation. The coefficient of determination R^2 is given by the squared value of Pearson's correlation coefficient, r (section 3.2.4). Hence;

$$R^2 = r^2 \qquad [3.22]$$

(Lavanya et al., 2013; Gopal, 2006)

As for the Pearson's correlation coefficient (section 3.2.4) its values are in the range $-1 < R^2 < +1$. A value of zero indicates no linear relationship, +1 indicates a perfectly linear positive relationship and -1 indicates a perfectly linear negative relationship. Hence linearity is achieved when R^2 is closest to +1 (Gopal, 2006; Sprent and Smeeton, 2007; Corder and Foreman, 2009). The concept of testing for linearity was discussed in Chapter 2 section 2.5.

OLS makes the following assumptions (de Souza and Junqueira, 2005; Miller, 1991; Tavernier et al., 2004);

a) Linearity: the relationship between the dependent variable y and the independent variable x is linear.
b) Independence: values of εi are linearly independent of each other, with a mean sum of zero, i.e., $E(\varepsilon_i \mid x_i) = 0$, if not autocolleration exists.

c) Independence: values of x_i are linearly independent of each other, if not multicolinearity exists.
d) Normality: the residuals, εi are normally distributed for all values of y_i,x_i hence y follows a normal distribution pattern.
e) Homoscedasticity: the variance of ε is constant for all values of x_i, i.e., $E(\varepsilon_i)^2 = \sigma^2$, if distribution is not constant then heterostadastacity exists.

If the assumptions are not met it implies there is a lack of fit and the model is not linear. As such these assumptions can be tested to evaluate linearity. If the condition of normality is met, there variance is constant as such the residual plots can be checked to see if variance is constant. Y values are independent indicates that the residuals follow a random distribution pattern (for further details refer to Chapter 2 section 2.5.1.2).

Regression analysis is one of the methods specified in validation guidelines to establish the performance of a method (Eurachem, 1998; CDER, 2004; ICH, 2005). As such it has been widely applied by several researchers in studies of drugs of abuse and other work in quantitative analysis (Byrska et al., 2010; Rambla-Alegre et al., 2012; Van Loco et al., 2002; Vorce et al., 2008). Consequently it was applied in validation of the method developed in this research (Chapter 7).

3.2.12 MANN KENDALL TREND TEST

The test is used for trend analysis. It can be applied to investigate the variation of a variable with time. Hence, it has found wide application in monitoring environmental trends with time (Gibbons and Coleman, 2001). A trend exists if there is variation of the data in a specific manner. Unlike most other researchers Aalberg et al. (2005b) applied the Mann Kendall test to evaluate stability studies. The study investigated the stability of impurities in organic solvents. The Mann Kendall was used to evaluate the existence of a trend in the stability data at a significance level of 5%. The test was successfully able to determine the existence of a trend for some of the analytes e.g. N-formylamphetamine (p = 3.01%).

It has been found that the t-test is more commonly applied than the Mann Kendal in monitoring trends (Onoz and Bayazit, 2003). Karinen et al. (2011) applied the t-test as a measure of stability in their study on stability of stock solutions of drugs of abuse.

However, it has been suggested by Gibbons and Coleman (2001) that the t-test for trend detection is based on linear regression, and therefore checks only for a linear trend. Onoz and Bayazit (2003) similarly discussed the use of the t-test to monitor trends and reported that it gives best results for normally distributed data. However, the authors further reported that it can still be successfully applied to moderately skewed data. It is therefore concluded that the t-test is limited in that if a non-linear trend exists it can miss it. It is possible that when an analyte becomes unstable degradation can occur in a non-linear manner. There is no such restriction for the Mann-Kendall test. Consequently, the Mann-Kendall is more versatile and will be able to detect the presence of even non-linear trends. The Spearman's rho test is another test that can also be applied however Yue et al. (2002) cited in Gibbons and Coleman (2001) showed that it provides results almost identical to those obtained for the Mann-Kendall test. Hence, it was not considered in this research for stability testing (Chapter 5). It is evident from the discussion above that the Mann Kendall is more appropriate than the t-test as such as such this test was applied in this research in addition to the chi square test. Furthermore, like the t-test it can be applied to small sample sizes ($n < 40$) hence was deemed ideal for this research ($n = 21$).

To conduct the test the null hypothesis is H_o: the data $x_1, x_2, \ldots, x_n$ in the time series are independent, identically distributed and alternative hypothesis is H_1: There is a monotonic (not necessarily linear) trend. Where $x_1, x_2, \ldots, x_n$ where x_i is the measured value on occasion i for the data arranged in order of sampling date (time) and x_j is the immediate subsequent value in the series. The Mann Kendall test statistic, S (equation 3.23) is calculated as follows;

The difference between subsequent measurements is given by $x_j - x_i$ giving a positive, negative or zero value for each difference. The signs of each of the possible differences $x_j - x_i$ are calculated as (Gibbons and Coleman, 2001; Aalberg et al., 2005b);

Sign $(x_j - x_i)$ = 1 if $x_j > x_i$

= 0 if $x_j = x_i$

= -1 if $x_j < x_i$

$$S = \sum_{i=1}^{n-1} \sum_{j=1}^{n} sign(x_j - x_i) \qquad [3.23]$$

However, it is necessary to compute the probability associated with S and the sample size, n so as to statistically quantify the significance of the trend. According to Gibbons and Coleman (2001) the variance of S, VAR(S), is calculated by the equation 3.24;

$$\mathrm{VAR(S)} = \frac{1}{18}\left[\mathrm{n(n-1)(2n+5)} - \sum_{\mathrm{p=1}}^{\mathrm{g}} \mathrm{t_p(t_p-1)(2t_p+5)}\right] \quad [3.24]$$

where: n is the number of data points,

g is the number of tied groups (a tied group is a set of sample data having the same value), and

t_p is the number of data points in the p^{th} group.

For n < 10 the probability is then evaluated from the literature Mann-Kendal tables for S. For n > 10 the normalized test statistic Z and its associated probability are applied. The Z value is computed as follows (Gibbons and Coleman, 2001);

$$\text{If } S > 0;\ Z = \frac{S-1}{\sqrt{VAR(S)}} \quad [3.25]$$

$$\text{If S} = 0;\ Z = 0 \quad [3.26]$$

$$\text{If S} > 0;\ \mathrm{Z} = \frac{\mathrm{S}+1}{\sqrt{\mathrm{VAR(S)}}} \quad [3.27]$$

The probability associated with the *Z* statistic is then computed as;

$$f(z) = \frac{1}{2\pi} e^{-\frac{z^2}{2}}$$

The computed *Z* values and associated probabilities, *p* are the compared to tabulated cirtical values (Onoz and Bayazit, 2003). The criteria applied to determine the existence of a trend is that at a specified probability level of significance (95% typically) a trend exists if $Z_{calculated} > Z_{\alpha/2}$ or if using probabilities $p < \alpha$ ($\alpha = 0.05$). In addition, the trend is decreasing if Z is negative and increasing Z if is positive. However if the computed probability is less

than the level of significance then there is no trend (Gibbons and Coleman, 2001; Onoz and Bayazit, 2003).

The strength of a trend can be measured in terms of trend size (Aalberg et al., 2005b).

$$\text{Trend size (\%)} = \frac{\text{(Final response – initial response)}}{\text{Initial response}} \times 100\% \qquad [3.28]$$

where:

Initial response is given by the average of the responses for the first three measurements at time t_0, t_1 and t_3 and

Final response is given by the average of the responses for the last three measurements at t_{19}, t_{20} and t_{21}.

3.2.13 RUNS TEST FOR RANDOMNESS

The Runs test evaluates whether the order of a series of events is random. A run is a sequence of one or more like observations, i.e., increasing (positive) events or decreasing (negative) events (Chatterjee and Simonoff, 2012). A sample with too many or too few runs suggests that the sample is not random. The test does not make any assumptions about the normality or distribution of the data. As such, it is applicable to the evaluation of residuals (Sprent and Smeeton, 2007), hence it was used in the validation study (Chapter 7) to evaluate the linearity of the calibration curves by checking the distribution pattern of the residuals. It was reported in section (3.3.11 and 2.4.1.1) that if the data follows a random distribution the residual term ε_i has a zero mean, constant variance and is independent. In its application in regression analysis the test proceeds by examination of the signs on the residuals, i.e., whether they are positive (+) or negative (-) and the distribution pattern of the positive and negative residuals.

Let y_i be the observed response (section 3.3.11, equation 3.16), i = 1, 2, 3,…., n with $y_i \neq \hat{y}_i$ and residuals ε_i. The randomness of ε_i is evaluated by the Runs the test statistic, R denoting the expected number of runs (equation 3.29), the mean and variance of the residuals by equations 3.30 and 3.31;

$$R = \left[\frac{n_{(+)}n_{(-)}}{n_{(+)} + n_{(-)}}\right] + 1 \quad [3.29]$$

$$\mu = \frac{2n_{(+)}n_{(-)}}{n} + 1 \quad [3.30]$$

$$Var(\varepsilon_i) = \sigma^2 = \frac{n_{(+)}n_{-}\left(n_{(+)}n_{(-)} - n\right)}{n^2(n-1)} \quad [3.31]$$

(Gopal, 2006; Chatterjee and Simonoff, 2012; Sprent and Smeeton, 2007)

where: n is the number of runs, $n = n_{(+)} + n_{(-)}$

$n_{(+)}$ is the number of positive runs

$n_{(-)}$ is the number of negative runs

μ is the mean

$Var(\varepsilon_i)$ is the variance of the residuals

σ is the standard deviation of the residuals

The significance of the test is determined from the probability using Z scores for $n > 10$ (or critical values for R ($n < 10$). If randomness is not achieved, the probability, $p < 0.05$ (significance level $\alpha_{/2} = 0.05$) (Gopal, 2006; Sprent and Smeeton, 2007).

$$Z = \frac{R - \mu}{\sigma} \quad [3.32]$$

3.2.14 CALCULATION OF IDENTIFICATION PARAMETERS

The relative retention time and Kovats retention index were calculated according to equations 3.32 and 3.33 (Kaur, 2010);

$$\text{Relative retentiontime} = \frac{\text{retention time of a compound}}{\text{retention time of the internal standard}} \quad [3.33]$$

$$\text{Relative retention index} = 100n + 100\left(\frac{t_x - t_n}{t_{n+1} - t_n}\right) \quad [3.34]$$

where: x is the analyte

n is the n-alkane eluting directly before analyte

t_x is the retention time of analyte

t_n is the retention time of preceding n-alkane to the analyte

t_{n+1} is the n-alkane eluting directly after analyte

3.2.15 QUANTITATIVE CALCULATIONS

The concentration of analytes was determined through the following equations 3.34 - 3.40 (Crockett, 1986; Kaur, 2010). All the quantitave analysis was conducted after conversion of any analytes existing in salt form to free base using equation 3.37.

The conversion of substances in salt form to free base is given by;

$$\frac{\text{Amount of substance(mg) as a salt x molecular mass of substance as free base}}{\text{molecular mass of substance as salt}} \quad [3.35]$$

$$\text{Concentration of analyte(mg/mL)} = \frac{\text{area of analyte} * \text{conc of internal standard}}{\text{area of Internal standard} * \text{RRF}} \quad [3.36]$$

$$\text{RRF} = \frac{\text{area of analyte standard} * \text{conc of internal standard}}{\text{area of Internal standard} * \text{conc of analyte standard}} \quad [3.37]$$

Where RRF is the Relative response factor, the RRF of an analyte is determined by analysing a known concentration of its standard and substituting into the equation 3.37.

Hence;

$$\text{Amount or mass(mg)} = \text{conc of analyte(mg/mL)x total dilution volume(mL)} \quad [3.38]$$

$$\%\ \text{Amount in tablet} = \frac{\text{Mass of unknown (mg) x 100\%}}{\text{mean tablet mass (mg)}} \quad [3.39]$$

The mass of drug on column (ng) is given by;

Concentration of the injected solution x injection volume x split ratio [3.40]

The yield for synthesis was calculated according to equation 3.41;

$$\%\ \text{Yield} = \frac{\text{amount obtained (g)}}{\text{stoichiometric expected amount (g)}}\ \text{x } 100\% \quad [3.41]$$

CHAPTER 4
DEVELOPMENT OF GC-MS METHOD: PRELIMINARY METHOD

4.1 INTRODUCTION TO PRELIMINARY INVESTIGATIONS

The need for development of a method for the analysis and characterisation of 4-FPP and 3-TFMPP street samples was identified in Chapter 1 (section 1.8). The method to be developed needed to selectively and simultaneously analyse for these drugs in a complex sample matrix containing positional isomers, congeners and any impurities present in street samples (Nikolova and Danchev, 2008; Kelleher et al., 2010; UNODC, 2013c). Furthermore, analysis of the isomers has been identified as a challenge due to their similar chemical characteristics (Inoue et al., 2004; Elliot and Smith, 2008; Takahashi et al., 2009).

The processes that were conducted in developing the method involved; a) theoretical considerations, b) development of a preliminary method, c) optimisation, d) validation, and e) its application to street samples. This study is therefore second in this sequence of events. The preliminary stage establishes the method variables that can then be further investigated to improve the method in further studies.

In this study, investigation of sample preparation techniques was conducted by evaluating the use of derivatising agents in comparison to non-derivatisation. Derivatisation was carried out according to the method by de Boer et al, (2001) using pentafluoropropionic anhydride (PFPA) as the derivatising agent (section 2.6). Selection of internal standard investigated quinoline and eicosane as potential internal standards. The investigation of instrumental parameters was carried out by applying different experimental conditions. These were oven temperature, injector port temperature, flow rate, MS interface temperature, MS scan rate and MS ionisation energy. This investigation however, was limited to capillary columns (30m x 0.25mm x 0.25um). These were deemed suitable as they had been successfully applied by other researchers (de Boer et al., 2001; UNODC, 2013b; Staack, 2007; Takahashi et al., 2009).

4.1.1 AIMS OF THE PRELIMINARY STUDY

The overall aim of this chapter was to establish a foundation of experimental conditions for development of the method. This study therefore, set out to find an appropriate sample preparation technique, column, internal standard and preliminary instrumental variables. As such, it will establish a preliminary method for application in stability studies and for further development in the optimisation and validation studies (Chapters 6 and 7).

4.2 MATERIALS AND METHODS

4.2.1 CHEMICALS/REAGENTS

1-(2-Methoxylphenyl)piperazine (2-MeOPP), 97%; lot number S33767 21007B2 was procured from Fluka. 1-(4-Methoxylphenyl)piperazine (4-MeOPP), 97%, batch number 871415 was procured from Sigma. The solvents/chemicals: ethyl acetate, methanol, 2-methyl-propan-2-ol, dichloromethane and pentane were procured from Fischer Chemicals. In addition, the drug reference standards used for this study were also used in subsequent studies (Chapters 5, 6, 7 and 8) and are shown in Table 4.1.

Table 4.1 List of drug standards and standards of other compounds used in the study

Substance	**Purity (%)**	**Supplier**	**Lot/Batch No.**
1-(2-fluorophenyl)piperazine	97	Sigma Aldrich	Lot: S39132
1-(3-fluorophenyl)piperazine	99	Fluorochem	DO8F
1-(4-fluorophenyl)piperazine	98	Sigma Aldrich	Lot: S47598
1-(2-trifluoromethylphenyl)piperazine	100	Chemos GMBH	AB148036
1-(3-trifluoromethylphenyl)piperazine	98	Alfa Aesar	LO5333
1-(4-trifluoromethylphenyl)piperazine	98	Fluka	1292205 21707187
1-benzylpiperazine	97	Fluka	0130991/1 40708251
1-(4-dibenzyl)piperazine	100	Sigma Aldrich	S983381
1-(4-methylbenzyl)piperazine	97	Aldrich	07602DJ
1-(3-chorophenyl)piperazine	100	Sigma Aldrich	Lot: 40796T7
1-(4-methylphenyl)piperazine	98	Sigma Aldrich	BCB0996
(±)3,4-methylenedioxy methamphetamine HCl	100	Fluka Sigma	1BCBC4747 22010P01 082M4033V
(+)-Amphetamine SO_4	100	Sgma	101K3351
(+)-Methamphetamine HCl	100	Sigma	31H0454
Cocaine HCl	100	Sigma	059K1139
Benzoylecgonine hydrate	100	Sigma	05M4010
Ecgonine methyl ester HCl hydrate	100	Sigma	050M4011
Diazepam	100	Sigma	105F0451
Dapoxetine HCl	100	Sigma	09M4737
Dextromethorphan HBr	100	Sigma Aldrich	090M1298V
Caffeine	100	Aldrich Fisher Chemicals	8595 5 0078218
Nicotinamide	100	Sigma	BCBD 0222V
Piperazine	98	Fluka	000143817
Eicosane (internal standard)	99.5	Agros chemicals	A0229 559
Quinoline (internal standard)	98	Aldrich	S61686-48

4.2.2 INSTRUMENTS

Preliminary studies were conducted using a Perkin Elmer GC-MS, Clarus Turbomass Gold 500MS fitted with a Zebron, ZB-1 capillary column (30m x 0.25mm x 0.25µm). The instrument was equipped with the NIST MS Search Version 2.0 library software. A Perkin Elmer 4mm quartz split/splitless (product number N6121001) injector liner was used. The injector was set at 250^{o}C with a split ratio of 20:1. The carrier gas was He (g) at a flow rate of 1mL/min. The initial oven temperature was set at 50^{o}C with a hold for 1min and ramped at 15^{o}C /min to 300^{o}C with a hold for 5min. The MS transfer line was set at 250^{o}C, source temperature 230^{o}C, ionisation energy 70eV and scan range at m/z 40 – 500. The total analysis run time was 22.67 minutes.

In addition, the following columns were used in the test for investigation of stationary a) Phenomenox, Zebron ZB-1 GC capillary column (30m x 0.25mm x 0.25µm), serial number 164044, b) Phenomenox, Zebron ZB-5 GC capillary column (30m x 0.25mm x 0.25µm), serial number 164043), and c) Supelco, Equity-5 GC capillary columns (30m x 0.25mm x 0.25µm), serial numbers 43734-04 and 169302. A Genevac Mivac was used to evaporate the solutions in derivatisation tests.

4.2.3 STATISTICAL SOFTWARE

Analysis of results was carried out using IBM SPSS Version 20 and MS Office Excel 2010.

4.2.4 PREPARATION OF STANDARD SOLUTIONS

4.2.4.1 Preparation of internal standard stock solutions

Internal standard stock solutions of eicosane and quinoline were individually prepared to a concentration of 1.0mg/mL in pentane for eicosane and in methanol for quinoline.

4.2.4.2 Preparation of analyte standard stock solution

Individually, stock solutions for the drug standards listed section 4.2.1 were prepared to a concentration of 2.50mg/mL free base in methanol. The solutions were sonicated for 15 minutes to dissolve samples where necessary.

4.2.4.3 Preparation of analyte standard working solutions

The standard solutions were prepared by diluting the stock solution as shown in Table 4.2.

Table 4.2 Preparation of analyte standard working solutions

Solution type	**Volume of standard stock solution taken (mL)**	**Volume of IS[1] stock solution taken (mL)**	**Final volume (mL)**	**Concentrations (mg/mL)**
a) Individual drug standard solutions	0.40mL of each standard	0.20mL of eicosane	10.0mL	0.10mg/mL analyte and 0.02mg/mL internal standard
b) Mixed standards solution 1	0.40mL of each of standard (all standards mixed)	0.20mL of eicosane	10.0mL	0.10mg/mL analyte and 0.02mg/mL internal standard
c) Mixed standards solution 2	0.40mL of each of standard all standards mixed)	1.0mL of quinoline	10.0mL	0.10mg/mL analyte and 0.1mg/mL internal standard

[1] Internal standard

4.2.5 INVESTIGATION OF SAMPLE PREPARATION TECHNIQUES: DERIVATISATION

The analysis of samples for this test was conducted with the GC-MS set up with method 1 (section 4.2.7 Table 4.3).

4.2.5.1 Derivatised standards

Derivatisation of BZP, 4-TFMPP, 2-MeOPP, 4-MeOPP, amphetamine, cocaine, diazepam, EME and methamphetamine was carried out with PFPA: Ethyl acetate (2:1) solution. Individual and mixed standard solutions with quinoline as the internal standard were prepared as outlined in Table 4.2. A volume of 200µL of the analyte working standard solution was pipetted into a derivatisation tube and evaporated to dryness using a Mivac (40/50^{o}C). PFPA solution (100µl) was then added. The solution was shaken to mix and placed in a heating block for 1hour 15 minutes at 70^{o}C. The solution was evaporated to dryness using a Mivac (40/50^{o}C). Ethyl acetate (200ul) was added to reconstitute and the solution was analysed by GC-MS (de Boer et al., 2001).

4.2.5.2 Un-derivatised standards

Drug standard solutions were prepared as for the derivatised standards (section 4.2.5.1) omitting addition of the PFPA solution.

4.2.6 SELECTION OF INTERNAL STANDARD

The test was conducted with the GC-MS set up with method 3b (section 4.2.7 Table 4.3). Solution 1 was prepared by diluting the eicosane stock solution with methanol to a concentration of 0.02mg/mL. Solution 2 was prepared by diluting the quinoline stock solution with methanol to a concentration of 0.10mg/mL. Solution 3 was prepared by diluting the eicosane and quinoline stock solutions with methanol to give a mixed solution of concentration 0.02mg/mL eicosane and 0.10mg/mL quinoline. Solutions 2, 3 and 4 were analysed by GC-MS.

4.2.7 INVESTIGATION OF COLUMN AND INSTRUMENTAL PARAMETERS

The GC-MS was set up with the injector at 250^{o}C and a split ratio of 20:1. The carrier gas was He (g) at a flow rate of 1mL/min. The MS transfer line was set at 280^{o}C, source temperature 230^{o}C, ionisation energy 70eV and scan range at m/z 40 - 500. The column and oven temperature were investigated by varying these parameters as shown in the Table 4.3. A mixed standard solution (Table 4.2 solution 2) was used for the test.

Table 4.3 Investigation of GC-MS column and instrumental parameters

Parameter	**Method 1**	**Method 2a**	**Method 2b**	**Method 3a**	**Method 3b**
Column	Zebron, ZB-1	Zebron, ZB-1	Zebron, ZB-1	Zebron, ZB-5	Supelco, Equity 5
Initial Temperature	50^{o}C	50^{o}C	90^{o}C	60^{o}C	60^{o}C
Hold	1 minute	1 minute	2 minute	1 minute	1 minute
Ramp 1	15^{o}C/min	10^{o}C/min	10^{o}C/min	10^{o}C/min	10^{o}C/min
Temperature	300^{o}C	150^{o}C	150^{o}C	150^{o}C	150^{o}C
Hold 2	5 minutes	2 minutes	2 minutes	2 minutes	2 minutes
Ramp 2	none	15^{o}C/min	15^{o}C/min	15^{o}C/min	15^{o}C/min
Temperature	-	300^{o}C	300^{o}C	280^{o}C	280^{o}C
Hold 3	-	5 minutes	5 minutes	2 minutes	2 minutes

In addition, a solution containing only piperazine standards was used to check if the method was capable of separation between isomers of FPP and TFMPP and other piperazines. Method 3b was applied.

4.2.8 DATA ANALYSIS

Relative retention time (RRT) and retention index (RI) were calculated according to equations 3.32 and 3.33 (Chapter 3 section 3.2.14) respectively.

4.3 RESULTS AND DISCUSSION

4.3.1 INVESTIGATION OF SAMPLE PREPARATION TECHNIQUES: DERIVATISATION

The total ion chromatographic profiles (TIC) are shown in Figures 4.1 and 4.2. The chromatographic data derived (retention times, RT, relative retention times, RRT and retention indices, RI) is shown in Table 4.4.

Table 4.4 Derivatisation versus un-derivatised chromatographic data

Substance	**Un-derivatised**			**PFPA derivatised**		
	RT/mins	**RRT**	**RI**	**RT/mins**	**RRT**	**RI**
(+)Amphetamine	6.76	0.88	1125	7.69	1.14	1217
(+)Methamphetamine	7.32	0.95	1180	9.06	1.34	1351
EME	9.96	1.29	1439	9.83	1.45	1427
BZP	10.10	1.31	1453	12.69	1.88	1707
4-TFMPP	10.8	1.40	1522	13.37	1.98	1774
2-MeOPP	11.19	1.45	1560	13.88	2.05	1824
4-MeOPP	12.09	1.57	1648	14.96	2.21	1930
Cocaine	15.19	1.97	1953	17.68	2.62	2197
Diazepam	16.55	12.14	2086	19.67	2.91	2392
Quinoline	7.72	1.00	1219	6.76	1.00	1125

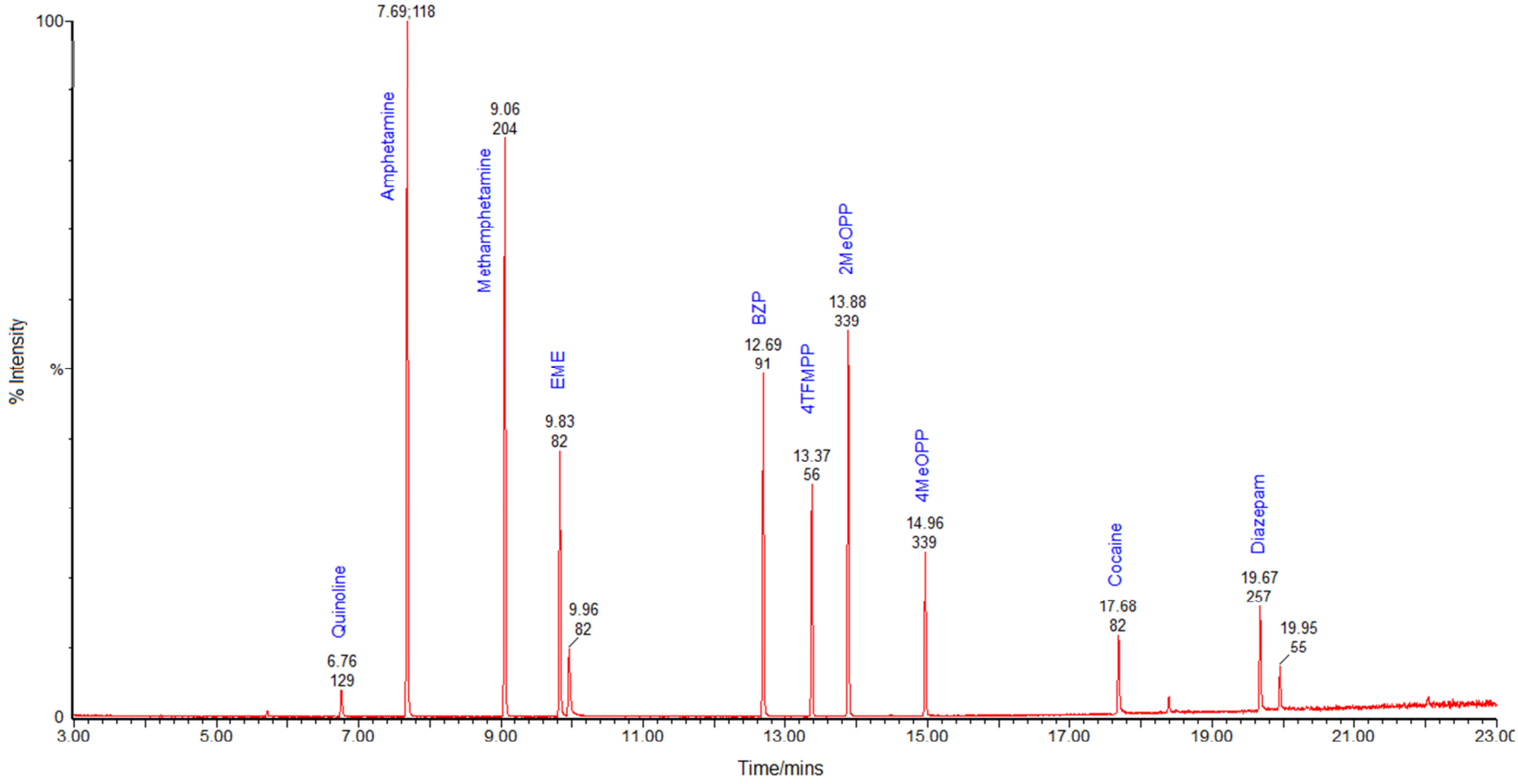


Figure 4.1 Total ion chromatogram of PFPA derivatised drugs.

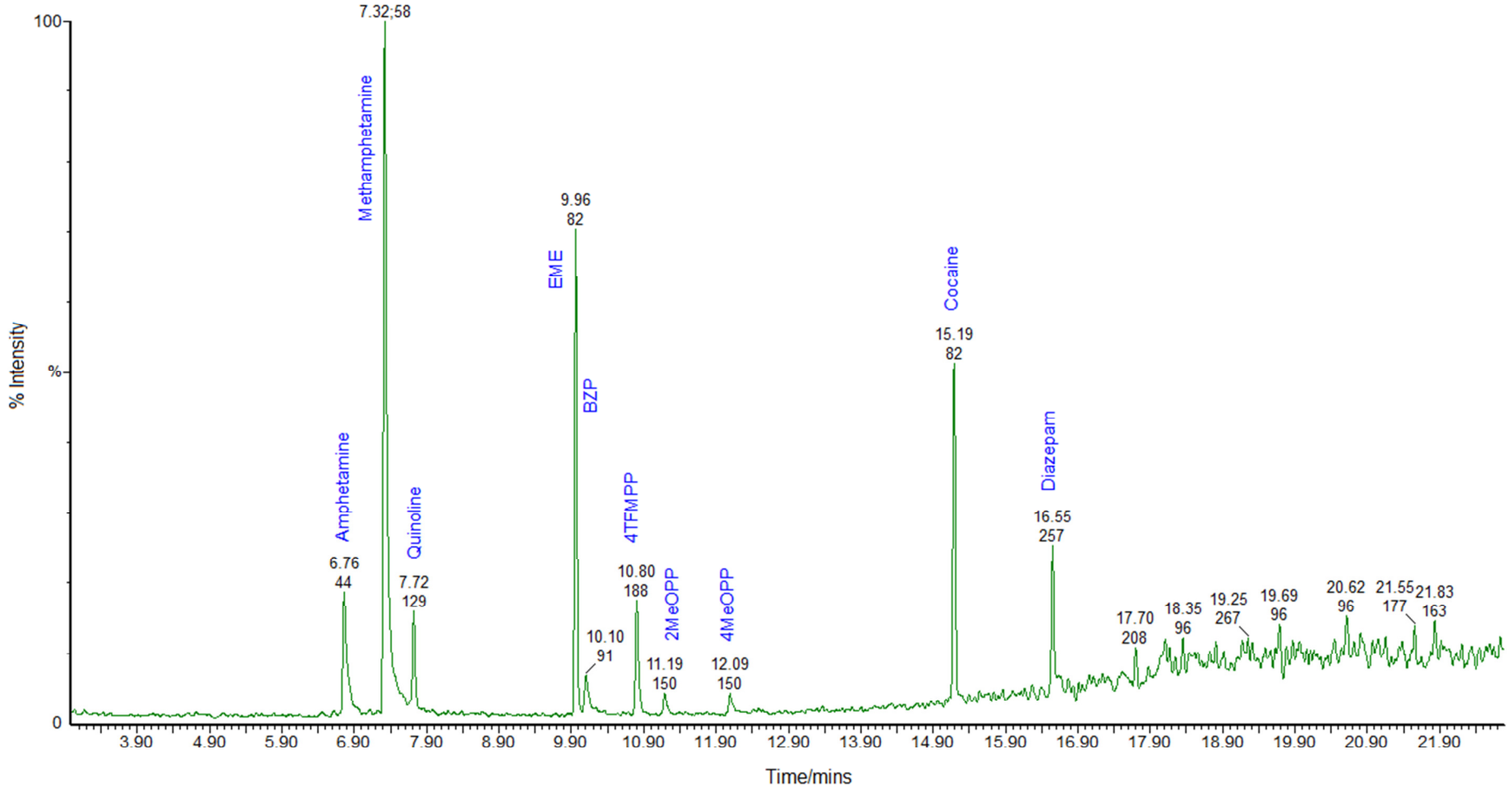


Figure 4.2 Total ion chromatogram of un-derivatised drugs.

Figures 4.1 and 4.2 show that derivatisation improved peak sensitivity and peak profiles in comparison to the un-derivatised analytes (Figure 4.1 and 4.2; Table 4.4). Both the peak intensities and peak separation are greater in Figure 4.1 relative to 4.2 for all the analytes; for example amphetamine, methamphetamine and BZP. Furthermore, the retention indices (RIs) routinely used to identify a compound (Takahashi et al., 2009; Kaur, 2010) also give an indication of the degree of retention of analytes; the closer the RIs the closer the analytes in elution. The RIs of the derivatised peaks are higher and there is a greater difference in the RIs between adjacent peaks showing greater separation, e.g. EME and BZP have RIs of 1439 and 1453 respectively compared to 1427 and 1707 derivatised (Table 4.4).This can be accounted for by the fact that derivatisation effectively increases the volatility of the drug (Chapter 2 section 2.6). Such results have commonly been found by other researchers such as de Boer et al. (2001) and Inoue et al. (2004). In these studies drugs of abuse including BZP and TFMPP were derivatised with silylation and acylation agents and sensitivity was improved. However, in the work of Inoue et al. (2004) it was indicated that derivatisation impaired peak separation between BZP and 3-TFMPP which had been not been observed in the un-derivatised samples. For a more comprehensive list of analytes and discussion, reference is made to Figure 4.14 and section 4.3.3. At the time of this investigation 3-TFMPP was not yet available hence could not be compared, however BZP (12.69 minutes) and 4-TFMPP (13.37 minutes) were observed to be well resolved (Figure 4.1). In Figure 4.2 column bleed and baseline drift were at higher retention times and temperatures (from 16 minutes). This could be due to decomposition of the column at the higher temperatures used as the analysis progresses, however this did not impact on the results as all the analytes had already eluted. In addition, this became a factor in selection of a more stable column (section 4.7.3).

The effect of derivatisation on the mass spectra fragmentation pattern is given for a representation of the drugs BZP and 4-TFMPP in Figures is 4.3 - 4.6.

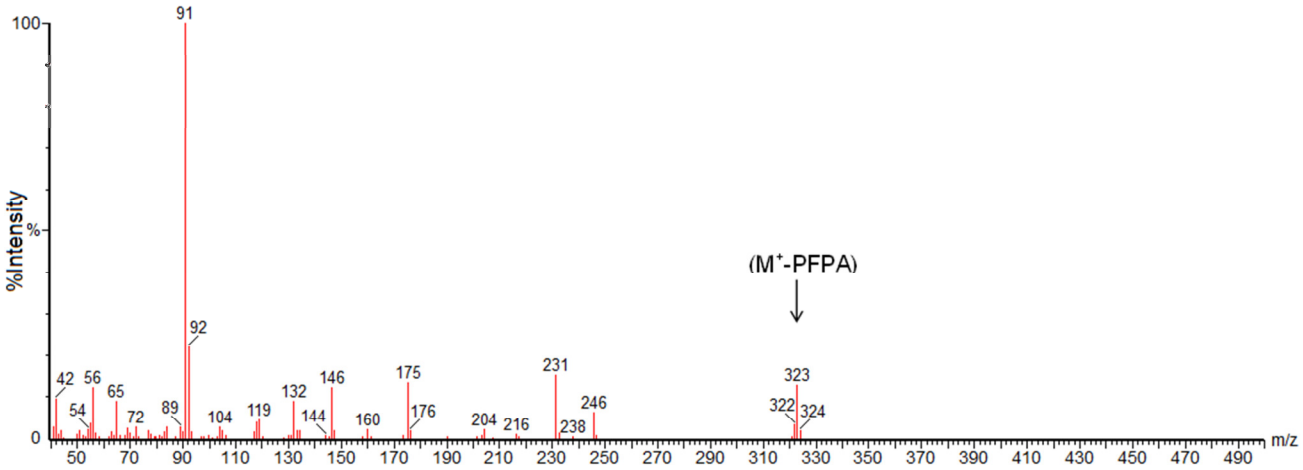


Figure 4.3 Mass spectrum of PFPA derivatised BZP.

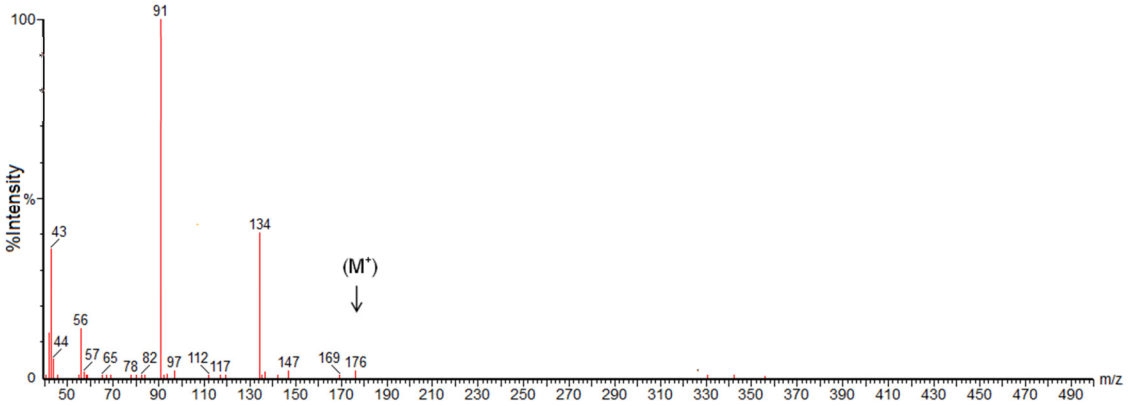


Figure 4.4 Mass spectrum of un-derivatised BZP spectrum.

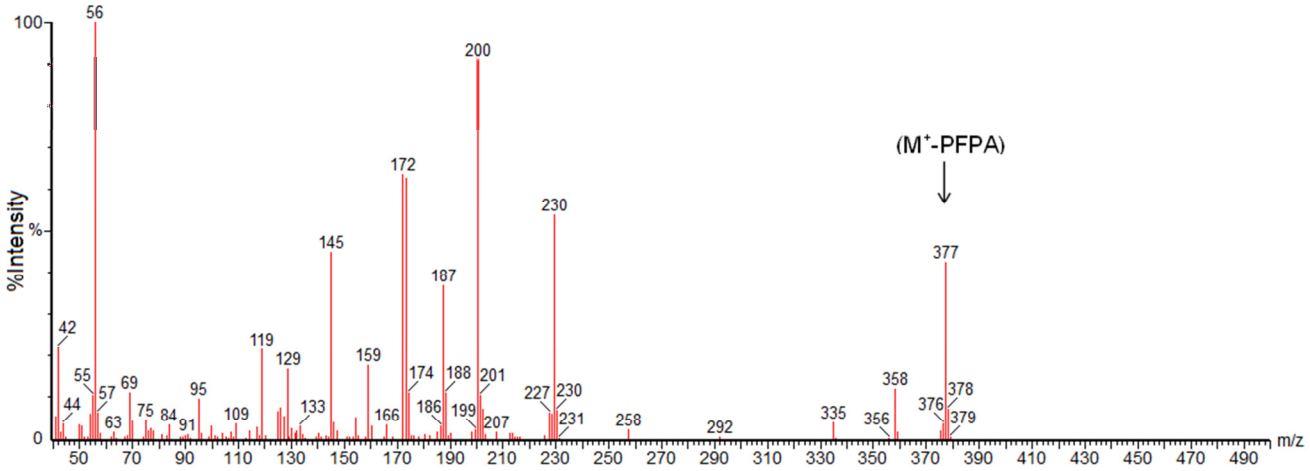


Figure 4.5 Mass spectrum of PFPA derivatised 4-TFMPP.

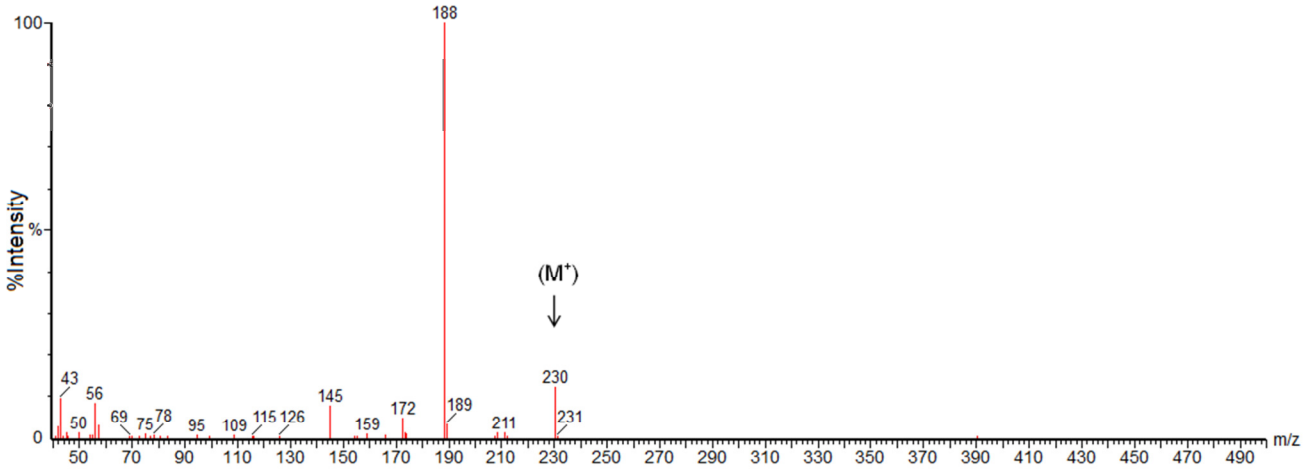


Figure 4.6 Mass spectrum of un-derivatised 4-TFMPP.

The results indicated that derivatisation changes the mass spectra fragmentation pattern for most compounds. High intensities for the ions and in addition ions due to derivatising agent adducts were observed. Derivatisation appears to stabilise the compounds as less fragmentation is observed, this evidenced by the prevalence of the parent ions in the derivatised samples e.g. BZP and 4-TFMPP at m/z 323 (M^+-PFPA and 176(M^+) for BZP for the derivatised and un-derivatised respectively. For 4-TFMPP these were at m/z 377 (M^+-PFPA and 230(M^+) for the derivatsed and un-derivatised respectively. For the piperazine compounds there is less fragmentation of the piperazine moiety as be seen by the higher prevalence of the molecular ion and fragments with the piperazine ring in the mass spectra (Figures 4.3 – 4.6). This is likely to be due to the presence of non-bonding electrons from the derivatising moiety, (-COC_2F_5) which can easily be delocalised. These are easier to remove than the covalent bond electrons resulting in a different fragmentation pattern to the un-derivatised form. A similar trend was observed by de Boer et al., (2001) with BSTFA and TFA as derivatising agents. In the chromatographic profile adducts (Figure 4.1) were observed (9.96, 18.40, 19.95 minutes) which could be the result of the reaction of the derivatisation agents with other materials in the sample matrix. de Boer et al. (2001), Takahashi et al. (2009), UNODC (2013c) in their work utilised both derivatisation and un-derivatised for the analysis of street samples. Both techniques gave good results with the un-derivatised mass spectra showing the expected fragmentation pattern and ions. Also fragmentation was not excessive; the parent ions were observed for example at m/z176 and m/z 230 for TFMPP drugs. Characteristic ions were identified for BZP at m/z 91 (100), 134, 56, 120, 176 (M+) and for TFMPP as was shown in Table 1.1 and were confirmed in this research in validation studies (Chapter 7). The un-derivatised samples gave chromatographic profiles and spectra that were satisfactory. All the characteristic ions were present. Therefore, the results are in agreement with those observed in other studies. The positive effects of derivatisation are counteracted by its limitations.

The disadvantage of derivatisation is that the derivatising agents might react with the impurities in the drugs or other compounds in the sample matrix. This is undesirable in characterisation and profiling of drugs and is an imminent factor in this research. Since the method developed is to be employed in analysing street samples which according to Davies et al. (2010); Yuk (2010) contains a cocktail of unknown substances. Of importance is the fact that un-derivatised samples also showed acceptable results. This was also observed in the works of de Boer et al. (2010), Inoue et al. (2004) and Takahashi et al. (2009). It was

observed that quinoline shows very low sensitivity on derivatisation hence would be a poor choice as an internal standard if derivatisation was to be applied. This is probably due to it not having a reactive H which is required for derivatisation reactions to occur as highlighted in Chapter 2 (section 2.6). Consequently, in such instances a deuterated standard of the drug is more effective as an internal standard. Furthermore, if the samples are derivatised, there is need to ensure all the analytes are 100% derivatised which might not always be feasible. Since the un-derivatised samples were successful it can therefore be suggested that not derivatising samples is an acceptable option for this study and as such was employed in all further studies.

Since this section entailed to investigate whether derivatisation will be advantageous or not to this research, the discussion on mass spectra is limited. A further discussion on mass spectra (un-derivatised) is included in the section on application of the developed method (Chapter 7 section 7.7.4).

4.3.2 SELECTION OF INTERNAL STANDARD

A summary of the results is shown in Table 4.5 below. Figure 4.7 shows a comparative chromatographic profile (total ion chromatogram, TIC) for the analysis of eicosane and quinoline. The characteristic ions used for their identification and comparison were from de Boer et al. (2001), Moffat et al. (2011) and NIST (2014) e.g. m/z at 129 for quinoline and for eicosane 57 and 71. The chromatographic profiles for the analysis of samples with quinoline and eicosane as internal standards respectively are shown in Figures 4.7 - 4.10 for a representation of the samples to be analysed.

Table 4.5 Qualitative data for selection of internal standards

Substance	**Retention time/mins**	**Characteristic ions (m/z, intensity)**	**Peak profile**
Quinoline C_9H_7N Mwt = 129	9.74	129(100 and M^+), 102, 76, 63, 51	Sharp symmetrical peaks. Resolution from adjacent peaks > 2, tailing <1.0. High sensitivity.
Eicosane C_9H_7N Mwt = 282	18.66	57(100), 71, 85, 99, 113, 127, 141, 283(M^+),	Sharp symmetrical peaks. Resolution from adjacent peaks > 2, tailing = 1. High sensitivity. Peak area ratio with analytes 0.1 – 1.9.
Synthetic 4-FPP	14.24	138(100), 180(M^+), 122, 56	No co-elution for any peaks was observed with either internal standard.
Synthetic 3-TFMPP	14.43	188(100), 230(M^+), 145, 56	No co-elution for any peaks was observed with either internal standard.

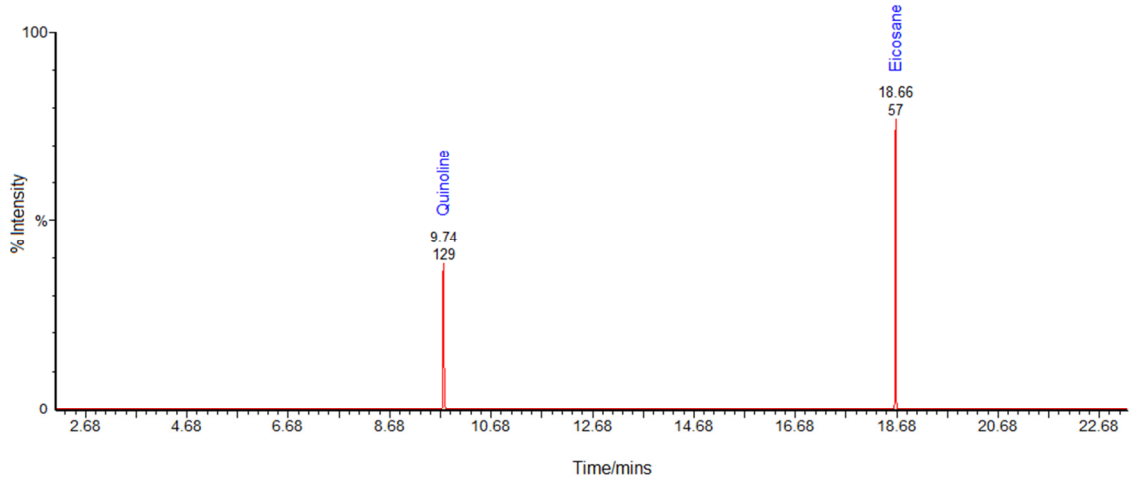


Figure 4.7 Selection of internal standard: Total ion chromatogram of mixed standards quinoline and eicosane.

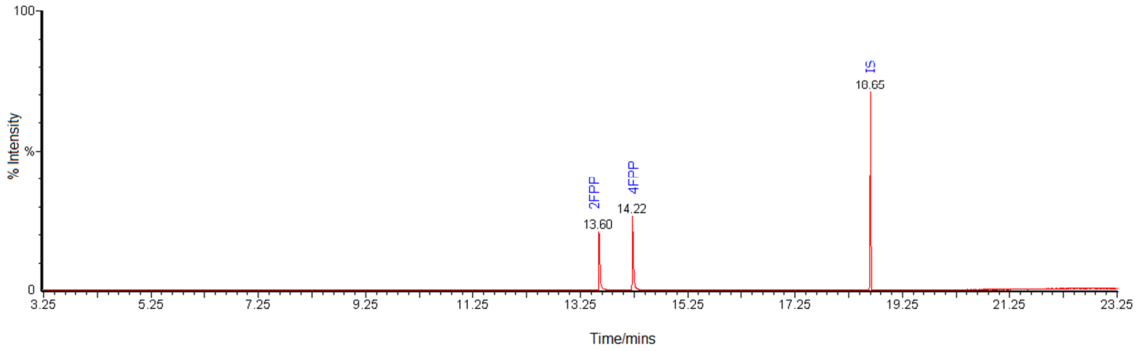


Figure 4.8 Selection of internal standard: Total ion chromatogram of FPP standards with eicosane as the internal standard (IS).

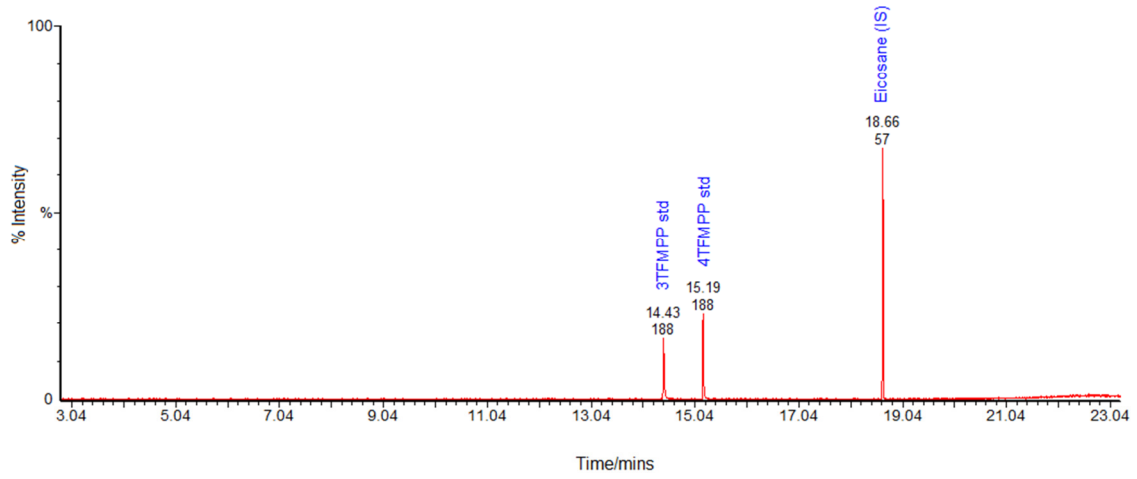


Figure 4.9 Selection of internal standard: Total ion chromatogram of TFMPP standards with eicosane as the internal standard.

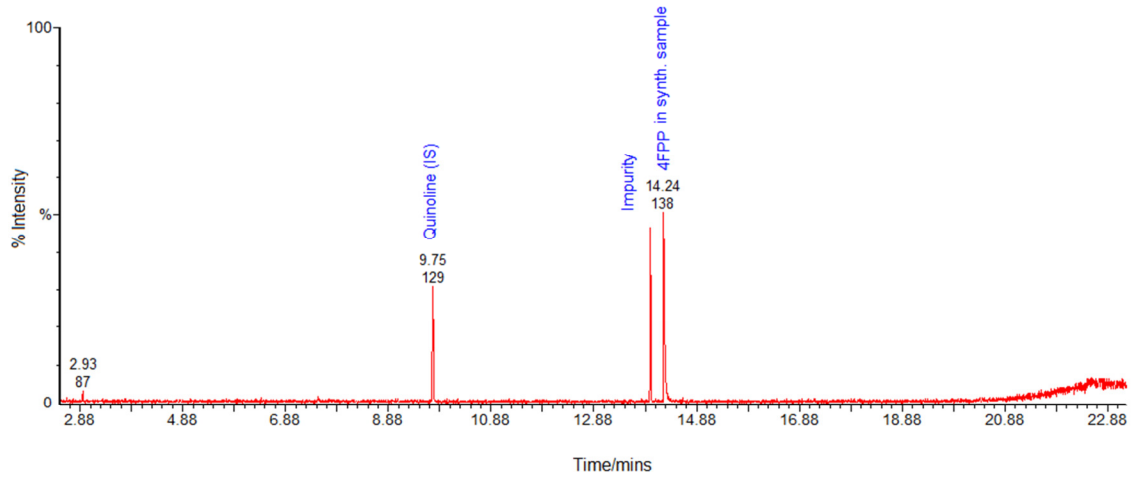


Figure 4.10 Selection of internal standard: Total ion chromatogram of 4-FPP with quinoline as the internal standard.

The total ion chromatograms (Figures 4.7 – 4.10) obtained with both internal standards were satisfactory. The peaks for both the internal standards were well defined, narrow, sharp, symmetrical peaks. Peak tailing was low (quinoline < 1 and eicosane = 1). Furthermore, they were well resolved from the analytes in the sample. Both internal standards had a resolution factor > 2 as such showed sufficient separation from other analytes (CDER, 2004). The peak area ratios for internal standard to analyte were also reasonable (0.2 - 2). It has been identified that such traits are indicative of good chromatographic properties in a compound (Horacio et al., 2008; IUPAC, 2014). Hence, they would generate accurate data. It has been identified that poor resolution and assymetric peaks reduce accuracy in quantitation of peak areas. In addition, they cause inconsistency in retention times (Andersson et al., 2007a; Khopkar, 2012). It therefore follows that both the internal standards can successfully be employed for both qualitative and quantitative analysis. Furthermore, they do not co-elute with any of the impurity peaks observed in the synthesised samples as shown Figure 4.10 for 4-FPP. This minimises interference during analysis and will be advantageous in characterisation of the drugs.

The results obtained are comparable with the work of other researchers. Inoue et al. (2004) in their study on analysis of benzylpiperazine-like compounds successfully employed eicosane as an internal standard. Furthermore, the quinoline and eicosane show a wide gap in their retention times (9.75 - 18.66 minutes; Table 3.4). This has the advantage that both can be used simultaneously as internal standards and as such cover a wider range of analytes. In this study the drugs of focus 4-FPP and 3-TFMPP have been identified to exist in combination with a variety of other drug substances which are often unknown (Davies et al., 2010; Yuk, 2010; Arbo et al., 2012).

It has been argued that drugs of abuse contain impurities (Andreasen et al., 2009; Bartos and Gorog, 2008; UN, 2001). Impurities are of unknown chemical properties and consequently there is need to limit or minimize potential reactions of other substances in the sample matrix with impurities. This method under development will also be utilised in characterisation and impurity profiling of 4-FPP and 3-TFMPP street samples. On this basis whilst both compounds are chemically stable, eicosane an n-alkane is chemically inert and as such was selected as the preferred internal standard in subsequent studies. Comparatively, quinoline a benzopyridine, is basic with a pKa of 4.9 (760mmHg, 20^{o}C), (Jones, 1982) and potentially can undergo reactions of both the benzene ring and the

pyridine ring. According to Jones (1982) quinoline can undergo electrophilic substitution in highly acidic conditions as shown below;

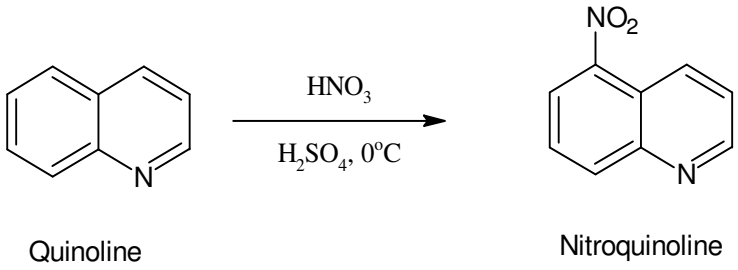


Figure 4.11 Reaction of quinoline in acidic conditions.

This has consequences if solvents, reagents, other drugs or chemicals in the sample are acidic. However, in this research the possibility was minimized through selection of a non-reactive solvent and investigating stability studies (Chapter 5). In addition the chromatographic profiles (Figures 4.1 and 4.2) show that quinoline elutes earlier than most of the drugs under investigation whilst eicosane (Figure 4.14) is in line with the other analytes. Consequently, better precision and accuracy in results is achived with eicosane. These are probably the reasons why eicosane has found favour as an internal standard in characterisation and profiling work (Andersson et al., 2007a; Inoue et al., 2008; Santali et al., 2011), as such eicosane was selected as the internal standard of choice for this research.

4.3.3 INVESTIGATION OF COLUMN AND INSTRUMENTAL PARAMETERS

In order to establish preliminary parameters, different oven temperature programs and columns of different stationary phase polarities were investigated (Table 4.3). A non polar 100%-dimethylpolysiloxane stationary phase column (Zebron ZB1) was used in methods 1 and 2 and polar, 5%-phenyl-95%-dimethylpolysiloxane stationary phase (Zebron ZB5 and Supelco Equity-5) in method 3a and b. The total ion chromatograms (TIC) generated are presented in Figures 4.12 - 4.14. In the results Figures 4.14a and b show more analytes than Figures 4.12 and 4.13 as some of the drug standards had not yet been procured (2-TFMPP, 3-FPP, MBZP and CPP). Figure 4.14a also shows results for preliminary application of the method to piperazine drugs only.

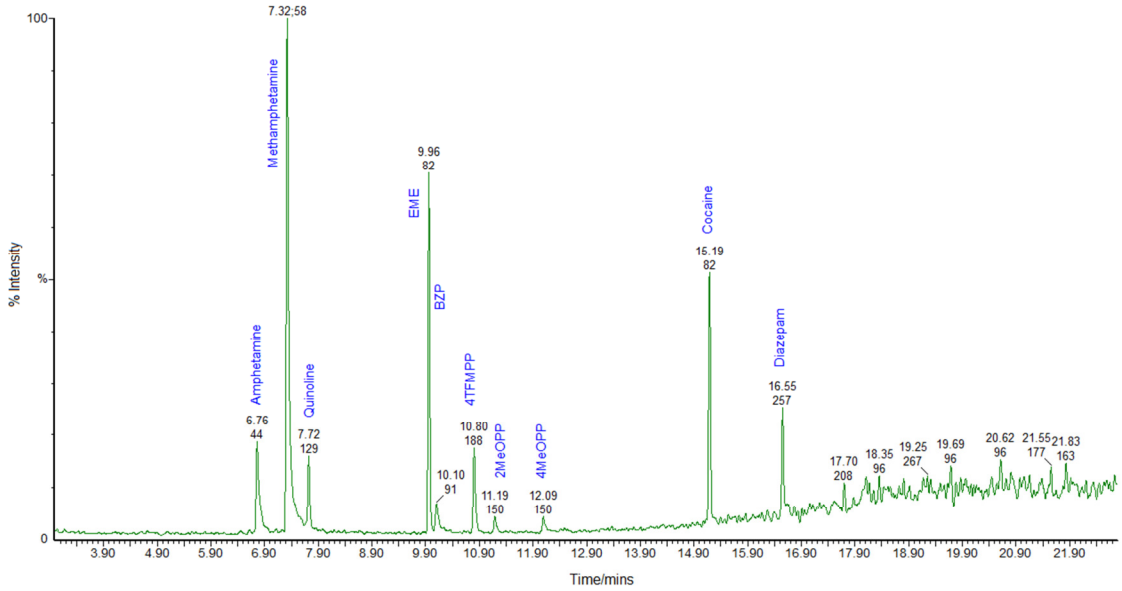


Figure 4.12 Chromatographic profiles (TIC) of mixed standards obtained with Method 1 and column Zebron ZB1 (Table 4.3).

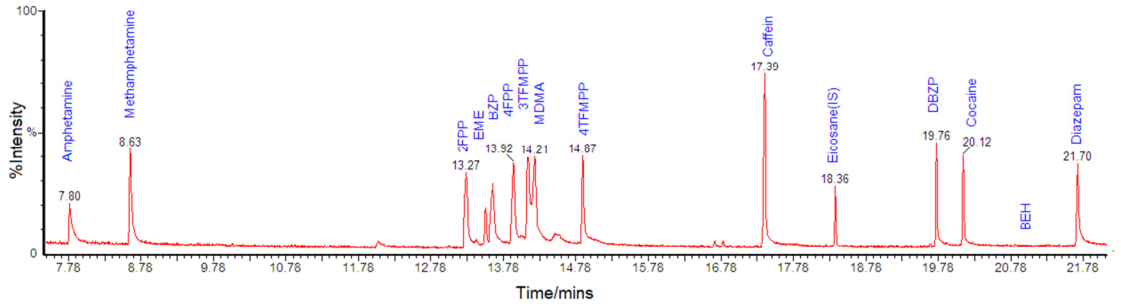


Figure 4.13a Chromatographic profiles (TIC) of mixed standards obtained with Method 2a and column Zebron ZB1 (Table 4.3).

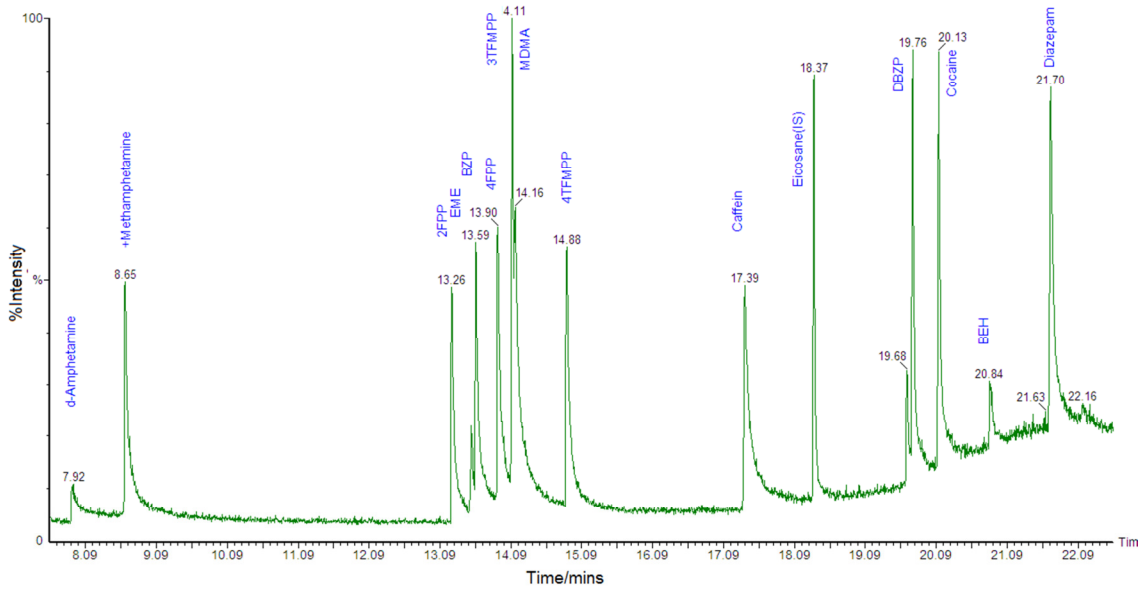


Figure 4.13b Chromatographic profiles (TIC) of mixed standards obtained with Method 2b and column Zebron ZB1 (Table 4.3)

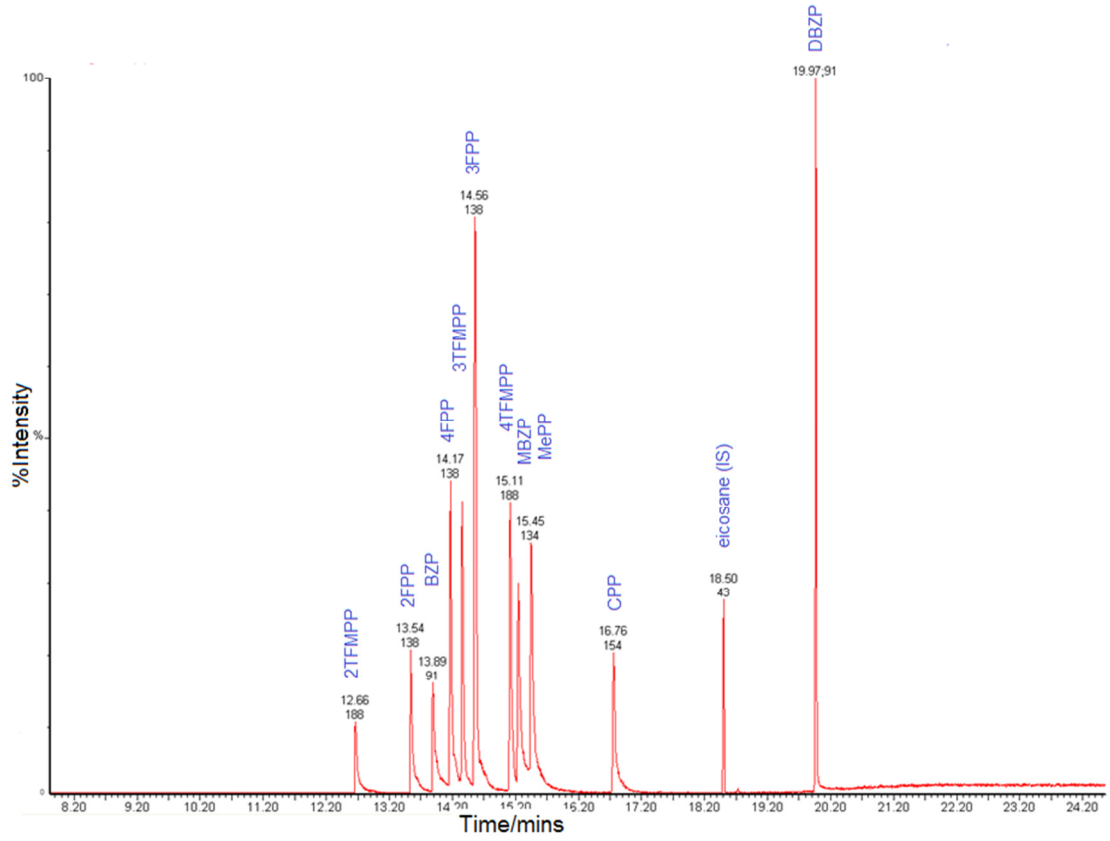


Figure 4.14a Chromatographic profiles (TIC) of mixed standards obtained with Method 3a and column Zebron ZB5 (Table 4.3).

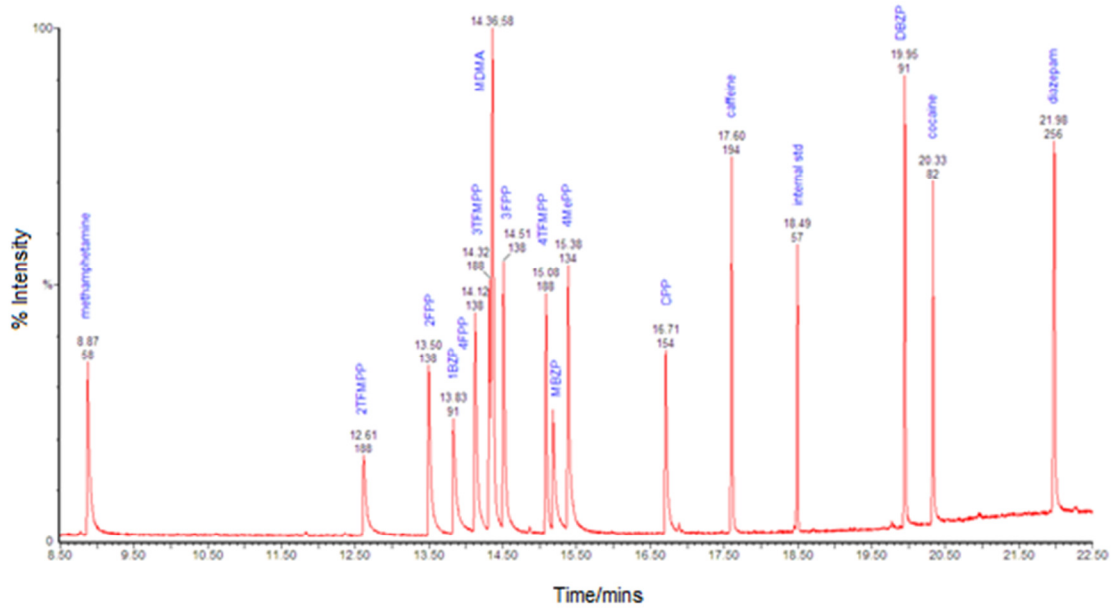


Figure 4.14b Chromatographic profiles (TIC) of mixed standards obtained with Method 3b and column Supelco Equity 5 (Table 4.3).

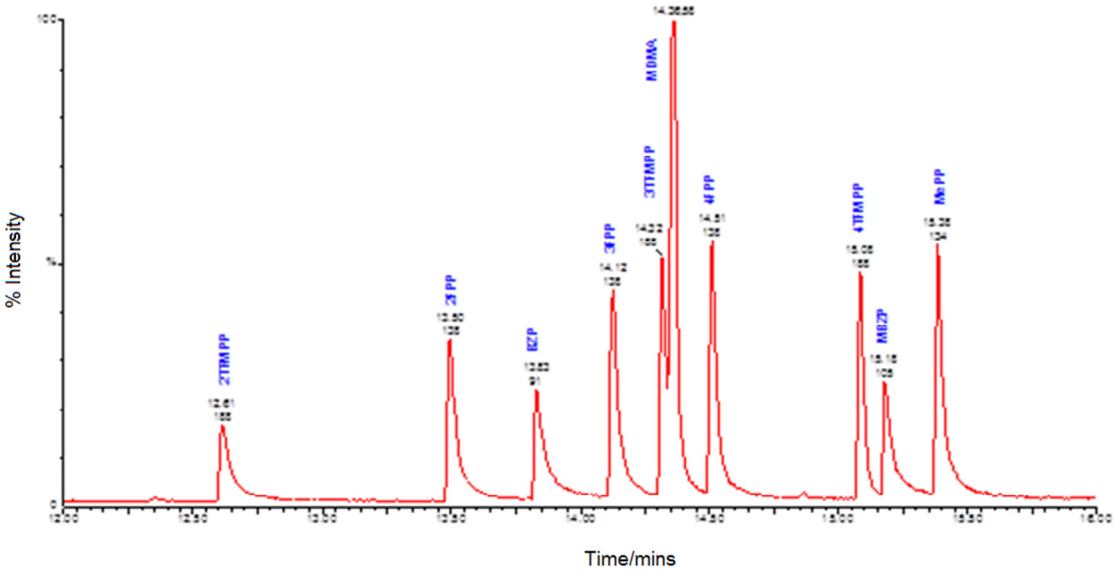


Figure 4.14c Method 3b TIC expanded view of the peaks in the range 12 - 16mins.

The results showed that all the methods gave similar chromatographic peak profiles in terms of the order of elution of the peaks. The main difference in the methods was in the degree of resolution, tailing and general peak shape (Figures 4.12 - 4.14). With both methods 1 and 2 poor peak profiles were obtained. However, it is evident in total ion chromatograms that the results progressively show improvement from Figures 4.12, 4.13a to 4.13b. This is due to the variation in the oven temperature programming (Table 4.2) as

these methods used the same column, ZB1. Whislt improved the peak profiles were still not ideal. There is noticeably poor resolution for the peaks eluting in the range 12 – 16 minutes with method 2 as shown by 2-FPP, EME, BZP, 4-FPP, 3-TFMPP and MDMA (Figures 4.13a and b). Hence a more polar stationary phase was applied (ZB5 column, method 3a in Table 4.2).

The results showed a significant improvement in peak shape, as narrower more symmetrical peaks with higher intensities were observed. In addition resolution and tailing were also better as evident for all the analytes in Figure 4.14a (method 3a). However, separation was poor between 3-TFMPP and MDMA due co-elution observed with both methods 2 and 3 (Figures 4.13b and 4.14a). A comparison could not be made with method 1 as MDMA was not available at the time of analysis. To potentially improve and also confirm the results, a polar column from a different manufacturer was applied (Supelco Equity-5) whilst maintaining other conditions smilar to method 3b. The Equity-5 column gave slightly better resolution than the ZB5 as shown by the peaks 4-TFMPP, MBZP and MePP in Figures 4.14a (ZB5) and 4.14b (Equity-5). In addition both ZB5 and Equity-5 columns gave less column bleed and relatively "cleaner" chromatograms (Figure 4.14 relative to 4.12 and 4.13a; b) allowing for better accuracy in peak detection (Barwick, 1999; McMaster, 2007).

Since the columns' physical properties were similar for all the columns the reason the ZB5 and Equity 5 columns gave better results can be attributed to the chemical properties of the stationary phase in the column. In accordance with the Plate theory (Grob and Barry, 2004; McNair and Miller, 2009; Skoog et al., 2007) the resolution between peaks (equation 2.11) is dependent on the capacity factor and selectivity which are measures of retention of the substance in the column. An increase in the capacity factor and selectivity increases the resolution. These factors are in turn governed by the chemical properties of the analytes. Given that the drugs under consideration are polar it follows that the analytes have a higher affinity for the polar stationary phase. Consequently they show better separation and peak shape with the ZB5 and Equity 5 columns. The changes in oven temperature programming (initial hold temperature, time and ramping) from method 1 to 3b allows for longer equilibration time of the drug between the stationary phase and mobile phase. This results in an increase in the partition coefficient and consequently plate number, N. An increase in N increases resolution (section 2.2 equations 2.2, 2.8 – 2.11).

The results obtained are in accordance with the work of Andersson et al. (2007a), where it was found that 'like separates like more efficiently'. In addition, in their study the column was selected on the basis of inertness and resolution and minimal bleed. A study by Aalberg et al., (2004) shows the importance of selecting an appropriate column to achieve adequate selectivity. Optimisation was through varying stationary phase polarities so as to increase specificity towards regioisomeric MDMA substances. The authors reported that polar stationary phases allowed for higher temperature and were able to identify 10 regioisomeric substances of MDMA. These results support the observed trend as the more polar column shows higher selectivity.

It could be for these reasons that stationary phases of the type 5%-Phenyl-95%-Dimethylpolysiloxane have been extensively used by other researchers in analysis of piperazines, amphetamines and other drugs of abuse (de Boer et al., 2001, Takahashi et al., 2009, Kenyon et al., 2010; Lecompte et al., 2008, UNODC, 2013c; Boumrah et al., 2014). In addition the Equity-5 column gave slightly better resolution than the ZB5 as shown by the peaks 4-TFMPP, MBZP and MePP in Figures 4.14a (ZB5) and 4.14b (Equity-5). Consequently, the Supelco Equity 5 column and method 3b was be applied in all subsequent studies. The preliminary chromatographic performance data is shown in Table 4.6.

Table 4.6 Preliminary Method 3b performance characteristics

Substance	**Mean values (n = 2)**					
	N x 10^5	**T/mins**	**α**	**R**	**Rt/mins**	**RRT**
Methamphetamine	3.831	5.872	1.422	5.603	8.880	0.480
2-FPP	4.432	5.872	1.070	9.629	12.620	0.683
3-FPP	13.636	3.174	1.040	12.607	14.515	0.785
4-FPP	10.429	4.055	1.013	3.638	14.130	0.764
3-TFMPP	13.740	ND[1]	1.003	1.058	14.320	0.774
4-TFMPP	21.225	1.911	1.007	1.994	15.090	0.816
BZP	6.244	6.677	1.021	4.615	13.840	0.749
DBZP	58.723	1.023	1.019	11.683	19.950	1.079
MBZP	10.672	4.473	1.013	3.711	15.190	0.822
3-CPP	23.566	2.814	1.054	22.398	16.715	0.904
4-MePP	15.870	3.672	1.086	28.646	15.390	0.832
MDMA	15.922	ND[1]	1.010	2.937	14.370	0.777
cocaine	61.013	0.986	1.074	45.053	20.335	1.100
diazepam	66.788	2.178	ND[1]	ND[1]	21.840	1.181
caffeine	37.654	0.933	1.050	26.919	17.610	0.952
eicosane (IS)	64.907	1.180	1.079	47.074	18.490	1.000

[1] ND stands for not determined. This was due to coelution between 3-TFMPP and MDMA hence tailing could not be calculated for these two analytes. In addition, diazepam is the last peak hence there is no resolution to a subsequent peak after it.

4.4 CONCLUSION

The preliminary method developed (method 3b, Table 4.3) was able to detect and simultaneously analyse for 4-FPP and 3-TFMPP and all their congeners in street samples. In addition, separation of FPP and TFMPP positional isomers was achieved. These compounds are likely to be present as impurities in a street sample of these drugs. Therefore, the preliminary method development established usable parameters which were a foundation for further investigation. Of the methods investigated method 3b (section 4.2.7) gave the best results in terms of general peak profile, resolution and tailing and therefore this method was selected for further development. The best column had a

stationary phase of the type 5%-Phenyl-95%-Dimethylpolysiloxane copolymer, hence this type of column was selected for use and Supelco Equity 5 is proposed as an ideal column in analysis of drugs of abuse. In the sample preparation stage, both derivatisation and non-derivatisation were investigated and it was concluded that for the work to be conducted in this research there were no advantages to derivatising the samples. Not derivatising the samples gave good results as such other studies were carried out without derivatisation.

It has been reported by other researchers (Andersson et al., 2007a; Inoue et al., 2008; Hibbert, 2007; Maher et al., 2009) that optimisation attains optimum conditions and as such the best results possible with the method. It would be desirable to increase resolution and reduce tailing for the drugs eluting in the 12 -16 minutes range and as such method 3b was further developed, i.e., optimised (Chapter 6) so as to improve its performance. Prior to the optimisation method 3b was used in the study on solvents and stability studies (Chapter 5), so as to generate stability conditions and select an appropriate solvent for use.

CHAPTER 5

INVESTIGATION OF THE STABILITY OF DRUGS AND EFFECT OF SOLVENTS DURING ANALYSIS

5.1 INTRODUCTION TO STABILTY INVESTIGATIONS

The conceptual framework for this was highlighted in Chapter 2 (section 2.7). Information derived from stability studies is used to set appropriate conditions, limits for analysis and storage during analytical investigations (FDA, 2008). Such information can be used to eliminate or minimise potential degradation and the resulting loss in stability during laboratory investigations.

To ensure the stability of the drugs under investigation this study carried out various tests;

a) The solubility of the analytes in different solvents (water, ethyl acetate, dichloromethane, methanol and 2-methylpropan-2-ol was investigated. This was to select a solvent capable of dissolving the analytes for further investigation,
b) The chemical stability of the analytes during analysis in different solvents (methanol, dichloromethane and 2-methyl-propan-2-ol) under routine environmental conditions (ambient temperature and pressure) was tested,
c) The stability of the analytes during analysis on the GC-MS auto-sampler during instrumental analysis was tested for the solvents methanol, dichloromethane and 2-methyl-propan-2-ol,
d) Data analysis and selection of the solvent in which the analytes were most stable,
e) The stability during storage (at different temperatures and dark or light storage conditions was also conducted. Storage stability was commenced only after selection of solvent from the other tests (section 5.2.6 – 5.2.7).

Unless stated, analysis was performed under routine ambient temperature and pressure. The drugs were handled according to their manufacturer's instructions, to avoid unforeseen decomposition of the analytes. The drugs were stated by the manufacturers to be stable for one year from date of opening under routine dry, out of sunlight ambient conditions. Stability was established by determining whether concentration and retention times

remained constant with time (Yoshioka and Stella, 2000; ICH Q1A(R2), 2003). The significance of the results was statistically determined. This was achieved by application of the Chi (χ^2) square test, Pearson's correlation and ANOVA and variance (sections 3.2.7, 3.2.4, 3.2.6 and 3.2.2).

5.1.1 AIMS OF THIS STUDY

The aim of this study was to investigate the effect of solvents on the analysis of drugs of abuse commonly found on the streets and to establish the stability of these drugs during routine laboratory analysis. Hence, define appropriate stability limits for their analysis under ambient environmental conditions, on the instrument during analysis and storage in the refrigerator (4^{o}C) and freezer (-20^{o}C). In addition also select an appropriate solvent for use in subsequent analytical work (Chapters 6, 7 and 8).

5.2 MATERIALS AND METHODS

In all the tests where 2-methyl-propan-2-ol was used as a solvent the temperature was maintained above its freezing point 20 - 25^{o}C, during preparation of solutions. The storage test (5.2.8) was only carried out after the selection of a suitable solvent from the solubility, solvent selection and auto-sampler stability tests

5.2.1 CHEMICALS/REAGENTS

The drug standards used were as listed in Chapter 4 (section 4.2.1). The solvents ethyl acetate, methanol, 2-methyl-propan-2-ol, dicholoromethane and chloroform were from Fisher Chemicals.

5.2.2 INSTRUMENTS

A Perkin Elmer GC-MS Clarus Turbomass Gold 500MS fitted with a Supelco, Equity-5 GC capillary column (30m x 0.25mm x 0.25um). The instrument was equipped with the NIST MS Search Version 2.0 library software. The instrument was initially set up according to Table 4.3 Method 3b (Chapter 4, section 4.2.7), i.e., the injector was set at 250^{o}C with a split ratio of 20:1. The carrier gas was He (g) at a flow rate of 1mL/min. The

initial oven temperature was set at 60 ^{o}C with a hold of 1min. The oven was ramped at 10 ^{o}C /min to 150^{o}C with a hold of 2min and at 10^{o}C /min to 280^{o}C, with a hold of 4min. The MS transfer line was set at 280^{o}C, source temperature 230^{o}C, ionisation energy 70eV and scan range at m/z 40 – 500. The total analysis run time was 23.67minutes.

A Zanus Electrolux, Tutella system 3 temperature monitoring refrigerator (4^{o}C) and a Scanfrost, Tutella system 6 temperature monitoring freezer (-20^{o}C) were used for storage stability tests.

5.2.3 STATISTICAL SOFTWARE

Analysis of results was carried out using IBM SPSS Version 20 and MS Office Excel 2010.

5.2.4 SOLUBILITY TEST

Each analyte (1.0mg) was weighed into a test tube. Ethyl acetate (1.0mL) was added. The solution was shaken to dissolve or sonicated for 20 minutes if the analytes were observed to have a solid residue on shaking. If soluble, incremental amounts of solute were added up to 10.0mg whilst observing to see whether higher amounts could be dissolved for the preparation of more concentrated solutions. The test was repeated for methanol, 2-methyl-propan-2-ol, dichloromethane and water.

5.2.5 PREPARATION OF STANDARD SOLUTIONS FOR THE STABILITY TESTS

Standard solutions were prepared as outlined in preliminary method development (Chapter 4, section 4.2.4.3) for individual drug standard solutions and mixed drug standards solution 1 using methanol as a solvent. Standard solutions were similarly prepared using dichloromethane and 2-methyl-propan-2-ol as solvents.

5.2.6 STABILITY OF THE DRUGS DURING ANALYSIS IN DIFFERENT SOLVENTS UNDER ROUTINE AMBIENT ENVIRONMENTAL CONDITIONS

Standards solutions in methanol were used prepared as outlined in section 4.2.4.3 (Table 4.2). The individual drug standard solution for each analyte was analysed 3 times on the GC-MS. The stability of the mixed drug standard solution was determined by analysing the

mixed drug standard solution 6 times on the GC-MS. The 6 replicates were used to determine repeatability so as to establish system suitability for quality control (section 2.5.2). The test was similarly conducted using the solvents dichloromethane and 2-methyl-propan-2-ol. The environmental conditions were ambient temperature and pressure.

5.2.7 STABILITY OF THE DRUGS IN DIFFERENT SOLVENTS ON THE GC-MS AUTO-SAMPLER DURING INSTRUMENTAL ANALYSIS

The standards listed inTable 4.1 (Chapter 4) were prepared in methanol as outlined in section 4.2.4.3. The final solution was split (50uL) into different vials (22) covering the stability period. The vials were all simultaneously placed on the auto-sampler programmed to inject hourly in sequence vial 1 to 22 for a 25 hours run cycle (time $t_0 - t_{25}$). The time of the first injection was recorded as t_0. The test was repeated using the solvents dichloromethane and 2-methyl-propan-2-ol.

The drugs were analysed as a) a mixed standard solution of all the piperazine based drugs and, b) a mixed drug standard solution of all the non-piperazines. For those analytes which did not show degradation the test was repeated and with an extended runtime of 36hours. Where a degradation product was observed in the mixed samples, the drugs were then individually analysed.

5.2.8 STORAGE STABILITY

Stock solutions of the mixed drug standards were prepared in 2-methylpropan-2-ol as outlined in section 4.2.4 (Chapter 4).The samples were placed in the refrigerator at 4^{o}C. The time and day were recorded. Sampling and analysis was conducted daily for a period of one week. The study was similarly conducted for storage in a freezer (-20^{o}C), except the samples were withdrawn every 3rd day for a period of a month or until degradation was observed in the analyte.

5.2.9 DATA ANALYSIS

5.2.9.1 Checking for stability

For all the stability tests (sections 5.2.6 - 5.2.8), the peak area ratios and retention times were determined. The peak area ratios were calculated according to equation 2.18 (Chapter 3). The trend in peak area ratios and retention times over the period of investigation was then analysed for each analyte as a measure of stability. To measure the stability, stability graphs (peak area ratios or retention time versus time) were plotted. In addition statistical analysis was conducted using Chi (χ^2) square and Mann-Kendall trend tests. The Chi square and Mann-Kendall analysis was conducted according to Chapter 3 sections 3.2.7 and 3.2.12 respectively.

Furthermore, the total ion chromatograms obtained in these tests were visual inspected for the presence of artefacts (secondary peaks).

5.2.9.2 Solvent comparison

Comparison of the solvents was conducted using the results from the stability of the drugs in different solvents during instrumental analysis test (section5.2.7). Pearson's correlation and ANOVA (2-way) and variance were applied. These parameters were calculated according to Chapter 3 sections 3.2.4, 3.2.6 and 3.2.2 respectively. In the tests if a drug was insoluble in solvent it was assigned a value of zero.

5.3 RESULTS AND DISCUSSION

5.3.1 SOLUBILITY

The solubility of the drugs in solvents commonly used during routine analysis of drugs of abuse is reported in Table 5.1 below. All 25 of the analytes tested dissolved in water, methanol and ethyl acetate. 23 were soluble in 2-methylpropan-2-ol. The least solubility was observed with dichloromethane with 21 analytes soluble. The insoluble analytes were detailed as; (+)-amphetamine and EME were found to be insoluble in both dichloromethane and 2-methylpropan-2-ol. In addition, CPP and nicotinamide were insoluble in dichloromethane.

Table 5.1 Solubility of drugs of abuse in different solvents.

Substance	**H_2O**	**MeOH**	**EtAc**	**DCM**	**MPOH**
1-(2-fluorophenyl)piperazine	+	+	+	+	+[1]
1-(3-fluorophenyl)piperazine	+	+	+	+	+
1-(4-fluorophenyl)piperazine	+	+	+	+	+
1-(2-trifluoromethylphenyl)piperazine	+	+	+	+	+
1-(3-trifluoromethylphenyl)piperazine	+	+	+	+	+
1-(4-trifluoromethylphenyl)piperazine	+	+	+	+	+
1-benzylpiperazine	+	+	+	+	+
1-(4-dibenzyl)piperazine	+	+	+	+	+
1-(4-Methylbenzyl)piperazine	+	+	+	+	+
1-(3-Chorophenyl)piperazine	+	+	+	−	+[1]
1-(4-Methylphenyl)piperazine	+	+	+	+	+
(±)3,4-Methylenedioxymethamphetamine HCl	+	+	+	+	+
(+)-Amphetamine SO_4	+	+[1]	+	−	−
(+)-Methamphetamine HCl	+	+	+	+	+
Caffeine	+	+	+	+	+
Cocaine HCl	+	+	+	+	+
Diazepam	+	+	+	+	+
Benzoylecgonine hydrate	+	+	+	+	+
Ecgonine methyl ester HCl hydrate	+	+[1]	+	−	−
Piperazine	+	+	+	+	+
Dapoxetine HCl	+	+	+	+	+
Nicotinamide	+	+	+	-	+
Dextromethorphan HBr	+	+	+	+	+
Eicosane (IS)	+	+	+	+	+
Quinoline (IS)	+	+	+	+	+

[1] Indicates that the analyte was soluble with shaking for 30mins or ultra-sonication for 10mins. Its solubility was ≤ 2mg/mL. All the other analytes were soluble up to 10mg/mL. MeOH denotes methanol, EtAc is ethyl acetate, DCM is dichloromethane, MPOH is 2-methylpropan-2-ol and IS denotes internal standard.

The results indicated that all the solvents have good solvation properties (Table 5.1), as the majority of the drugs (≥ 84%) were soluble in all the solvents. Solubility was in the order water > ethyl acetate > methanol > 2-methylpropan-2-ol > dichloromethane. This is in

accordance with both the properties of the solvents (Gokel, 2004) and the polarity of the drugs. The solvents' dipole and dielectric constants were reported in Table 2.1. A solvent's dipole moments and dielectric constants affect its ability to solvate ions thereby to dissolve solutes. The analytes are polar and as such would dissolve more readily in the more polar solvent. Ethyl acetate and water have the highest dipole moment and as expected the highest solubility, whilst dichloromethane has the lowest. Methanol and 2-methylpropan-2-ol have almost similar dipole moments, 1.68 and 1.67 respectively; consequently their solubilities are expected to be similar. However, a higher solubility is observed with methanol. This can be attributed to the effect of molecular structure; 2-methylpropan-2-ol is a tertiary alcohol (structure is shown in Table 2.3), hence it exhibits steric hindrance, with the methyl substituents adjacent to the functional group hindering solvation (Lawrence, 2004). Consequently, based on solubility, ethyl acetate and methanol would be the solvents of choice as they dissolve a higher number of the drugs. Whilst there is no record of solubility studies of these drugs in the various solvents, methanol has been extensively, confirming the results of this study. Ethyl acetate has also been extensively applied for extraction and isolation of drugs and for general analysis especially when derivatised (de Boer et al., 2001; Inoue et al., 2008). Water has also been routinely used to dissolve street samples in the sample preparation stage, from which the analytes are then extracted by the organic solvent. In this study the street samples were to be dissolved in the organic solvent hence it was not investigated further.

5.3.2 STABILITY OF THE DRUGS DURING ANALYSIS IN DIFFERENT SOLVENTS UNDER ROUTINE AMBIENT ENVIRONMENTAL CONDITIONS

A typical total ion chromatogram (TIC) for the drugs obtained under routine conditions is shown in Figure 5.1. This was used as a comparator to the chromatograms generated under different stability conditions (sections 5.2.6 – 5.2.8).

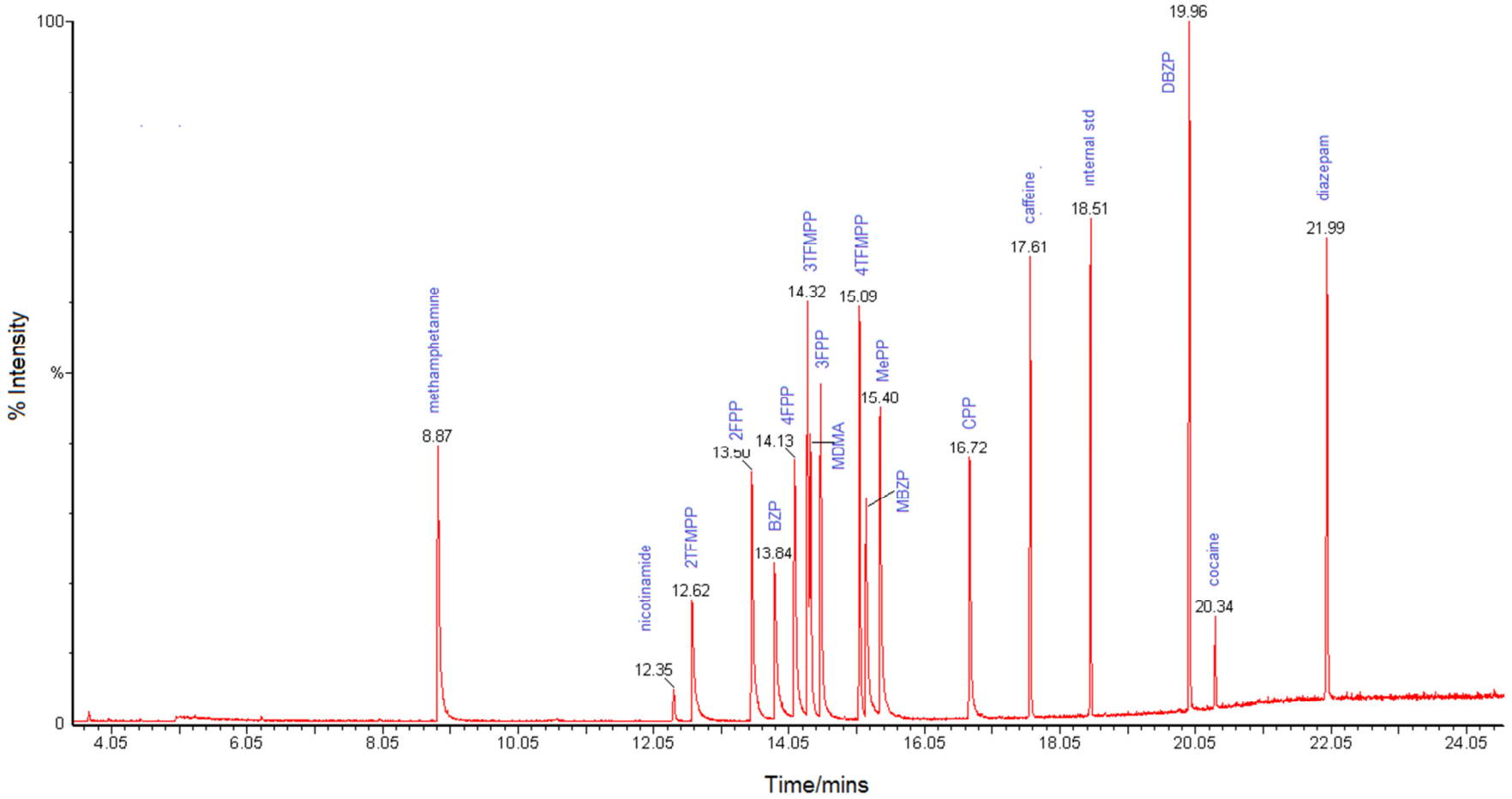


Figure 5.1 Total ion chromatogram of the drugs under ambient conditions at time t_0 (reference chromatogram).

No artefacts were observed in the freshly run samples under routine environmental conditions. It can therefore be deduced that the sample preparation step and the prevailing environmental conditions in the laboratory do not cause degradation. These results were used as a comparator for the rest of the stability studies. Very few studies were encountered on the stability of drugs of abuse; in addition these were mostly on stability during bioanalytical studies. However, the results obtained were as expected and in line with the previous findings by other researchers. Moody et al., (1999) investigated the long-term stability of drugs of abuse and chemotherapeutic agents at different storage conditions had comparatively similar results. They observed no degradation at ambient conditions prior to storage (at time t_0).

5.3.3 STABILITY OF THE DRUGS IN DIFFERENT SOLVENTS ON THE GC-MS AUTO-SAMPLER DURING INSTRUMENTAL ANALYSIS

The stability of an analyte was determined by monitoring the change in concentration with time (Yoshioka and Stella, 2000) as peak area ratio. This was depicted graphically as stability profiles of the drugs. The stability profiles for each analyte for each of the solvents methanol, dichloromethane and 2-methylpropan-2-ol were determined and are exemplified by 2-FPP and DBZP in Figures 5.2 and 5.3 respectively. For the other drugs analysed reference is made to Appendix 1.

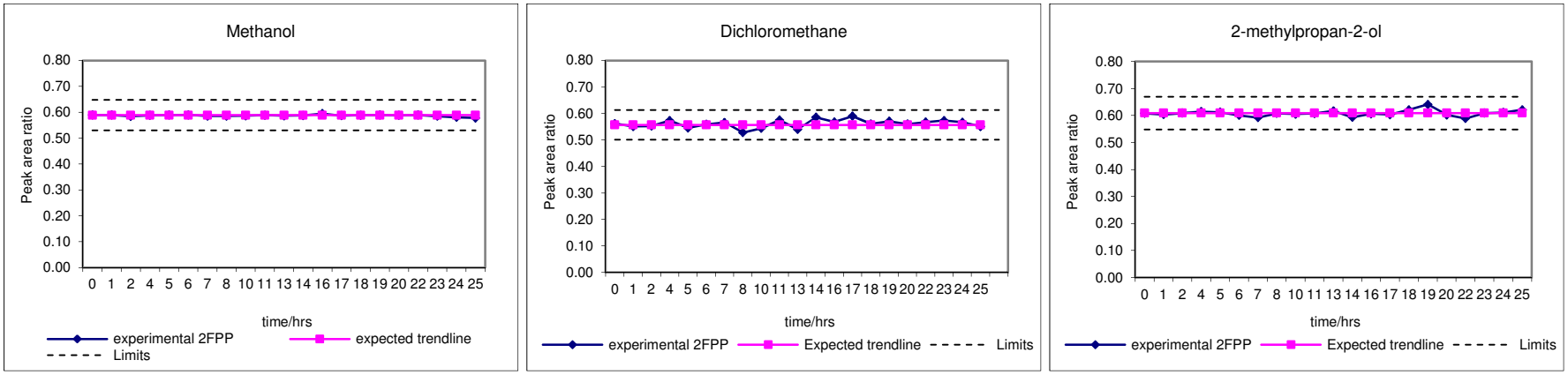


Figure 5.2 2-FPP stability profiles in different solvents over 25hours on the GC-MS autosampler.

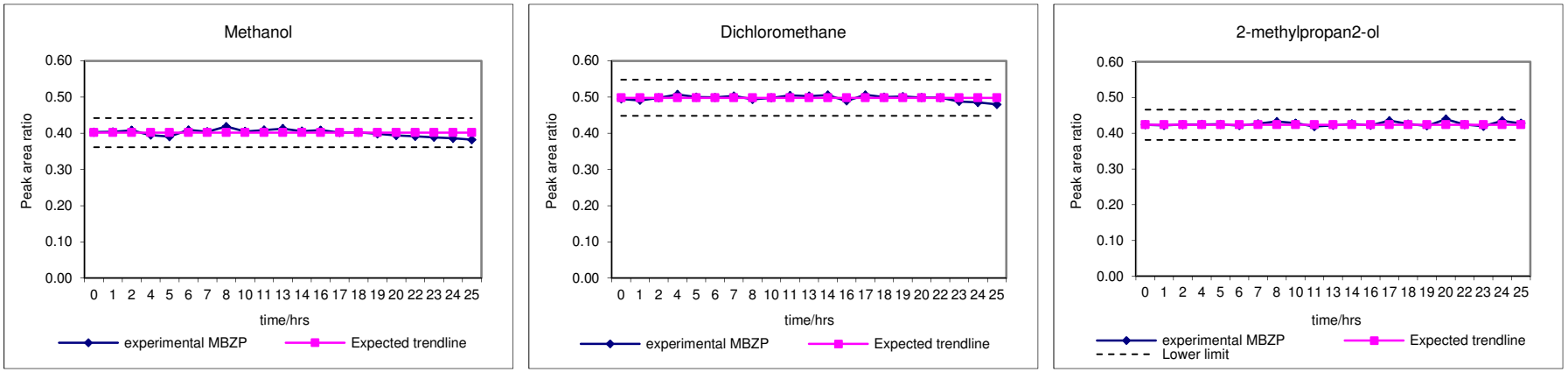


Figure 5.3 MBZP stability profiles in different solvents over 25hours on the GC-MS autosampler.

Limits: acceptable limits of variation for stability (± 10% of Expected value).

It is evident in Figure 5.2 that for 2-FPP the peak area ratio is constant over the whole analysis time period. Consequently, the drug was deemed stable on the GC-MS auto-sampler during instrumental analysis for all the solvents used. In comparison, for MBZP (Figure 5.3) in methanol and dichloromethane the peak area ratio is initial constant and then the trend decreased slightly after 18 hours. Hence, MBZP was deemed stable for 18 hours in methanol and 20 hours in dichloromethane. In addition, MBZP was stable for the whole analysis period (25 hours) in dichloromethane.

A similar analysis was conducted for all the other drugs (stability graphs in Appendix 1). On average most of the drugs were stable in all the solvents on the GC-MS auto-sampler during instrumental analysis. It was found that the stability ranged from 14 – 25 hours and was in the order 2-methylpropan-2-ol > dichloromethane > methanol. For easier evaluation and comparison a summary of the results are depicted graphically in Figure 5.4 and tabulated in Appendix 2. In the graph the duration the drug was stable is shown in hours. Amphetamine and EME were insoluble in 2-methylpropan-2-ol whilst 3-CPP, amphetamine and EME were insoluble in dichloromethane. Hence, these drugs were not analysed for these two solvents, therefore their stability appears as zero in the graph.

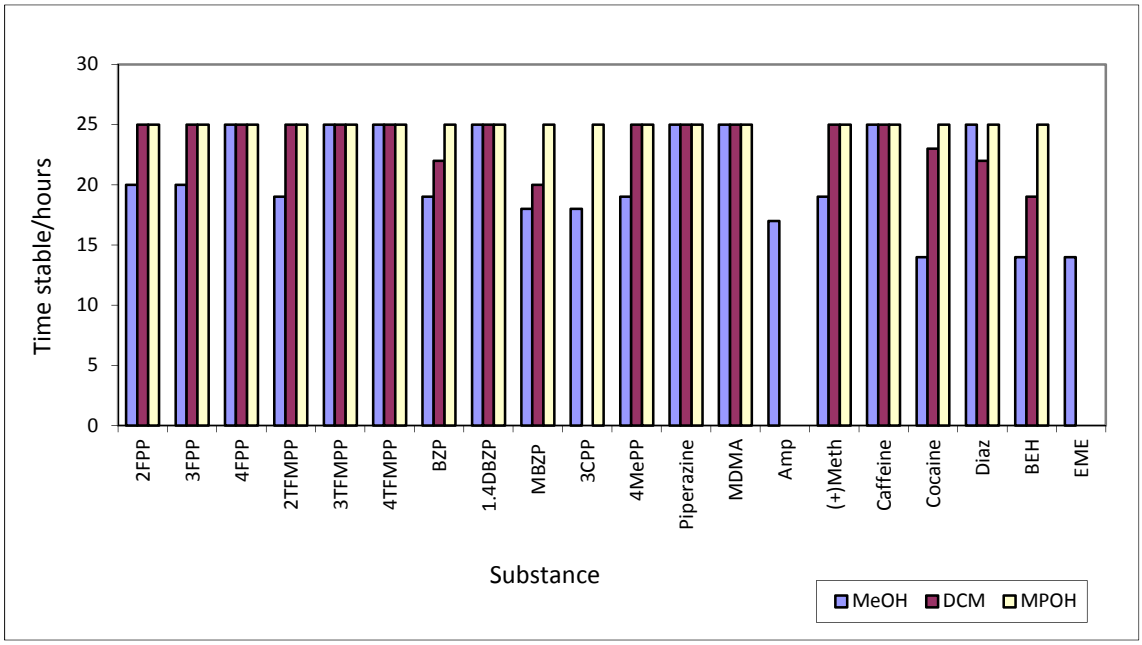


Figure 5.4 Graphical comparison of stability of drugs in different solvents on the auto-sampler (n = 2). The solvents are represented as methanol (MeOH), dichloromethane (DCM) and 2-methylpropan-2-ol (MPOH).

The analytes were least stable in methanol, in which the drugs were stable for a period of 14 – 25 hours. For the piperazines 4-FPP had the lowest stability period, 15hours, whilst 3-TFMPP, 4-TFMPP, and DBZP were stable for 25hours. The non piperazines MDMA, caffeine, diazepam, and nicotinamide were stable in methanol for 25 hours, whilst cocaine and EME and BEH had the lowest stability (14hours).

In comparison, the drugs when in dichloromethane show a higher stability period. All the drugs were stable in dichloromethane for a period of 19 - 25 hours, with all the piperazines stable for 25 hours, except for 4-TFMPP (23 hours), BZP (22 hours) and MBZP (20 hours), whilst for the non piperazines, cocaine (23 hours), BEH (19 hours) and diazepam (22 hours). All the non piperazines were stable in dichloromethane and all the drugs were stable in 2-methylpropan-2-ol in the range for 25 hours.

It can therefore be deduced that the duration of stay of analytes on the instrument auto-sampler has an impact on the stability of the drug. Extensively long periods exacerbate degradation. This is likely to be due to thermal effects as auto-samplers can warm up during analysis. Several studies have shown that solutions are more stable for a longer period if maintained at lower temperatures, such as in the refrigerator or freezer (Karinen et al., 2011; Moody et al., 1999; Nowatzke and Woolf, 2007). In the current study the auto-sampler was found to be around ambient temperature (25 - 28^{o}C). In addition, since the same drug analytes were analysed for each of the solvents, the observed results can also be attributed to the chemical properties of the solvent and its effect on the stability of the drug analytes (Yoshioka and Stella, 2000). No studies on stability of drugs of abuse during instrumental analysis were found, hence this limited comparative review of the results. To confirm the findings discussed above statistical analysis was conducted using Chi square and Mann-Kendal trend test, in accordance with Chapter 3 sections 3.2.7 and 3.2.12.

5.3.4 STATISTICAL ANALYSIS OF STABILITY DATA

5.3.4.1 Chi-square (χ^2) and Standard deviations

The results of the χ^2 test on the stability data for each of the solvents methanol, dichloromethane and 2-methylpropan-2-ol were in the ranges shown in Table 5.2.

Table 5.2 Statistical analysis: Chi-square (χ^2) and Standard deviations (n = 21)

Test	**Dichloromethane**	**Methanol**	**2-methylpropan-2-ol**
Chi-square	$\chi^2 = P(\chi^2_{0.05}) = 0.975$ - 0.995	$P(\chi^2_{0.05}) = 0.975$ - 0.995	$P(\chi^2_{0.05}) = 0.975 – 0.995$
Std dev	0.05 – 0.16	0.01 – 0.78	0.02 – 0.07, N = 3

Statistical evaluation of the stability profile of each drug gave Chi square values that were less than the tabulated value ($\chi^2_{0.05,21} = 31.41$) with probabilities above 97.5% (Table 5.2) and as such were significant. Consequently, this implies there is no difference between experimental and expected values, with any variations observed due to chance, and thus insignificant. This was also evidenced by the small values observed in the standard deviations (range 0.01 – 0.78). It can be said that generally analyte concentration did not vary greatly with time since the standard deviations were low. However, the results indicated that the analytes were most stable in 2-methylpropan-2-ol, $\sigma = 0.02 – 0.07$ with the highest standard deviation observed for DBZP very small ($\sigma = 0.07$). The retention times and retention indices analysed for selected drugs 2-FPP, 4-FPP, 3-TFMPP and 4-TFMPP were stable over 25 hours (RSD < 1%). It can be argued that these solvents have found use with other researchers (Baker and Phillips, 1983; Dayright, 2001; Staack et al., 2003; Inoue et al. 2004, Takahashi et al., 2009) due to the fact that they are stable enough for routine use (14 hours minimum from placement of fresh samples on the GC-MS auto-sampler) and can be applied for longer periods with planning. It can also be argued based on the stability results, that 2-methylpropan-2-ol has potential for more application in analysis of drugs of abuse; currently its use is not common.

5.3.4.2 Mann-Kendal trend analysis

To confirm the statistical findings further analysis using the Mann-Kendal trend test (Chapter 3 section 3.2.12) was conducted. The results are shown in Tables 5.3 - 5.5. The Mann-Kendal test statistic (*S*), the corresponding cumulative normal distribution test statistic (*Z*), the probabilities, *p* corresponding to the *Z* values and the Trend size are given for the solvents methanol, dichloromethane and 2-methylpropan-2-ol.

Table 5.3 Summary of methanol Mann-Kendall statistical data for drug auto-sampler/solvent stability (n = 21).

Compound	**Test statistic *S***	***Z***	***P (%)***	**Trend Y/N**	**Trend size (%)**
2-FPP	+3	+0.0143	98.86	N	-1.08
3-FPP	+6	+0.0356	97.16	N	+7.81
4-FPP	-9	-0.0571	95.45	N	-2.78
2-TFMPP	-3	-0.0143	98.86	N	-5.94
3-TFMPP	-6	-0.0356	97.16	N	-1.36
4-TFMPP	-13	-0.0857	93.17	N	-0.52
BZP	-11	0.0071	99.43	N	-3.96
DBZP	-7	-0.0427	96.59	N	-0.18
MBZP	+4	+0.0358	97.15	N	-4.77
3-CPP	-4	-0.0214	98.29	N	-3.59
4-MePP	-2	-0.0071	99.43	N	-3.53
MDMA	-9	-0.0570	95.46	N	+0.84
Amphetamine	0	+0.0071	99.43	N	-6.84
Methamphetamine	-5	-0.0285	97.72	N	-4.00
Caffeine	-6	-0.0356	97.16	N	+0.56
Cocaine	-18	-0.1211	90.36	N	-4.48
Diazepam	-1	0	100.00	N	+2.17
Dapoxetine	+12	+0.0784	93.75	N	-0.71
Nicotinamide	0	±0.0077	99.94	N	-0.26

Table 5.4 Summary of dichloromethane Mann-Kendall statistical data for drug auto-sampler/solvent stability (n = 21).

Compound	**Test statistic *S***	***Z***	***P (%)***	**Trend Y/N**	**Trend size (%)**
2-FPP	-4	-0.0214	98.30	N	+1.56
3-FPP	-10	-0.0641	94.89	N	-1.14
4-FPP	+7	+0.0427	96.59	N	0.00
2-TFMPP	-8	-0.0499	96.02	N	+3.26
3-TFMPP	-8	-0.0498	96.03	N	+0.22
4-TFMPP	-14	-0.0927	92.62	N	-1.52
BZP	-4	-0.0214	98.30	N	-3.30
DBZP	-8	-0.0499	96.02	N	+0.04
MBZP	-16	-0.1071	91.47	N	-2.09
3-CPP	Insoluble			N	
4-MePP	-4	-0.0214	98.30	N	+1.67
MDMA	+4	+0.0214	98.30	N	+2.41
Amphetamine	Insoluble				
Methamphetamine	+9	+0.0571	95.45	N	-0.80
Caffeine	+6	+0.0356	97.16	N	-0.16
Cocaine	-8	-0.0500	96.01	N	-4.34
Diazepam	+2	+0.0214	98.29	N	+7.34
Dapoxetine	+5	+0.0285	97.73	N	-2.56
Nicotinamide	Insoluble				

Table 5.5 Summary of 2-methylpropan-2-ol Mann-Kendall statistical data for drug auto-sampler/solvent stability (n = 21).

Compound	**Test statistic *S***	***Z***	***P (%)***	**Trend Y/N**	**Trend size (%)**
2-FPP	+10	+0.0640	94.90	N	+1.10
3-FPP	-9	-0.0570	95.45	N	+2.24
4-FPP	-3	-0.0142	98.86	N	+0.90
2-TFMPP	-4	-0.0215	98.29	N	+2.08
3-TFMPP	-12	-0.0784	93.75	N	+0.09
4-TFMPP	+12	+0.0783	93.76	N	+0.35
BZP	+3	+0.0143	98.86	N	+0.32
DBZP	+4	+0.0214	98.30	N	-0.32
MBZP	-2	-0.0071	99.43	N	+1.02
3-CPP	-3	-0.0142	98.86	N	+1.70
4-MePP	-2	-0.0071	99.43	N	+0.95
MDMA	-5	-0.0285	97.73	N	-0.55
Amphetamine	Insoluble			N	
Methamphetamine	-10	-0.0642	94.89	N	+0.11
Caffeine	-4	-0.0214	98.30	N	-1.80
Cocaine	-6	-0.0356	97.16	N	-0.12
Diazepam	-8	-0.0498	96.03	N	+0.10
Dapoxetine	+4	+0.0214	98.29	N	-0.94
Nicotinamide	-4	-0.0215	98.29	N	0.00

In the Mann-Kendal results Tables 5.3 - 5.5 the existence of signs indicates that potentially a trend exists, hence potentially there is loss of stability. In accordance with Onoz and Bayazit (2003) and Aalberg et al. (2005b) the trend in the degradation is increasing for the drugs with a positive sign and decreasing for those with a negative sign. However, evaluation of the Z values and probabilities so as to investigate the statistical significance of the results negates this deduction. The calculated Z values were all less than Z critical value ($Z_{\alpha/2} = 1.96$). The ranges were -0.1211 to 0.0356 for methanol, with MBZP and cocaine showing the highest value (0.0356 and -0.1211 respectively). For dichloromethane 0 to -0.0927 (4-TFMPP was highest) and for 2-methylpropan-2-ol 0 to -0.07836 (3-TFMPP was highest). This implied that no trend exists. Furthermore, the probability, *p* values are all greater than the significance level ($\alpha = 5\%$). This can be seen in the tables that for all the

solvents on average $p > 90\%$. In the discussion of statistical concepts (Chapter 3) it was highlighted that if $p > \alpha$, the null hypothesis for this test (H_o: no trend exists) is rejected. This implies that no statistically significant trend exists. As such the analytes can be considered stable for the time period investigated (25 hours on the auto-sampler). Any variations observed in the concentration are due to chance. Investigation of the trend size (Aalberg et al., 2005b) confirms the findings. These findings confirm the statistical observations made using Chi square analysis. The trend sizes are in range magnitude 0.26 – 20.36 for methanol, 0 – 12.53 for dichloromethane and 0 – 2.24 for 2-methylpropane-2-ol, of which MBZP had the highest trend size values. This showed it had the highest potential for degradation. Similarly, cocaine also indicated degradation with a trend size of 11.21 for methanol relative to 5.54 for dichloromethane and 0.12 for 2-methylpropan-2-ol (Tables 5.2 -5.4). However, it is suggested that whislt there is no statistically significant trend it is evident in the stability graphs for MBZP, cocaine (Appendix 1) that the stability of these compounds show potential degradation. The reason why this is not statistically could be that the time period when they went out of specification (i.e., start of degradation) is minimal compared to the overall stability period. In addition, the limits are narrow 10%. In other studies 15 – 20 % was used (Karinen et al., 2011; Nowatzke and Woolf, 2007).

5.3.4.3 Statistical comparison of solvents

Pearson's correlation was used to determine if there were any differences on the effects of these solvents on the stability of the drugs. The results suggested that there was no correlation between the solvents methanol and dichloromethane ($r = 0.37$, $n = 22$, $p = 0.05$). There was no correlation between the solvents methanol and 2-methylpropan-2-ol ($r = 0.42$, $n = 22$, $p = 0.05$). There was moderate correlation between the solvents dichloromethane and 2-methylpropan-2-ol ($r = 0.66$, $n = 22$, $p = 0.05$).

Results from ANOVA analysis confirm these findings, $F_{(21, 42)} = 3.50$, $F_{crit} = 1.81$, $p = 2.68 \times 10^{-4}$. This implies statistically there is a significant difference in stability of the drugs in the solvents tested. As such, it can be concluded that the drugs show differences in stability depending on the solvent used. Therefore, it is imperative to select a solvent for use which minimises instability of the analytes under investigation.

5.3.5 DEGRADATION PRODUCTS

The chromatographic profiles for the drugs that showed the presence of artefacts are exemplified by 1,4-dibenzylpiperazine in Figure 5.5. Appendix 3 shows the chromatographic profiles of the other drugs.

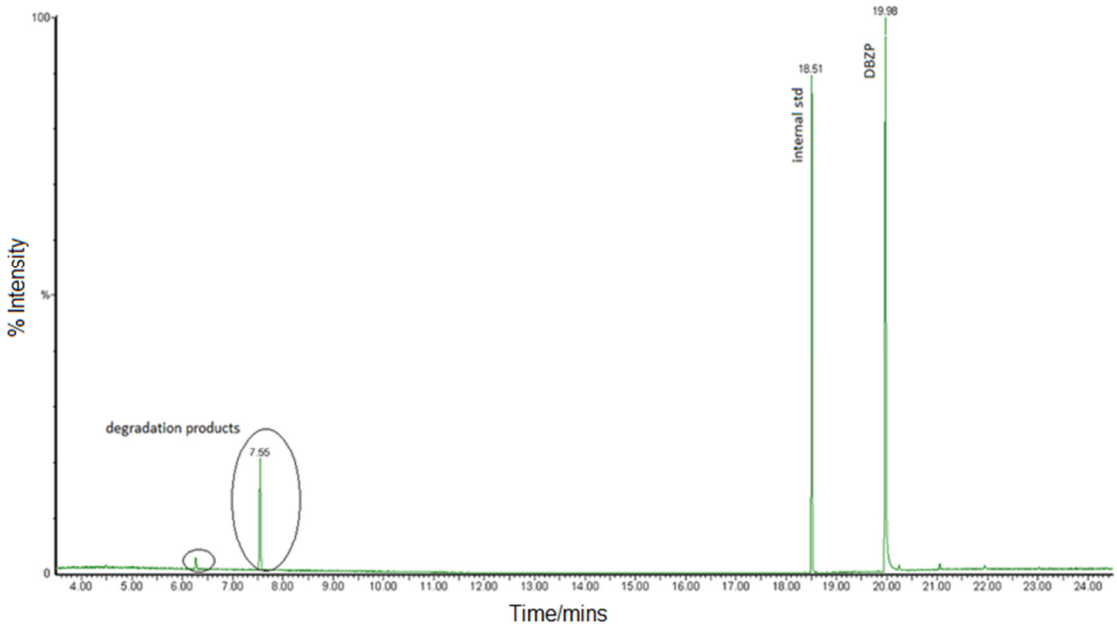


Figure 5.5 Chromatographic profile (TIC) of DBZP stability study.

A summary of the degradation products observed on the GC-MS auto-sampler for the drugs in the different solvents is given in Table 5.6.

Table 5.6 Degradation products observed during stability studies on the GC-MS auto-sampler in different solvents.

Reference	Rt/mins	Degradation compound observed	Formula
Solvent 1- methanol			
	12.00	Ecgonine Ecgonidine methyl ester.	
Solvent 2- dichloromethane			
4-FPP (not mixed)	6.27	4-fluoroaniline	C_6H_6NF
DBZP (not mixed)	6.27 7.55	Benzyl chloride Benzyl chloroformate	C_7H_7Cl $C_8H_7O_2Cl$
MBZP (not mixed)	15.03	Banzamide N-(3-amino-3-hydroxyminopropyl)-N-methyl- Or Piperazine,1-(2-trifluoromethylbenzyl)-4-(3-methylbenzyl)-	$C_{11}H_{15}N_3O_2$ $C_{20}H_{23}N_{2F3}$
mixture-all piperazines	6.29 7.57 15.03	Benzyl chloride Benzyl chloroformate Not identified	
mixture-all nonpiperazines		No artefacts	
Solvent 3- 2-methylpropan-2-ol			
mixture-all piperazines	6.27 7.54	4-fluoroaniline Benzoyl chloride - trace	
MBZP		No artefacts	
DBZP (not mixed)	6.27 7.54	Benzyl chloride Benzyl chloroformate	
4-FPP	6.27	4-fluoroaniline	

For most of the drugs, degradation was not observed even though potentially from the chemistry of the drug and the solvents such reactions were expected (section 2.7). This could be due to the fact that conditions prevailing during analysis were not able to sustain such reactions. For example esterification was not observed with methanol as high temperatures maybe required.

Analysis of the chromatographic data revealed the presence of artefacts arising in some of the samples during the stability studies (Table 5.6; Figure 5.5 and Appendix 3). With the solvent methanol the piperazines showed the absence of degradation products. It has been reported that methanol undergoes oxidation to formaldehyde, formic acid, carbon dioxide and water (Tanabe and Matsuda, 1961). These products can react with piperazines where the nitrogen acts as a base, consequently with the piperazines esterification was expected with methanol/H^+ (Figure 2.15). Its absence is likely to be due to the need for catalysis and higher temperature highlighted in section 2.7.3 for the reaction to occur. The non-piperazines were also mostly stable, with only cocaine showing marked degradation. The presence of prominent amounts of degradation products as can be seen in its chromatographic profile (TIC) in Appendix 3. Ecgonine methyl ester and benzylecgonine were identified as the degradation products (Table 5.6) and in addition ecgonidine was present. This could be attributed to breakdown of cocaine through hydrolysis as shown in Figure 5.6. This is further confirmed by extrapolation of the results metabolic studies on cocaine were similar substances were identified as degradation products (Cole, 2003; Yoshioka and Stella, 2000).

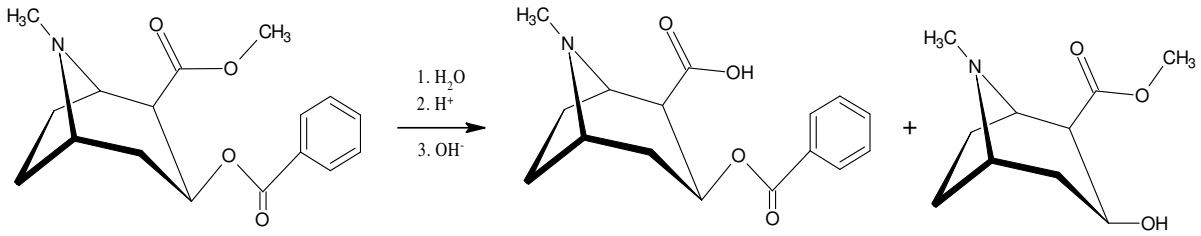


Figure 5.6 Degradation of cocaine through hydrolysis.

Even though hydrolysis was also expected for diazepam, no degradation was observed. This result is in accordance with literature; amides are less susceptible to hydrolysis than esters due to the C=O bond being less electrophilic and in addition the rate of degradation reactions are reported to be minimal (Yoshioka and Stella, 2000).

The use of dichloromethane showed the presence of more degradation products than methanol or 2-methylpropan-2-ol (Table 5.6). 4-Fluoroaniline was also identified as being present, but this was attributed to it being residual precursor from the 4-FPP synthesis of the reference standard or breakdown of the 4-FPP. Stability of drugs in the solvent

dichloromethane results showed the presence of benzyl chloride and benzyl chloroformate, (Figure 5.6) for 1,4 dibenzylpiperazine (DBZP) and also in composite drug mixture. This correlates with its loss of stability after 25 hours. This can be attributed to DBZP on prolonged analysis (36 hours on the auto-sampler), being unstable in the solvent dichloromethane. Tanabe and Matsuda (1961) showed that dichloromethane undergoes hydrolysis, the reactions were shown in Figure 2.12 (section 2.7), the products of the hydrolysis; HCl, CH_2ClOH in the presence of moisture facilitate degradation of DBZP (I) as shown in the reaction scheme below, Figure 5.7. The degradation results in the formation of benzyl chloride (II) and Benzyl chloroformate (IV).

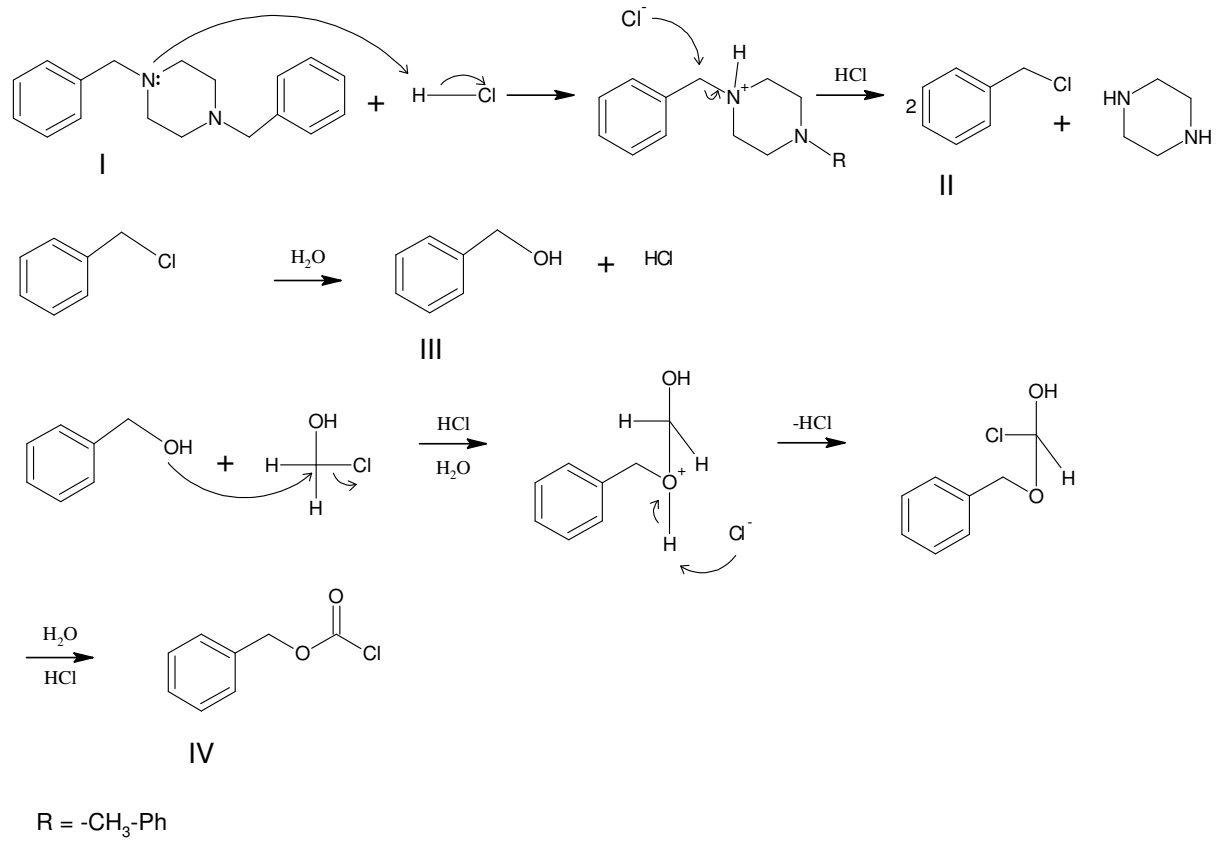


Figure 5.7 Proposed degradation pathway of benzylpiperazines, an exemplar of DBZP showing the formation the benzyl chloride and benzyl chloroformate.

The absence or presence of this degradation pathway with the other piperazines can be attributed to electronic (inductive and resonance) and steric effects due to their molecular structure and the impact on basicity and consequently reactivity (Jones, 1982; Yoshioka and Stella, 2000). The presence of the methyl group attached to the nitrogen atom has a

positive inductive effect (+I) and a positive resonance effect (+R) resulting in an increase in electron density about the nitrogen atom, which increases its basicity and consequently reactivity. It can therefore be suggested that the lack of such a methyl group in the non-benzylpiperazines makes them less susceptible to degradation. DBZP is more likely to undergo this reaction than the other benzylpiperazines due to presence of two such methyl groups. This is supported by the results obtained, where benzylchloride and benzylchloroformate were absent in BZP and MBZP. This is in line with the basicity of amines (Lawrence, 2004).

An un-identified peak was also observed at 15.03mins (Table 5.6 and Appendix 3) mixture arising from MBZP, tentatively identified as benzamide N-(3-amino-3-hydroxyminopropyl)-N-methyl- or Piperazine,1-(2-trifluoromethylbenzyl)-4-(3-methylbenzyl)-. The solvent 2-methylpropan-2-ol showed good results in both solvation and non-reactivity with analytes, as evidenced by the lack of degradation products on analysis using this solvent. This can be attributed to steric effects as it is a tertiary alcohol. This same property is somewhat of a hindrance as it has less solubility. However, stability of drugs in the solvent 2-methylpropan-2-ol results showed evidence of degradation on prolonged analysis in the solvent, artefacts were observed at 36 hours, trace amounts of benzyl chloride and benzyl chloroformate were observed. It was concluded that there was a strong possibility of esterification if ethyl acetate is used taking into consideration its chemical properties and those chemistry of the drugs to be analysed. This was confirmed by the presence of impurities due to esterification during synthesis (section 8.3.2), ethyl acetate was one of the solvent used as part of purification in one of the routes of synthesis routes. Furthermore, it was recorded that ethyl acetate was used extensively in derivatisation of samples whilst the other solvents were mainly applied to un-derivatised samples. Since in this research project the solvent was applied to characterisation of street samples expected to contain impurities, no derivatisation of samples was conducted and further use of ethyl acetate was considered unsatisfactory compared to the other solvents. However, it is highlighted that for work which does not involve impurity profiling of drugs ethyl acetate can be of use.

It is evident from the discussions above, that of the solvents investigated the analytes were most stable in 2-methylpropan-2-ol, hence this solvent was deemed suitable for use in this

research. Consequently, the stability of the analytes on storage in this solvent was investigated.

It was discussed in chapter 2 (section 1.6) that currently no studies have been reported on the stability of drugs the auto-sampler during analysis and or the influence of solvents on that stability. Hence, no comparative analysis could be conducted. The data generated in this study can therefore be of use to other researchers.

5.3.6 STABILITY OF DRUGS ON STORAGE IN THE REFRIGERATOR (4^{o}C)

The stability of all the analytes on storage in the fridge at 4^{o}C in 2-methylpropan-2-ol is depicted in Figure 5.8. The graph shows the period the analytes were stable in days.

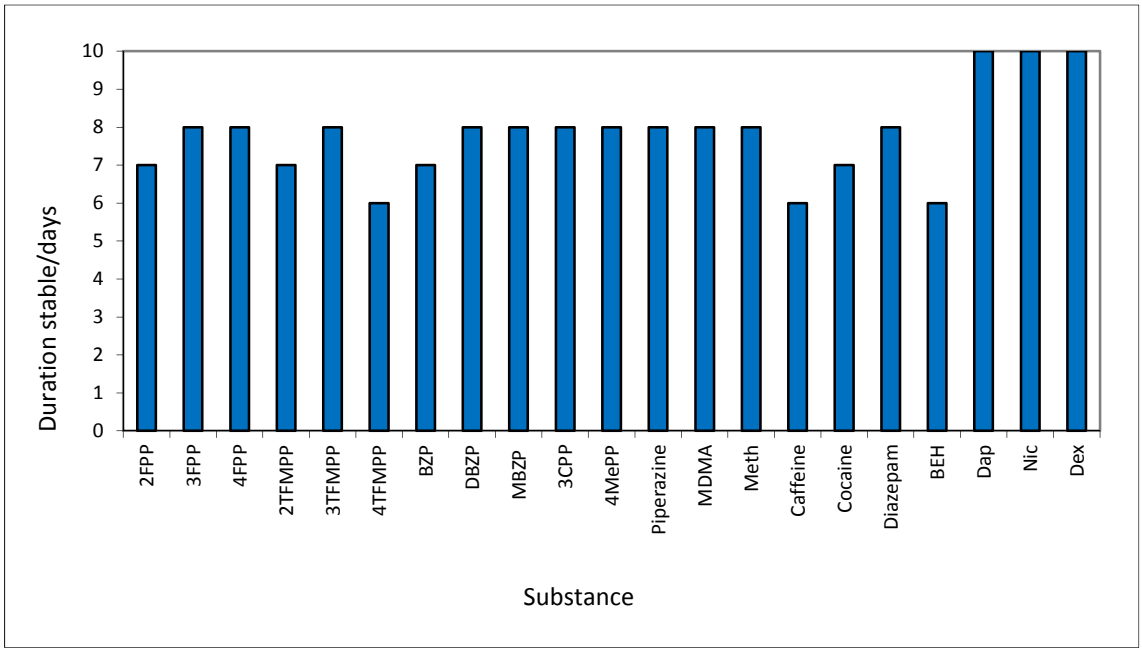


Figure 5.8 Storage stability graph of all the drugs at 4^{o}C (n = 2).

5.3.7 STABILITY OF DRUGS ON STORAGE IN THE FREEZER (-20^{o}C)

The stability of all the drugs on storage in the freezer (-20^{o}C) in 2-methylpropan-2-ol is depicted in Figure 5.9. The graph shows the period the analytes were stable in days.

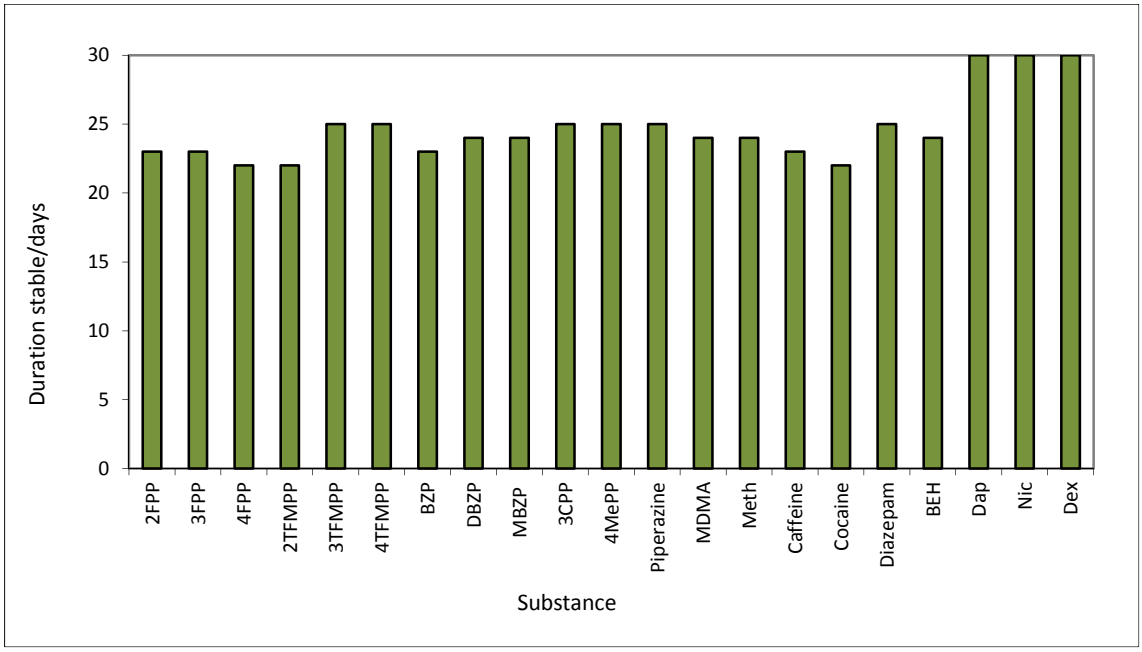


Figure 5.9 Aggregate storage stability graph of all the drugs at (-)20^{o}C (n = 2).

The refrigerator (4^{o}C) results indicated that the drugs are stable for 6 - 10 days whislt the freezer (-20^{o}C) results indicated that drugs are stable up to a month (Figures 5.8 and 5.9). This therefore implies that with time even frozen drugs can undergo degradation. However, the degradation is much slower than that observed at higher temperatures. It was found on average to be about 24 hours on the auto-sampler and up to 8 days in the refrigerator. This is due to the very low temperatures which reduces the rate of degradation. The low temperature decreases the kinetic energy of the molecules and furthermore results in the molecules having inadequate energy to meet the activation energy required for any degradation reactions. However, the presence of moisture in the freezer can result in slow hydrolysis (Yoshioka and Stella, 2000) and is the likely reason for the observed degradation after a period of storage time. Few studies were encountered on stability of drugs during storage and these were mainly in biological studies, but some of the results could be extrapolated to this study. The work of Karinen et al., (2011) was found to be closely related to this study. They conducted a comparative stability of stock solutions of drugs of abuse stored in the refrigerator, freezer and at ambient conditions involving an extensive range of drugs. In their study the results of amphetamine, BEH, MDMA, methamphetamine, diazepam and cocaine were of interest as these drugs were also investigated in this study. It was observed that whilst both studies reported the presence of degradation, the results were rather divergent with respect to the period/duration the drug

was stable. In their study the drugs were stable for longer periods; the least stable was diazepam which showed degradation at 6 months (refrigerator). This could be due to tighter limits used in this study (±10%) compared to theirs (±20%). In addition, in this study the chromatographic profiles were also reviewed for any changes as confirmation. In other study studies Gunnar et al. (2004), Moody et al. (1999) and Nowatzke and Woolf (2007) indicated that for stability studies, an analyte was considered stable if its concentration remained within ±15 – 20% of the nominal value.

5.4 CONCLUSION

The solvents water, ethyl acetate, methanol, 2-methylpropan-2-ol and dichloromethane were investigated for their ability to dissolve the analytes. Good solubilities were observed for all the solvents, dissolving at least 84% of the 25 analytes investigated. As such any of the solvents could be applied in further analytical studies. The influence of the solvents methanol, 2-methylpropan-2-ol and dichloromethane on the stability of the analytes was investigated. All drugs analysed (22) were found stable in all the solvents at ambient temperature and pressure. It has been discussed that studies on the stability of drugs on the auto-sampler during routine laboratory investigations had not been encountered. Consequently, it was deemed imperative to study this aspect of stability, especially since analytes might spend a long duration on the auto-sampler awaiting analysis. In addition, auto-samplers can warm up with time hence this might have thermal effects on the stability (Yoshioka and Stella, 2000; ICH Q1A(R2), 2003; FDA, 2008). The 22 drugs analysed showed the highest stability in 2-methylpropan-2-ol. All the analytes were stable in this solvent on the GC-MS auto-sampler for the period investigated (25 hours). Whilst in methanol, stability was in the range 14 – 25 hours and for dichloromethane 19 – 25 hours. Cocaine, BEH and EME were the drugs that were the least stable in the solvents. Artefacts were observed with dichloromethane (i.e., benzylchloride and benzylchloroformate). The formation of artefacts is undesirable (Aalberg et al., 2005b), since the solvent was also to be used in impurity profiling, 2-methylpropan-2-ol was the solvent selected for further use in all the studies. Further investigation of the stability of the analytes on storage in solution in 2-methylpropan-2-ol showed that the analytes were stable for 6 – 10 days in the refrigerator and 22 – 30 days in the freezer. This showed that the analytes can be retained for an adequate period during laboratory investigations.

It can be concluded that the following stability limits were established and can be set for piperazine drugs of abuse and their congeners; a) for routine ambient laboratory conditions (ambient temperature and pressure): stable in the solvents methanol, dichloromethane and 2-methylpropan-2-ol are stable, b) on the GC-MS auto-sampler: stable in methanol for 14 hours, dichloromethane 19 hours and 2-methylpropan-2-ol 25 hours, c) for storage in the refrigerator at 4^{o}C in 2-methylpropan-2ol: stable for 6 - 10 days, and d) storage in the freezer at (-)20^{o}C in 2-methylpropan-2ol: stable for 22 - 30 days.

The results of this research provide novel information on the stability profile of piperazines and other drugs of abuse prevailing on the market, set stability limits for storage and analysis, i.e., provided data for other chapters of the research project. It was also identified that 2-methylpropan-2-ol has not been extensively used in the analysis of drugs of abuse; hence it is proposed as a solvent for use especially in profiling or impurity analysis, mainly due to its non-reactivity.

It was highlighted that no record was found of a study on the stability of FPP, TFMPP and other piperazines or drugs of abuse during routine laboratory analysis, or as a research study in its own entirety. Information derived from stability studies can be used to eliminate or minimise potential degradation and the resulting loss in stability during laboratory investigations. Furthermore, in view of the increasing use of phenylpiperazines and other psychoactive substances (Kenyon et al., 2010; King and Kicman, 2011; Arbo et al., 2012; Philip et al., 2013; EMCDDA, 2014) which will likely increase analytical investigations, the information derived from this study will therefore be of use in future investigations by other researchers.

CHAPTER 6

OPTIMISATION OF THE METHOD

6.1 INTRODUCTION TO THE OPTIMISATION STUDY

In Chapter 4 it was discussed in that whilst the preliminary method of analysis developed (method 3b, Table 4.3) generated good chromatographic profiles for the drugs (Figure 4.16, and 4.17), it was desirable to improve resolution for those peaks between 14 to 15.5 minutes. This was evidenced by the co-elution between 3-TFMPP and MDMA (14.22 and 14.36 minutes respectively). The resolution was R = 1.06 whilst for all the other drugs R > 2 and there was need to reduce the tailing. As such, there is need to investigate whether or not the variables in method 3b can be optimised to give better results. It was identified in the discussion on theoretical concepts (Chapter 2 sections 2.3 and 2.4) that method operational variables have a profound effect on the quality of the chromatographic result. Furthermore, these variables can be manipulated so as to enhance the method performance, i.e., optimised (Lavanya, 2013). In the investigation of instrumental parameters in Chapter 4 the drugs identified as needing further work (resolution) elute in the temperature range 12 - 16 minutes (temperature range 150 - 200^{o}C). Consequently, in this study optimisation of column oven temperature focused on the mid ramp temperature of 150^{o}C (section 6.2.2) as this was anticipated to have a higher effect on the results. However. it was also deemed desirable if all aspects of the chromatographic profile were improved as this would enhance accuracy.

In this study, optimisation was conducted through investigation of the effect of injector port and oven temperatures, mobile phase flow rate, MS scan rate and ionisation energy on method 3b performance characteristics. Similar analysis successfully optimised methods in the works of Andersson et al. (2007a), Maher et al. (2009), Byrska et al. (2010) and Santali et al. (2011). The performance characteristics were measured in terms of plate number (N), resolution (R_s), tailing (T), capacity (retention) factor (k), selectivity (α) and retention time (R_t). The concepts behind these parameters were discussed in Chapter 2 (section 2.3).

The data generated was then evaluated so as to select the injector temperature and oven temperature which gave optimum performance. Optimum variables were those that gave

the highest N, decreased T, increased k, increased selectivity, reduced mass spectral fragmentation, maintained or decreased analysis run time and generally resulted in sharp narrow peaks (Andersson et al., 2007a; Hibbert, 2007; Inoue et al., 2008). To determine the validity of the results, statistical analysis was conducted using Correlation analysis, T-test (paired), Friedman test and Wilcoxon signed-rank test (Chapter 3).

This study was conducted as depicted in Figure 6.1.

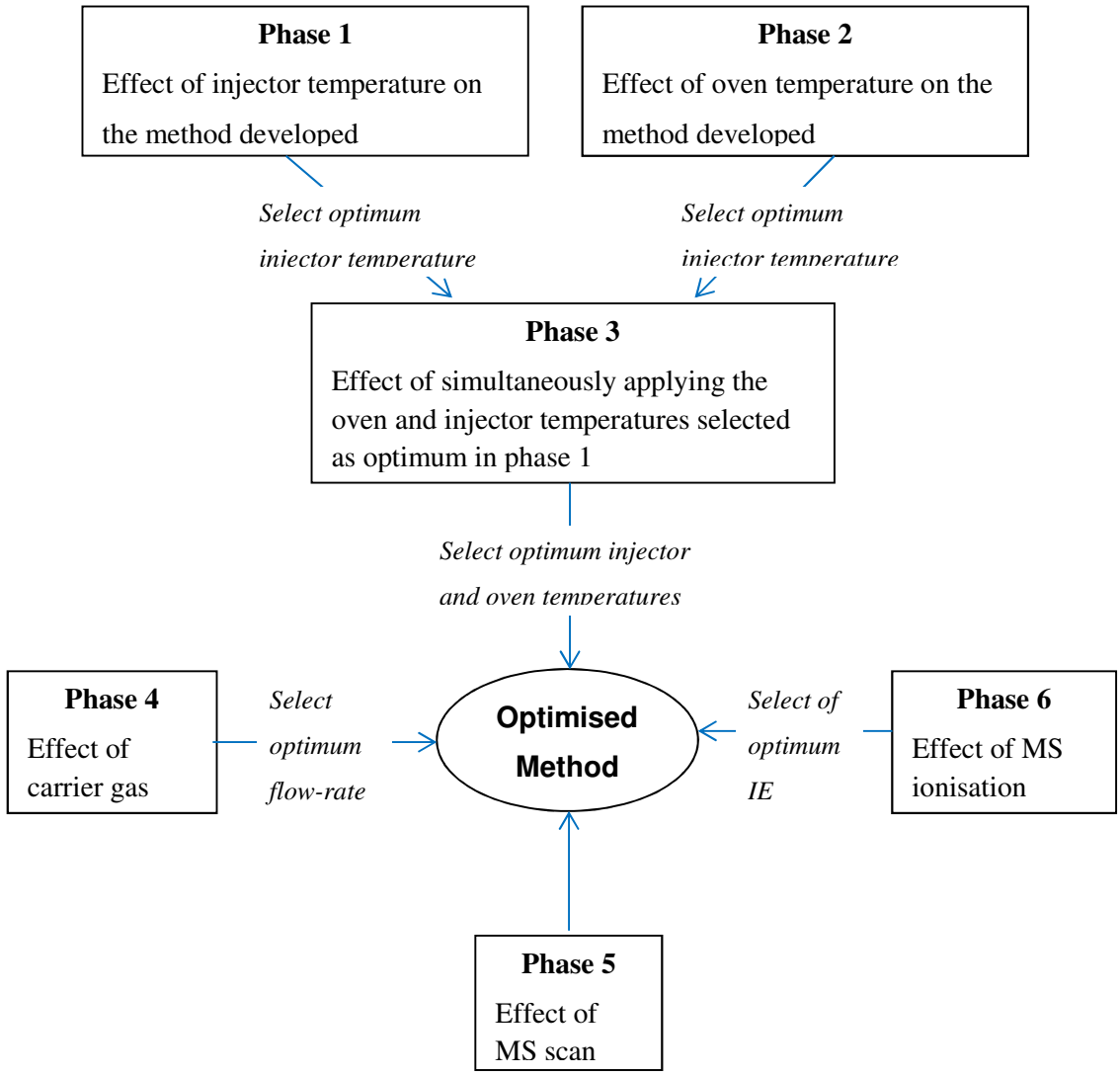


Figure 6.1 Structure of the experimental work for the method optimisation study.

6.1.1 AIMS

The aims were to a) investigate the effect of variation of the method operational variables; injector port and oven temperatures, mobile phase flow rate, MS scan rate and ionisation energy on the on the quality of the chromatographic profile, i.e., plate number, resolution, tailing, capacity factor, selectivity and retention time, b) to select the optimum values of these parameters so as to improve method performance, and c) to generate an optimised method for validation and use in later studies (Chapter 7 and 8).

6.2 MATERIALS AND METHODS

All the tests (sections 6.2.6 – 6.2.11) were run in duplicate.

6.2.1 CHEMICALS/REAGENTS

The drug standards used were as listed in Chapter 4, section 4.2.1.

6.2.2 INSTRUMENTS

Optimisation was carried out using Perkin Elmer GC-MS, Clarus Turbomass Gold 500MS fitted with a Supelco, Equity-5 GC capillary column (30m x 0.25mm x 0.25µm). The instrument was equipped with a Perkin Elmer 4mm quartz split/splitless injector liner and with the NIST MS Search Version 2.0 library software. The instrument was initially set up with the un-optimised method parameters (Method 3b Table 4.3 Chapter 4), i.e., the injector was set at 250^{o}C with a split ratio of 20:1. The carrier gas was He (g) at a flow rate of 1mL/min. The initial oven temperature was set at 60 ^{o}C with a hold of 1min. The oven was ramped at 10 ^{o}C /min to 150^{o}C with a hold of 2min and at 15^{o}C /min to 280^{o}C, with a hold of 4min. The MS transfer line was set at 280^{o}C, source temperature 230^{o}C, ionisation energy 70eV and scan range at m/z 40 – 500. The total analysis run time was 23.67minutes.

6.2.3 STATISTICAL SOFTWARE

Statistical analysis of results was carried out using IBM SPSS Statistics version 20 and MS Office Excel 2010.

6.2.4 PREPARATION OF STANDARD SOLUTIONS

The drug standards listed in Table 4.1 (Chapter 4) were used to prepare standard solutions for use in the optimisation tests. Individual drug standard solutions and a mixed drug standard solution were prepared in 2-methylpropan-2-ol using the method outlined in Chapter 4 (section 4.2.4.3) with eicosane as the internal standard.

6.2.5 CONTROL: UN-OPTIMISED METHOD ANALYSIS FOR COMPARISON

The individual and mixed standard solutions were analysed by GC-MS. This test was carried out using the method specified in section 6.2.2 above without any variations.

6.2.6 INVESTIGATING THE EFFECT OF INJECTOR PORT AND OVEN TEMPERATURES, MOBILE PHASE FLOW RATE, MS SCAN RATE AND MS IONISATION ENERGY

Investigation of the method variables was carried out from phases 1 to 6 as shown in Table 6.1. The parameter values were applied to the method in section 6.2.2 one at a time for each of the variables investigated.

Table 6.1 Optimisation of method variables

Analysis	**Variable investigated**	**Parameter value**[1]
Phase 1	Injector port temperature	240, **250,** 260, 270 ^{o}C
Phase 2	Oven temperature (1st ramp)	140, ***150***, 160, 170, 180 ^{o}C
Phase 3	Injector port, oven temperature	Optimum values from phases 1 and 2
Phase 4	Carrier gas flow rate	0.75, **1.0** mL/min
Phase 5	MS scan rate: A = 1246Da/s	scan time 0.349s; inter scan delay 0.02s; scan range m/z 40 – 500
	B = 2277Da/s	scan time 0.2s; inter scan delay 0.02s; scan range m/z 40 – 620
	C = 4509Da/s	scan time 0.1s; inter scan delay 0.02s; scan range m/z 40 – 500
Phase 6	MS ionisation energy	60, ***70***eV

[1]Values in bold are those in the original un-optimised method, these are the controls

6.2.7 DATA ANALYSIS

Plate number, tailing, selectivity and resolution were calculated using the equations outlined in Chapter 2 (section 2.3). For plate number equation 2.8, tailing equation 2.12, selectivity equation 2.10 and resolution equation 2.11 were used.

Statistical analysis of Phases 1 and 2 results was conducted using the Friedman test in accordance with section 3.2.8 (Chapter 3).

Percentage gain or loss graphs were used to evaluate Phase 3 data. In addition statistical analysis was conducted using Correlation analysis, T-test (paired), Friedman test and Wilcoxon signed-rank test. The statistical calculations were done as outline in Chapter 3. For % Gain and loss graphs section 3.2.10, Correlation analysis section 3.2.4, T-test (paired) section 3.2.5, Friedman test section 3.2.8 and Wilcoxon signed-rank test section 3.2.9.

6.3 RESULTS AND DISCUSSION

6.3.1 PHASE 1 INVESTIGATING THE EFFECT OF INJECTOR TEMPERATURE ON THE METHOD DEVELOPED

The total ion chromatographic profile (TIC) is shown in Figure 6.2 for a representation of the injector temperatures investigated in comparison to the control. There was a slight change in the chromatographic profile for all the analytes, as can be seen in the total ion chromatogram (TIC), Figure 6.2. The peaks at one of the experimental injector port temperatures (260^{o}C) compared to the control (250^{o}C) showed less tailing. This was observed, for example, for methamphetamine, 2-TFMPP and 3-FPP and 4-TFMPP. However, the resolution between peaks and retention time were observed to be unaffected by changes in injector port temperature.

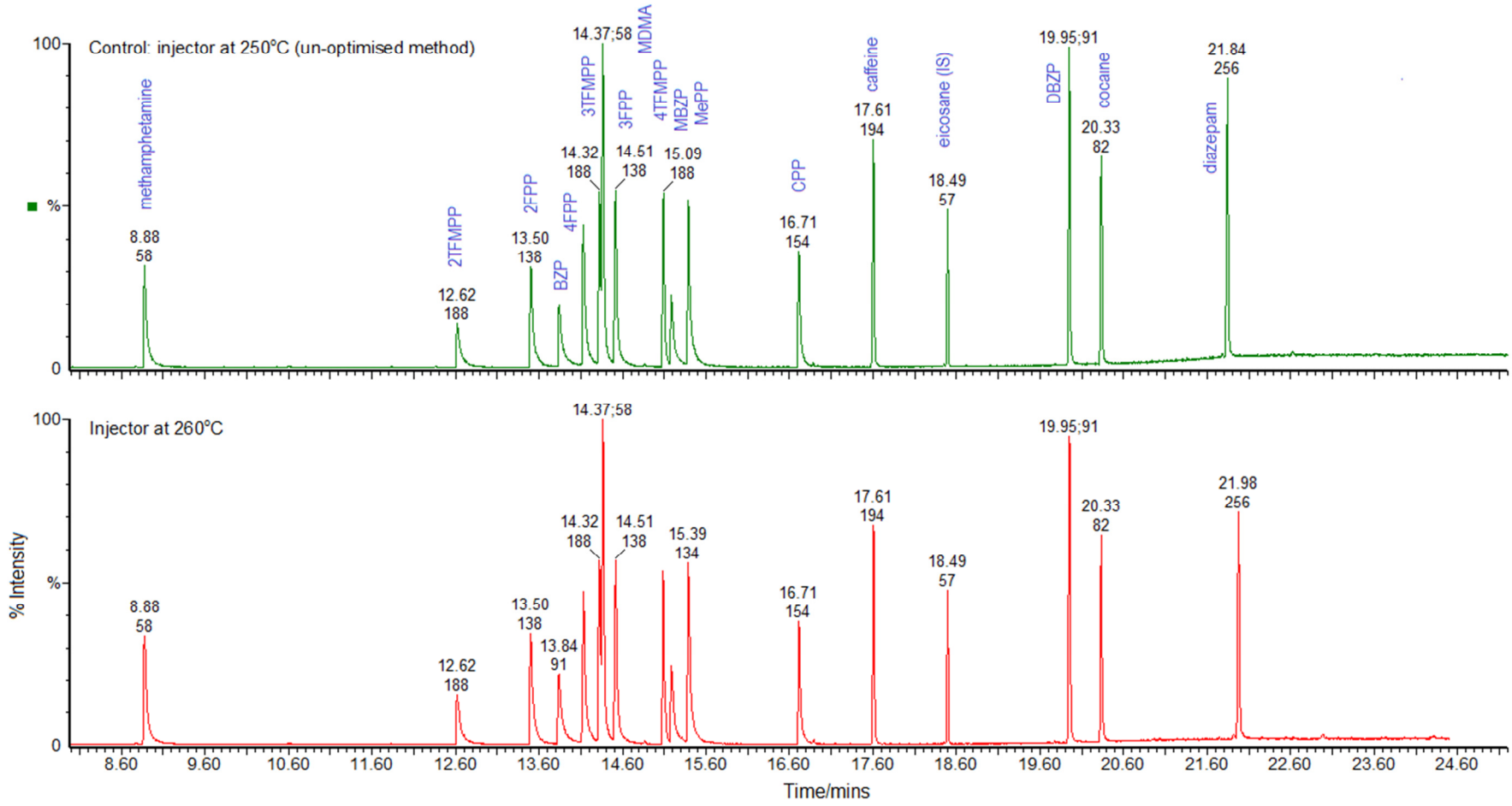


Figure 6.2 Phase 1 Chromatographic profiles (TIC) showing effect of injector temperature for selected temperatures (250 and 260°C).

To confirm the observations the chromatographic quality characteristics plate number N, tailing T, selectivity α, resolution R_s and retention time R_t were determined and analysed. The effect of injector temperature on N, T, α, R_s and R_t is shown below in Table 6.2 and graphically represented in Figures 6.3 - 6.6.

Table 6.2 Phase 1 optimisation: Effect of injector port temperature.

Temp/°C	**Mean values (n = 2)**						**Temp/°C**	**Mean values (n = 2)**			
	N x 10^5	T	α	R_s	R_t/min			N x 10^5	T	α	R_s
Meth							MBZP				
240	4.015	4.224	1.422	5.938	8.880		240	11.629	4.211	1.013	3.768
250	3.831	4.000	1.422	5.603	8.880		250	10.672	4.211	1.013	3.711
260	3.613	3.038	1.422	5.476	8.880		260	13.545	4.188	1.014	4.086
270	3.798	3.353	1.422	5.938	8.880		270	11.629	4.048	1.013	3.740
280	3.785	3.394	1.422	6.318	8.860		280	12.024	3.810	1.014	3.897
2-FPP							CPP				
240	5.265	3.947	1.070	9.660	12.620		240	22.813	3.000	1.054	21.248
250	4.432	3.750	1.070	9.629	12.620		250	23.566	2.875	1.054	22.398
260	4.265	3.491	1.070	9.354	12.620		260	23.551	2.875	1.054	22.398
270	5.478	3.182	1.070	9.745	12.620		270	23.551	2.875	1.054	22.849
280	6.914	3.210	1.070	11.439	12.605		280	23.551	2.764	1.054	22.273
3-FPP							MePP				
240	13.627	3.001	1.040	12.270	14.510		240	15.330	3.142	1.086	28.282
250	13.636	2.513	1.040	12.607	14.515		250	15.870	2.960	1.086	28.646
260	14.107	2.500	1.040	12.835	14.510		260	15.860	2.788	1.086	28.646
270	13.627	2.466	1.040	12.716	14.510		270	14.846	2.700	1.086	28.037
280	14.087	2.611	1.040	13.202	14.500		280	15.310	2.850	1.087	28.496
4-FPP							MDMA				
240	12.107	3.401	1.013	3.951	14.130		240	15.341	ND[1]	1.010	2.911
250	10.429	3.250	1.013	3.638	14.130		250	15.922	ND[1]	1.010	2.937
260	12.107	2.953	1.013	3.819	14.130		260	14.813	ND[1]	1.010	3.015
270	10.426	3.275	1.013	3.727	14.130		270	14.307	ND[1]	1.010	2.861
280	12.904	3.300	1.013	3.883	14.120		280	17.826	ND[1]	1.011	3.260
2-TFMPP							caffeine				
240	5.724	4.000	1.070	13.560	12.620		240	34.968	1.045	1.050	26.346
250	4.432	5.875	1.070	13.140	12.620		250	37.654	1.000	1.050	26.919
260	4.264	3.240	1.070	13.544	12.620		260	37.654	1.019	1.050	25.277
270	5.022	3.583	1.071	13.889	12.620		270	37.654	1.000	1.050	27.883
280	4.620	3.989	1.070	13.440	12.620		280	37.611	1.000	1.051	27.225
3-TFMPP							cocaine				
240	16.389	ND	1.003	1.096	14.320		240	57.872	1.000	1.081	43.899
250	13.740	ND	1.003	1.058	14.320		250	61.013	1.000	1.074	45.053
260	14.208	ND	1.003	1.049	14.320		260	57.872	1.000	1.081	43.218
270	15.234	ND	1.003	1.058	14.320		270	57.872	1.000	1.081	43.381
280	14.188	ND	1.003	0.863	14.310		280	57.872	1.000	1.081	43.349
4-TFMPP							diazepam				
240	18.199	1.842	1.007	1.977	15.090		240	45.127	1.273	ND[1]	ND[1]
250	21.225	1.803	1.007	1.994	15.090		250	66.788	1.193	ND[1]	ND[1]
260	21.211	1.745	1.007	1.910	15.085		260	43.277	1.150	ND[1]	ND[1]
270	21.225	1.715	1.007	2.044	15.090		270	43.277	1.110	ND[1]	ND[1]

280	21.197	1.625	1.006	2.010	15.080		280	43.238	1.036	ND[1]	ND[1]
BZP							eicosane				
240	5.511	5.880	1.021	4.602	13.840		240	64.907	1.000	1.079	47.924
250	6.244	5.557	1.021	4.615	13.840		250	64.907	1.000	1.079	47.074
260	6.501	4.950	1.021	4.911	13.835		260	56.236	1.000	1.079	44.882
270	5.511	5.068	1.021	4.427	13.840		270	64.907	1.000	1.079	47.074
280	7.541	5.278	1.022	5.284	13.820		280	64.907	1.000	1.079	46.117
DBZP											
240	55.729	1.100	1.019	11.233	19.955						
250	58.723	1.023	1.019	11.683	19.950						
260	55.729	1.013	1.019	11.233	19.950						
270	55.729	1.034	1.019	11.233	19.950						
280	55.673	1.011	1.020	11.528	19.940						

[1] ND stands for not determined.

In Table 6.2 and all subsequent tables and graphs the analytes were not determined (ND) is indicated. This was due to the co-elution between 3-TFMPP and MDMA hence tailing could not be calculated for these two analytes. In addition diazepam is the last peak hence there is no resolution to a subsequent peak after it.

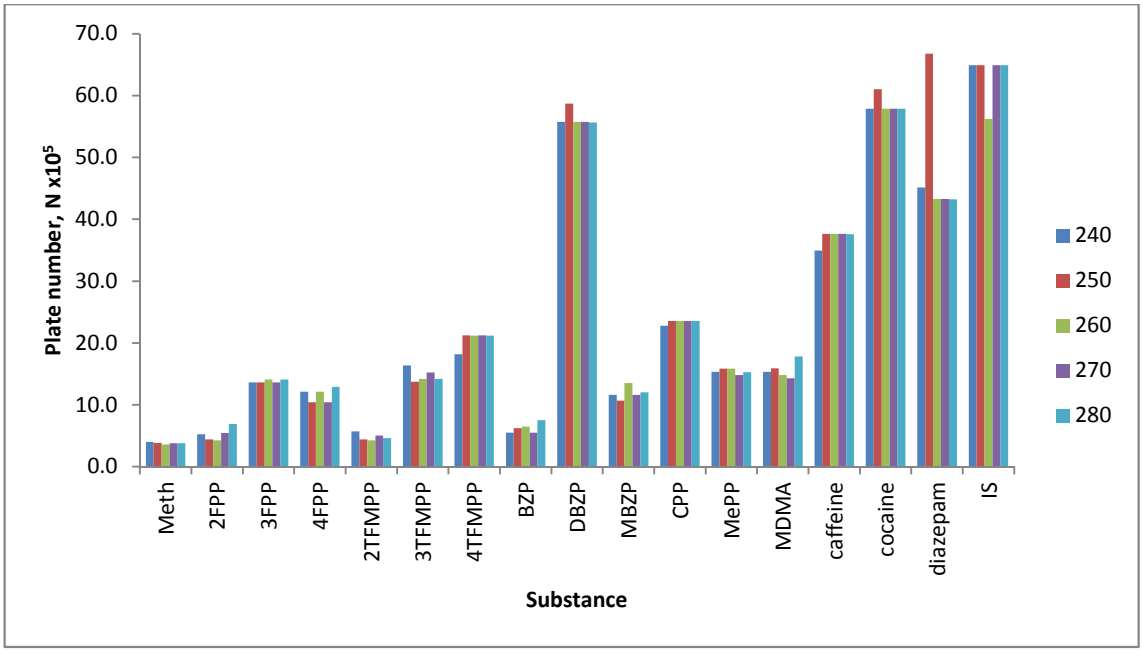


Figure 6.3 Phase 1 Effect of injector temperatures on plate number (N x 10^5).

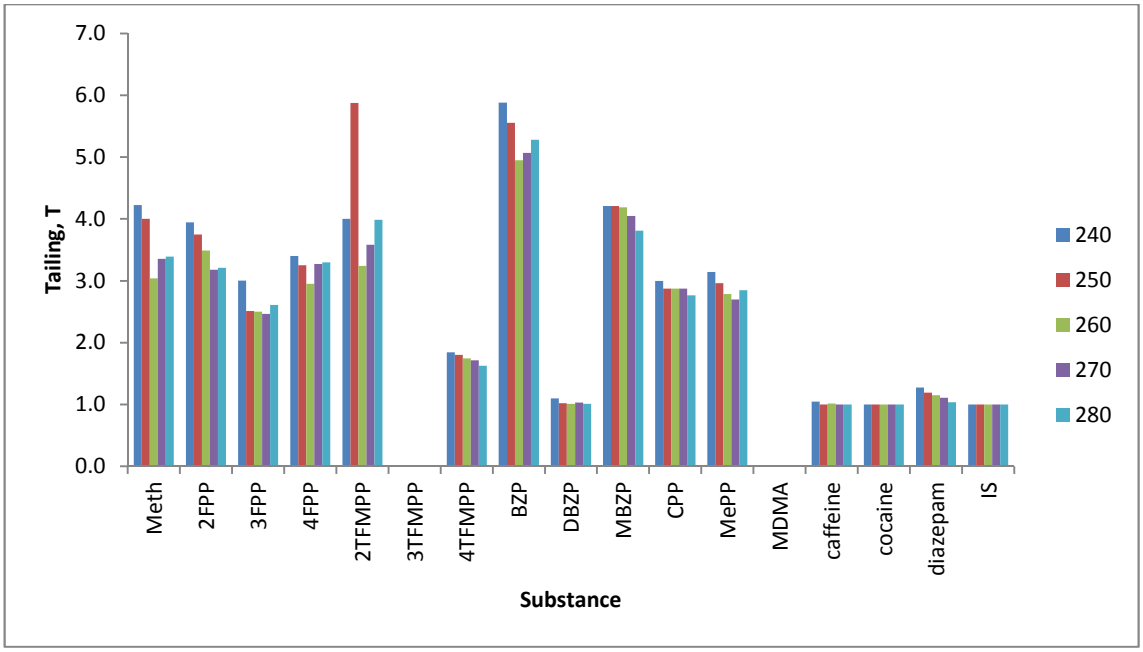


Figure 6.4 Phase 1 Effect of injector temperatures on tailing.

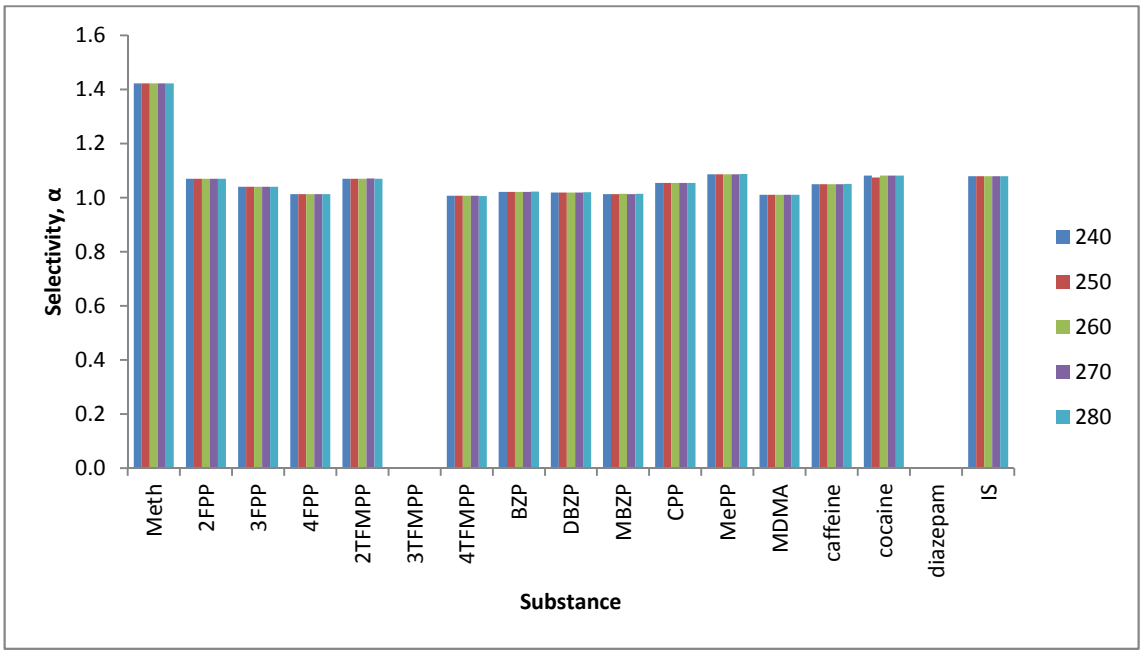


Figure 6.5 Phase 1 Effect of injector temperatures on selectivity.

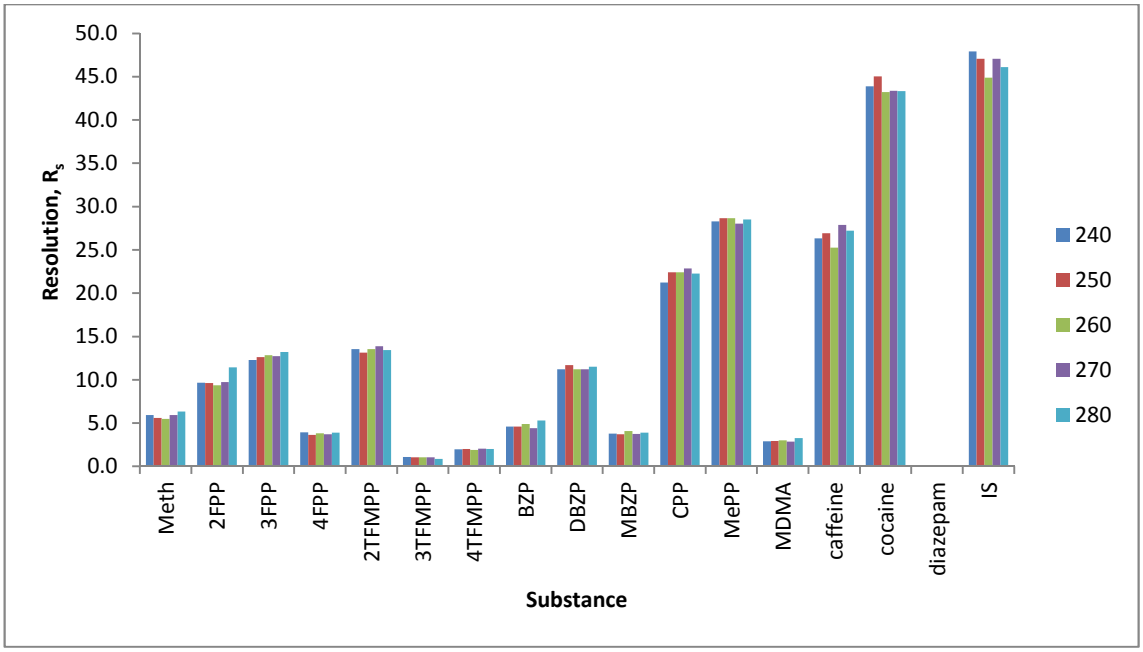


Figure 6.6 Phase 1 Effect of injector temperatures on resolution.

The following general trends were observed on the effect of injector port temperatures on plate number, tailing, selectivity, resolution and retention time. The changes observed can be attributed to the influence of injector port temperature on volatilisation of the sample during injection (Barwick, 1999; Grob, 1994; Kaur, 2010). In Figure 6.3 plate number, N increased with increase in temperature. According to equation 2.8, plate number increases with increase in retention time and decreases with with increase in peak width. If the sample is not flash volatilised on injection band broadening can occur, thereby reducing plate number. For DBZP, cocaine and diazepam plate number, N increased from 240^{o}C to 250^{o}C, theafter it decreased to 260^{o}C and remained constant (260 – 280^{o}C). Figure 6.3 shows that the degree of change increased from DBZP to diazepam and is significantly large for diazepam. This can be attributed to the boiling points of the compounds which influence their chromatographic properties. The elution order of the analytes (Figure 6.2) followed the trend in their boiling points with compounds with higher boiling points having higher retention times, e.g. the last 5 are caffeine, eicosane, DBZP, cocaine and diazepam, with diazepam which has the highest boiling point eluting last. With increase in injector port temperature volatilisation of the sample increases until it reaches optimum after which it can remain constant, e.g. for caffeine, N is constant. Thereafter with further increase in temperature loss of sample from the injector port can occur due to vapourisation if the temperature is too high. Barwick (1999) reported that as much as a 30% loss can occur.

In Figure 6.4 tailing decreased with increase in temperature. Selectivity (Figure 6.5) and retention time (Table 6.2) were marginally affected by injector port temperature and remained relatively constant. In confirmation the variance in these parameters was zero for methamphetamine, 2-TFMP and 3-FPP. Resolution was observed to be very high for all the drugs except the co-eluting substances (3-TFMPP and MDMA), with R for most drugs > 3 (Table 6.2). It has been stated that a resolution of R > 2 implies good separation between peaks (IUPAC, 2014; CDER, 2004; Horacio et al., 2008). However, whilst there is adequate resolution generally it was observed to be constant and only marginally varied with injector temperature (Figure 6.6). However, for a few of the analytes the trend in resolution was not consistent as can be seen for 2-TFMPP and cocaine (Table 6.2; Figure 6.6).

According to Hibbert (2007) the purpose of optimisation is to select those variables which improve the chromatographic peak profile and consequently the results generated by the method. As such the graphs (Figures 6.3 – 6.6) were reviewed so as to evaluate which of the injector temperatures investigated resulted in optimum plate number, tailing, selectivity, resolution and retention time (the criteria for acceptability was discussed in Chapter 2 section 2.4). Inoue et al. (2008) and Maher et al. (2009) used similar approaches in optimising their methods. It was found that overall the injector temperature at 260^{o}C gave the best results in terms of highest values of plate number, selectivity and resolution and lowest values for tailing and retention times.

The influence of injector temperatures can be attributed to the fact that to achieve the best result flash vapourisation of the compound is required on injection. This reduces leading peaks and band broadening (Barwick, 1999; Kronstrand and Jones, 2000; Andersson, 2007a) and as such sharper, narrower peaks, decreased tailing, improved peak separations are obtained.

Further evaluation was conducted by statistical analysis so as to confirm the above findings and also determine the actual trends and their significance. Thus determine the injector temperature which gave optimum results.

6.3.1.1 Statistical analysis of Phase 1 investigating the effect of injector temperature

The statistical results are shown in Table 6.3.

Table 6.3 Statistical analysis of effect of injector results (Phase 1): Friedman Test.

	Plate number			**Tailing**				**Selectivity**			**Resolution**		
Temp °C	Median rank	Upper quartile (75%)	Mean rank	Median rank	Lower quartile (25%)	Upper quartile (75%)	Mean rank	Median rank	Upper quartile (75%)	Mean rank	Median rank	Upper quartile (75%)	Mean rank
240	3.00	3.75	2.88	5.00	4.50	5.00	4.63	3.00	3.00	2.81	2.50	4.00	2.91
250	3.50	5.00	3.47	4.00	3.00	4.00	3.50	2.75	3.00	2.66	3.00	3.875	2.88
260	3.00	3.75	2.85	3.00	1.00	3.00	2.33	3.00	3.00	2.97	2.50	4.00	2.56
270	2.50	3.75	2.74	2.00	2.00	3.00	2.20	3.00	3.00	2.97	3.25	4.75	3.09
280	3.00	5.00	3.06	3.00	1.00	3.00	2.33	3.00	5.00	3.59	4.00	5.00	3.56
Test statistic	Fr = 2.530, N = 17, df = 4, p = 0.639			Fr = 31.88, N = 15, df = 4, p = 0.000				Fr = 10.947, N = 16, df = 4, p = 0.027			Fr = 3.563, N = 16, df = 4, p = 0.468		

In the table Fr is the Friedman test statistic.

A Friedman test was conducted to determine whether the analytes had a differential rank response on being subjected to different injector port temperatures. With the objective of choosing the optimum value for further analytical investigation. The response was measured in terms of plate number, tailing, selectivity and resolution. The criterion for the Friedman test is; if $p > 0.05$ the results are insignificant, if Fr < Fr (critical) reject H_o (Sprent and Smeeton, 2007, Corder and Foreman, 2009, Best et al., 2009). The injector results (Table 6.3) indicate that injector temperature has a statistically insignificant effect on plate number ($Fr = 2.530$, N = 17, df = 4, $p = 0.639$) and resolution ($Fr = 3.563$, N = 16, df = 4, $p = 0.468$). As evidenced by both values for p being greater than the limit 0.05. In addition, the Fr observed was lower than Fr(critical) = 9.49 (Corder and Foreman, 2009; Best et al., 2009; Martin et al., 1993) consequently Ho was retained. This means any variations in plate number and resolution are statistically found to be unaffected by changes in injector temperature. Any variations observed are therefore due to chance.

This statistical outcome is consistent with the discussion graphical analysis Figures 6.3 – 6.6 conducted above where it was highlighted changes were observed for example, even small variations such as for 4-FPP plate number, N is virtually constant. Considering that for all the analytes the N values are large ($x10^5$), the changes observed are minimal. In contrast, the results show that there are significant differences in tailing ($Fr = 31.88$, N = 15, df = 4, $p = 0.000$) and selectivity ($Fr = 10.947$, N = 16, df = 4, $p = 0.027$) at various injector and as such H_o is rejected. This implies that both these parameters were statistically found to be variable with temperature. A further review of the statistical results was conducted so as to ascertain the magnitude of the changes and hence select the optimum temperature which gave the highest changes.

In the review of the descriptive data in Table 6.3 the criteria is that the upper quartile range (75% + range) encompasses the highest tailing. As such, for the injector at 240 and 250^{o}C should be avoided as these temperatures fall in this range. The lower quartile range encompasses the lowest tailing results. It can be seen that the lowest median (= 1) is observed with temperatures 260 and 280^{o}C. For selectivity, resolution and plate number an increase is desirable unlike tailing. Hence, for the criteria for these parameters the upper quartile implies good effect whilst the lower quartile implies bad effect. A similar analysis for selectivity gives the highest median at 240, 260, 270 and 280^{o}C; however the highest upper quartile range is observed with 280^{o}C. It is therefore proposed that an injector

temperature of 260°C maximises the chromatographic profile to a higher degree than the other temperatures investigated, hence was investigated in further optimisation (Phase 3). This view confirms the observations made on review of the quality of the chromatographic profile for 260°C (TIC) (Figure 6.1) where it was observed that this temperature gave better peak profiles than the control (250°C). Since both the 250°C and 280°C gave good results, further use of 280°C was not recommended since a higher temperature causes evaporation of some of the sample from the needle during injection (Grob, 1994). Barwick (1999) reported that for very high boiling point components losses of up to 80% can occur. In addition a higher temperature would shorten the life span of the septum in the injector.

6.3.2 PHASE 2 INVESTIGATING THE EFFECT OF OVEN TEMPERATURE ON THE METHOD DEVELOPED

The chromatographic profiles (TIC) resulting from variation of oven temperatures are shown in Figure 6.7 for a representation of the oven temperatures investigated. The Figure shows the result for the oven at 160°C, 180°C and the control (150°C) for comparison. There were a marked changes in the chromatographic profile for all the analytes, as can be seen in the total ion chromatogram (TIC), Figure 6.7 for oven at 160°C and 180°C in comparison to the control (150°C). It can be observed in Figure 6.7 that the TIC with the injector temperature at 180°C shows has a more marked effect. Peak separation was observed to increase with temperature from 150 to 180°C. This was more apparent for the peaks eluting in the range 12 - 16 minutes. The peak shapes are narrower, less broad at the base and tail less. This indicates a reduction in band broadening, hence the reduced tailing. Furthermore, the retention time decreased with increase in oven temperature for the peaks eluting in the said range, whilst it had the opposite effect for those eluting thereafter. In this chapter it was previously discussed (section 6.1) that the optimisation of oven temperature was to focus on the mid-ramp temperature so as to try and improve resolution between the analytes in the range 12 - 16 minutes. It was further highlighted that due their boiling points optimising the mid ramp temperature within 150 - 200°C would have more impact on their chromatographic behaviour. As such, it can be suggested that the optimisation of oven temperature gave the desired outcome i.e improved the method perfomance.

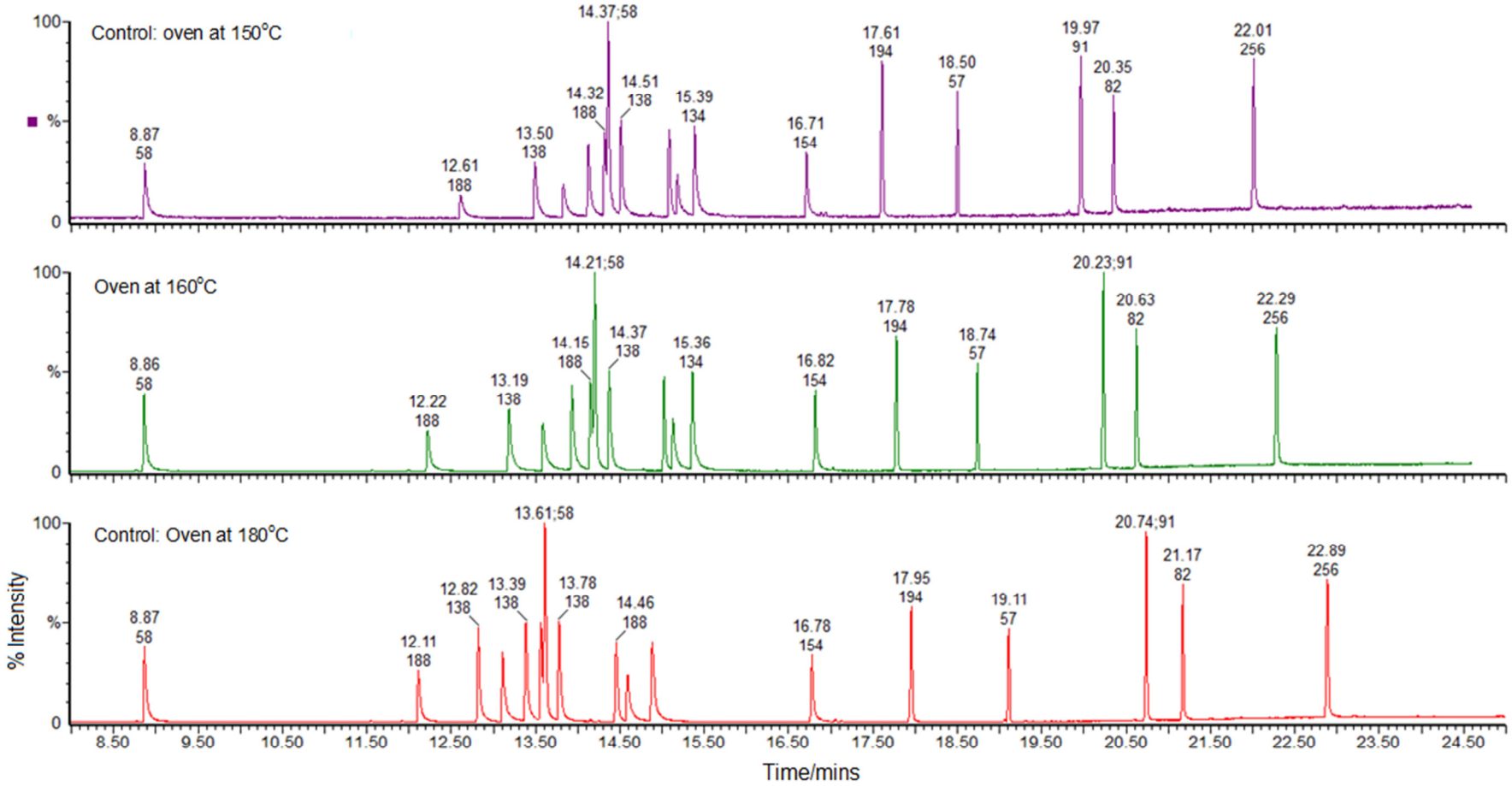


Figure 6.7 Phase 2 chromatographic profiles (TIC) showing effect of the selected oven temperatures, 160°C and 180°C in comparison to the control (up-optimised method at 150°C).

The plate number (N), tailing (T), selectivity (α), resolution (R_s) and retention time (R_t) were determined so as to further evaluate the changes in the chromatographic peak profile of the analytes, with oven temeperature and confirm the observations made earlier on Figure 6.7. The parameters were evaluated for all the oven temperatures investigated 140 - 200^{o}C) and the data is given in Appendix 4 and depicted in Figures 6.8 - 6.11 for easier evaluation.

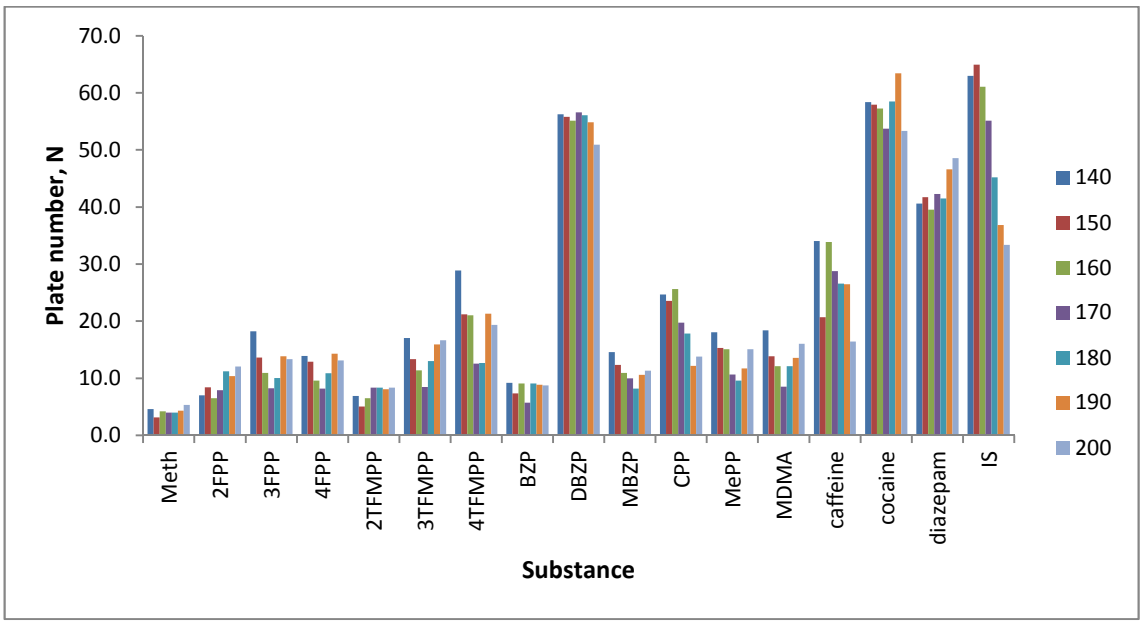


Figure 6.8 Phase 2 Effect of oven temperatures on plate number (N x 10^5) comparative to control 150^{o}C.

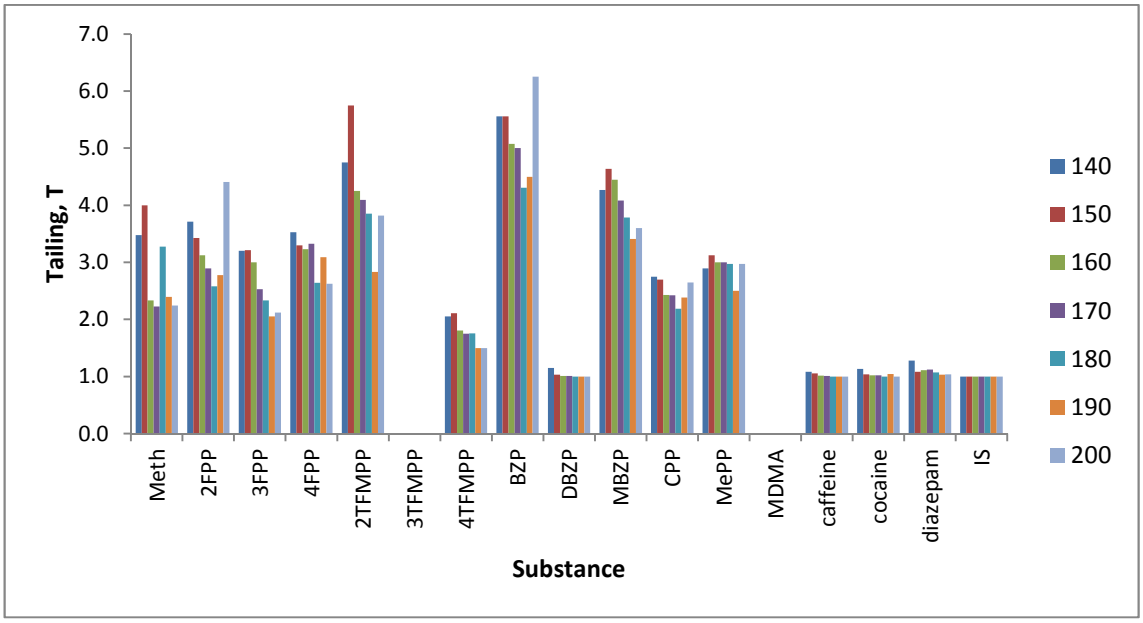


Figure 6.9 Phase 2 Effect of oven temperatures on tailing comparative to control 150^{o}C.

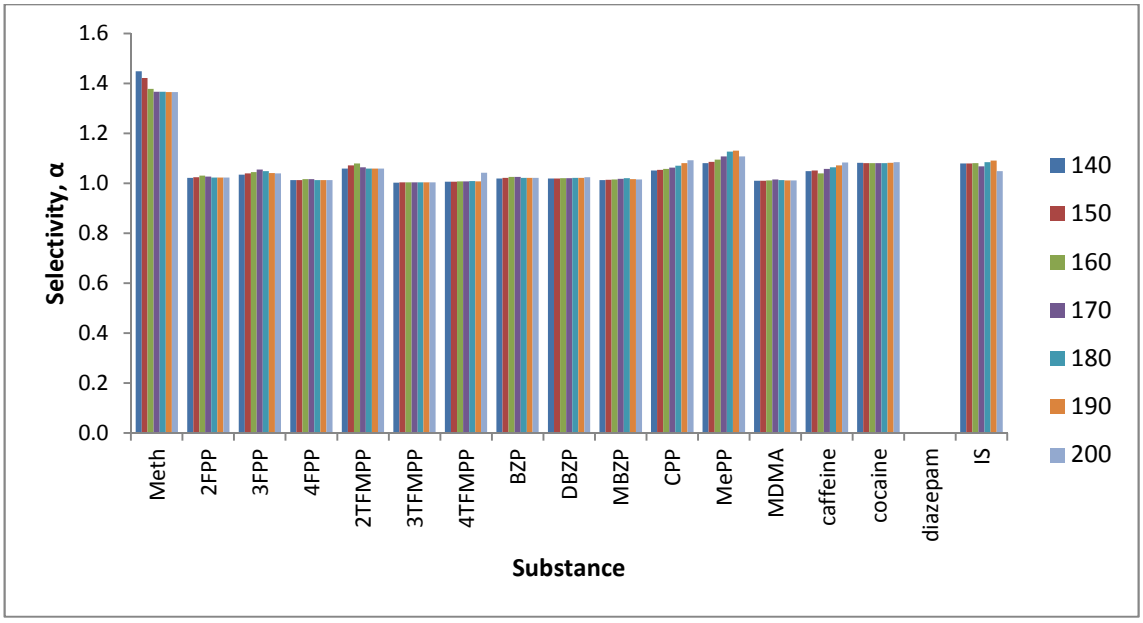


Figure 6.10 Phase 2 Effect of oven temperatures on selectivity comparative to control 150°C.

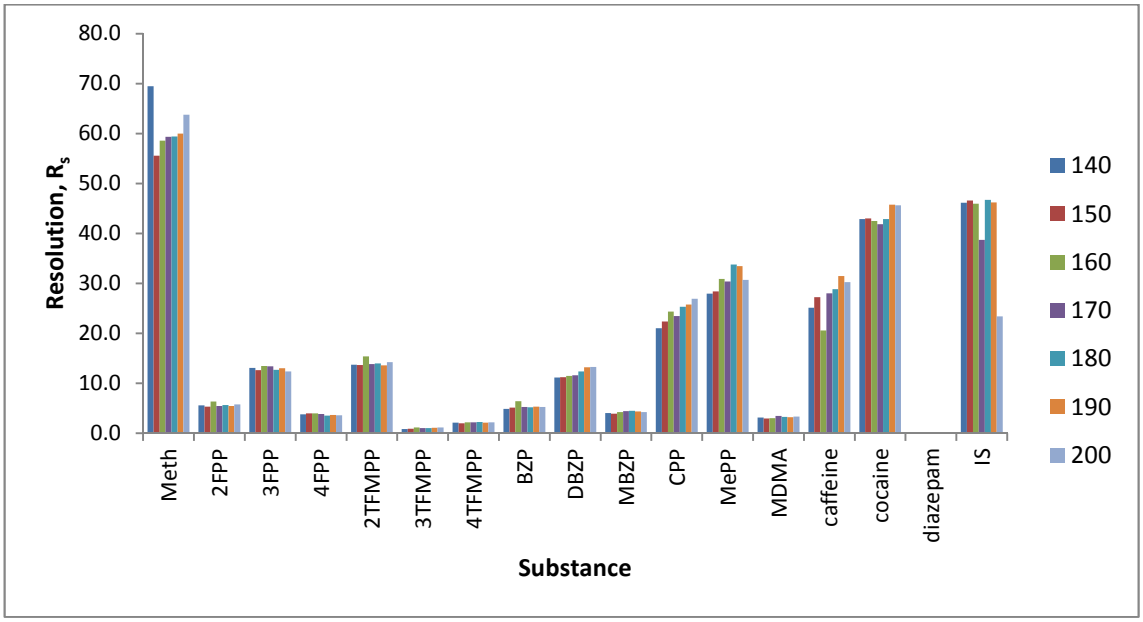


Figure 6.11 Phase 2 Effect of oven temperatures on resolution comparative to control 150°C.

In contrast to the injection port temperature, variation of oven temperature (Figures 6.7 – 6.11 and Appendix 4) resulted in more marked changes in N, α, R and T. The changes were much higher with the oven than with injector variation. This is evidenced by the variation for plate number for the injection port temperature effect (Figure 6.3) in comparison to that for oven temperature effect (Figure 6.8) and for tailing Figure 6.3 relative to 6.8. Whilst

selectivity changed minimally, it followed a similar trend as evidenced for example by CPP (Appendix 4); with increase in temperature, selectivity increased from 1.051 to 1.092. In line with this, resolution simultaneously increased from 21.027 to 26.903. In addition, selectivity changes whilst small were more than those observed with the injector. This was evidenced by the changes in Figure 6.10 (oven) in comparison to Figure 6.5 (injector) for the selectivity of 3-FPP, 2-TFMPP, CPP and MePP. However, statistical analysis was required to confirm the significance of these observations. Furthermore, this would also enable selection of the oven temperature that gives the best results in plate number, tailing, selectivity, resolution and retention time.

The greater impact of oven temperature is due to the fact that the oven temperature has a direct impact on the partitioning processes inside the column. Mainly oven temperature influences the vapour pressure inside the column and consequently the partition coefficient. An increase in oven temperature results in an increase in vapour pressure thereby equilibration of the analyte between the stationary phase and mobile phase is achieved faster. This reduces the partition coefficient, which in turn reduces the retention factor and consequently increases plate number. This is in accordance with the chromatographic concepts and their influence on optimisation described in sections 2.3 and 2.4(Chapter 2). An increase in oven temperature increases the kinetic energy of the analyte molecules, thereby reducing resistance to mass transfer (C term in the van Deemeter r equation) in accordance with the Rate theory (section 2.4 equations 2.13 - 2.16). This increases the elution rate hence the decrease in retention time. Furthermore, the increased migration of the molecules reduces longitudinal diffusion (B-term), (Moody, 1982; Kaur, 2010; Khopkar, 2012; IUPAC, 2014) as such this reduces the height of a theoretical plate (HETP). This term is inversely proportional to plate number hence reduction of HETP increases plate number. Changes in column plate number impact on resolution. This affects separation between substances (equation 2.11) as was discussed in section 2.3 for resolution. Therefore, the increase in plate number resulted in the improved resolution observed in the results. This equation also explains the similarity in behaviour of selectivity and plate number. The fact that selectivity only changed marginal is due to selectivity being dependent on capacity factor (retention factor) hence is more subject to changes in the type of stationary phase.

With the oven at 180^{o}C resolution was observed to be lower for those drugs with lower retention times eluting before 16 minutes and more improved for the drugs eluting after 16mins (Figure 6.7, 6.11 and Appendix 4). In contrast, 160^{o}C gave better resolution for the early eluting drugs. It was discussed in section 6.1 that drugs eluting within this range would be influenced more by investigation of the oven temperatures under consideration (1st oven ramp temperature) as their boiling points fall within this temperature range. As such these eluted faster at higher temperatures hence the observed reduction in their in resolution at 180^{o}C.

6.3.2.1 Statistical analysis of Phase 2 investigating the effect of oven temperature.

The Friedman test results for statistical analysis of the oven data are summarised in Table 6.4.

Table 6.4 Statistical analysis of effect of oven temperature results (Phase 2): Friedman Test.

	Plate number			**Tailing**				**Selectivity**			**Resolution**		
Temp °C	Median rank	Upper quartile (75%)	Mean rank	Median rank	Lower quartile (25%)	Upper quartile (75%)	Mean rank	Median rank	Upper quartile (75%)	Mean rank	Median rank	Upper quartile (75%)	Mean rank
140	6.00	7.00	5.76	6.00	6.00	7.00	5.93	1.50	2.375	2.13	3.00	4.00	2.88
150	4.00	5.00	4.06	6.00	5.00	7.00	5.87	2.75	4.25	3.09	2.00	2.75	2.44
160	3.00	4.50	3.47	5.00	4.00	5.00	4.57	4.50	5.75	4.41	4.45	7.00	4.50
170	2.00	4.50	2.82	4.00	3.00	4.00	3.83	5.00	6.75	4.81	4.00	5.95	4.00
180	3.00	5.00	3.50	2.50	1.00	3.00	2.57	5.00	6.00	4.81	5.00	6.65	4.75
190	4.00	6.00	4.29	2.00	1.00	2.00	2.23	4.50	6.00	4.47	5.00	6.00	4.63
200	5.00	6.50	4.09	2.00	1.50	4.00	3.00	3.75	7.00	4.28	6.00	6.00	4.81
Test statistic	*Fr* = 18.714, N = 17, df = 6, *p* = 0.005			*Fr* = 48.016, N = 15, df = 6, *p* = 0.000				*Fr* = 22.710, N = 16, df = 6, *p* = 0.001			*Fr* = 19.098, N = 16, df = 6, *p* = 0.004		

In the table *Fr* is Friedman test statistic.

The Friedman test results for variation of oven temperature (Table 6.4) show that all the parameters plate number (Fr = 18.714, N = 17, df = 6, p = 0.005), tailing (Fr = 48.016, N = 15, df = 6, p = 0.000), selectivity (Fr = 22.710, N = 16, df = 6, p = 0.001) and resolution (Fr = 19.098, N = 16, df = 6, p = 0.004) are significant. This is in agreement to the discussion above where it was highlighted that the oven has a greaterr effect on the parameters than injector temperature. An analysis of the descriptive data indicated the highest two temperatures (in order) were; for plate number 140>200°C, tailing 140>150°C (lowest at 190 and 180°C), selectivity 170>180°C, resolution 200>180>160°C. As such it is proposed that an oven temperature of 180°C maximises the chromatographic profile to a higher degree than the other temperatures investigated and that 160°C also be investigated in further optimisation (Phase 3).

6.3.3 PHASE 3 INVESTIGATING THE EFFECT OF SIMULTANEOUSLY APPLYING THE OVEN AND INJECTOR TEMPERATURES SELECTED AS OPTIMUM IN PHASES 1 AND 2

The temperatures investigated were those chosen as giving optimum results in Phase 1 (injector port temperature 260°C) and Phase 2 (oven 160 and 180°C). The purpose was to determine which of the oven temperatures gave the best result when applied together with the optimum injector temperature from Phase 1. The chromatographic profiles (TIC) observed are shown in Figures 6.12 and 6.13 below.

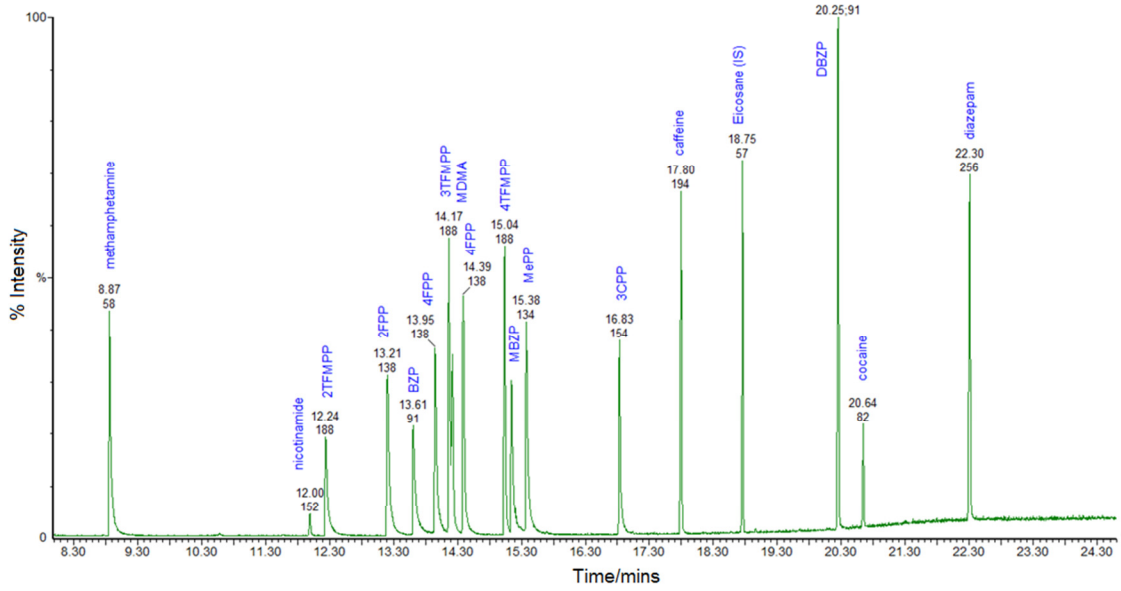


Figure 6.12 Phase 3 Effect of simultaneously implementing the temperatures oven at 160°C and injector at 260°C selected in Phases 1 and 2 on the chromatographic profile.

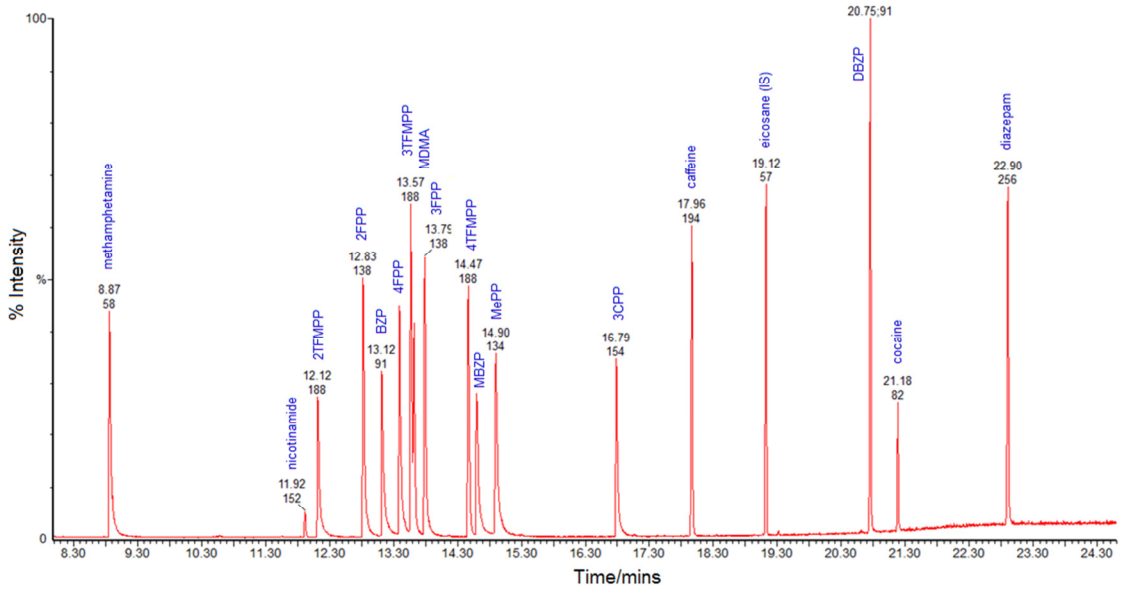


Figure 6.13 Phase 3 Effect of simultaneously implementing the temperatures oven at 180°C and injector at 260°C selected in Phases 1 and 2 on the chromatographic profile.

The results of simultaneously implementing the selected injector and oven temperature from Phases 1 and 2 of the studies show a general improvement in the chromatographic peak profile, Figures 6.12 (oven 160/injector 260°C) and 6.13 (oven 180/injector 260°C) comparative to the TICs for the individual variables, Figure 6.7 (oven 160°C and 180°C) and Figure 6.2 (injector 260°C). In both cases the simultaneous use of injector and oven temperatures resulted in improved peak shapes; the peaks are narrower and tailing appears reduced. However, the TICs for the simultaneous implementation appear similar. Hence, it cannot be accurately confirmed which is the better profile by visual inspection of the TICs. To enable an evaluation of the optimisation on the chromatographic profile of the analytes the performance parameters plate number, tailing, selectivity and resolution were determined from the TICs and the arising values are shown in Table 6.5.

Table 6.5 Phase 3 Investigating the effect of simultaneously applying the injector and oven temperatures selected as optimum in phases 1 and 2 on column plate number N, resolution R_s, tailing T, selectivity α and retention time R_t (For Mean, n = 2).

Temp/°C		Mean values					Temp/°C		Mean values			
	N x 10^5	T	α	R_s	R_t/min			N x 10^5	T	α	R_s	R_t/min
Methamphetamine							**MBZP**					
150	4.554	2.518	1.423	64.935	8.870		150	17.791	3.570	1.014	4.414	15.190
160	6.198	2.330	1.380	60.706	8.870		160	11.786	2.685	1.015	4.148	15.150
180	5.345	2.116	1.367	62.829	8.870		180	7.922	3.989	1.021	4.568	14.605
2-FPP							**3-CPP**					
150	10.710	3.618	1.025	6.121	13.500		150	23.580	2.301	1.053	22.273	16.720
160	6.564	3.562	1.030	6.081	13.210		160	23.342	2.645	1.058	23.849	16.830
180	11.309	2.909	1.023	5.510	12.830		180	15.390	2.331	1.070	24.558	16.790
3-FPP							**4-MePP**					
150	16.245	2.638	1.040	13.371	14.515		150	17.014	3.302	1.086	29.170	15.395
160	12.360	2.150	1.045	13.827	14.395		160	12.570	1.709	1.094	29.284	15.380
180	10.422	2.052	1.049	12.706	13.795		180	7.789	3.493	1.126	30.956	14.905
4-FPP							**MDMA**					
150	12.922	4.022	1.013	4.056	14.130		150	14.353	ND[1]	1.010	3.096	14.370
160	9.958	3.346	1.015	4.236	13.955		160	10.383	ND[1]	1.012	3.246	14.220
180	11.402	2.682	1.013	3.854	13.400		180	9.824	ND[1]	1.013	3.116	13.625
2-TFMPP							**Caffeine**					
150	6.465	4.025	1.070	15.287	12.620		150	37.654	1.149	1.051	27.225	17.610
160	5.524	3.998	1.079	14.785	12.240		160	36.627	0.833	1.053	28.082	17.800
180	7.775	3.324	1.058	13.644	12.125		180	30.592	1.258	1.065	30.286	17.960
3-TFMPP							**Cocaine**					
150	17.028	ND[1]	1.004	1.088	14.320		150	63.816	1.015	1.081	44.723	20.340
160	15.563	ND[1]	1.004	1.005	14.170		160	62.059	1.136	1.084	44.486	20.640
180	16.349	ND[1]	1.004	1.025	13.575		180	66.434	1.197	1.081	45.158	21.180
4-TFMPP							**Diazepam**					
150	22.133	1.672	1.007	2.320	15.090		150	44.910	1.316	ND[1]	ND[1]	21.990
160	21.085	1.353	1.007	2.259	15.040		160	46.354	1.275	ND[1]	ND[1]	22.300
180	12.335	1.616	1.010	2.291	14.470		180	44.860	1.125	ND[1]	ND[1]	22.900
BZP							**Eicosane(IS)**					
150	9.373	8.403	1.021	5.516	13.853		150	65.012	0.940	1.079	47.483	18.505
160	6.824	7.606	1.025	5.645	13.610		160	60.909	1.205	1.080	48.441	18.750
180	8.502	6.073	1.021	5.224	13.120		180	46.496	1.028	1.085	46.488	19.120
DBZP												
150	61.453	0.838	1.019	11.790	19.960							
160	66.150	1.001	1.019	12.058	20.250							
180	57.538	1.038	1.021	12.736	20.750							

[1] ND stands for not determined. This was due to co-elution between 3-TFMPP and MDMA hence tailing could not be calculated for these two analytes. In addition diazepam is the last peak hence there is no resolution to a subsequent peak after it. This applies to all other Tables and Figures where this notation is used

The data is comprehensive as such graphical analysis was conducted for easier evaluation of trends in the data. A graphical representation of the data for plate number and tailing is shown in Figures 6.14 and 6.15 respectively and the other parameters in Appendix 5.

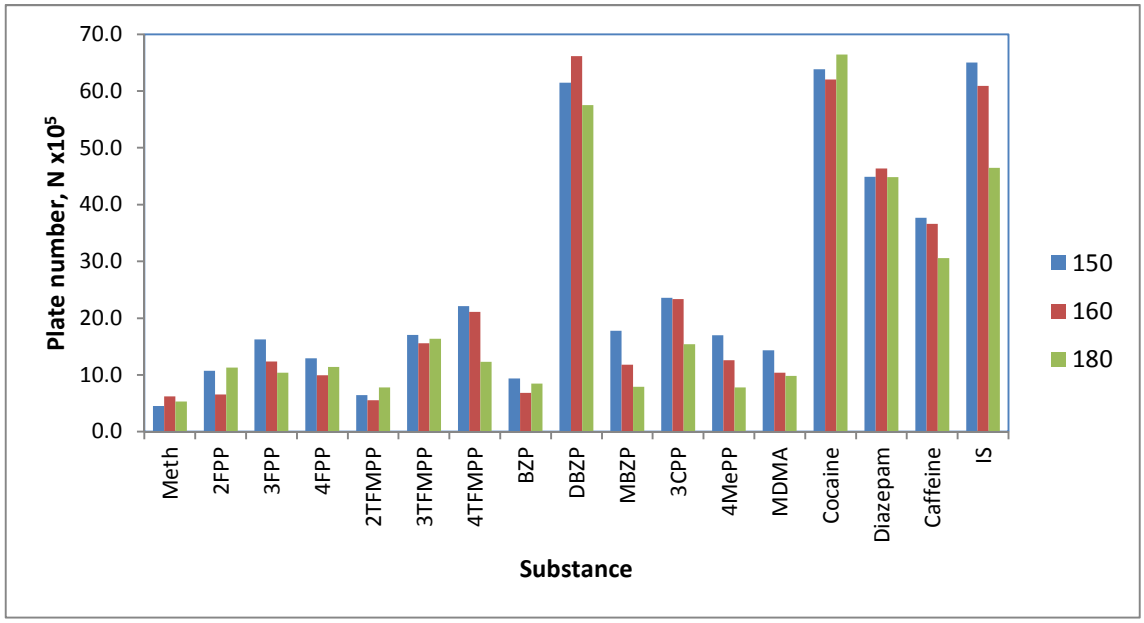


Figure 6.14 Phase 3 Effect of simultaneously applying selected optimum temperatures (injector port 260°C; oven 160°C and 180°C; control 150°C) on plate number, N (values of N x 10^5).

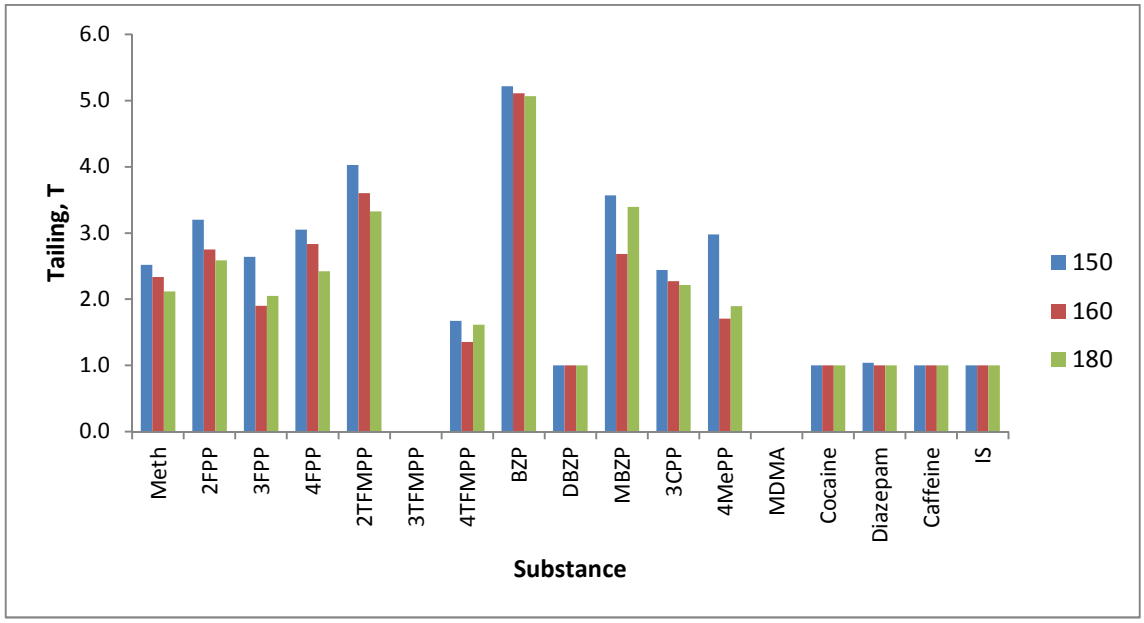


Figure 6.15 Phase 3 Effect of simultaneously applying selected optimum temperatures (injector port 260°C; oven 160°C and 180°C; control 150°C) on tailing, T.

A review the data for plate number, tailing, selectivity and resolution (Table 6.5) and the graphical analysis (Figures 6.14; 6.15 and Appendix 5) showed that there is a general decrease in plate number, decrease in tailing, increase in selectivity and a mixed response for resolution for both temperatures comparative to the control (150^{o}C). However, it is not easy to elucidate magnitude of the response from the data or determine which temperature achieved the best results. To overcome this limitation and for effective comparison of the results of the two oven temperatures investigated; 160^{o}C and 180^{o}C (with optimum injector port temperature 260^{o}C) gain and loss graphs (Chapter 3 section 3.3.10) were employed. These simplify data by showing the percentage change in the response (Bersimis and Parakis, 2007; Cheung et al., 2012).

The results of analysis of the data in Table 6.5 (Phase 3 data) using Gain or Loss graphs to show the percentage change in plate number, N and tailing, T are shown in Figures 6.16 and 6.17 respectively. The other parameters are given in Appendix 6.

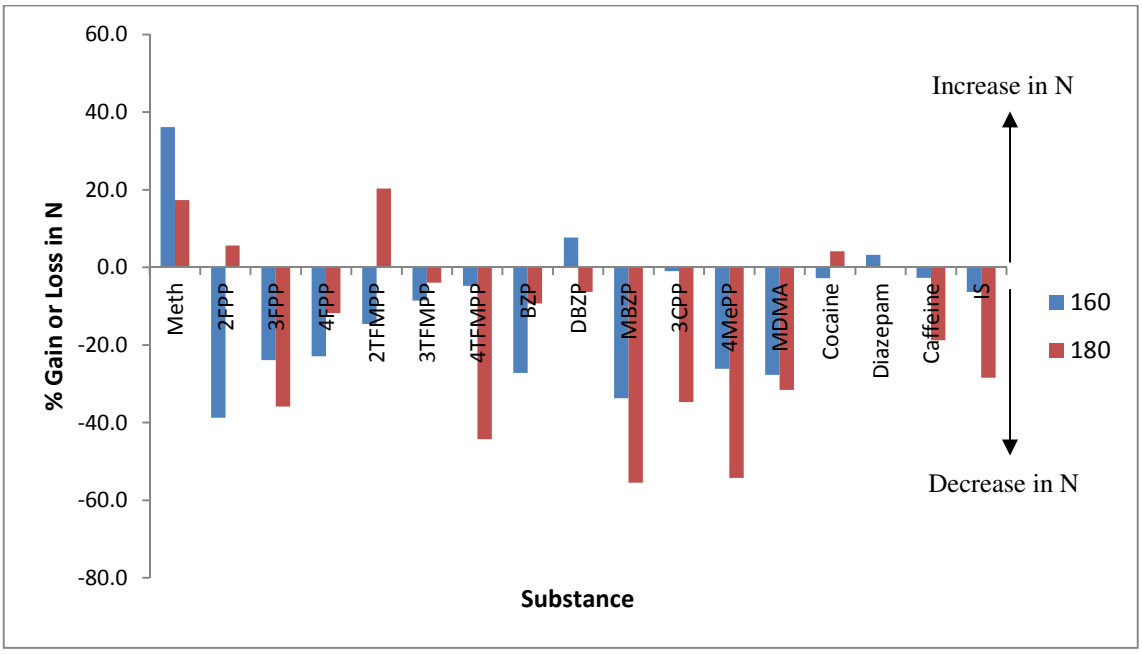


Figure 6.16 Phase 3 Gain or Loss graph for effect of selected injector and oven temperatures on plate number, N.

From the graphs a gain in plate number was desirable. As such it can be argued that generally both the selected oven temperatures (160^{o}C and 180^{o}C) do not achieve the desired outcome, as Figure 6.16 shows that for most (>76%) of the analytes plate number, N has decreased.

The magnitude of the change in plate number for the drugs was very variable ranging from 0 to the highest 54 % observed in 4-MePP with the oven at 180°C. For those drugs that had a gain in plate number (methamphetamine, 2-FPP, 2-TFMPP, cocaine DBZP and diazepam) the oven at 180°C had a higher number of the drugs gaining in plate number than at 160°C (4 compared to 3). However, the plate number after Phase 1 optimisation was sufficiently high. This is evidenced by the good peaks observed in Figures 6.12 and 6.13, consequently the emphasis was on reducing tailing so as to get narrower, more symmetrical peaks and improving resolution. It has been shown that symmetrical peaks with less tailing increase separation between analytes. In accordance with the Plate theory of chromatography (Chapter 2, section 2.3) equations 2.8 (plate number) and 2.11 (resolution), reduced tailing results in a reduction in peak widths and hence increases the value of N and subsequently increases resolution.

The effect (gain or loss) on tailing is shown in Figure 6.17. A loss in tailing, T was deemed desirable as it implies a lower tailing factor.

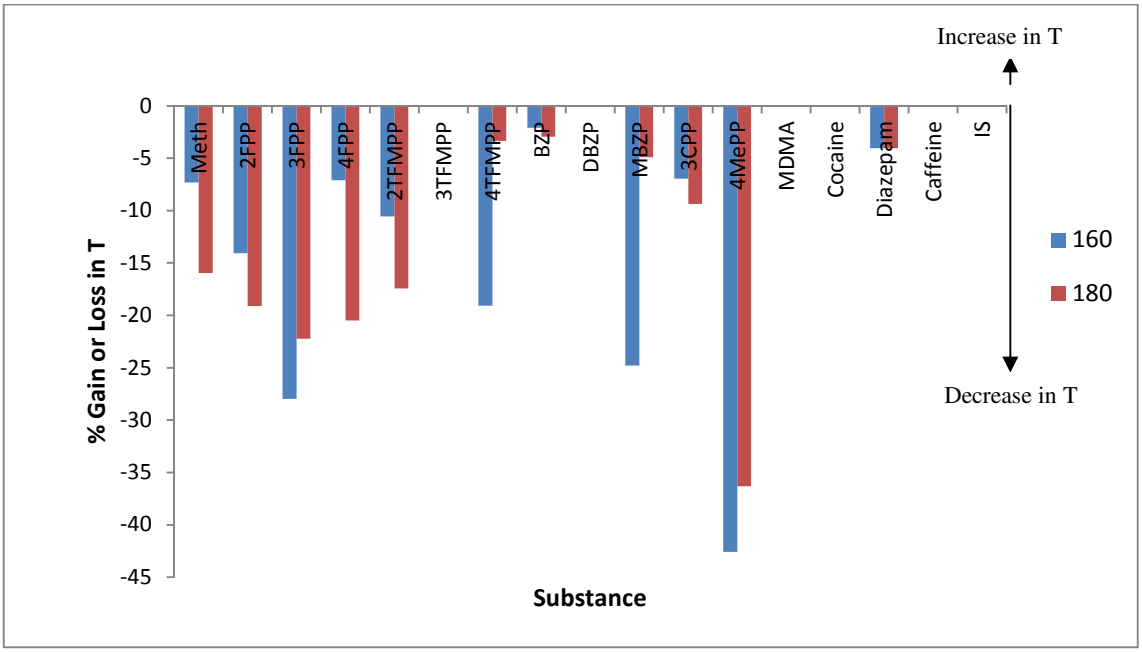


Figure 6.17 Phase 3 Gain or Loss graph for effect of selected injector and oven temperatures on tailing, T.

From the graph (Figure 6.17) it is evident there is generally a loss in tailing for the analytes as can be seen by the negative % values. Comparative to plate number, both temperatures

(160°C and 180°C) were remarkably effective as most of the drugs (67%) had a loss in tailing and 33% were unchanged. The oven at 180°C gave better results than at 160°C, with a greater number of drugs (60%) showing a higher degree of loss than the160°C. Gain and loss graphs for the other parameters (Appendix 6) show that there was a gain in selectivity for 75% of the analytes with the oven at 160°C compared to 56% with the oven at 180°C. For resolution 56% of the analytes gained with the oven at 160°C, whilst the oven at 180°C showed a decrease (44 % gain). It should be noted that the degree of gain in selectivity (highest 3%) and resolution (highest 11%) were smaller than for plate number and tailing. However, resolution was remarkably high for all the drugs (Table 6.5) in the range, R = 3 - 45, except the coe-luting substances, 3-TFMPP and MDMA in comparison the acceptable criteria for resolution, R > 2 (ICH, 2005; Horacio et al., 2008). It has also been reported that a resolution of R > 1.25 is acceptable for GC-MS (Bowers et al., 2002). Of concern is the fact that baseline separation is unlikely to be achieved with such a low resolution factor. Consequently, for plate number and tailing the oven temperature 180°C is best, whilst for selectivity and resolution 160°C is the better option.

Retention times were longer at 160°C than at 180°C except for drugs eluting later in the run (after 16minutes), caffeine, eicosane, cocaine, DBZP and diazepam. This could be due to the fact that these drugs have higher boiling points above the temperatures under investigation and as such are not appreciably affected by them. This is also evidenced by the smaller impact these temperatures have on the peak profile parameters for these drugs comparative to the others (Appendix 5) especially for the lower temperature.

6.3.3.1 Statistical analysis of Phase 3 results

The results for statistical analysis of Table 6.5 Phase 3 data are shown below. For the T-test Table 6.6, Wilcoxon Signed rank test in Table 6.7 and Friedman test in Table 6.8.

Table 6.6 Statistical analysis of Phase 3 data: T-test (paired, α = 0.05), pair variables 160°C and 180°C.

T-test (paired)	Plate number	Tailing	Selectivity	Resolution
Pair differences				
Mean	2.587	-0.00960	-0.00169	-0.1757
Std. deviation	5.253	0.261	0.01168	1.163
Std. error mean	1.274	0.0673	0.00292	0.2908
Test statistic	t = 2.030, N = 17, df = 16, p = 0.059	t = -0.143, N = 15, df = 14, p = 0.889	t = -0.578, N = 16, df = 15, p = 0.572	t = -0.604, N = 16, df = 15, p = 0.555
Pearson's correlation coefficient	r = 0.9713	r = 0.9753	r = 0.9916	r = 0.9983

The T-test (paired) was conducted in order to ascertain the influence of the two selected oven temperatures (160 and 180°C) on chromatographic performance. The hypothesis postulated, H_o: there is no difference in the effect of the two temperatures. The level of significance was 95%. The criteria were if $p < 0.05$ and if t(calculated) > t(critical), H_o was rejected (Laurencelle and Frangois, 2002; Corder and Foreman, 2009). The results (Table 6.6) showed that there is no statistically significant difference between two variables in their effect on all the parameters, i.e., for plate number ($t(16)$ = 2.030, p = 0.059), tailing (t(14) = 0.143, p = 0.889, selectivity ($t(15)$ = 0.578, p = 0.572 and resolution ($t(15)$ = -0.604, p = 0.555. As evidenced by both values for p being greater than the limit 0.05. Consequently H_o was retained. This means statistically the two temperatures exert similar effects, with any differences due to chance. Hence, either 160°C or 180°C can be used with the same result. This supports the observation made on discussion of the total ion chromatograms (TICs) obtained with the two temperatures (Figures 6.12 and 6.13) that visually it was difficult to distinguish between the TICs.

Statistical analysis by Pearson's correlation coefficient shows that the results for plate number are marginal whilst those for the other parameters show high correlation in the effects of the two temperatures as evidenced by the high Pearson's correlation coefficients (Table 6.6) which are in the range 0.9713 – 0.9983.

Table 6.7 Statistical analysis of Phase 3 data: Wilcoxon Signed rank (paired, $\alpha = 0.05$), pair variables 160°C and 180°C.

	Plate number			**Tailing**			**Selectivity**			**Resolution**		
	N	Mean	Sum ranks	N	Mean	Sum ranks	N	Mean	Sum ranks	N	Mean	Sum ranks
(-) ranks	11	10.18	112.00	6	5.00	30.00	6	8.42	50.50	7	8.14	57.00
(+) ranks	6	6.83	41.00	4	6.25	25.00	9	7.72	69.50	9	8.78	79.00
Ties	0			5			1			0		
Total	17			15			16			16		
Test statistic	*Z* = -1.681*, *p* = 0.093 (2-tailed) *Based on (+) ranks			*Z* = -2.55*, *p* = 0.799 (2-tailed) *Based on (+) ranks			*Z* = -0.540*, *p* = 0.589 (2-tailed) *Based on (-) ranks			*Z* = -0.569*, *p* = 0.569 (2-tailed) *Based on (-) ranks		

Table 6.8 Statistical analysis of Phase 3 data: Friedman Test: comparison of 160, 180 and the control 150°C.

	Plate number			**Tailing**				**Selectivity**			**Resolution**		
Temp °C	Median rank	Upper quartile (75%)	Mean rank	Median rank	Lower quartile (25%)	Upper quartile (75%)	Mean rank	Median rank	Upper quartile (75%)	Mean rank	Median rank	Upper quartile (75%)	Mean rank
150	17.0280	41.282	2.59	2.510	1.000	3.200	2.73	1.50	2.375	2.13	3.00	4.00	2.88
160	2.570	41.491	1.82	1.908	1.000	2.750	1.70	2.75	4.25	3.09	2.00	2.75	2.44
180	11.402	37.726	1.59	2.052	1.000	2.588	1.57	4.50	5.75	4.41	4.45	7.00	4.50
Test statistic	*Fr* = 9.294, N = 3, df = 2, *p* = 0.010			*Fr* = 48.016, N = 15, df = 6, *p* = 0.000				*Fr* = 22.710, N = 16, df = 6, *p* = 0.001			*Fr* = 19.098, N = 16, df = 6, *p* = 0.004		

Fr is the Friedman test statistic and *p* is the asymptotic significance at 95%level of significance.

Further statistical analysis was done to confirm that there were no differences in the distribution of data using Wilcoxon signed rank test (Table 6.7). It has been discussed in chapter 3 that Wilcoxon tests ranks the data and that consequently it gives a better analysis than the t-test. The advantages derived from ranking were discussed in Chapter 3 (section 3.4.8) for the Friedman test which also makes use of ranking and was applied for Phase 1 data (section 6.3.1.1). However, the results of the Wilcoxon signed rank test followed a similar pattern as for the t-test, there is no statistical significance for all the parameters; for plate number (Z = -1.681, p = 0.093), tailing (Z = -2.55, p = 0.799), selectivity (Z = -0.540, p = 0.589) and resolution (Z = -0.569, p = 0.569). Examining the ranks shows that on average plate number decreased (negative ranks), which is in agreement with the gain and loss analysis. Compared to the 160^{o}C, plate number and Tailing were lower for 180^{o}C selectivity and whilst the resolutions were higher.

The Wilcoxon signed rank test showed that the effect is the same between the 160 and 180^{o}C but did not divulge whether the two temperatures resulted in any increase or decrease chromatographic performance. As such, further statistical analysis was conducted. Since the Wilcoxon signed rank test can only compare 2 subjects the Friedman test was applied on the two temperatures and the control temperature (150^{o}C). This was to elucidate whether there was any statstistically significant change in the results of each of the two oven temperatures in comparison to the control temperature. The results of the Friedman test (Table 6.8) indicate that for all the parameters there are significant differences in their distribution when subjected to a change in temperature from 150^{o}C. For plate number (Fr = 9.294, N = 3, df = 2, p = 0.010), tailing (Fr = 48.016, N = 15, df = 6, p = 0.000), selectivity (Fr = 22.710, N = 16, df = 6, p = 0.001), and resolution (Fr = 19.098, N = 16, df = 6, p = 0.004). Investigation of the descriptive statistics (Table 6.8) to elucidate the degree of the effect revealed that there are marked changes; plate number decreases on increase of temperature the median rank is lowest at 180^{o}C hence 180^{o}C results in the lowest plate number. Tailing follows a similar trend, however the median rank is lowest at 160^{o}C, hence it results in the lowest tailing. Selectivity increases with temperature and is highest for 180^{o}C, resolution decreases at 160^{o}C whilst it is enhanced at 180^{o}C. It can be seen that a compromise has to be reached as no one temperature has an optimum effect on all the parameters. Consequently, basing on plate number and tailing the oven at 160^{o}C is the better of the two whilst basing on selectivity and resolution 180^{o}C is best. On average 170^{o}C was proposed to be the statistically correct

optimum temperature. It offers a compromise in performance such an approach was applied by Maher et al. (2009).

In evaluation of the results there was no similar study on developing and optimising a method for the drugs under investigation. Nor was there a method for simultaneous analysis of such a number of mixed drugs together. There was no data such as tailing, resolution available for a more subjective analysis of the results. As such, a reference was made of research work which had a bearing on the objectives of this study, and could be extrapolated for the benefit of this work or to provide insight into this study.

Santali et al. (2011) in their study on developing a method for mephedrone and Inoue et al. (2008) in a study on developing a method for profiling methamphetamine by GC-MS also observed that both plate number and tailing were temperature dependent. Furthermore, in optimising their methods variation of both injector and column temperatures similarly improved plate number and tailing

Andersson et al. (2007a), Inoue et al. (2008) developed and optimised methods for profiling of amphetamine and methamphetamine. They applied similar concepts as in this study and also reported improved chromatographic results. This proves that the method is dependent on operational variables as theorised in the Plate theory. The work of Maher at al. (2009) was found to be highly significant to this study, as it was on the differentiation of regioisomers of TFMPP, which is one of the drugs of focus for the overall research project. The results were comparable since they also resolved all three (2, 3, 4) TFMPP isomers. It was observed that the order of elution of the substances was similar to this study 2-TFMPP>3-TFMPP>4-TFMPP. The analytical methodology similarly to this study was carried out without derivatisation, using a capillary column. However, in contrast to this study the column was non-polar (Rtx-1). Preliminary method studies (Chapter 4) evaluated a non-polar column and found that whilst it resolves the TFMPP isomers, resolution of these compounds to other drugs was poor; in addition a more polar column gave better peak profiles.

Optimisation as with other researchers (Andersson et al., 2007a; Inoue et al., 2008; Maher et al., 2009; Byrska et al., 2010; Santali et al., 2011) used the approach of evaluating different columns and several temperature programs. A compromise was reached between resolution and analysis time. The performance characteristics such as resolution or tailing were not

stated for comparison. However, interestingly the chromatographic profile was said to have excellent resolution and on observation the total ion chromatogram supported this. It is important to note that this study was on TFMPP and its isomers only. In drugs of abuse this substance is found in a sample matrix in combination with other drug substances (Baron et al., 2011; Davies et al., 2010; Yuk, 2010; King and Kicman, 2011; Arbo et al., 2012) and as such depending on the intended use, the method might have limitations. The method under development in this research is therefore advantageous as it overcomes these limitations. Congeners and impurities expected to be present have been evaluated. In addition, complete resolution of the FPP and TFMPP isomers was achieved. Furthermore, it simultaneously analyses for all the substances investigated. Therefore, it can be applied for the analysis of any of these drugs individual or in combination in street samples. This imparts it with versatility. It has been identified that drugs of abuse exist in a variety of combinations and dosages in street samples (Yeap et al., 2010; Kelleher et al., 2011).

Byrska et al. (2010) studied 6 piperazines MePP, BZP, TFMPP, MBZP, MeOPP and 3-CPP. Except for MeOPP these are part of the drugs under investigation. The column stationary phases are similar (5-MS) as were the column physical properties. Resolution and tailing were not specified. However, it was observed that the peaks were not baseline resolved; in addition MeOPP and 3-CPP showed considerable tailing. This could be the reason why in their study GC-MS was used only for qualitative analysis and HPLC for quantitative analysis. Whilst this study provided an important insight into optimisation there is need to ensure it is capable of simultaneously meeting both qualitative and quantitative aspects, if it is to have more diverse application e.g. characterisation of street samples.

6.3.4 PHASE 4 EFFECT OF CARRIER GAS FLOW-RATE

The chromatographic profiles resulting from variation of flow rate are represented by Figure 6.18.

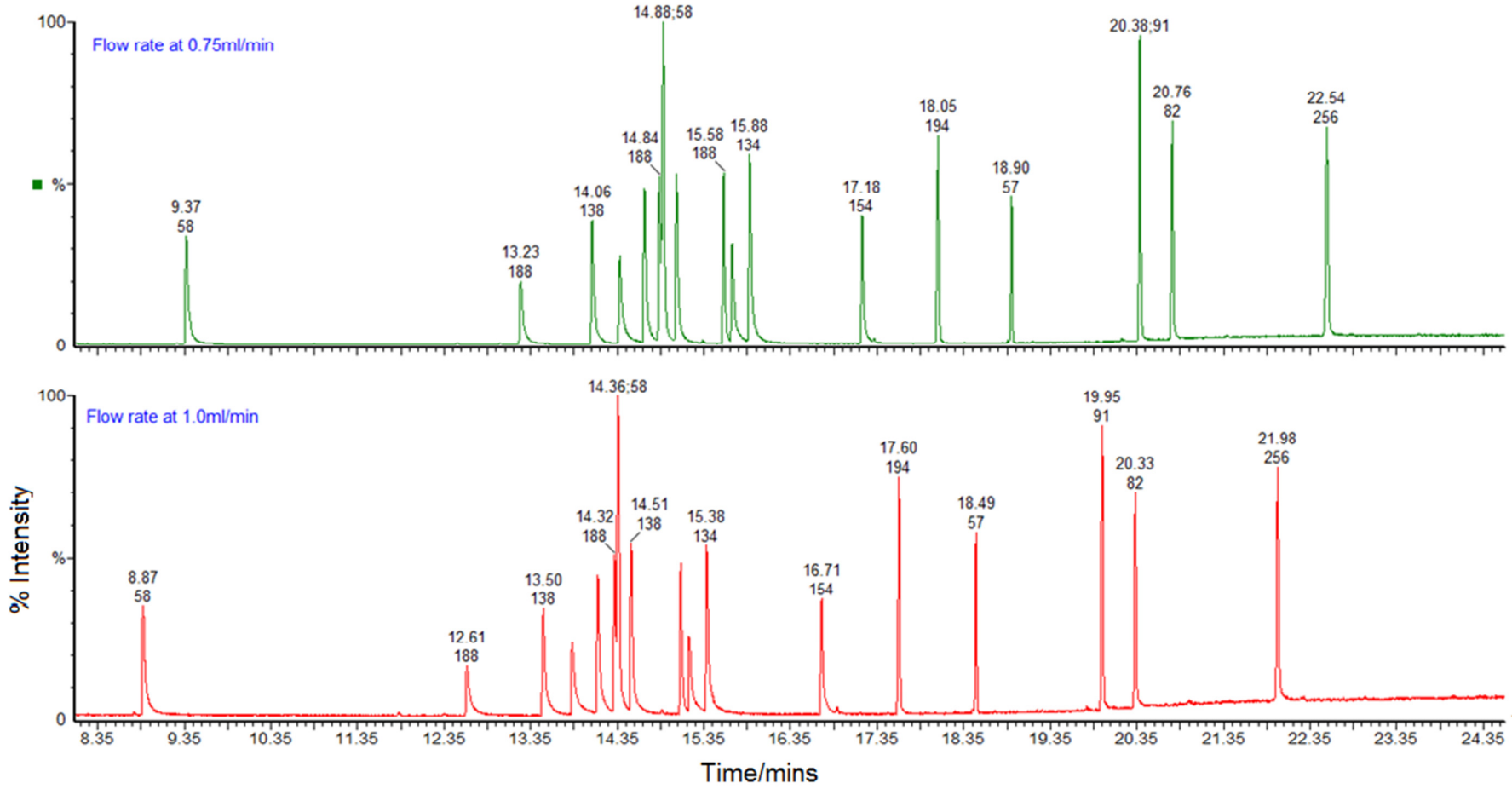


Figure 6.18 Phase 4 Effect of flow rate. Showing the total ion chromatomatographic profiles observed at different flow rates (0.75 and 1.0ml/min) for comparison.

The retention times increased at lower flow rates as evident in Figure 6.18 at a flow rate of 0.75mL/min longer retention times were observed. However, the effect on resolution was marginal and not as pronounced and the peaks appeared slightly broader at 0.75mL/min in comparison to flow rates of 1mL/min (control) and 1.25mL/min. At 1.25mL/min the peaks started to show tailing. It has been discussed that retention time and peak shape increased with reduced flow rate, which is in line with the observed results. In fact, they advocated that slow is best. Andersson et al. (2007a) also made a similar observation. This is in partial agreement with this study where flow rate at 0.75mL/min was found to slightly improve resolution; however the benefits were counteracted by tailing. This was deemed counterproductive to the objective of optimisation and as such the flow rate of 1.0mL/min was proposed as giving optimum results. The increase in retention time with flow rate can be attributed to the fact that flow rate is a measure of mobile phase velocity, μ. A decrease in flow rate results in the analyte being retained longer in the column hence its lower retention time. Longitudinal diffusion (B term) was identified as one of the causes of band broadening. It is evident from the equation for B (equation 2.15 Chapter 2) that a decrease in velocity increases B hence band broadening. This results in the observed tailing at lower flow rates.

6.3.5 PHASE 5 EFFECT OF MS SCAN RATE

The total ion chromatogram obtained is shown in Figure 6.19.

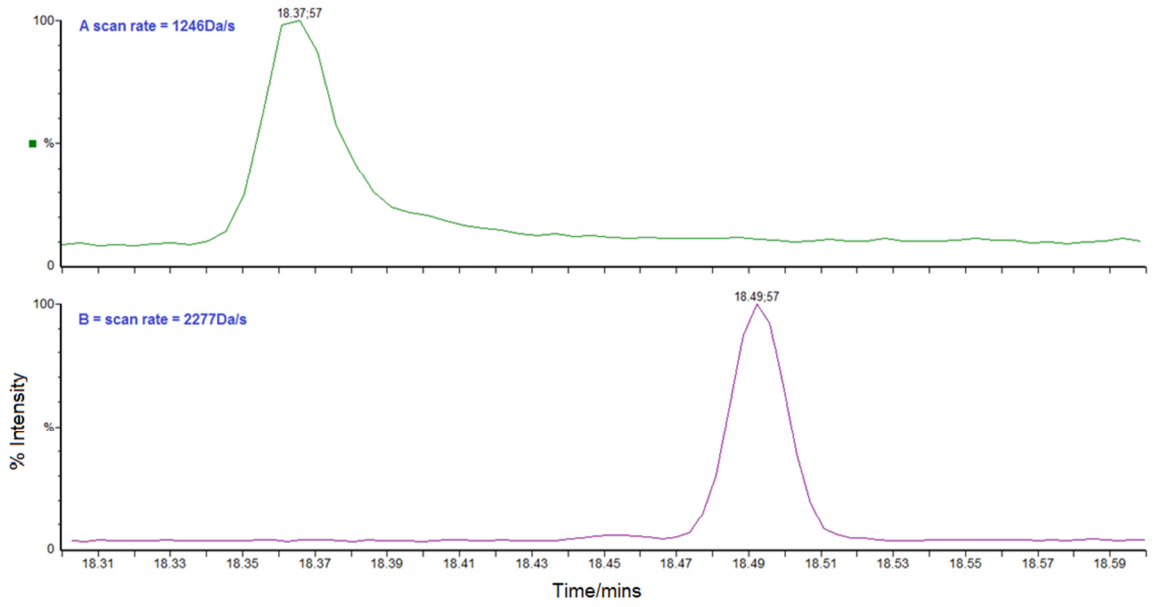


Figure 6.19 Phase 5 Effect of MS scan rate, showing the impact on peak shape.

The results for optimisation of the detector MS scan rate (Figure 6.19) showed that higher scan rates give an improved peak shape in terms of symmetry at the apex of the peak. This can be attributed to faster sampling giving more data points, hence the peak shape is smoother. This is important as peak shape is critical in peak processing, e.g. poor peak shape reduces accuracy in quantitation of the area under the peak (Barwick, 1999; Kaur, 2010). Studies on optimisation of detectors focus mainly on selection of detector systems as such these results show that a chosen detector system can be enhanced to achieve optimum results is especially important in reducing instrument errors.

6.3.6 PHASE 6 EFFECT OF MS IONISATION ENERGY

The effect of ionisation energy (EI) on the mass spectra of a compound was investigated using 3-TFMPP and BZP as representatives of the analytes. The effect of EI on their mass spectra is shown in Figures 6.20 and 6.21. The figure shows the mass spectra observed at 50, 60, 70 and 80eV.

The ideal ionisation energy gives optimum abundances for all the ions and a mass spectrum with adequate fragmentation for easy interpretation and structural derivation. A visual insepection of Figures 6.20 and 6.21 indicated that all the ionisation energies investigated show a similar fragmentation pattern. The relative intensities appeared slightly different as evident in Figure 6.20 for the ions at m/z 145, 172 and the parent ion 230(M^+). This implies that ionisation affected the abundancies of the ions. Further evaluation of the mass spectral data (ions and their abundance) was conducted. The results are given in Table 6.9 (3-TFMPP) and Table 6.10 (BZP) corresponding to the spectra in Figures 6.20 and 6.21.

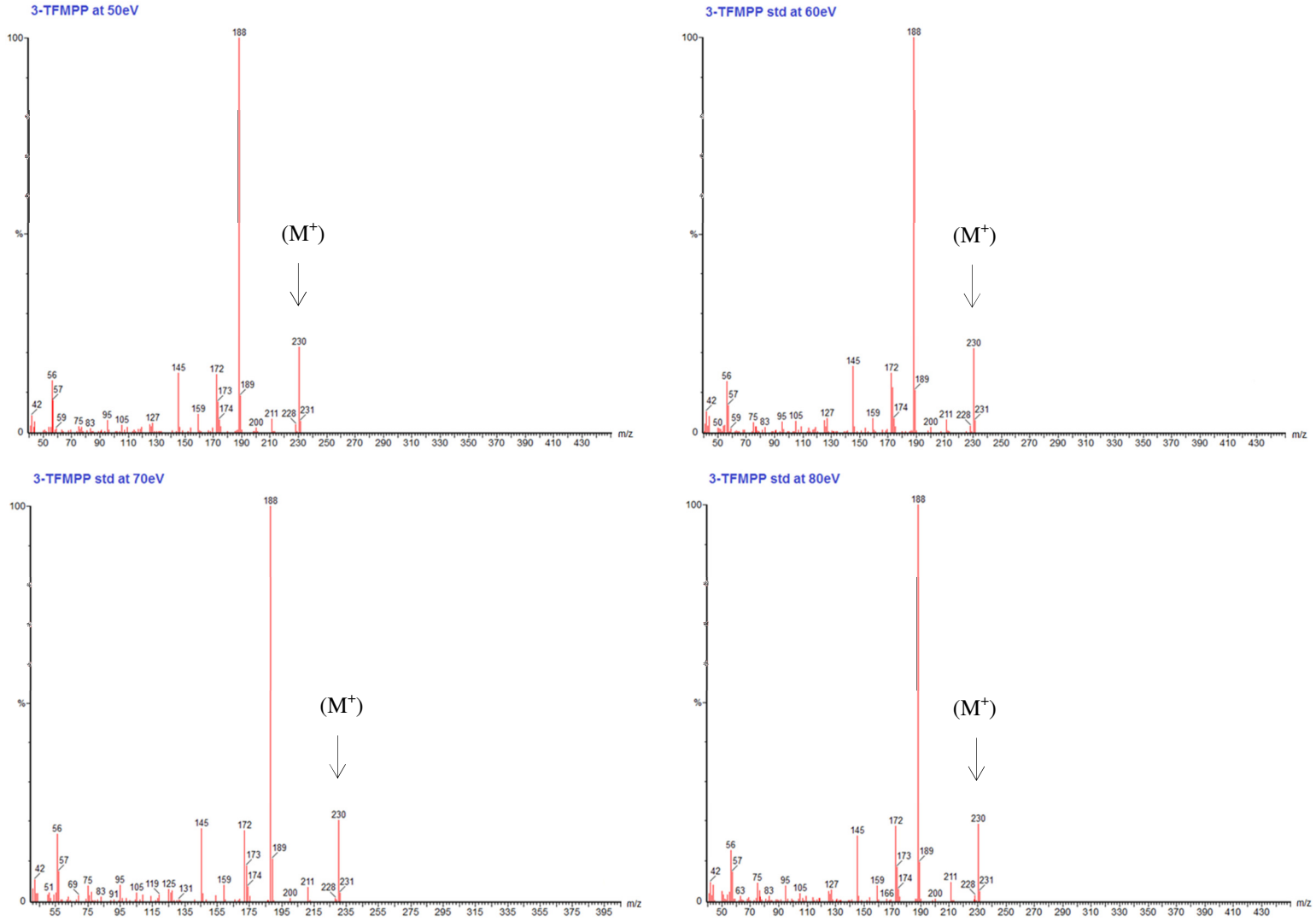


Figure 6.20 Phase 6 Effect of ionisation energy (EI) on the mass spectra of 3-TFMPP showing the mass spectra at different EI (50, 60, 70 and 80eV) for comparison.

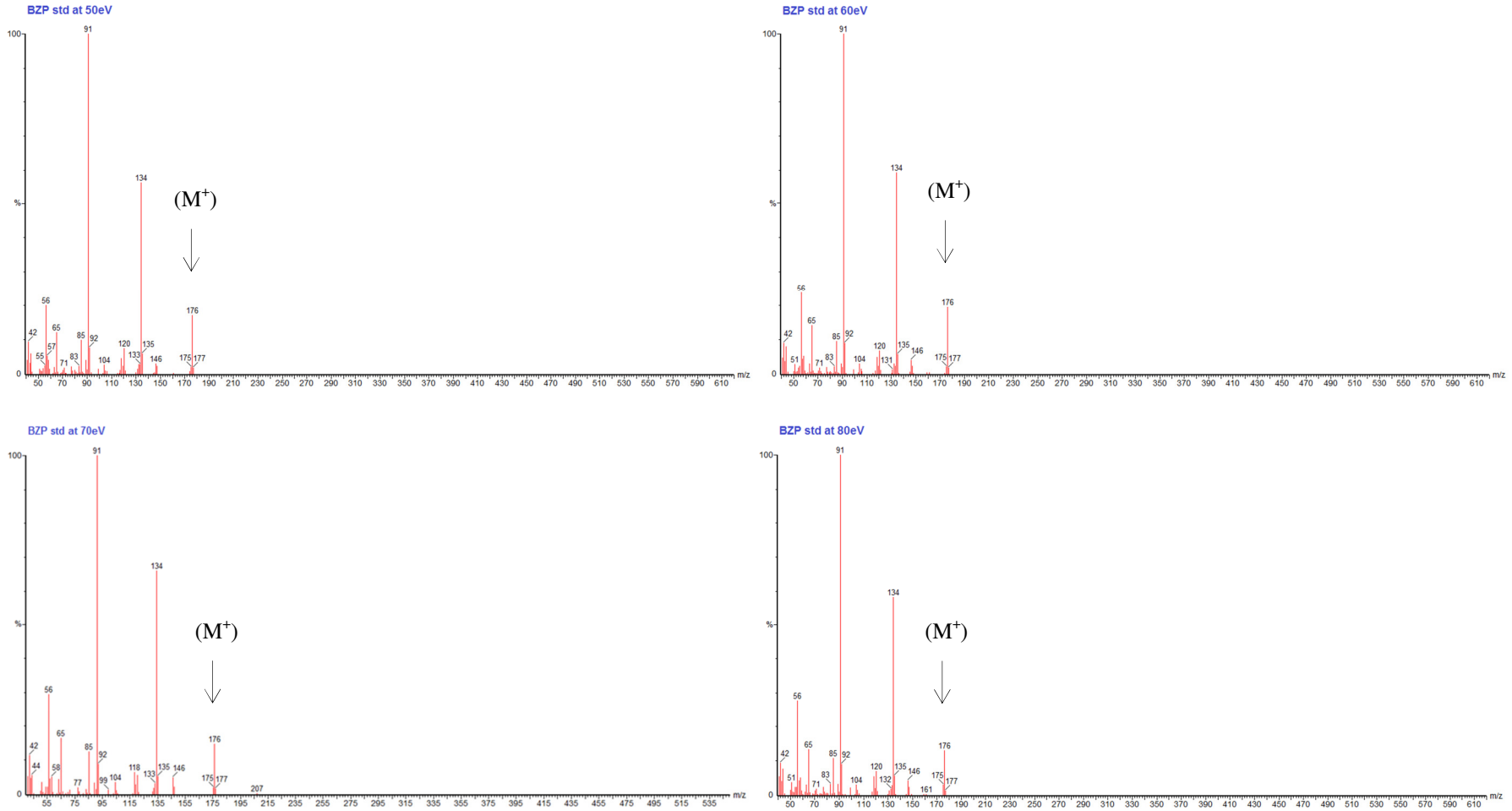


Figure 6.21 Phase 6 Effect of ionisation energy (EI) on the mass spectra of BZP showing the mass spectra at different EI (50, 60, 70 and 80eV) for comparison.

Table 6.9 Phase 6 Mass spectra data for effect of ionisation energy on 3-TFMPP.

	Intensity (%TIC)									
Ionisation Energy (eV)	**Fragment Ion (m/z)**									
	230(M^+)	**188**	**172**	**159**	**145**	**127**	**109**	**95**	**75**	**42**
50	8.38	39.08	5.68	1.77	5.78	0.91	0.46	1.12	0.54	1.6
60	7.67	36.46	5.43	1.29	6.02	1.24	0.51	0.94	0.86	1.9
70	7.23	35.7	6.28	1.43	6.46	0.91	0.54	1.43	1.37	1.89
80	6.92	36.13	6.73	1.4	5.84	0.99	0.46	1.38	1.6	1.68

Table 6.10 Phase 6 Mass spectra data for effect of ionisation energy on BZP.

	Intensity (%TIC)									
Ionisation Energy (eV)	**Fragment Ion (m/z)**									
	176 (M^+)	**134**	**120**	**119**	**118**	**91**	**85**	**77**	**65**	**56**
50	5.16	17.03	2.24	0.68	1.38	30.24	2.95	0.64	3.63	6.06
60	5.58	16.98	1.92	0.65	1.38	28.67	2.75	0.54	4.06	6.82
70	4.16	18.67	1.54	0.79	1.80	28.28	3.54	0.52	4.63	8.37
80	3.71	16.64	1.95	0.55	1.52	28.57	3.05	0.63	3.78	7.93

It was observed that at lower ionisation energies (50eV) there is a higher abundance of the ions with larger masses. Such as the molecular ion as evidenced by 3-TFMPP in Table 6.9 and BZP in Table 6.10. For 3-TFMPP the intensity of the molecular ion at m/z 230 decreased from 8.38 to 6.92 %TIC with increase in ionisation energy from 50 to 80eV. The principal ion at m/z 188 showed a similar trend. At higher ionisation energies more fragmentation of the molecular ion occurs as a result of higher energies and velocities of the bombarding electrons resulting in a higher abundance of the low molecular mass ions. Ionisation energy at 70eV gave the most optimum results as it shows the highest intensity values for the majority of the lower molecular ions e.g. 5.29, 4.00 and 1.53% at m/z 42, 95 and 109 for 3TFMPP. Furthermore, adequately high abundances for the high molecular mass compounds in addition the molecular ion was also observed making identification of compounds easier. The differences lie in the intensities of the observed ions. A similar trend was observed by Vekey (1996) in a study on internal energy effects on mass spectrometry. The study highlighted that very low ionisation energies (20eV and lower) have a more profound impact on the mass spectra due to very little fragmentation occurring, whereas higher ionisation energies result in similar fragmentation patterns. Furthermore, it has been discussed (Vekey, 1996) that

ionisation energy at 70eVgives optimum intensity values and is typically used in mass spectra libraries such as NIST. This could be the reason why it has found favour in most research studies (Takahashi et al 2009; de Boer et al, 2001; Inoue et al., 2004; Staack, 2007). As such in further investigations for this research 70eV will be used.

6.3.7 OPTIMISED METHOD

The chromatographic profile (TIC) of the final method after optimisation is shown in Figure 6.22 and the method parameters in the conclusion (section 6.4).

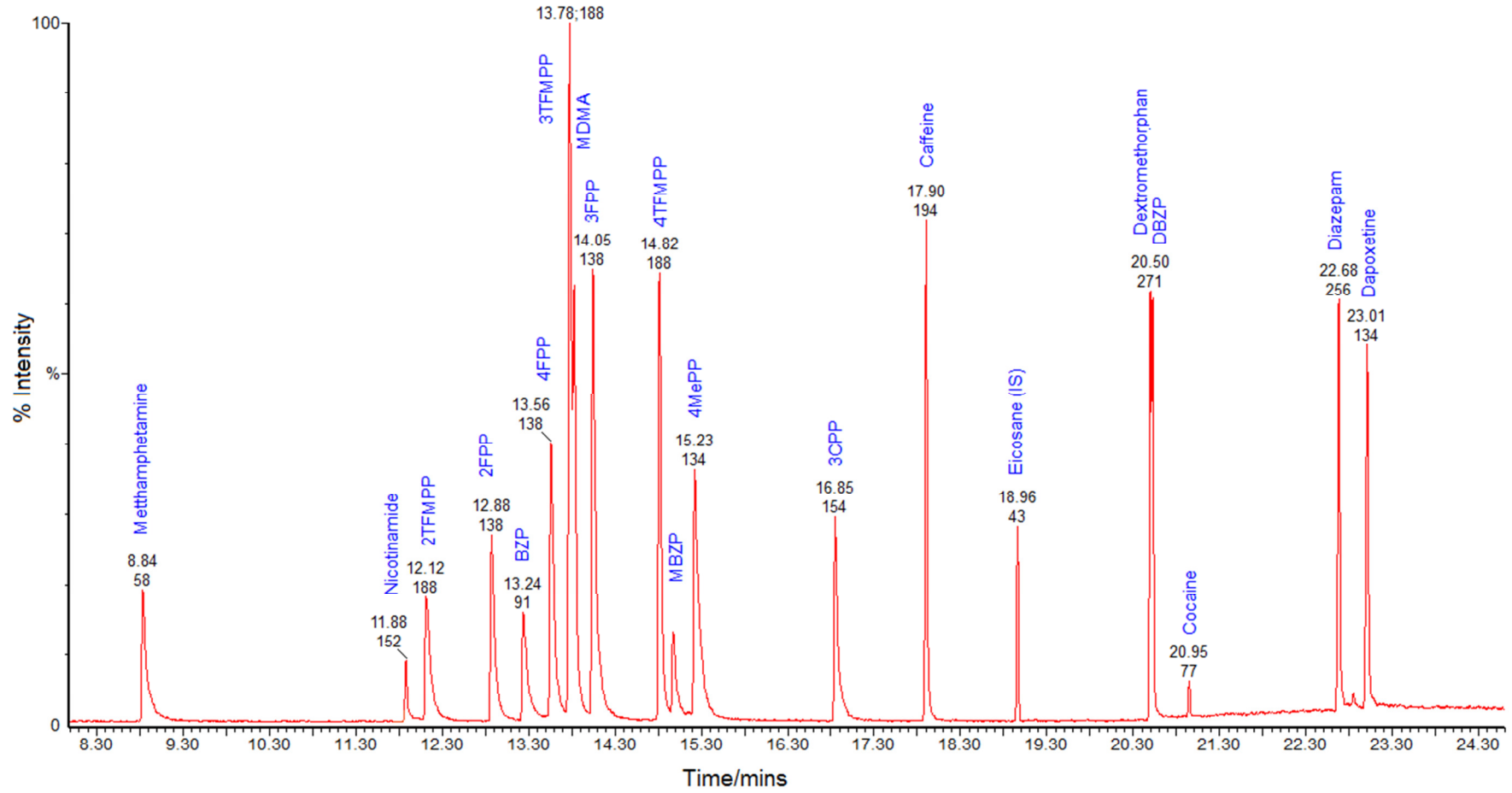


Figure 6.22 Chromatographic profile (TIC) of the optimised method. The mass of the principal ion for each analyte is shown below the retention time.

The optimised method's total ion chromatogram (Figure 2.22) showed improved peak profiles. The peaks in the range 12 - 16 minutes were more resolved. This range of peaks was discussed in section 6.1 as requiring improvement in resolution and tailing. The tailing was also less for the all the peaks from methamphetamine to CPP. This is in agreement with observations made in the discussions on optimisation and the related statistical analysis.

6.4 CONCLUSION

Optimisation of the method operational variables; injector port temperature, oven temperature, carrier flow-rate, MS scan rate, and MS ionisation was investigated with the aim of improving performance of the method in terms of the quality of chromatographic peak profiles generated. The injector and oven temperatures improved the quality characteristics of the chromatograms generated, i.e., plate number, tailing, selectivity and resolution. The method improved to give good peak shapes, high resolution, $R > 2$ and reduced tailing.

Variation of oven temperature was found to change the parameters to a greater degree than the injector port temperatue. This is due to the direct impact of changes in oven temperature on the stationary phase. Variation of flow rate showed that fast flow rates reduced the resolution. Optimising the MS scan rate showed that peak shape was dependent on scan rate. Too slow scan rates broadened the peaks. Investigating MS ionisation energies indicated that ionisation energy (EI) affects the ion abundances. Use of EIs which were too high decreased the intensity of the peaks and also showed slightly more fragmentation. This has the potential to make the mass spectra complex and its evaluation less easy e.g. the parent ion can disappear. It was found that 70eV gave the best results. This confirmed the works of de Boer et al. (2001), Maurer (2004) and UNODC (2013c), and in addition generally most spectral libraries (e.g. NIST) apply EI 70eV.

It was found that the co-eluting peaks could not be completely resolved upon optimisation. This was not a limitation as for these analytes (3-TFMPP and MDMA, DBZP and dextromethorphan) extracted ions would be applied in the analysis of samples containing both the analytes (Takahashi et al., 2009; de Boer et al., 2001). Furthermore, the peaks were

sufficiently distinct for identification using retention times (3-TFMPP 13.78minutes and MDMA 13.83 minutes).

Consequently, it can be concluded that optimisation was successful and resulted in the following optimised method; the initial oven temperature was set at 60^{o}C with a hold for 1min and ramped at 10^{o}C /min to 170^{o}C with a hold for 2min. The oven was further ramped at 15^{o}C /min to 280^{o}C, with a hold for 4min. The MS transfer line was set at 280^{o}C, source temperature 230^{o}C, ionisation energy 70eV and scan range at m/z 40 - 500. The carrier gas was He (g) at a flow rate of 1mL/min. The injector was set at 260^{o}C with a split ratio of 20:1. The instrument was equipped with a Supelco, Equity-5 (30m x 2.5mm x 2.5µm) capillary column. The total analysis run time was 25.33 minutes.

There was a rather limited amount of previous research on method development and or optimisation for similar drugs, especially involving FPP, one of the two main drugs of focus for the overall research. This is probably due to the drugs being relatively new on the market and this was a limitation in this study for comparative purposes. In depth data on optimisation of the drugs was found to be mainly on amphetamines. In addition, the few studies found reported optimisation mostly on qualitative methods on a limited number of drugs. Therefore, this study will provide relevant data on optimisation of a method for the analysis and profiling of phenylpiperazines, benzylpiperazines and other drugs of abuse in street samples. According to Eurachem (1998) a method requires validation before its application so as to establish its reliability. The need for validation was established in section 2.5 (Chapter 2) and therefore, the method optimised will undergo validation in a later study (Chapter 7) of this research.

CHAPTER 7

VALIDATION OF THE OPTIMISED METHOD

7.1 INTRODUCTION TO THE VALIDATION STUDY

The previous chapters saw the development and optimisation of a method for the analysis, chemical characterisation and profiling of piperazine drugs of abuse. The need for validation was established in Chapter 1 (section 1.9) and the theoretical considerations and definitions underpinning method validation were discussed in Chapter 2 (section 2.5). In this study validation was conducted as per ICH (2005) and Eurachem (1998) guidelines. The tests conducted (outlined in Appendix 8) were linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision. The test for accuracy was determined through the method of %recovery of unknown. Precision of the method was determined as a) repeatability and b) intermediate precision.

The need for quality control (QC) was established in Chapter 2 (section 2.5.2). As such, throughout the life of the research project quality control was assured through monitoring instrument and column performance so as to establish there suitability for use. This was achieved by monitoring the detector response, precision and variation in retention time and peak shape. The analytes used for QC were n-alkanes (carbon number C8 to C24) and 1-(4-methylphenyl)piperazine 4-MePP and Shewhart quality control charts were used as a monitoring tool. Corrective action was taken when a parameter was found to be out of limits. For example, if the peak shape deteriorated, inconsistent results or high column bleed were obtained, these were indications of an aging column and as such the column was changed. The MePP was monitored every 3 months; this period was used due to the instrument being robust, i.e., giving consistent results. The general background in a chromatogram might show the presence of distinct peaks or an abundance of small peaks due to column bleed. In this study, contaminant mass ions due to column bleed (section 2.5.2) were monitored using the ion at m/z = 207. This ion was selected for monitoring as it is characteristic of polysiloxane based columns (McMaster, 2007).

7.1.1 AIMS OF THIS CHAPTER

The aims of this study were to investigate and establish linearity (linearity range and working range), limit of detection, limit of quantitation, accuracy and, precision. Thereby confirm suitability of the GC-MS method developed for its use in qualitative and quantitative analysis. This will therefore validate its use for the chemical characterisation and impurity profiling of 4-FPP and 3-TFMPP street drugs.

7.2 MATERIALS AND METHODS

All the work in this study was conducted at 25^{o}C (above the freezing point of the solvent, 22^{o}C)

7.2.1 CHEMICALS/REAGENTS

The drug standards used were as listed in Chapter 4, section 4.2.1.

7.2.2 INSTRUMENTS

Validation was conducted using Perkin Elmer GC-MS, Clarus Turbomass Gold 500MS fitted with a Supelco, Equity-5 GC capillary column (30m x 0.25mm x 0.25µm). The instrument was equipped with a Perkin Elmer 4mm quartz split/splitless injector liner and with the NIST MS Search Version 2.0 library software and was set up with the method to be validated. The injector was set at 260^{o}C with a split ratio of 20:1. The carrier gas was He (g) at a flow rate of 1mL/min. The initial oven temperature was set at 60^{o}C with a hold of 1min. The oven was ramped was at 10^{o}C /min to 170^{o}C with a hold of 2min and at 15^{o}C /min to 280^{o}C with a hold of 4min. The MS transfer line was set at 280^{o}C, source temperature 230^{o}C, ionisation energy 70eV and scan range at m/z 40 - 500. The total analysis run time was 25.33minutess. A Shimadzu GC-MS and a Phenomenox, Zebron ZB-5 GC capillary column (30m x 0.25mm x 0.25um), serial number 164043) were used in the test for robustness.

7.2.3 STATISTICAL SOFTWARE

Analysis of results was carried out using IBM SPSS Version 20 and MS Office Excel 2010.

7.2.4 PREPARATION OF STANDARD SOLUTIONS

The drug standards listed in Chapter 4 (Table 4.1) and ephedrine were used to prepare standard solutions for use in validation. Stock solutions of each analyte were prepared at a concentration of 1mg/mL free base in 2-methyl-propan-2-ol as outlined in Chapter 4 (section 4.2.4). For studies of retention time, precision and accuracy standards at 0.1mg/mL containing eicosane (0.02mg/mL) as the internal standard were used.

7.2.5 LINEARITY AND METHOD DETECTION LIMITS TEST

For linearity studies the stock solutions (section 7.2.4) were diluted to give 6 to 10 calibration standards for each analyte in the concentration range 0.01 – 1.0mg/mL free base (equivalent to 0.5 – 50.0µg/mL on column) containing 0.02 mg/mL of the internal standard eicosane. This was used to generate calibration, limit of detection (LOD) and limit of quantitation (LOQ) data.

7.2.6 ACCURACY TEST

A standard solution to be used for determing the %recovery was prepared for each analyte. The standard was weighed (7.00 - 10.0mg) to a 10.0mL volumetric flask. The procedure followed was as outlined in Chapter 3 section 4.2.4.3 for individual drug standard solutions. The recovery solution was prepared in triplicate.

Samples were also created to simulate real street samples by adding standards of the analytes to powdered tablets and extracting the analytes in the same manner as for street samples (section 8.2.4.2.2.2). Control samples were also analysed without standards added. The samples were analysed by GC-MS and the amount recovered was calculated by determination of the difference between the samples with standards added and the control.

7.2.7 PRECISION

The test was carried out concurrently with linearity tests (section 7.2.5). The test was carried as a) repeatability: The mixed standard solution was analysed six times by GC-MS, b) intermediate precision: The test was carried out using a total of 7 - 9 standard solutions at different concentrations. The calibration solutions specified in section 7.2.5 above were used. Each solution was analysed 3 times by GC-MS.

7.2.8 METHOD ROBUSTNESS

The test was conducted by using two different analysts and instruments; a) different analyst conducted linearity tests on a selected number of drugs (3-TFMPP and caffeine) and b) The mixed standard solution (0.10mg/mL) was analysed 6 times using a Shimadzu GC-MS.

7.2.9 QUALITY CONTROL

The n-alkanes (octane, decane, dodecane, tetradecane, hexadecane, octadecane, eicosane, docosane and tetracosane were individually weighed (10.0mg) and dissolved in pentane to give stock solutions of 1mg/mL. The solutions were diluted to give a mixed QC solution containing 0.1mg/mL each analyte. The n-alkanes solution was analysed by GC-MS prior to any analysis of analytes and also analysed at the end of each run sequence.

A solution of 4-MePP standard (0.1mg/mL) was prepared as outlined in Chapter 4, section 4.2.4.3 for individual standards. The solution was injected into the GC-MS six times every 3 months. However, for any quantitative determination where the mixed standard solution was used, the mixed standard solution was analysed 3 times and 4-MePP was monitored from this solution. As a measure of precision, the standard deviation and %RSD were monitored.

Column bleed was monitored by generating a background scan. A blank solution, i.e., only the solvent with no analytes was analysed by GC-MS. The chromatogram generated was observed for peak abundance and the presence of the peaks at m/z 207 and 281.

System suitability was determined prior to any quantitative work by injecting a standard solution of the analyte 6 times and determining the standard deviation and % RSD.

7.2.10 DATA ANALYSIS

7.2.10.1 Linearity and method detection limits

Calibration graphs were plotted for each analyte for both the total ion chromatogram (TIC) data and extracted ion (m/z) data. In the graphs peak area ratio versus concentration (µg base on column) were plotted. The confidence interval was also depicted for each data point using error bars. The peak area ratio was calculated according to equation 2.18 (Chapter 2), confidence interval equation 3.5 and concentration equations 3.35 - 3.40 (Chapter 3).

The linearity (lack of fit) was tested by the following methods; a) visual inspection of the calibration line plots, b) regression analysis and determination of correlation coefficients, c) use of residual plots (Thompson et al. 2002, ICH, 2005; Thompson, 2005). Regression analysis and correlation coefficients were determined according to section 3.12 (Chapter 3) and residual analysis according to Chapter 2 section 2.5.1.1.2. The randomness of the residuals was evaluated according to the Runs test section 3.2.13 (Chapter 3). The linearity range was also determined as per section 2.5.1.2.

Method detection limits were calculated as per equations 2.19 and 2.20 Chapter 2 (section 3.0)

7.2.10.2 Accuracy

The recovery (%) of the known standard was determined. The criteria for accuracy was that recovery = 100 ± 5%. The standard deviation and relative standard deviations were also calculated. The recovery was calculated according to 2.22 (Chapter 2)

7.2.10.3 Precision

The standard deviation and relative standard deviations were evaluated (ICH, 2005). These were calculated according to Chapter 3 section 3.2.2.

7.2.10.4 Robustness

Statistical comparative analysis of the results was conducted through Pearson's correlation coefficient, T-test (paired) and ANOVA (2-way). These parameters were calculated according to Chapter 2 sections 3.2.4, 3.2.5 and 3.2.6.

7.2.10.5 Extracted ion data

Linearity, range, limit of detection, limit of quantitation, accuracy and precision were determined using extracted ions for the drugs and compared to those derived from total ion chromatograms. For the comparison ANOVA (2-way) was applied. The ions used were 3-TFMPP (m/z 188), MDMA (m/z 135), DBZP (m/z 91 or 266) and dextromethorphan (m/z 271).These ions were selected from the mass spectra and were distinct to the compound (Table 7.9 and Appendix 10).

7.2.10.6 Quality control

The variation in detector response (peak height), retention times and precision through the course of the research projected were calculated and monitored. To determine precision, peak height ratios (analyte to internal standard) were calculated for each of the repeated injections. The standard deviation and %RSD were then determined. QC charts were plotted.

7.2.10.7 Method application GC-MS confirmation of drug identity data parameters

Qualitative data for each drug was derived as retention time, relative retention time, retention index and mass spectra. Relative retention time and retention index were calculated according to equations 3.33 and 3.34 (Chapter 3).

7.3 RESULTS AND DISCUSSION

7.3.1 LINEARITY AND METHOD DETECTION LIMITS

7.3.1.1 Linearity: Calibration graphs

A typical calibration graph is shown in Figure 7.1 for 3-TFMPP as an exemplar. The calibration graphs for all the analytes are shown in Appendix 9. In the graphs the error bars represent the confidence interval (95% CL) for each peak area ratio value. In the graphs the concentration is for the free base on column.

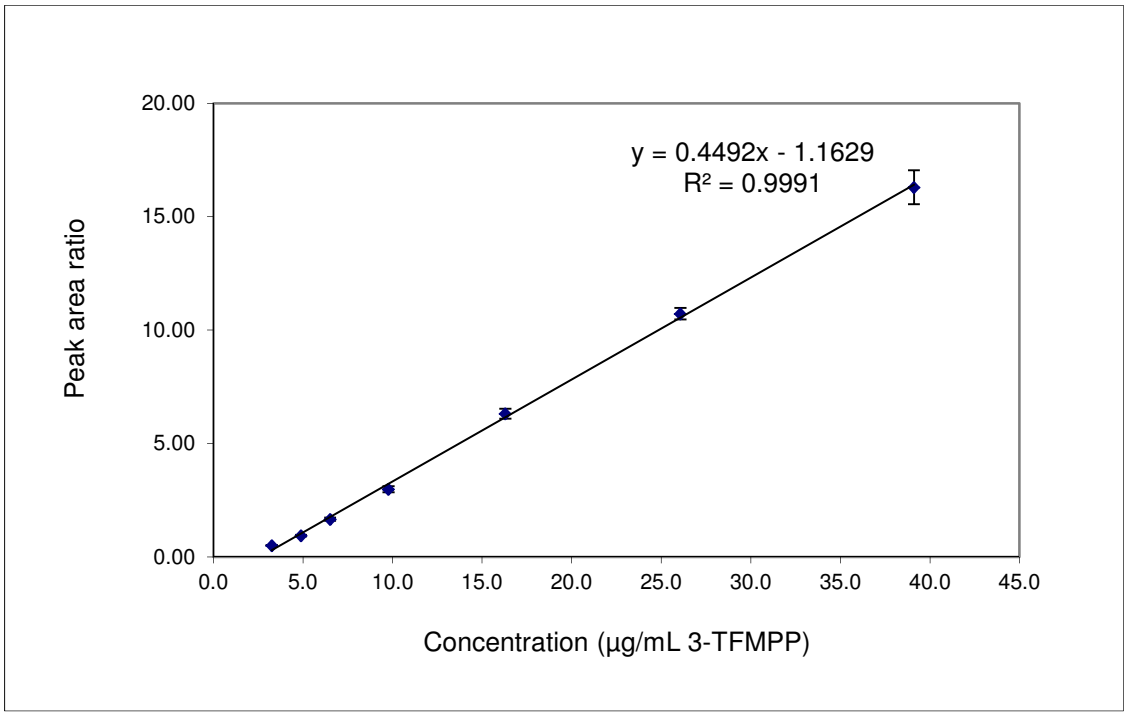


Figure 7.1 Calibration graph for 3-TFMPP.

A visual inspection of the calibration graphs (Appendix 9) indicated a linear trend for all the drugs. According to ICH (2005) and Thompson et al. (2002) linearity should be tested. The need and how to establish linearity was discussed in Chapter 2. Consequently, analysis of regression coefficients and residuals was conducted (section 7.6.2.8) to verify whether linearity was achieved.

7.3.1.2 Testing for linearity

7.3.1.2.1 Analysis of regression coefficients

The regression coefficients (R^2) are shown in Table 7.1 and were used to determine the linearity of the calibration graphs (Appendix 9).

Table 7.1 Testing for linearity: regression coefficients, R^2.

Analyte	**R^2**	**n[1]**
2-FPP	0.9992	8
3-FPP	0.9989	7
4-FPP	0.9998	8
2-TFMPP	0.9991	8
3-TFMPP	0.9991	7
4-TFMPP	0.9989	7
BZP	0.9999	7
DBZP	0.9996	8
MBZP	0.9990	7
3-CPP	0.9986	8
4-MePP	0.9990	6
MDMA	0.9995	7
Methamphetamine	0.9990	8
Caffeine	0.9975	7
Cocaine	0.9968	7
Diazepam	0.9946	6
Dapoxetine	0.9970	7
Dextromethorphan	0.9997	6
Nicotinamide	0.9998	8
Ephedrine	0.9991	5

[1]n is the sample size: number of calibration standards analysed

According to Chan et al., (2010) an $R^2 > 0.99$ implies linearity is achieved. This was evidenced by the correlation coefficients which were $R^2 > 0.995$ for all the analytes. This implied a highly linear trend. It was discussed in Chapter 2 section 2.5.1.1.2) that use of correlation coefficients on their own to test for linearity is inadequate and might give misleading results (Thompson et al., 2002; Rambla-Alegre et al., 2012). As such, the analysis of residuals reported below was conducted to further test for linearity.

7.3.1.2.2 Analysis of residuals

An exemplar of the residual plots is given in Figures 7.2 and 7.3 for 2-FPP and nicotinamide.

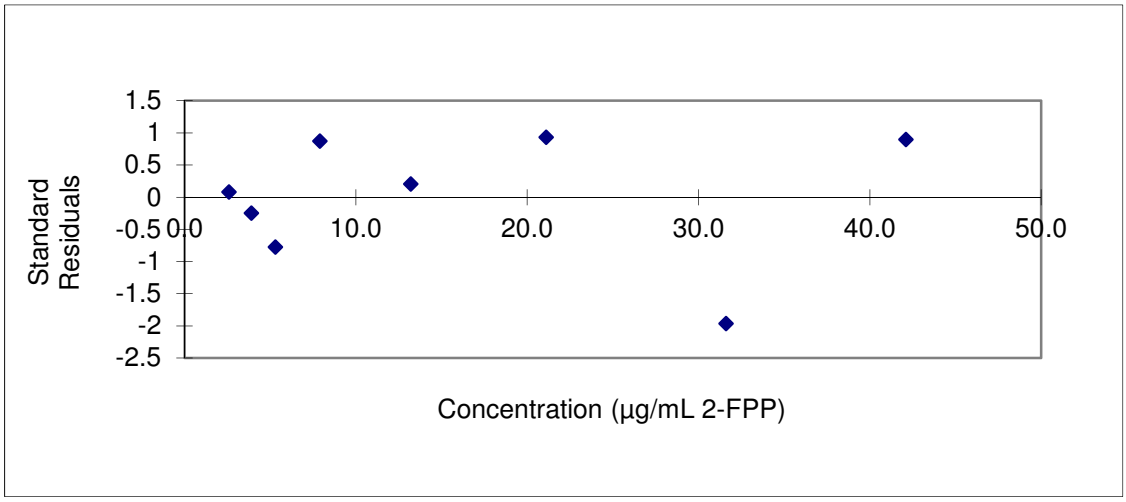


Figure 7.2 Residual analysis to test for linearity of the 2-FPP calibration plot.

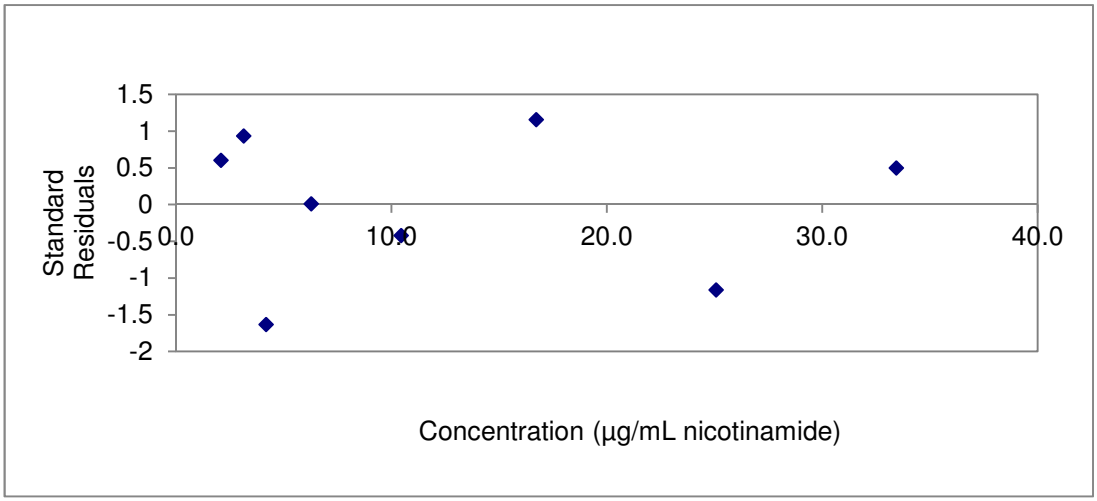


Figure 7.3 Residual analysis to test for linearity of the nicotinamide calibration plot.

A visual analysis of the plots of the residuals was conducted and the distribution patterns were observed to have no particular trend for all the analytes as evidenced by Figures 7.2 and 7.3. It was discussed in Chapter 2 that if the distribution pattern of the residuals is random it indicates a linear trend. Furthermore, the deviations from linearity are not statistically significant if the sum of the residuals has a mean of zero (Thompson, 2005; Van Loco et al., 2002). The mean of the residuals was calculated and is given in Table 7.2. The mean values of all the analytes were in the range of -0.0017 to +0.0014 (Table 7.2),

these can therefore be approximated as zero. Therefore, both the distribution patterns observed and the mean are in agreement with the criteria for linearity. As such, the residuals are random indicating the calibration plots are linear. This confirms the observations made using correlation coefficients. To further statistically confirm the randomness of the residuals the Runs test was conducted. The results are also given in Table 7.2.

Table 7.2 Testing for linearity: Residuals randomness.

	Mean[1]	**RUNS TEST**						
		$n_{(-)}$	$n_{(+)}$	N	No. of Runs	Z	*p* Asymp. (2-tailed)	*p* Exact (2-tailed)
2-FPP	0.0013	3	5	8	5	0.00	1.00	1.00
3-FPP	-0.0014	3	4	7	5	0.06	0.95	0.66
4-FPP	0.0000	4	4	8	6	0.38	0.70	0.74
2-TFMPP	0.0000	3	5	8	5	0.00	1.00	1.00
3-TFMPP	0.0000	4	3	7	4	0.00	1.00	1.00
4-TFMPP	0.0000	4	3	7	4	0.00	1.00	1.00
BZP	0.0000	3	4	7	6	0.91	0.36	0.26
DBZP	0.0000	5	3	8	6	0.62	0.54	0.43
MBZP	0.0014	4	3	7	4	0.00	1.00	1.00
CPP	0.0000	5	3	8	4	-0.21	0.84	0.71
MePP	0.0000	3	3	6	4	0.00	1.00	1.00
MDMA	-0.0014	3	4	7	6	0.91	0.36	0.26
Methamphetamine	0.0000	4	4	8	4	-0.38	0.70	0.74
Caffeine	0.0014	5	2	7	4	0.00	1.00	1.00
Cocaine	0.0000	3	4	7	4	0.00	1.00	1.00
Diazepam	0.0000	2	4	6	3	-0.18	0.86	0.60
Dapoxetine	0.0000	4	3	7	4	0.00	1.00	1.00
Dexetromethorphan	-0.0017	3	3	6	5	0.46	0.65	0.60
Nicotinamide	0.0000	3	5	8	7	1.44	0.15	0.11

[1]Test value = mean, $n_{(-)}$ is observations < Test value and $n_{(+)}$ is observations ≥ Test value, N is the total number of observation (number of residuals).

The total number of runs observed R, relative to the sample sizes N, for all the analytes indicates randomness (section 3.2.13). This is also evidenced by the probability ($p > 0.05$).

According to Sprent and Smeeton (2007) and (Chatterjee and Simonoff, 2012) too few or too many runs imply a departure from randomness and if randomness is not achieved, the probability is less than the significance level, i.e., $p < 0.05$ (significance level $\alpha_{/2} = 0.05$). Hence, the residuals are random. This confirms the observations made using the mean and visual inspection.

The application of residual analysis to test for linearity is commonly used in research and Byrska et al. (2010), Rambla-Alegre et al. (2012), Van Loco et al. (2002) and Vorce et al. (2008) successfully applied residual analysis in addition to correlation coefficients to verify linearity in their studies. It can therefore be said this test is complimentary to use of correlation coefficients. It can therefore be concluded that the calibration plots are linear and any departures from linearity are insignificant and due to random chance.

7.3.1.3 Linearity ranges and method detection limits

The linearity data (linearity range and working range), limit of detection (LOD) and limit of quantitation (LOQ) corresponding to the calibration graphs (Appendix 9) were determined and are shown in Table 7.3. In addition, the concentration range in which linearity was achieved is also given in terms of the linearity and working range (ICH, 2005; Rambla-Alegre et al., 2012). The detection limits indicated that the method has good sensitivity (ng range) for all the drugs, with detection limits in the range 0.26 - 1.95 x 10^{-3} µg/mL free base on column. The quantitation limits for all the drugs were in the range 0.77 - 5.90µg/mL free base on column. As such, the drugs can be reliably detected and analysed qualitatively and quantitatively in various concentration ranges, even for samples containing very low concentrations.

Table 7.3 Calibration results: linearity range and method detection limits.

Analyte	**Linearity range (µg/mL free base on column)**		**Method detection limits (free base on column)**	
	Linearity range	***Working range***	***LOD (x 10^{-3}* µg/mL)**	***LOQ* (µg/mL)**
2-FPP	2.6 – 42.1	3.9 – 31.6	0.82 ± 0.037	2.48 ± 0.113
3-FPP	4.6 – 49.5	6.2 – 37.1	0.93 ± 0.049	2.79 ± 0.150
4-FPP	1.3 – 41.7	3.9 – 41.7	0.36 ± 0.022	1.09 ± 0.065
2-TFMPP	2.5 – 40.0	3.8 – 40.0	0.97 ± 0.044	2.93 ± 0.133
3-TFMPP	3.3 – 39.1	4.9 – 39.1	0.85 ± 0.040	2.58 ± 0.120
4-TFMPP	3.8 – 40.8	7.7 – 30.6	1.16 ± 0.10	3.50 ± 0.317
BZP	3.8 – 40.7	4.0 – 40.0	0.28 ± 0.014	0.86± 0.043
1.4DBZP	1.0 – 32.0	4.0 – 32.0	0.45 ± 0.005	1.36 ± 0.015
MBZP	3.1 – 33.4	4.2 – 33.4	0.83± 0.046	2.54 ± 0.140
4CPP	2.9 – 47.1	5.9 – 47.1	1.18 ± 0.059	3.58 ± 0.179
4-MePP	3.8 – 40.0	6.0 – 40.0	1.01 ± 0.008	3.06 ± 0.023
MDMA	1.0 – 16.2	2.0 – 16.2	0.26 ± 0.015	0.77 ± 0.069
Methamphetamine	1.0 – 33.3	4.2 – 33.3	0.69 ± 0.040	2.10 ± 0.122
Caffeine	3.8 – 30.0	5.0 – 30.0	1.10 ± 0.13	3.35 ± 0.40
Cocaine	3.7 – 39.3	6.0 – 29.5	1.74 ± 0.11	5.26 ± 0.340
Diazepam	5.0 – 30.0	7.5 – 30.0	1.95 ± 0.035	5.90 ± 0.105
Dapoxetine	2.3 – 37.5	7.0 – 28.1	1.62 ± 0.062	4.90 ± 0.189
Dextromethorphan	2.0 – 31.4	2.5 – 30.0	0.52 ± 0.003	1.5 ± 0.010
Nicotinamide	2.1 – 33.4	5.0 – 33.4	0.32 ± 0.016	0.98 ± 0.048
Ephedrine	2.5 – 20.2	3.0 – 20.0	0.56 ± 0.030	1.71 ± 0.09

It was found that on average the common working range was 5 - 35.0µg/mL free base on column. It was shown that street samples containing piperazine drugs have relatively high drug content for example 50 - 200 mg BZP, 90 110mg 3-CPP (Kenyon et al., 2010). Hence, the observed detection, quantitation limits and working range are ideal as since they are sensitive enough (µg range) to be applicable to analysis of street samples. Furthermore, the wide working range is advantageous since the method is to be used for analysis of street samples as these drugs are expected to be found in various combinations and dosages with 3-TFMPP and 4-FPP (Yeap et al., 2010, Vorce et al., 2008). In addition, the wide range implies they can be simultaneously analysed since it encompasses the individual working ranges of the drugs.

7.3.2 ACCURACY AND PRECISION

The simulated street samples and the standards gave similar results with a correlation of 100 ± 1.5%. Hence any differences were deemed statistically insignificant and as such either could be applied in this study. The results for accuracy and precision determined using the standards are tabulated (Table 7.4). In accordance with section 7.2.10 (Data analysis) the recovery (%) is given as a measure of accuracy and the standard deviation (SD) and relative standard deviations (%RSD) as a measure of precision. In addition the SD is also given show the error in the accuracy determinations.

Table 7.4 Accuracy and Precision.

	Accuracy	**Precision**			
	%Recovery (n = 3)	***Repeatability (intra-day), n = 6***		***Intermediate (inter-day), n = 9***	
		SD	**%RSD**	**SD**	**%RSD**
2-FPP	98.7% ± 0.03	0.023	1.40	0.10	1.72
3-FPP	100.7% ± 0.04	0.017	1.23	0.31	1.41
4-FPP	100.4% ± 0.03	0.024	1.15	0.10	1.46
2-TFMPP	100.1% ± 0.02	0.016	1.15	0.06	1.45
3-TFMPP	99.23% ± 0.24	0.090	1.38	0.34	1.50
4-TFMPP	98.8% ± 0.02	0.020	0.83	0.14	1.25
BZP	100.02% ± 0.01	0.090	1.10	0.09	1.10
1.4DBZP	100.3% ± 0.04	0.012	1.47	0.14	1.25
MBZP	99.6% ± 0.03	0.010	1.43	0.07	1.24
3-CPP	101.7% ± 0.02	0.037	1.90	0.23	1.73
4-MePP	99.1% ± 0.02	0.003	0.62	0.50	1.41
MDMA	100.2% ± 0.02	0.040	1.31	0.04	1.17
Methamphetamine	101.3% ± 0.02	0.009	1.86	0.63	1.44
Caffeine	98.2% ± 0.03	0.160	1.70	0.23	1.69
Cocaine	100.0% ± 0.01	0.010	1.31	0.66	1.40
Diazepam	99.4% ± 0.01	0.130	1.32	0.32	1.53
Dapoxetine	99.4% ± 0.02	0.018	1.88	0.22	1.73
Dextromethorphan	99.8% ± 0.09	0.020	1.48	0.08	1.28
Nicotinamide	100.9% ± 0.03	0.014	1.13	0.06	1.18
Ephedrine	99.3% ± 0.01	0.006	1.04	0.94	1.39

The results (Table 7.4) show that accuracy on average was 99.8% (range 98.5 - 102. 9%). Precision for all the drugs was less than 2%, with RSD in the range of 0.83 - 1.90% for repeatability as a measure of intra-day precision. The range was 1.10 -1.73% for intermediate precision also known as inter-day precision (ICH, 2005). 4-TFMPP (RSD = 0.83%) and 3-CPP (RSD = 1.90%) showed the lowest and highest deviations respectively. The criterion for achieving acceptable accuracy was reported as 98 - 102% for an analyte ratio of 0.1 (Gonzalez and Herrador, 2007). For precision, the critera was stated as RSD < 2.0% (Eurachem, 1998; Gonzalez and Herrador, 2007). Therefore, it is evident from the results that good method accuracy and precision were achieved for all the analytes.

Comparison of the validation results with the work of other researchers was rather limited as only a few research studies has been conducted on these drugs, especially for 4-FPP. However, Byrska et al. (2010) in a study on determination of piperazines developed and validated a GC-MS method for qualitative analysis. The study investigated BZP, 3-CPP, MBZP, MePP and 3-TFMPP. The LODs obtained were in the range 2.5 - 5.0µg/mL (this was also stated as mg/mL), hence the sensitivity of their method is lower than that observed with the method in this research (LODs were 0.26 – 1.95 x 10^{-3}µg/mL. Whilst their methods and the one for this study can be applied for qualitative investigation, the analysis of street samples this would not be a limitation due to relatively high drug content (mg/tablet) but the method might face a challenge in analysis of samples with very low concentrations, e.g. if the method is extrapolated to toxicological investigations. Vorce et al. (2008) obtained comparable linearity data for BZP and 3-TFMPP; R^2 = 0.9991 and 0.9990 respectively. Their accuracy data (97.8% for BZP and 101.4% for 3-TFMPP) was comparable to those in this study 100.2% and 99.23%. However, their method was less sensitive with detection limits in the range of 0.1mg/L for both BZP and 3-TFMPP. It should be taken into account that the method was for analysis of drugs of abuse in a biological matrix, urine and not street samples, or the combination of analytes used in this study. Boumrah et al. (2014) developed a method for piperazines (BZP, TFMPP, CPP, MeOPP, MDBP) and amphetamines (MDMA, MDA, MDEA, MBDB) in seized drugs. They reported LODs in the range 71.5 - 116µg/mL for the piperazines. Whilst like this study the method was for street samples as for this study, the LODs were lower. However, they would still be applicable to street samples as these contain high dosages of drugs (section 1.2.2, Table 1.3). The limitation is the range/number of drugs investigated was less including the congeners in street samples. Also the common diluents were not investigated,

e.g., caffeine nor were isomers of TFMPP. In addition, the authors recommended derivatisation to improve resolution. Furthermore other validation parameters were not reported unlike this study which reports more holistic data (Kuleya et al., 2014).

7.3.3 COMPARISON OF TOTAL ION CHROMATOGRAM (TIC) AND EXTRACTED ION DATA (M/Z DATA)

A comparison of linearity data derived using TIC with that from extracted ion data was conducted, so as to verify if the results are comparable. This is especially important if there are substances which co-elute in the sample, for example in this study, 3-TFMPP and MDMA, DBZP and dextromethorphan. In addition, new additives and sample combinations are increasingly appearing on the street market. Consequently, there is a possibility of co-elution of such substances on analysis. This limitation is overcome by use of extracted ions characteristic to the drug only (de Boer et al., 2001; Takahashi et al., 2009). The linearity results using extracted ion data are shown in Tables 7.5 and 7.6.

The data derived from total ion chromatograms (TICs) of the analytes (Tables 7.1 - 7.4) in comparison to the extracted ion (Tables 7.5 and 7.6) show similar linearity, LOD, LOQ, accuracy and precision trends. For example, for 3-TFMPP, LOD = 0.85 x 10^{-3}µg/mL free base on column, LOQ = 2.58µg/mL free base on column for TIC data, whilst for extracted ion data, LOD = 0.72 x 10^{-3}µg/mL free base on column, LOQ = 2.18µg/mL free base on column. In addition, the regression coefficients were comparable. This suggested that either method could be used without any significant differences in the results.

Statistical analysis by ANOVA (2-way without replication) was conducted to determine whether the results from TIC data were statistically significantly different to those from the extracted ion data. The statistical results on LOD and accuracy data show that there was no significance difference; $F(1) = 0.79$, $p = 0.39$, Fcrit = 4.45 for LOD and $F(1) = 3.39$, $p = 0.08$, Fcrit = 4.45 at 5% significance level. The $p < 0.05$ the significance level and The F value observed is less than the critical value. This implies statistically the TIC and m/z show similar results and any deviations are due to chance.

Table 7.5 Calibration results using extracted ion (mz) data.

Substance	**Ion used (m/z)**	**R^2**	**n**	**Linearity** (µg/mL free base **on column)**		**Method detection limits (free base on column)**	
				Linearity range	***Working range***	***LOD* (x 10^{-3}µg/mL)**	***LOQ* (µg/mL)**
2-FPP	138	0.9991	8	2.6 – 42.1	3.9 – 31.6	0.82 ± 0.016	2.5 ±0.049
3-FPP	138	0.9988	7	4.6 – 49.5	6.2 – 37.1	0.96 ± 0.017	2.92 ± 0.050
4-FPP	138	0.9998	8	1.3 – 41.7	3.9 – 41.7	0.36 ± 0.008	1.08 ± 0.023
2-TFMPP	188	0.9985	8	2.5 – 40.0	3.8 – 40.0	1.04 ± 0.017	3.15 ± 0.220
3-TFMPP	188	0.9993	7	3.3 – 52.1	4.9 – 39.1	0.72 ± 0.009	2.18 ± 0.027
4-TFMPP	188	0.9985	7	3.8 – 40.8	7.7 – 30.6	1.22 ± 0.036	5.40 ± 0.108
BZP	91	0.9999	7	3.8 – 40.7	4.0 – 40.0	0.29 ± 0.003	0.87 ± 0.010
DBZP	91 and 266	0.9996	8	1.0 – 32.0	4.0 -32.0	0.44 ± 0.019	1.33 ± 0.0573
MBZP	105	0.9989	7	3.1 – 33.4	4.2 – 33.4	0.86 ± 0.003	2.62 ± 0.003
3-CPP	154	0.9987	8	2.9 – 47.1	5.9 – 47.1	1.15 ± 0.011	3.48 ± 0.050
4-MePP	134	0.9989	6	7.5 – 40.0	12.5 – 40.0	1.04 ± 0.03	3.18 ± 0.100
MDMA	135	0.9996	7	1.0 – 16.2	2.0 – 16.2	0.24 ± 0.001	0.73 ± 0.004
Methamphetamine	91	0.9962	8	1.0 – 33.3	4.2 – 33.3	0.67 ± 0.004	2.04 ± 0.013
Caffeine	194	0.9938	7	3.8 – 30.0	5.0 – 30.0	1.74 ± 0.057	5.26 ± 0.176
Cocaine	82	0.9959	7	3.7 – 39.3	6.0 – 29.5	1.97 ± 0.019	5.98 ± 0.056
Diazepam	256	0.9963	6	5.0 – 30.0	7.5 – 30.0	1.62 ± 0.025	4.90 ± 0.078
Dapoxetine	134	0.9967	7	2.3 – 37.5	7.0 – 28.1	1.69 ± 0.074	5.11 ± 0.223
Dextromethorphan	271	0.9995	6	2.0 – 31.4	2.5 – 30.0	0.58 ± 0.003	1.74 ± 0.010
Nicotinamide	122	0.9998	8	2.1 – 33.4	5.0 – 33.4	0.33 ± 0.003	1.00 ± 0.009

Table 7.6 Accuracy and Precision –determined from extracted ion data.

Substance	**Accuracy**		
	%Recovery (Mean, n = 3)	***SD***	***%RSD***
2-FPP	99.8 ± 0.034	0.014	1.37
3-FPP	100.3 ± 0.038	0.014	1.44
4-FPP	99.8 ± 0.027	0.011	1.08
2-TFMPP	99.5 ±5 0.035	0.014	1.40
3-TFMPP	100.1 ± 0.020	0.008	0.79
4-TFMPP	99.1 ± 0.021	0.008	0.85
BZP	100.9 ± 0.020	0.007	0.74
DBZP	99.6 ± 0.021	0.011	1.14
MBZP	100.2 ± 0.026	0.011	1.06
3-CPP	102.8 ± 0.021	0.008	0.81
4-MePP	98.5 ± 0.015	0.006	0.62
MDMA	100.0 ± 0.029	0.012	1.18
Methamphetamine	101.0 ± 0.032	0.013	1.25
Caffeine	102.9 ± 0.042	0.017	1.62
Cocaine	102.8 ± 0.025	0.020	1.92
Diazepam	100.5 ± 0.048	0.002	1.91
Dapoxetine	100.7 ± 0.010	0.004	0.39
Dextromethorphan	100.9 ± 0.005	0.009	0.88
Nicotinamide	100.4 ± 0.028	0.011	1.11

It has been discussed that co-elution was observed between MDMA and 3-TFMPP; DBZP and dextromethorphan, making selective determination of these analytes difficult if they co-exist in the same sample. Furthermore, it was proposed that this could be overcome through use of extracted ions as their mass spectra are sufficiently different to discriminate between them (Chapter 4). As such, it can be confirmed that either TIC or extracted ion data can be used in the method. Therefore, the extracted ions for 3-TFMPP (m/z 188), MDMA (m/z135), DBZP (m/z 91 or 266) and dextromethorphan (m/z 271) can be employed in analysis of these substances. Since drugs often exist as combinations in street samples (Yeap et al., 2010) this will be advantageous.

7.3.4 METHOD ROBUSTNESS

7.3.4.1 Robustness using different analysts

The chromatographic profiles obtained by two different analysts working independently are shown in Figures 4 and 5.

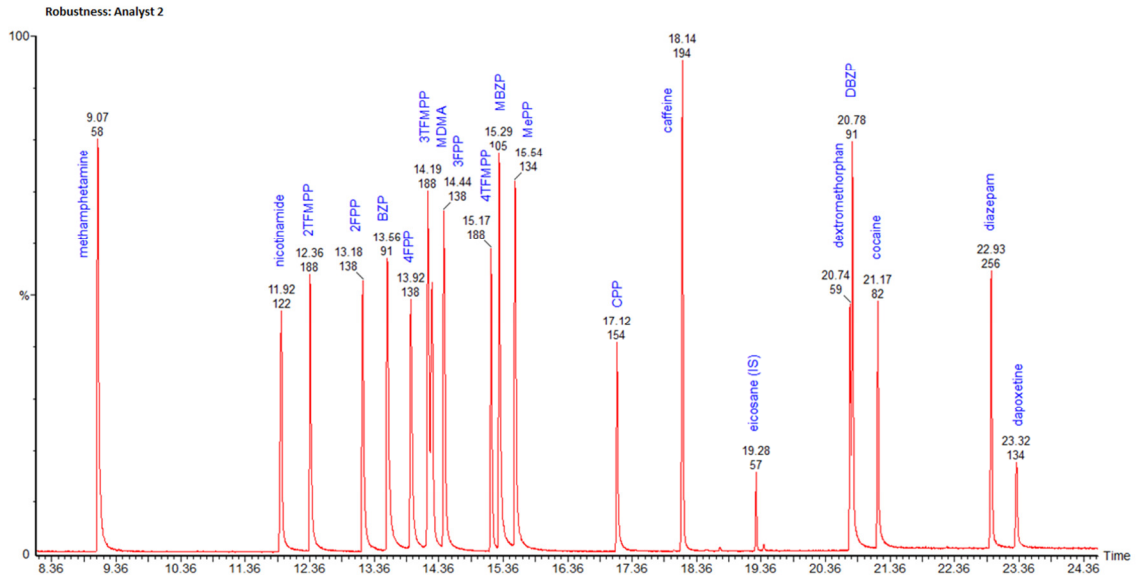


Figure 7.4 Total ion chromatogram of mixed drug standard: Analyst 2.

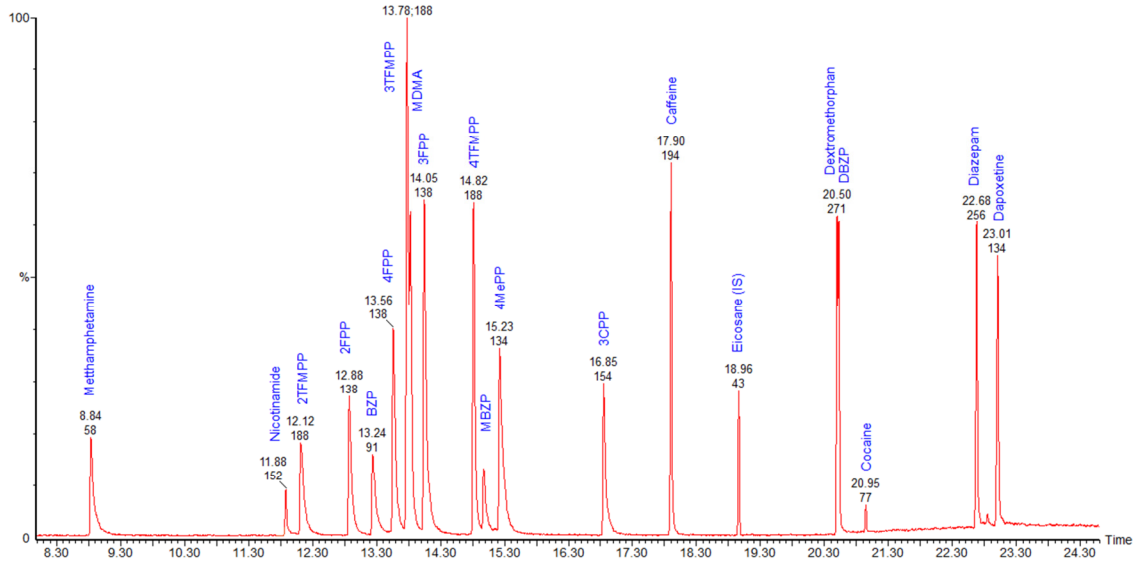


Figure 7.5 Total ion chromatogram of mixed drug standard: Analyst 1.

The chromatographic profiles generated by different analysts (Figures 7.4 and 7.5) were similar in terms of the order of elution of the analytes and peak shapes. This was confirmed

by the qualitative data; retention times, relative retention times and retention indices. The data for 2 is presented in Table 7.7 (derived from Figure 7.4) together with that for analyst 1 (extracted from Table 7.8) for comparison.

Table 7.7 Method robustness: Comparison of GC-MS data generated by different analysts.

	Analyst 2			Analyst 1		
Compound	**Rt/mins**	**RRT**	**RI (±0.37%)**	**Rt/mins**	**RRT**	**RI**
Methamphetamine	9.07	0.470	1199	8.84	0.466	1201
Nicotinamide	11.92	0.618	1392	11.88	0.627	1412
2-TFMPP	12.36	0.641	1421	12.12	0.639	1427
2-FPP	13.18	0.684	1477	12.88	0.679	1479
BZP	13.56	0.703	1503	13.24	0.698	1505
4-FPP	13.92	0.722	1527	13.56	0.715	1527
3-TFMPP	14.19	0.736	1545	13.78	0.727	1542
MDMA	14.25	0.739	1549	13.83	0.729	1545
3-FPP	14.44	0.749	1562	14.05	0.741	1548
4-TFMPP	15.17	0.787	1611	14.82	0.782	1614
MBZP	15.29	0.793	1619	14.98	0.790	1624
4-MePP	15.54	0.806	1636	15.23	0.803	1642
3-CPP	17.12	0.888	1743	16.85	0.889	1754
Caffeine	18.14	0.941	1822	17.90	0.944	1867
Dextromethorphan	20.74	1.076	2210	20.50	1.081	2208
DBZP	20.78	1.078	2216	20.53	1.083	2212
Cocaine	21.17	1.098	2275	20.95	1.105	2268
Diazepam	22.93	1.189	2537	22.68	1.196	2494
Dapoxetine	23.32	1.210	2596	23.01	1.214	2179
Eicosane (IS)	19.28	1.000	1993	18.96[1]	1.000	2006[1]

[1]To verify the accuracy of the calculations, the accuracy of Retention index (RI) calculations was determined. The expected retention index of eicosane is 2000, the actual value obtained was 1993 hence error in experimental RI values = 0.35%. Consequently the calculations were deemed highly accurate.

A comparative analysis of analyst 1 and analyst 2 results (Table 7.7) by ANOVA (2 factor without replication) indicated that there were no significant differences in the results

obtained by the 2 analysts. $F(1, 24) = 2.20$, $p = 0.16$, Fcrit = 4.41, for relative retention time $F(19,20) = 3697$, $p < 0.001$, Fcrit = 2.14 and for retention indices $F(1,18) = 0.81$, $p < 0.38$, Fcrit = 4.41. Consequently, any variations observed were due to chance. Therefore, the method can be deemed robust as it is able to consistently give similar results independent of changes in the analyst.

7.3.4.2 Robustness using different instruments

To further test for robustness analysis was conducted on a different CG-MS instrument (Shimadzu GC-MS). The total ion chromatogram generated is given in Figure 7.6. The peak profile in terms of order of elution, peak shape, retention time and resolution were observed to be similar with those from the Perkin Elmer instrument routinely used for this research (Figure 7.5 and Table 7.8). The relative retention times of the 17 analytes shown in Appendix 11 were statistically evaluated by ANOVA (single factor) and t-test which indicated that there were no significant differences in the results obtained by the 2 instruments. $F(1, 17) = 0.38$, $p = 0.54$, Fcrit = 4.15. For the t-test (2-tailed) $t(16) = -14.25$, $p = 1.7 \times 10^{-10}$, $t$crit = 2.12. Consequently, it was concluded that the method is unaffected by the type of GC-MS used and is robust and therefore capable of transferring the method to different instruments and analysts. This is very useful as it means the method can be used under different laboratory conditions.

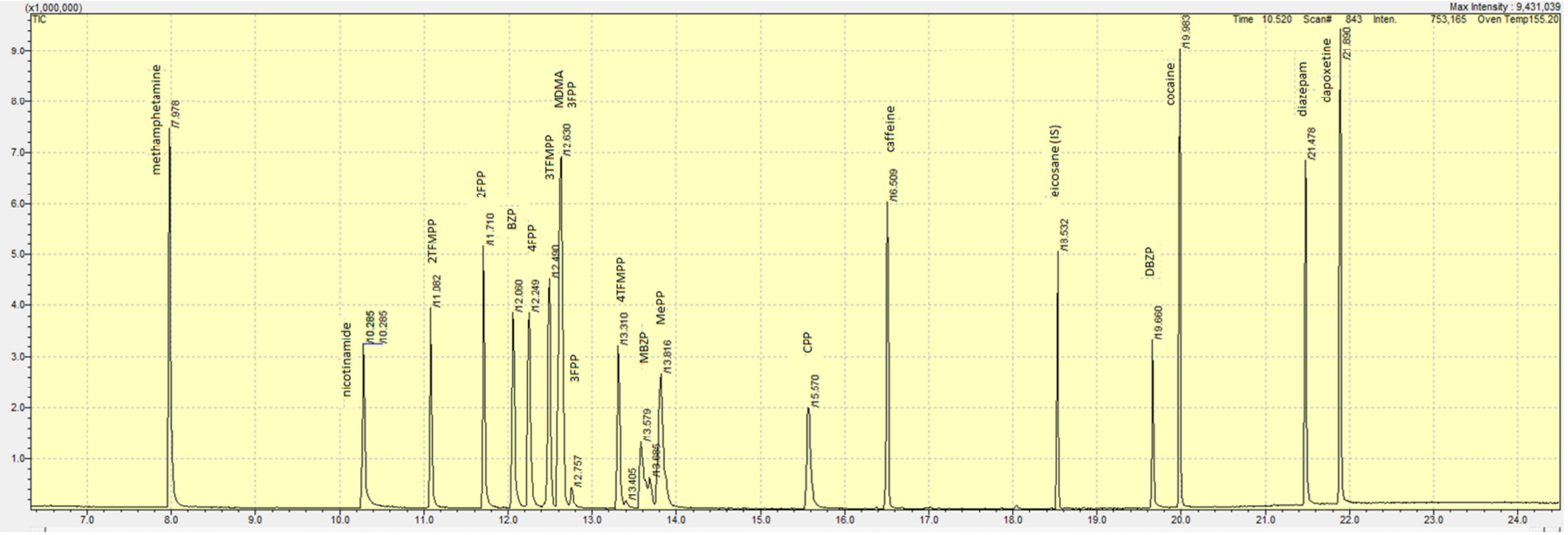


Figure 7.6 Test for robustness: Total ion chromatogram generated on a different GC-MS instrument (Shimadzu GC-MS).

7.3.5 METHOD APPLICATION: CONFIRMATION OF DRUG IDENTITY PARAMETERS

7.3.5.1 GC-MS analytical profile of drugs

The chromatographic peak profile of the mixed drugs using the validated method is shown in Figure 7.5.The qualitative data for confirmation of drug identity is shown in Table 7.8. In the table RRT is relative retention time and RI is relative retention index.

Table 7.8 Qualitative data for confirmation of drug identity.

Compound	Retention time/mins	RRT	RI
Methamphetamine	8.84	0.465	1201
Nicotinamide	11.88	0.626	1412
2-TFMPP	12.12	0.638	1427
2-FPP	12.88	0.678	1479
BZP	13.24	0.698	1505
4-FPP	13.56	0.714	1527
3-TFMPP	13.78	0.726	1542
MDMA	13.83	0.729	1545
3-FPP	14.05	0.731	1548
4-TFMPP	14.82	0.781	1614
MBZP	14.98	0.789	1624
MePP	15.23	0.802	1642
CPP	16.85	0.888	1754
Caffeine	17.90	0.943	1867
Dextromethorphan	20.50	1.080	2208
DBZP	20.53	1.081	2212
Cocaine	20.95	1.104	2268
Diazepam	22.68	1.195	2494
Dapoxetine	23.01	1.212	2179
Eicosane (IS)	18.96	1.00	2006[1]

[1] Verification of Retention index (RI) calculations: The expected retention index of eicosane is 2000, the value obtained wass 2006, hence error in experimental RI values = 0.30%).

The chromatographic profile (TIC, Figure 7.5), retention times, relative retention times and retention indices (Table 7.8) for each analyte indicated that it is possible to isolate and

selectively analyse these substances by the method developed CDER (2004). According to USP (2011) system suitability tests were 5 – 6 consecutive injections are made should show an RSD < 2 if the variability between the measurements is acceptable. As such, the results indicated that the instrument and method was consistent and had good repeatability.

Inoue et al., (2008) discussed that if good selectivity is not achieved, then resolution between peaks is reduced and this in turn reduces accuracy in the results. It is evident that good specificity was achieved by the method, since the resolution between analytes was R > 2 for all the analytes except the co-eluting substances (Chapter 7). Hence, the method is confirmed to have achieved validation. As such these GC-MS chromatographic parameters were used to identify substances in later investigations (analysis of street samples, Chapter 8). In addition, the chromatographic profile shows that the method is able to simultaneously analyse for all the drugs investigated. It has been reported that confirmation of identification of a compound is also conducted through evaluation of mass spectra (de Boer et al., 2001; Kaur, 2010) and the ionisation energy which achieved the best spectral data was determined during optimisation (Chapter 6). Therefore, consideration will be given to the mass spectra that can be applied to the identification of the drugs under investigation as an identification tool.

7.3.5.2 Mass spectral data of (2, 3, 4) FPP and TFMPP isomers

A representation of mass spectra results is shown for (2, 3, 4) isomers of FPP and TFMPP in Figures 7.7 and 7.8 respectively. The mass spectra of all the drugs investigated are shown in Appendix 10. In addition the mass spectra ions are given in Table 7.9.

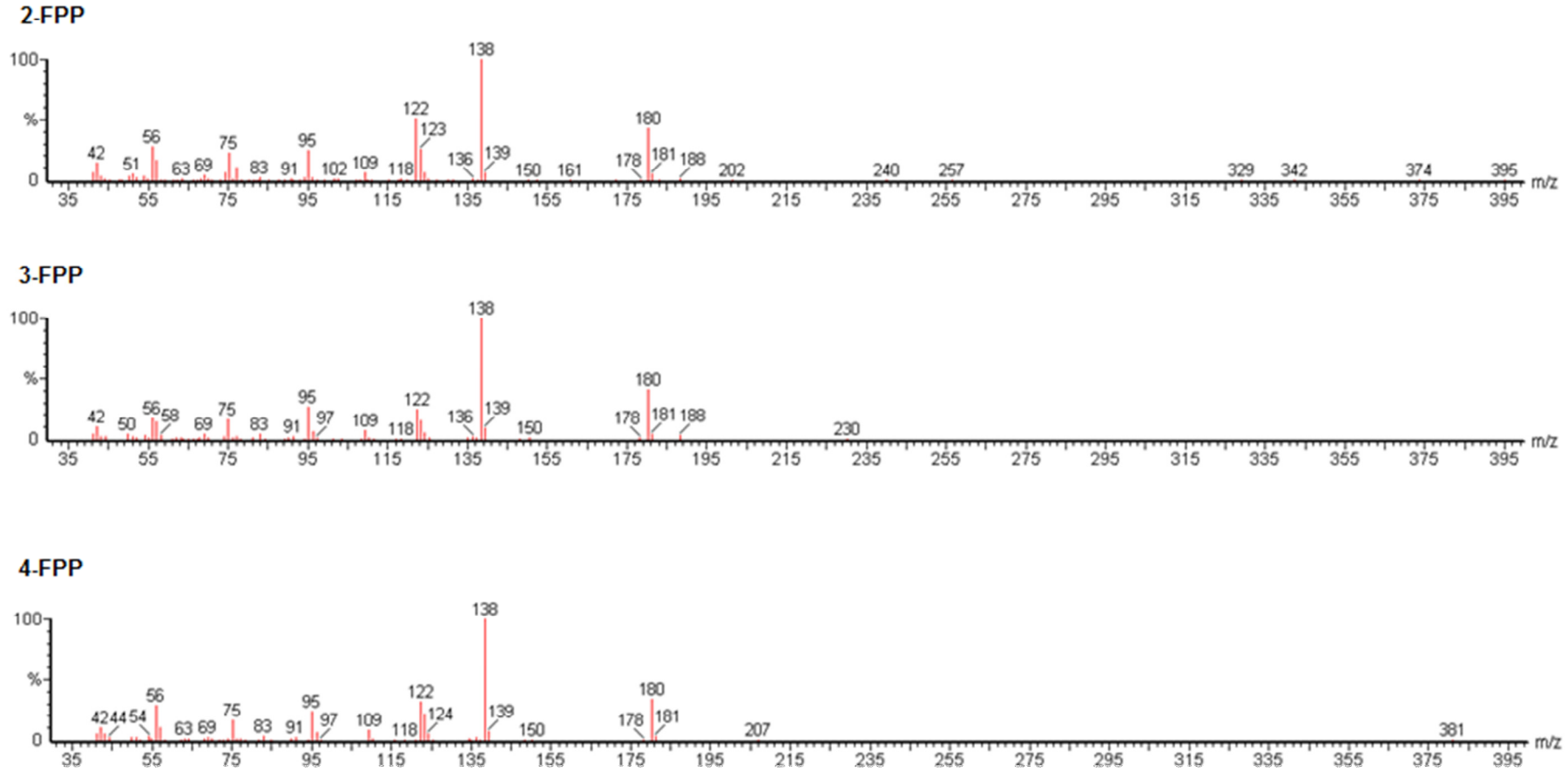


Figure 7.7 Mass spectra of (2, 3, 4) FPP positional isomers.

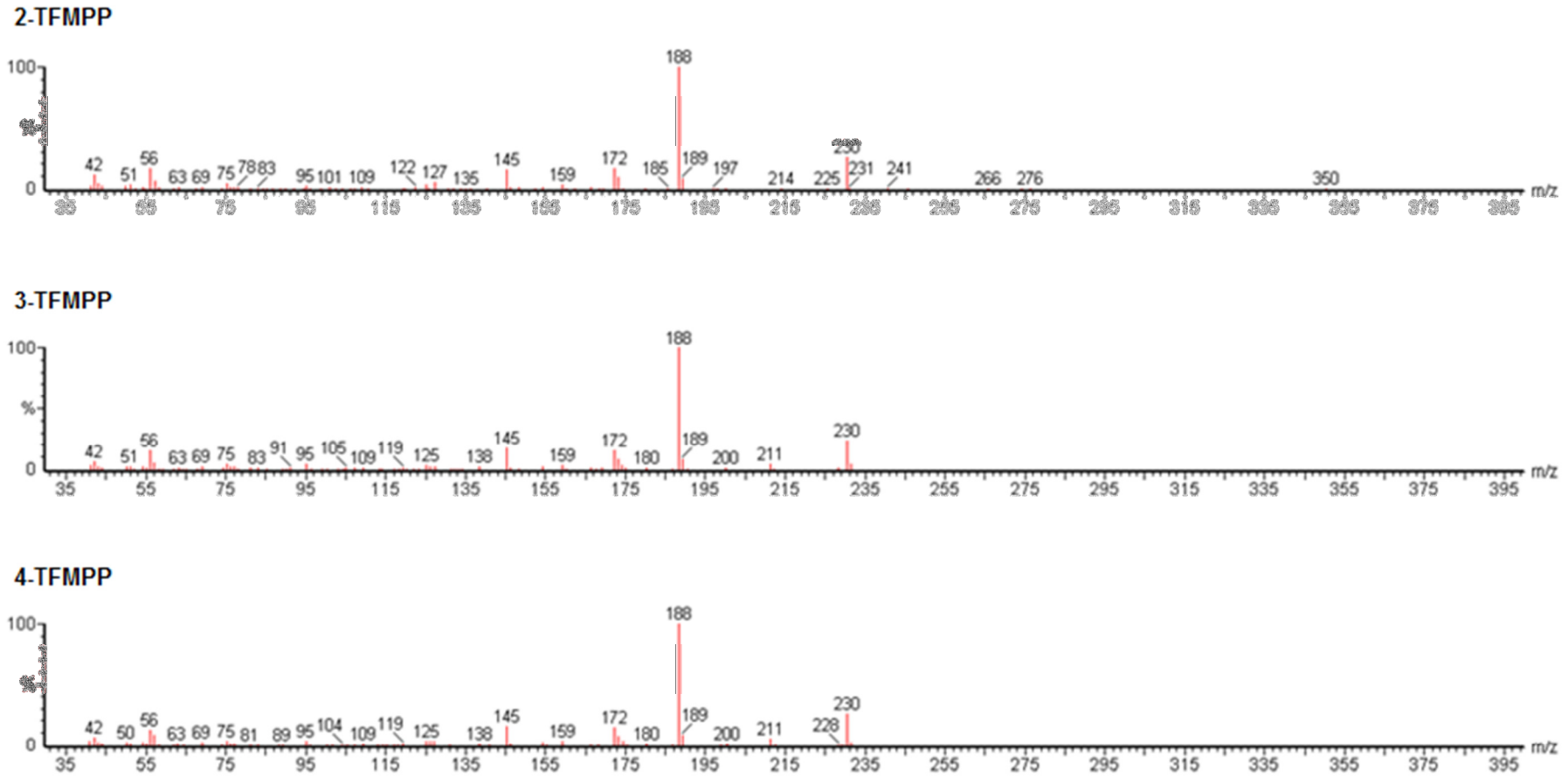


Figure 7.8 Mass spectra of (2, 3, 4) TFMPP positional isomers.

Table 7.9 Mass spectra data for the analytes

Substance	**Mass/ Da**	**Observed Principal ions, m/z(%intensity)**
2-FPP	180.2	138(100), 180(M^+, 25.69), 122(21.11), 123(14.62), 56(14.44), 95(11.26), 75 (7.63), 109(6.99), 77(4.30), 83(2.57), 91(1.58), 69(1.60), 150(0.61)
3-FPP	180.2	138(100), 180(M^+, 25.75), 122(17.45), 123(13.63), 56(13.16), 95(16.88), 75 (8.97), 109(6.41), 77(1.11), 83(1.84), 91(0.69), 69(1.59), 150(0.96)
4-FPP	180.2	138(100), 180(M^+, 24.81), 122(25.56), 123(16.37), 56(13.93), 95(18.98), 75 (8.93), 109(6.08), 77(1.06), 83(2.32), 91(0.85), 69(1.56), 150(1.15)
2-TFMPP	230.2	188(100), 230(M^+, 18.94), 56(19.48), 145(15.87), 172(19.33), 173(9.49), 174(1.42), 175(0.33), 189(13.82), 127(8.44), 95(3.74), 75(4.03), 109(1.15), 154(2.77), 159(4.60), 88(0.26)
3-TFMPP	230.2	188(100), 230(M^+, 20.26), 56(16.69), 145(18.10), 172(17.58), 173(8.81), 174(3.60), 175(1.13), 189(10.49), 127(2.55), 95(4.00), 75(3.85), 109(1.53), 154(1.34), 159(3.99), 88(0)
4-TFMPP	230.2	188(100), 230(M^+, 20.38), 56(15.01), 145(17.20), 172(16.70), 173(8.35), 174(3.29), 175(1.41), 189(9.16), 127(2.87), 95(4.59), 75(3.07), 109(1.42), 154(2.23), 159(4.79), 88(0.20)
BZP	176.3	91(100), 134(66.03), 56(29.59), 65(16.36), 176(M^+, 14.71), 120(5.45), 118(6.38), 119(2.80), 77(1.84), 85(12.51)
1.4DBZP	266.4	91(100), 120(22.96), 65(10.11), 135(1.51), 105(2.15), 106(2.04), 51(1.71), 77(1.38), 89(2.20), 63(1.39), 41(2.54), 175(20.24)
MBZP	190.3	105(100), 56(26.25), 148(29.45), 42(7.89), 77(13.86), 103(9.20), 85(15.11), 190(M^+, 2.63), 79(8.98), 134(8.95), 106(8.69), 118(4.54), 160(2.46), 65(2.35), 119(0.70), 120(0.25)
3-CPP	196.7	154(100), 156(32.26), 196(M^+, 28.15), 138(13.57), 111(12.56), 56(17.19), 75(11.17), 77(6.14),113(5.28), 119(4.30), 125(3.83), 63(2.56), 89(2.00), 117(1.56), 98(1.52), 104(1.49), 85(1.40), 91(1.21), 166(1.05)
4-MePP)	176.3	134(100), 176(M^+, 31.05), 91(21.05), 118(12.84), 135(9.96), 56(10.17), 65(8.80), 120(6.10), 77(3.65), 89(3.20), 119(14.46), 105(3.64), 146(0.84)
MDMA	193.2	58(100), 135(5.78), 77(6.13), 51(5.66), 55(0.41), 136(2.69), 105(1.87), 63(1.83) 193(M^+,0.31), 178(0.28), 56(6.12), 120(0.32), 148(0.27)

(+)Methamphetamine	149.2	58(100), 91(10.07), 65(5.89), 56(8.07), 42(5.77), 59(4.05), 51(2.42), 63(2.06), 77(1.94), 89(1.54), 134(1.73), 115(1.36), 92(0.79, 119(0.85), 148($(M-H)^+$, 0.26)
Caffeine	194.2	194(M^+, 100), 109(54.26), 55(37.07), 67(46.38), 82(30.96), 195(8.22), 42(17.22), 110(7.53)
Cocaine HCl	303.4 (base)	82(100), 182(68.51), 94(37.19), 77(37.69), 105(31.04), 42(23.56), 198(8.00), 303(M^+, 10.03), 122(9.19), 272(4.40), 51(10.89), 68(5.46), 59(4.83), 152(4.79), 166(2.98), 140(0.40)
Diazepam	284.7	256(100), 283(80.88), 284(M^+,61.43), 285(37.28), 257(46.66), 255(40.52), 258(33.90), 286(21.03)
Dextromethorphan HBr	271.4 (base)	59(100), 271(M^+, 49.77), 150(52.35), 270(27.98), 214 (27.88), 42(21.36), 171(0.83)
Nicotinamide	122.1	122(100, M^+), 78(98.51), 106(73.08), 51(52.01), 50(29.34), 52(26.38), 44(21.47), 123($(M+H)^+$, 6.40)
Dapoxetine	305.4	134(100), 115(11.57), 91(8.67), 58(9.53), 117(7.17), 127(5.23), 84(3.23), 77(3.00), 162(2.22), 160(1.98), 183(0.33), 177(0.30), 306($(M+H)^+$, 0.91,
Ephedrine	165.2	58(100), 77, 42, 105, 146, 117, 91

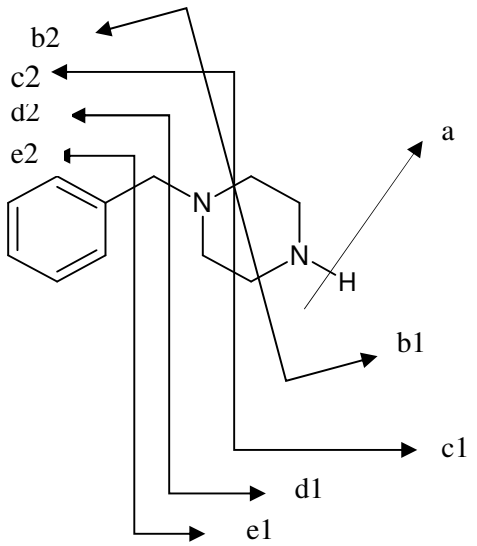
The results (Appendix 10 and Table 7.9) show that typical mass spectra, characteristic of the analytes were obtained for all the drugs (Moffat et al., 2011; NIST, 2014; de Boer et al., 2001; Takahashi et al., 2009). For all the analytes except DBZP and MDMA the molecular ion $(M)^+$ was observed which further aids in identification. This also confirms that the ionisation energy selected under optimisation (EI 70eV) is suitable. The absence of the molecular ion can be attributed to its fragmentation. For example, for DBZP the molecular ion is expected at m/z 266, however, cleavage of the benzyl groups results mainly in formation of the ion at m/z 91 (benzyl group) as evidenced by its principal abundance in the mass spectra of DBZP. The route of fragmentation for the cleavage is similar to that of BZP presented in Figure 7.29 (route d).

It has been reported the fragmentation pattern is characteristic of a compound and can be utilised in its identification (Kaur, 2010; Khopkar, 2012). Hence, the mass spectra and ions observed listed in Table 7.9 typically identify these compounds and can be utilised in future identification of unknowns. de Boer et al. (2001) in one of the earliest studies on piperazines and of recent UNODC (2013c) gives a limited data on the mass spectra of

piperazines and other drugs. UNODC gives the top 5 principal ions only, de Boer et al., on the other hand gives more extensive data but only for BZP, TFMPP and MeOPP. This study brings in additional spectral data and study gives more comprehensive mass spectra by identifying ion m/z values for the piperazines and also all the congeners. Furthermore, the route of fragmentation and the corresponding fragment structures for benzylpiperazines and phenylpiperazines represented by BZP and 3-TFMPP respectively were reported (Figures 7.10 – 7.11). This aids in understanding the processes involved.

It is evident that the isomers of FPP and TFMPP, Figures 7.7 and 7.8 respectively have similar mass spectra. The fragmentation pattern and ions observed were similar. The ions at mz 180(100), 230 (M^+), 122, 56 and 95 were observed for FPP. The ions 188(100), 230(M^+), 56, 145, 172 were observed for TFMPP. A review of the mass spectral ions in Table 7.9 confirmed this observation as the intensities of the ions were not sufficiently different enough to be used for identification. This is typical of isomers. It was established that positional isomers show similar chemical properties, hence they cannot be differentiated on the basis of mass spectra only (Maher et al., 2009; Takahashi et al., 2009). It was earlier discussed that the total ion chromatogram (Figure 7.5) and qualitative data (Table 7.9) showed that the isomers were well resolved from each other with resolution, R > 2.0. Consequently, even though on the basis mass spectra the FPP and TFMPP cannot be identified indiscriminately, with this method the isomers can be distinguished according to their retention time, relative retention times and mass indices.

The structures for the ion fragments given in Table 7.9 are proposed for phenylpiperazines and benzylpiperazines. Also proposed are the corresponding routes of fragmentation (de Boer et al., 2001, Inoue et al, 2004). BZP (Figures 7.9 and 7.10) and 4-TFMM (Figures 7.11 and 7.12) are used as exemplars respectively.



Route	Fragment	Structure
a	176M^+)ion	I
b2	134	II
c1	56	V
c2	120	III
d1	85	
d2	91	IV
e2	77	V1

Figure 7.9 Proposed routes of fragmentation of un-derivatised BZP.

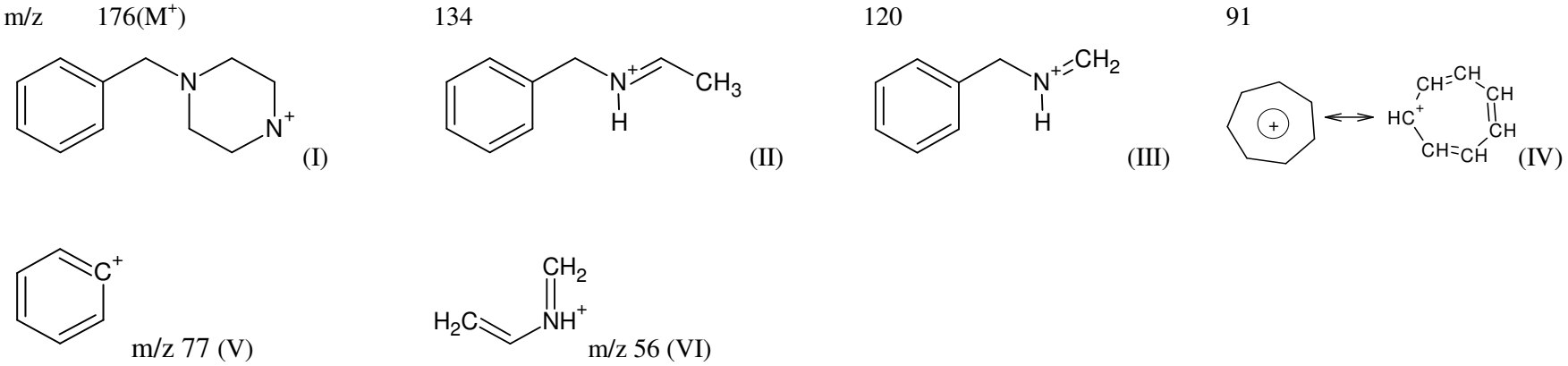
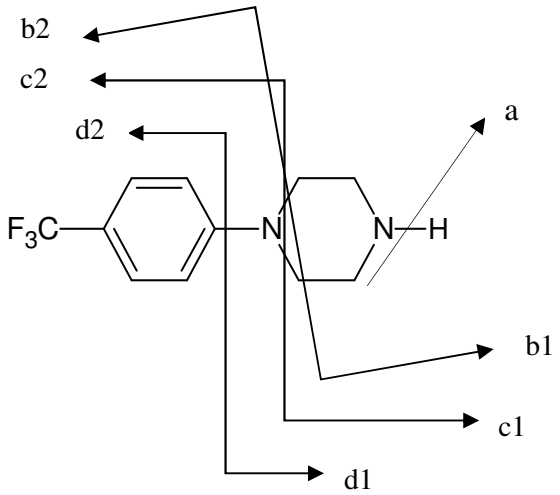


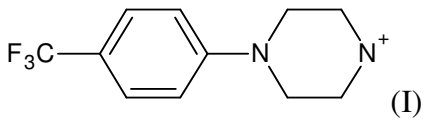
Figure 7.10 Structures of GC-Mass spectra ion fragments for un-derivatised BZP (EI, 70eV).



Route	Fragment	Structure
a	M+ ion	I
b2	188	II
c1	56	VI (as for BZP)
c2	174	III
c2	173(174-H)	IV
c2	172(173-H)	V
d1	85	
d2	145	VI

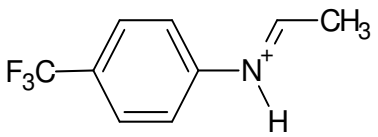
Figure 7.11 Proposed routes of fragmentation of un-derivatised 4-TFMPP (Similarly for 2 and 3-TFMPP).

m/z 230(M+)



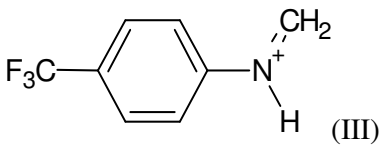
(I)

188



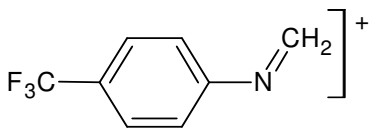
(II)

174



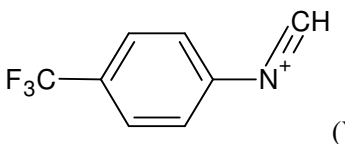
(III)

173



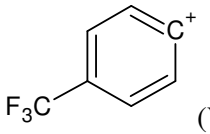
(IV)

172



(V)

145



(VI)

(VIII) m/z/56-as for BZP

Figure 7.12 Structure of GC-Mass spectra ion fragments for un-derivatised selected drugs (EI, 70eV).

Figures 7.9 – 7.10 showed that some of the ions are common such as at m/z56. These ions were generated by both routes of fragmentation for benzylpiperazines and phenylpiperazines. The more specific ions can then be applied for more selective distinction as routinely practiced in most studies (Khopkar, 2012). It is therefore suggested that the proposed routes of fragmentation and the fragment structures aid in understanding the process behind the mass spectra obtained. Studies showing routes of fragmentation and fragments ion structures are very limited, more for piperazine drugs (de Boer et al., 2001, UNODC, 2013c, Inoue et al., 2004; Takahashi et al., 2009). For both 3-TFMPP and BZP, limited information on fragmentation ions and structures have been reported for selected ions only (de Boer et al., 2001; Inoue et al., 2004; Elie et al., 2013). de Boer et al. in his study gave structures for BZP, TFMPP and MeOPP. But the routes of fragmentation were not reported. Such data is reported in this study (Table 7.9, Figures 7.8 – 7.11).

There were a limited number of studies on validation of similar drugs of abuse by other researchers for comparative purposes. However, Inoue et al. (2004) in their study on piperazine like compounds investigated BZP, 3-TFMPP, 3-CPP, 4-FPP. No detection limits were specified; however the study did a comprehensive qualitative analysis and generated comparable mass spectra in terms of the ions observed. In addition, the retention indices observed are also similar to those obtained in this study. However, a limitation to their methods was inability to separate the FPP isomers. The challenge posed by isomers was also encountered in other studies (Takahashi et al., 2009; Elliot and Smith, 2008). In addition the range of drug substances was rather limited as adulterants, such as caffeine have been commonly observed in most legal highs (Davies et al, 2010). These shortcomings were overcome with this method, as the isomers were not only resolved, but a wider range of drugs was investigated. This encompassed 4-FPP, 3-TFMPP and their congeners in street samples in which the drugs exist as combinations (Kelleher et al., 2011). In addition, this method gives holistic data (retention times, relative retention times, retention indices, mass spectra, linearity, LOD, LOQ, accuracy, precision and selectivity) for 19 drug substances for use in both qualitative and quantitative analysis.

A study by UNODC (2013) developed methods (three) for the analysis of materials containing piperazine drugs (BZP, MeOPP, FPP, TFMPP). The method gives similar results in terms of the trend in elution. In addition, the methodology is basically similar. These methods can successfully be employed for qualitative analysis. Differences lie in the

solvent used, diversity of the drugs analysed and practical applicability. This investigation has a more practical approach, as it not only gives a method which can be used for both analysis of drug standards, but also for actual street samples. It has been said street samples of piperazine drugs contain a mixture of other drugs and or adulterants and diluents (Davies et al, 2010; Yuk, 2010, Yeap et al., 2010, Kenyon et al., 2010). This method was developed with this information in mind and has been able to overcome the complexity of a street sample matrix. The drugs studied were those expected to be found in a street sample with FPP, TFMPP or other piperazines. In addition, it investigated potential residual precursors and by-products of synthesis and therefore, can be applied to a street sample which might contain these impurities. Furthermore, it can analyse for these impurities thereby fulfilling one of its objectives; characterisation and profiling. The method has successfully been applied in the analysis of street samples (Chapter 8). Consequently, the methods by UNODC may face limitations when applied to actual street samples.

In the UNODC (2013c) method the solvent was methanol unlike this study where 2-methylpropan-2-ol was used, though similar columns were used in both cases. The use of methanol can affect the stability of the analytes if they are left on the auto-sampler for prolonged periods. It was established in Chapter 5 (Stability studies) that some analytes show degradation in methanol if left on the auto-sampler for a prolonged duration. For example, the minimum stability period was 15 hours for cocaine. For the GC-MS method the authors did not report linearity, limits of detection, accuracy and precision. This information is critical for a new method as it gives the performance characteristics of the method, consequently such data from this study Tables 7.1 – 7.6 will be of use to other researchers.

7.3.6 QUALITY CONTROL

The results of monitoring the variability in detector response and method performance (retention time) for the QC standard (alkane mix and QC sample 4-MePP, section 7.2.9) are graphically represented in Figures 7.13 and 7.14. The limits on the chart define the boundaries within which the results are acceptable. When the results exceed the limit or are lower than the lower limit then corrective action such as servicing the instrument is taken, so as to bring the variable back to acceptable standards.

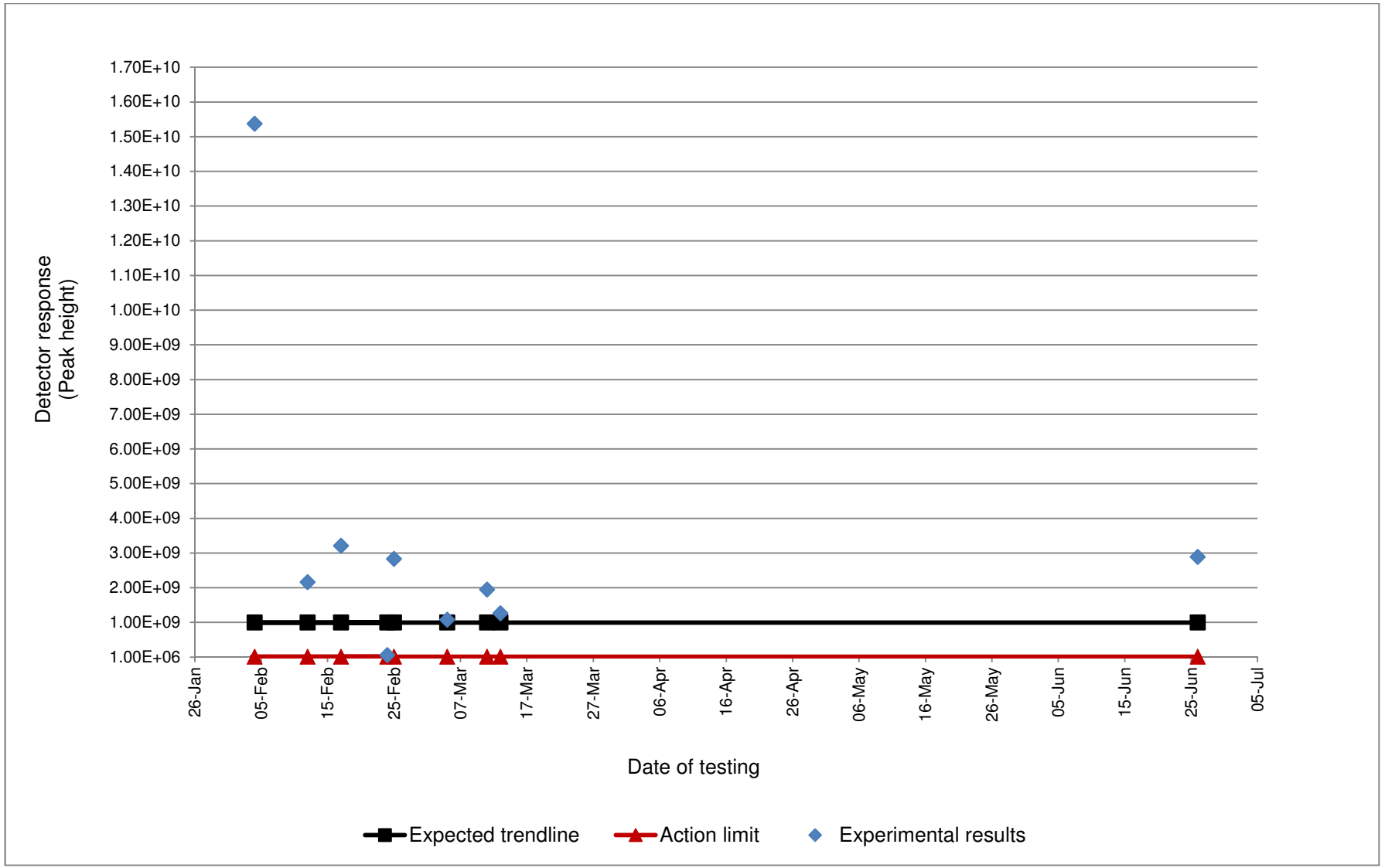


Figure 7.13 Quality control chart for variation in detector response using peak height.

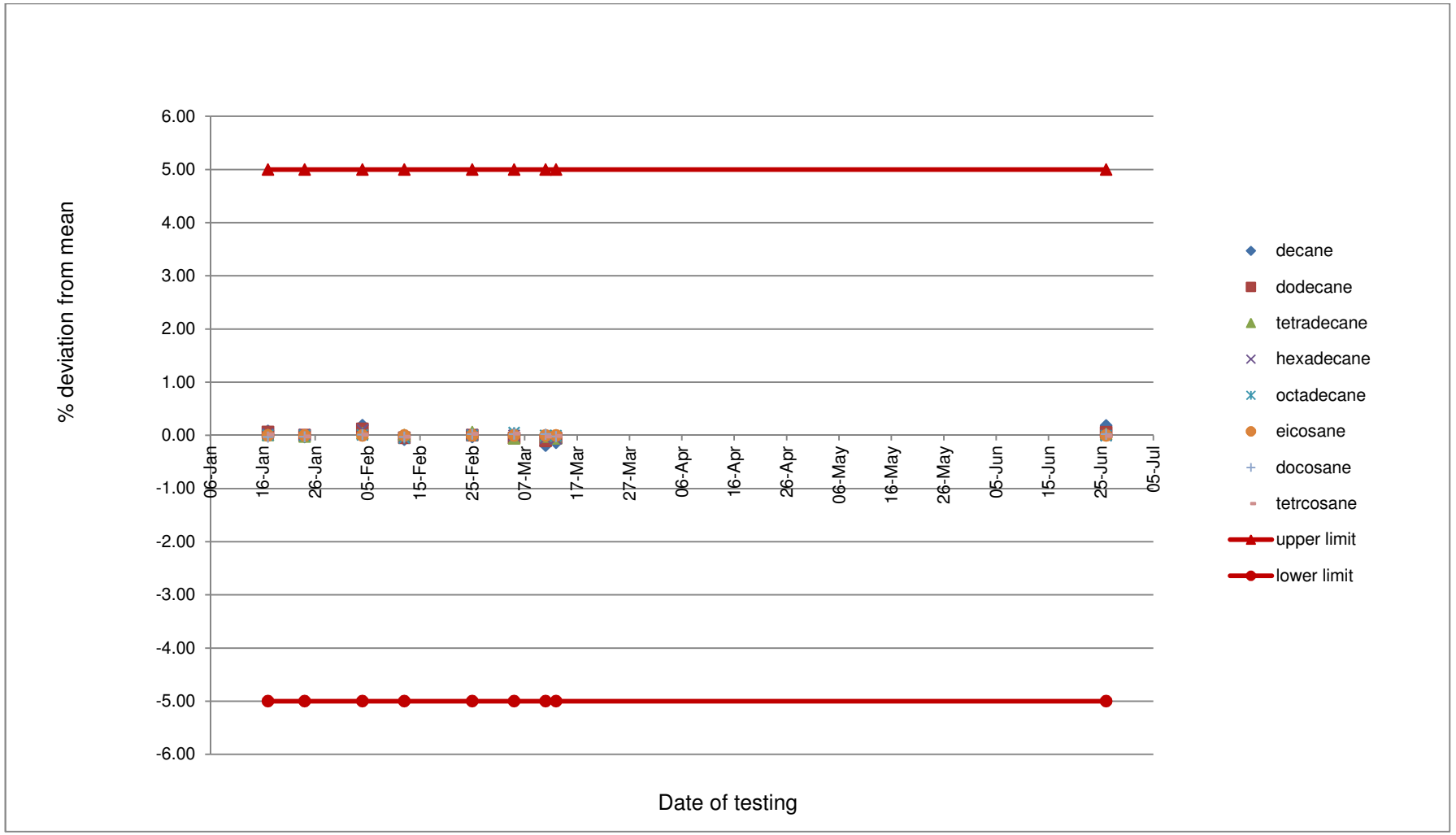


Figure 7.14 Quality control chart: variation in retention time (n-alkanes C8 – C24, section 7.2.9).

The QC data for n-alkanes was within the limits indicated on the quality control chart (Figure 7.13), indicating consistency of the detector response throughout the life of the project. However, the detector response was observed to give low values below the limit (Figure 7.14 end of February value). This resulted in corrective action, i.e., instrument service maintenance was conducted and the performance improved. The retention times of n-alkanes were monitored as well as the ion at m/z 207 for column bleed (sections 7.2.9 and 2.4.2). This was to verify the stability of the column since aging can result in retention times lengthening and the column increasingly bleeds. The retention times were found to be consistent as shown by their lack of significant variation on QC chart, i.e., for the period investigated they were within the limits depicted on the chart (Figure 7.14). In addition, it also gave an indication of instrument repeatability. Column bleed was monitored through checking for ion m/z 207 in analyte mass spectra. It was identified in the discussion in chapter 2 (section 2.5.2), that an aging column can cause inconsistent results (Hibbert et al., 2007; McNair and Miller, 2009). As such the column was changed when excessive column bleed was observed. McNair and Miller highlighted that column bleed can cause too high a background in chromatography, which can interfere with the peaks of interest by enhancing the peaks. In their study in developing a GC method for profiling amphetamines Andersson et al. (2007b) similarly monitored column bleed as part of quality control.

The precision of the instrument was monitored using 4-MePP, one of the drugs under investigation, as an additional method of maintaining variability of the instrument's response. 4-MePP was used, as it elutes mid-way through the chromatographic profile of the analytes (Figure 7.5). In addition, it is also relatively stable, as it has been successful analysed and optimised in previous studies (Chapter 6).The data is shown in Table 7.10.

Table 7.10 QC trend in 4-MePP retention times and instrument precision (detector response) on repeated injections.

Date	**RT**	**RRT**	**Precision (N =6)**	
			SD	%RSD
15/04/2011	15.477	0.813	0.100	1.35
21/05/2011	15.463	0.836	0.011	1.10
29/05/2011	15.400	0.832	0.023	1.43
04/06/2011	15.410	0.833	0.010	1.12
08/09/2011	15.220	0.803	0.015	1.24
04/02/2013	15.540	0.806	0.006	1.31
05/03/2013	15.547	0.806	0.006	0.80

According to Gonzalez and Herrador (2007); Eurachem (1998) and ICH (2005) precision is normally expressed as standard deviation or relative standard deviation (%RSD) and RSD $\leq$ 2% to be acceptable. The precision was below the limit throughout the period investigated. Hence, it can be concluded that the instrument performance was consistent. This is also evidenced by the low standard deviation and %RSD values in Table 7.10. Furthermore, system suitability results gave %RSD of 6 injections in the range 0.8 – 1.94 for all the drugs investigated prior to any quantitative investigations. According to Horacio et al. (2008) system suitability tests (were 5 – 6 consecutive injections are made) should show an RSD < 2 for the variability between the measurements to be acceptable. As such, the results further confirmed that the instrument was consistent and had good repeatability.

The QC results helped to highlight systematic errors resulting in their minimisation during the course of the research. Consequently, neither the instrument nor the column contributed adversely to the analytical work. This established reliability in the investigations conducted and aided in generating accurate data.

7.4 CONCLUSION

It was established that the method developed is suitable for its intended use, i.e., analysis and profiling of piperazine based street drugs. Method accuracy was reported on average as 99.8% and precision was RSD < 2%. Detection limits were in the range 0.5 – 1.95 x 10^{-3}µg/mL free base on column. On average, the common working range was 5 – 35.0µg/mL

free base on column. This was ideal for the analysis of street samples, as they have high dosages of drugs (mg range) (Kelleher et al., 2011). Furthermore, it can also be applied to very low concentrations. This could prove useful if the method is extrapolated to toxicological studies. In such studies drug concentrations in the biological samples are usually low (Stack and Maurer, 2005). In addition it was found that the method developed was able to simultaneously analyse for 22 drugs which can be found in different combinations with the target analytes 4-FPP and 3-TFMPP in street samples. In addition, the method was able to completely resolve (baseline resolution) the (2, 3, 4) isomers of both FPP and TFMPP thereby being the first method available able to simultaneously analyse these isomers and congeners in street samples. The isomers 2-FPP, 3-FPP and 4-FPP were separated with $R > 2$ and retention times of 12.88 min (2-FPP), 14.05 min (3-FPP) and13.56 min (4-FPP) respectively

It was observed that there is co-elution between 3TFMPP/MDMA and also DBZP/dextromethorphan. However, whilst the ideal situation is complete resolution, it was established that there is statistically no significant difference between using total ion chromatographic data and extracted ion data. Hence, this limitation was overcome through use of extracted ions for these particular drugs (3-TFMPP m/z 188), MDMA m/z135, DBZP m/z 91 or 266 and dextromethorphan m/z 271). Consequently, the method developed was found to work satisfactorily and will be employed in the characterisation and impurity profiling aspect of the research, in profiling of 3-TFMPP and 4-FPP.

Currently, there was no record of the drugs having been chemically profiled. However, in this study comprehensive data for all the 22 drugs investigated was reported (retention times, relative retention times, retention indices, and mass spectra) for all the 3-FPP and 2-TFMPP isomers and the other drugs investigated. Furthermore, this study brings in additional spectral data in terms of more comprehensive mass spectra and identifying ion m/z values for the piperazines and also all the congeners. In addition, the routes of fragmentation and fragment structures for benzylpiperazines and phenylpiperazines were identified. It is suggested this data will aid further investigation of these drugs and can be used by other researchers, law enforcement agencies and in toxicity studies.

CHAPTER 8

ANALYSIS, CHARACTERISATION AND SYNTHESIS OF STREET SAMPLES

8.1 INTRODUCTION TO THIS STUDY

It has been identified that characterisation can provide a chemical picture of the make up or composition of a drug (Bartos and Gorog, 2008; UN, 2001). This entails physical and chemical analysis, and identification of potential routes of synthesis. The need for characterisation and profiling of piperazine drugs was identified in Chapter 1.

In this study, the morphology, dimensions, colour, logos and any other physical traits of street samples were analysed (Milliet et al., 2009). Identification and quantitative determination of the components of street samples was conducted through presumptive testing and GC-MS analysis. In addition, the identity of impurities present in the street samples was investigated. Presumptive testing employed the Marquis and Simon's tests, these methods were identified in section 1.8 (Chapter 1) as appropriate to the drugs under investigation. These tests were conducted according to the methods by UNODC (2006) and Takahashi et al. (2009). Identification by GC-MS was on the basis of retention times, relative retention times, retention indices and mass spectra, applying the parameter values identified in the method developed as a reference (section 7.3.5).

The synthesis of 4-FPP and 3-TFMPP was conducted as a representative of the synthesis of phenylpiperazines. The drugs were synthesised according to the methods by Liu and Robichaud (2005) and Kiritsy et al. (1978). These routes were identified as potential routes of clandestine synthesis from literature (section 1.7 Chapter 1). Analysis of the samples synthesised was conducted by UV-Vis, FTIR and GC-MS so as to ascertain their identity. These techniques had been identified in Chapter 1 (section 1.8) as the appropriate techniques for this research. In addition, the impurity profile was investigated (Aalberg et al., 2005a; Bartos and Gorog, 2008). This was achieved through identification of precursors, isomers and by-products of synthesis. Furthermore, the impact of reaction time on yield was conducted. Residual precursors were identified (Chapter 1) as potential impurities that can be present in the sample after synthesis and as such there is need to test

whether when in street samples they would not react or degrade. Hence, the test for stability was conducted on the precursors.

A comparison of the total ion chromatographic profiles of the street samples and the synthesised samples was conducted. This involved the comparison of not only the drug components but their impurity profiles especially those arising from the synthesis routes as this would indicate any links to the synthesis routes (UN, 2001; Aalberg et al., 2005a. This study mainly focused on characterisation consequently, impurity profiling was only partially covered.

The street samples were provided courtesy of Cambridgeshire Constabulary. The samples were illicit drug seizures by the police. Each sample consisted of three tablets with the exception of two which had one tablet. The GC-MS method (Kuleya et al., 2014) of analysis developed, optimised and validated in previous chapters was applied in this study.

8.1.1 AIMS

The aims of this study were to carry out the physical and chemical characterisation of street samples thought to contain the piperazines 3-TFMPP and 4-FPP. To achieve this, the following objectives were implemented; to a) identify potential viable routes of clandestine synthesis of phenylpiperazines (4-FPP and 3-TFMPP), b) synthesise and analyse 4-FPP and 3-TFMPP and their chemical impurities arising from the synthesis (by-products, positional isomers, residual solvents and precursors and c) compare street samples and the laboratory synthesised samples.

8.2 MATERIALS AND METHODS

8.2.1 CHEMICALS/REAGENTS

The drug standards and solvent, 2-methylpropan-2-ol used were as outlined in Chapter 4 (section 4.2.1). In addition anhydrous piperazine, 4-fluoroaniline, 3-trifluoromethylaniline, bis(2-chloroethyl)amine hydrochloride, diethylene glycol monomethyl ether, 3-bromo-l-(trifluoromethy1)benzene and anhydrous methanol were all purchased from Sigma. Sodium carbonate, diethyl ether, ethyl acetate, sodium hydroxide, benzene, anhydrous magnesium

sulphate, anhydrous sodium sulphate, anhydrous methanol, acetaldehyde, sodium nitroprusside, sulphuric acid, Whatman ashless 542 filter paper, and petroleum ether were all purchased from Fischer Chemicals.

8.2.2 INSTRUMENTS

The GC-MS instrument and set up were as per the optimised and validated method outlined in Chapter 7 (section 7.2.2). In addition an Agilent Cary 300 UV-Vis spectrophotometer and a Perkin Elmer Spectrum One FTIR Spectrometer equipped with a Perkin Elmer Universal ATR sampling accessory were utilised for additional identification of synthesised samples. A Nikon D90 digital SLR camera was used to take photographs of the street samples. An Eppendorf 5810 centrifuge was used for clarification of solutions of samples.

8.2.3 STATISTICAL SOFTWARE

Analysis of results was carried out using IBM SPSS Version 20 and MS Office Excel 2010.

8.2.4 METHODS: STREET SAMPLES

8.2.4.1 Physical characteristics

A physical description and photograph of the street samples were recorded. The characteristics analysed were colour, logos, shape, diameter, thickness, mass and any other markings observed such as damage.

8.2.4.2 Chemical characteristics

8.2.4.2.1 Presumptive tests

8.2.4.2.1.1 Marquis test

The Marquis reagent was prepared by mixing 40% formaldehyde (5.0mL) with concentrated sulphuric acid (100.0mL) (UNODC, 2006; Takahashi et al., 2009). The test was carried out by addition of a small amount (≈3.0mg) of the drug standard onto a reaction plate. The control was an empty well on the reaction plate. One drop of the Marquis reagent was added to the sample and the control. The colour changes were observed and noted. The test was repeated using the street samples A1 - A11.

8.2.4.2.1.2 Simon's test

The following solutions were prepared; a) Solution 1: 20% aqueous sodium carbonate, b) Solution 2: 50% ethanolic acetaldehyde and, c) Solution 3: 1% aqueous sodium nitroprusside (UNODC, 2006; Takahashi et al., 2009). The test was carried out as for the Marquis test, sequentially adding a drop of each of the solutions 1 - 3.

8.2.4.2.2 Qualitative and quantitative analysis of street samples

8.2.4.2.2.1 Standard solutions

Individual drug standard and mixed drug standard solutions were prepared as outlined in Development of the method (Chapter 4, section 4.2.4.3) using 2-methyl-propan-2ol as a solvent.

8.2.4.2.2.2 Sample preparation

The sample was crushed to a fine powder and homogenised using the "cone and quarter" method. A sample of the powder (30.0mg) was dissolved in 7.0 mL 2-methyl-propan-2-ol in a volumetric flask; the sample was placed in an ultrasonic bath for 30 minutes and then diluted to volume. After thorough mixing the solution was centrifuged at 3000 rpm for 3 minutes to remove the solid material. A sample of the supernatant (1mL) was diluted tenfold and analysed by GC-MS. The samples were prepared in triplicate.

8.2.5 METHODS: SYNTHESIS

The substances 4-FPP and 3-TFMP were synthesised individually using methods identified in literature as possible routes of synthesis in clandestine laboratories (Liu and Robichaud, 2005, Kiritsy et al., 1978).

8.2.5.1 Synthesis Route 1: Synthesis of 4-FPP and 3-TFMPP

4-FPP and 3-TFMPP were synthesized from fluoroanilines (Chapter 1, Figures 1.17a and 1.17b) according to previously published procedures (Liu and Robichaud, 2005). 4-Fluoroaniline 0.3334g (3.0 mmol), bis(2-chloroethyl)amine hydrochloride 0.5355g (3.0 mmol), and diethylene glycol monomethyl ether (0.75mL) were weighed into a reaction vessel and mixed under dry N_2(g). The mixture was heated at 150^{o}C for 7.5 hours, (6 - 12 hours recommended) and then cooled to room temperature. Methanol (0.4mL) was added to dissolve substance and followed by addition of diethyl ether (Et_2O) (150.0mL). The

precipitate was filtered and washed with the ether to provide hydrochloride salt. Sodium carbonate solution (10%) was added until alkaline to litmus paper, so as to convert the salt to the free amine. This was extracted with ethyl acetate (10.0mL) twice. The organic layers were collected dried over anhydrous sodium sulphate (2.00g). This was followed by filtration using Whatman ashless 542 filter paper. The filtrate was concentrated in using rotary evaporator.

The same procedure was used for the synthesis of 3-TFMPP except 3-trifluoromethylaniline was used instead of 4-fluoroaniline. To ascertain the impact of the length of reaction time on product yield, the drugs were synthesised under varying reaction times; 6, 8, 12hours. The method stated 6 - 12 hours (Liu and Robichaud, 2005)

8.2.5.2 Synthesis Route 2 (Kiritsy et al., 1978) Method A: Synthesis of 3-TFMPP

3-bromo-l-(trifluoromethy1)benzene 22.50g (0.10 mol) and 43.1g (0.50 mol) of anhydrous piperazine were weighed to a reaction vessel. The mixture was refluxed at 100 ^{o}C for 45 hours. The hot melt was poured into a 250.0mL of 10% NaOH solution to give 18.70g (0.08 mol). The product was extracted with petroleum ether and the solvent was removed with a rotary evaporator.

8.2.5.3 Synthesis Route 2 (Kiritsy et al., 1978) Method B: Synthesis of 4-FPP

39.60g (0.22 mol) bis(2-chloroethy1)amine hydrochloride, 47.00g (0.44 mol) of Na_2CO_3, and 30.00g (0.22 mol) of 4-fluoroaniline were weighed into a reaction vessel. To this ethanol (150.00 mL) was added. The mixture was refluxed at 100^{o}C for 46 hours. The solvent was concentrated and the residue re-dissolved in water. The aqueous solution was extracted with benzene. The extracts were dried over $MgS0_4$ and the solvent was removed under reduced pressure using a rotary evaporator.

8.2.5.4 Identification of synthesised samples by UV-Vis analysis

Reference standard solutions were individually prepared for each FPP drug isomer (2-FPP, 3-FPP and 4-FPP). The standard was weighed (10.0mg) and dissolved in methanol and in 2-methylpropan-2-ol (10.0mL). The solution was shaken to dissolve and diluted to a concentration of 0.02mg/mL. A solution of the synthesised FPP sample was similarly prepared. The UV-Vis spectra of the solutions were determined with the solvent as a blank. The scan range was set to 200 - 800nm wavelength.

The test was similarly conducted for the synthesised TFMPP samples using standards of TFMPP isomers as the reference solutions.

8.2.5.5 Identification of synthesised samples by FTIR-Attenuated Total Reflectance (ATR)

The synthesised FPP sample was sampled using the ATR technique and its FTIR spectrum determined. The scan range was set at 650 - 4000cm^{-1} wave numbers. For each sample 5 scans were taken at a resolution of 4 cm^{-1}. The spectra of the standards (2-FPP, 3-FPP and 4-FPP) were similarly determined. The sample and standards spectra were comparatively analysed.

The test was similarly conducted for the synthesised TFMPP samples

8.2.5.6 Identification of synthesised samples by GC-MS analysis

The samples were prepared according to the procedure outlined in Chapter 4 (section 4.2.4.3) for individual drug standard solutions and mixed drug standard solution1 using 2-methyl-propan-2-ol as a solvent. The following set of samples were prepared a) 2-FPP, 3-FPP, 4-FPP reference standards and the synthesised FPP and b) 2-TFMPP, 3-TFMPP, 4-TFMPP reference standards and the synthesised TFMPP. All the samples were analysed by GC-MS.

8.2.5.7 Analysis of precursors from synthesis

8.2.5.7.1 Identification of precursors

Solutions of the precursors, 4-fluoroaniline, 3-trifluoromethyl aniline, 2-Bischloroethylamine hydrochloride, diethylene glycol monomethyl ether and piperazine were individually prepared. The solutions were prepared as per the procedure outlined in Chapter 4 (section 4.2.4.3) for individual drug standard solutions and mixed drug standard solution1 using 2-methyl-propan-2-ol as a solvent and the precursor as the analyte.

8.2.5.7.2 Test for precursor reactivity- stability of precursors

Stability testing of the precursors to the synthesis (routes 1 and 2) was done as per the method outlined in Chapter 5 (section 5.2.7) on GC-MS auto-sampler stability. The following were prepared a) individual solutions of each precursor and b) mixed combinations of precursors as tabulated below in Table 8.1.

Table 8.1 Precursor combinations for stability/reactivity testing.

Combinations (synthesis route 1 precursors)	
4-FPP precursors	***3-TFMPP precursors***
2-bischloroethylamine 4-fluoroaniline	2-bischloroethylamine 3-trifluoromethyl aniline
diethyl glycol monomethyl ether 4-fluoroaniline	diethylene glycol monomethyl ether 3-trifluoromethyl aniline
2-bischloroethylamine diethyl glycol monomethyl ether 4-fluoroaniline	2-bischloroethylamine diethyl glycol monomethyl ether 3-trifluoromethyl aniline
diethyl glycol monomethyl ether and 2-bischloroethylamine	

8.2.6 DATA ANALYSIS METHODS

8.2.6.1 Qualitative analysis and identification of street samples

Identification of illicit drugs in the street samples was achieved by comparison to the drug reference standards using the confirmation of identity parameters established during validation (Chapter 7, section 7.3.5).

In addition, the street samples were checked for impurities. The total ion chromatograms were checked for the presence of precursors, isomers of FPP and TFMPP and any other artefacts. Identification of sample components was achieved by comparison to reference standards. Where these were unavailable the NIST library software was applied.

8.2.6.2 Quantitative analysis of street samples

Quantification of the identified analytes was determined according to section 3.35 equations 3.35 - 3.40 (Chapter 3).

8.2.6.3 Synthesis

Identification of the synthesised sample was by comparison of the FPP isomers to the synthesised FPP sample and comparison to TFMPP isomers for the synthesised TFMPP sample. In addition, the synthesised sample was checked for impurities. The total ion chromatograms were checked for the presence of precursors, isomer and by-products.

Identification of sample components was achieved by comparison to reference standards. Where these were unavailable the NIST library was applied.

To statistically confirm the identity of the synthesised samples comparative analysis by Pearson's correlation (Chapter 3 section 3.2.4) was conducted between the isomers and the synthesised samples. The test was applied on the UV-Vis and FTIR results. The statistical data was analysed to determine the isomer that showed the strongest correlation to the synthesised sample.

8.2.6.3.1 Determination of yield for the synthesis

The yield was calculated according to equation 3.41 (Chapter 3). Comparison was made to the expected yields. According to the authors the expected yield for route 1 was 67% 3-TFMPP and 87% 4-FPP (Liu and Robuchaud; 2005). The expected yield for route 2 was 81.2% 3-TFMPP and 30% 4-FPP (Kiritsy et al.; 1978).

8.2.6.4 Precursor reactivity- stability of precursors

The total ion chromatograms both for the individual precursor and for the combinations were checked for the presence of secondary peaks (artefacts).

8.2.6.5 Comparison of street samples and synthesised samples

The total ion chromatograms of the street samples and the synthesised samples were compared for any similarity in the peaks observed. Furthermore, they were checked for the presence of synthesis precursors so as to identify the route of synthesis used in manufacture of the street sample and also similarities in other impurities observed.

8.3 RESULTS AND DISCUSSION

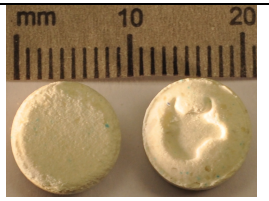
8.3.1 STREET SAMPLES

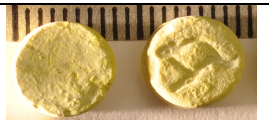
8.3.1.1 Physical characteristics

The physical characteristics of the street samples investigated are shown in Table 8.2. The 11 street samples received for investigation were all tablets. These showed a variety of physical traits. As shown in Table 8.1 this was mainly in the colour and logos inscribed on the Tablets. The sizes of the tablets were in the range 7.5mm diameter x 3.0mm width to

10.0mm diameter x 2.5mm width with 8mm diameter x 4mm width being the most common (36% of the samples). The tablet mass ranged from 229.9 – 354.5mg with a mean mass of 286 ± 31.9mg. The relative standard deviations showed that for each of the samples the tablets were reasonably consistent in mass (RSD 2.02 – 15.3%). It was established in Chapter 2 (section 2.8.1) that physical characteristics such as the shape, logo, colour, dimensions or any other markings can provide links between different samples of tablets (Milliet et al., 2009; Cheng et al., 2003; Makino et al., 2003). In studies on profiling of MDMA, Makino et al, (2003) and Zingg (2005) investigated the physical characteristic of tablets and similarly concluded that physical characteristics can be characteristic of manufacturers for example logos. In addition, defects on the tablets can be characteristic of the metal dies used during manufacture. Consequently, the samples were inspected for any common similarities in physical traits.

Table 8.2 Physical characteristics of the street samples analysed tablets (Tablets courtesy of Cambridgeshire Constabulary).

Name	**Tablet (mg)**	**Size (mm) diameter x thickness**	**Description**	**Image**
A1	333.2 N= 3, RSD = 5.4%	8.0mm x 5.5mm	Round, cream coloured tablets with a clown face inscribed on one side.	
A2	303.3 N = 3, RSD = 4.7%	7.5mm x 4.0mm	Round, pale blue cream coloured tablets. With a single score line on one side and a smiley face inscribed on the other side.	
A3	309.7 N = 3, RSD = 9.8%	8.5mm x 4.0mm	Round, green coloured tablets with the Mercedes car logo inscribed on one side.	
A4	306.5 N = 2, RSD = 12.8%,	8.5mm x 3.0mm	Round, off-white coloured tablets with slight blue and brown flecks. A bull's head was inscribed on one side.	
A5	223.9 N = 3, RSD = 4.1%,	8.5mm x 4.0mm	Round beige coloured tablets with an in-twined 3D design on one side.	

A6	298.3 N = 1, RSD = N/A	8.0mm x 3.0mm	Round, pink coloured tablets with a single score line on one side. The tablets were slightly discoloured.	
A7	265.5 N = 3, RSD = 2.1%	8.0mm x 4.0mm	Round, yellow coloured tablets with a single score line and copyright sign on one side. A star was inscribed on the other side.	
A8	203.5 N = 3, RSD = 12.1%	7.5mm x 3.0mm	Flat, round, pale yellow coloured tablets. A kangaroo was inscribed on one side. The texture was not smooth and the colour intensity inconsistent.	
A9	229.9 N = 1, RSD = N/A	8.0mm x 4.0mm	Round, pink coloured tablets. The colour intensity was inconsistent. A dolphin was inscribed on one side.	
A10	354.5 N = 2, RSD = 15.3%	8.5mm x 4.5mm	Round, pink coloured tablets with a single score line one side and a fairy inscribed on the other side.	
A11	290.8 N = 3, RSD = 2.1%	10.0mm x 2.5mm	Round, bevel edged, speckled, orange coloured tablets with a star inscribed on one side.	

The samples were found to have similarity in size, but no similarity in colour and logos. In addition defects were observed on samples A2, A3, A9 and A10. These tablets were chipped. The patterns of the damage on the tablets were not similar. It is therefore suggested that the damage arose out of poor tablet quality and not die defects. This view was taken as a result of observing that the tablets were of poor quality, i.e., samples A9 and A8 were observed to be easily friable with A8 also having a rough texture. This implies poor methods of manufacture and skill. It is likely not enough binders were used. This also confirms that tablets were made in a non-professional laboratory such as a clandestine manufacture and hence are a potential health risk.

8.3.1.2 Chemical characteristics

8.3.1.2.1 Presumptive tests

The results of presumptive testing with Marquis and Simon's reagent are shown in Table 8.3 and 8.4 for the drug standards and street samples respectively.

Table 8.3 Presumptive tests results of the drug standards.

Substance	**Marquis reagent**	**Simon's reagent**
2-FPP	No change, turned pale yellow with time	Pale violet
3-FPP	No change, turned pale yellow with time	Brown
4-FPP	No change, turned pale yellow with time	Pale violet
2-TFMPP	No change	Pale blue-green
3-TFMPP	No change	Pale pink
4-TFMPP	No change	No change
BZP	No change	Blue
DBZP	No change	No change
MBZP	Pink	Violet
3-CPP	No change	No change
4-MePP	No change, turned pale yellow-green with time	Violet
MDMA.HCl	Black	Dark blue
(+)Amphetamine SO_4	Orange	No change
(+)Methamphetamine HCl	Orange	Dark blue
Caffeine	No change	No change
Cocaine HCl	No change	Pale pink
Diazepam	Pale yellow	No change
Dapoxetine HCl	Black	No change
Dextromethorphan HBr	Black	Pale pink
Nicotinamide	No change	brown
Control	No change	No change

Table 8.4 Presumptive tests results of the street samples.

Sample	**Marquis reagent**	**Simon's reagent**
A1	No change	No change
A2	No change	Pale pink
A3	Black	Dark blue
A4	Black	Dark blue
A5	No change	~~Slightly~~ Pale violet
A6	No change	No change
A7	Black	Dark blue
A8	No change	No change
A9	Black	Dark blue
A10	No change	Pale pink
A11	Black	Dark blue
Control	No change	No change

The results for presumptive tests (Table 8.3) on the reference standards were similar to those prescribed in UNODC guides for National Drug Testing Laboratories (UNODC, 2006; 2013c; Kovar and Laudsazun, 1989; Cole, 2003). This can be seen for amphetamine, methamphetamine, MDMA, dapoxetine and dextromethorphan giving a positive result to the Marquis reagent. This is due to the reaction of aromatic compounds with the Marquis reagent. The mechanism was shown in the theoretical concepts (Chapter 2, Figure 2.16). The formation of the carbenium ion (VII) is responsible for the colour observed with aromatic compounds. The reaction and final colour also depend on the substituents present. It is suggested that the reason piperazines do not react with the Marquis reagent is due to the fact that the substituent, R in the reaction in Figure 2.16 is a piperazine ring. This has electron donating properties which reduces the charge on the first carbenium ion (IV) thereby decreasing the reaction from occuring. According to the UNODC, (2013a) and Inoue et al., (2004) piperazines do not give a positive reaction to Marquis reagent. This can be seen for the benzyl and phenyl piperazine based drugs, BZP, FPP, and TFMPP which showed no reaction (Table 8.4).

The response to the Simon's reagent was also in line with that observed by other researchers. Simon's reagents tests for secondary amine, consequently as can be seen in Table 8.4 the compounds which are secondary amines such as methamphetamine, MDMA,

BZP, FPP, TFMPP produced a colour change with Simon's reagent. The blue colour is due to the formation of the ion (VII) shown in the reaction in Figure 2.17 (Chapter 2). However, most of the piperazines showed poor sensitivity. The piperazines which contain a secondary nitrogen atom (all except DBZP) are heterocyclic amines. Hence, the lone pair of electrons on the nitrogen is delocalised in the ring making them less available for bonding and as such this reduces reactivity. This is probably the reason they produce no reaction or a faint colour change. Comparatively, the aliphatic secondary amines (amphetamines) show better reactivity and produce the standard dark blue colour.

Inoue et al. (2004) conducted presumptive tests on a number of piperazine drugs and obtained similar results with the piperazines giving less sensitivity to Simon's reagent. Similarly UNODC (2013c) investigated BZP, 4MePP, 3CPP, (2, 3, 4) FPP and TFMPP, methamphetamine and MDMA and obtained similar results with both reagents.

Street samples A3, A4, A5, A7, A9 and A11 (Table 8.4) showed a positive reaction to both the Marquis reagent and Simon's reagent. This clearly suggests the presence of an amphetamine derivative which is also a secondary amine. This was potentially MDMA since it is one of the most commonly found drugs of abuse on the street (Yeap et al., 2010; Davies et al., 2010). The other street samples (A1, A2, A5, A6, A8 and A10) showed no reaction to the Marquis reagent. Also, the observed absence of the standard blue colour to Simon's reagent suggested the absence of amphetamines. It was discussed (section 2.8.2) that according to the UNODC (2013c) piperazines were less sensitive than secondary amines, as such these samples may contain piperazines. On comparison to the standards (Table 8.3) these samples showed similar reactions to piperazines, e.g. the piperazines generally gave no reaction with both the Marquis and Simon's reagent except for 3-TFMPP which gave a pale pink colour with Simon's reagent. This was also observed for the samples A2 and A10 further suggesting the samples to contain piperazine substances.

8.3.1.2.2 Qualitative and quantitative analysis of street samples

The total ion chromatograms (TICs) arising from analysis of the street samples are shown below in Figures 8.1 - 8.11. A typical mixed standard TIC used for comparison and identification of the samples components is given in Figure 8.12.

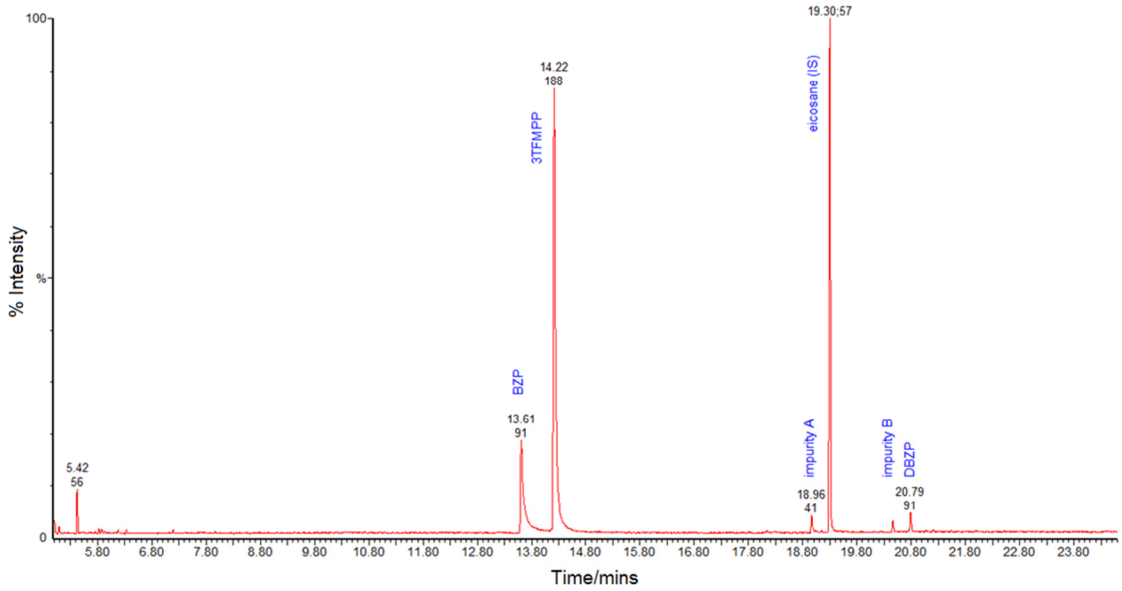


Figure 8.1 Total ion chromatogram of street sample A1.

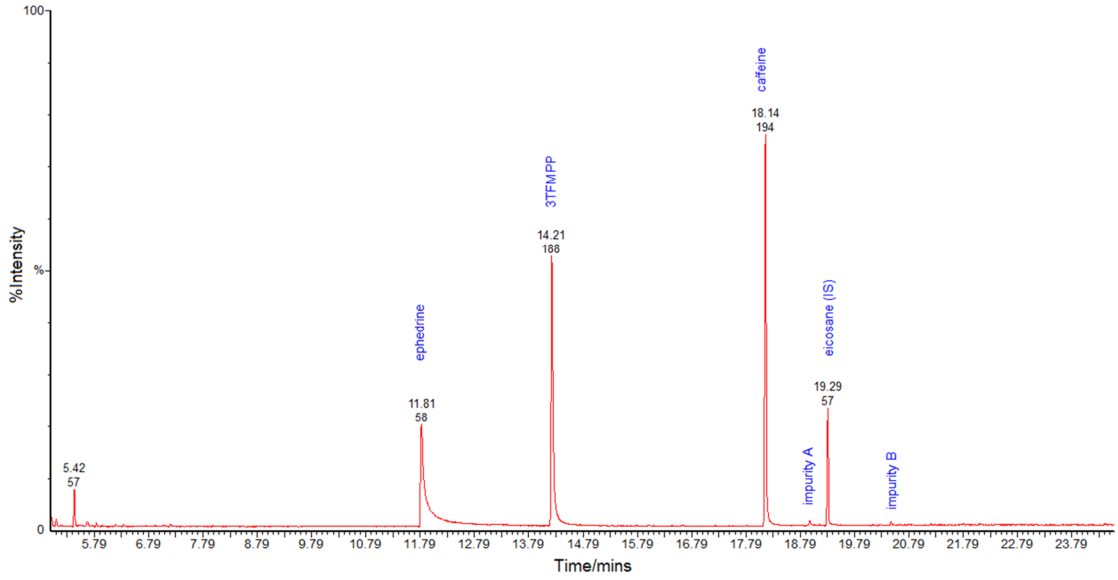


Figure 8.2 Total ion chromatogram of street sample A2.

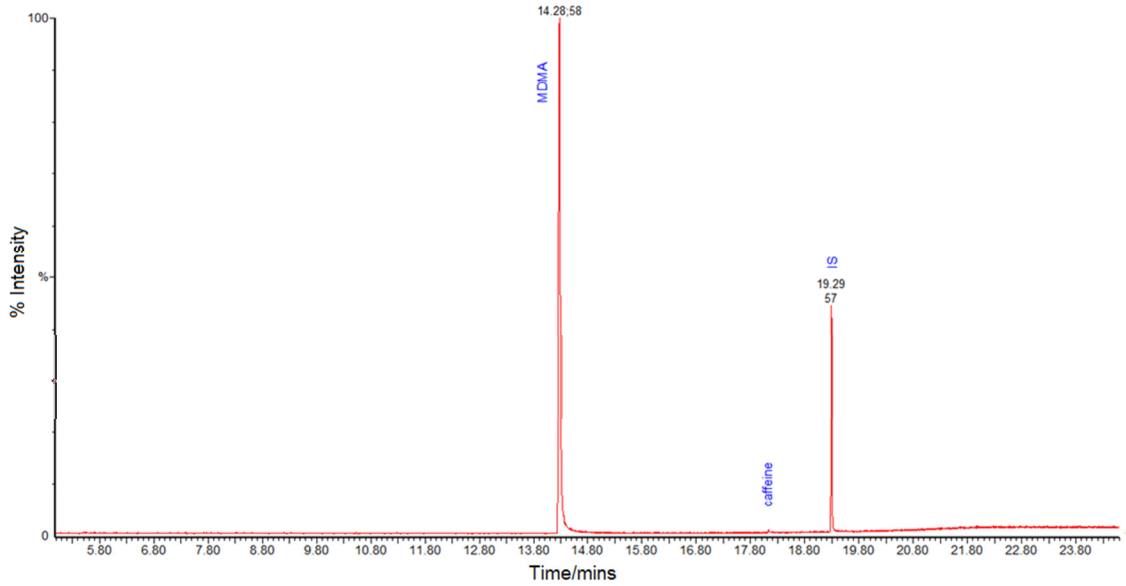


Figure 8.3 Total ion chromatogram of street sample A3.

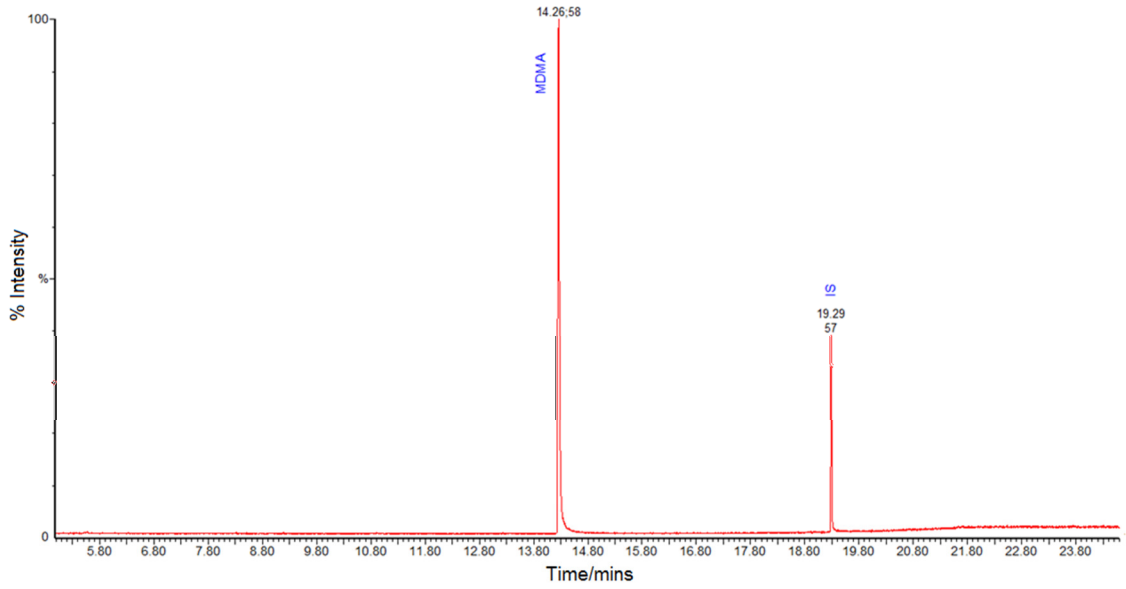


Figure 8.4 Total ion chromatogram of street sample A4.

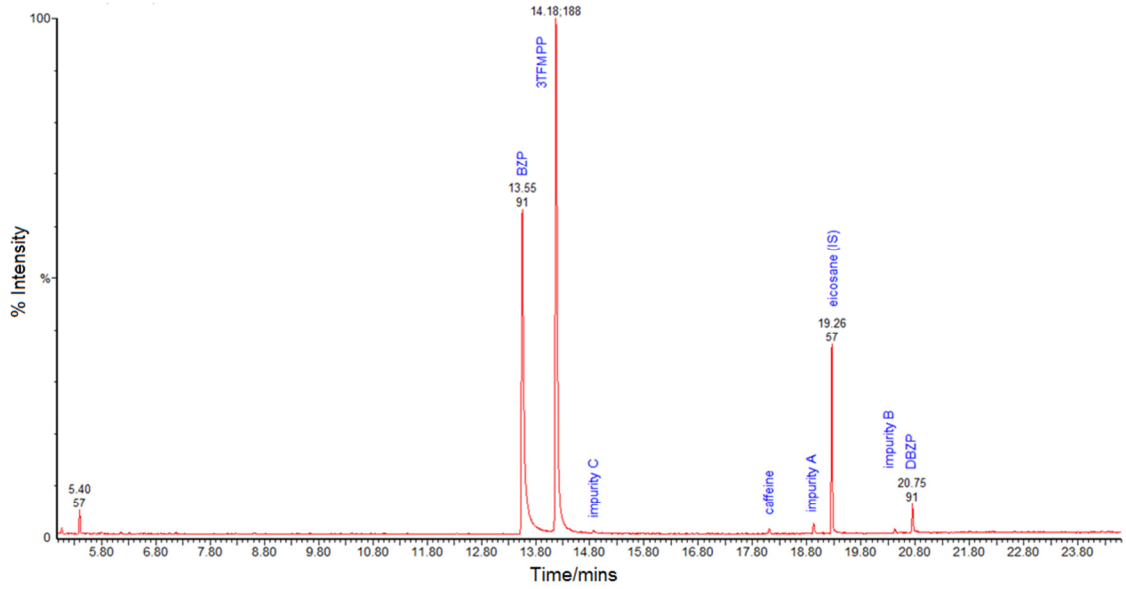


Figure 8.5 Total ion chromatogram of street sample A5.

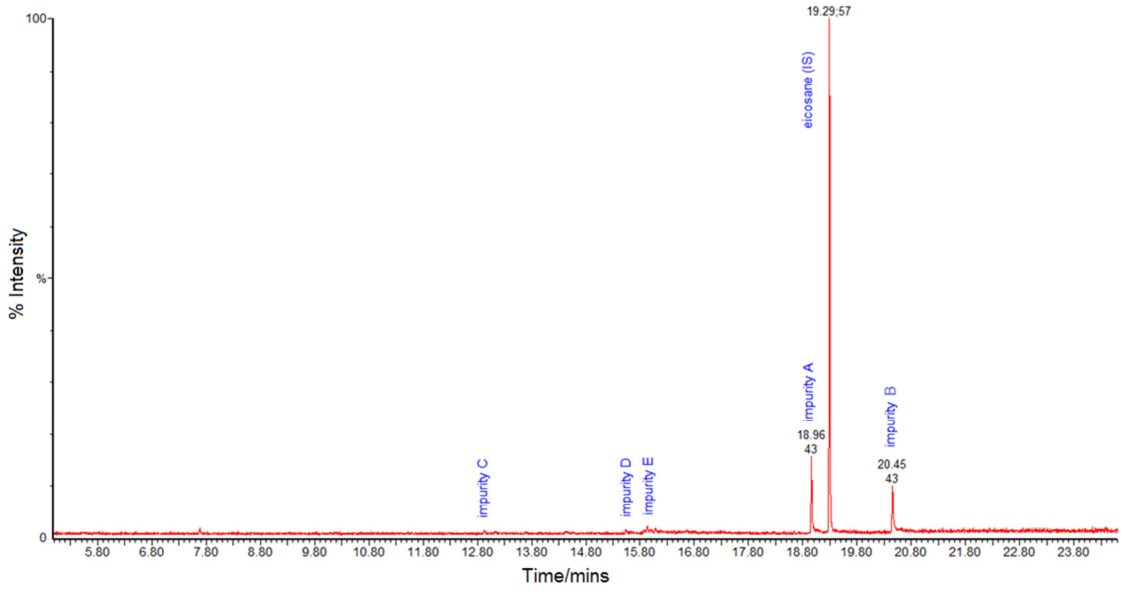


Figure 8.6 Total ion chromatogram of street sample A6.

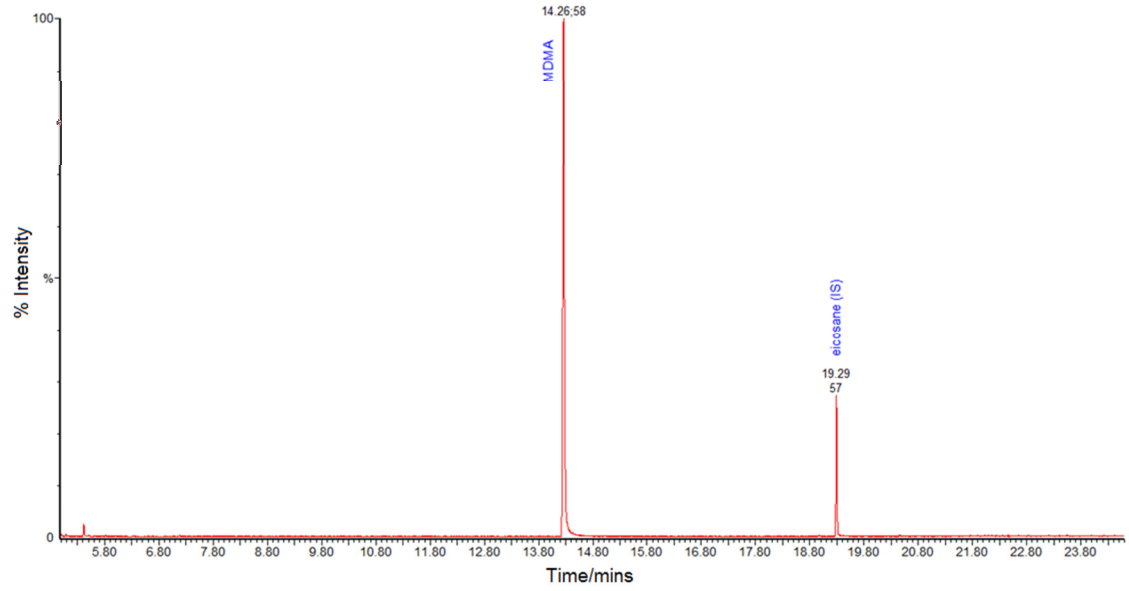


Figure 8.7 Total ion chromatogram of street sample A7.

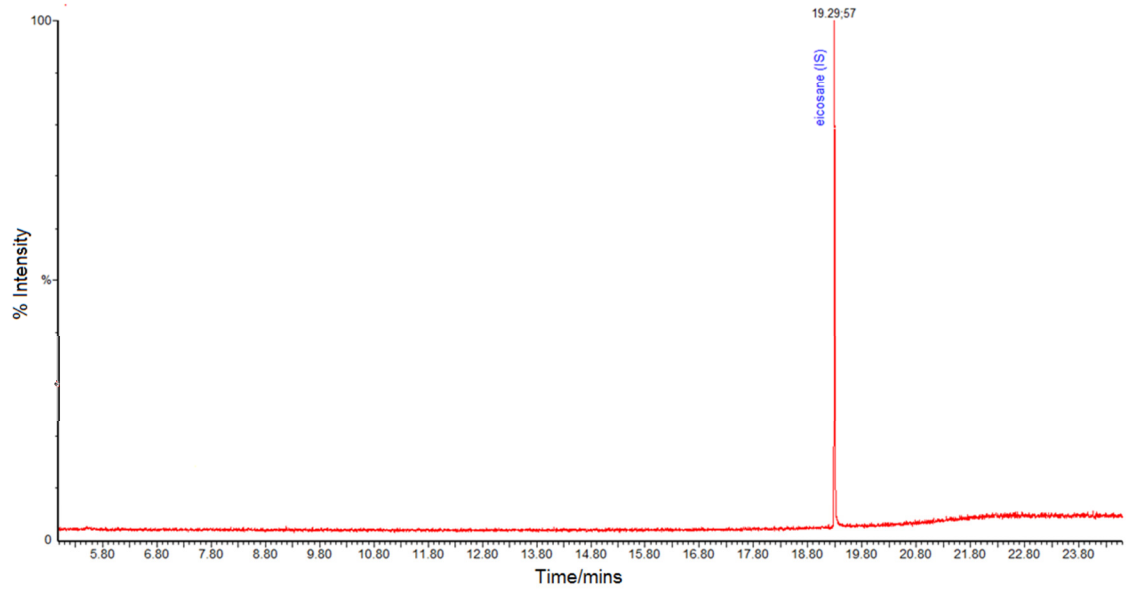


Figure 8.8 Total ion chromatogram of street sample A8.

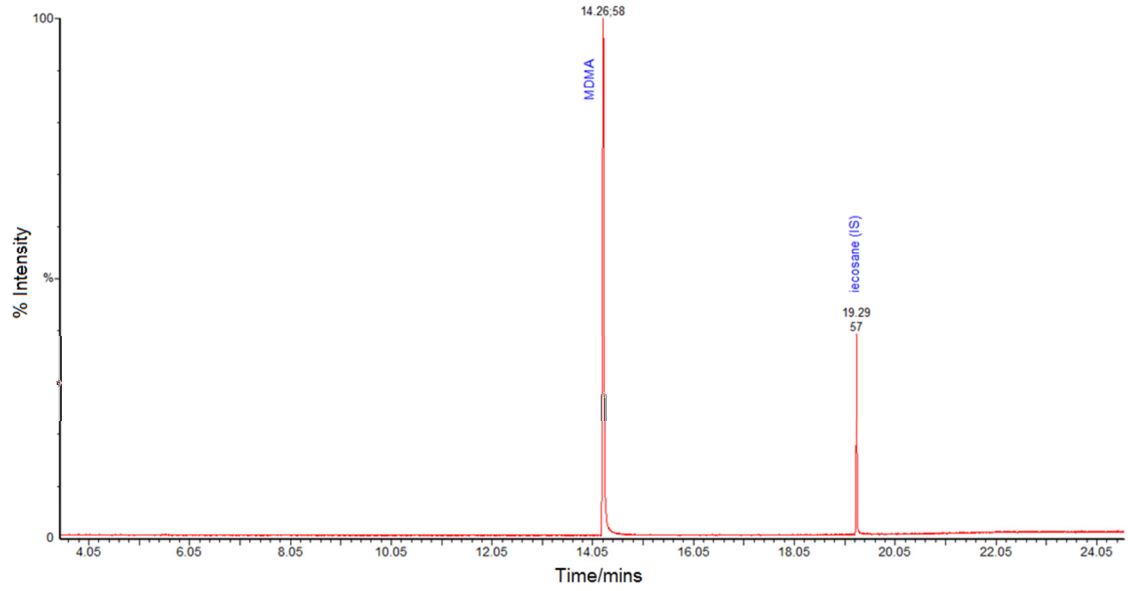


Figure 8.9 Total ion chromatogram of street sample A9.

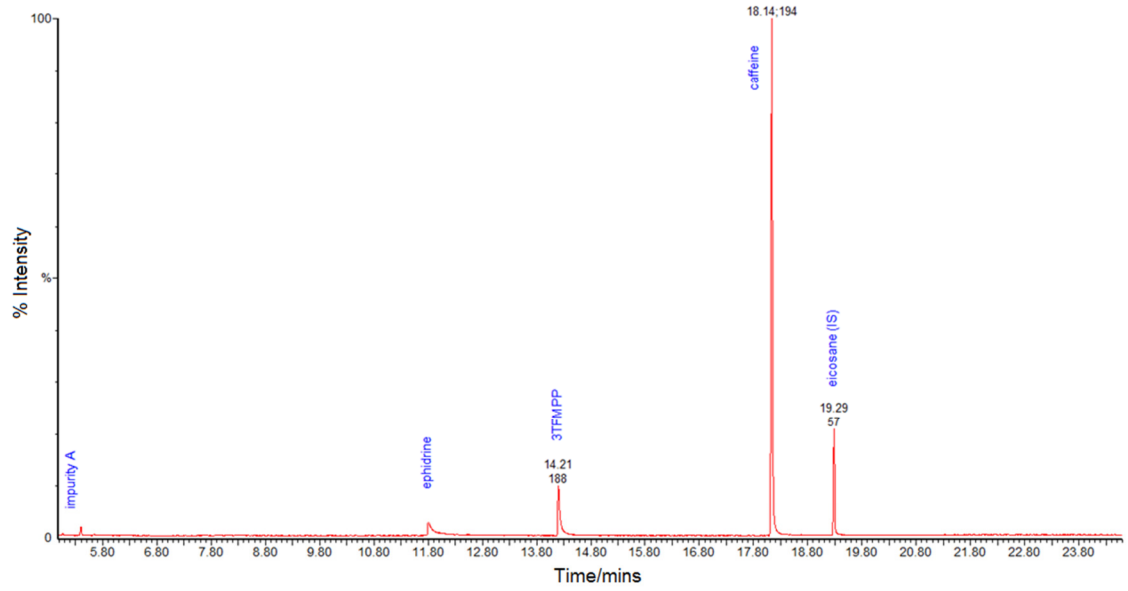


Figure 8.10 Total ion chromatogram of street sample A10

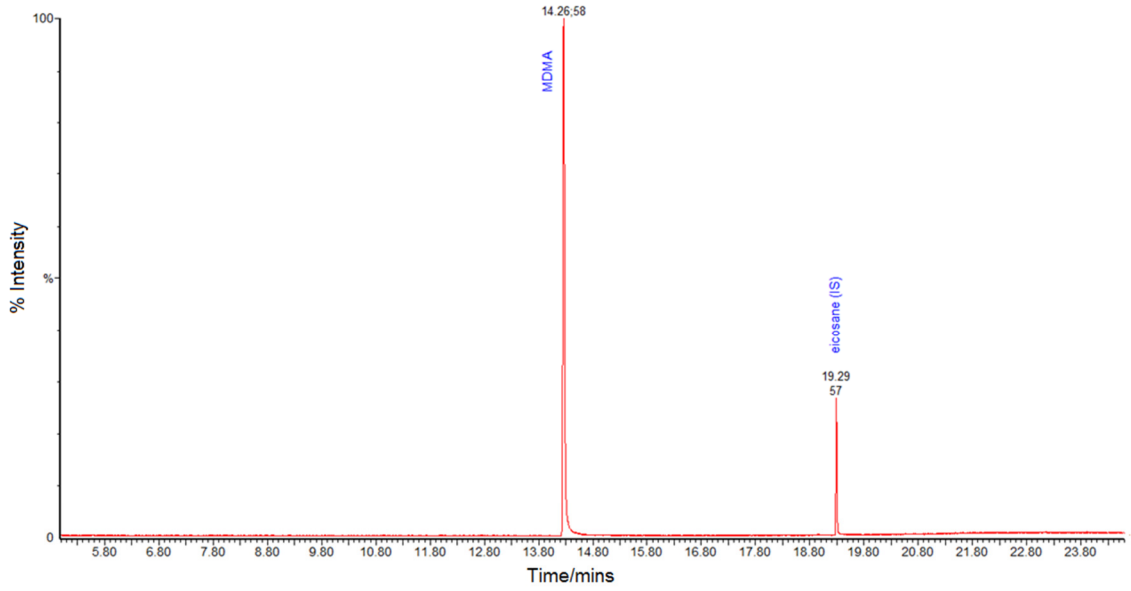


Figure 8.11 Total ion chromatogram of street sample A11.

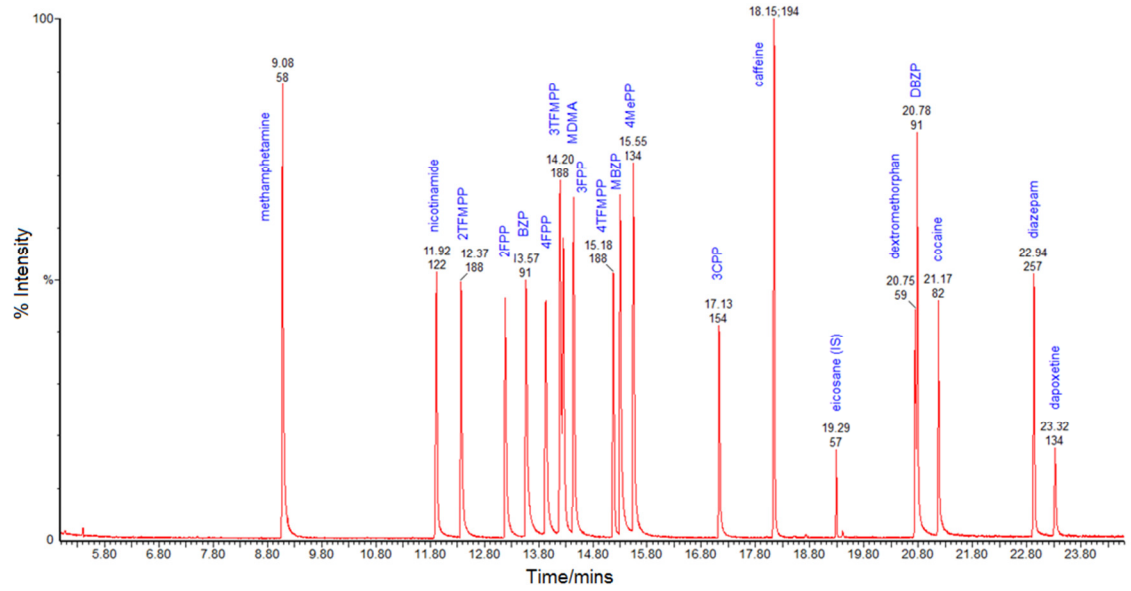


Figure 8.12 Total ion chromatogram of the standards.

The total ion chromatograms (Figures 8.1 - 8.11) show that one or more substances exist in the street samples. Identification of these substances was done on the basis of retention time (RT), relative retention time (RRT), retention index (RI) and mass spectra comparative to the reference standards (Figure 8.12). The data is shown in Table 8.5 below and the mass spectra in Appendix 12. In addition, the components were quantified and the data is also given in Table 8.5.

Table 8.5 Chemical characteristics of the street samples analysed tablets.

Name	**Substances observed in sample**				
	RT (mins)	**RRT**	**Principal ions observed in mass spectra (GC-MS, EI, 70eV), m/z**	**Identity**	**Content per tablet in mg (%mass)**
A1	13.61 14.22 20.79 18.97 20.46	0.705 0.737 1.077 0.983 1.060	91(100), 134, 56, 65, 176(M^+) 188(100), 230(M^+), 56, 145, 172 91(100), 120, 65, 135, 106, 266(M^+)	BZP 3-TFMPP DBZP Impurity A Impurity B	56 (17%) 106 (32%) 8 (2%)
A2	11.81 14.21 18.14 18.96 20.46	0.612 0.218 0.940 0.983 1.061	58(100), 77, 105, 117, 146, 165(M^+) 188(100), 230(M+), 56, 145, 172 194(M+, 100), 109, 55, 67, 82	Ephedrine 3-TFMPP Caffeine Impurity A Impurity B	55 (18%) 70 (23%) 92 (30%)
A3	14.28 18.13	0.741 0.940	58(100), 135, 77, 51, 55, 193(M^+) 194(M+, 100), 109, 55, 67, 82	MDMA Caffeine	50 (16%) 0.3 (0.1%)
A4	14.26	0.739	58(100), 135, 77, 51, 55, 193(M+)	MDMA	55(18%)
A5	13.55 14.28 20.75 18.11 18.93 20.42 14.87	0.704 0.741 1.077 0.940 0.983 1.060 0.772	91(100), 134, 56, 65, 176(M+) 188(100), 230(M+), 56, 145, 172 91(100), 120, 65, 135, (266, M+) 194(M+, 100), 109, 55, 67, 82	BZP 3-TFMPP DBZP Caffeine Impurity A Impurity B Impurity C	17 (8%) 21 (9%) 1 (0.5% Trace
A6	18.96 20.45 12.92 15.54 15.93	0.983 1.060 0.670 0.806 0.826	No psychoactive substance was detected.	Impurity A Impurity B Impurity D Impurity E Impurity F	
A7	14.26	0.739	58(100), 135, 77, 51, 55, 193(M^+)	MDMA	100 (38%)
A8	No analytes were detected.				
A9	14.26	0.739	58(100), 135, 77, 51, 55, 193(M^+) 194(M+, 100), 109, 55, 67, 82	MDMA Caffeine	50 (22%) 0.1 (0.04%)
A10	11.81 14.21	0.612 0.737	58(100), 77, 105, 117, 146, 165(M^+) 188(100), 230(M+), 56, 145, 172	Ephedrine 3-TFMPP	6 (2%) 36 (10%)

	18.14 5.65	0.940 0.293	194(M+, 100), 109, 55, 67, 82	Caffeine Impurity A	101 (28%)
A11	14.26	0.739	58(100), 135, 77, 51, 55, 193(M^+)	MDMA	69 (24%)

Table 8.6 Qualitative GC-MS data for the standards for confirmation of unknown identities.

Compound	**Retention time/mins**	**Relative retention time**	**Retention index**
Methamphetamine	9.08	0.471	1198.31
Nicotinamide	11.92	0.618	1390.61
2-TFMPP	12.37	0.641	1421.01
2-FPP	13.18	0.683	1475.74
BZP	13.57	0.703	1502.09
4-FPP	13.93	0.722	1526.42
3-TFMPP	14.20	0.736	1544.66
MDMA	14.26	0.739	1548.72
3-FPP	14.44	0.749	1560.88
4-TFMPP	15.18	0.787	1610.88
MBZP	15.30	0.793	1618.99
4-MePP	15.55	0.806	1635.88
3-CPP	17.13	0.888	1742.64
Caffeine	18.15	0.941	1865.00
Dextromethorphan	20.75	1.076	2190.00
DBZP	20.78	1.077	2193.75
Cocaine	21.17	1.097	2242.50
Diazepam	22.94	1.189	2463.75
Dapoxetine	23.32	1.209	2511.25
Eicosane (IS)	19.29	1.000	2007.50

The retention times, relative retention times and retention indices of the substances found in the street samples identified the peak at 13.61 minutes as BZP, 14.22 minutes as TFMPP, 20.79 minutes as DBZP, 11.81 minutes as ephedrine, 18.14 minutes as caffeine and 14.28 minutes as MDMA (Figures 8.1 - 8.10 and 8.10). This was due to the analytes having similar characteristics to those of the reference standards for these drugs (Table 8.6 comparative to Table 8.5). This was further confirmed by the similarity in the mass spectra (Table 8.5 and Appendix 12). For example, for sample A it is evident that the peak at

13.61minutes with a relative retention time of 0.705 and principal mass spectral ions at mz/ 91(100), 176(M^+), 134, 56 and 65 corresponds to the BZP reference standard (Table 8.6 and Appendix 12). The standard has similar retention and mass spectra data.

However, for the (2, 3, 4) TFMPP isomers the mass spectra was similar and could not be distinguished. This was a limiting factor in specifically identifying the peak at 14.22 minutes using mass spectra. This challenge was also observed in other studies involving positional isomers (Takahashi et al., 2009, Inoue et al., 2004; Elliot and Smith 2008). In this study the isomers showed complete peak separation (Figure 8.12), hence this limitation was overcome by investigating the retention times, relative retention times and retention indices of the unknown substances comparative to the drug standards (Tables 8.5 and 8.6). Consequently, the composition of the samples was confirmed as containing a combination of the following a) BZP, 3-TFMPP, DBZP for samples A1 and A5 in addition sample A5 contained trace amounts of caffeine. This could be due to contamination during manufacture since the amount was very low (trace level) compared to the caffeine content in other samples b) a combination of ephedrine, 3-TFMPP and caffeine for samples A2 and A10, c) MDMA for samples A3, A4, A7, A9 and A11 and d) no psychoactive substances for samples A6 and A8. In addition impurities were observed in most of the drugs with the exception of MDMA samples which had no impurities (samples A8, A9 and A11). The results obtained are in-line with the presumptive tests above and as such this confirms presumptive tests are an important aid in drug identification. The concentration was observed to be in the range 17 – 56mg BZP, 21 – 106mg 3-TFMPP, 1 – 8mg DBZP, 6 – 55mg ephedrine, 0.1 – 101mg caffeine and 50 – 100mg MDMA (Table 8.5). The total mass contributions ranged from 0% (samples A6 and A8) to 71% (sample A6). The solubility of the analytes in the solvent used was confirmed in sections 5.2.4 and 5.3.1. Consequently, the concentrations obtained can be attributed to the manufacturers adding low amounts of the drugs and or adulterants and making up with other non psychoactive bulking agents such as starch. This is most likely to increase profits, e.g. samples A6 and A8 had no psychoactive substances at all.

Yeap et al. (2010) in a review on 'legal highs' reported the prevalence of BZP and TFMPP combination in designer drugs with a variety of dosages such as 75mg BZP and 5mg TFMPP in Jet tablets. The authors reported the dosage range for BZP was 50 – 200mg and for TFMPP 5 – 25mg. They further stated that the drug seizures were tablets and capsules

of various colours and composition containing such drugs as cocaine, dextromethorphan, caffeine, steroids, ephedrine and vitamins among others. Caffeine was found to be the most common adulterant (9 9%). DEA (2009a) reported doses of BZP 50 - 200mg, CPP 90 - 110mg. The findings are similar to those obtained in this study in terms of the variety in the drugs. In this study it was found that TFMPP is the most common piperazine appearing in all the samples not containing MDMA (excluding those without psychoactive drugs), i.e., 4 out of 11 samples (36.4%) contained 3-TFMPP and 2 contained BZP (18.2%). However, the concentration of 3-TFMPP varied with caffeine. When 3-TFMPP was lower (A2, A10) it was observed that the caffeine content was higher and furthermore was also mixed with ephedrine.

Europol and EMCDDA (2007) reported that twenty six capsules and tablets were analysed by a team from St Georges University of London. BZP and TFMPP was also found to be the most common piperazine combination with a mean BZP content of 65mg and 22mg for 3-TFMPP. However the actual range reported varied widely 28 – 138mg BZP and 4 - 72mg TFMPP. As can be seen in Table 8.5 both BZP and TFMPP were found to be in combination, however these were also mixed with other adulterants. The concentration ranges also fall within those generally found by other researchers (de Boer et al, 2001; Kelleher et al., 2011, Takahashi et al., 2009). Takahashi et al (2009) investigated 205 different psychoactive drugs so as to create a library of psychoactive designer drugs. Whilst the study was purely qualitative the drugs identified in the study were also found to be present in the street samples investigated in this study. It is interesting to note that such diversity in drug concentration exists; hence the user is unaware of what they are getting.

Table 8.7 Qualitative analysis of impurities in sample.

Sample	**Impurity**			**Substances observed in sample**	
		RT (mins)	**RRT**	**Principal ions observed in mass spectra (GC-MS, EI, 70eV), m/z**	**Preliminary identity**
A1	Impurity A	18.97	0.983	57(100), 55, 43, 60, 73, 69, 129, 87, 83, 282, 96	N-hexanedecanoic acid
	Impurity B	20.46	1.060	41(100), 60, 73, 43, 69, 57, 71, 129, 83, 97, 171	Octanedecanoic acid
A2	Impurity A	18.96	0.983	41(100), 43, 73, 60, 69, 55, 71, 83,129, 171, 282	N-hexanedecanoic acid
	Impurity B	20.46	1.061	57(100), 55, 43, 41, 73, 60, 69, 83, 87, 96, 129	Octanedecanoic acid
A5	Impurity A	18.93	0.983	43(100), 73, 60, 57, 69, 83, 115, 129, 186, 157, 141	N-hexanedecanoic acid
	Impurity B	20.42	1.060	60(100), 55, 43, 69, 129, 97, 79, 209, 115, 191, 283	Octanedecanoic acid
	Impurity C	14.87	0.772	120(100), 44, 65, 92, 165, 137, 96, 106, 84, 84, 141	Ethyl P-acetamoobenzoate or benzocaine
A6	Impurity A	18.96	0.983	43(100), 41, 73, 55, 60, 57, 69, 71, 83, 129,61	N-hexanedecanoic acid
	Impurity B	20.45	1.060	43(100), 55, 57, 41, 60, 73, 69, 97, 129, 83,185	Octanedecanoic acid
	Impurity D	13.10	0.679	129(100), 77, 117, 56, 45, 132, 61, 156, 103, 65, 87	
	Impurity E	15.54	0.806	117(100), 75, 44, 133, 56, 50, 159, 89, 234, 98, 149	
	Impurity F	15.93	0.826	75(100), 129, 117, 133, 203, 103, 143, 175, 149, 159, 185	
	Impurity G	18.28	0.947	73(100), 74, 43, 103, 61, 55, 85, 133, 117	Sorbitol
	Impurity H	12.96	0.672	44(100), 103, 86, 73, 61, 57, 117, 133, 146	1,4-anhydro-glucitol
A10	Impurity A	5.65	0.293	105(100), 77, 50, 41, 56, 78, 191, 59, 67, 88, 145, 171	

The analysis shows the presence of some impurities In the total ion chromatograms (Figures) showed, the peak sizes for the impurities varied from trace to visually visible peaks and is an indication of the amount of the impurity present. In all the samples were impurities were observed (A1, A2, A5, A6, A10) impurities at retention times 18.96 minutes; relative retention time (RRT) at 0.983) and 20.46 minutes (relative retention times (1.061) were the most abundant giving small prominent peaks in the total ion chromatograms (Figures 8.1, 8.2, 8.5 and 8.6) above. The other impurities were at trace level. The mass spectra of the impurities is given in Appendix 12 (Figures 3, 4, 8, 9 and 11 - 19) and also for the other observed substances. A typical representation of the mass spectra is shown below for the common impurities (Impurities A and B).

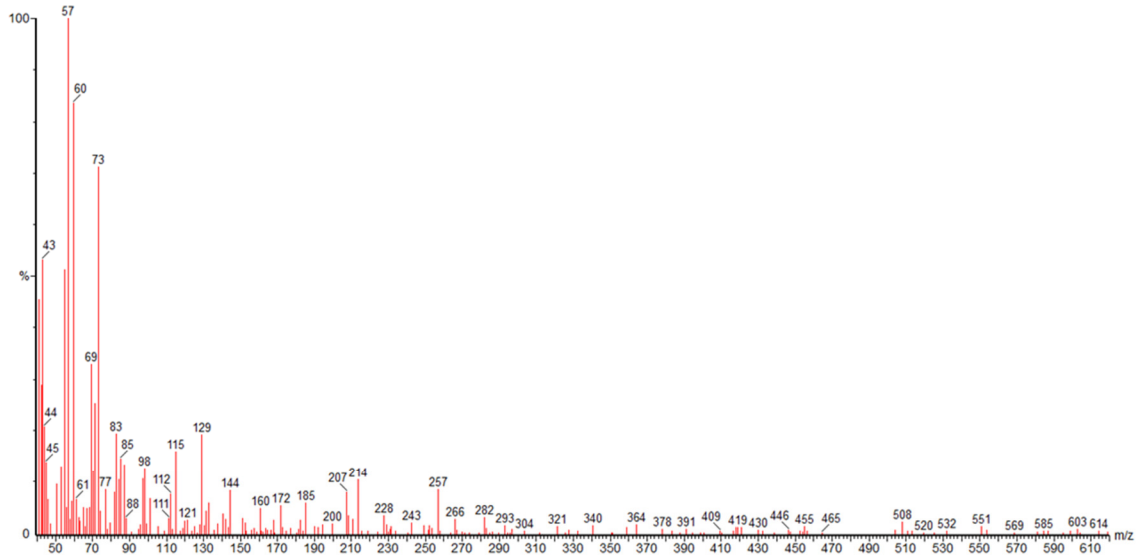


Figure 8.13 Mass spectrum of sample A1 peak at 18.97mins (RRT = 0.983) identified as N-hexanedecanoic acid (impurity A).

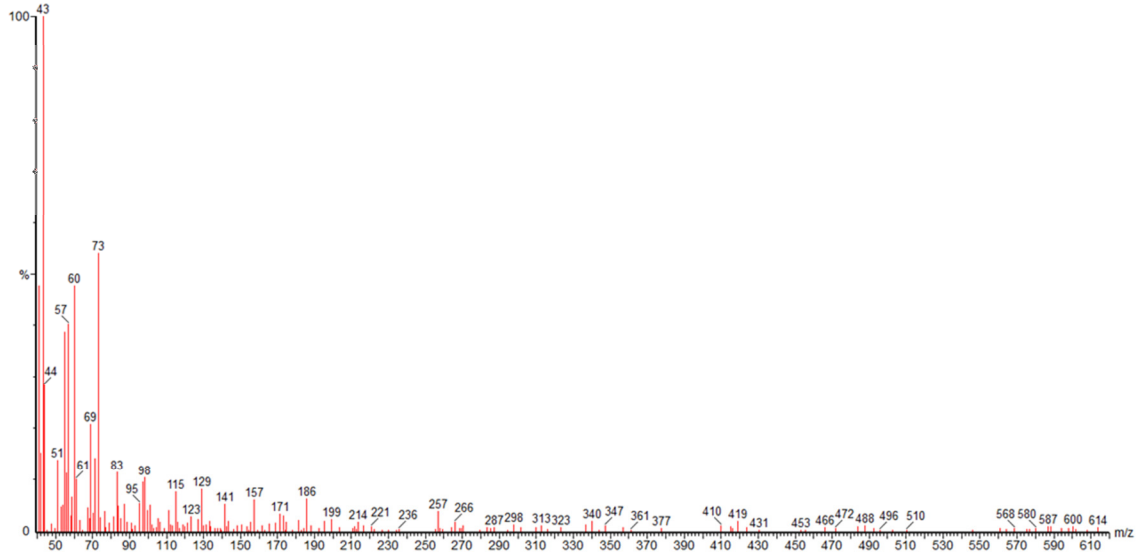


Figure 8.14 Mass spectrum of sample A5 peak at 18.93mins (RRT = 0.983) identified as N-hexanedecanoic acid (impurity A).

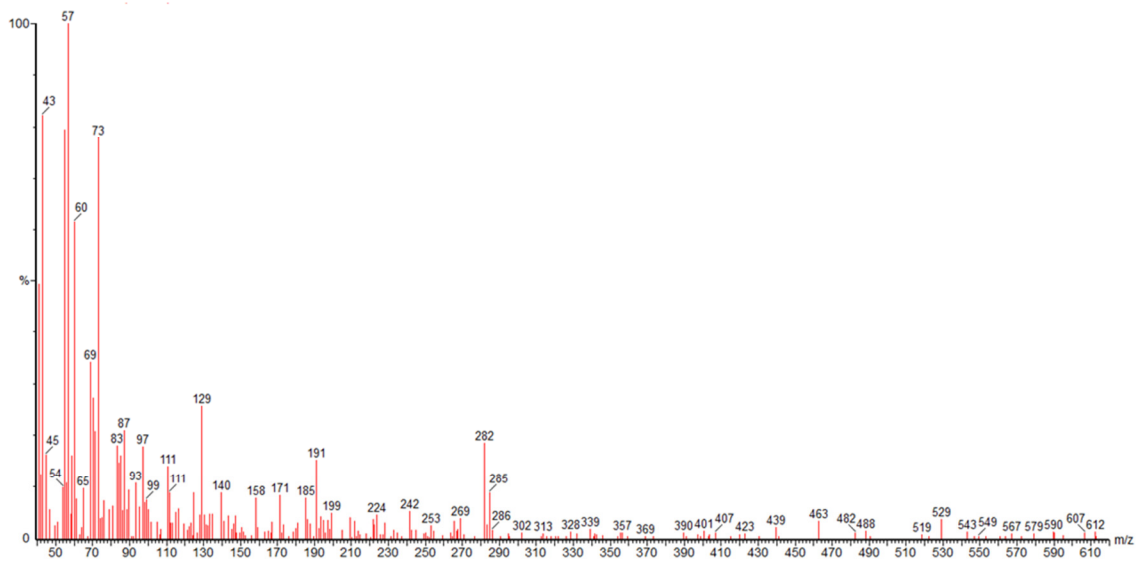


Figure 8.15 Mass spectrum of sample A1 peak at 20.46mins (RRT = 1.060) identified as octanedecanoic acid (impurity B).

The impurities were preliminary identified using the NIST spectral library. The most common impurities were identified as N-hexanedecanoic acid (impurity A) and octanedecanoic acid (impurity B). These are palmitic acid and stearic acid respectively; common fatty acids which are routinely used as lubricants in manufacture of pharmaceuticals such as tablets (King, 2009; Simth and Webb, 2007). These have also been identified in investigation of street drugs (Kelleher et al., 2011). In addition sorbitol and

1,4-anhydro-glucitol (sample A6 impurity G and H) were present in the sample with no psychoactive substances (sample A6). These are sugars also commonly found in tablets, capsules and other pharmaceutical preparations where they act as sweeteners (Smith and Webb, 2007). Consequently, it is suggested that the samples with no psychoactive substances are likely for the purpose of misleading the user into thinking they contain the real drugs. Baron et al. (2011), Davies et al. (2010) and Kelleher et al. (2011) had similar findings in their studies in addition they also reported the presence of tablets with no pyschoactive substances.

Some of the impurities observed in the different samples show similar chemical properties in terms of retention times, hexanedecanoic acid and octanedecanoic acid (impurities A and B) in samples A1, A2, A5 and A6 as shown by their similar retention times (Table 8.7). It is therefore possible that samples A1, A2, A5 and A6 similar reagents were used in the manufacture of these samples or they were potentially manufactured from the same source. This is also confirmed by the similarity in the composition of drugs highlighted in the discussion of Table 8.5 above. A1 was identified as having similar composition to A5 and A2 to A10, however the dosages were different. The MDMA samples were observed not to have any impurities. However, similarities in dosage were observed between samples A3 and A9. Both samples contained the adulterant caffeine. In addition, it was identified above that the tablet quality was poor for these samples. Potentially, they could also be from the same source.

8.3.2 SYNTHESIS

8.3.2.1 Synthesis Route 1 and 2 product description and yields

The synthesised 4-FPP was a dark brown viscous liquid. The synthesised 3-TFMPP was observed to be a dark brown liquid. The amounts of reagents used and the yields generated on synthesis are shown in the tables below. For the synthesis conducted by route 1 (Liu and Robichaud, 2005) the results are in Tables 8.8 and 8.9 and for route 2 (Kiritsy et al., 1978) Table 8.10. Also given in tables is the variation of yield with reaction time.

Table 8.8 4-FPP synthesis yields: Route 1.

Reference	**Amount/Run**					
Synthesis 4-FPP	1 (trial)	2	3	4	5	6
4-fluoroaniline (g)	0.6737	0.3340	0.3334	0.6105	0.6658	0.6702
Bis(2-chloroethyl)amine HCl (g)	1.072	0.5355	0.5355	0.4906	0.5347	1.0761
Product amount (g)	0.7431	0.3495	0.3479	0.8100	0.8770	0.8950
Expected stoichiometric amount(g)	1.0823	0.5407	0.5407	1.0814	1.0814	1.0865
Product yield (%)	68.66	64.64	64.34	74.90	81.10	82.37
Reaction time (hours)	7.5	7.0	7.5	8.0	10.0	12.0

Table 8.9 3-TFMPP synthesis yields: Route 1.

Reference	**Amount/ Run**				
Synthesis 3-TFMPP	1(trial)	2	3	4	5
3-Trifluoromethylaniline (g)	0.4834	0.9670	0.4836	0.9675	0.9677
Bis(2-chloroethyl)amine hydrochloride (g)	0.5357	1.0720	0.5358	1.0740	1.0730
Product amount (g)	- [1]	0.7538	0.4018	0.8785	0.8569
Expected stoichiometric amount (g)	0.6907	1.3817	0.6910	1.3824	1.3826
Product yield (%)	N/A	54.56	58.15	63.55	61.98
Reaction time (hours)	7.0	7.5	8.0	10.0	12.0

[1] Trial sample not determined due to leakage of during extraction.

The yields generated using route 2 synthesis (Kiritsy et al., 1978) results are shown in Table 8.10 below.

Table 8.10 3-TFMPP synthesis yields: Route 2.

Reference	Amount/ Run	
Synthesis 3-TFMPP	1	2
Anhydrous piperazine/g	4.3535	4.3535
3-bromo-l-(trifluoromethy1)benzene/g	2.2727	2.7400
Product amount (g)	1.4272	1.4568
Expected stoichiometric amount (g)	1.8511	1.8511
Product yield (%)	77.1%	78.7%

The product yields obtained were comparable but slightly lower than the expected yields (Tables 8.8 – 8.10), e.g. 82.37% 4-FPP compared to the expected 87%. In their studies the

authors reported yields of 87% 4-FPP and 67% 3-TFMPP (Liu and Robichaud, 2005). According to Kiritsy et al. (1978) synthesis by their procedure (route 2) should give expected of 30% 4-FPP and 81% 3-TFMPP. For the synthesis of 4-FPP route 1 (Liu and Robichaud, 2005) gave relatively high yields, comparatively the results showed a much higher disparity to that of synthesis route 2 (Kiritsy et al., 1978), 87 % versus 30%. However, for 3-TFMPP the method by Kiritsy et al. (1978 as expected gave higher yields for 3-TFMPP, 81 % versus 67% respectively. The disparities could be due to the presence of competitive side reactions, consequently reducing the product yield. In the reaction (Chapter 1; Figures 1.18a,b) of anilines (I) with bis(2-chloroethylamine) (II), a competitive side reaction of the product of the reaction (III) and reactant (II) occurs resulting in low yields of the product. This is possible if the reaction is carried out in the presence of a base. In synthesis route 1, diethylene glycol monomethyl ether is used so as to minimise the competitive side reaction. Consequently, the use of sodium carbonate as part of the reactants in Kiritsy et al. (1978), (Chapter 1; Figure 1.16b) is likely to have resulted in low yields by driving the side reaction shown in Figure 1.15. Assuming that the side reaction occurs, the following products (II and III) could be produced as impurities (Figure 8.16);

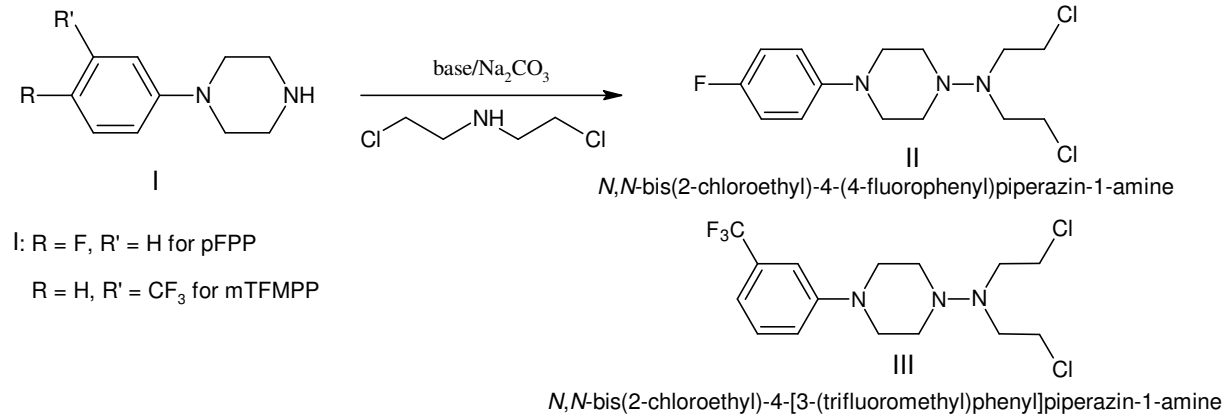


Figure 8.16 Reaction for potential impurities arising from Route 1 synthesis (section 1.7).

Both methods were found easy to use with the synthesis route 2, being much simpler as it involved fewer steps in the procedure but its reflux period was about 3 times longer (12 hours versus 45 - 46hours). In addition, synthesis route 1 method for is more amenable to the synthesis of a wider range of 1-arylpiperazine, in the study by Liu and Robichaud (2005) a total of 11 1-arlypiperazines were synthesised both psychoactive and non-psychoactive. It can therefore be concluded that both the routes of synthesis are viable and

have potential for the synthesis of 4-FPP and 3-TFMPP drug illicitly. Furthermore, they have potential for application to other phenylpiperazine drugs of abuse.

The impact of reaction time on yield is shown by the variation in product yield with reaction time (Tables 8.8 and 8.9). The yield increased with increase in reaction time. In the tables it can be seen that with lower reaction times lower yields are obtained, at 7 hours 64.6% 4-FPP was obtained comparative to 74.9% at 8 hours and 82.4% at 12 hours. Liu and Robichaud (2005) in their study reported the reaction time as 6-12hours and expected yields of 87% 4-FPP. Consequently, it is evident that this was achieved at the longest reaction time stated (12 hours). 3-TFMPP followed a similar trend (54.56 to 61.98%). As such it was concluded advantageous to use the longest reaction time so as to obtain better yields. This may impact on the procedure followed by other researchers in future studies.

8.3.2.2 Identification of synthesised samples by UV-Vis analysis

Results for the confirmation of identity of synthesised samples by UV-Vis are shown in Tables 8.11 for the FPP sample and 8.12 for the TFMPP. The UV-Vis spectra are given in Figures 8.17 and 8.18.

Table 8.11 UV-Vis analysis of synthetic 4-FPP.

Name	**Wavelength, λmax (nm)**
2-FPP standard	241.0 -
3-FPP standard	250.0 279.9
4-FPP standard	241.0 290.0
Synthesised FPP sample	241.0 293.0

Table 8.12 UV-Vis analysis of synthetic 3-TFMPP.

Name	**Wavelength, λmax (nm)**
2-TFMPP standard	254.0 -
3-TFMPP standard	263.0 -
4-TFMPP standard	254.0 297.0
Synthesised 4-FPP sample	254.9 298.0

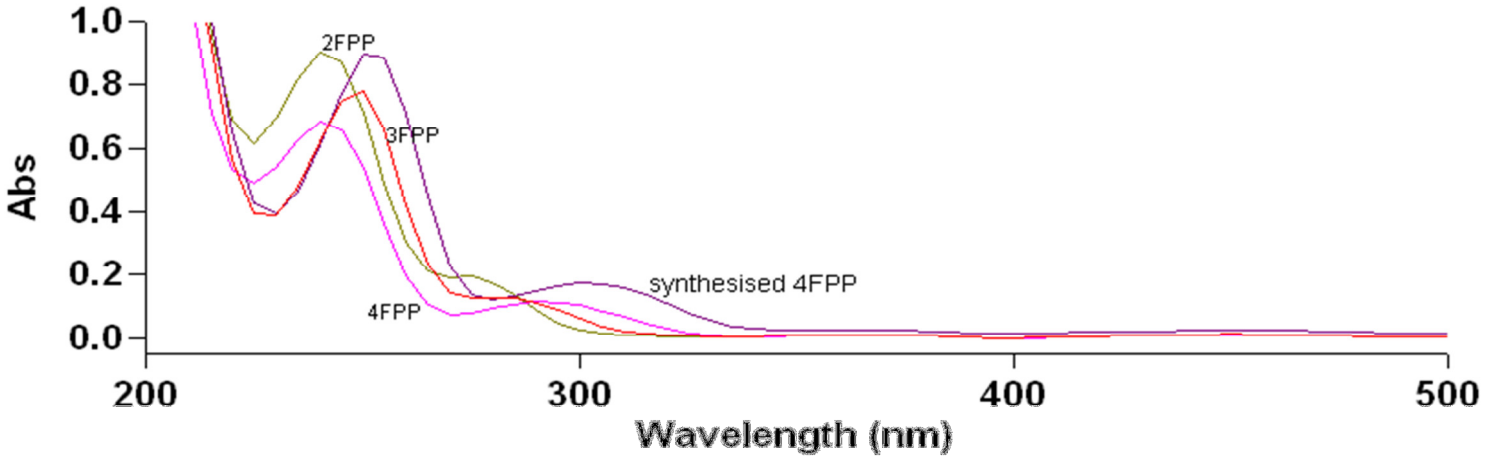


Figure 8.17 UV-Vis spectra of the synthesised FPP relative to (2, 3, 4) FPP standards.

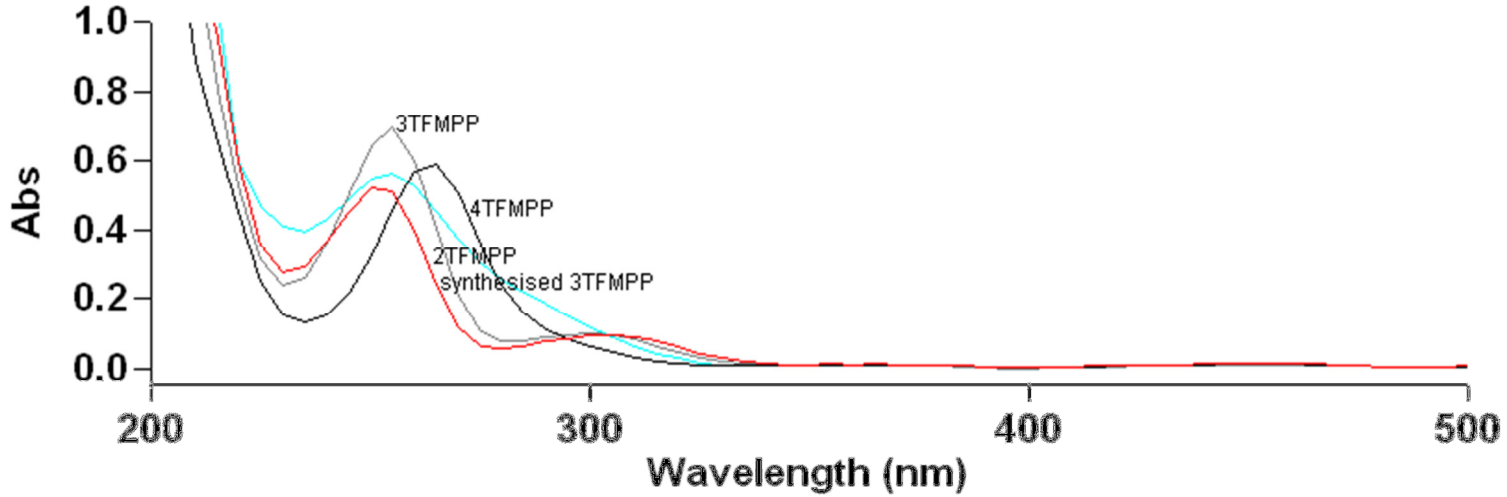


Figure 8.18 UV-Vis spectra of the synthesised TFMPP relative to (2, 3, 4) TFMPP standards.

The synthesised samples had similar UV-Vis spectral profiles to 4-FPP and 3-TFMPP standards (Tables 8.11 and 8.12; Figures 8.17 and 8.18). This is evidenced by their similar spectra and wavelengths, e.g. the synthesised FPP sample and 4-FPP standard both show λmax 241nm and 290nm which are absent in the other isomers. As such the UV-Vis results suggest that the synthesised samples are 4-FPP and 3-TFMPP.

8.3.2.3 Identification of synthesised samples by FTIR-ATR analysis

Results for confirmation of identity of synthesised FPP sample (route 1) by FTIR are shown in Tables 8.13, 8.14 and Figure 8.19. The 4-FPP reference spectrum is given in Figure 8.20 for comparison. Statistical analysis by Pearson's correlation was determined on the entire spectrum. This gave an indication of the significance of the relationship (similarity) between the sample and reference spectra. This was of use in determining the identity of the sample relative to the isomers.

Table 8.13 Identification of synthesised 4-FPP by FTIR: correlation of spectrum.

Compound	**Correlation coefficient**
Synthesised FPP sample to 2-FPP standard	0.32
Synthesised FPP sample to 3-FPP standard	0.14
Synthesised FPP sample to 4-FPP standard	0.95

Table 8.14 FTIR peak table for 4-FPP showing characteristic peaks for identification of sample.

Name	**Characteristic peaks (wavenumber cm^{-1}) (UNODC, 2013c)**	**Observed peaks (wavenumber cm^{-1})**
2-FPP standard	764, 1149, 1209, 1252, 1500 [1]	748, 1139, 1207, 1233, 1498
3-FPP standard	Not given	759, 1165, 1177, 1250, 1494
4-FPP standard	845, 1165, 1228, 1423, 1512 [2]	812, 1161, 1228, 1416, 1505
Synthesised TFMPP sample	816, 1121, 1224, 1414, 1508	816, 1121, 1224, - 1508

[1] 2-FPP.HCl; [2] 4-FPP.2HCl: UNODC data was given for the salt form.

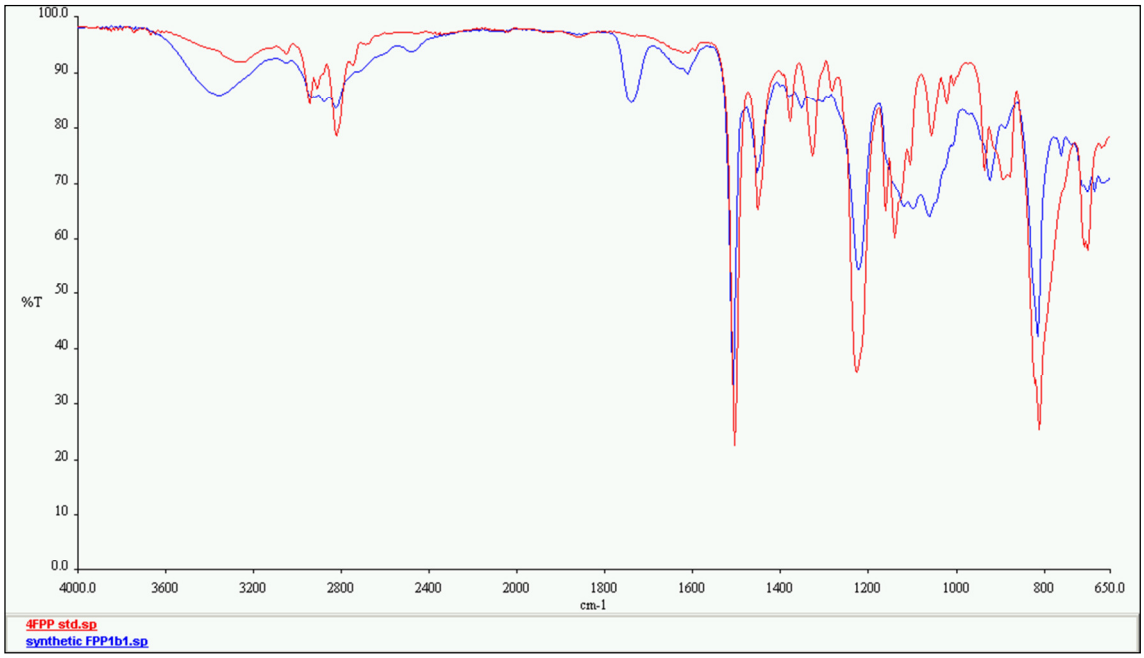


Figure 8.19 FTIR spectra of synthesised FPP comparative to 4-FPP standard.

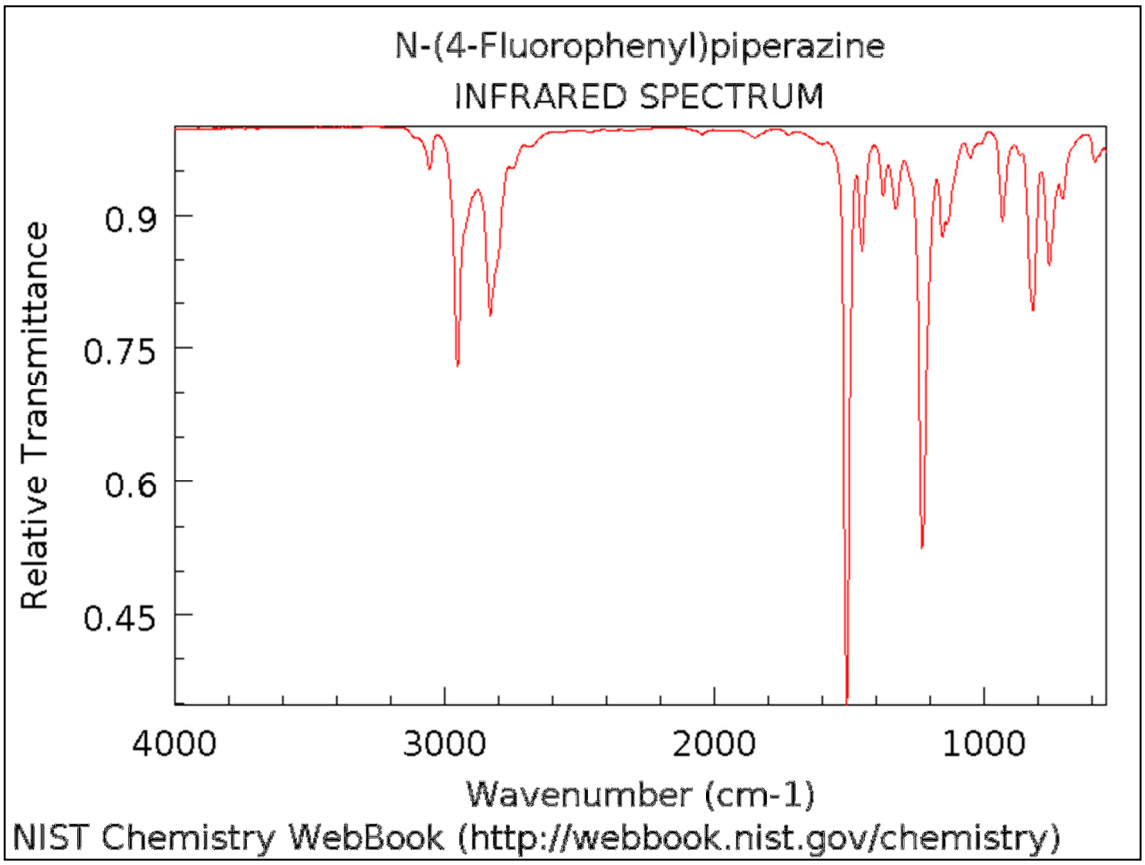


Figure 8.20 Literature reference FTIR spectra for identification of the synthesised 4-FPP (NIST, 2014).

Results for confirmation of identity of synthesised TFMPP sample (route 1) by FTIR are shown in Tables 8.15, 8.16 and Figure 8.21. The reference spectrum is given in Figure 8.22 for comparison. The correlation coefficients were determined on the entire spectrum.

Table 8.15 Identification of synthesised 3-TFMPP by FTIR: correlation of spectrum.

Compound	**Correlation coefficient**
Synthesised TFMPP sample to 2-TFMPP standard	0.28
Synthesised TFMPP sample to 3-TFMPP standard	0.93
Synthesised TFMPP sample to 4-TFMPP standard	0.32

Table 8.16 FTIR peak table for 3-TFMPP showing characteristic peaks for identification of sample.

Name	**Characteristic peaks (wavenumber cm^{-1}) (UNODC, 2013c)**	**Observed peaks (wavenumber cm^{-1})**
2-TFMPP standard	1036, 1109, 1136, 1315, 1454	1034, 1105, 1131, 1310, 1454
3-TFMPP standard	1120, 1163, 1319, 1354, 1450	1114, 1160, 1318, 1351, 1448
4-TFMPP standard	1068, 1109, 1244, 1325, 1614	1069, - 1242, 1321, 1614
Synthesised TFMPP sample	-	1113, 1158, 1318, 1343, 1450

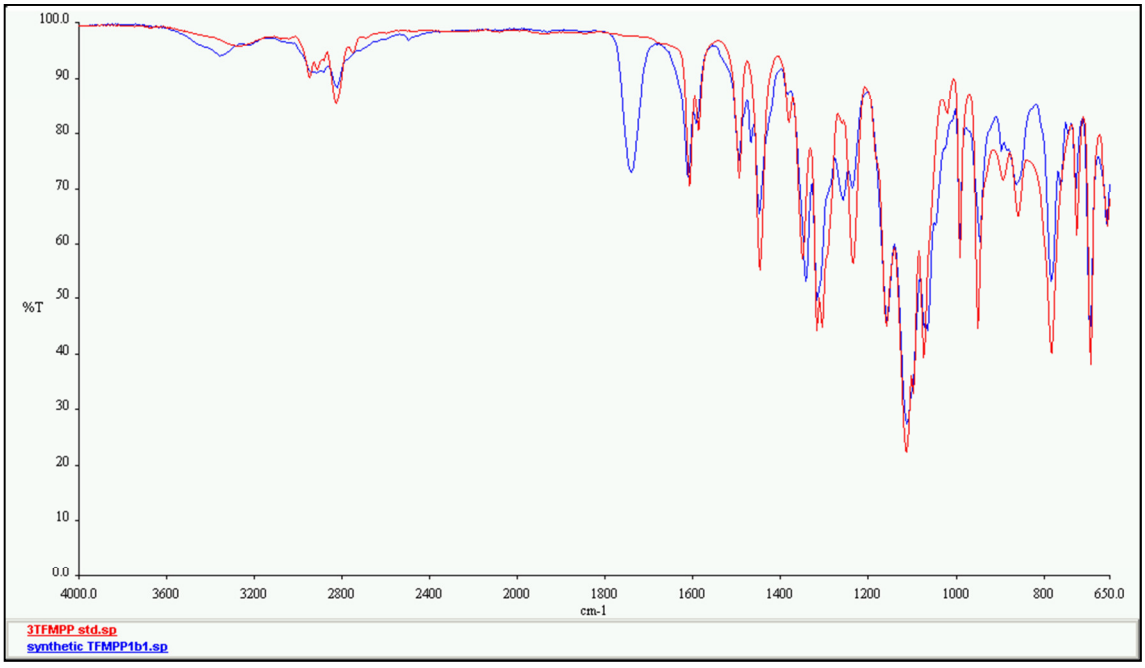


Figure 8.21 FTIR spectra of synthesised TFMPP comparative to 3-TFMPP standard.

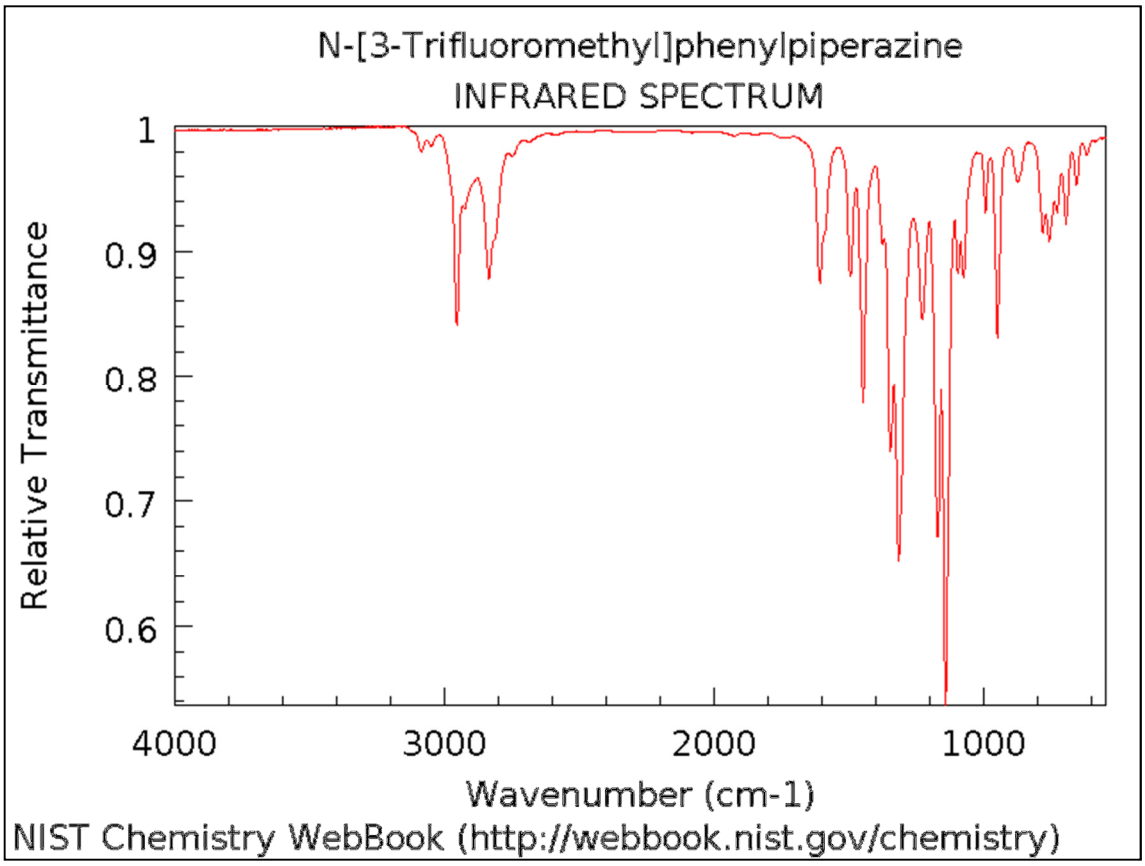


Figure 8.22 Literature reference FTIR spectra for identification of the synthesised TFMPP (NIST, 2014).

The FTIR spectra of the isomers of both FPP and 3-TFMPP were sufficiently different to allow distinction of 4-FPP and 3-TFMPP from its isomers (Tables 8.13 – 8.16 and Figures 8.19 - 8.22). The synthesised FPP samples showed the highest correlation to the 4-FPP standard with a correlation coefficient, *r* of 0.95. The correlation to the other isomers was significantly lower, $r = 0.14$ relative to 3-FPP and $r = 0.32$ relative to 2-FPP (Table 8.13). For the TFMPP synthesised sample, the correlation was 0.28 relative to 2-TFMPP, 0.32 to 4-TFMPP and 0.93 to 3-TFMPP (Table 8.15). However, the FTIR spectra of the 2-TFMPP and 4-TFMPP isomers were relatively similar as can be seen from their close correlation coefficients (Table 8.15). It was discussed in Chapter 2 (section 3.3.4) that the higher the correlation coefficient the greater the similarity. A correlation coefficient of 1.0 implies that the substances being compared are the same. This was also confirmed by the characteristics peaks observed in the sample (Tables 8.14 and 8.16). It can therefore be suggested that the synthesised samples are 4-FPP and 3-TFMPP. The results also confirm the study by

UNODC (2013c) were it was reported that 4FPP and 3-TFMPP could be distinctly identified by FTIR.

8.3.2.4 Identification of synthesised samples by GC-MS analysis

To further confirm the identity of the synthesised samples, analysis by GC-MS was conducted. The data is given in Table 8.17 and the masss spectra are given in Figures 8.25 (FPP sample) and 8.26 (TFMPP sample).

Table 8.17 GC-MS confirmation data for the identification of the synthesised substances.

Compound	**Retention time/mins**	**Relative retention time**	**Retention indices**
2-FPP standard	13.50	0.730	1511.69
3-FPP standard	14.51	0.785	1591.85
4-FPP standard	14.13	0.764	1561.69
Synthesised 4-FPP sample	14.12	0.764	1560.90
2-TFMPP standard	12.61	0.682	1441.06
3-TFMPP standard	14.32	0.774	1576.77
4-TFMPP standard	15.09	0.816	1637.88
Synthesised 3-TFMPP sample	14.33	0.775	1577.56
Eicosane	18.49	1.000	2010.53[1]

[1]Accuracy 99.5% (expected eicosane retention indices = 2000)

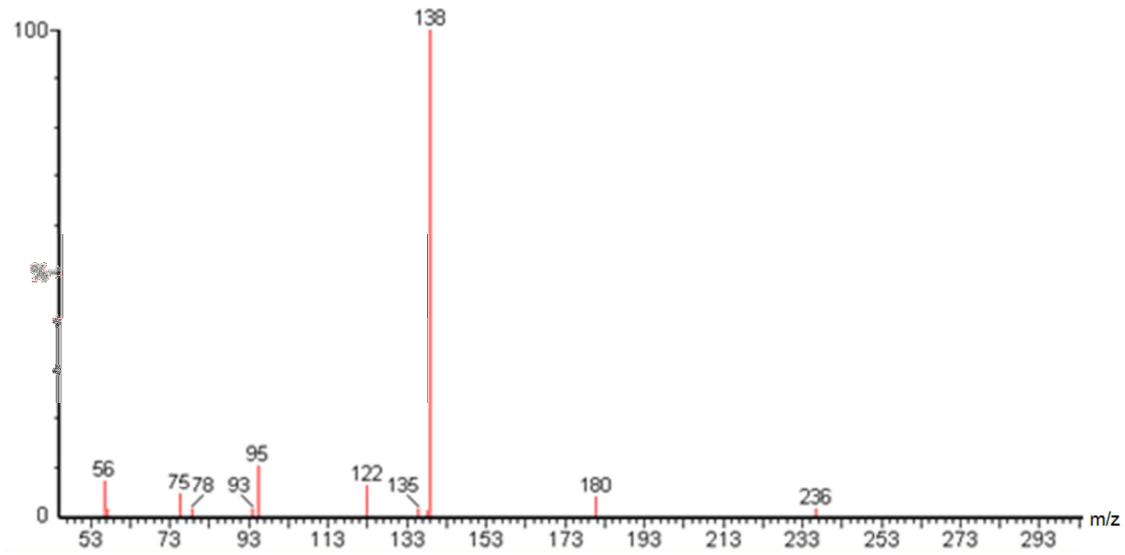


Figure 8.23 Mass spectra of peak at 14.25mins in the synthesised samples (Figure 8.25).

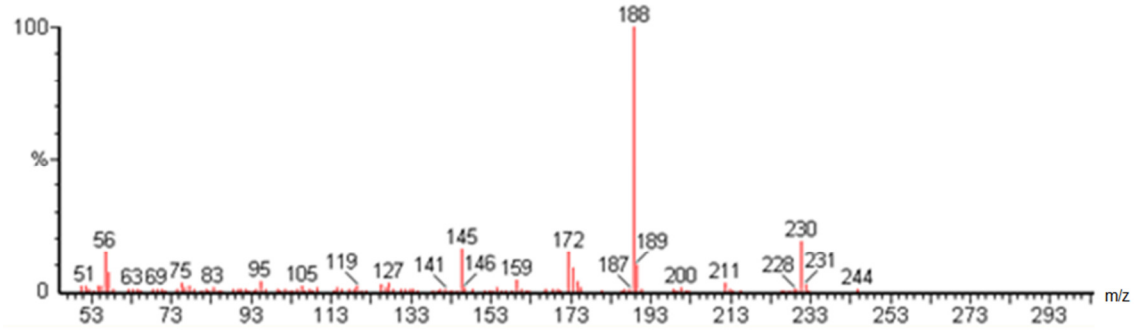


Figure 8.24 Mass spectra of the peaks 14.33mins in the synthesised samples (Figure 8.26).

Comparison of the synthesised sample mass spectra to those of the standards for FPP and TFMPP isomers (section 7.3.5.2 and Appendix 7.5) showed that in the samples ions characteristic to FPP and TFMPP (de Boer et al., 2001; Maurer, 2004; Takahashi et al., 2009) were observed. These were at m/z 138(100), 180(M+), 122 and 56 for the FPP sample in Figure 8.23. The characteristic ions observed for the TFMPP sample were at m/z 188(100), 230(M^+), 145 and 56 (Figure 8.24). Consequently, this confirmed the samples as containing FPP and TFMPP. However, on the basis of mass spectra alone it could not be distinguished which isomer had been synthesised. However, further identification on the basis of retention times and retention indices (Table 8.17) identified the synthesised substances as 4-FPP and 3-TFMPP. It is evident from the table that the retention times and retention indices of the synthesised samples are similar to the 4-FPP and 3-TFMPP

isomers. It can be seen, that for example the same relative retention times were observed for the synthesized sample and 4-FPP standard (0.764). In addition, their retention indices were similar (1562 and 1561 respectively). Thereby, confirming the samples as 4-FPP and 3-TFMPP.

Baker and Phillips (1983) in their review of analysis of drugs of abuse identified FTIR and UV-Vis spectrometry as techniques which can successfully be applied for identification. This is also evidenced by the work of Inoue et al. (2004) in their study on an analysis of seized BZP like compounds and Takahashi et al. (2009). The GC-MS results confirmed the results by UV-Vis and FTIR. Hence, it was concluded that the routes of synthesis investigated synthesised 4-FPP and 3-TFMPP. This is in agreement with the works of Liu and Robichaud (2005) and Kiritsy et al. (1978) where these substances were synthesised. Consequently, it was concluded that the routes of synthesis are viable and could be of use in clandestine synthesis of these substances.

Several impurities were observed in the synthetic samples as is evident in the chromatographic profiles of the synthesised samples, Figures 8.25 and 8.26.

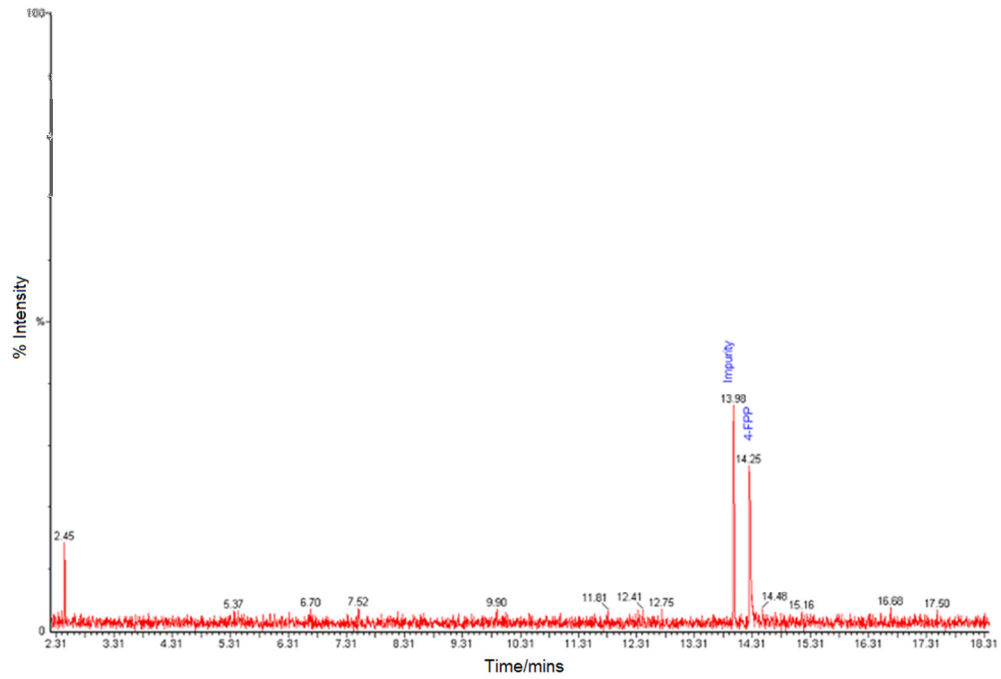


Figure 8.25 GC-MS Total Ion Chromatogram for identification of synthesised FPP (route 1) showing the peak identified as 4-FPP and the main impurity peak.

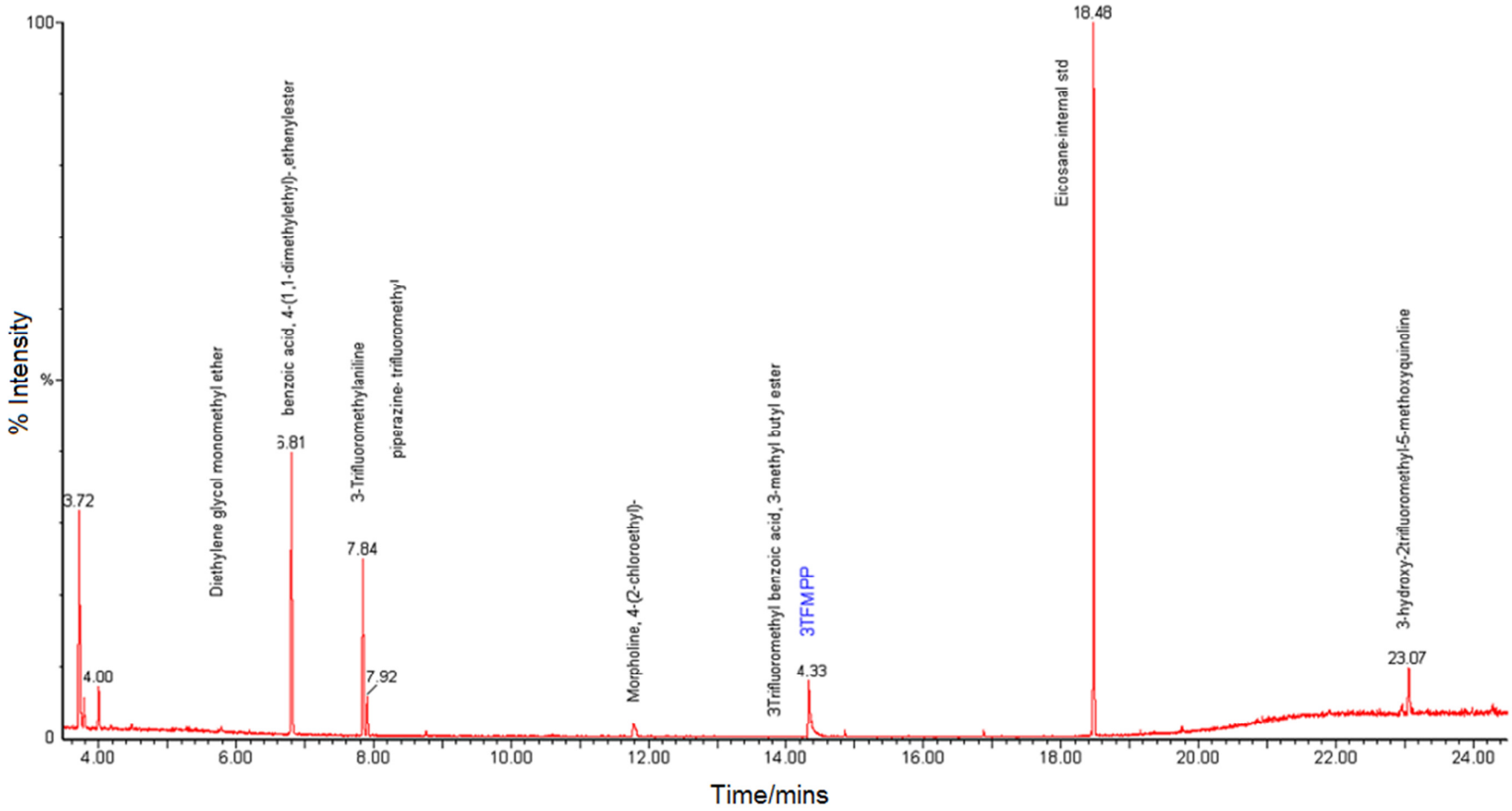


Figure 8.26 GC-MS Total Ion Chromatogram for identification of the synthesised TFMPP (route 1) showing the peak identified as 3-TFMPP and the major impurities (some of the impurities were preliminary identified from NIST mass spectra library).

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Preliminary identification using GC-MS mass spectral search (NIST library) identified the impurities (Appendices 13 and 14). These impurities were grouped into precursors, isomers of 4-FPP, 3-TFMPP and other by-products; the main impurities were preliminary identified by GC-MS as 1-(4-fluorophenyl)-4-piperidone and 3-trifluoromethylbenzoic acid, 3-methylbutyl ester for 4-FPP and 3-TFMPP samples respectively. Further testing using reference standards confirmed the presence of;

a) 3-Trifluoromethylaniline and dimethyl glycol monoethyl ether, precursors to the synthesis routes. This is likely to be due to incomplete reaction, since stoichiometric amounts of reagents were used. This view was supported by the lack of precursors as impurities in the synthesis done with longer reaction time. It can therefore be argued that reaction time not only affects yield as was discussed in section 8.3.2.1 but also quality of the drug (Lawrence, 2004).
b) Trace amounts of isomers of 4-FPP and 3-TFMPP. This can be due to resonance effects that were discussed and shown in Figure 2.21 (Chapter 2 section 2.8.3).
c) By-products of synthesis such as 1-(4-fluorophenyl)-4-piperidone (4-FPP synthesis), morpholine,4-(2-chloethyl)- and 3-trifluoromethylbenzoic acid,3-methylbutyl ester (3-TFMPP synthesis)**.** It was noted that the number and amount of impurities varied depending on duration of synthesis. Some of the impurities are still to be confirmed and therefore this was suggested for future research work.

Precursors used in the synthesis of 4-FPP and 3-TFMPP were also analysed individually and in different combinations. The results indicated (Appendix 13) that if present in the street sample, precursors can selectively be analysed as they are sufficiently resolved from each other and from the target substances, their isomers and the other impurities observed. The precursors do not show any secondary peaks when mixed, hence no side reactions occurred (Figures 8.25 and 8.26); this could be because the syntheses require an elevated temperature. These findings are ideal as it implies that no interference from any residual precursors can occur during analysis of samples.

8.3.3 COMPARISON OF STREET SAMPLES AND IN-HOUSE SYNTHESISED 4-FPP AND 3-TFMPP DRUGS

The total ion chromatograms of the street samples (Figures 8.1 - 8.12) were compared to that of synthesized sample (Figures 8.26 and 8.27). In addition, the mass spectra of the street sample components (Appendix 12) were compared to the mass spectra of the synthesis impurities (Appendix 14). It was observed that the main impurities were not similar. In the street samples the main impurities were those arising from tableting or adulteration (adulterants, binders, sugars). The major impurities that had been observed in the synthesized were absent. However, traces of 3-trifluoroaniline were found in some of the street samples containing 3-TFMPP when analysed at very high concentration (above 0.1mg/mL used in the study, at 0.5mg/mL). This was evidenced by the presence of a peak with similar retention time to the 3-trifluoroaniline standard (Appendix 12). Furthermore, some of the mass spectral ions (m/z 161, 142, 114, 111, 65, 81, 91, 120 and 133) characteristic of 3-trifluoromethylaniline were observed in samples A1 and A2. The ions at m/z161, 114, 111, 65 and 81 were evident in the mass spectra for sample A1 (Appendix 16 Figure 4). Due to the very low concentration of the impurity observed (trace) interference from the background was evident in the spectrum. This compound is a precursor in route 1 synthesis. This implies that the 3-TFMPP in the street samples (A1 and A2) was mostly likely synthesized by this route. However, it is suggested that the 3-TFMPP was commercially synthesized using this route and not in a clandestine manner since the profile of impurities obtained with the in-house synthesized 3-TFMPP sample was not similar to the street samples. The street samples contained less of the synthesis impurities. The observed impurities were mainly those due to tableting such as binders, sugars and starch.

8.4 CONCLUSION

Street samples were successfully characterised by the method developed, optimised and validated in previous studies of this research (Kuleya et al., 2014). The method selectively analysed for the different piperazine, their congeners, adulterants and also other impurities which were present in the street samples. The 11 street samples investigated were all tablets of various colours and logos. The sizes of the tablets ranged from 7.5mm diameter x 3.0mm width to 10.0mm diameter x 2.5mm width with a mean mass of 286 ± 31.9mg.

On analysis the samples were found to be MDMA (45.5%), piperazines (36.4%) and samples containing no psychoactive substance (18.2%). The piperazine samples were found to contain more than one psychoactive substances, i.e., BZP, DBZP, 3-TFMPP, caffeine and ephedrine. 3-TFMPP was found to be the most common piperazine in street samples. Street samples containing MDMA did not contain any other psychoactive substances. Caffeine was found to be the most common adulterant, present in 45.5% of the samples. Drug concentrations were found to be diverse. The dosages were in the range 17 - 56mg BZP, 21 - 106mg 3-TFMPP, 1 - 8mg DBZP, 6 - 55mg ephedrine, 0.1 - 101mg caffeine and 50 - 100mg MDMA. Impurities were also found to be present in most of the street samples (72.7%) with the tablet binders N-hexanedecanoic acid and octanedecanoic acid being the most common.

4-FPP and 3-TFMPP were successfully synthesised. The percentage yields were 64.34% 4-FPP and 58.15% 3-TFMPP. The identity of the synthesised samples was confirmed by several techniques; FTIR and UV-Vis and GC-MS. Several impurities were observed in the synthesised drugs with the number of impurities dependent on the extent of the purification of the product. The main impurities were preliminary identified as 1-(4-fluorophenyl)-4-piperidone and 3-trifluoromethylbenzoic acid, 3-methylbutyl ester for 4-FPP and 3-TFMPP samples respectively. Both methods of synthesis investigated have the potential to be used on the street, single drug manufactures of 3-TFMPP are likely to use synthesis route 2 for its high yield, were as those who manufacture a variety are more likely to use synthesis route 1 as it is amenable to a wider spectrum of drugs. This is the more likely situation as the drugs are found in combination with other substances. It can be argued that viable routes of synthesis exist which can be used be used or adapted for clandestine synthesis these substances, giving good product yields. Especially in view of the reported continued rise in prevalence of these drugs (UNODC, 2014) and also increasing legislative controls (King and Kicman, 2011) might drive suppliers to clandestine synthesis.

Traces of 3-trifluoroaniline were found in some of the street samples when analysed at very high concentration (1mg/mL). This compound is a precursor to one of the routes of synthesis investigated (route 1). This suggests a link between the route of synthesis investigated and the route used in the manufacture of the street sample. As such, this further confirms that it is a viable route of synthesis. However, it is proposed that the 3-TFMPP was most likely commercially synthesized using this route and not in a clandestine manner

since the profile of impurities obtained with the in-house synthesized 3-TFMPP sample was not similar to the street samples. The street samples contained less of the synthesis impurities. The observed impurities were mainly those due to tableting such as binders and cutting agents such as sugars, starch and also common adulterants such as caffeine and ephedrine.

It is proposed that even though the methods highlighted in this study were for 4-FPP and 3-TFMPP these methods can be applied for illicit synthesis of other phenylpiperazine drugs. Furthermore, the routes of syntheses identification in this study and the method of analysis developed prove will be a useful tool in profiling clandestine drugs found on the market by other researchers.

CHAPTER 9

CONCLUSION AND FUTURE RECOMMENDATIONS

9.1 GENERAL CONCLUSIONS

9.1.1 INTRODUCTION

Phenylpiperazines are prevalent as drugs of abuse (UNODC, 2013b). It was discussed in Chapter 1 that these drugs exist in combination with other drugs. These were found to be other piperazine drugs of abuse and also other psychoactive drugs such as (+) methamphetamine and dextromethorphan. In addition adulterants such as caffeine and cutting agents are commonly present (Kelleher et al, 2011; UN, 2001). Studies by Baron et al. (2011) and Kelleher et al. (2011) highlighted that the labelling on the street compounds is often misleading with drugs labelled as herbal highs, vitamin supplements etc. when in actual fact they contain a psychoactive drugs such as piperazines. In some cases those stated as piperazine street samples are sold as ecstasy. A case in study was a report by DEA (2010) where a drug labelled as ecstasy was actually on analysis found to be 4-FPP. In addition, the dosages of the drugs are often not labelled on the street samples. Furthermore, these have been found to be variable. LTG (2006) and Kenyon et al. (2010) reported ranges of 50 - 200mg for BZP and 5 - 75mg for TFMPP. However, dosages for 4-FPP or any of the other drugs have not yet been reported. This study had similar findings; the dosages of the street samples analysed were variable. Also, it has been highlighted that impurities arising from the synthesis during manufacture of the drug are likely to be present. These were identified as precursors and by-products of synthesis and were determined by several complexities such as duration of synthesis, skill/technique of the manufacturer. It is therefore evident that the purity of most of the illicit drugs on the streets inclusive of 4-FPP and 3-TFMPP is unknown. In addition analytical information on piperazines is still limited. It was therefore found imperative to investigate and characterise piperazines and generate data to potentially overcome these limitations.

It has been established that characterisation of piperazines has not yet been done (Chapter 1, section 1.8.3). In order to characterise and profile the drugs requires the development of

methods of analysis. It was discussed in Chapter 1 (section 1.8) that there are limitation to currently existing methods (de Boer et al., 2001; Staack et al., 2003; Inoue et al., 2004; LTG, 2006; Vorce et al., 2008; Takahashi et al, 2009; Maher et al., 2009; Kelleher et al., 2011; UNODC, 2013c). In these studies whilst the methods are useful for qualitative analysis and to a limited extent to quantitative analysis it was found that they were limited as they had not been applied to actual profiling of street samples. In addition, they did not take into account all the congeners identified in this study in street drugs containing 4-FPP and 3TFMPP. Furthermore, the presence of positional isomers of FPP and TFMPP posed a challenge iterated by the authors.

Consequently, this research project developed, optimised and validated a GC-MS method of analysis. It investigated the stability of drugs during analysis, clandestine routes of synthesis and carried out analysis, characterisation and profiling of 4-FPP and 3-TFMPP drugs of abuse. The results of this research provides novel information and, a) address the aforementioned gaps in research, b) provide methods for analysis which can be used in future investigation of illicit drugs in identification and quantification, c) provide insight into the profile of psychoactive phenylpiperazines prevailing on the market- the impurities present and their potential route for clandestine synthesis, d) identified precursors and chemicals which can potentially be controlled as a means to limit availability of these drugs, hence may impact on future analytical, pharmaceutical and forensic studies and regulations.

It is also worth noting the socioeconomic impact that drugs of abuse have on communities. With increases in the abuse of so called 'safe drugs' such as 4-FPP and 3-TFMPP, unknown purity and the limited research currently available on their toxicity, these may have unknown detrimental health effects.

9.1.2 KEY FINDINGS

9.1.2.1 Investigation of the stability of drugs and use of 2-methylpropan-2-ol as a solvent

Twenty two drugs were investigated for stability in the solvents methanol, dichloromethane and 2-methylpropan-2-ol. In addition under different environmental conditions; ambient temperature, on the GC-MS auto-sampler during analysis, storage at 4^{o}C in the refrigerator

and storage at (-20^{o}C) in the freezer. It was found that whilst on the GC-MS auto-sampler the least stability was observed in methanol. In methanol the drugs were stable for a period of 14 – 25 hours. In dichloromethane the drugs were stable for a period of 19 – 25 hours and in 2-methylpropan-2-ol for 25 hours. Cocaine, BEH and EME showed the lowest stability (14 hours in methanol). The formation of artefacts due to a solvent is undesirable, especially since in this research where the solvent will also be used in impurity profiling. Artefacts were observed with dichloromethane and methanol (mainly benzyl chloride, benzychloroformate, ecgonine methyl ester and benzylecgonine hydrate). Consequently the 2-methylpropan-2-ol was the solvent selected for further use in all the studies.

Storage stability of drug analytes in 2-methylpropan-2-ol in the fridge and refrigerator indicated that the drugs were stable for 6 - 10 days in the refrigerator and for 22 - 30 days in the freezer.

9.1.2.2 GC-MS method development, optimisation and validation

The operational variables injector and oven temperatures, carrier gas flow rate, MS scan rate and MS optimisation energy were investigated so as to improve performance of the preliminary method developed. The results confirmed findings by Santali et al, (2011), Maher et al., (2009), Andersson et al, (2007a) and Byrska et al., (2010) where it was found that variation of similar operational variables resulted in better quality chromatographic profiles. It was observed that optimisation of the preliminary method developed improved its performance in terms of the quality of chromatographic peak profiles generated. The method improved to give better peak shapes, high resolution, $R > 2$ and reduced tailing.

The need for method validation was established in section 2.5 (Chapter 2). On validation (Chapter 7) it was established that the method developed is suitable for its intended use, i.e., analysis and profiling of piperazine based street drugs. Method accuracy was reported on average as 99.8% and precision was RSD < 2%. Detection limits were in the range 0.5 - 1.95 x 10^{-3}µg/mL free base on column. On average, the common working range was equivalent to 5 - 35.0µg/mL free base on column. This was ideal for the analysis of street samples were concentratios are high (mg range) (Kelleher et al., 2011). Furthermore, it can also be applied to very low concentration. This could prove useful if the method is extrapolated to toxicological studies. In such studies concentrations in the biological

samples are usually low (Stack and Maurer, 2005). In addition, it was found that the method developed was able to simultaneously analyse for 20 drugs which can be found in different combinations with the target analytes 4-FPP and 3-TFMPP in street samples. Also, the method was able to completely resolve (baseline resolution) the (2, 3, 4) isomers of both FPP and TFMPP thereby being the first method available able to simultaneously analyse these isomers and congeners in street samples. Hence, the method developed was found to work satisfactorily and will be employed in the characterisation and impurity profiling aspect of the research, in profiling of 3TFMPP and 4-FPP.

However, it was observed that there is co-elution between 3-TFMPP/MDMA and also DBZP/dapoxetine. Whilst it would be ideal to achieve baseline resolution, it was established that there is statistically no significant difference between using total ion chromatographic data and extracted ion data. Hence, this limitation was overcome through use of extracted ions for these particular drugs.

9.1.2.3 Analysis, characterisation and synthesis of street samples

9.1.2.3.1 Street samples

Street samples (11) were successfully analysed and of these 5 were found to contain 1 or more piperazines in addition to other substances. 4 were found to contain MDMA as the only psychoactive substance and 2 were found to contain no psychoactive substance at all. The street samples which contained piperazines were found to contain one or more of BZP, 3-TFMPP, ephedrine and caffeine. 3-TFMPP was found to the most common piperazine in street samples and caffeine as the most common adulterant. The street samples containing MDMA did not contain any other psychoactive substances. Drug concentrations were found to be diverse. The concentrations were in the range 17 – 56mg BZP, 21 – 106mg 3-TFMPP, 1 – 8mg DBZP, 6 – 55mg ephedrine, 0.1 – 101mg caffeine and 50 – 100mg MDMA. Impurities were also found to be present in street samples.

9.1.2.3.2 Synthesis

4-FPP and 3-TFMPP were successfully synthesised. It can therefore be suggested that viable routes of synthesis exist for potential clandestine synthesis of these substances. The percentage yields were 64.34% 4-FPP and 58.15% 3-TFMPP. The identity of the synthesised samples was confirmed by several techniques; FTIR-ATR, UV-Vis and GC-MS. Several impurities were observed in the synthesised samples. The main impurities

were preliminary identified as 1-(4-fluorophenyl)-4-piperidone and 3-trifluoromethylbenzoic acid, 3-methylbutyl ester for 4-FPP and 3-TFMPP samples respectively. The number of impurities depends on how far purification is carried out.
Both methods of synthesis investigated have the potential to be used on the street, single drug manufactures of 3-TFMPP are likely to use synthesis route 2 for its high yield, where as those who manufacture a variety are more likely to use synthesis route 1 as it is amenable to a wider spectrum of drugs. This is the more likely situation as the drugs are found in combination with other substances. It can be argued that the routes of synthesis in literature can be used or adapted for clandestine synthesis, giving good product yields. The methods highlighted in this study were for 4-FPP and 3-TFMPP these methods can be applied for illicit synthesis of other phenylpiperazine drugs. Furthermore, the identification of routes of syntheses and development of methods of analysis prove will be a useful tool in profiling clandestine drugs found on the market by other researchers

On comparison of street samples and in-house synthesized samples it was observed that the impurity profiles were not similar. However, traces of 3-trifluoromethylaniline were found in some of the street samples containing 3-TFMPP when analysed at very high concentration. 3-Trifluoromethylaniline is a precursor in route 1 synthesis. As such, this confirms route 1as a viable route of synthesis. However, it is most likely the 3-TFMPP was commercially synthesized using this route and not in a clandestine manner and then used in the manufacture of the illicit drugs. This deduction was made on the basis that the profile of impurities obtained with the in-house synthesized 3-TFMPP sample was not similar to the street samples.

9.1.2.4 The method developed

The final optimised and validated method was as follows; the initial oven temperature was set at 60^{o}C with a hold for 1min and ramped at 10^{o}C /min to 170^{o}C with a hold for 2min. The oven was further ramped at 15^{o}C /min to 280^{o}C, with a hold for 4min. The MS transfer line was set at 280^{o}C, source temperature 230^{o}C, ionisation energy 70eV and scan range m/z 40 – 500. The carrier gas was He (g) at a flow rate of 1ml/min. The injector was set at 260^{o}C with a split ratio of 20:1. The instrument was equipped with a Supelco, Equity-5 (30m x 2.5mm x 2.5um) capillary column. The total analysis run time was 25.33 minutes.

9.1.3 CONCLUSION

The results of this research provided novel information in the stability profile of psychoactive phenylpiperazines and other drugs of abuse prevailing on the market. It was also noted that 2-methylpropan-2-ol has not been extensively used in literature hence it is proposed as a solvent for use to other researchers, especially in profiling or impurity analysis mainly due to its non-reactivity.

There was a limited amount of previous research on method development and or optimisation for similar drugs, especially involving FPP, one of the two main drugs of focus for the overall research. This was probably due to the drugs being relatively new on the market and this was a limitation in this study for comparative purposes. In depth data on optimisation of the drugs was found to be mainly on amphetamines. In addition, the few studies found mostly reported optimisation on qualitative methods and on a limited number of drugs. Therefore, this study provides relevant data on optimisation and validation of a method for the analysis and profiling of phenylpiperazines, benzylpiperazines and other drugs of abuse in street samples.

The data for all the 22 drugs investigated was also reported. Currently, there was no record of the drugs having been chemically profiled. Furthermore, the drugs can be reliably detected and analysed qualitatively and quantitatively in a wide concentration range. Hence, this will prove useful in application to both samples containing very low concentrations and high doses with good accuracy precision. Also, the GC-MS analytical data (retention times, relative retention times, retention indices, and mass spectra) for all the FPP and TFMPP isomers and the other drugs investigated was generated for the first time. This study brings in additional spectral data in terms of more comprehensive mass spectra and identifying ion m/z values for the piperzines and also all the congeners. In addition, the routes of fragmentation and fragment structures for benzylpiperazines and phenylpiperazines. It is suggested this data will aid further investigation of these drugs and can be used by other researchers, law enforcement agencies and in toxicity studies.

9.2 FUTURE WORK

In the review on methods of analysis (Chapter 1, section 1.8) it was found that researchers highlighted the need for HPLC for the separation of isomers of FPP and TFMPP (Elliott and Smith, 2008; Vorce et al., 2008; Takahashi et al., 2009). Consequently, it is suggested that such techniques as LC-MS will have the potential to distinguish between the analytes. Investigation of the use of HPLC, both in characterisation of piperazines is therefore proposed for future work. It is hoped that an attempt will be made to develop an HPLC/DAD or HPLC/MS method for the separation of the isomers and also for characterisation. Schurenkamp (2010) successfully developed a chiral HPLC/DAD method for analysis of CPP isomers in seized tablets.

Preliminary analysis for impurities of the in-house synthesised 4-FPP and 3-TFMPP indicated that phenylpiperazines street drugs potentially contain several impurities dependent not only on the route but also technique. Therefore, it is suggested that a more in depth investigation of impurities including extraction of trace components be conducted. However, application of this method to the analysis of trace impurities might require carrying out an additional test, i.e., extraction of trace impurities by liquid-liquid extraction and or solid-phase extraction (discussed in section 1.8.3). This study can therefore be a foundation for further investigations, such as to develop methods for other drugs and to carry out impurity profiling using radio labelled precursors. This will assist law enforcement agents in establishing links between samples, thereby identifying the origin of the drugs. Furthermore, investigation of the piperazine drugs which are fluoro compounds using stable isotopes of fluorine, nitrogen or chlorine for (CPP and related substances) could be investigated as this study identified some the compounds and was not exhaustive. It is also suggested that analysis and impurity profiling of street samples containing other piperazine drugs be conducted using the method developed. The method was found to selectively analyse for 20 drug substances and as such it has the potential to be applied for the profiling of not just the drugs investigated. Furthermore, future investigation of other routes of synthesis or the same routes on other drugs would be useful. It is likely that with increasing control on piperazines clandestine manufacture may arise. In addition, it has been identified that profiling of piperazines has not been done. Hence, such a study will generate useful data which can be utilised by law enforcement agencies and other researchers.

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APPENDIX 1 Auto-sampler stability graphs in different solvents (Limits are ± 10% of expected value).

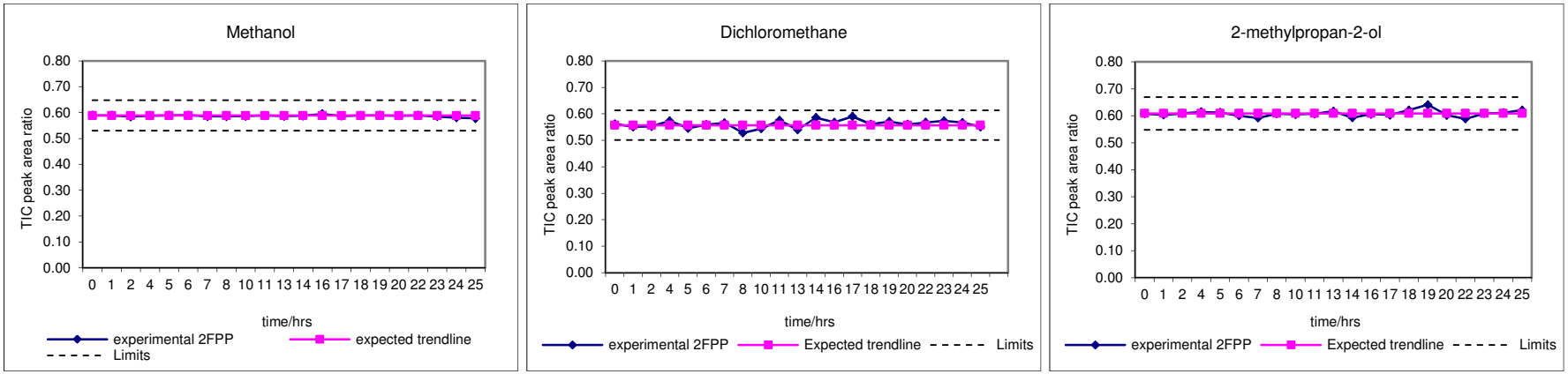


Figure 1 2-FPP stability profiles in different solvents over 25 hours on the GC-MS autosampler.

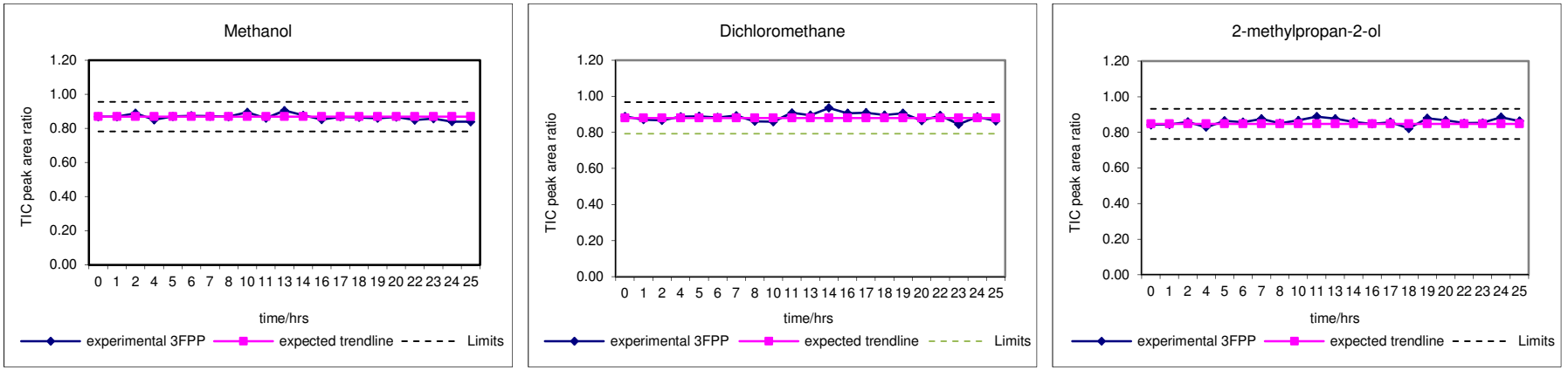


Figure 2 3-FPP stability profiles in different solvents over 25 hours on the GC-MS autosampler.

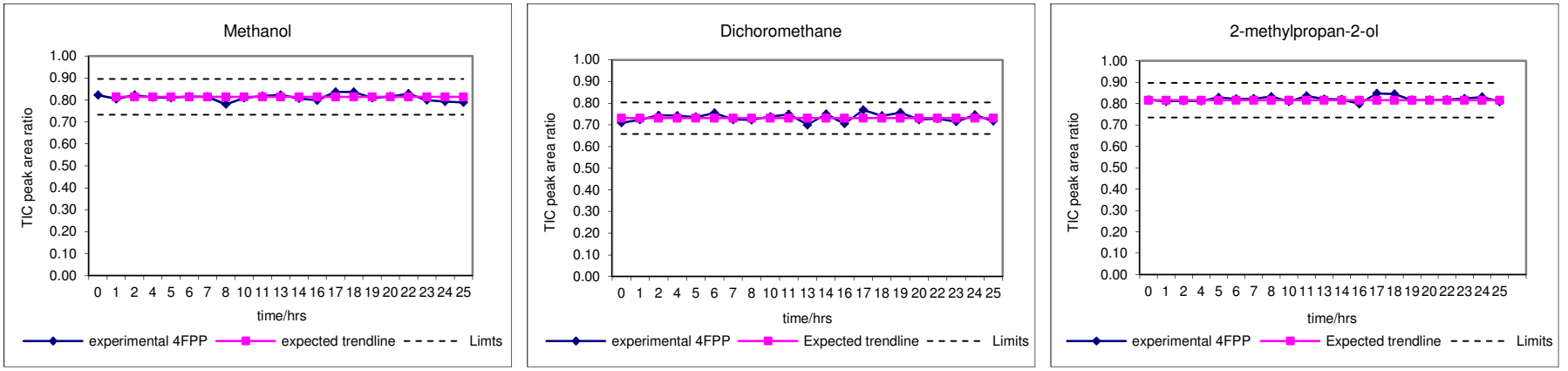


Figure 3 4-FPP stability profiles in different solvents over 25hours on the GC-MS autosampler.

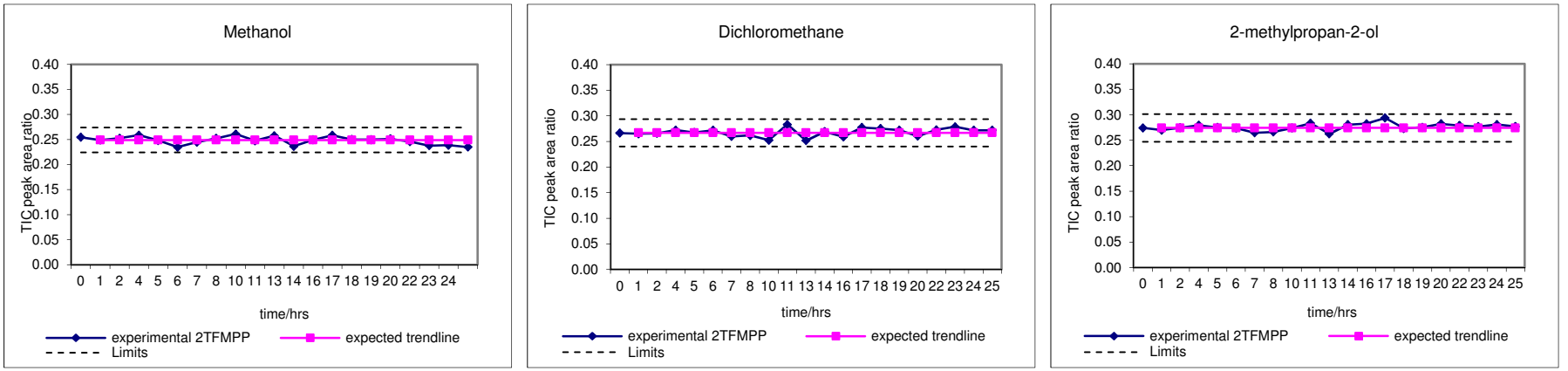


Figure 4 2-TFMPP stability profiles in different solvents over 25 hours on the GC-MS autosampler.

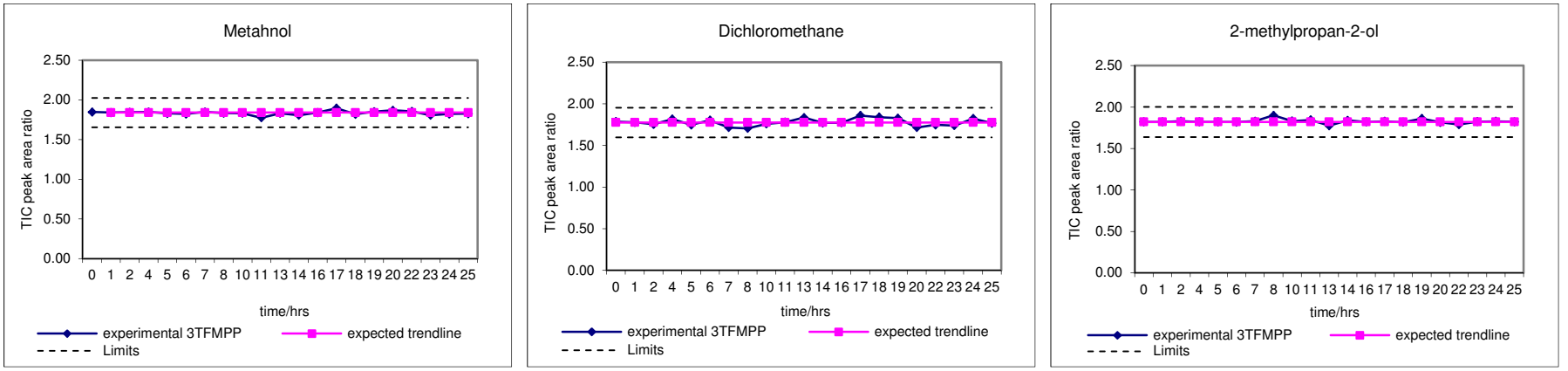


Figure 5 3-TFMPP stability profiles in different solvents over 25 hours on the GC-MS autosampler.

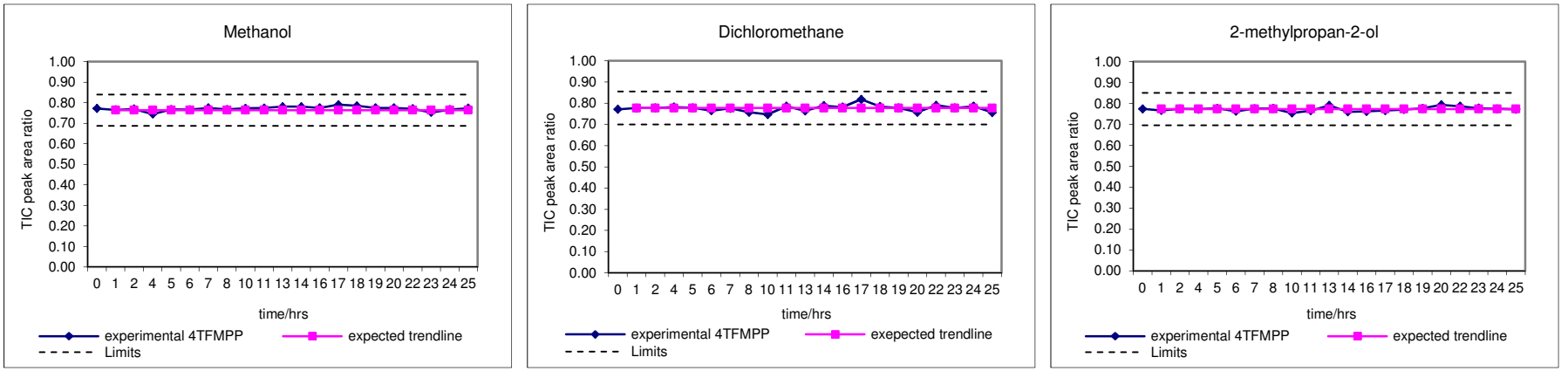


Figure 6 4-TFMPP stability profiles in different solvents over 25hours on the GC-MS autosampler.

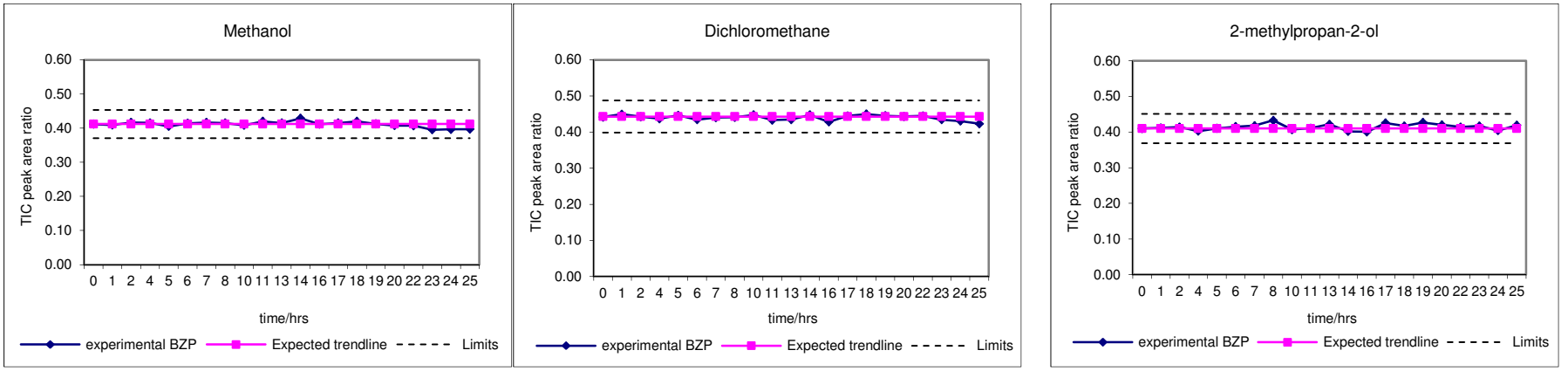


Figure 7 BZP stability profiles in different solvents over 25hours on the GC-MS autosampler.

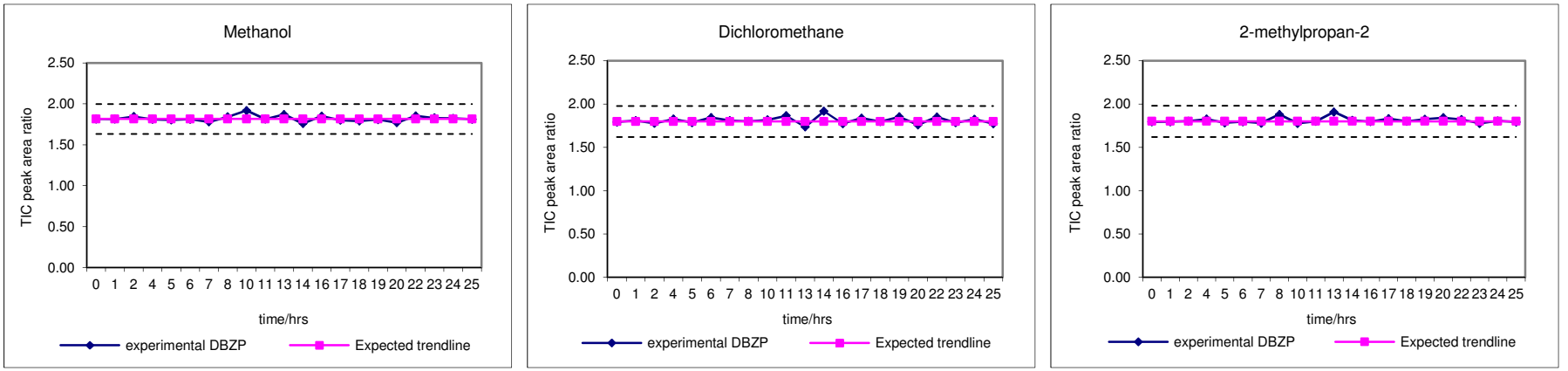


Figure 8 DBZP stability profiles in different solvents over 25hours on the GC-MS autosampler.

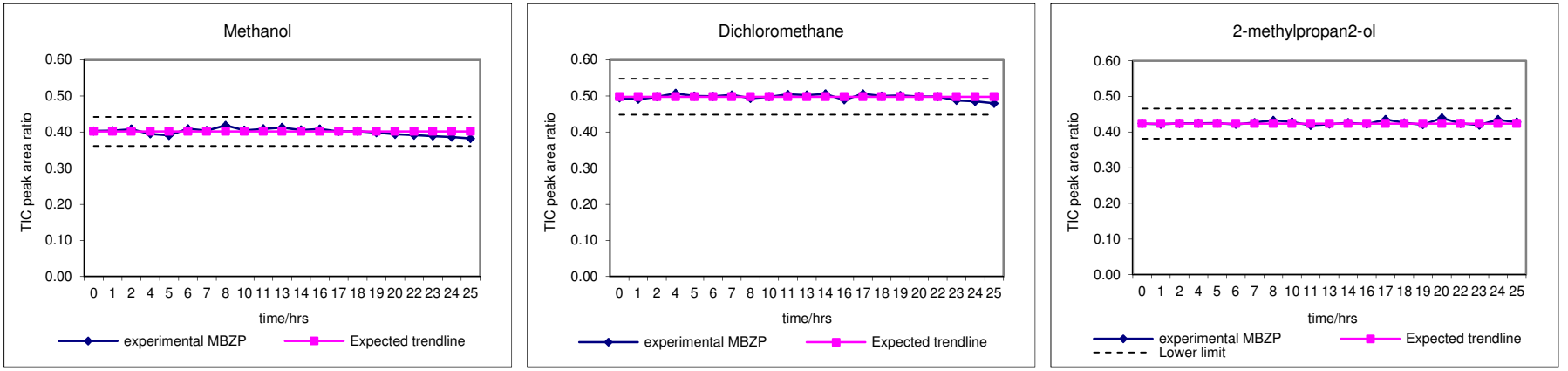
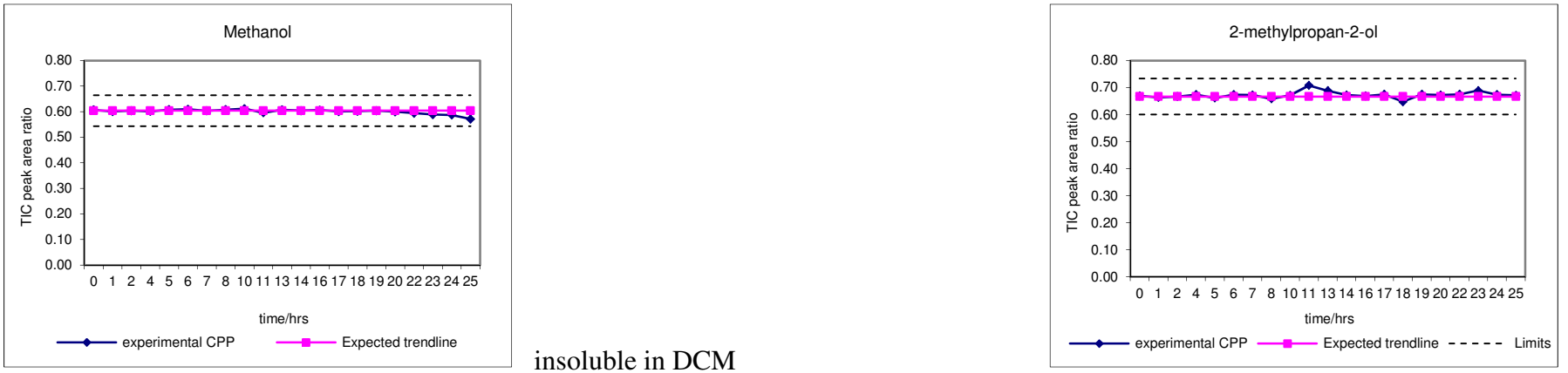


Figure 9 MBZP stability profiles in different solvents over 25hours on the GC-MS autosampler.



insoluble in DCM

Figure 10 CPP stability profiles in different solvents over 25 hours on the GC-MS autosampler.

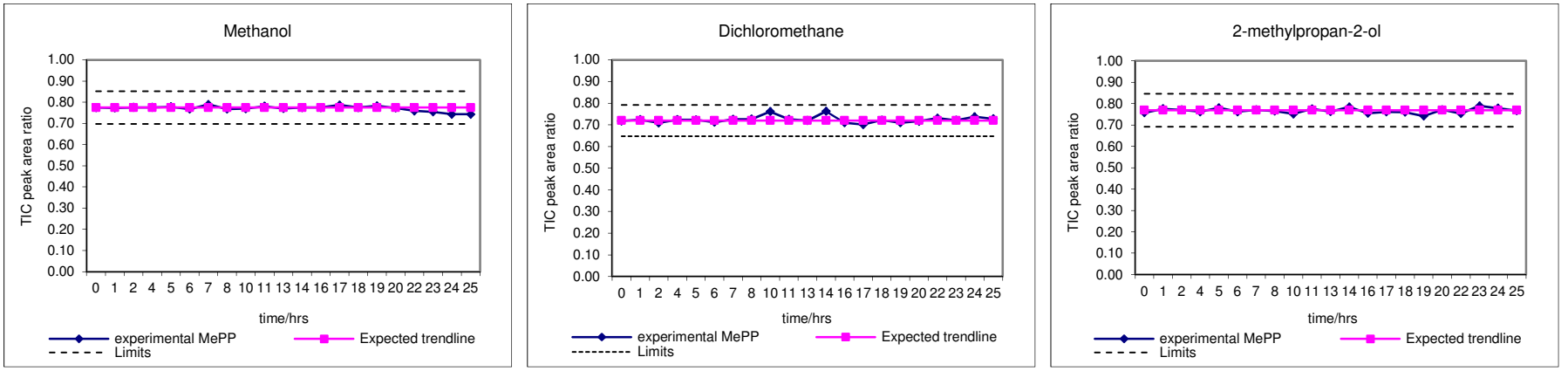
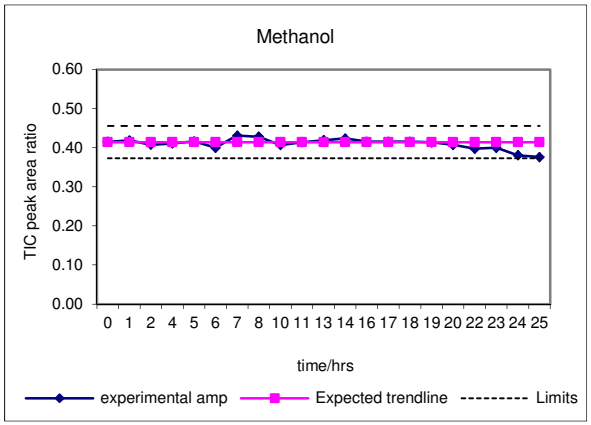


Figure 11 MePP stability profiles in different solvents over 25 hours on the GC-MS autosampler.



insoluble in DCM and MPOH

Figure 12 Amphetamine stability profiles in different solvents over 25 hours on the GC-MS autosampler

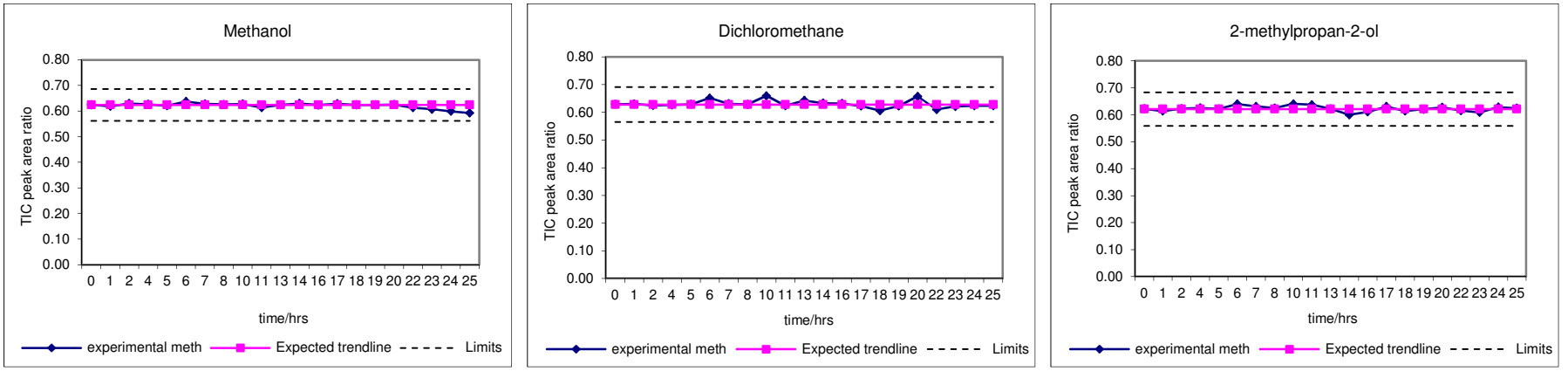


Figure 13 Methamphetamine stability profiles in different solvents over 25 hours on the GC-MS autosampler

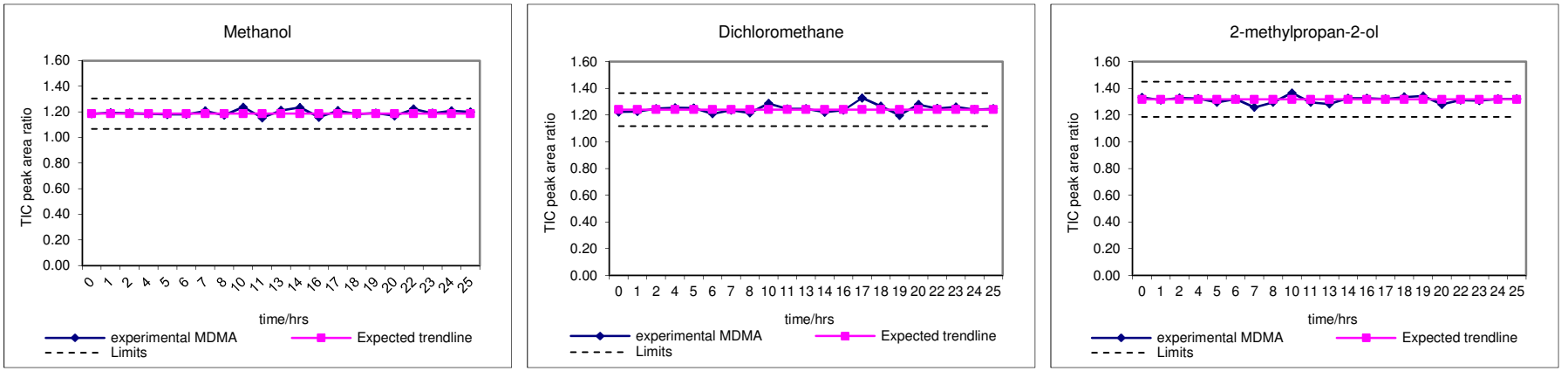


Figure 14 MDMA stability profiles in different solvents over 25 hours on the GC-MS autosampler

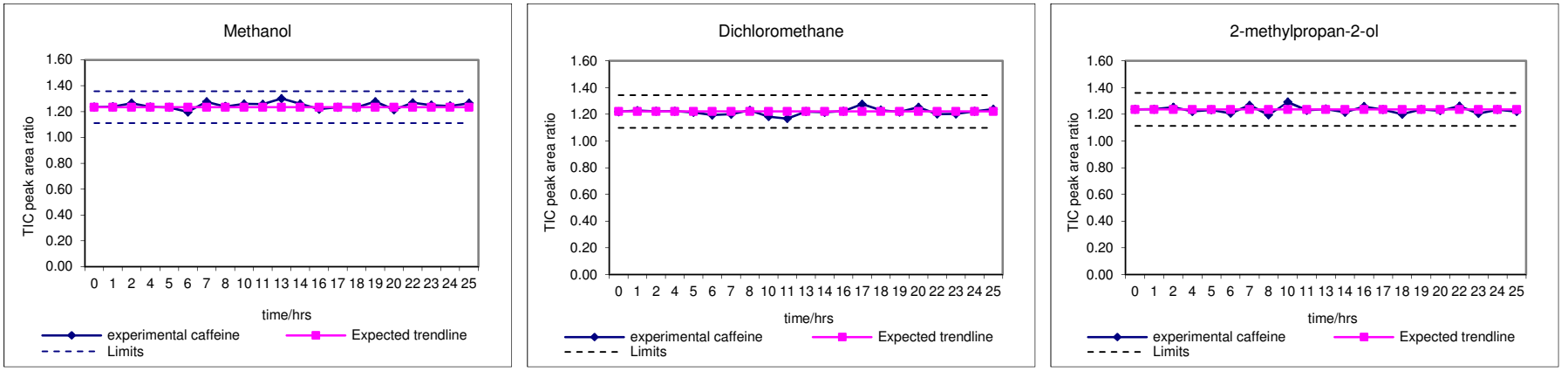


Figure 15 Caffeine stability profiles in different solvents over 25 hours on the GC-MS autosampler

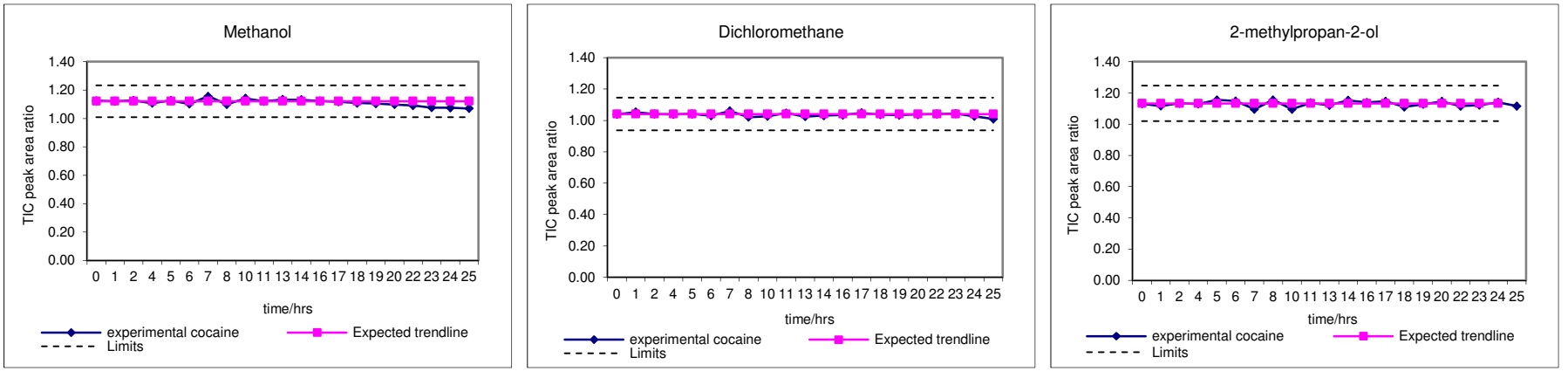


Figure 16 Cocaine stability profiles in different solvents over 25 hours on the GC-MS autosampler

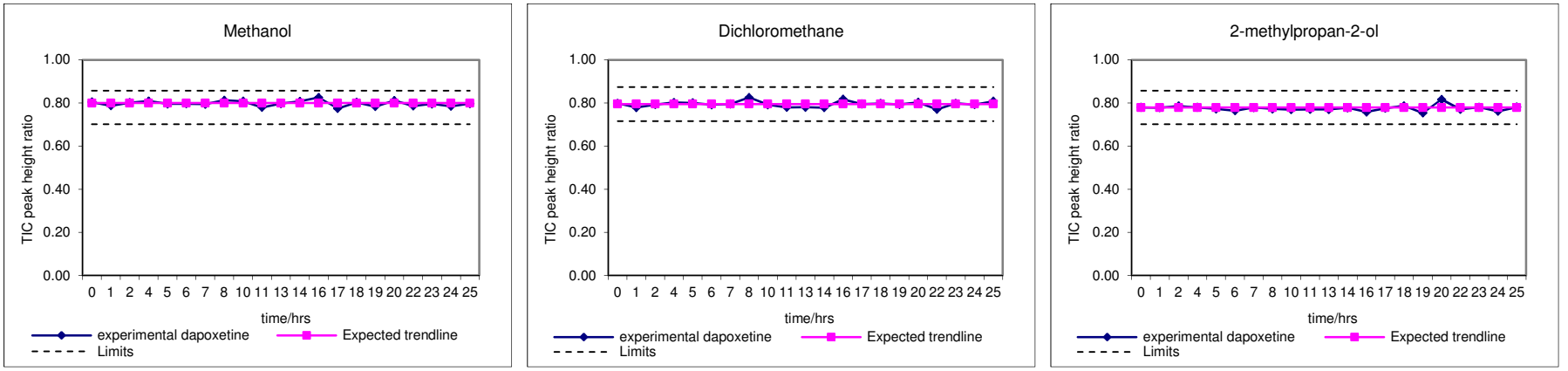


Figure 17 Dapoxetine stability profiles in different solvents over 25 hours on the GC-MS autosampler

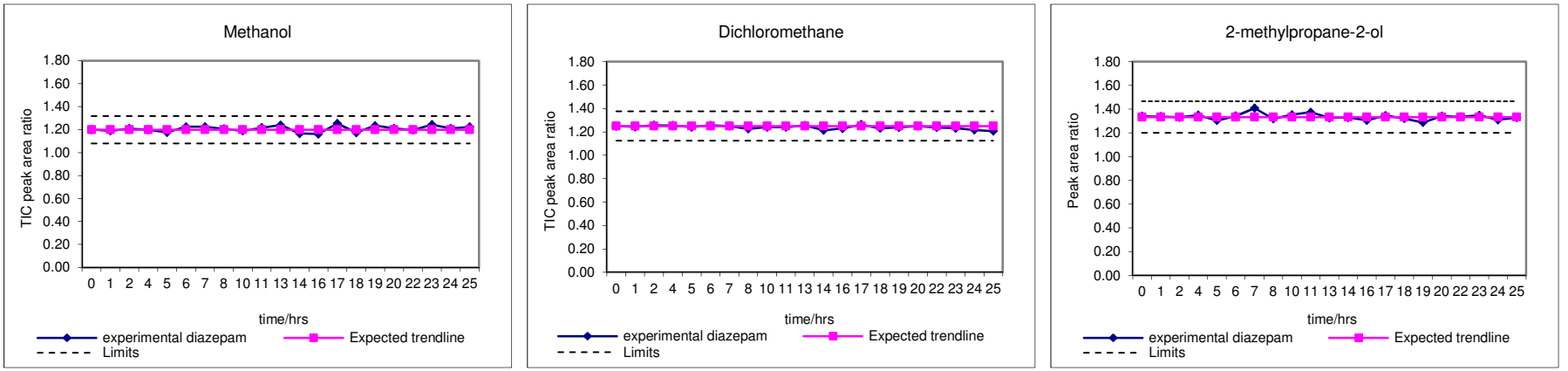


Figure 18 Diazepam stability profiles in different solvents over 25 hours on the GC-MS autosampler

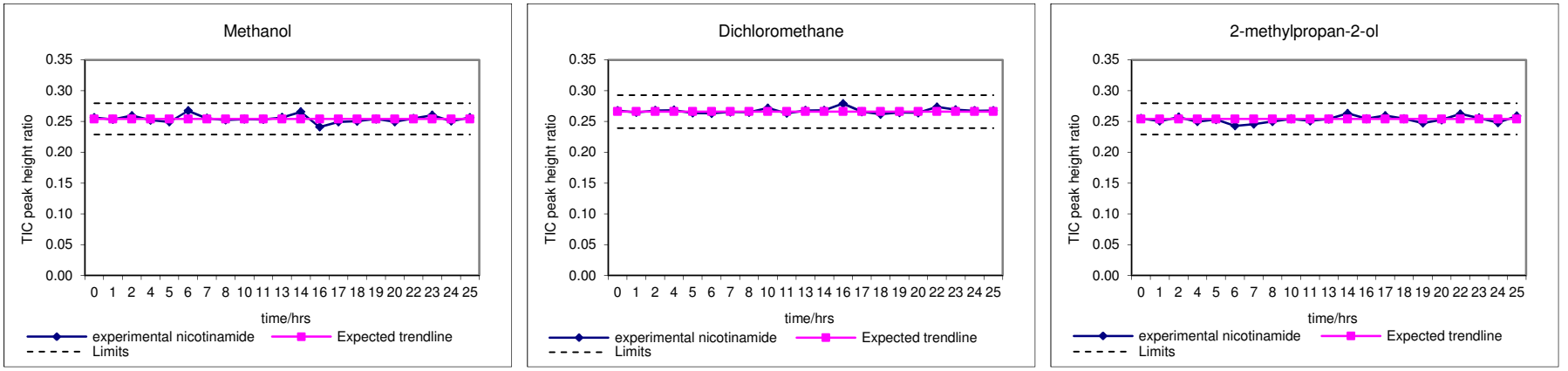


Figure 19 Nicotinamide stability profiles in different solvents over 25 hours on the GC-MS autosampler

Limits: acceptable limits of variation for stability (± 10% of Expected value)

APPENDIX 2 Stability of drugs in different solvents on the GC-MS auto-sampler

In the Table MeOH is methanol, DCM is dichloromethane and MPOH is 2-methyldichoromethane.

Table 1 Stability data for solvents/auto-sampler analysis

	Time stable/hours			
Substance	MeOH	DCM	MPOH	Expected
2-FPP	20	25	25	25
3-FPP	20	25	25	25
4-FPP	25	25	25	25
2-TFMPP	19	25	25	25
3-TFMPP	25	25	25	25
4-TFMPP	25	25	25	25
BZP	19	22	25	25
DBZP	25	25	25	25
MBZP	18	20	25	25
3-CPP	18	0	25	25
4-MePP	19	25	25	25
Amphetamine	17	0	0	25
Methamphetamine	19	25	25	25
Caffeine	25	25	25	25
Cocaine	14	23	25	25
MDMA	25	25	25	25
Diazepam	25	22	25	25
Dapoxetine	25	25	25	25
Nicotinamide	25	0	25	25
BEH	14	19	25	25
EME	14	0	0	25
Piperazine	25	25	25	25

APPENDIX 3 Degradation products observed on investigation of stability of the drugs in different solvents on the auto-sampler

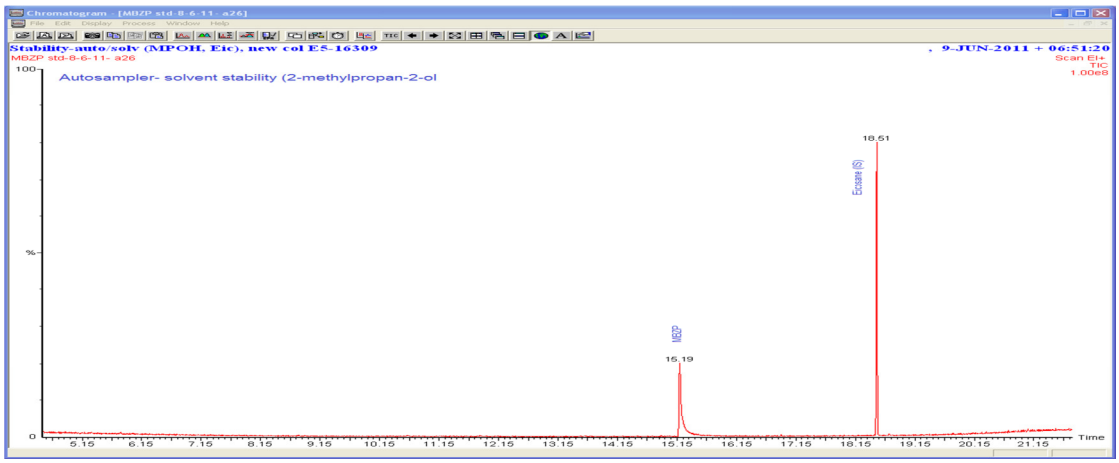


Figure 1 MBZP in 2-methylpropan-2-ol

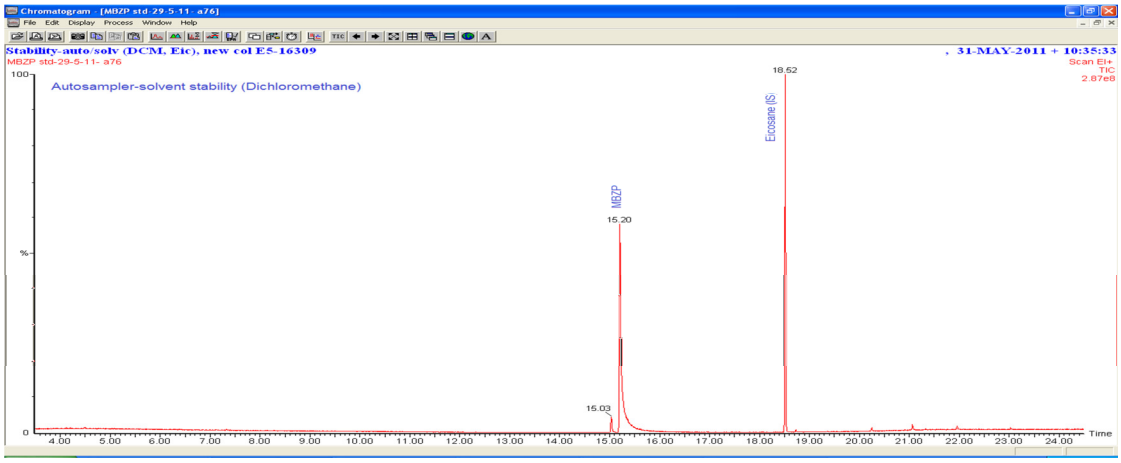


Figure 2 MBZP in dichloromethane

Figure 3 Mass spectra of degradant peak at 15.03 mins in MBZP (Figure 2)

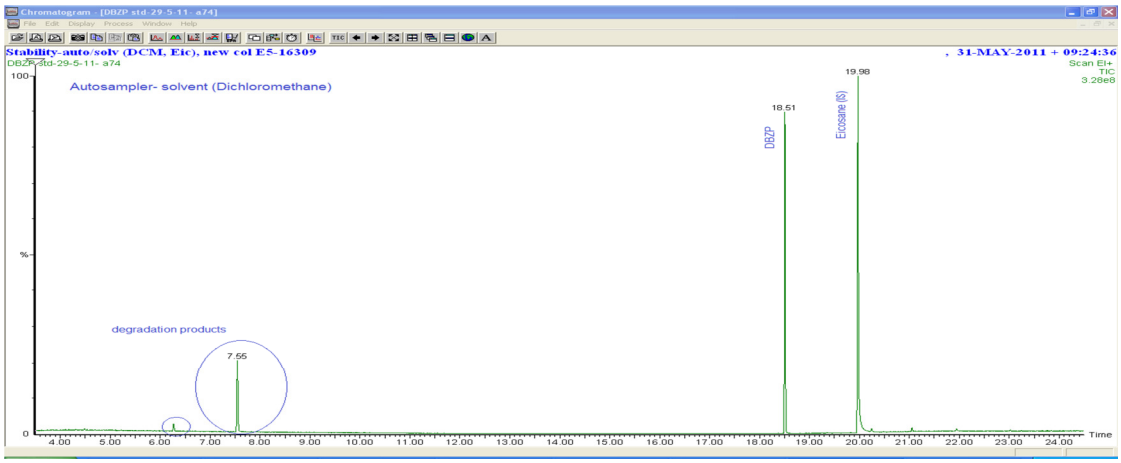
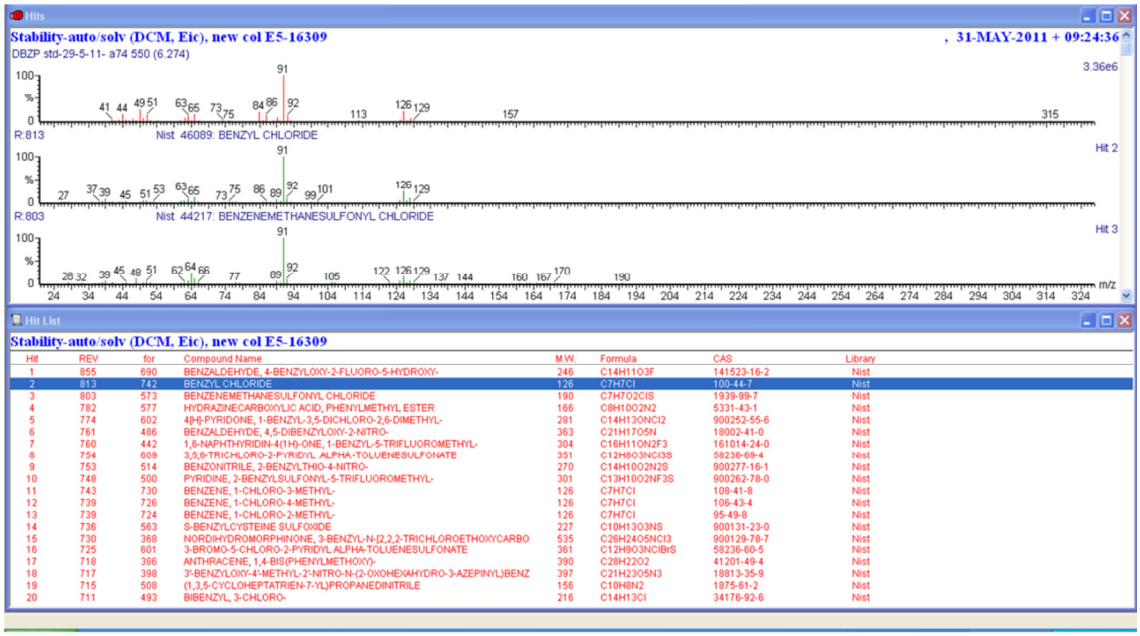
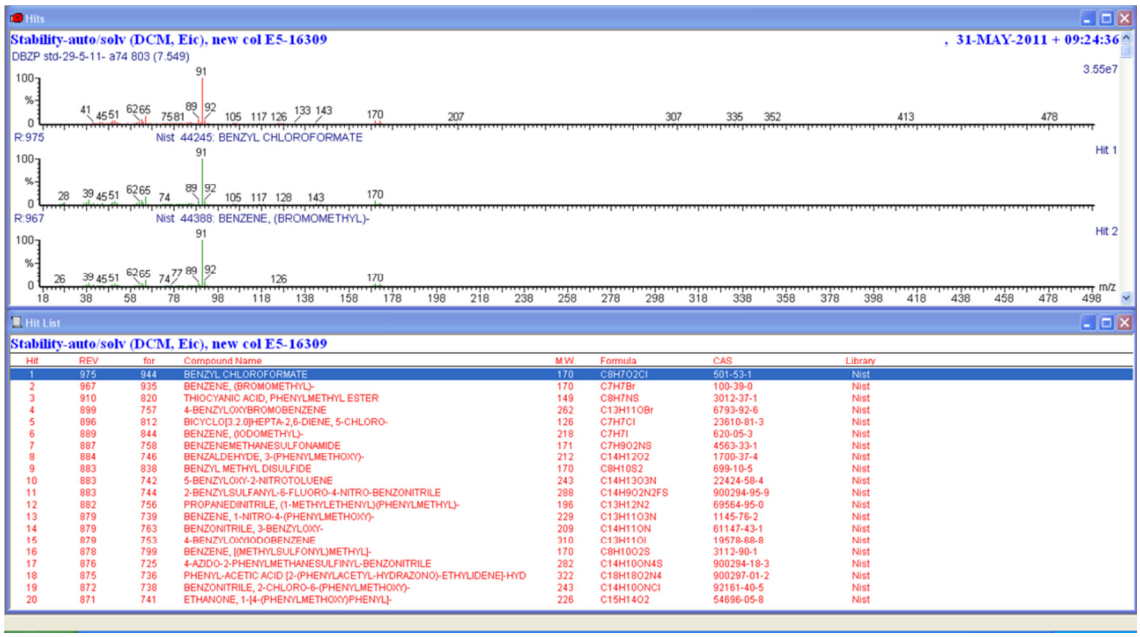


Figure 4 DBZP in dichloromethane



Hit	REV	for	Compound Name	M.W.	Formula	CAS	Library
1	855	690	BENZALDEHYDE, 4-BENZYLOXY-2-FLUORO-5-HYDROXY-	246	C14H11O3F	141523-16-2	Nist
2	813	742	BENZYL CHLORIDE	126	C7H7Cl	100-44-7	Nist
3	803	573	BENZENEMETHANESULFONYL CHLORIDE	190	C7H7O2ClS	1939-99-7	Nist
4	782	577	HYDRAZINECARBOXYLIC ACID, PHENYLMETHYL ESTER	166	C8H10O2N2	5331-43-1	Nist
5	774	602	4[H]-PYRIDONE, 1-BENZYL-3,5-DICHLORO-2,6-DIMETHYL-	281	C14H13ONCl2	900252-55-6	Nist
6	761	486	BENZALDEHYDE, 4,5-DIBENZYLOXY-2-NITRO-	363	C21H17O5N	18002-41-0	Nist
7	760	442	1,6-NAPHTHYRIDIN-4(1H)-ONE, 1-BENZYL-5-TRIFLUOROMETHYL-	304	C16H11ON2F3	161014-24-0	Nist
8	754	609	3,5,6-TRICHLORO-2-PYRIDYL ALPHA-TOLUENESULFONATE	351	C12H8O3NCl3S	58236-69-4	Nist
9	753	514	BENZONITRILE, 2-BENZYLTHIO-4-NITRO-	270	C14H10O2N2S	900277-16-1	Nist
10	748	500	PYRIDINE, 2-BENZYLSULFONYL-5-TRIFLUOROMETHYL-	301	C13H10O2NF3S	900262-78-0	Nist
11	743	730	BENZENE, 1-CHLORO-3-METHYL-	126	C7H7Cl	108-41-8	Nist
12	739	726	BENZENE, 1-CHLORO-4-METHYL-	126	C7H7Cl	106-43-4	Nist
13	739	724	BENZENE, 1-CHLORO-2-METHYL-	126	C7H7Cl	95-49-8	Nist
14	736	563	S-BENZYLCYSTEINE SULFOXIDE	227	C10H13O3NS	900131-23-0	Nist
15	730	368	NORDIHYDROMORPHINONE, 3-BENZYL-N-[2,2,2-TRICHLOROETHOXYCARBO	535	C26H24O5NCl3	900129-78-7	Nist
16	725	601	3-BROMO-5-CHLORO-2-PYRIDYL ALPHA-TOLUENESULFONATE	361	C12H9O3NClBrS	58236-60-5	Nist
17	718	366	ANTHRACENE, 1,4-BIS(PHENYLMETHOXY)-	390	C28H22O2	41201-49-4	Nist
18	717	398	3'-BENZYLOXY-4'-METHYL-2'-NITRO-N-(2-OXOHEXAHYDRO-3-AZEPINYL)BENZ	397	C21H23O5N3	18813-35-9	Nist
19	715	508	(1,3,5-CYCLOHEPTATRIEN-7-YL)PROPANEDINITRILE	156	C10H8N2	1875-61-2	Nist
20	711	493	BIBENZYL, 3-CHLORO-	216	C14H13Cl	34176-92-6	Nist

Figure 5 Mass spectra and preliminary identification of degradant peak at 6.27mins in DBZP (Figure 4)



Hit	REV	for	Compound Name	M.W.	Formula	CAS	Library
1	975	944	BENZYL CHLOROFORMATE	170	C8H7O2Cl	501-53-1	Nist
2	967	935	BENZENE, (BROMOMETHYL)-	170	C7H7Br	100-39-0	Nist
3	910	820	THIOCYANIC ACID, PHENYLMETHYL ESTER	149	C8H7NS	3012-37-1	Nist
4	899	757	4-BENZYLOXYBROMOBENZENE	262	C13H11OBr	6793-92-6	Nist
5	896	812	BICYCLO[3.2.0]HEPTA-2,6-DIENE, 5-CHLORO-	126	C7H7Cl	23610-81-3	Nist
6	889	844	BENZENE, (IODOMETHYL)-	218	C7H7I	620-05-3	Nist
7	887	758	BENZENEMETHANESULFONAMIDE	171	C7H9O2NS	4563-33-1	Nist
8	884	746	BENZALDEHYDE, 3-(PHENYLMETHOXY)-	212	C14H12O2	1700-37-4	Nist
9	883	838	BENZYL METHYL DISULFIDE	170	C8H10S2	699-10-5	Nist
10	883	742	5-BENZYLOXY-2-NITROTOLUENE	243	C14H13O3N	22424-58-4	Nist
11	883	744	2-BENZYLSULFANYL-6-FLUORO-4-NITRO-BENZONITRILE	288	C14H9O2N2FS	900294-95-9	Nist
12	882	756	PROPANEDINITRILE, (1-METHYLETHENYL)(PHENYLMETHYL)-	196	C13H12N2	69564-95-0	Nist
13	879	739	BENZENE, 1-NITRO-4-(PHENYLMETHOXY)-	229	C13H11O3N	1145-76-2	Nist
14	879	763	BENZONITRILE, 3-BENZYLOXY-	209	C14H11ON	61147-43-1	Nist
15	879	753	4-BENZYLOXY-IODOBENZENE	310	C13H11OI	19578-68-8	Nist
16	878	799	BENZENE, [(METHYLSULFONYL)METHYL]-	170	C8H10O2S	3112-90-1	Nist
17	876	725	4-AZIDO-2-PHENYLMETHANESULFINYL-BENZONITRILE	282	C14H10ON4S	900294-18-3	Nist
18	875	736	PHENYL-ACETIC ACID [2-(PHENYLACETYL-HYDRAZONO)-ETHYLIDENE]-HYD	322	C18H18O2N4	900297-01-2	Nist
19	872	738	BENZONITRILE, 2-CHLORO-6-(PHENYLMETHOXY)-	243	C14H10ONCl	92161-40-5	Nist
20	871	741	ETHANONE, 1-[4-(PHENYLMETHOXY)PHENYL]-	226	C15H14O2	54696-05-8	Nist

Figure 6 Mass spectra and preliminary identification of degradant peak at 7.5mins in DBZP (Figure 5)

The chromatographic profiles of 4-fluoroaniline, benzyl chloride and benzyl chloroformate standards for the identification of degradants are shown below.

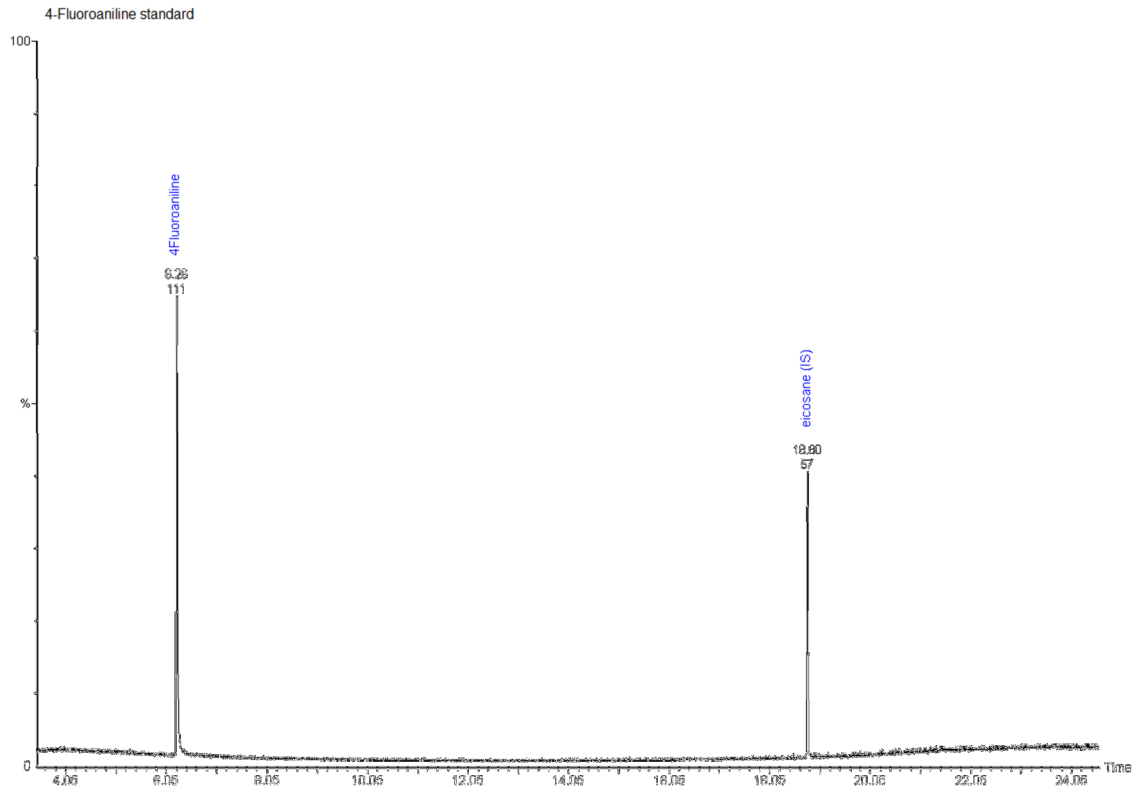


Figure 7 Chromatographic profile of 4-fluoroaniline stability study

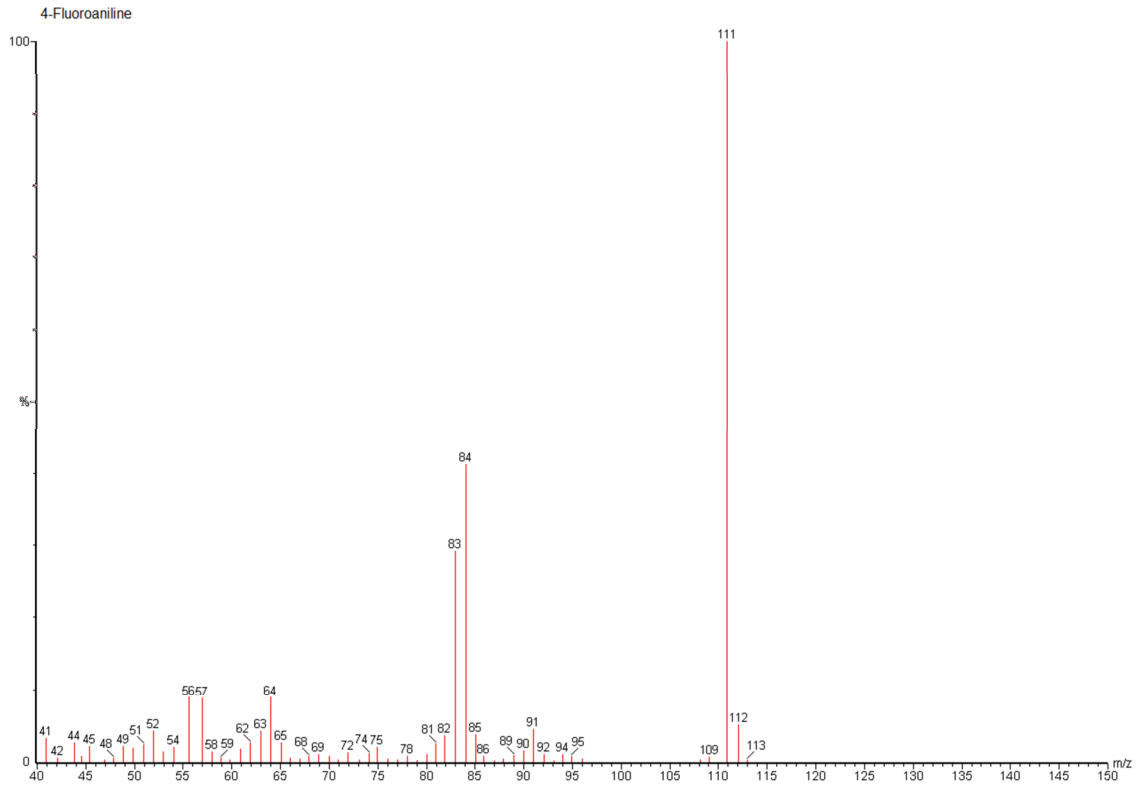


Figure 8 Mass spectra of 4-fluoroaniline stability

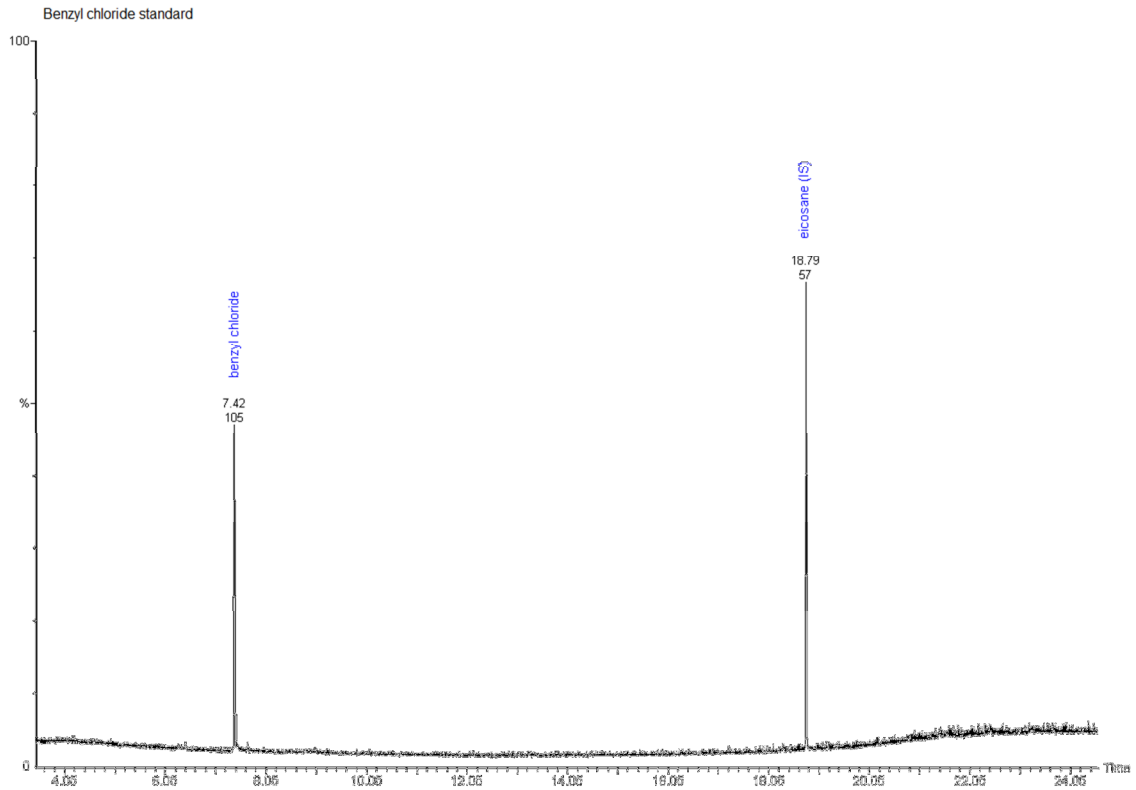


Figure 9 Chromatographic profile of benzyl chloride stability study

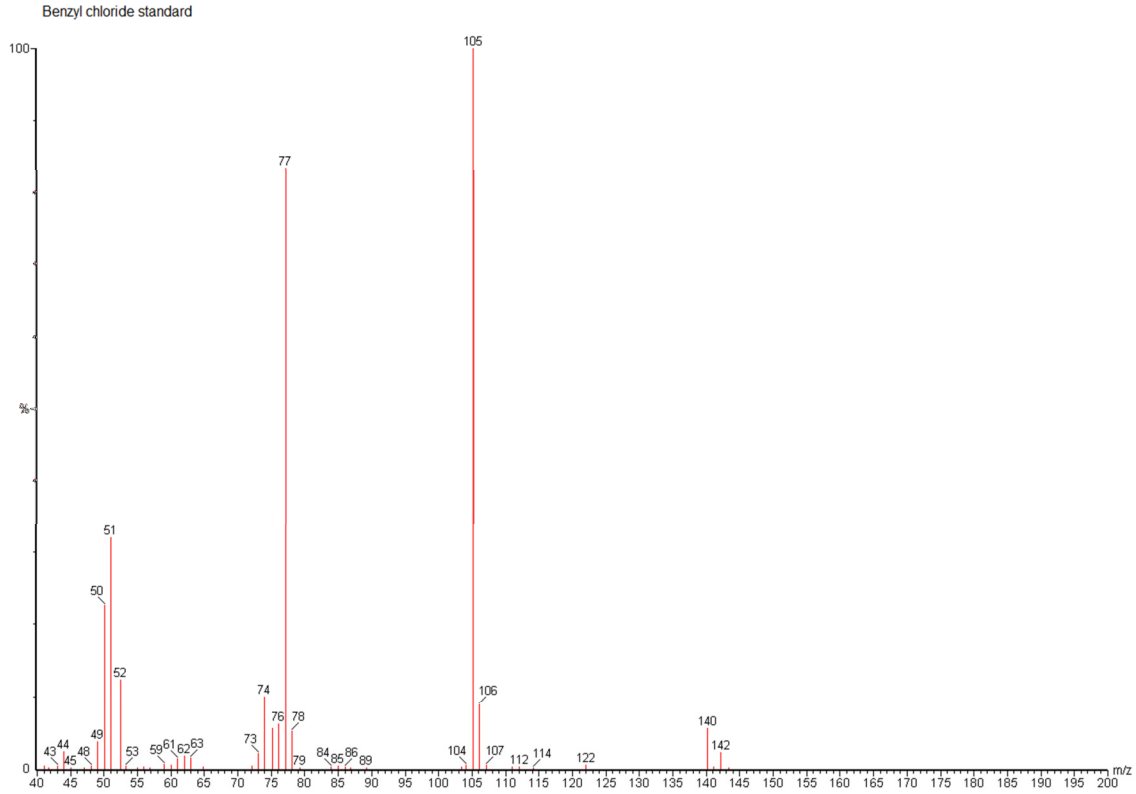


Figure 10 Mass spectra of benzyl chloride stability study

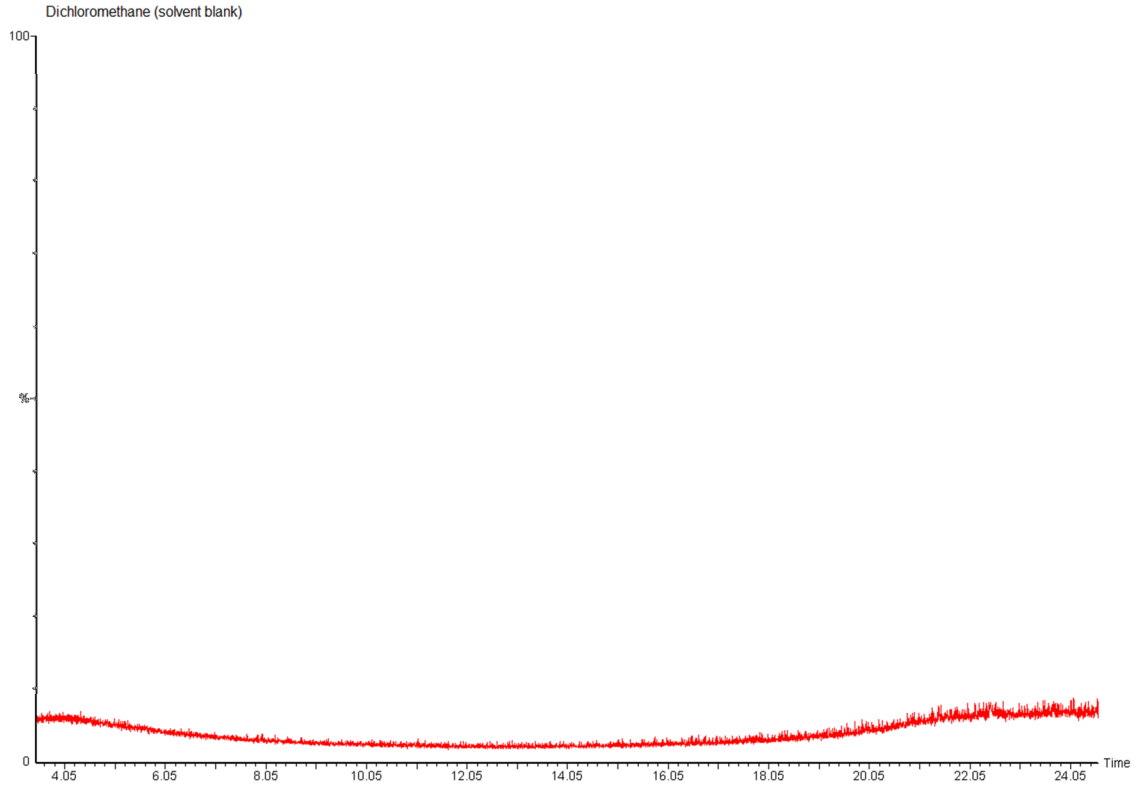


Figure 11 Chromatographic profile of dichloromethane solvent

APPENDIX 4 Phase 2 Investigating the effect of oven temperature on the method developed.

The table below shows the effect of oven temperature on column efficiency N, resolution R, tailing T, selectivity α and retention time Rt.

Table 1 Phase 1 Effect of oven temperature

Temp/^{o}C		Mean values (n = 2)					Temp/^{o}C	Mean values				
	N x10^5	T	α	R	Rt/mins			N x10^5	T	α	R	Rt/mins
Methamphetamine							MBZP					
140	4.596	3.475	1.448	69.464	8.870		140	14.574	4.268	1.013	4.060	15.115
150	3.124	4.001	1.422	55.546	8.870		150	12.365	4.637	1.014	3.927	15.180
160	4.219	2.333	1.378	58.565	8.865		160	10.930	4.447	1.015	4.254	15.135
170	4.002	2.228	1.366	59.341	8.865		170	9.965	4.081	1.018	4.454	14.960
180	4.002	3.273	1.366	59.435	8.865		180	8.218	3.784	1.020	4.512	14.590
190	4.325	2.393	1.365	60.009	8.870		190	10.578	3.409	1.017	4.355	14.400
200	5.339	2.245	0.365	63.738	8.865		200	11.344	3.600	1.015	4.225	14.390
2-FPP							CPP					
140	7.006	3.713	1.022	5.609	13.600		140	24.654	2.750	1.051	21.027	16.530
150	8.429	3.428	1.024	5.344	13.500		150	23.552	2.700	1.054	22.398	16.710
160	6.484	3.125	1.030	6.377	13.195		160	25.629	2.427	1.057	24.374	16.820
170	7.926	2.896	1.027	5.473	12.875		170	19.735	2.425	1.063	23.470	16.850
180	11.215	2.579	1.023	5.653	12.820		180	17.842	2.190	1.070	25.295	16.775
190	10.352	2.778	1.023	5.447	12.820		190	12.195	2.381	1.081	25.772	16.550
200	12.068	4.409	1.023	5.743	12.815		200	13.777	2.647	1.092	26.903	16.185
3-FPP							MePP					
140	18.226	3.202	1.035	13.106	14.510		140	18.061	2.892	1.080	27.923	15.305
150	13.631	3.213	1.040	12.608	14.510		150	15.320	3.125	1.086	28.389	15.385
160	10.938	3.000	1.045	13.461	14.375		160	15.064	3.000	1.095	30.877	15.365
170	8.247	2.527	1.055	13.393	14.065		170	10.681	3.000	1.107	30.367	15.225

180	10.026	2.332	1.049	12.683	13.780		180	9.575	2.971	1.127	33.783	14.885
190	13.873	2.053	1.041	13.033	13.740		190	11.701	2.500	1.131	33.458	14.640
200	13.339	2.121	1.040	12.398	13.735		200	15.083	2.972	1.108	30.732	14.605
4-FPP							MDMA					
140	13.937	3.529	1.012	3.802	14.160		140	18.423	ND	1.010	3.123	14.370
150	12.913	3.297	1.013	3.953	14.125		150	13.827	ND	1.010	2.938	14.365
160	9.594	3.228	1.016	3.971	13.930		160	12.105	ND	1.011	2.991	14.215
170	8.193	3.327	1.017	3.834	13.565		170	8.547	ND	1.015	3.443	13.855
180	10.889	2.642	1.013	3.535	13.385		180	12.126	ND	1.013	3.251	13.610
190	14.276	3.088	1.012	3.645	13.380		190	13.571	ND	1.011	3.212	15.590
200	13.145	2.625	1.012	3.607	13.375		200	16.025	ND	1.011	3.312	15.585
2-TFMPP							Caffeine					
140	6.896	4.750	1.059	13.715	12.840		140	34.038	1.080	1.048	25.152	17.370
150	5.068	5.750	1.071	13.680	12.610		150	20.688	1.056	1.051	27.225	17.605
160	6.499	4.250	1.079	15.354	12.225		160	33.883	1.018	1.040	20.564	17.785
170	8.340	4.094	1.064	13.873	12.105		170	28.767	1.009	1.058	28.019	17.951
180	8.347	3.850	1.059	13.964	12.110		180	26.605	1.000	1.064	28.861	17.950
190	8.052	2.833	1.059	13.593	12.110		190	26.497	1.000	1.072	31.467	17.890
200	8.340	3.820	1.059	14.229	12.105		200	16.421	1.000	1.083	30.261	17.670
3TFMPP							Cocaine					
140	17.063	ND	1.002	0.821	14.335		140	58.358	1.131	1.082	42.887	20.015
150	13.370	ND	1.003	0.900	13.320		150	57.929	1.036	1.081	43.009	20.340
160	11.388	ND	1.004	1.143	14.155		160	57.267	1.023	1.081	42.523	20.630
170	8.479	ND	1.004	1.000	13.379		170	53.734	1.021	1.080	41.871	20.915
180	13.001	ND	1.004	1.029	13.560		180	58.507	1.000	1.081	42.851	21.170
190	15.919	ND	1.004	1.115	13.540		190	63.425	1.042	1.082	45.749	21.390
200	16.648	ND	1.004	1.177	13.535		200	53.326	1.000	1.085	45.659	21.555
4-TFMPP							Diazepam					
140	28.870	2.053	1.006	2.110	15.025		140	40.576	1.280	ND	ND	21.650

150	21.210	2.111	1.006	1.974	15.085		150	41.707	1.081	ND	ND	21.995
160	21.009	1.806	1.007	2.212	15.025		160	39.529	1.113	ND	ND	22.290
170	12.569	1.750	1.008	2.214	14.835		170	42.282	1.121	ND	ND	22.595
180	12.700	1.753	1.009	2.261	14.455		180	41.479	1.074	ND	ND	22.885
190	21.286	1.500	1.007	2.099	14.300		190	46.573	1.034	ND	ND	23.150
200	19.353	1.500	1.042	2.203	14.285		200	48.558	1.037	ND	ND	23.385
BZP							Eicosane					
140	9.186	5.559	1.019	4.900	13.900		140	62.956	1.000	1.079	46.127	18.210
150	7.369	5.556	1.021	5.158	13.830		150	64.942	1.000	1.079	46.594	18.495
160	9.096	5.074	1.025	6.413	15.590		160	61.081	1.000	1.080	45.937	18.745
170	5.719	5.000	1.026	5.231	13.225		170	55.149	1.000	1.068	38.709	18.955
180	9.075	4.306	1.021	5.168	13.110		180	45.203	1.000	1.085	46.702	19.105
190	8.877	4.500	1.021	5.353	13.110		190	36.843	1.000	1.091	46.183	19.180
200	8.767	6.250	1.021	5.256	13.105		200	33.350	1.000	1.048	23.426	19.140
DBZP												
140	56.220	1.150	1.019	11.156	19.645							
150	55.785	1.030	1.019	11.233	19.960							
160	55.095	1.012	1.020	11.446	20.235							
170	56.576	1.011	1.020	11.598	20.505							
180	56.099	1.000	1.021	12.401	20.735							
190	54.820	1.000	1.022	13.183	20.930							
200	50.905	1.000	1.024	13.248	21.060							

ND = not determined due to 1) 3TFMPP and MDMA co-elute and 2) diazepam is the last peak hence there is no resolution to a subsequent peak after it.

APPENDIX 5 Phase 3 Investigating the effect of simultaneously applying the injector and oven temperatures selected as optimum in phase 1

Note:

The temperatures investigated were those chosen as giving optimum results in Phase 1 (injector port temperature 260°C) and Phase 2 (oven temperatures 160 and 180°C). The purpose was to determing which of the oven temperatures gave the best result when applied together with the optimum injector temperature from Phase 1. The effect on plate number, tailing, selectivity resolution and retention time are in Figures 1 – 5. For plate number N = N x10^5. For 3-TFMPP and MDMA tailing not determined peaks highly co-eluting. IS = internal standard (eicosane).

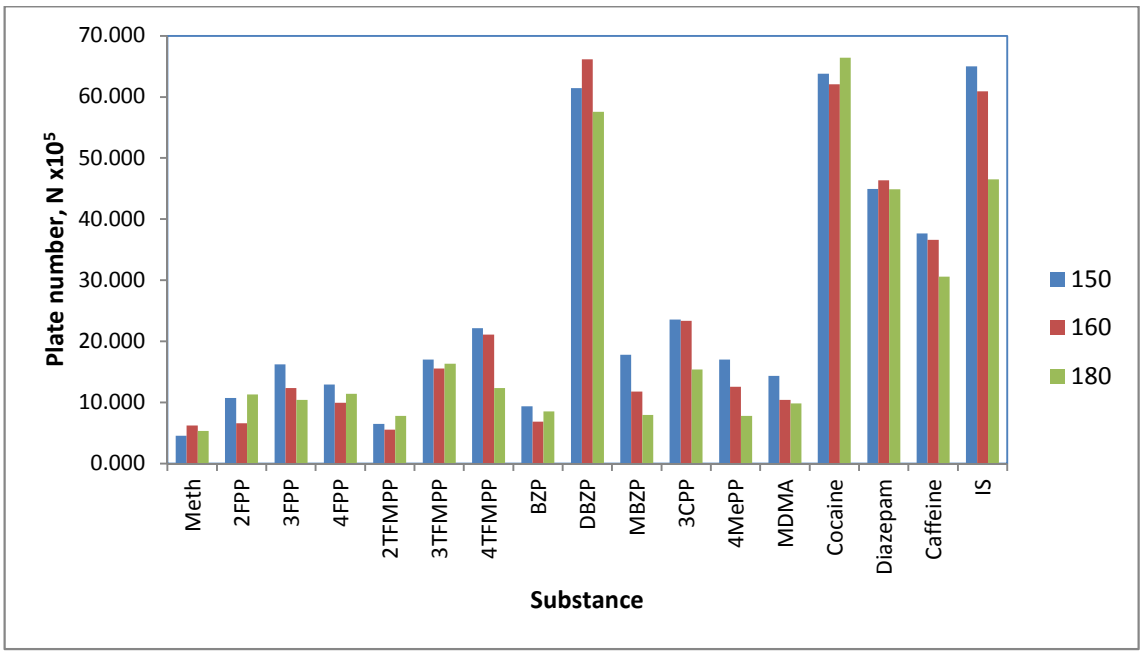


Figure 1 Effect of simultaneous applying selected injector and oven temperatures on number, N

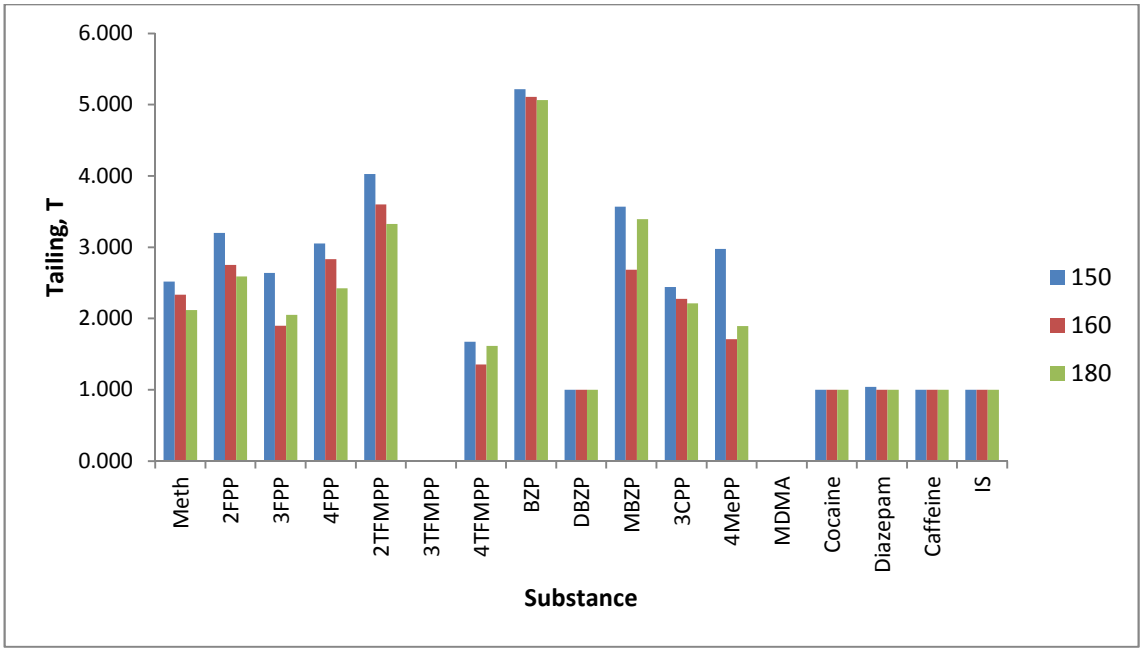


Figure 2 Effect of simultaneous applying selected injector and oven temperatures on tailing factor, T

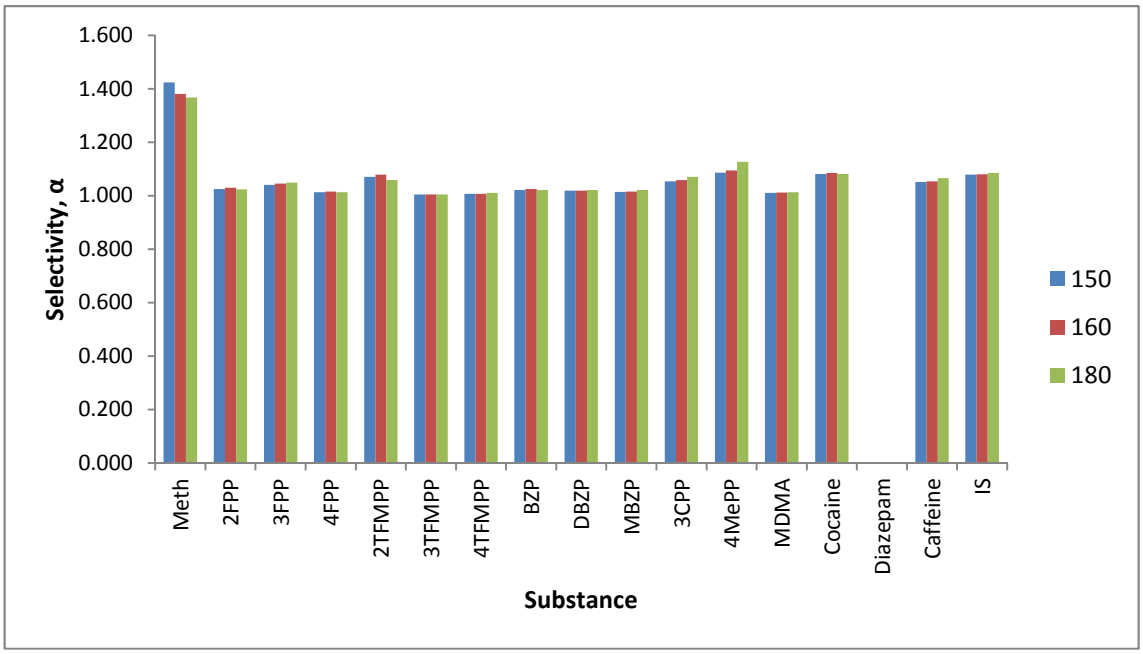


Figure 3 Simultaneous effect of injector and oven temperatures on Selectivity, α

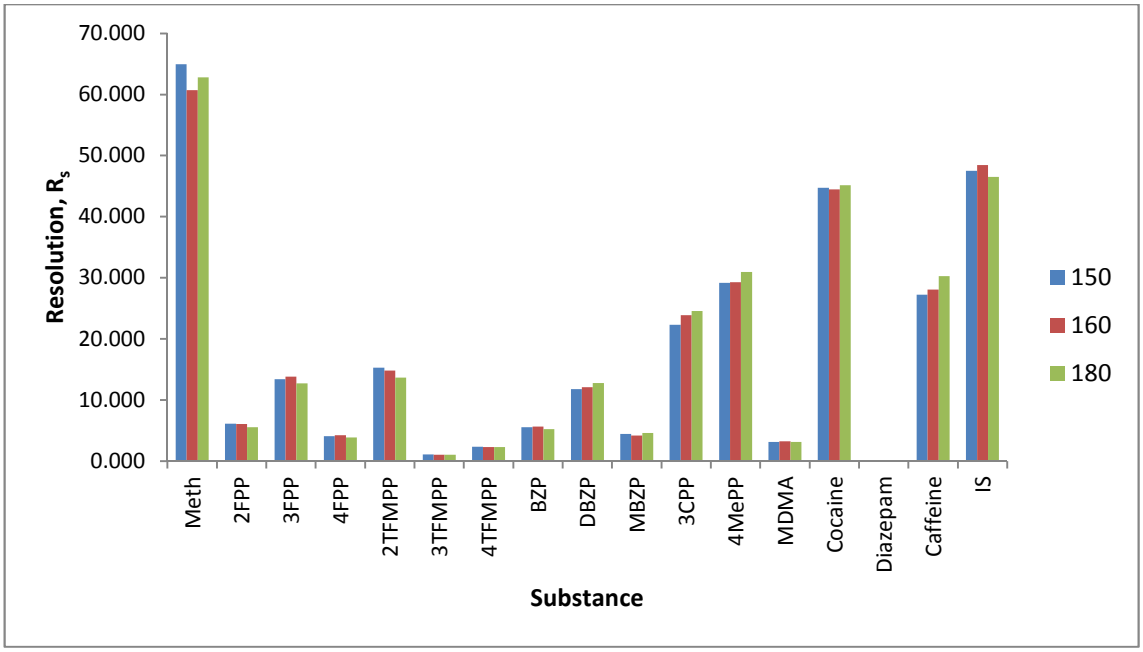


Figure 4 Simultaneous effect of injector and oven temperatures on Resolution, R

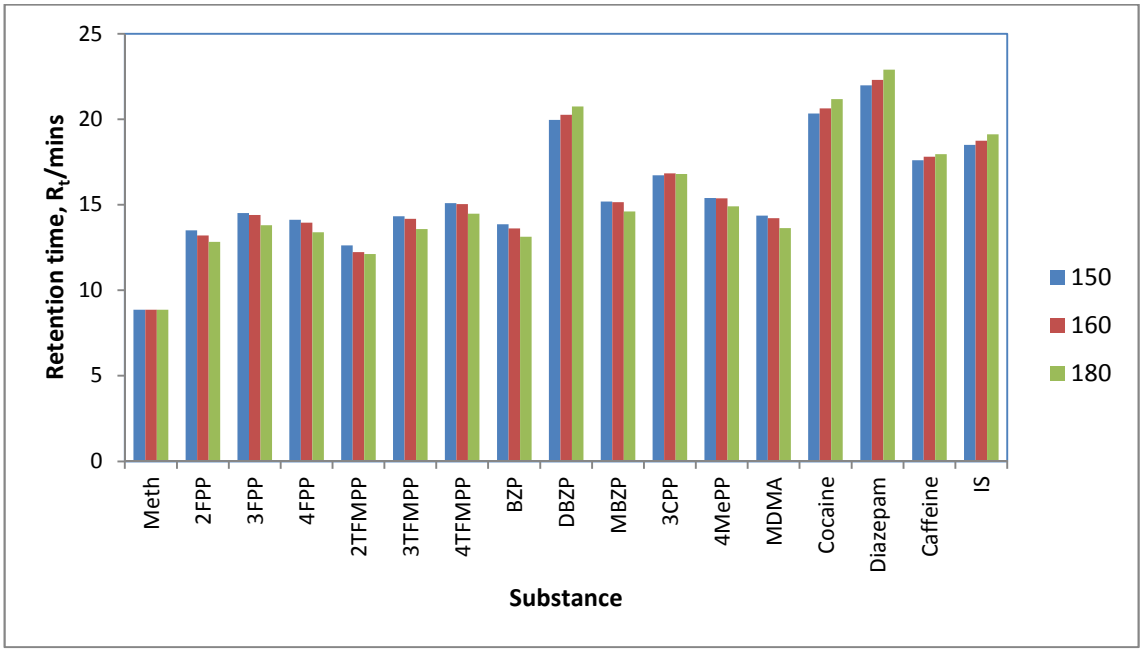


Figure 5 Simultaneous effect of injector and oven temperatures on Retention time, Rt

APPENDIX 6 Phase 3 Investigating the effect of simultaneously applying the oven and injector temperatures selected as optimum in phases 1 and 2

Figures 1 - 4 show the % gain or loss graphs for the effect of simultaneously applying injector and oven temperature vasriations on plate number, tailing, selectivity and resolution.

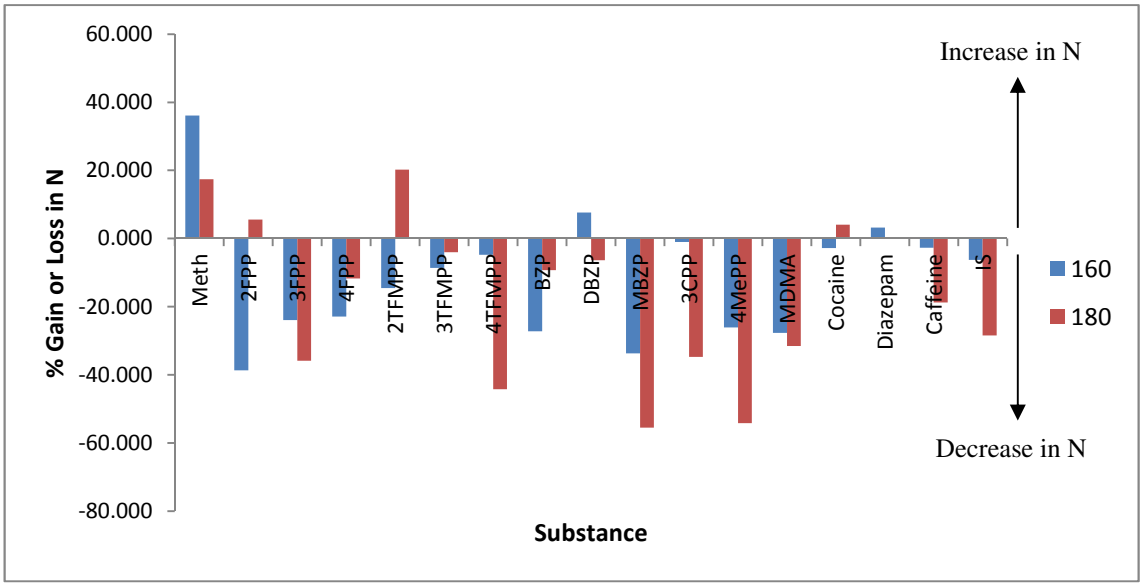


Figure 1 %Gain or loss for effect of injector and oven temperatures on plate number, N

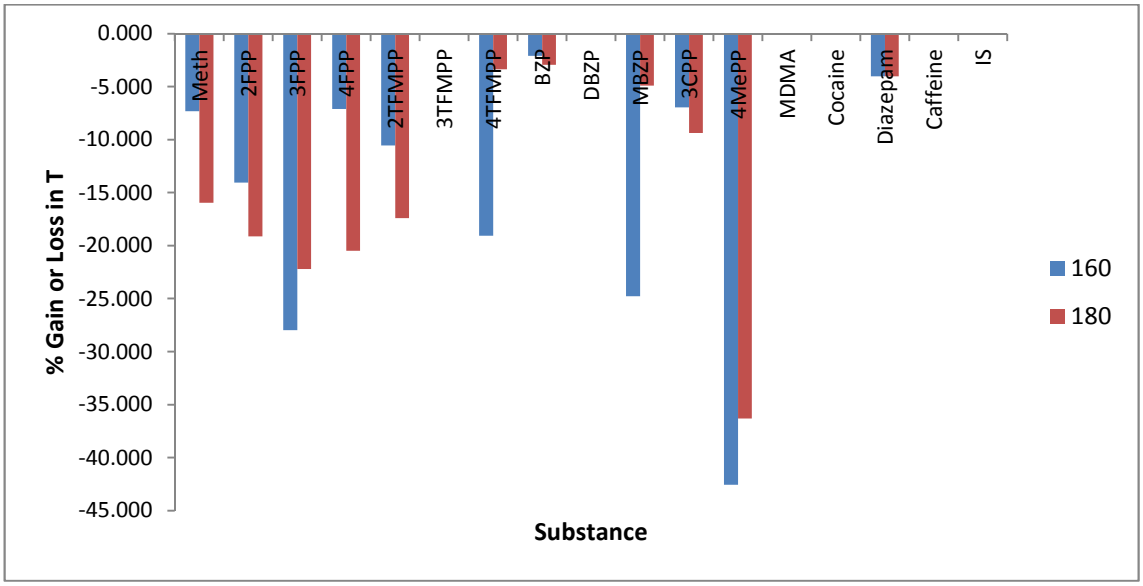


Figure 2 %Gain or loss for effect of injector and oven temperatures on tailing, T

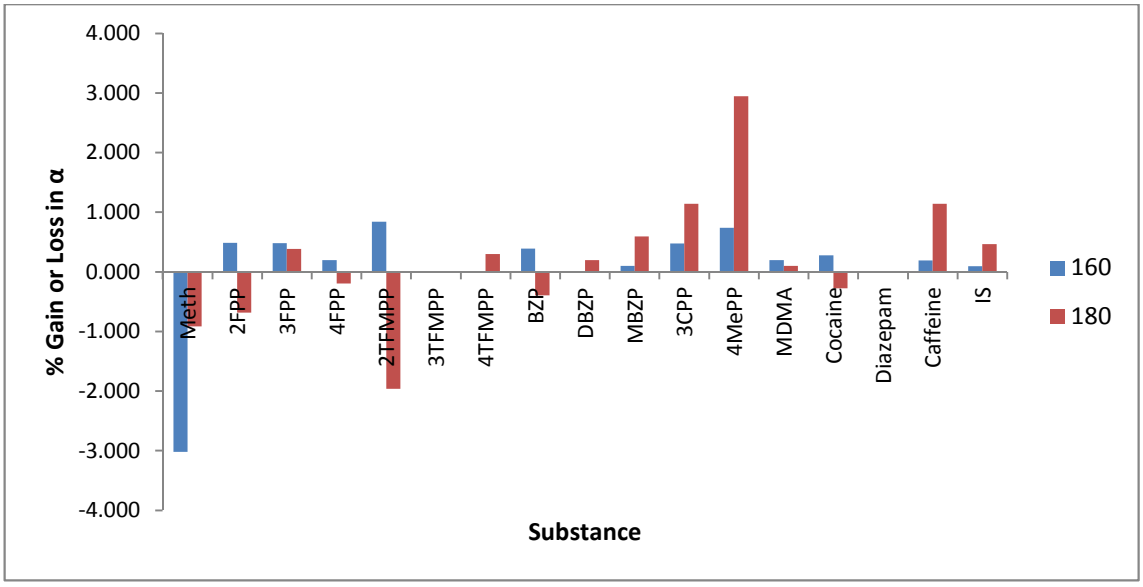


Figure 3 %Gain or loss for effect of injector and oven temperatures on selectivity, α

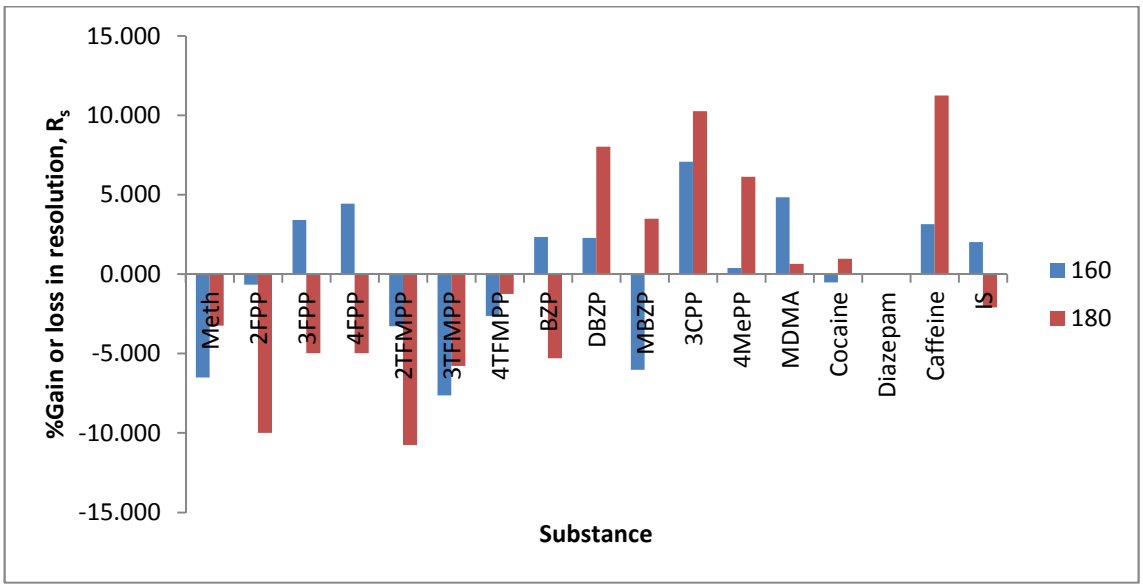


Figure 4 % Gain or loss for effect of injector and oven temperatures on resolution, R_s

APPENDIX 7 Phase Investigating the ffect of optimisation of ionisation energy on mass spectra of BZP

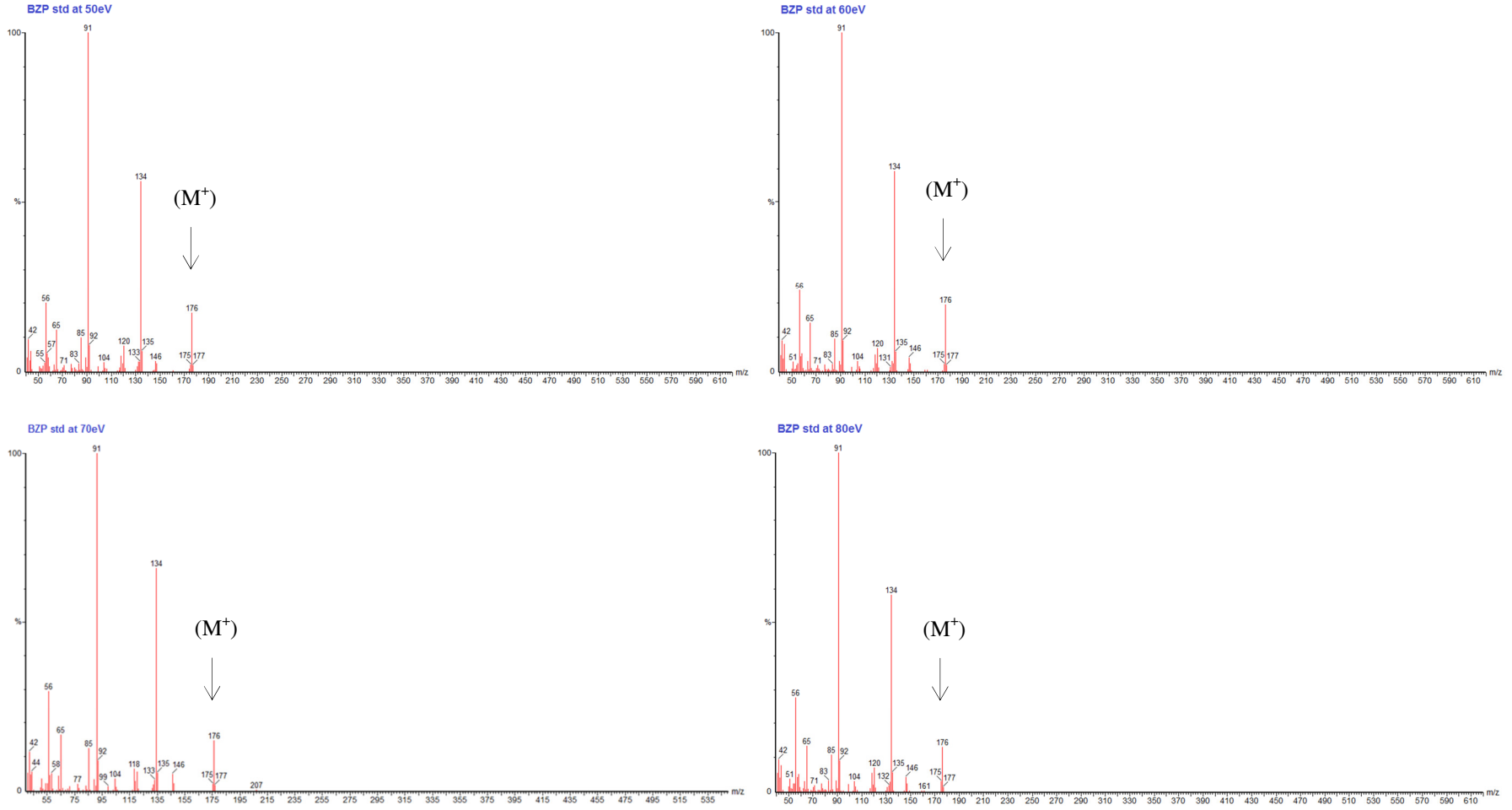


Figure 1 Phase 6 Effect of ionisation energy (EI) on the mass spectra of 3-TFMPP showing the mass spectra at different EI (50, 60, 70 and 80eV) for comparison.

APPENDIX 8 Method validation characteristics investigated in the study (Horacio et al, 2008; ICH, 2005; Thompson, 2002; Eurachem, 1998)

Validation characteristics	**Analytical procedure**	
	Identification (including impurities)	Quantitative tests
Specificity	x	x
Linearity		x
Quantitation limit		x
Detection limit	x	x
Range : linearity range working range		x
Accuracy	x	x
Precision- repeatability - intermediate precision	x	x
Robustness	x	x

APPENDIX 9 Calibration graphs

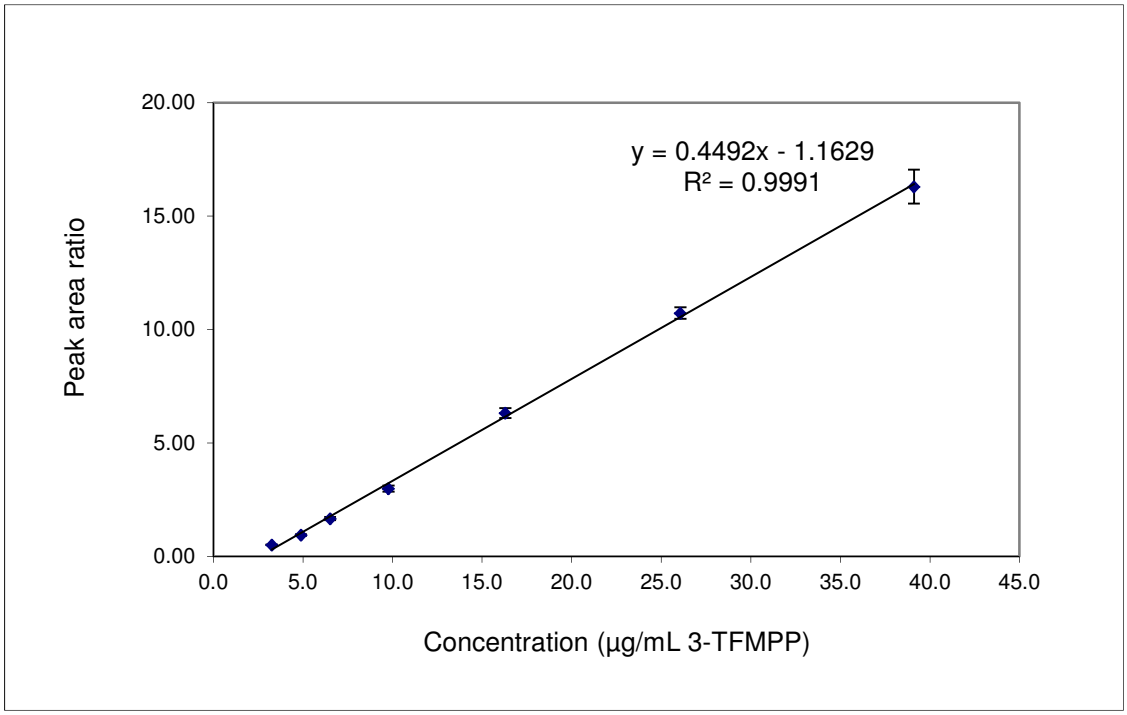


Figure 1 Calibration graph for 3-TFMPP.

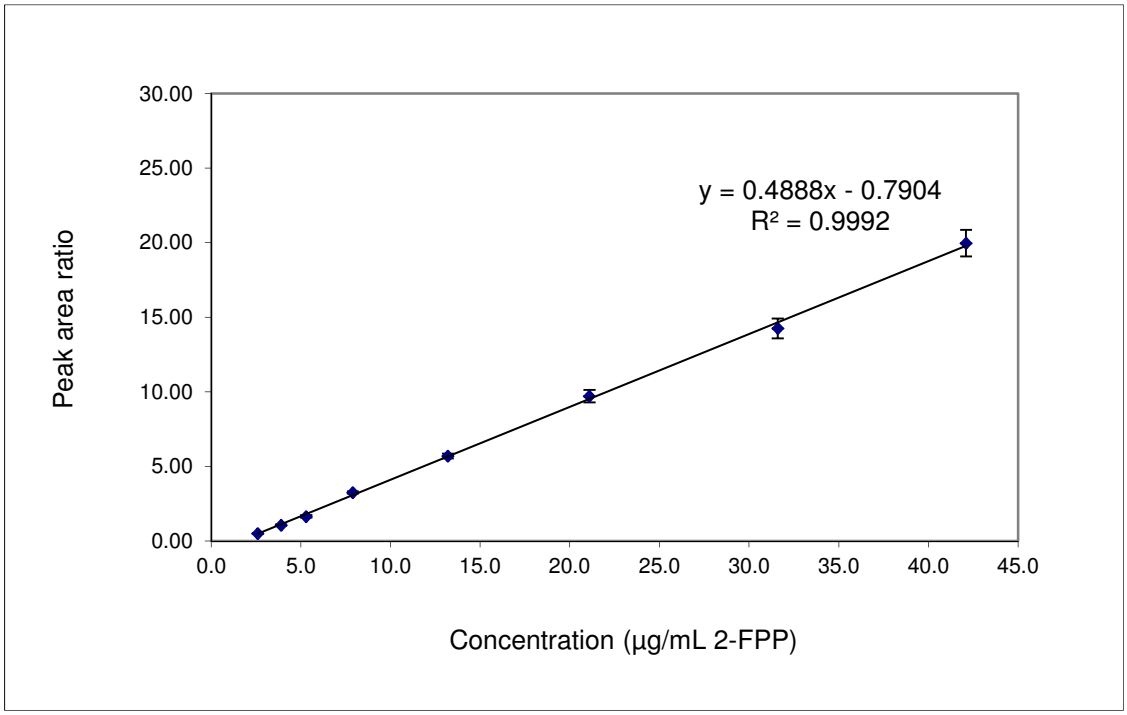


Figure 2 Calibration graph for for 2-FPP.

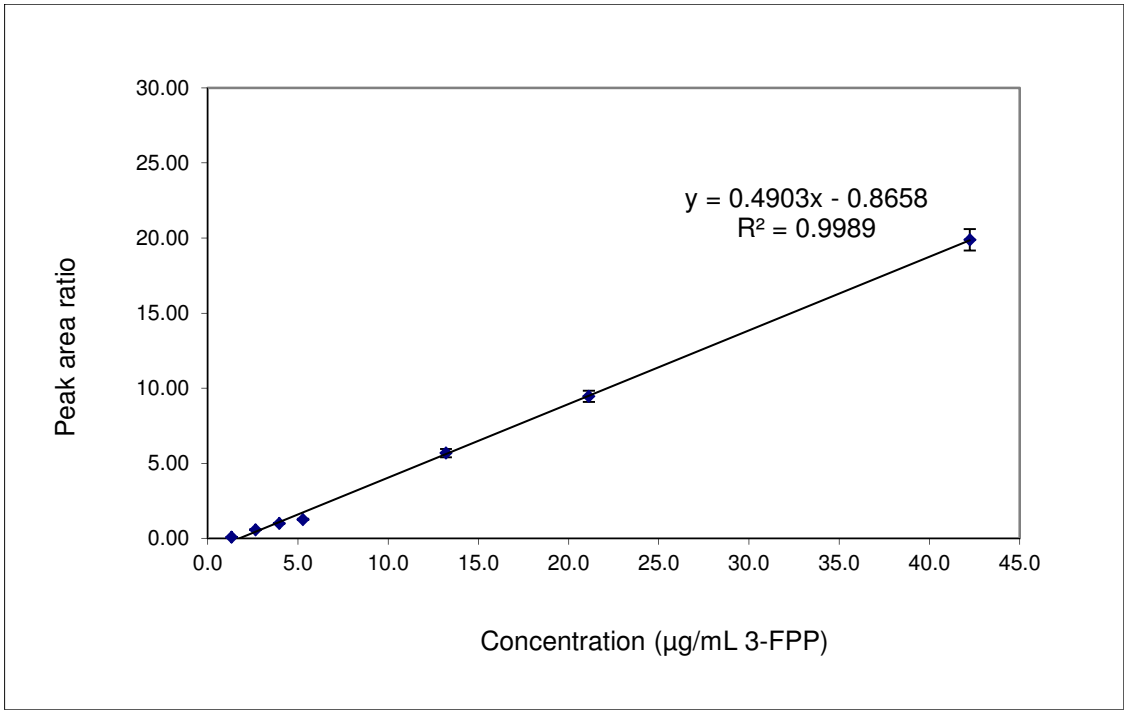


Figure 3 Calibration graph for 3-FPP.

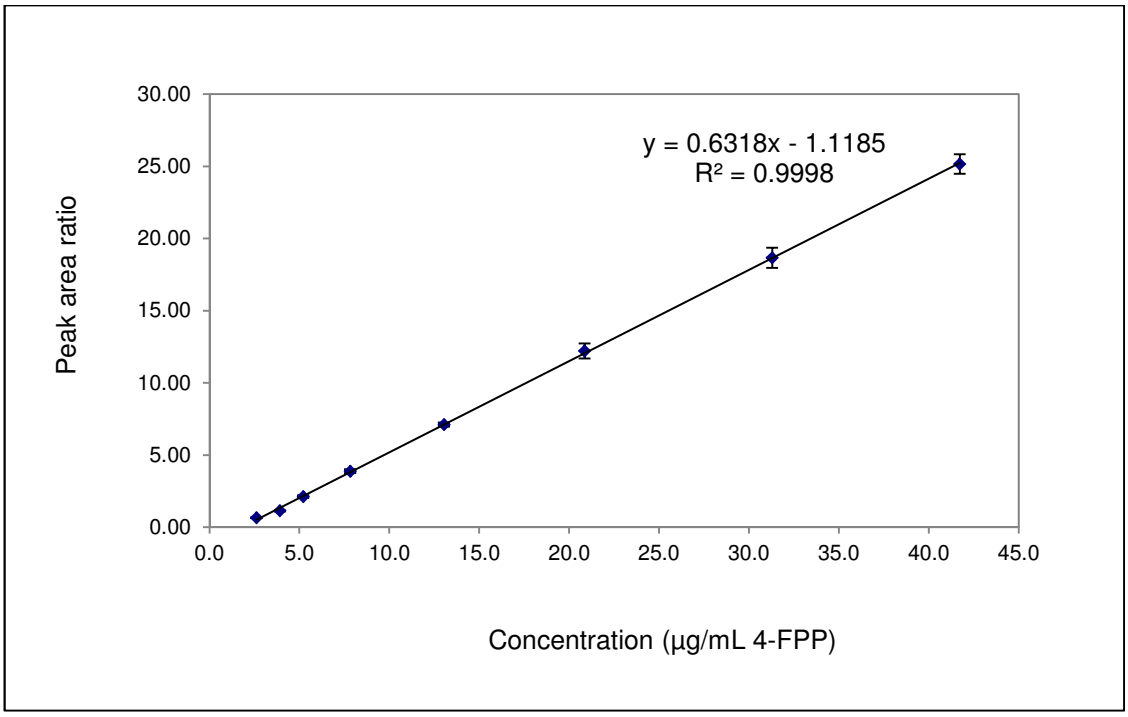


Figure 4 Calibration graph for 4-FPP.

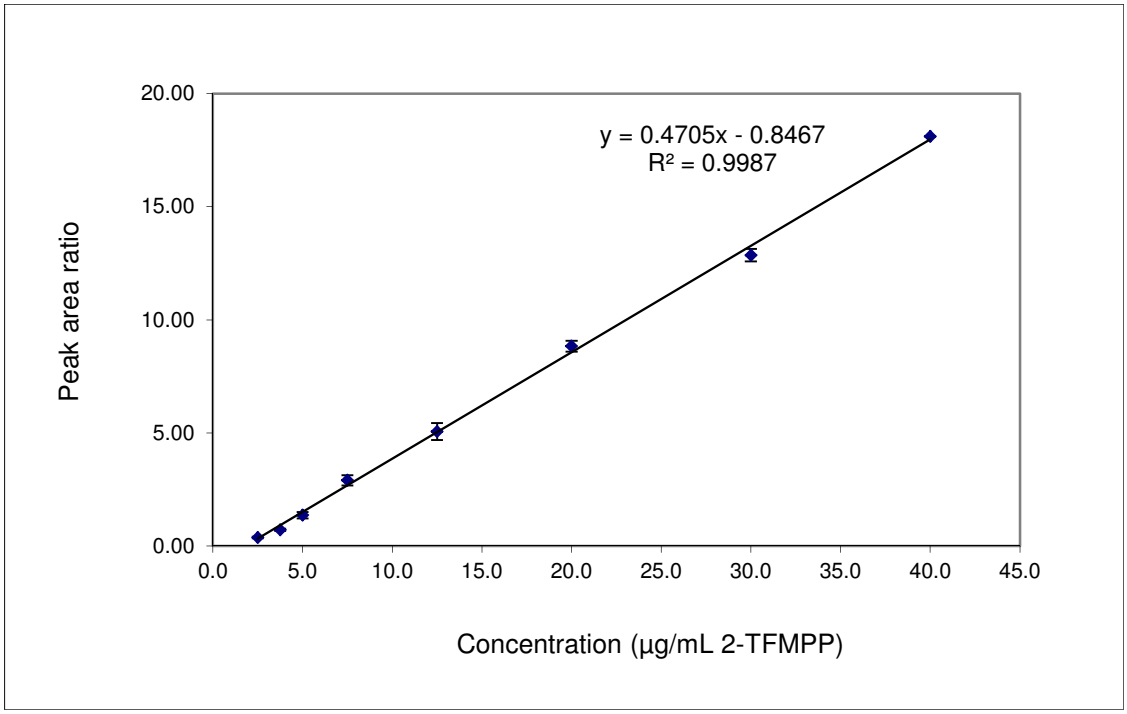


Figure 5 Calibration graph for 2-TFMPP.

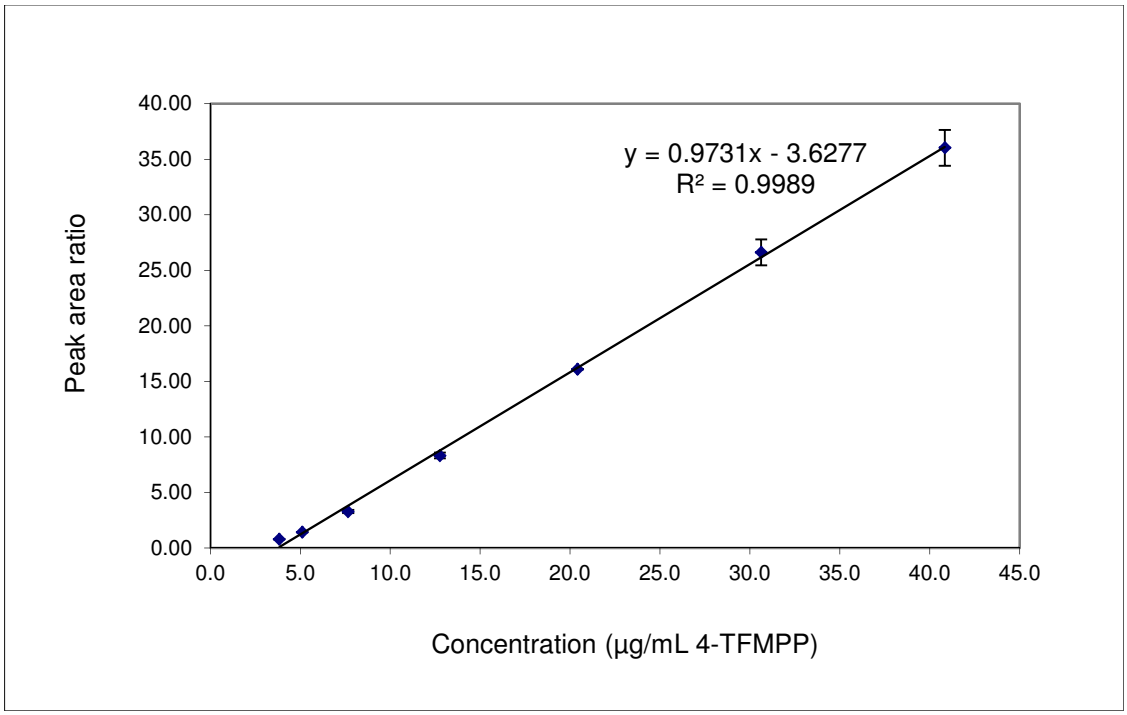


Figure 6 Calibration graph for 4-TFMPP.

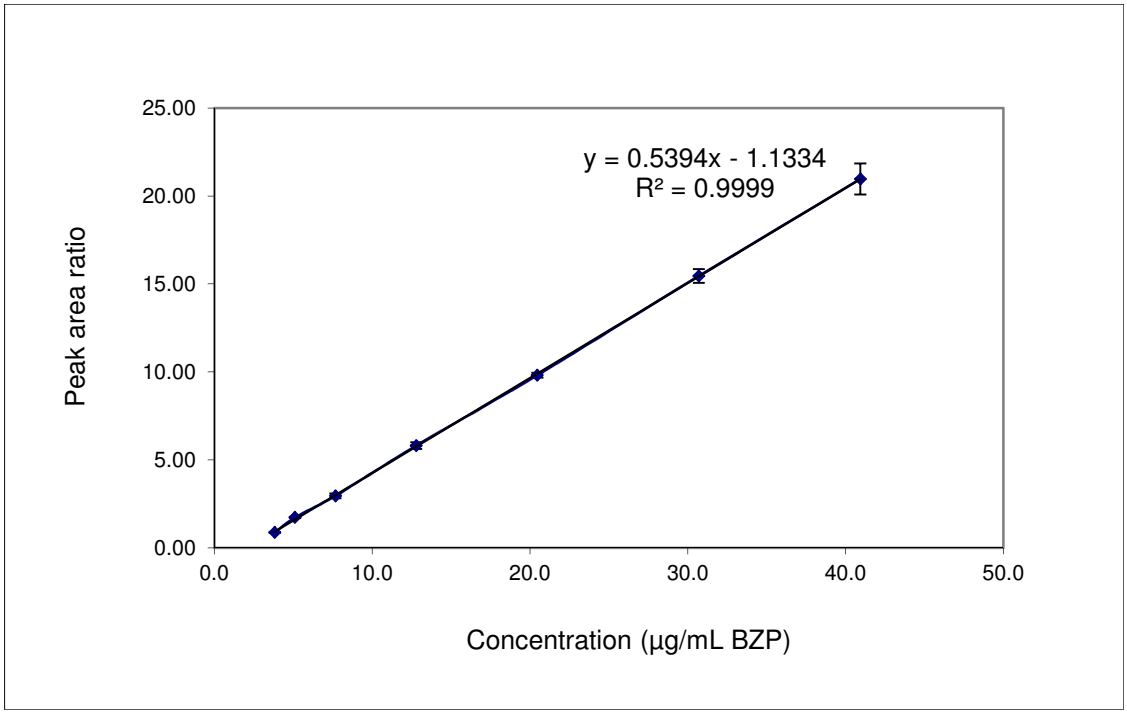


Figure 7 Calibration graph for BZP.

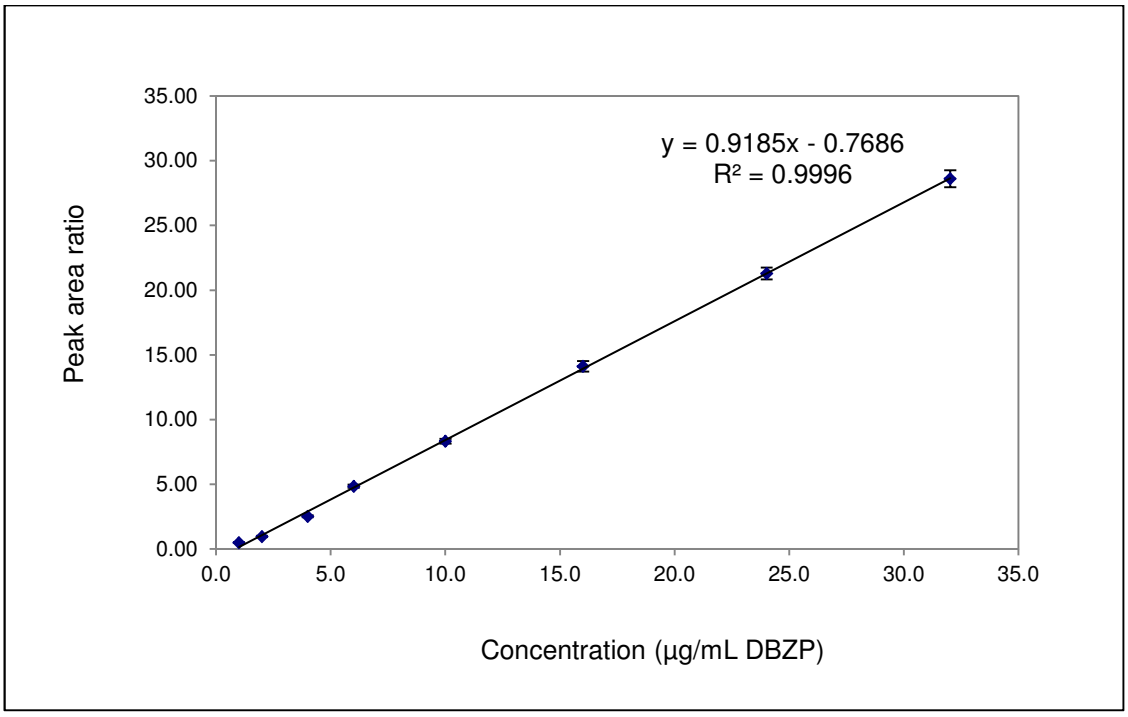


Figure 8 Calibration graph for DBZP.

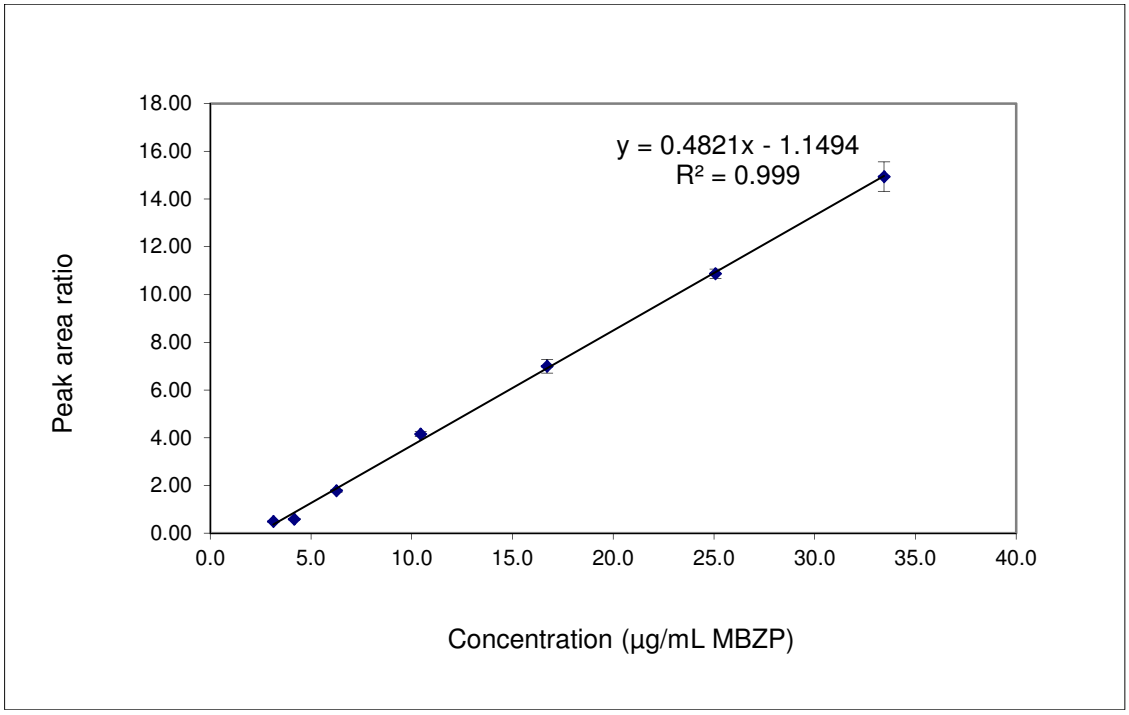


Figure 9 Calibration graph for MBZP.

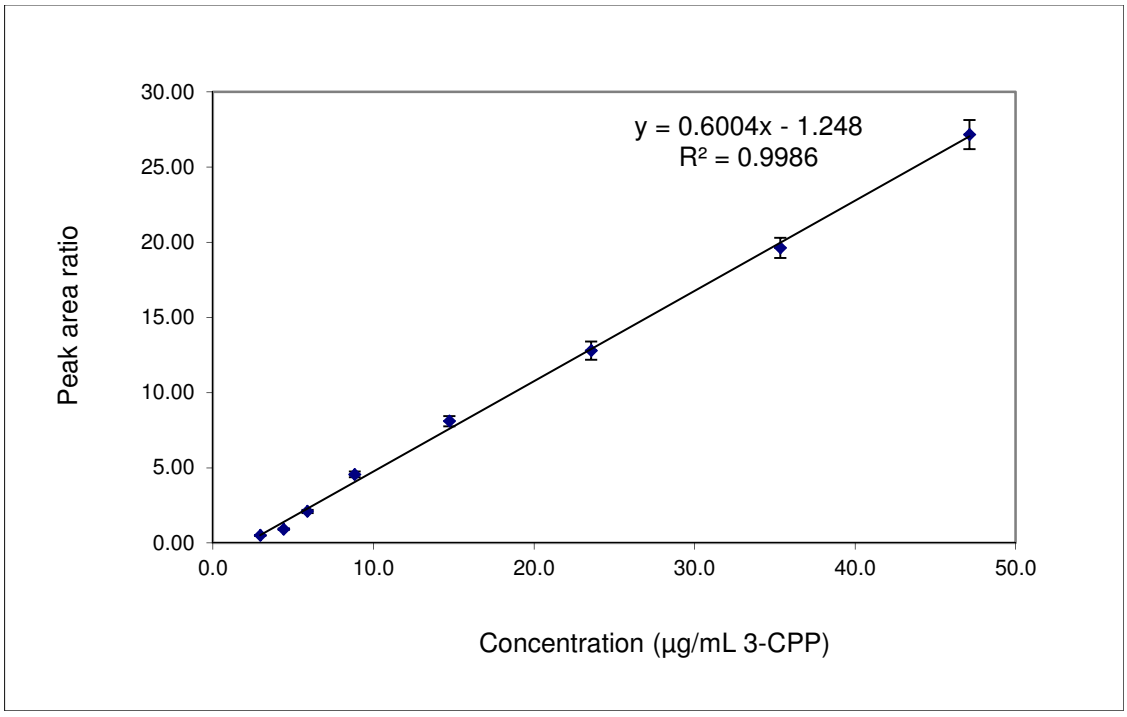


Figure 10 Calibration graph for 3-CPP.

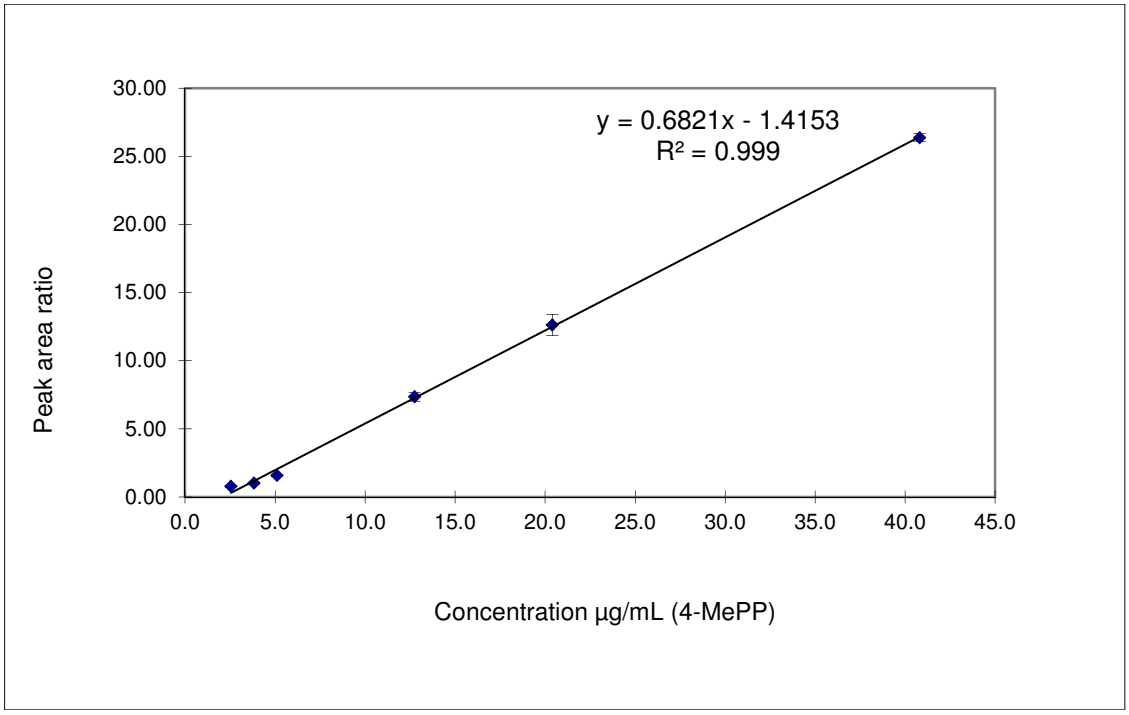


Figure 11 Calibration graph for 4-MePP.

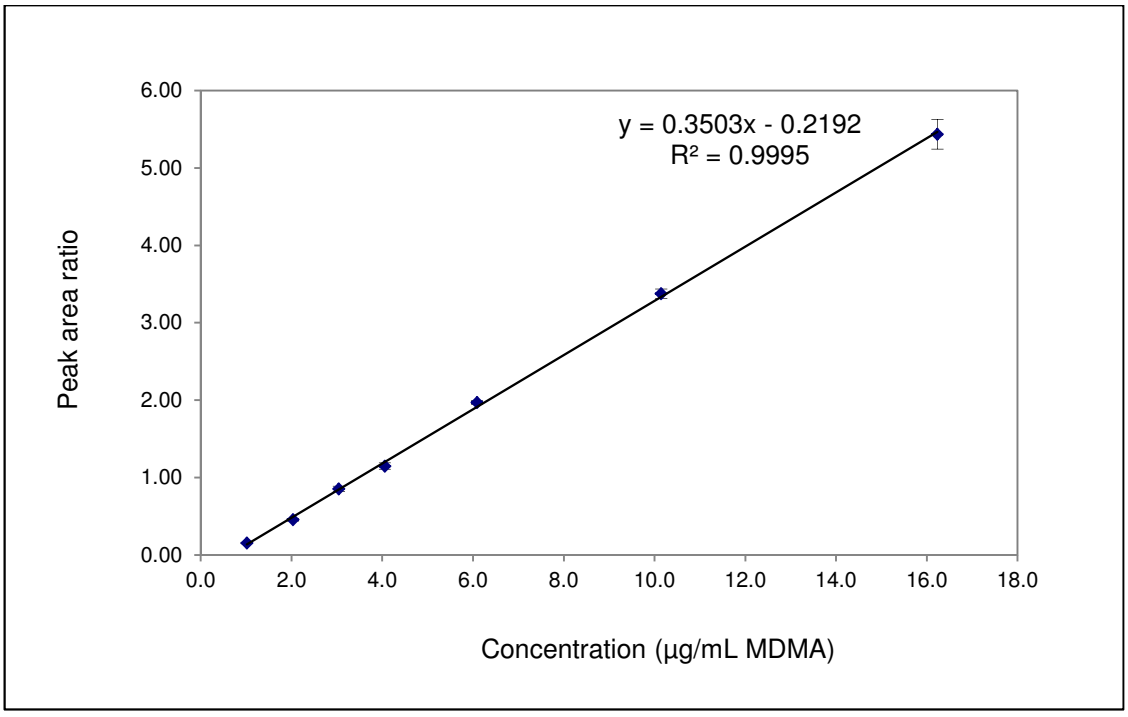


Figure 12 Calibration graph for MDMA.

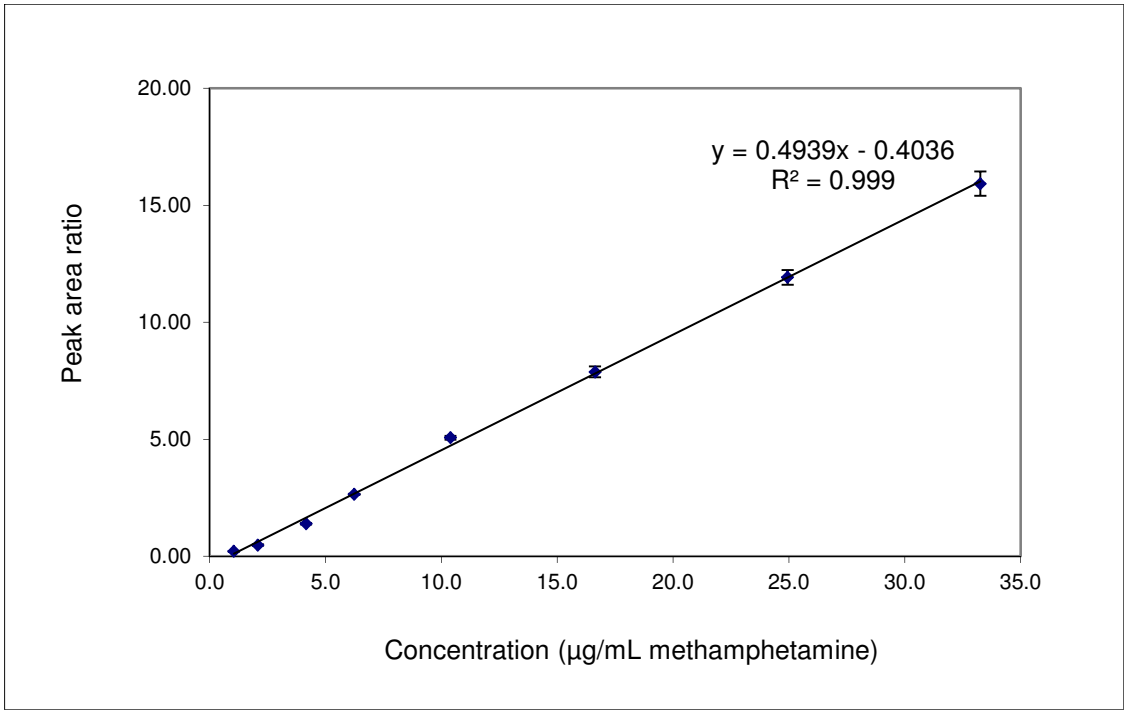


Figure 13 Calibration graph for methamphetamine.

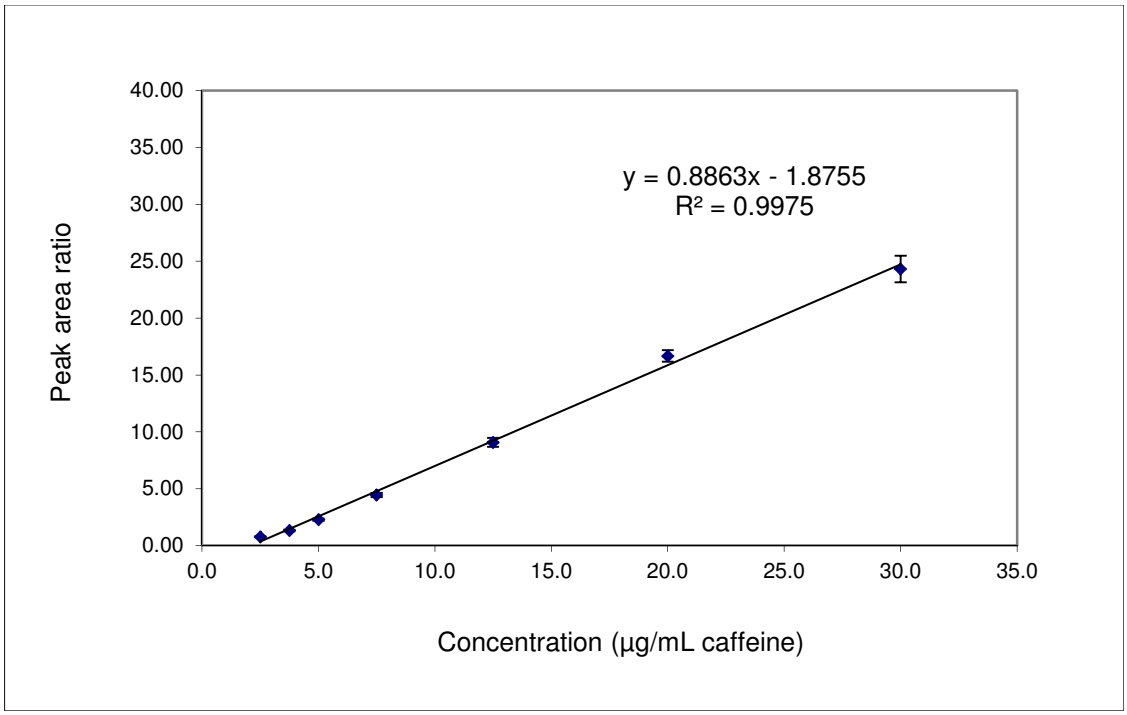


Figure 14 Calibration graph for caffeine.

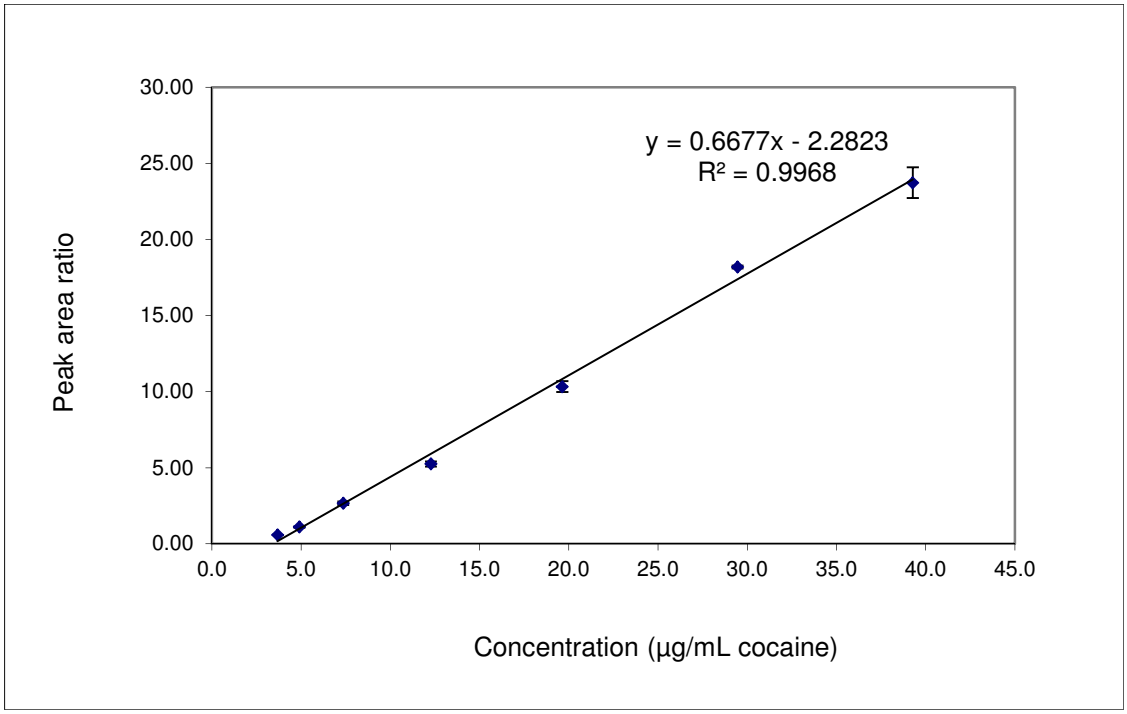


Figure 15 Calibration graph for cocaine.

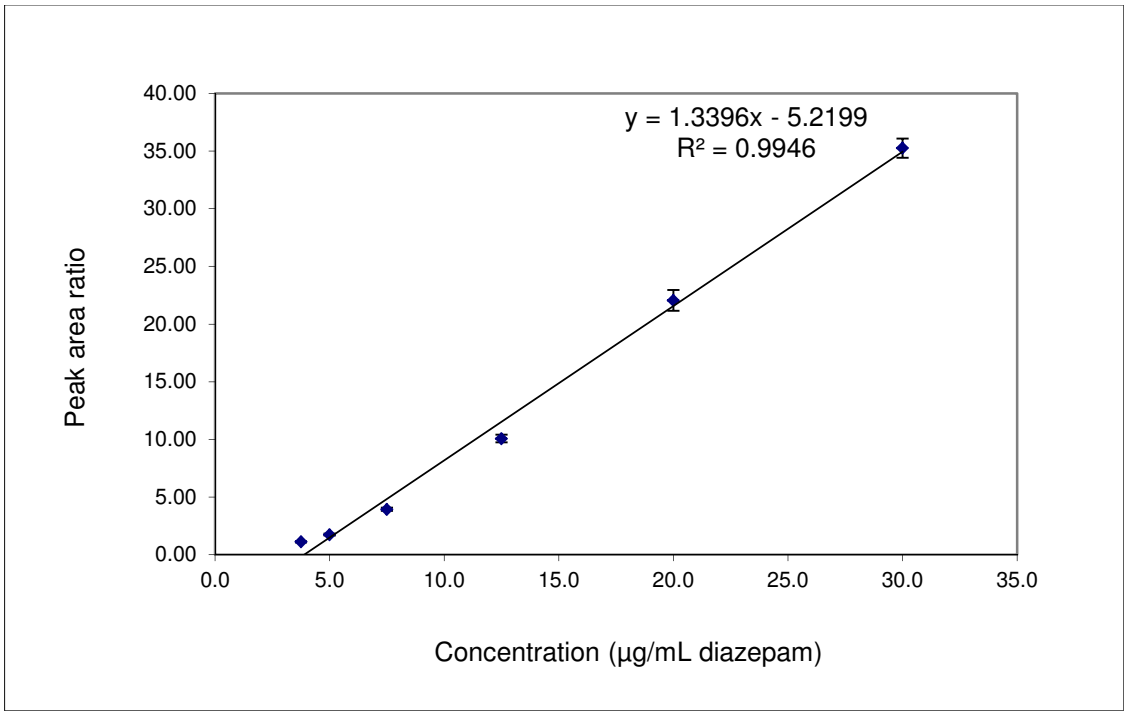


Figure 16 Calibration graph for diazepam.

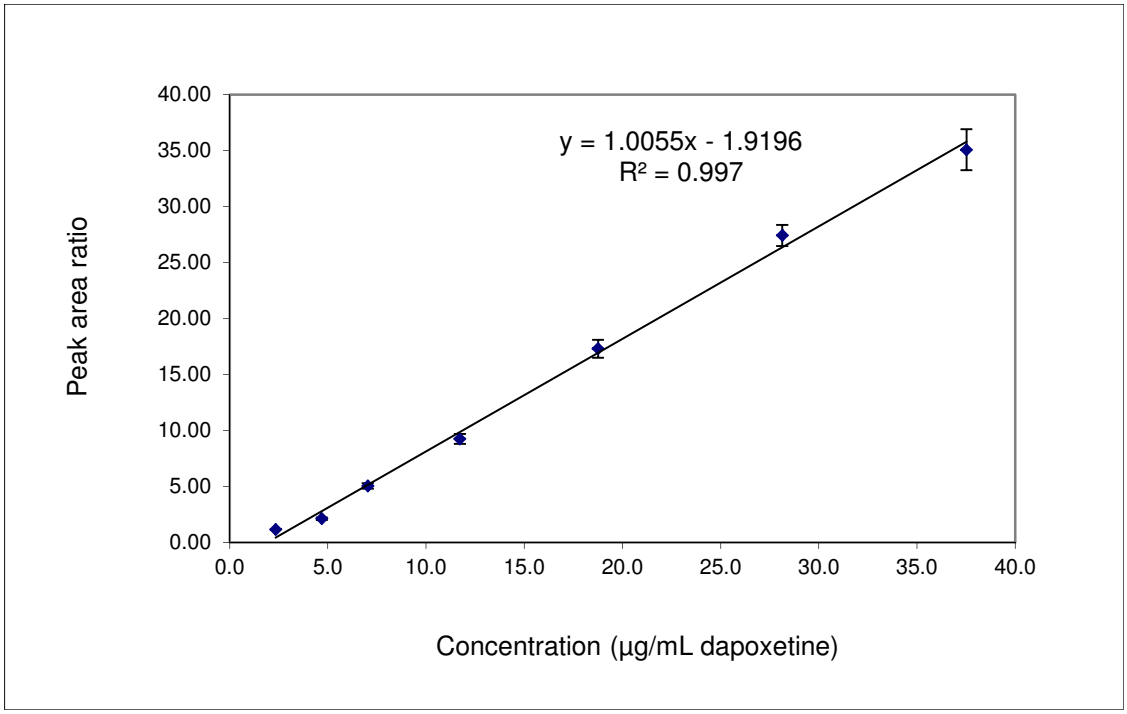


Figure 17 Calibration graph for dapoxetine.

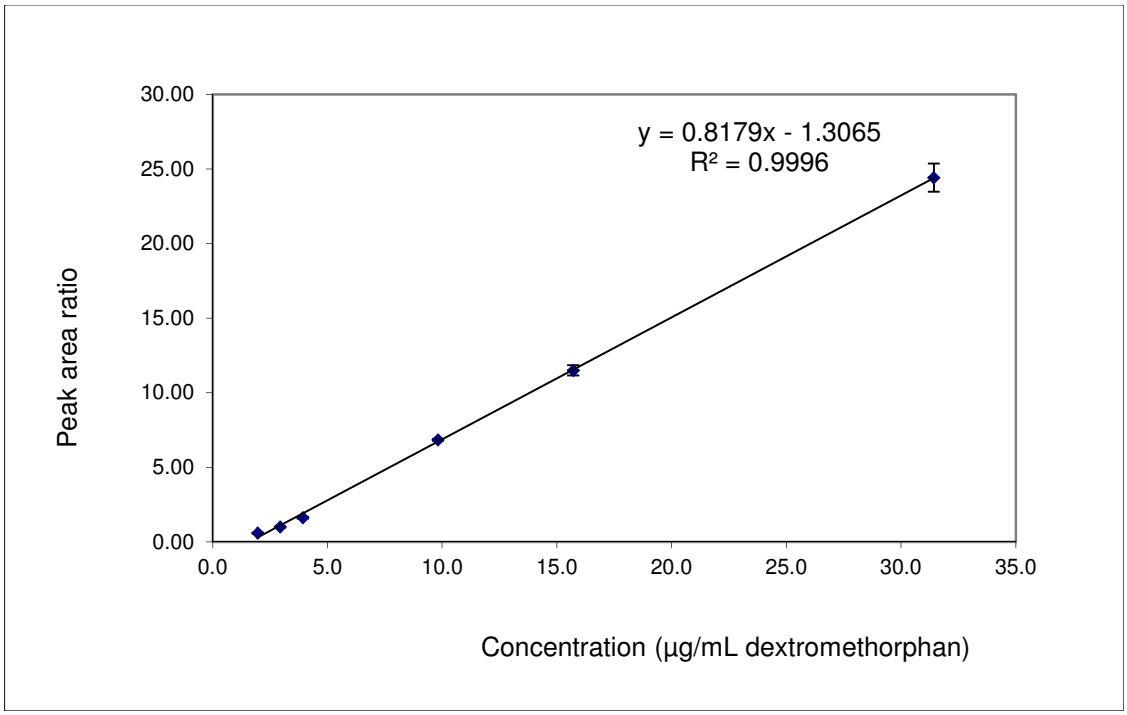


Figure 18 Calibration graph for dextromethorphan.

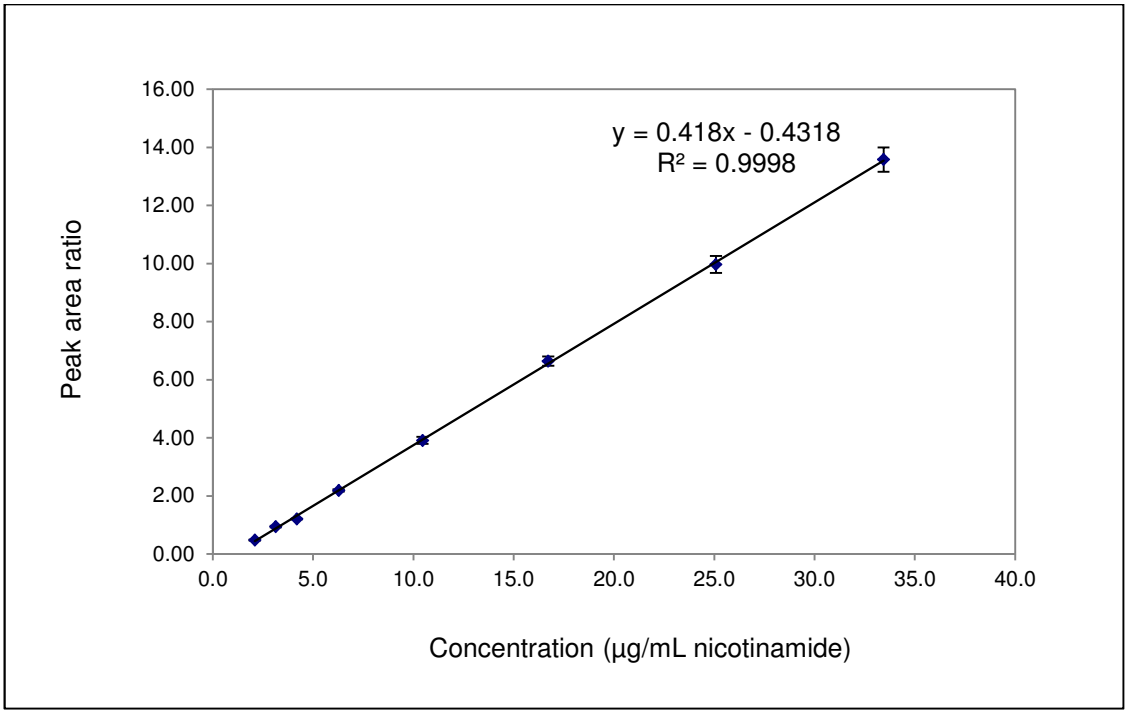


Figure 19 Calibration graph for nicotinamide.

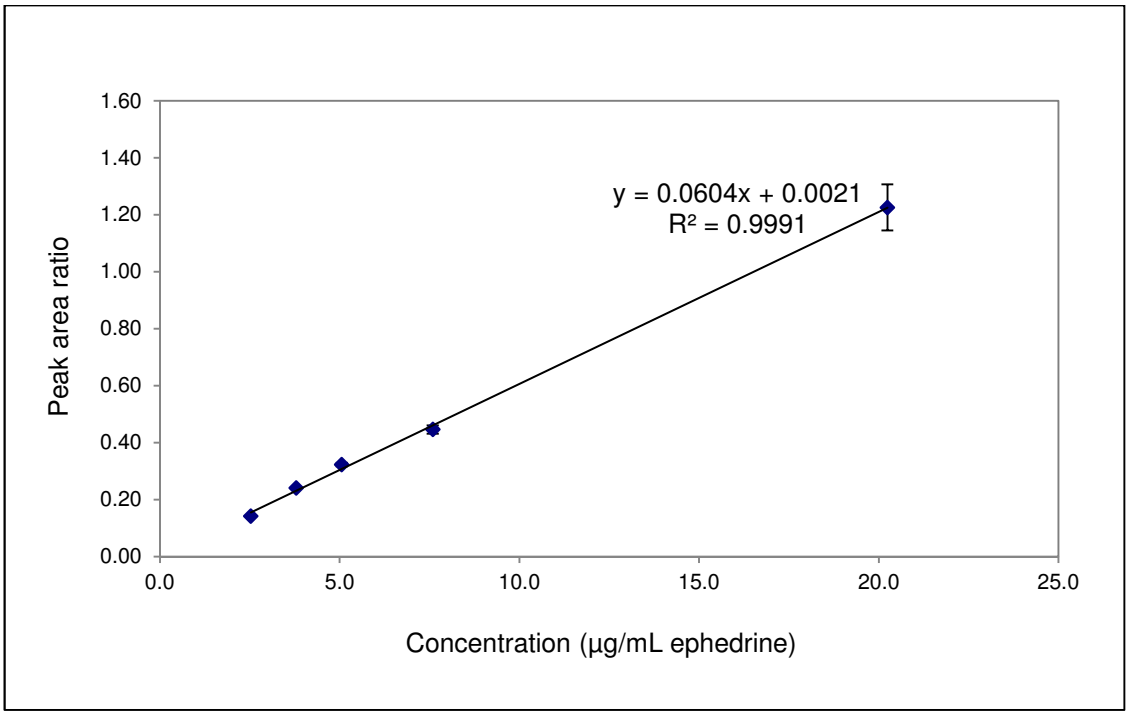


Figure 20 Calibration graph for ephedrine.

APPENDIX 10 GC-MS mass spectra of analyte drug standards (GC-MS/EI 70eV)

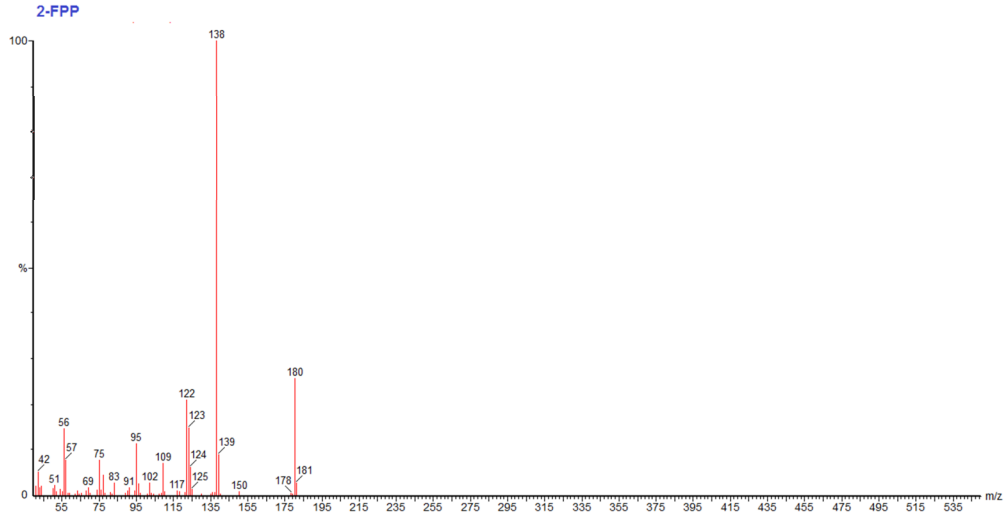


Figure 1 Mass spectrum of 2-FPP

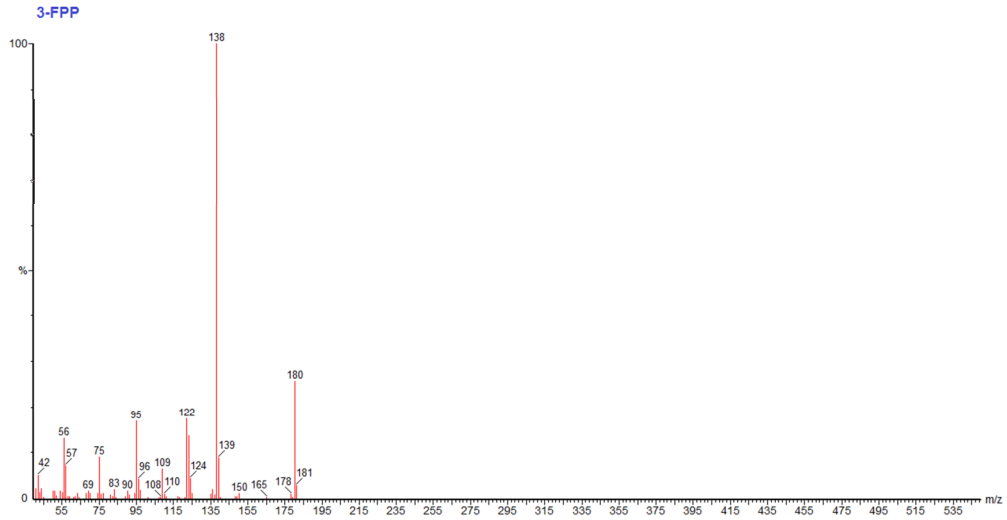


Figure 2 Mass spectrum of 3-FPP

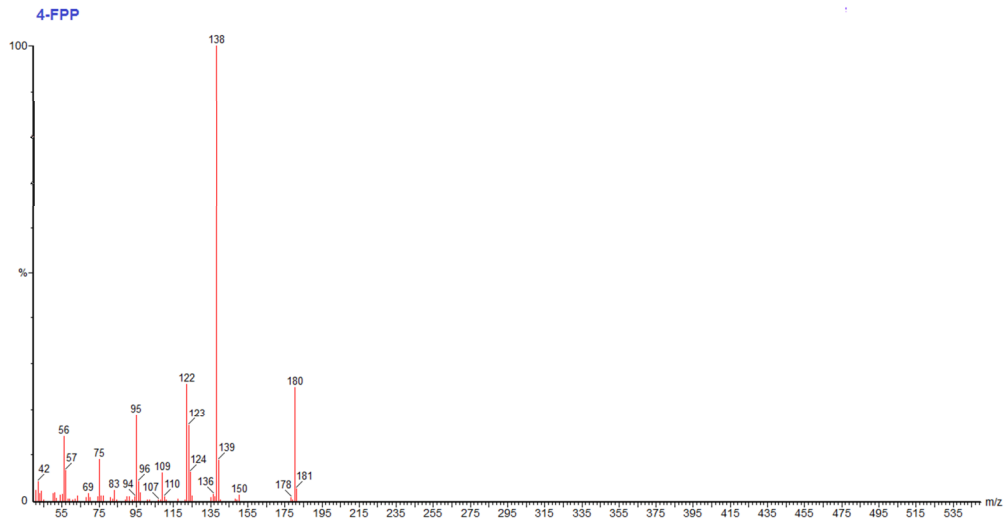


Figure 3 Mass spectrum of 4-FPP

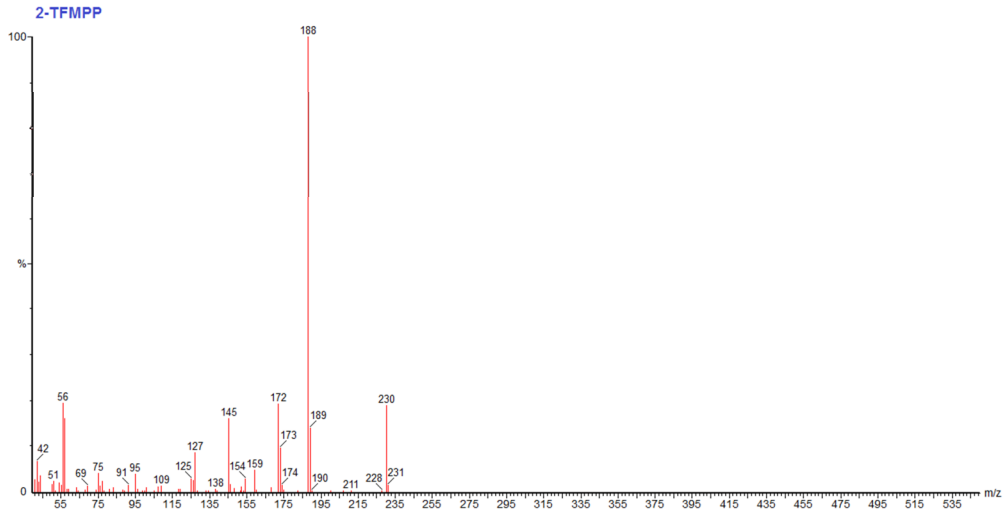


Figure 4 Mass spectrum of 2-TFMPP

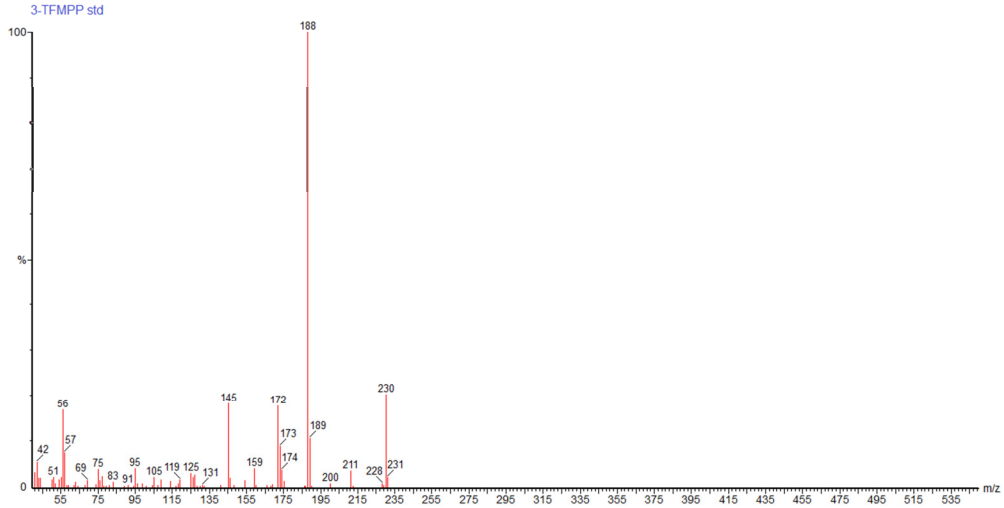


Figure 5 Mass spectrum of 3-TFMPP

Figure 6 Mass spectrum of 4-TFMPP

Figure 7 Mass spectrum of BZP

Figure 8 Mass spectrum of DBZP

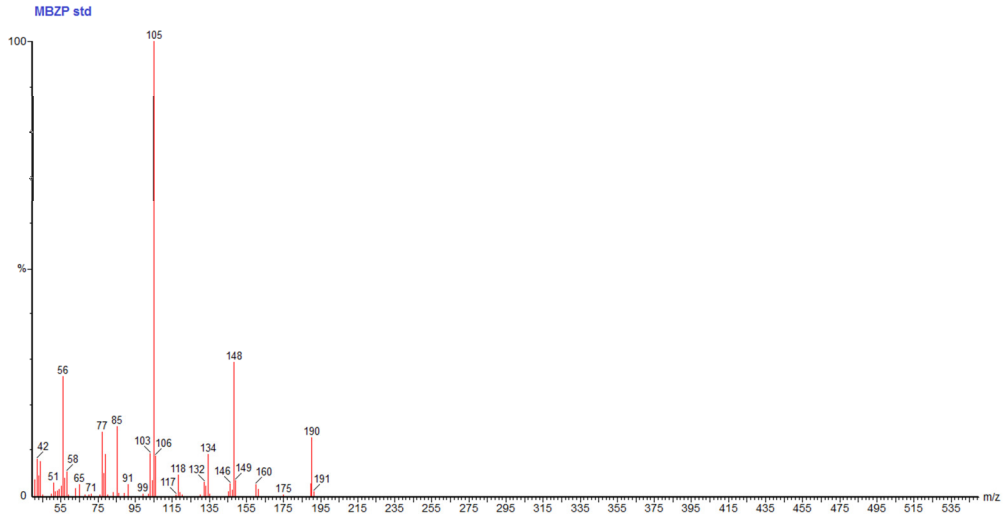


Figure 9 Mass spectrum of MBZP

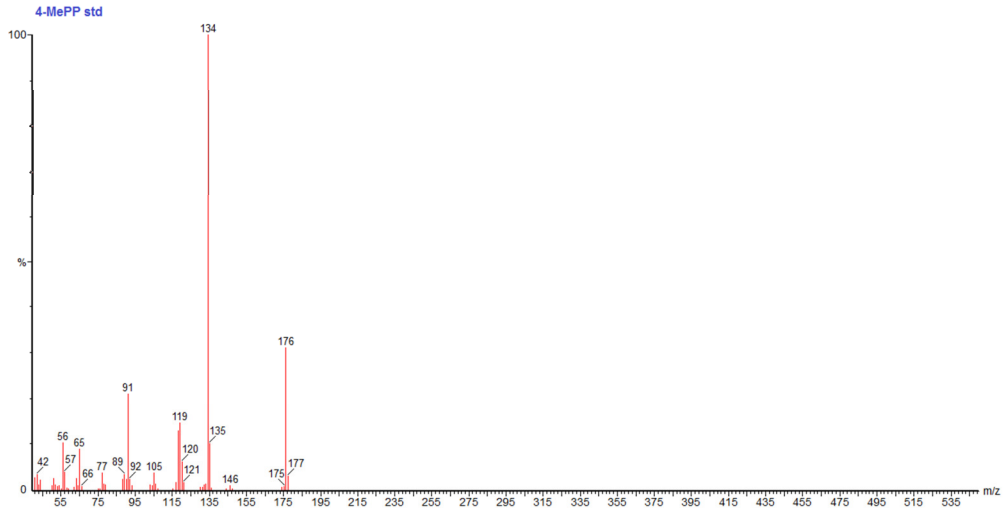


Figure 10 Mass spectrum of 4-MePP

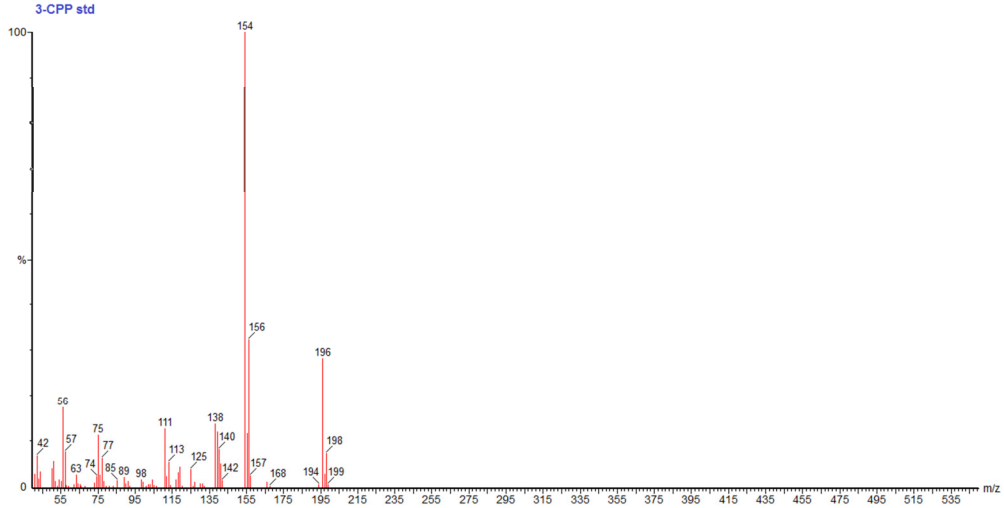


Figure 11 Mass spectrum of 3-CPP

Figure 12 Mass spectrum of MDMA

Figure 13 Mass spectrum of (+) Methamphetamine

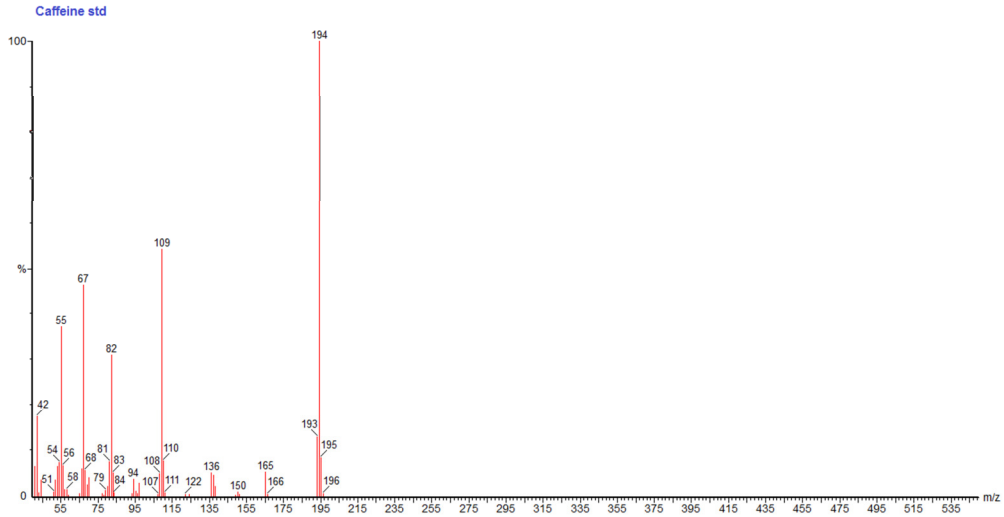


Figure 14 Mass spectrum of Caffeine

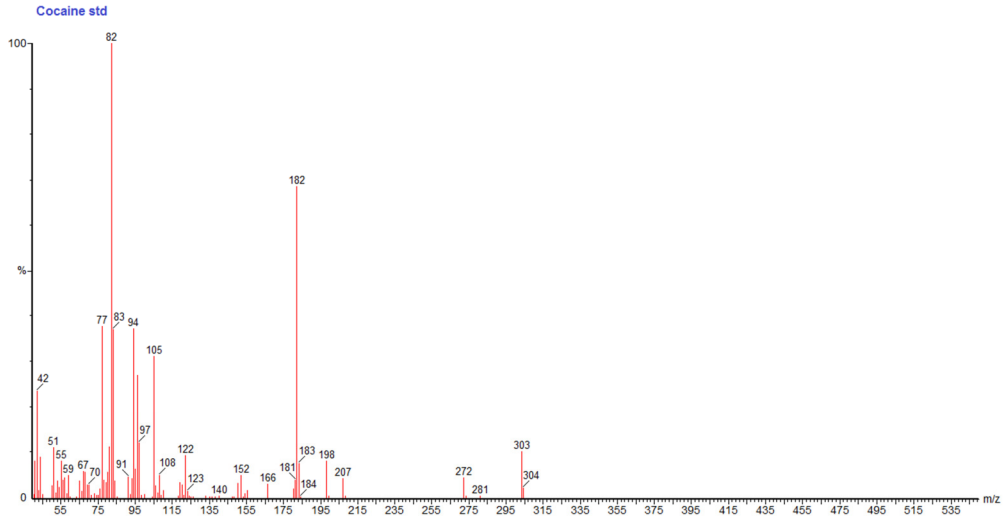


Figure 15 Mass spectrum of Cocaine

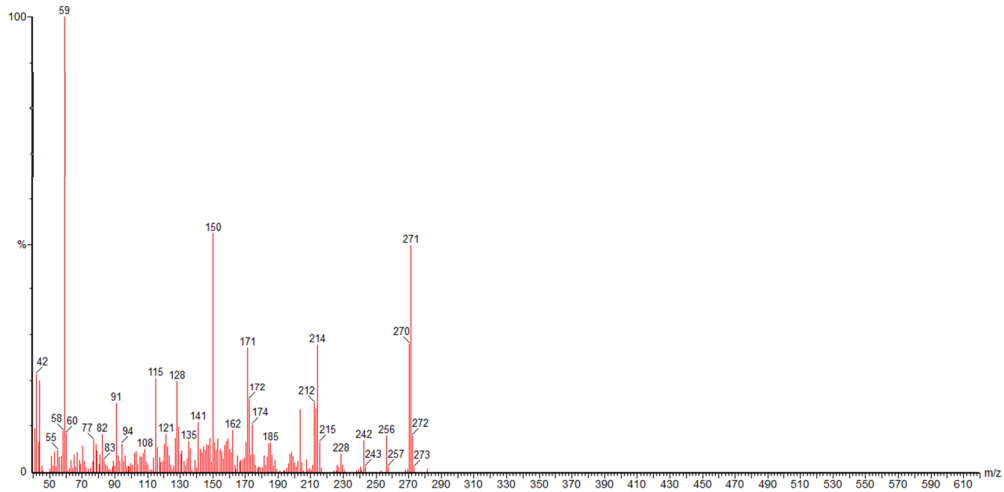


Figure 16 Mass spectrum of Dextromethorphan

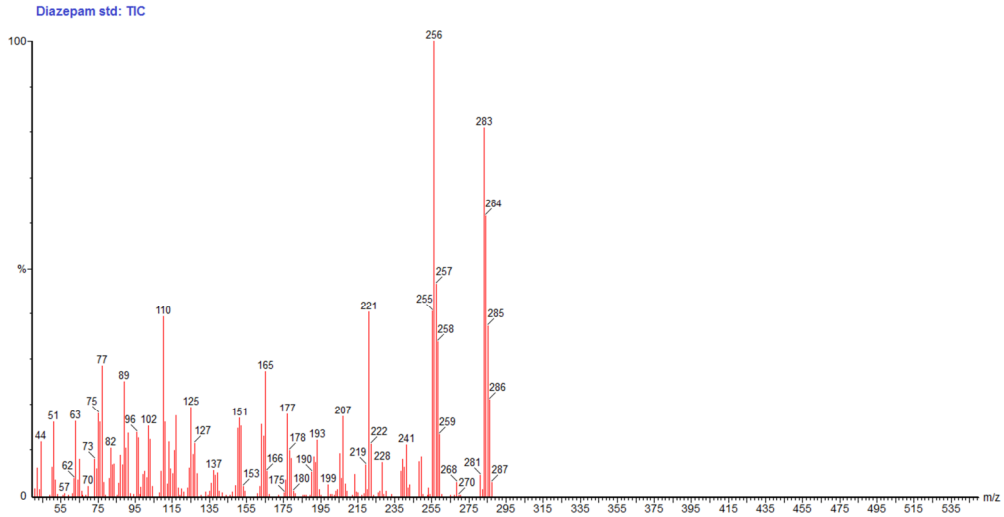


Figure 17 Mass spectrum of Diazepam

Figure 18 Mass spectrum of Dapoxetine

Figure 19 Mass spectrum of Nicotinamide

Figure 20 Mass spectrum of Ephedrine

APPENDIX 11 Test for Robustness using different GC-MS instruments

	Shimadzu			**Perkin Elmer**		
Compound	RT/mins $\bar{x} = \pm 0.005$	RRT	RI	RT/mins	RRT	RI
Methamphetamine	7.979	0.431	1173.86	8.84	0.465	1201
Nicotinamide	10.287	0.555	1337.53	11.88	0.626	1412
2-TFMPP	11.084	0.598	1394.07	12.12	0.638	1427
2-FPP	11.715	0.632	1438.81	12.88	0.678	1479
BZP	12.072	0.651	1464.16	13.24	0.698	1505
4-FPP	12.260	0.662	1477.5	13.56	0.714	1527
3-TFMPP	12.499	0.674	1494.47	13.78	0.726	1542
MDMA	12.643	0.682	1504.68	13.83	0.729	1545
4-TFMPP	13.315	0.718	1552.3	14.82	0.781	1614
MBZP	13.587	0.733	1571.57	14.98	0.789	1624
4-MePP	13.838	0.747	1589.4	15.23	0.802	1642
3-CPP	15.59	0.841	1713.63	16.85	0.888	1754
Caffeine	16.507	0.891	1778.71	17.9	0.943	1867
DBZP	19.66	1.061	2171.37	20.53	1.081	2212
Cocaine	19.983	1.078	2216.86	20.95	1.104	2268
Diazepam	21.478	1.159	2427.45	22.68	1.195	2494
Dapoxetine	21.89	1.181	2485.48	23.01	1.212	2179
Eicosane (IS)	18.532	1.000	2013	18.96	1.000	2006

RT is retention time, RRT is relative retention time and RI is relative retention index

APPENDIX 12 Mass spectra of the components in street samples (GC-MS/EI 70eV)

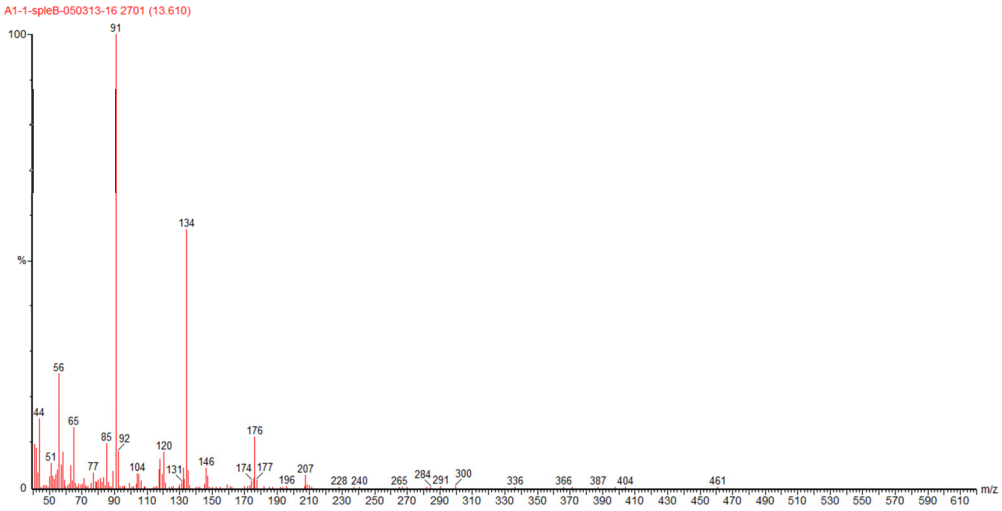


Figure 1 Mass spectrum of sample A1 peak at 13.61mins

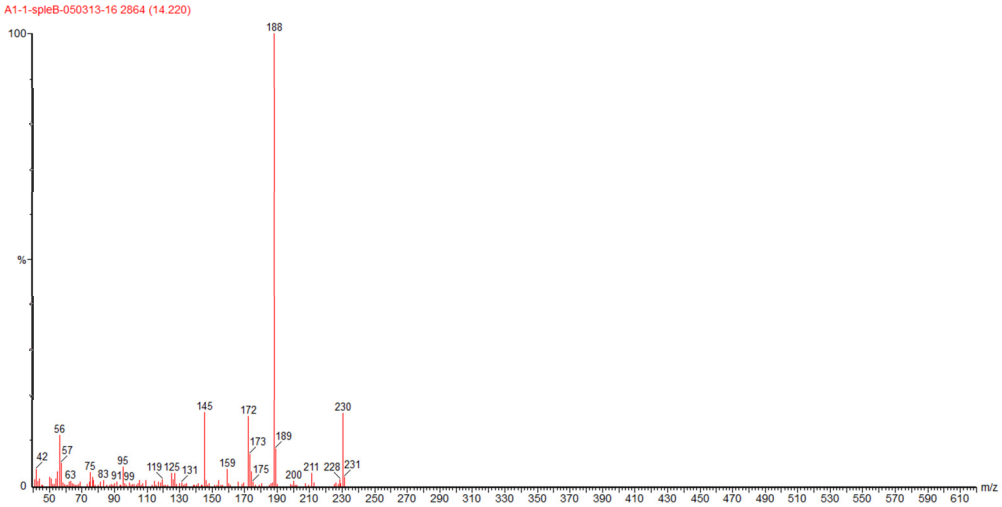


Figure 2 Mass spectrum of sample A1 peak at 14.22mins

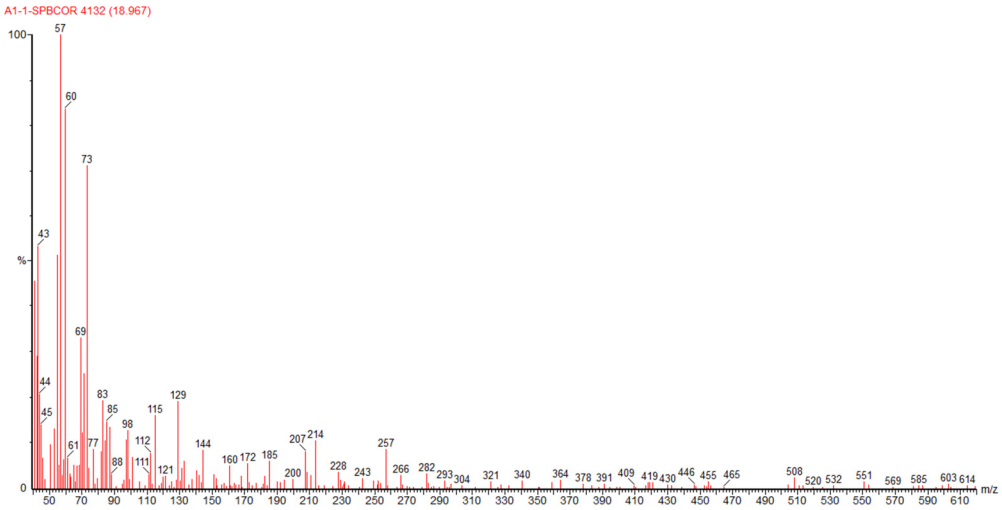


Figure 3 Mass spectrum of sample A1 peak at 18.97mins

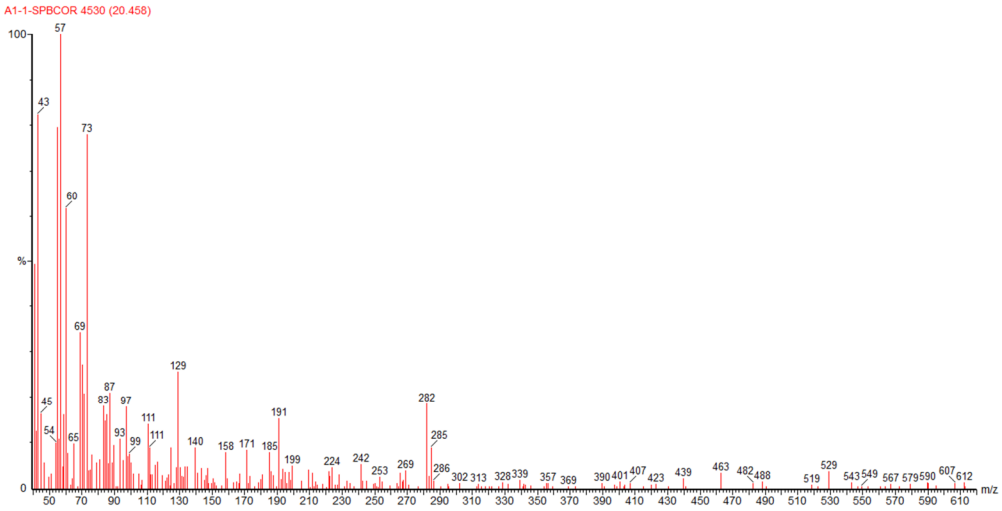


Figure 4 Mass spectrum of sample A1 peak at 20.46mins

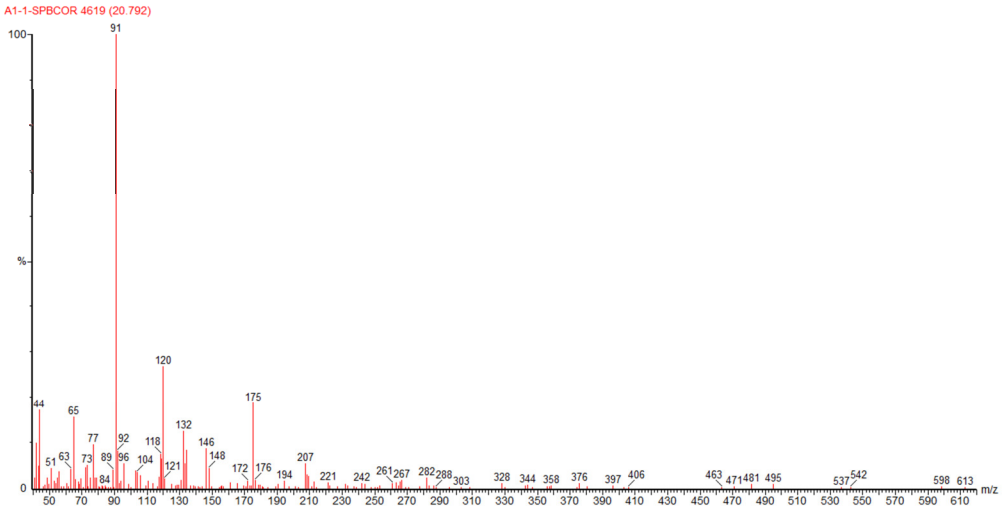


Figure 5 Mass spectrum of sample A1 peak at 20.79mins

Figure 6 Mass spectrum of sample A2 peak at 11.81mins

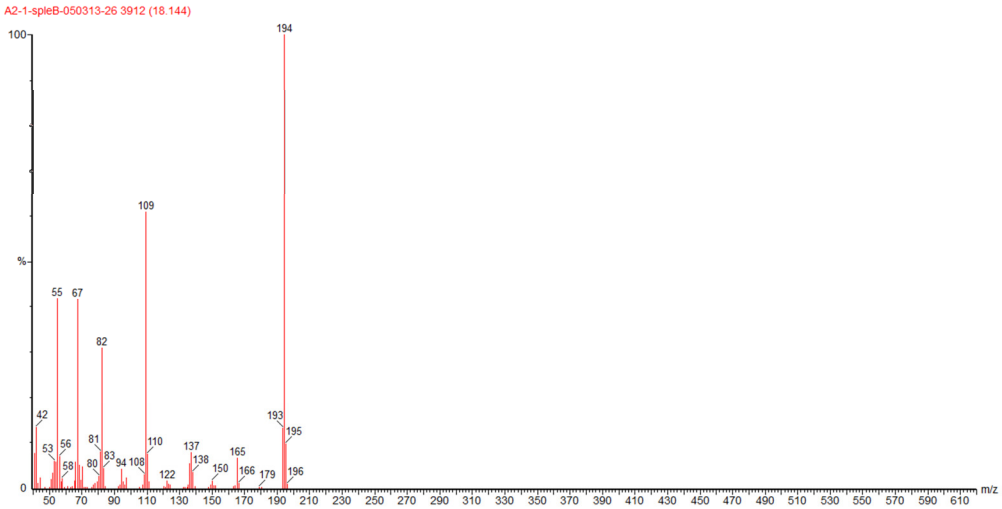


Figure 7 Mass spectrum of sample A2 peak at 18.14mins

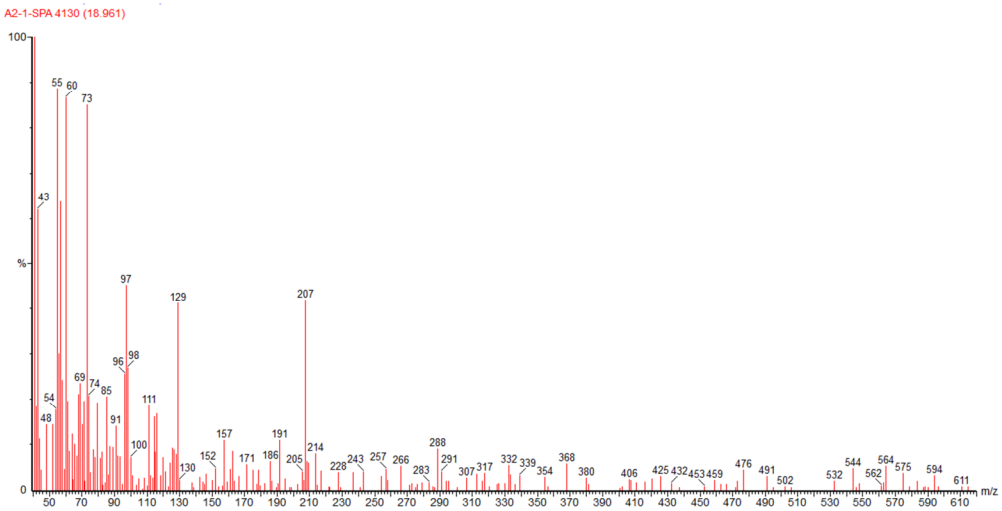


Figure 8 Mass spectrum of sample A2 peak at 18.96mins

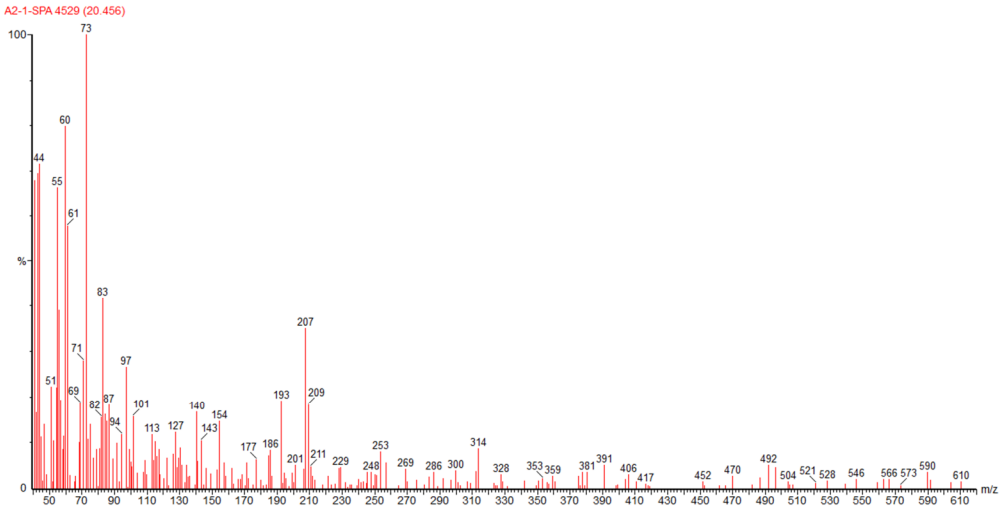


Figure 9 Mass spectrum of sample A2 peak at 20.46mins

Figure 10 Mass spectrum of sample A2 peak at 20.28mins (similar for A4, A7, A9, A11)

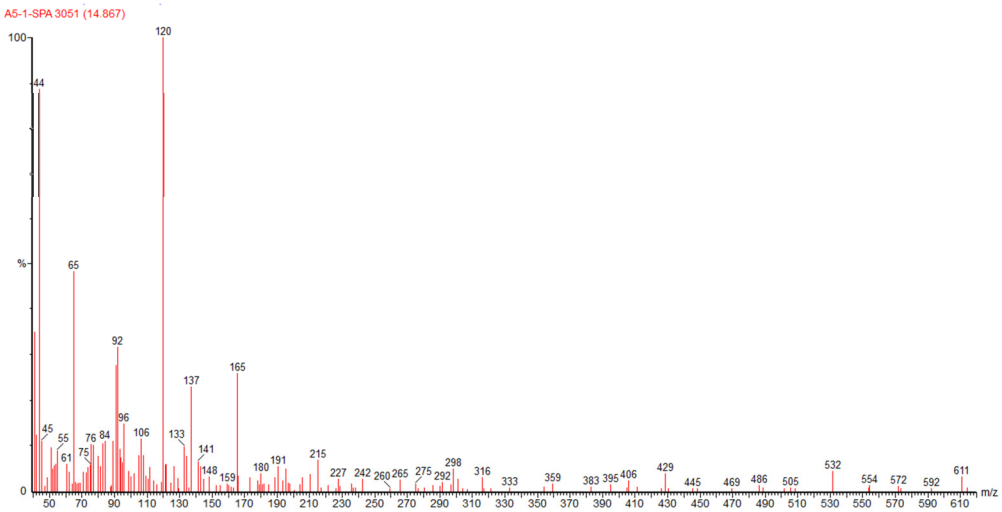


Figure 11 Mass spectrum of sample A5 peak at 14.87mins

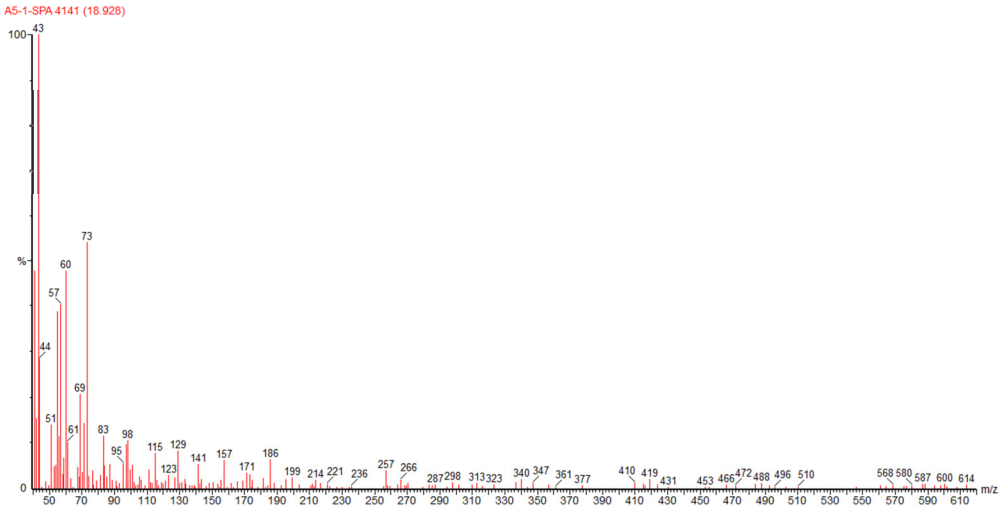


Figure 12 Mass spectrum of sample A5 peak at 18.93mins

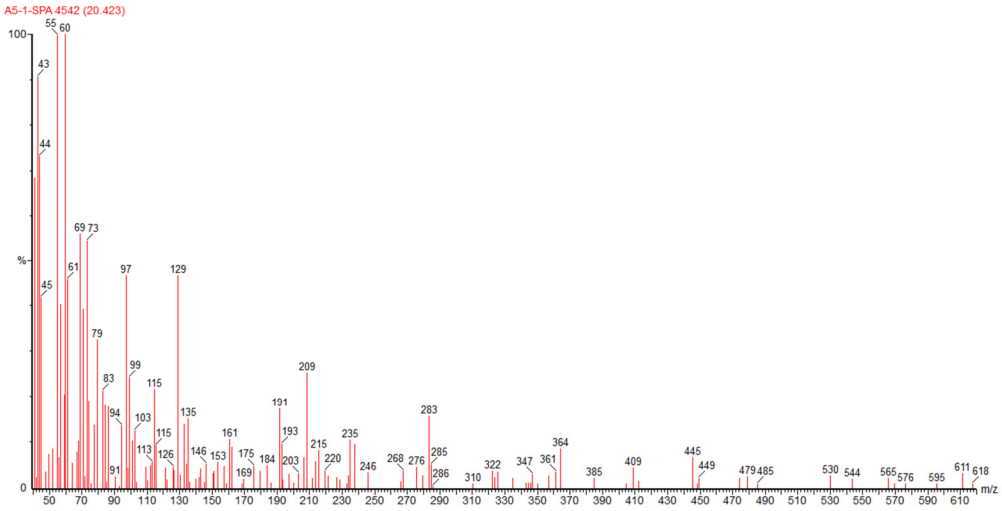


Figure 13 Mass spectrum of sample A5 peak at 20.42mins

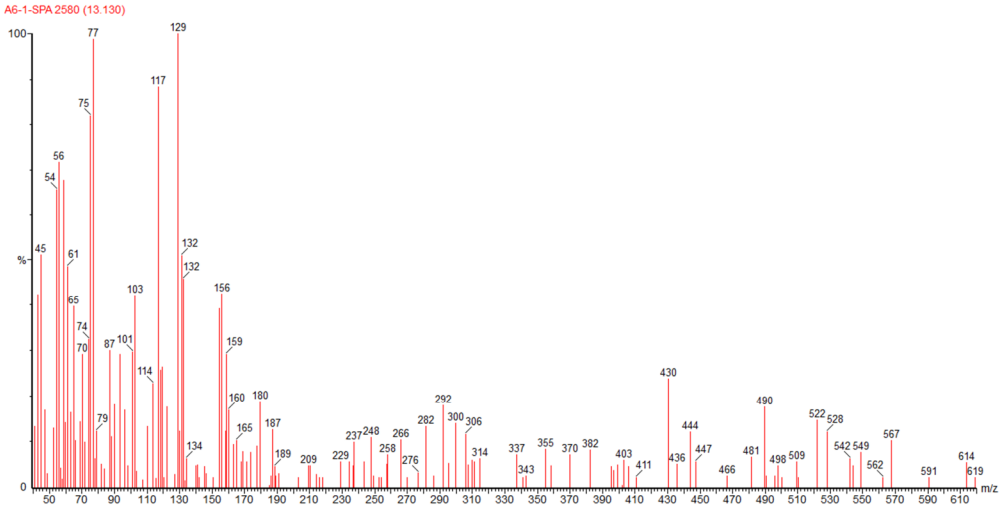


Figure 14 Mass spectrum of sample A6 peak at 13.10mins

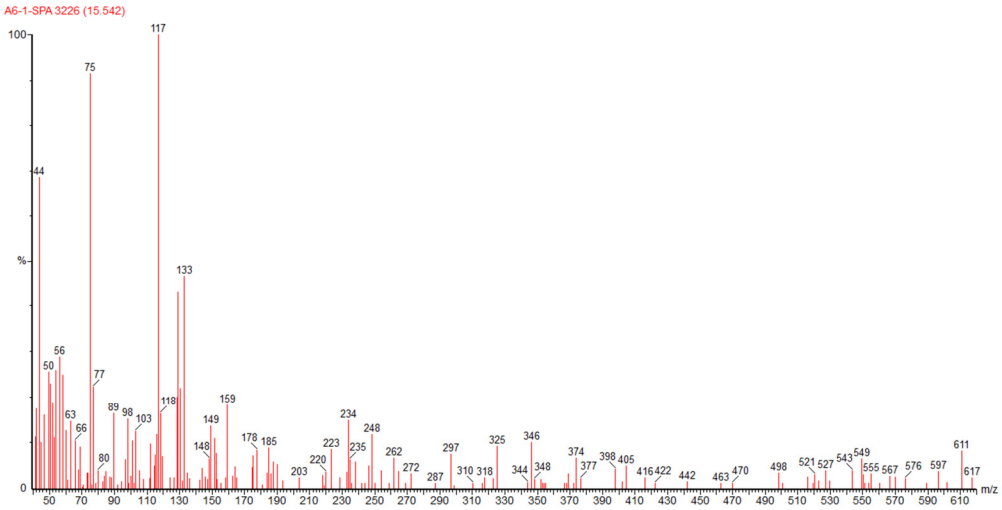


Figure 15 Mass spectrum of sample A6 peak at 15.54mins

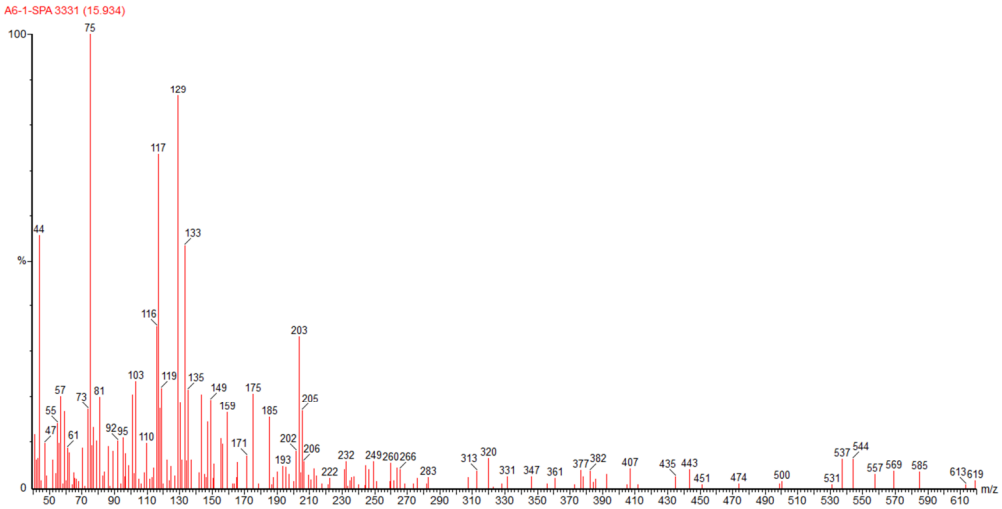


Figure 16 Mass spectrum of sample A6 peak at 15.93mins

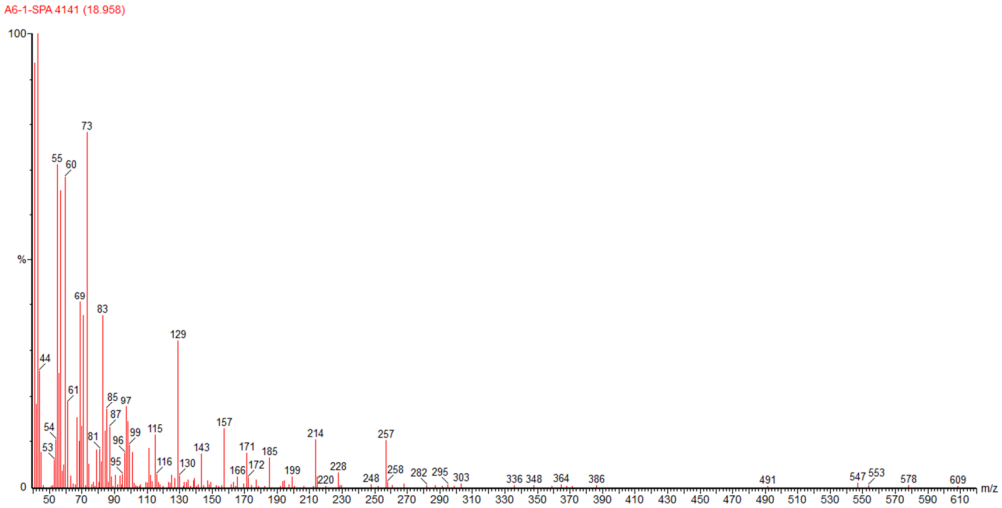


Figure 17 Mass spectrum of sample A6 peak at 18.96mins

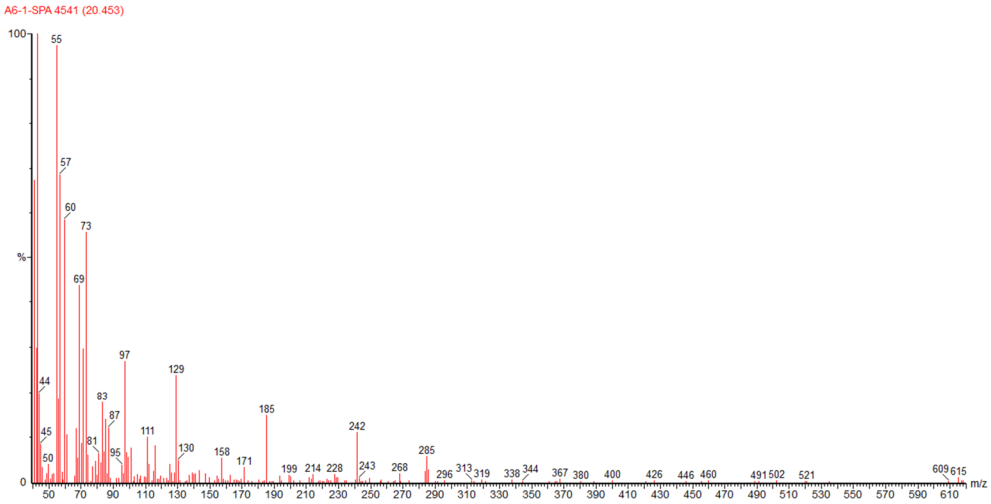


Figure 18 Mass spectrum of sample A6 peak at 20.45mins

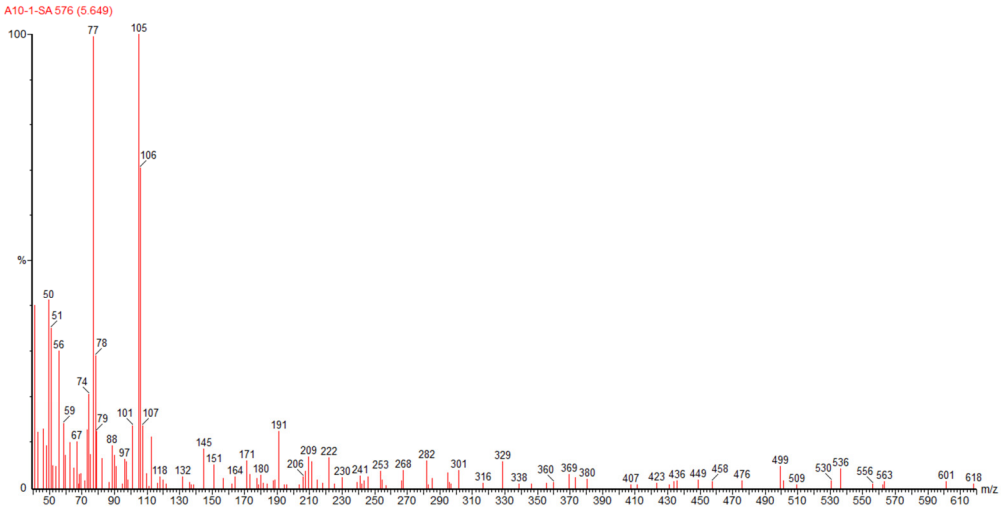
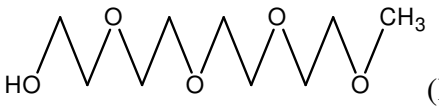


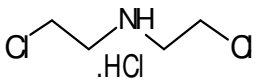
Figure 19 Mass spectrum of sample A10 peak at 5.65mins

APPENDIX 13 Qualitative data for analysis of precursors

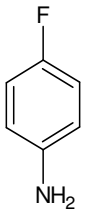
The precursors for the synthesis of 4-FPP were diethylene glycol monomethyl ether (DGME; I), 2-bischloroethylamine HCl (2-Bis; II) and 4-fluoroaniline (4Fa, III). For analysis of 3-TFMPP 3-trifluoromethyl aniline (3TFa, IV) was used instead of 4-fluoroaniline.



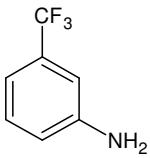
(I)



(II)



(III)



(IV)

Figure 1 Structures of synthesis precursors (Route 1)

Table 1 Qualitative data for analysis of precursors (*Quantifier ion highlighted in red)*

Substance	**Retention time/mins**	**Characteristic ions (m/z)**
DGME (Mwt = 208)	4.99	45(100),75, 90, 207(M^+)
3-Bis (Mwt = 141) (base)	6.78	92(100), 105, 119, 141(M^+)
4Fa (Mwt = 111)	6.03	84(100), 91, 111(100)(M^+)
3TFa (Mwt = 161)	6.74	114, 142, 161(100)(M^+)
Combinations		**Comments**
2Bis + DGME	4.99- DGME 6.77- 2bis 7.45	These precursors are common for both 4-FPP and 3-TFMPP
For 4-FPP		
2Bis + 4Fa	6.03- 4Fa 6.77- 2bis 7.45	No co-elution, impurity at 7.45mins
D + 4Fa	4.99- DGME 6.04- 4Fa 7.45	

2Bis + DGME + 4Fa	4.98- DGME 6.03- 4Fa 6.77- 2Bis	
For TFMPP		
2Bis + 3TFa	6.74- 3TFa 6.77- 2bis 7.45	Co-elution
D + 3TFa	4.99- DGME 6.74- 3TFa 7.45	
2Bis + DGME + 3TFa	4.98- DGME 6.74- 3TFa 6.77- 2Bis 7.45	Co-elution between 2Bis and 3TFa
Synthetic 4-FPP	14.28	138(100), 180(M^+), 122, 56
Synthetic mTFMPP	14.43	188(100), 230(M^+), 145, 56

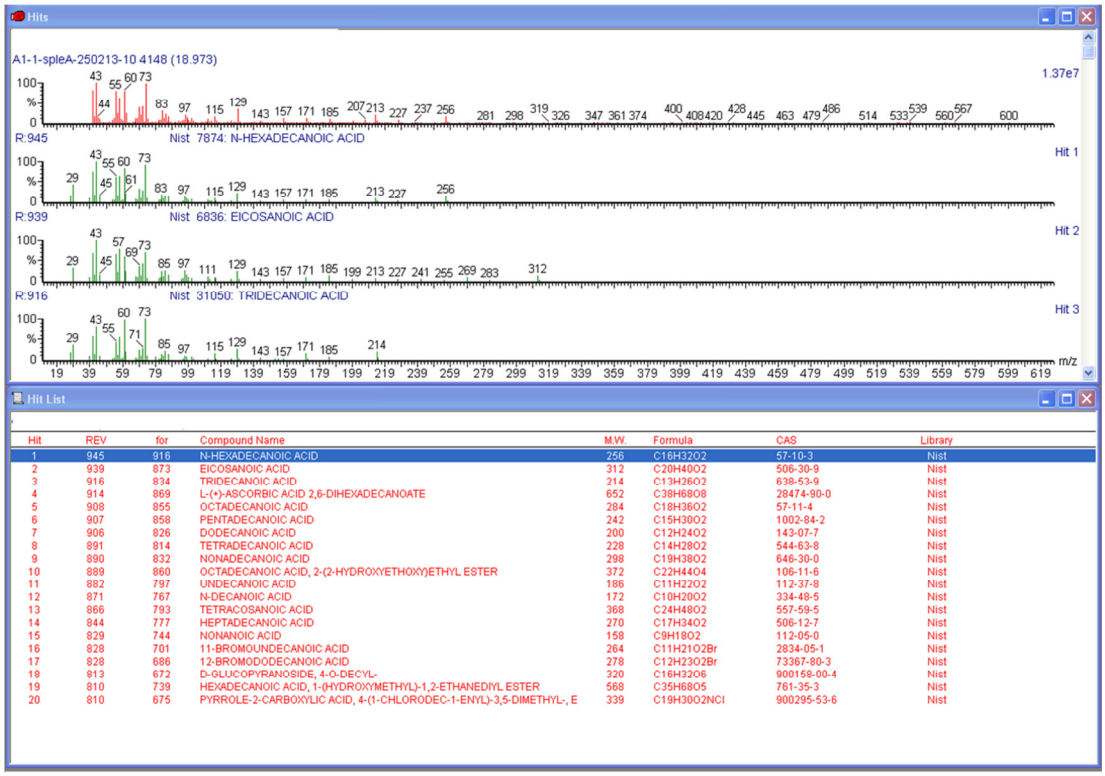
APPENDIX 14 Analysis of impurities in synthesised samples

Table1: Confirmation data

Compound	**FORMULA**	**Mwt**	**Characteristic ions m/z** *quantifier in red*	**Rt/mins**	**Rt of IS/min**	**RRT**	**RI**
Preliminary Identification of Impurities in synthetic FPP							
trans 1,4 di-tert butyl cyclohexane(nd)	$C_{14}H_{28}$	196	57(100), 94, 121, 278	7.15	18.64	0.384	1064.09
1-p(fluorophenyl)-4-piperidone	$C_{11}H_{12}ONF$	193	95, 123(100), 194	13.99	18.64	0.751	1520.09
1-m(fluorophenyl)piperazine (isomer)	$C_{10}H13N_2F$	180	95, 123, 138(100), 180(M^+)	14.30	18.64	0.767	1540.75
Preliminary Identification of Impurities in synthetic FPP							
Benzenamine, N-ethyl	$C_8H_{11}N$	121	77, 106(100),121(M^+)	6.37	18.58	0.343	1012.35
3-trifluoromethyl aniline (precursor)		161	114, 142, 161(100)(M^+)	6.74	19.58	0.344	1036.75
1.3.5-triazine,2-methyl-4.6-bis(2-4 morpoline)ethylamino (nd)	$C_{16}H_{29}O_3N_7$	367	100(100), 200, 207, 243	7.38	18.58	0.397	1079.42
Dodecane-1-fluoro	$C_{12}H_{2SF}$	188	57(100), 71, 88	7.45	18.58	0.401	1084.09
Piperazine, (1-3) trifluoro methyl (= isomer-2-TFMPP)	$C_{11}H_{13}F_3$	230	56, 145, 175, 188(100), 189	7.91	18.58	0.426	1114.75
2 or 3- trifluoromethyl benzoic acid-3 methyl butyl ester	$C_{13}H_{15}O_2F_3$	260	71, 145, 173, 244(100)	14.05	18.58	0.756	1524.09
eicosane (internal standard, IS)				18.64	18.64	1.000	2021.11

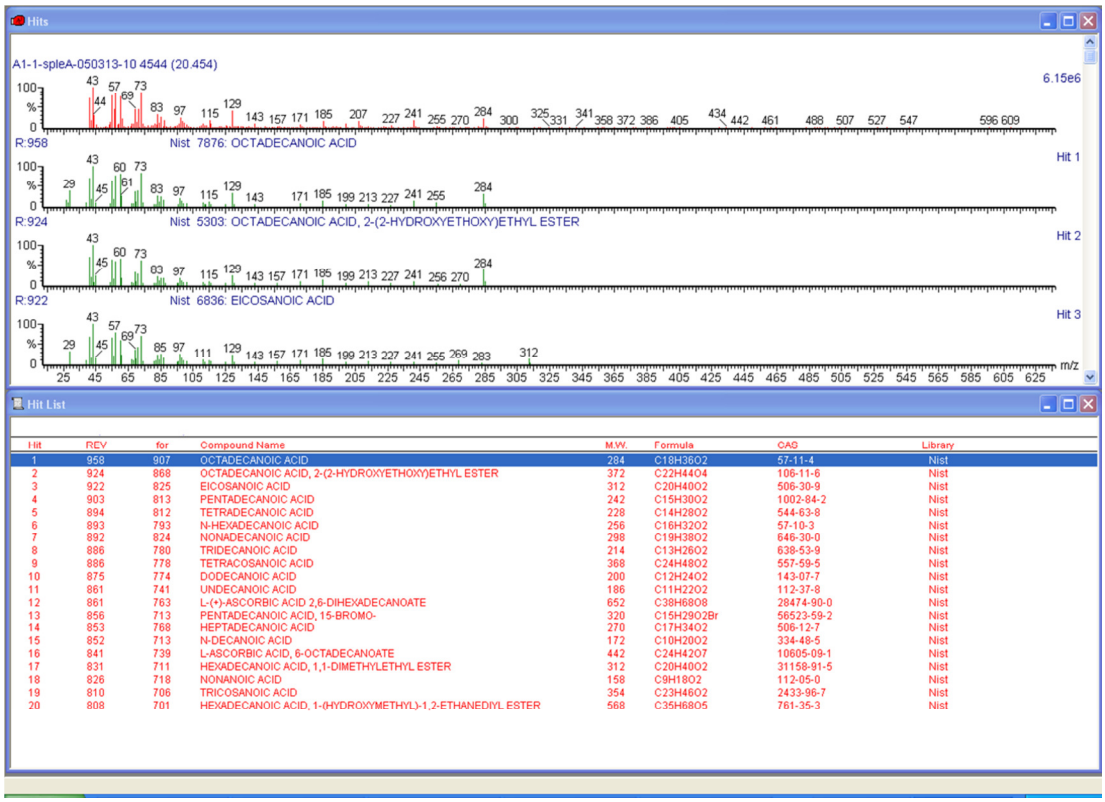
APPENDIX 15 Reference spectra for the identification of impurities in street samples

The spectra was generated from analysis of standards were these were not available literature spectra are given (NIST, 2014) identification. The mass spectrum of 3-Trifluoromethylaniline is shown in Appendix 16.



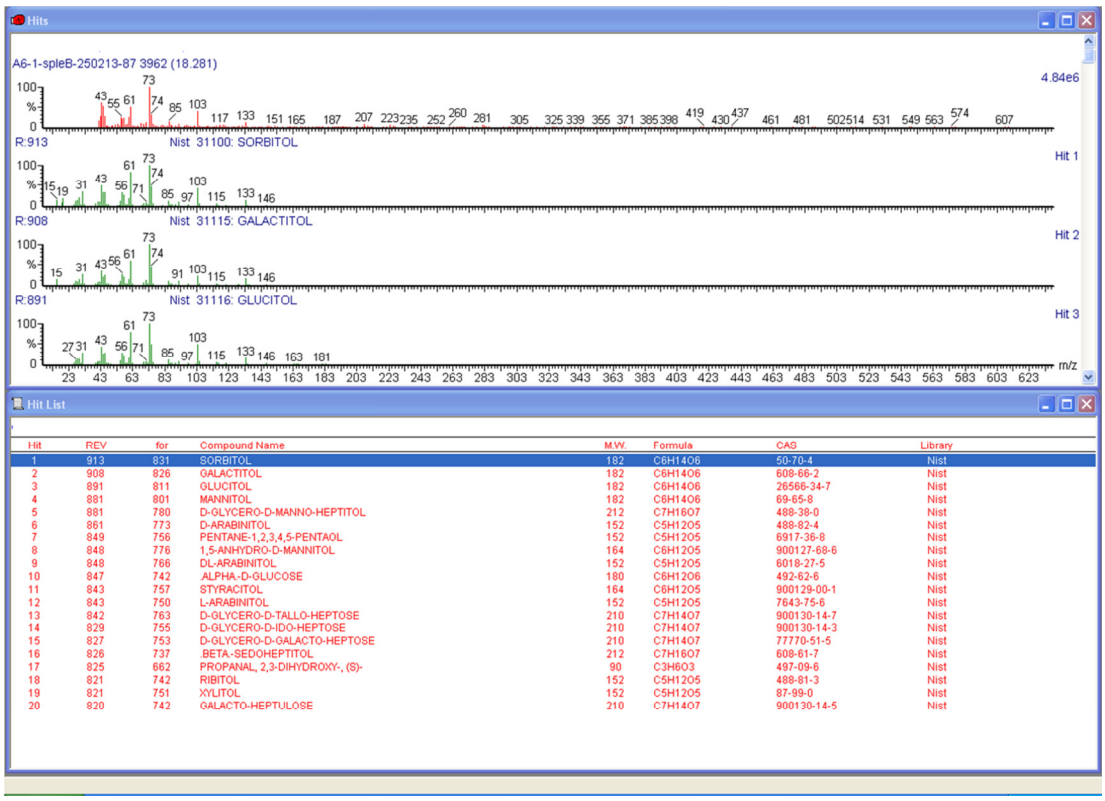
Hit	REV	for	Compound Name	M.W.	Formula	CAS	Library
1	945	916	N-HEXADECANOIC ACID	256	C16H32O2	57-10-3	Nist
2	939	873	EICOSANOIC ACID	312	C20H40O2	506-30-9	Nist
3	916	834	TRIDECANOIC ACID	214	C13H26O2	638-53-9	Nist
4	914	869	L-(+)-ASCORBIC ACID 2,6-DIHEXADECANOATE	652	C38H68O8	28474-90-0	Nist
5	908	855	OCTADECANOIC ACID	284	C18H36O2	57-11-4	Nist
6	907	858	PENTADECANOIC ACID	242	C15H30O2	1002-84-2	Nist
7	906	826	DODECANOIC ACID	200	C12H24O2	143-07-7	Nist
8	891	814	TETRADECANOIC ACID	228	C14H28O2	544-63-8	Nist
9	890	832	NONADECANOIC ACID	298	C19H38O2	646-30-0	Nist
10	889	860	OCTADECANOIC ACID, 2-(2-HYDROXYETHOXY)ETHYL ESTER	372	C22H44O4	106-11-6	Nist
11	882	797	UNDECANOIC ACID	186	C11H22O2	112-37-8	Nist
12	871	767	N-DECANOIC ACID	172	C10H20O2	334-48-5	Nist
13	866	793	TETRACOSANOIC ACID	368	C24H48O2	557-59-5	Nist
14	844	777	HEPTADECANOIC ACID	270	C17H34O2	506-12-7	Nist
15	829	744	NONANOIC ACID	158	C9H18O2	112-05-0	Nist
16	828	701	11-BROMOUNDECANOIC ACID	264	C11H21O2Br	2834-05-1	Nist
17	828	686	12-BROMODODECANOIC ACID	278	C12H23O2Br	73367-80-3	Nist
18	813	672	D-GLUCOPYRANOSIDE, 4-O-DECYL-	320	C16H32O6	900158-00-4	Nist
19	810	739	HEXADECANOIC ACID, 1-(HYDROXYMETHYL)-1,2-ETHANEDIYL ESTER	568	C35H68O5	761-35-3	Nist
20	810	675	PYRROLE-2-CARBOXYLIC ACID, 4-(1-CHLORODEC-1-ENYL)-3,5-DIMETHYL-, E	339	C19H30O2NCl	900295-53-6	Nist

Figure 1 Identification of impurity at 18.96 mins (Relative retention time 0.983) present in samples A1, A2, A5, A6.



Hit	REV	for	Compound Name	M.W.	Formula	CAS	Library
1	958	907	OCTADECANOIC ACID	284	C18H36O2	57-11-4	Nist
2	924	868	OCTADECANOIC ACID, 2-(2-HYDROXYETHOXY)ETHYL ESTER	372	C22H44O4	106-11-6	Nist
3	922	825	EICOSANOIC ACID	312	C20H40O2	506-30-9	Nist
4	903	813	PENTADECANOIC ACID	242	C15H30O2	1002-84-2	Nist
5	894	812	TETRADECANOIC ACID	228	C14H28O2	544-63-8	Nist
6	893	793	N-HEXADECANOIC ACID	256	C16H32O2	57-10-3	Nist
7	892	824	NONADECANOIC ACID	298	C19H38O2	646-30-0	Nist
8	886	780	TRIDECANOIC ACID	214	C13H26O2	638-53-9	Nist
9	886	778	TETRACOSANOIC ACID	368	C24H48O2	557-59-5	Nist
10	875	774	DODECANOIC ACID	200	C12H24O2	143-07-7	Nist
11	861	741	UNDECANOIC ACID	186	C11H22O2	112-37-8	Nist
12	861	763	L-(+)-ASCORBIC ACID 2,6-DIHEXADECANOATE	652	C38H68O8	28474-90-0	Nist
13	856	713	PENTADECANOIC ACID, 15-BROMO-	320	C15H29O2Br	56523-59-2	Nist
14	853	768	HEPTADECANOIC ACID	270	C17H34O2	506-12-7	Nist
15	852	713	N-DECANOIC ACID	172	C10H20O2	334-48-5	Nist
16	841	739	L-ASCORBIC ACID, 6-OCTADECANOATE	442	C24H42O7	10605-09-1	Nist
17	831	711	HEXADECANOIC ACID, 1,1-DIMETHYLETHYL ESTER	312	C20H40O2	31158-91-5	Nist
18	826	718	NONANOIC ACID	158	C9H18O2	112-05-0	Nist
19	810	706	TRICOSANOIC ACID	354	C23H46O2	2433-96-7	Nist
20	808	701	HEXADECANOIC ACID, 1-(HYDROXYMETHYL)-1,2-ETHANEDIYL ESTER	568	C35H68O5	761-35-3	Nist

Figure 2 Identification of impurity at 20.46mins (Relative retention time 1.060) present in samples A1, A2, A5, A6



Hit	REV	for	Compound Name	M.W.	Formula	CAS	Library
1	913	831	SORBITOL	182	C6H14O6	50-70-4	Nist
2	908	826	GALACTITOL	182	C6H14O6	608-66-2	Nist
3	891	811	GLUCITOL	182	C6H14O6	26566-34-7	Nist
4	881	801	MANNITOL	182	C6H14O6	69-65-8	Nist
5	881	780	D-GLYCERO-D-MANNO-HEPTITOL	212	C7H16O7	488-38-0	Nist
6	861	773	D-ARABINITOL	152	C5H12O5	488-82-4	Nist
7	849	756	PENTANE-1,2,3,4,5-PENTAOL	152	C5H12O5	6917-36-8	Nist
8	848	776	1,5-ANHYDRO-D-MANNITOL	164	C6H12O5	900127-68-6	Nist
9	848	766	DL-ARABINITOL	152	C5H12O5	6018-27-5	Nist
10	847	742	.ALPHA.-D-GLUCOSE	180	C6H12O6	492-62-6	Nist
11	843	757	STYRACITOL	164	C6H12O5	900129-00-1	Nist
12	843	750	L-ARABINITOL	152	C5H12O5	7643-75-6	Nist
13	842	763	D-GLYCERO-D-TALLO-HEPTOSE	210	C7H14O7	900130-14-7	Nist
14	829	755	D-GLYCERO-D-IDO-HEPTOSE	210	C7H14O7	900130-14-3	Nist
15	827	753	D-GLYCERO-D-GALACTO-HEPTOSE	210	C7H14O7	77770-51-5	Nist
16	826	737	.BETA.-SEDOHEPTITOL	212	C7H16O7	608-61-7	Nist
17	825	662	PROPANAL, 2,3-DIHYDROXY-, (S)-	90	C3H6O3	497-09-6	Nist
18	821	742	RIBITOL	152	C5H12O5	488-81-3	Nist
19	821	751	XYLITOL	152	C5H12O5	87-99-0	Nist
20	820	742	GALACTO-HEPTULOSE	210	C7H14O7	900130-14-5	Nist

Figure 3: Identification of impurity at 18.28mins (Relative retention time 0.947) present in sample A6

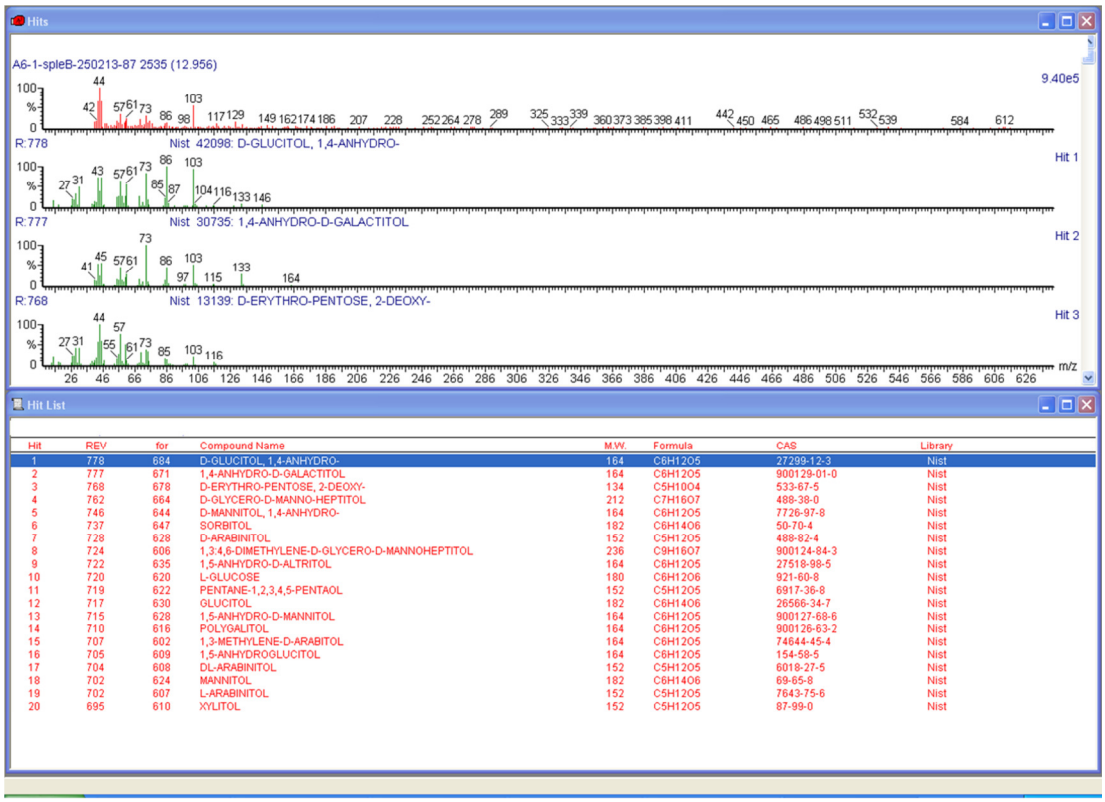


Figure 4 Identification of impurity at 12.96 mins (Relative retention time 0.672) present in sample A6

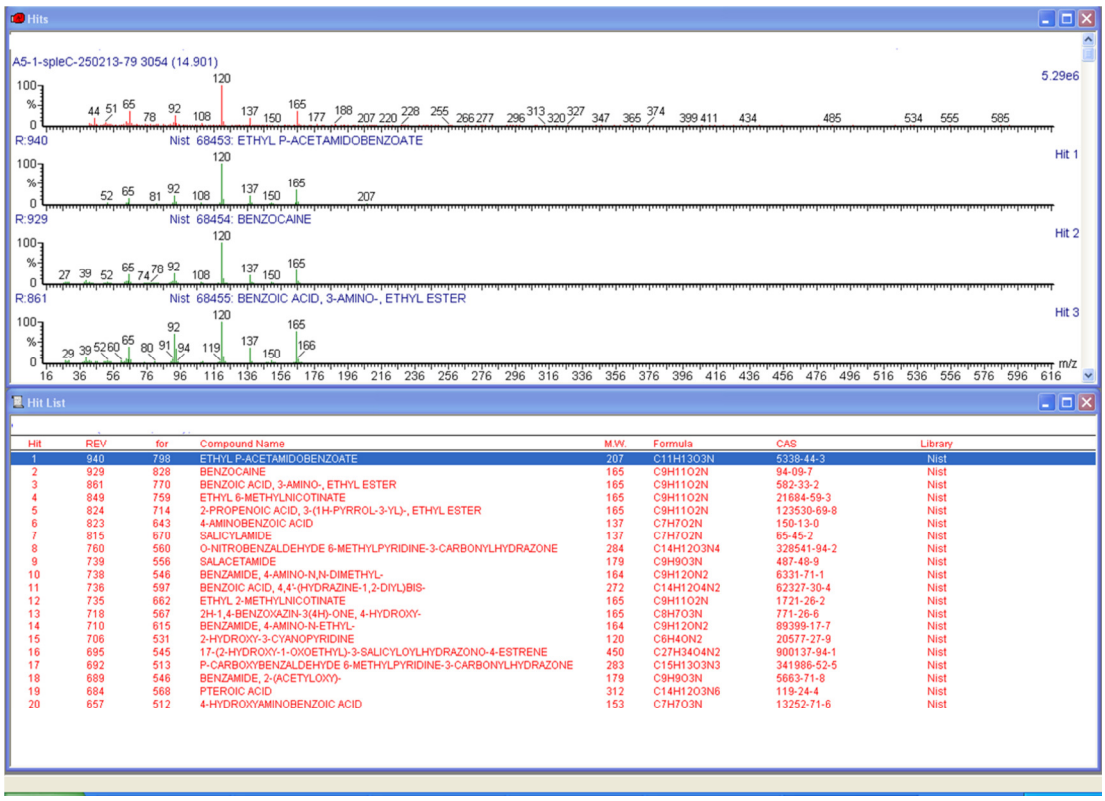


Figure 5 Identification of impurity at 14.90 mins (Relative retention time 0.772) present in sample A5

APPENDIX 16 Comparison of street samples and in-house synthesised samples impurity profile

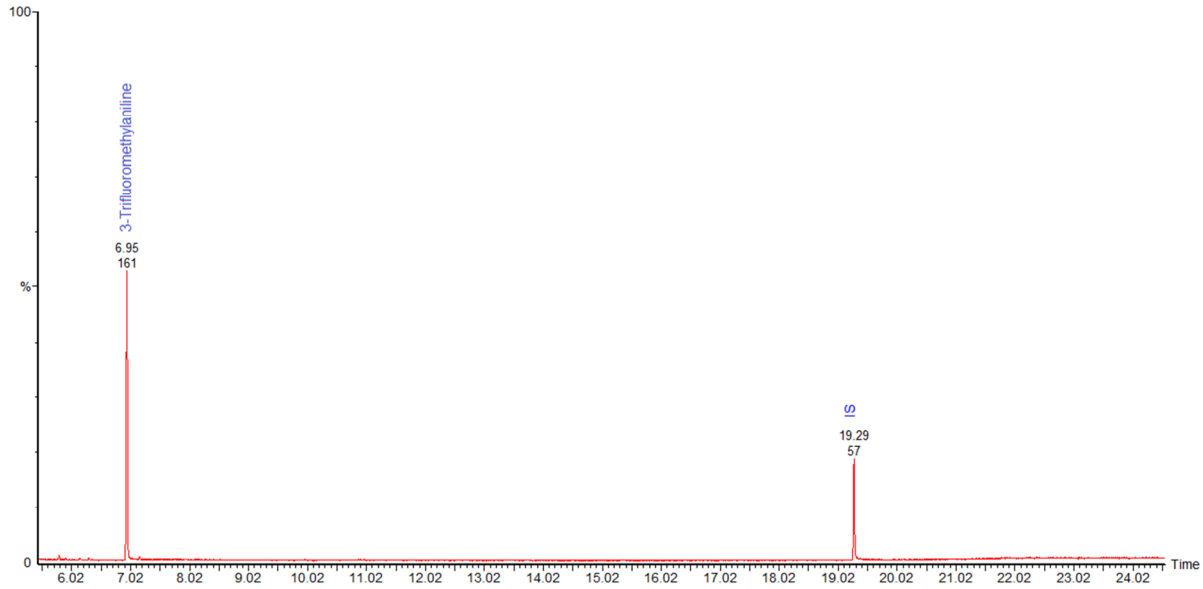


Figure 1 Total ion chromatogram of 3-Trifluoromethylanilie (a synthesis precursor)

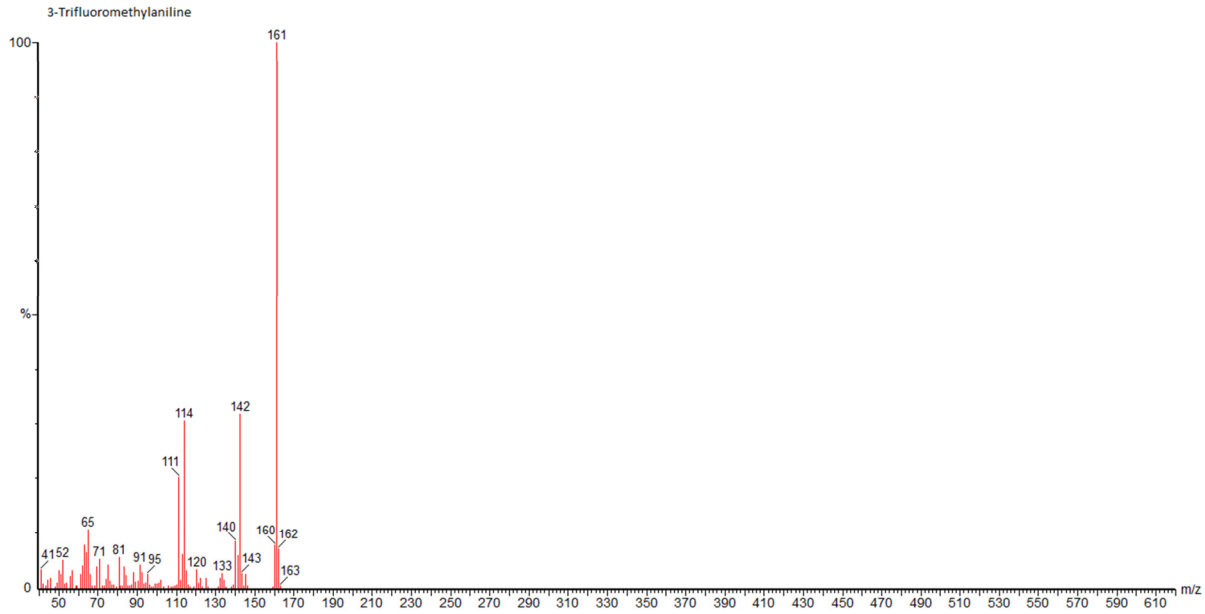


Figure 2 Mass spectra of 3-Trifluoromethylanilie

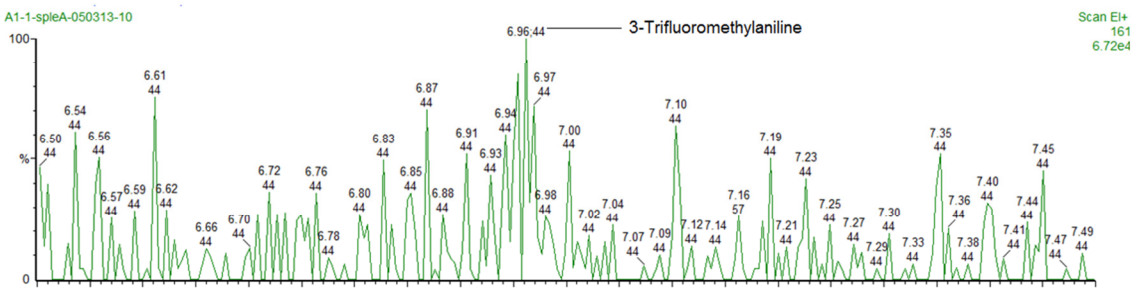


Figure 3 Total ion chromatogram of street sample A1 on trace analysis for impurities (3-trifluoromethylanilie)

A similar chromatogram was obtained with sample A2.

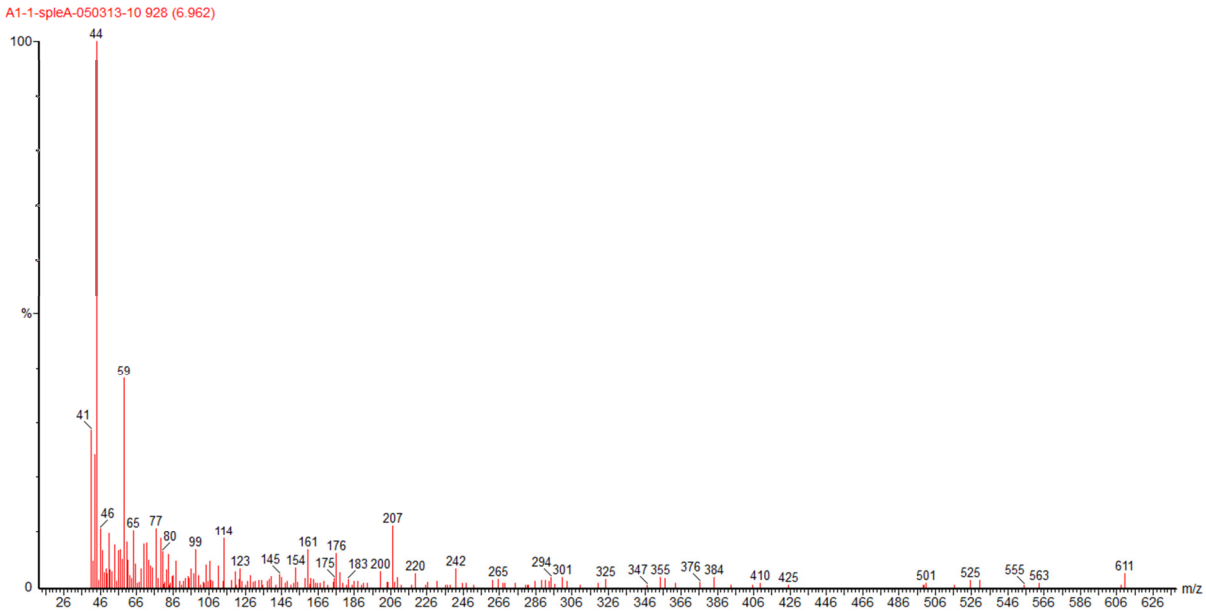


Figure 4 Mass spectra of street sample A1 showing traces of 3-Trifluoromethylanilie at 6.95 mins

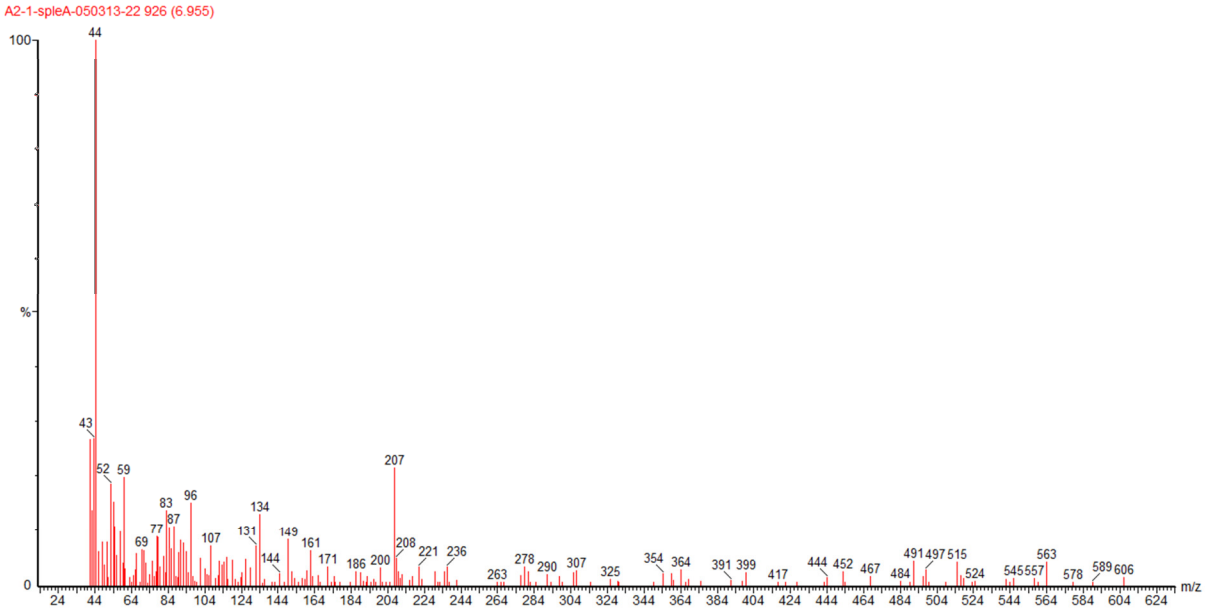


Figure 5 Mass spectra of street sample A2 showing traces of 3-Trifluoromethylanilie at 6.95 mins

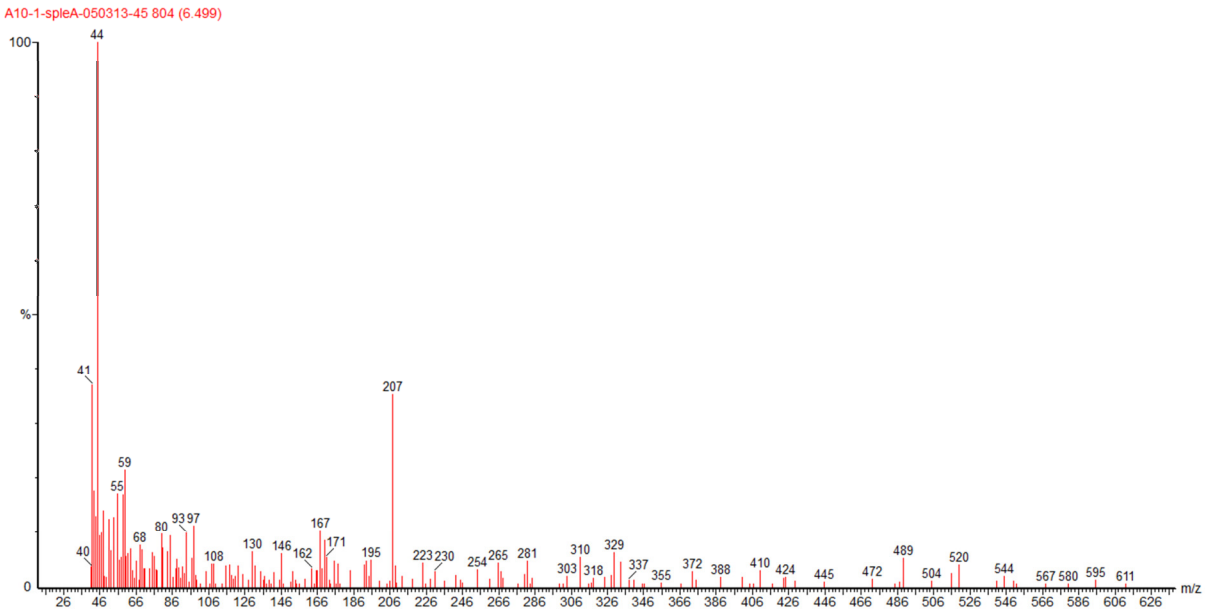


Figure 6 Mass spectra of street sample A10- checking for traces of 3-Trifluoromethylanilie at 6.95 mins.

PUBLICATIONS

Kuleya, C., Hall, S., Gautam, L. and Cole, M.D., 2014. An optimised gas chromatographic-mass spectrometric method for the chemical characterisation of benzylpiperazine and 1-arylpiperazine based drugs. *Analytical Methods,* 6 (1), pp.156-163.

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Poster presentation: The synthesis, analysis and characterisation of piperazine based drugs. FORREST (Forensic science research and Teaching) conference. Nottingham, UK. 29 – 30 June 2011.

Poster presentation: The synthesis, analysis and characterisation of piperazine based drugs. Anglia Ruskin University 5th Annual research student conference. Chelmsford, UK. 17 June 2011.

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