Synthesis, characterisation and development of novel, validated methods for the detection and quantification of diphenidine-derived New Psychoactive Substances

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Synthesis, characterisation and development of novel, validated methods for the detection and quantification of diphenidine-derived New Psychoactive Substances

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Abbreviations and Acronyms

AA	Ammonium acetate
AB-CHMINACA	(*N*-[1-Amino-3-methyl-oxobutan-2-yl]-1-[cyclohexylmethyl]-1*H*-indazole-3-carboxamide)
2-AI	2-Aminoindane
ATR-FTIR	Attenuated total reflection- Fourier Transform Infrared Spectroscopy
A_s	peak asymmetry
BrDP	Bromodiphenidine
BZP	Benzylpiperazine
CHMINACA	An indole-based synthetic cannabinoid
CLDP	Chlorodiphenidine
COSY	Correlation Spectroscopy
CYP	Cytochrome P450 enzymes
Dd	Doublet of doublets
Ddd	Doublet of doublet of doublets
DAD	Diode-Array Detector
DAMP	4-Dimethylaminopyridine
DEPT	Distortionless Enhancement by Polarization Transfer
DET	*N,N*-Diethyltryptamine
DIPH	Diphenidine
DMSO-d_6	Dimethyl sulfoxide-d_6
DP	Diphenidine
DPD	Diphenidine
DPH	Diphenidine

2-EAPB	2-(2-Ethylaminopropyl) benzofuran
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EU	European Union
EWS	Early Warning System
ε	Molar absorptivity
FDP	Fluorodiphenidine
FCEP	Fluorocyanoephenidine
FEP	Fluephenidine
FTIR	Fourier-transform infrared spectroscopy
GC	Gas Chromatography
GC-EI-MS	Gas chromatography electron ionisation mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
LC-HR-MS	Liquid chromatography–high resolution mass spectrometry
LC-MS(n)	Liquid chromatography coupled with multistage accurate mass spectrometry (LC–MS^n)
GDS	Global Drug Survey
H	Height equivalent per theoretical plate
halo DP	Halogenated diphenidine
HCG	Human Chorionic Gonadotropin
HCl	Hydrochloride
HIV	Human Immunodeficiency Virus
H(m)	Height equivalent to a theoretical plate
HMBC	Heteronuclear Multiple Bond Coherence

HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IDP	Iododiphenidine
ICH	International Conference on Harmonisation of Technical Requirements of Pharmaceutical for Human Use
i.d.	Internal diameter
IR	Infra-Red
IUPAC	International Union of Pure and Applied Chemistry
K	Capacity factor
LC	Liquid Chromatography
LCMS	Liquid Chromatography Mass Spectroscopy
LOD	Limit of Detection
LOQ	Limit of Quantification
LSD	Lysergic acid diethylamide
M	Molar
M	Metre(s)
MAA	Methacrylic acid
MALDI-Q-TOF/MS	Matrix-assisted laser desorption ionization (MALDI) quadrupole time-of-flight (Q-TOF)/MS
MANDRAKE	MANchester DRug Analysis & Knowledge Exchange
MBOMe	*N*-Methoxybenzyl
MDA	Misuse of Drugs Act
MDMA	3,4-Methylenedioxymethamphetamine
mM	Millimolar

MHz	Megahertz
Min	Minute(s)
MIP	Molecularly imprinted polymer
MDDP	Methylenedioxydiphenidine
MDMA	4-Methylenedioxymethamfetamine
$MgSO_4$	Magnesium sulfate
MMC	Mephedrone (or 4-methyl methcathinone)
MP	Mobile phase
MPA	Methiopropamine
Mpt	Melting point
MS	Mass Spectroscopy
MXE	Methoxetamine
MXP	Methoxphenidine
m/z	Mass-to-charge ratio
N	Column efficiency
NMR	Nuclear magnetic resonance
NMDAR	*N*-methyl-D-aspartate receptor
NPS	New psychoactive substances
PCP	Phencyclidine
pH	Logarithm of the reciprocal of the hydrogen ion concentration
PMMA	*p*-Methoxymethamphetamine
Ppm	Parts per million
PSA	Psychoactive Substances Act
r^2	R-squared (regression)

R_f	Retention factor
RP	Reverse (or reversed) phase
RPLC	Reverse (or reversed) phase liquid chromatography
RR_f	Relative Retention factor
RRT	Relative retention time
R_s	Resolution
RSD	Relative standard deviation
t_R	Retention time
SS	Street sample
t_0	HPLC Column Dead Time
TEAP	Triethylammonium phosphate
TFA	Trifluoroacetic acid
TFMXP	Trifluoromethoxphenidine
THC	Tetrahydrocannabinol
TLC	Thin-Layer Chromatography
UNAIDS	United Nations Programme on HIV and AIDS
UNODC	United Nations Office on Drugs and Crime
UPLC-ESI-MS	Ultra-performance liquid chromatography-electrospray tandem mass spectrometry
UHPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS
UV	Ultra-violet
UV-DAD	Ultra-violet/Diode Array Detection
UV-vis	Ultra-violet-visible
v/v	Volume by volume

WHO	World Health Organisation
w/v	Weight by volume

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Abstract

There is a worldwide increase in the abuse of new psychoactive substances, which pose a threat to public health. The fast-paced nature of the NPS market and increased availability is drawing international concern. There is a general lack of comprehensive evidence on the toxicity and abuse risks associated with long-term use. The rapid pace of NPSs development means that they remain as an area of concern and interest; this shows the technical challenges in terms of development of analytical methods for the detection and determination of these substances.

There is a knowledge gap in terms of chromatographic methods of detection, separation and quantification of diphenidine and its derivatives, in particular HPLC approaches. Currently, there is more research applying GC methods for NPSs analysis. The aim of this project is to develop a reliable, rapid, sensitive and robust HPLC method for the analysis of this group of NPSs.

Regioisomeric compounds, 2-, 3- and 4-methoxphenidine (MXP) were used to develop a robust high performance liquid chromatography (HPLC) method using mobile phase (acetonitrile:ammonium acetate 55:45% v/v) whilst the stationary phase was ACE-5 C18 AR column (150 x 4.6 mm, 5 µm particle size). The method was validated according to the International Conference on Harmonisation of Technical Requirements of Pharmaceutical for Human Use (ICH) guidelines and shown to be both selective and sensitive (Limit of Detection, LOD = 0.04-0.15 µg mL^{-1}, Limit of Quantification, LOQ = 0.38-0.47 µg mL^{-1}). The reference materials used for this study were characterised using ^{1}H NMR, ^{13}C NMR, IR, UV and GC-MS.

The scope of the study was applied to the recently reported diphenidine derivatives, 2-, 3- and 4-fluephenidines, in addition to 2-, 3- and 4-fluorocyanophenidines and the halogenated diphenidine compounds.

Accuracy, precision, robustness and specificity of these substances were investigated.

As an average, limit of detection (LOD) was between 0.05 and 0.60 μg mL^{-1}, whereas, limit of quantification (LOQ) was between 0.16-1.84 μg mL^{-1} for all the diphenidine derived regioisomers tested in this study. Additionally, total run time of just 10 minutes with resolution values (R_s) of greater than 2 in the case of both MXP isomers and all tested halogenated diphenidine isomers indicates that the applied HPLC method was rapid and sensitive, therefore it can be implemented to examine any samples that might contain these substances.

Chapter 1: Introduction and literature review

There are many reasons for substance use including positive impact on performance and experience, such as pleasure, fun, improved concentration and attention, enhanced work performance, relaxation and sleep.[1] Drug abuse and addiction have negative consequences on human health and on society. Drug addiction can be prevented effectively via programmes that include families, schools, communities, and the media. Thus, education and outreach are key in helping youth and the general public understand the risks of drug abuse in general and the new emerging classes of new psychoactive substances (NPS) in particular. Education is one of the most important preventive interventions to counter destructive NPS marketing approaches. It is essential to inform people that there is not anything “smart” concerning these drugs. Because these are the product of profit-motivated individuals with the technical knowledge to produce, market and distribute them exploiting the gaps and cracks of the legal systems.

In order to control substances of concern (including NPS), reducing the supply and reducing the demand are two basic strategies/policies, besides, the burden falls on national and international legal for controlling these drugs, in terms of adding, transferring of the substance from one schedule to another, or removal from any of the schedules of the corresponding Convention. Several countries have early warning systems that aim to offer timely information so that policymakers will be able to make evidence-based judgements. Therefore, these drugs need to be regulated to supress unlawful drug abuse. [2]

1.1 Laws controlling substances of concern in the UK

The biggest challenge of substances with the potential for misuse or abuse is having in place a legislative response that can respond to emerging new substances in a timely and effective way. The UK Government’s existing

approach is defined within the Misuse of Drugs Act (1971), although it is essential to review the current legal framework available to authorities to govern the possession, production and supply of controlled substances. This section provides an overview about the development of the legal framework for the control of harmful substances in the United Kingdom. The history of drug related legislation in the United Kingdom began with the Pharmacy Act (1868); this Act is the first regulation of poisons and dangerous substances. Five decades later, the Dangerous Drugs Act (1920) came into force, to limit the production, import, export, possession, sale and distribution of opium, cocaine, morphine or heroin to licensed persons. Five years later, The Dangerous Drugs Act (1925) was amended, which regulated the controlled importation of coca leaf and cannabis, and after three years this Act was amended by criminalising possession of cannabis. However, doctors still have the ability to prescribe any drugs as treatments (including for addiction). Later in 1961, the United Nations 1961 Single Convention on Narcotic Drugs was passed as a law to combat drug abuse by coordinated international action, it banned countries from treating addicts by prescribing illicit drugs, permitting only scientific and medical uses of drugs. This act was not itself obligatory on countries, which had to pass their specific laws. Three years later, The Dangerous Drugs Act (1964) was amended, this Act criminalised cultivation of cannabis.[3].[4]

In the same year (1964), the Drugs Prevention of Misuse Act criminalized possession of amphetamines. In 1967, in an amendment to the Dangerous Drugs Act, doctors were then required to inform the Home Office of addicted patients and also included restriction on the prescription of heroin and cocaine for addiction treatment. In 1971, The Misuse of Drugs Act was announced.[5]

The laws controlling drug use are complicated but there are three main statutes regulating the availability of drugs in the UK:

(i) The Medicines Act (1968), this law governs the manufacture and supply of medicine. It divides medical drugs into three categories: prescription only medicines, pharmacy medicines and general sales list medicines.[6]

(ii) The Misuse of Drugs Act (1971) and the Misuse of Drugs Regulations (2001) outlines the law around drugs that have been deemed to be harmful and are therefore 'controlled' by law.[5]

(iii) The Psychoactive Substances Act (2016) is intended to restrict the production, sale and supply of a new class of psychoactive substances often referred to as "legal highs".[7]

1.1.1 The Misuse of Drugs Act 1971 (MDA 1971)

The Misuse of Drugs Act (MDA) was passed in the UK parliament in 1971.[5] It represents action in line with treaty commitments under the Single Convention on Narcotic Drugs (1961) [4] and the Convention on Psychotropic Substances (1971).[8] The main purpose of the MDA 1971 is to prevent the misuse of controlled drugs and achieves this by imposing a complete ban on the possession, supply, manufacture, import and export of controlled drugs except as allowed by regulations or by licence from the Secretary of State.[5] The Misuse of Drugs (Safe Custody) Regulations 1973 supports the MDA and prescribes the steps individuals (or organisations) need to undertake to facilitate the storage and safe custody for Controlled Drugs.

The MDA 1971 places drugs into A, B, or C classification - according to how harmful they are considered to be. Higher classification is associated with stricter penalties for possession, supply and importation. The MDA 1971, as amended, prohibits certain activities in relation to 'Controlled Drugs', in particular their manufacture, supply, and possession (except where permitted by the 2001 Regulations or under licence from the Secretary of State). The penalties applicable to offences involving the different drugs are graded

broadly according to the harmfulness attributable to a drug when it is misused and for this purpose the drugs are defined in the following three classes:

Class A includes: alfentanil, cocaine, diamorphine hydrochloride (heroin), dipipanone hydrochloride, fentanyl, lysergide (LSD), methadone hydrochloride, 3,4-methylenedioxymethamfetamine (MDMA, 'ecstasy'), morphine, opium, oxycodone hydrochloride, pethidine hydrochloride, phencyclidine, remifentanil, and class B substances when prepared for injection.

Class B includes: oral amphetamines, barbiturates, cannabis, *Sativex*® (nabiximols), codeine phosphate, dihydrocodeine tartrate, ethylmorphine, glutethimide, ketamine, nabilone, pentazocine, phenmetrazine, and pholcodine.

Class C includes: certain drugs related to the amphetamines such as benzphetamine and chlorphentermine, buprenorphine, mazindol, meprobamate, pemoline, pipradrol, most benzodiazepines, tramadol hydrochloride, zaleplon, zolpidem tartrate, zopiclone, androgenic and anabolic steroids, clenbuterol, chorionic gonadotrophin (HCG), non-human chorionic gonadotrophin, somatotropin, somatrem, and somatropin.

1.1.2 Misuse of Drugs Regulations 1985

These schedules are similar to those in MDA 1971 with some modifications as shown at the end of this section. The 1973 regulations were revoked after the Misuse of Drugs Regulations 1985 were presented, the latter enabling certain classes of person to possess, produce, supply, prescribe or administer controlled drugs. For the purposes of these regulations, drugs are divided into Schedules as follows:

Schedule 1 Prohibited drugs except with a Home Office licence, no medical uses, e.g. cannabis, LSD, MDMA.

Schedule 2 Only available as prescription medicines with some restrictions (full controlled drug requirements in relation to prescribing and safe custody, keeping of registers), most opiates and cocaine.

Schedule 3 Only available on prescription e.g. most barbiturates, temazepam, flunitrazepam. It is an offence to possess the drug unless the individual can prove that it was lawfully supplied.

Schedule 4 Benzodiazepines (except temazepam and flunitrazepam), anabolic steroids. Can be lawfully possessed without a prescription.

Schedule 5 Available without prescription, preparations containing a small amount of controlled drugs.[9]

The Misuse of Drugs Regulations 1985 restructure the provisions of the Misuse of Drugs Regulations 1973, which were overridden. They offer certain exemptions from the provisions of the MDA 1971 that prohibit the production, importation, exportation, possession and supply of controlled drugs. The Regulations also create provision with regard to prescriptions, records and the furnishing of information relating to controlled drugs and for the supervision of the destruction of such drugs. The changes of substance made by the Regulations can be summarised below:

(i) the addition of those substances which are made subject to control under the Act of 1971 by virtue of the MDA 1971 (modification) Order 1985; the new Class B, and a number of Class C, drugs are included in Schedules 2 and 3 to the 1973 Regulations) but the largest group (benzodiazepines) compromise a new Schedule 4 and are exempted from the prohibition on importation and exportation.

(ii) the extension of the general authority to possess controlled drugs supply, and return to the supplier of the drug and, for destruction, (Regulation 6).

(iii) amendments regarding supply and possession of controlled drugs on ships and off-shore installations (Regulation 8 to 10).

(iv) an increase in the range of controlled drugs which midwives are authorised to possess and administer (Regulation 11).

(v) an additional exemption from labelling requirements for controlled drugs used in clinical trials and animal tests (Regulation 18).

(vi) the application of certain record-keeping and destruction provisions to drugs specified in Schedules 3 and 4, with exemptions for certain categories of people (Regulation 22, 24 and 26).

(vii) a new requirement that certain persons, on demand by the Secretary of State, should finish records and information concerning dealings in controlled drugs (Regulation 25).[10]

1.1.3 The Misuse of Drugs Regulations 2001

The Misuse of Drugs Regulations 2001 allow for the lawful possession and supply of controlled (illegal) drugs for legitimate purposes. They cover prescribing, administering, safe custody, dispensing, record keeping, destruction and disposal of controlled drugs to prevent diversion for misuse.[11] Two changes of substance are made by the regulations, the addition of 35 phenethylamine derivatives which are made subject to control under the MDA 1971 (Modification). The second change is that the 33 benzodiazepines and 8 other substances formerly in Schedule 4 Part II are now in Part I of that Schedule. In addition, the 54 anabolic substances formerly in Schedule 4 Part I are now in Part II of that Schedule.[11]

The Misuse of Drugs Regulations (2001) (MDR) and its subsequent amendments, retains drugs in five schedules (1-5) and specifies the requirements governing such activities as import, export, production, supply, possession, prescribing, and record keeping which apply to them. The Regulations also define the types of professional person(s) who are authorised to supply and possess Controlled Drugs and stipulates the conditions under which these activities may be carried out. In the 2001 regulations, drugs are

divided into five Schedules, each specifying the requirements governing such activities as import, export, production, supply, possession, prescribing, and record keeping which apply to them.

Schedule 1 includes drugs not used medicinally such as hallucinogenic drugs (e.g. LSD), ecstasy-type substances, raw opium, and cannabis. A Home Office licence is generally required for their production, possession, or supply. A Controlled Drug register must be used to record details of any Schedule 1 Controlled Drugs received or supplied by a pharmacy.

Schedule 2 includes opiates (e.g. diamorphine hydrochloride (heroin), morphine, methadone hydrochloride, oxycodone hydrochloride, pethidine hydrochloride), major stimulants (e.g. amphetamines), quinalbarbitone (secobarbital), cocaine, ketamine, and cannabis-based products for medicinal use in humans. Schedule 2 Controlled Drugs are subject to the full Controlled Drug requirements relating to prescriptions, safe custody (except for quinalbarbitone (secobarbital) and some liquid preparations), and the need to keep a Controlled Drug register, (unless exempted in Schedule 5). Possession, supply and procurement is authorised for pharmacists and other classes of persons named in the 2001 Regulations.

Schedule 3 includes the barbiturates (except secobarbital, now Schedule 2), buprenophine, mazindol, meprobamate, midazolam, pentazocine, phentermine, temazepam, and tramadol hydrochloride. They are subject to the special prescription requirements. Safe custody requirements do apply, except for any 5,5 disubstituted barbituric acid (e.g. phenobarbital), mazindol, meprobamate, midazolam, pentazocine, phentermine, tramadol hydrochloride, or any stereoisomeric form or salts of the above. Records in registers do not need to be kept (although there are requirements for the retention of invoices for 2 years).

Schedule 4 includes in Part I drugs that are subject to minimal control, such as benzodiazepines (except temazepam and midazolam, which are in Schedule 3), non-benzodiazepine hypnotics (zaleplon, zolpidem tartrate, and zopiclone) and Sativex®. Part II includes androgenic and anabolic steroids, clenbuterol, chorionic gonadotrophin (HCG), non-human chorionic gonadotrophin, somatotropin, somatrem, and somatropin. Controlled drug prescription requirements do not apply and Schedule 4 Controlled Drugs are not subject to safe custody requirements. Records in registers do not need to be kept (except in the case of Sativex®).

Schedule 5 includes preparations of certain Controlled Drugs (such as codeine, pholcodine or morphine) which due to their low strength, are exempt from virtually all Controlled Drug requirements other than retention of invoices for two years.

The illicit drugs have been a noticeable concern in public policy in the United Kingdom at least since the 1970s, much debated by politicians and the media.[12] In the 1980s, reduction of supply of controlled drugs was emphasised in UK government policy, while in the 1990s the focus was on minimising demand and in the 2000s the emphasis was on reducing harm.[13]

One of the most important reasons why the drug policy makers in the UK intervened by introducing a new legislation (the Psychoactive Substance Act (PSA) 2016) was due to the unprecedented popularity of NPS linked with reported death and toxicity cases not only in the UK but also all over the world. The widespread use of these harmful/dangerous substances is for a number of reasons. Some users had the misconception that NPS were legal, thus meaning they are safe to use, whereas other people are “novelty seekers” who want to try new and exciting things. Due to NPS being easy to obtain, these novelty seekers have been readily exposed.[14] At present, the Internet is considered as a perfect platform to promote and market these substances, forming a worldwide platform for marketing them.[15] In addition to their ease of synthesis and low cost, resourceful marketing/advertising have contributed

to the problem. Information available *via* the Internet, plus negligible difficulty in the manufacturing and transportation from distant areas, together with careless legal prosecution/enforcement, has led to high prevalence of these substances.[16] All these reasons led to a change to the law in the UK regarding NPS. Resultantly, the PSA (2016) was passed as a law. It was intended to plug gaps in the existing legislation, which did not deal sufficiently with NPS.

In the last decade, New Psychoactive Substances (NPS) have emerged on the (inter)national drug scene.[17, 18] These substances are similar in their chemical structures (with minor structural modifications) to other controlled drugs, and as a result produce similar pharmacological effects, albeit that these effects can be either up- or down-regulated depending on the NPS in question (e.g. amphetamines vs. synthetic cathinones). There are many terms given to unregulated NPS, which are mislabelled intentionally to circumvent the existing laws of controlled drugs. “Legal highs”, “herbal highs”, “bath salts”, “plant food”, “plant feeders”, “research chemicals”, “designer drugs”, “synthetic drugs”,” and “smart drugs” are some of the names/terms used for NPS. In some cases, NPS have been labelled ‘not for human use’ or ‘not tested for hazards or toxicity’ in an effort to further circumvent law.[17, 19, 20] It is noteworthy that the highlighting of potential new dangers is not just applicable to healthcare professionals, but also to the general public, as the latter also need to be clearly informed and aware of dangers resulting from NPS spread and use.[20, 21] This situation is exacerbated when the legislative process for prohibiting individual compounds is considered; it is regarded as being too slow, especially as the manufacturers can rapidly replace newly prohibited compounds with new uncontrolled substances.[22] This thesis will be concerned with a specific class of NPS known as the dissociative anaesthetics, such as diphenidine (**1**) and its substituted derivatives e.g. methoxphenidine (2-, 3- and 4-MXP, **2, 3** and **4** respectively). Before we discuss the specific class of NPS (dissociative anaesthetics) that this study focuses on, and its

chemistry, pharmacology and methods of analysis, it will be useful to contextualise the NPS situation and provide an overview about popularity, prevalence and classification of these substances.

1.2 Popularity of NPS

As mentioned earlier, despite the existing laws and regulations governing drug availability and use, nonetheless NPS are still emerging nearly on a daily basis. The appearance of new substances mean new toxicological threats for body organs, new analytical challenges in detecting them and new questions for emergency doctors and toxicologists about the treatments to carry out for adverse drug reactions to their consumption and overdose.[23]

In the last ten years, NPSs have extensively dominated the drug scene in Europe and the US. Many drug users have switched from their traditional drugs to NPS use. Several factors have contributed to their increasing popularity including, as mentioned earlier, their falsely legal image, their more reasonable costs, and their distribution based on the new technologies. However, major health issues have emerged in relation to the somatic, mental, and addictive consequences of their use with persistent unknowns for the future. All this explains the urgent requirement to developing clinical research and improving the management of addiction and poisonings attributed to these NPS.[24] In parallel with the physical drugs market, the last decade has seen the development of online marketplaces, facilitated by the emergence of new internet technologies. Some online vendors utilise the surface web, typically retailing non-controlled precursor chemicals, NPS or medicines, which may be falsified or counterfeit. This shows the complexity and therefore the difficulty of controlling the current drug market.

On one hand, modern technology such as social media, YouTube and smartphone applications are used for selling NPS. On the other hand, it is important to bear in mind that these substances are sold under many names. To conceal both transactions and physical locations of servers, many

strategies can be used. Anonymization techniques such as Tor and I2P that encrypt the computer internet protocol address and cryptocurrencies such as bitcoin [25] and litecoin are utilised to make communications between drug dealers and consumers undetectable.[26] Consequently, the same technology can be utilised for raising awareness among people particularly youth about the harm caused by these drugs of abuse.[15]

1.3 Prevalence of NPS

The data of NPS spread can be obtained from UNODC World Drug Report and from the other UNODC programmes in the region on the production and use of recreational drugs.[27] According to UNODC, in 2018, opioids are causing the most harm to public health in comparison to other drugs of misuse. Besides, the use of analgesics such as fentanyl in North America and tramadol in regions of Africa and Asia remain a big issue because these drugs are made by traffickers who manufacture them illegally and promote these substances in unlawful markets causing significant harm to health.

In February 2019, substances that have stimulant effects were the largest reported NPS drug group reported to UNODC (36%). Synthetic cannabinoid receptor agonists (30%) and classic hallucinogens (15%) were the next largest classes. Other groups, such as dissociative substances and sedatives/hypnotics, remained constant at 3%. The number of opioids nearly doubled in the course of 2018 to reach 62 different opioids or 7% of all substances listed in the system as of December 2018. The rising importance of synthetic opioids is also revealed in the current opioid crisis (Figure 1).[28]

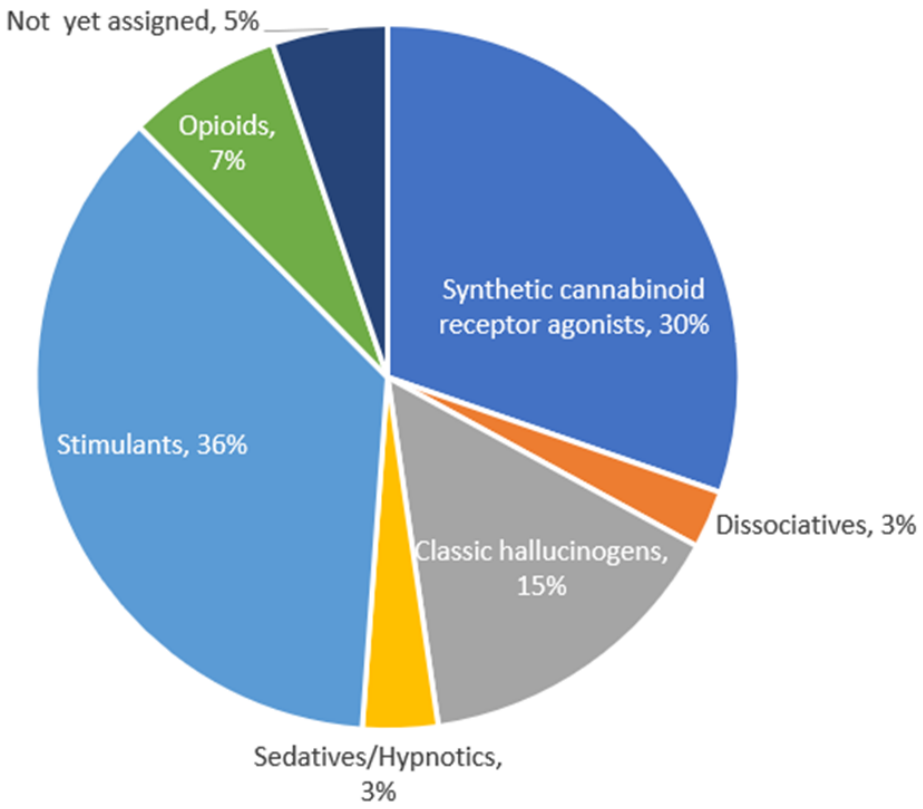


Figure 1. Proportion of NPS, by psychoactive effect group, as of December 2018 [56]

Although, the use of NPS is primarily a Western phenomenon, Asian countries are considered to be a fertile region for the production of NPS, particularly in China and India.[29] At a country-level, prevalence of NPS can be available but the lack of international data increases a knowledge gap that limits the capacity for agencies such as the World Health Organisation (WHO), UNODC, United Nations Programme on HIV and AIDS (UNAIDS) and other international non-governmental organizations to produce the highest possible impact.[30]

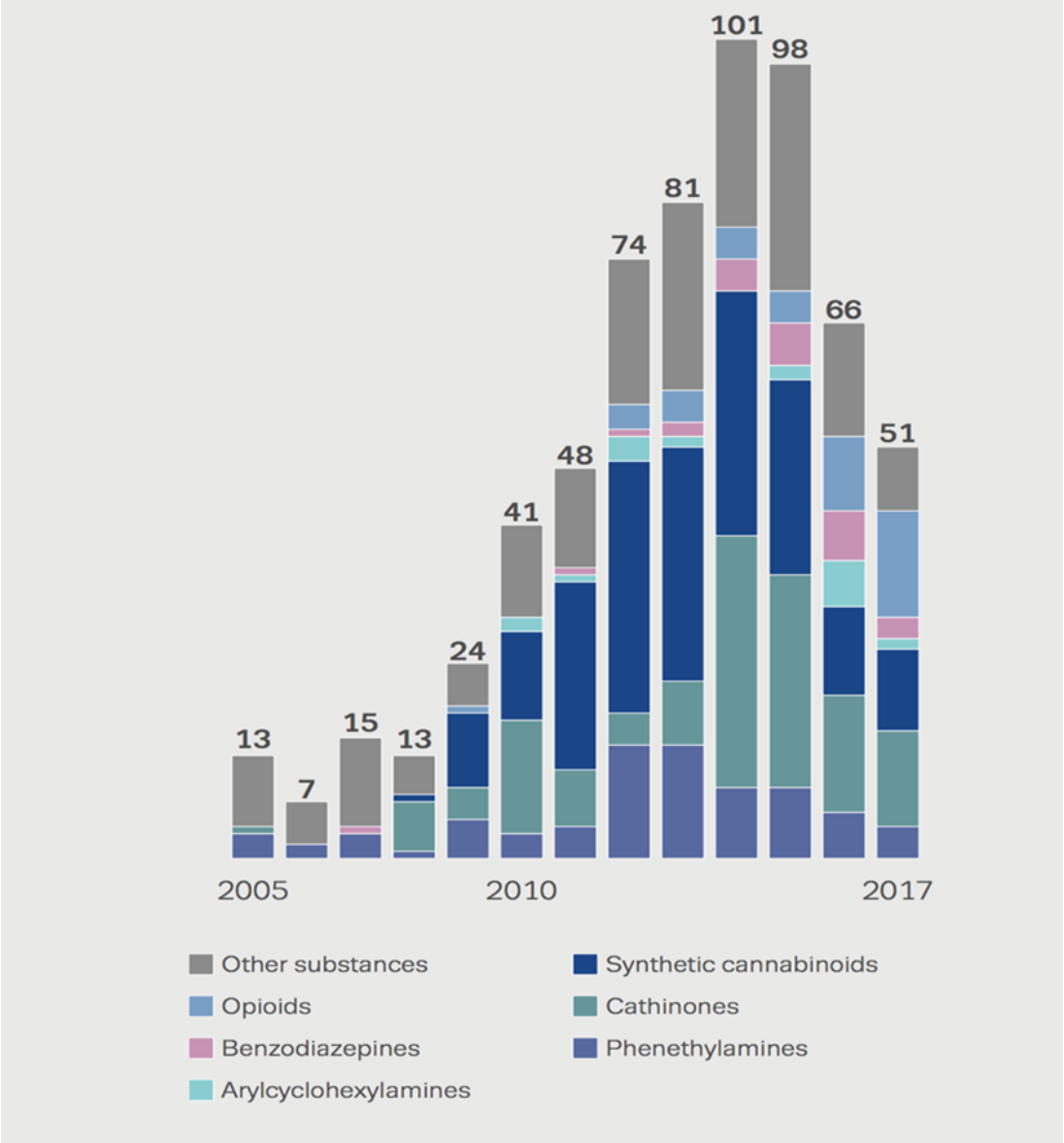


Figure 2. Number and categories of NPS notified to the EU Early Warning System for the first time, 2005-2017[59]

In 2017, according to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), more than 670 NPS were detected in Europe. The issue is that these substances are not under control by international drug regulations and include a wide range of drugs, for example synthetic cannabinoids, opioids, stimulants, and benzodiazepines. From Figure 2, the main trend

observed is the increase in the number of NPS, with about 100 substance reported to the EMCDDA in both 2014 and 2015, followed by subsequent drop to just below 70 in 2016 and just over 50 in 2017. In addition, there is a significant rise in the percentage of opioids and benzodiazepines, particularly between 2014 and 2017 in comparison to the years from 2005 to 2013.[31] The efforts made by European countries in terms of controlling NPS in general is one reason behind the decrease in the total number of NPS reported in 2016 and 2017. Another cause might be due to the closure of many laboratories that synthesise these substances in China following applying law enforcement systems.[32]

In 2018, the number of substances reported in Europe was approximately 650 substances in total and 300 in France. In a recent ten-year (2008-2018) overview of the situation in France compared with Europe (Figure 3) has shown that the number of NPS identified per year in Europe has declined in recent years: 67 in 2016, 51 in 2017, and 32 in 2018. The 32 compounds identified in Europe in the first semester of 2018 is considered as an increase in the number of NPS. A similar trend was seen in France with only two substances reported in the first quarter of 2018. Overall, this decline does not seem to be linked to law enforcement service activities, which have stated a growing number of seizures each year with approximately 900 in 2015 and over 2,000 in 2017. Again, this decline might be as a result of the measures taken by authorities in producer countries, in addition to the international control of precursors may have hindered NPS production and that producers eventually focus on making substances most wanted by users/consumers.[33]

While in the UK, in a report published by the Home Office, in total, there were 1,523 seizures of NPS in 2017/18. Synthetic cannabinoids were most often seized (858 seizures, followed by other NPS (448 seizures), NPS powders (135 seizures) and nitrous oxide (96 seizures).

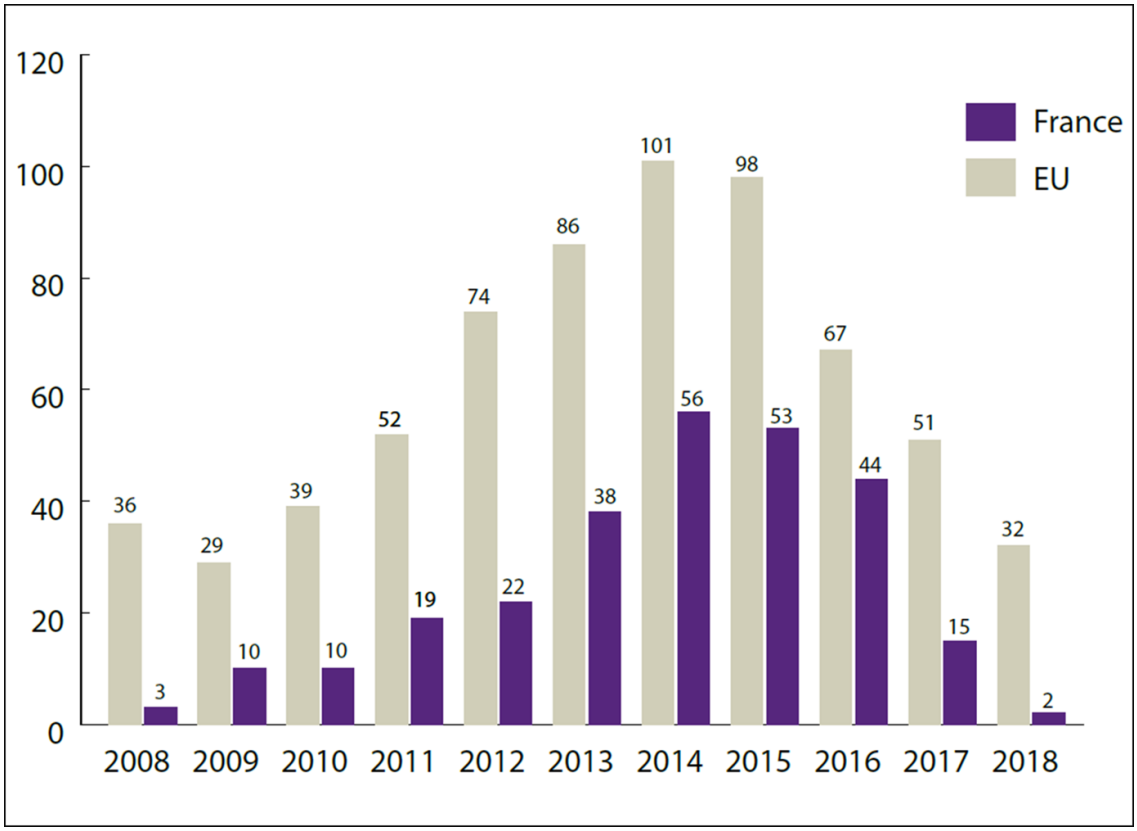


Figure 3. Number of NPS identified per year, in the EU and in France

All the above mentioned data and statistics on prevalence, drug use, drug harmful effects and the level of NPS usage show the importance of carrying out chemical analysis and pharmacological studies on these drugs. International and local authorities should take all the appropriate actions and measures to avoid the expansion of this new threat.

1.3.1 Psychoactive Substances Act (2016)

In response to the rapidly proliferating new psychoactive substance market, the UK Government implemented steps to restrict the supply, production and import of psychoactive substances. The resulting legislation was the Psychoactive Substances Act (PSA). The PSA (2016) came into force on 26 May 2016 and made a blanket ban on the production, distribution, sale and supply of psychoactive substances in the United Kingdom for human consumption with the exemption of caffeine, nicotine, alcohol and medicinal

products as defined by Human Medicines Regulations (2012). PSA (2016) does not replace the MDA (1971); controlled drugs laws are still the same.

The PSA (2016) gives police and other enforcement agencies a range of powers including: powers to seize and destroy psychoactive substances as defined by the PSA; search persons, premises and vehicles; and enter premises by warrant. It also includes a number of civil sanctions to enable a proportionate enforcement response.[7, 34]

In general, the Act demonstrates many aspects such as possession, importation, supply, production, penalties (Table 1), powers to stop and search; whereas the police will have powers for that and also for premises and prohibition notices.

Table 1. Penalties under the PSA (2016) [34]

Offence	**Summary (Magistrates Court)**	**Indictment (Crown Court)**
Possession	Not an offence	Not an Offence
Possession in a custodial institution	Up to 12 months and/or a fine	Up to 2 years and/or a fine
Possession with intent to supply	Up to 12 months and/or a fine	Up to 7 years and/or a fine
Supply/offer to supply etc.	Up to 12 months and/or a fine	Up to 7 years and/or a fine
Production	Up to 12 months and/or a fine	Up to 7 years and/or a fine
Importation/exportation	Up to 12 months and/or a fine	Up to 7 years and/or a fine
Failure to comply with a Prohibition or Premises notice	Up to 12 months and/or a fine	Up to 2 years and/or a fine

In the UK, the maximum penalties for scheduled drug possession, supply (selling, dealing or sharing) and production depend on what type or 'class' the

drug is. For class A drug possession, up to 7 years in prison, unlimited fine or both. While, for class B drug possession, up to 5 years in prison, unlimited fine or both. The penalty of possession of class C drug is up to 2 years in prison, unlimited fine or both. Penalties of supply and production of class A are up to life in prison, an unlimited fine or both. Whereas, for classes B and C the penalties are up to 14 years, an unlimited fine or both.

At a national level, an official Home Office review (November 2018) assessed the influence of the UK's PSA (2016) that banned the production, distribution, sale, and supply of majority of psychoactive substances. The review found that the sales of NPS in shops and online had been "fundamentally eliminated" by the legislation, but this had led to the sale of these substances underground. Nowadays, street dealers are the key source of supply for NPS, in particular with regard to synthetic cannabinoids.

The review also reported there had been a substantial decrease in the number of NPS users among the overall adult population since PSA was introduced. However, the use of these substances among homeless people and children had not decreased, similarly, there was no reduction in the use of nitrous oxide by adults. Additionally, the use of these substances in prisons showed no reduction, what is more is that synthetic cannabinoids were identified as being dominant. The violence in prisons (because of NPS use) has not dropped since the introduction of the Act. Despite adults' use of NPS decreasing, this finding could not be attributed to overall drug use having fallen as a result of the Act, as people may have moved to other illicit substances instead. Among vulnerable users, including homeless people, it found that many users had shifted from synthetic cannabinoids to "traditional" controlled drugs. Since the introduction of PSA, deaths directly related to NPS appear to have dropped in England and Wales but have increased in Scotland. However, there is no evidence regarding a decrease in social harm, such as violence, resulting from

the PSA, this is down to the fact that some NPS users may have used other drugs instead.

From the findings, the new substances, which are not controlled under the MDA 1971, have continued to appear since the Act was introduced.[35] One of the goals of the act was to reduce the various health and social harms associated with NPS, this aim seems to has been mainly reached. There have been considerable reductions in the numbers of medical enquiries, concerns reported by users, and persons presenting to treatment since the Act was introduced.[36]

1.3.2 Changes in the NPS market since the introduction of the PSA (2016)

A recent review of the PSA (2016) presented by the Home Office (November 2018) provided an assessment of the changes in the market of NPS associated to the application of the PSA. According to the review, the act has led to a rise in NPS prices and reduction in their availability. A shift away from vendors to street sellers has been observed, in particular for synthetic cannabinoids, with the Internet remaining a key source for obtaining NPS for users.

Approximately 330 retailers were identified as having stopped the sale of NPS; 490 arrests related to NPS were made in the months prior to December 2016 and 990 seizures prior to March 2017. This proposes that the Act has not totally eradicated the supply of NPS, because of the large numbers of offences and seizures of alleged NPS that have been documented. Additionally, the supply of NPS by street dealers is currently another issue, the continuous development of new substances, the possible shift from NPS to other harmful substances, and continued high levels of synthetic cannabinoid use amongst the homeless and prisoners.[37]

At an international level, the UNODC World Drug Report 2018 entitled “NPS pose great harm to vulnerable user groups”, detailed that in numerous countries, ways of NPS use among marginalized, vulnerable and socially

disadvantaged people, including homeless persons and individuals with mental health conditions, continues to be extensively recognized. Administration of stimulant NPS by injections also represents a concern, especially due to reported related high-risk injecting practices. Using NPS in prison remains an issue of concern in some European countries, North America and Oceania.[38]

In Europe, a new update from the EU Early Warning System (EWS) on NPS, led to the EMCDDA releasing its latest insights into NPS in Europe entitled “Fentanils and synthetic cannabinoids: driving greater complexity into the drug situation” (report covers the time-frame January 2016 until December 2017). One of the biggest issues is that laboratory personnel may be at danger of poisoning from work-related contact with substances such as the new synthetic opioids (particularly the fentanyls) and the synthetic cannabinoids. The latter are also easy to smuggle, with a few grams adequate to create many thousands of doses/amounts for the drug market.[39] Indeed, detection abilities and screening tools affect various fields and settings, involving seized products analysis, emergency departments, workplace, drug addiction treatment surgeries, autopsy and criminal caseworks, health involvements and law enforcement.[40]

In November 2018, a new legislation — bringing faster response to new drugs — strengthens the EU Early Warning System (EU EWS) and risk assessment processes on NPS and shortens control procedures. Europe’s ability to rapidly respond to public health and social threats caused by new psychoactive substances (NPS/new drugs’) will be significantly strengthened. The legislation was introduced in response to the recent growth in the availability of NPS. This new legislative package involves: a Regulation about information exchange on, and an early-warning system and risk-assessment process for, NPS; a Directive, which permits NPS to be controlled at EU level as 'drugs'.[41]

According to the Global Drug Survey (GDS 2018), the risk profile of NPS is different due to their inconsistent composition and potency. e.g. the deaths in recent months associated with the use of extremely potent hallucinogens (e.g. the substituted phenethylamine compound, NBOMe) and potent amphetamine analogues (e.g. 4-flouro-amphetamine) are of real concern across Europe and Australia.[42] This contrasts with the findings of GDS 2017, which suggested powerful novel opioid drugs such as carfentanyl and acetyl fentanyl were responsible for numbers of deaths in Canada.[42]

1.4 Classification of NPS

The United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory on New Psychoactive Substances (2019) [43] detailed the main substance groups of NPS:

(i) phencyclidine-type substances e.g. methoxetamine (MXE) [44]
(ii) phenethylamines e.g. *p*-methoxymethamphetamine (PMMA) [45]
(iii) piperazines e.g. 1-benzylpiperazine (BZP) [46]
(iv) plant-based substances e.g. *Khat* [47]
(v) aminoindanes (such as *2-aminoindane (2-AI))* [48]
(vi) synthetic cannabinoids e.g. 'HU-210' synthetic analogue of THC [49]
(vii) synthetic cathinones e.g. mephedrone [50]
(viii) tryptamines e.g. *N,N*-diethyltryptamine (DET) [51]
(ix) other substances (structurally diverse) e.g. benzodiazepines

This study will specifically focus on developing methods of analysis for emerging substances within the phencyclidine-type – which are colloquially known as dissociative anaesthetics. [52] Dissociative anaesthetics are examples of these types of substances e.g., Diphenidine (**1**), 2-methoxphenidine (**2**), 3-methoxphenidine (**3**), 4-methoxphenidine (**4**), phencyclidine (PCP, **5**) [53], methoxetamine (MXE, **6**) and ketamine (**7**) [54], [55], (Figure 4).

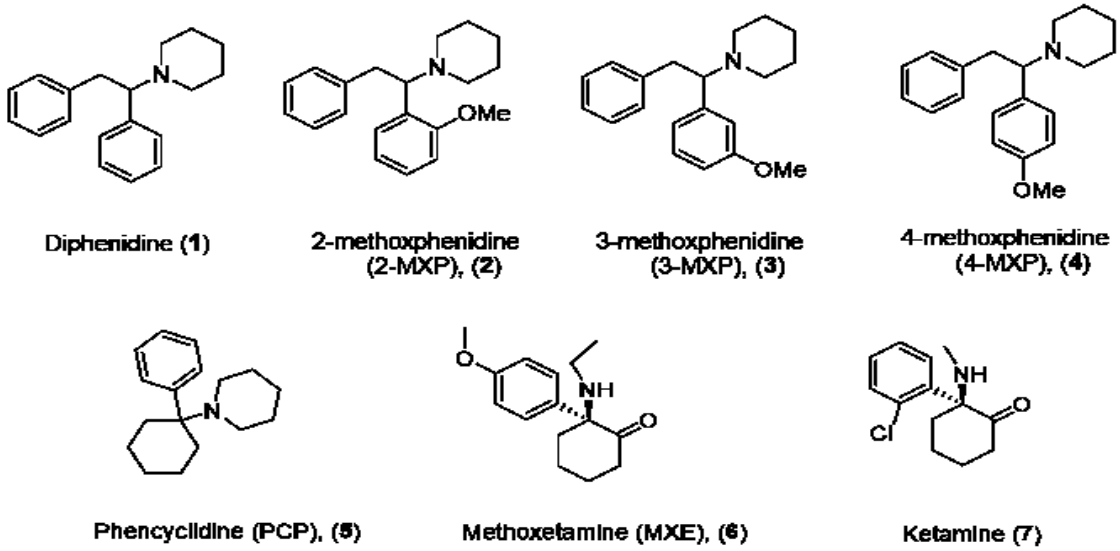


Figure 4. Chemical structures of common dissociative anaesthetics

1.5 Diphenidine (legal status, chemistry, synthesis, pharmacology, toxicology, metabolism and intoxication)

At present, **1** is used illegally as an NPS.[56] In addition to **1**, the category of dissociative substances also includes many drugs such as **2**-**7**, as outlined in 1.4.1. These drugs are a kind of hallucinogen that modify perceptions of sight and sound and create emotional state of detachment from reality.[57, 58] Diphenidine has entered the European, American and Japanese drug scene throughout the last years.[59, 60] In Europe (since 2013), seizures of **1** have been reported,[61] and similarly for Japan.[62, 63] **1** is not approved to be used as a medicine.[64] Additionally, **1** is unlawfully sold via the Internet; many drug users tend to discuss its use at fora and websites.[65] Intoxications of various degrees of severity caused by **1** have been documented in many countries such as Sweden,[66] Italy [67] and Japan.[62, 63, 68] **1** is now controlled in the United Kingdom due to the reported cases linked to its misuse and also the concerns raised by the authorities.[69] It is difficult to predict the exact figure of the increasing cases of abuse related to **1** and its derivatives, therefore,

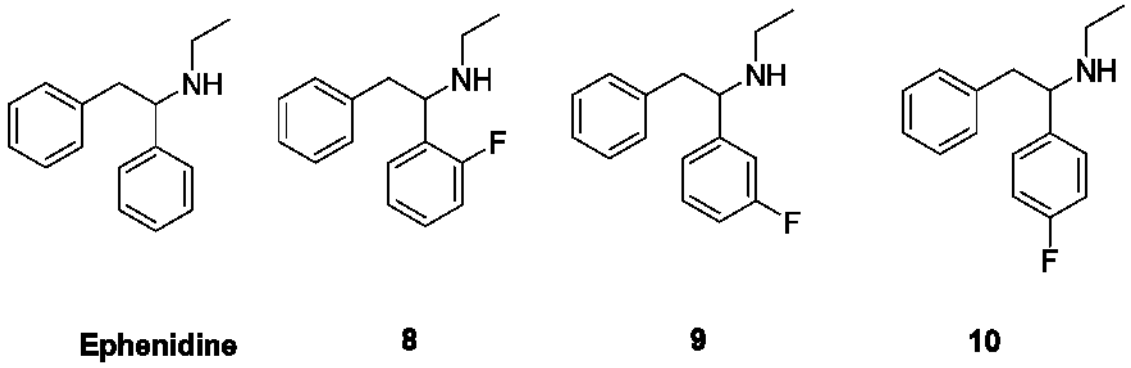
determination of these compounds by applying specific analytical approaches is urgently required.

1.5.1 The legal status of diphenidine and its derivatives

At the international level, **1** is currently considered as a legal grey area drug, as **1** can be purchased straightforwardly from many websites. Possession of this drug is illegal in many countries.

In the United kingdom, it is illegal to produce, supply, or import **1** and methoxphenidine (MXP) under the Psychoactive Substance Act, 2016.[7] Whereas, in Italy MXP is banned according to the Italian legislative instrument 'Table of Drugs' since 2016, similarly in Sweden (2015) this substance is banned and it is a controlled substance in China since October 2015. While, in Canada since March 2016, **1** is a Schedule I controlled substance, [70] and also MXP is a Schedule I controlled substance that can only be possessed by authorised persons.[71]

In the United Kingdom, ephenidine (Figure 5) is illegal to produce, supply, or import under PSA, while, in the United States, ephenidine might feasibly be considered a positional isomer of lefetamine, which is a Schedule 4 drug.[71] Ephenidine (the derivative of diphenidine where the piperidine ring is replaced with a simple N-ethyl side chain) is also prohibited in many countries as a structural isomer of the banned opioid substance lefetamine; in Canada, as of March 2016, ephenidine is Schedule 1 controlled substance, while in Sweden, ephenidine became a scheduled drug as of August 2015, [72] (see ephenidine structure and the structures of the 2-, 3- and 4-fluoronated isomers of ephenidine **8**, **9** and **10,** Figure 5, and see also the study of these three compounds outlined in chapter 4).



Ephenidine **8** **9** **10**

Figure 5. Chemical structure of ephenidine and its fluorinated regioisomers **8**, **9** and **10**

1.5.2 Chemistry of diphenidine

In the structure of **1**, there is a phenyl group and a piperidine ring linked to the phenylethylamine. The IUPAC name of **1** is 1-(1,2-diphenylethylethyl) piperidine, additionally there are many acronyms for diphenidine such as DIPH, DPH and DPD. Diphenidine (**1**) is a molecule of the diarylethylamine class which includes **5**-**7**. In addition, **1** is structurally analogous to MXP, lacking a 2-methoxy substitution on one of its phenyl rings. **5** was discovered in 1956 and soon became a popular street drug. Dissociative anaesthetics including **1**, **5** and **6** have similar chemical structures of phencyclidine.[73]

The diphenidine derivatives (compounds that contain a diphenylethylamine nucleus in their structures) are available currently as NPS include, methoxphenidine (MXP), trifluoromethoxphenidine (TFMXP), mesophenidine, IAS-013, 2-chlorodiphenidine (2-CLDP), methylenedioxydiphenidine, naphthadine and ephenidine. Ephenidine is a lesser-known novel dissociative substance of the diarylethylamine class and is an *N*-Methyl-D-aspartate receptor (NMDAR) antagonist.[74],[71] (See Figure 5).

Compound **1** and its derivatives are phenylethylamine substances, (see Figure 6), examples include methoxphenidines (MXP), (**2, 3** and **4**),[75] phencyclidine (**5**), methoxetamine (**6**), ketamine (**7**), fluephenidines (FEP, **8**, **9** and **10,** See

Chapter 4), fluorocyanoephenidines (FCEP, **11**, **12** and **13**, See Chapter 5) and the halogenated diphenidine compounds (halogenated DP, **14-25** See Chapter 6). The compounds **5**, **6** and **7** were not tested/investigated in this study.

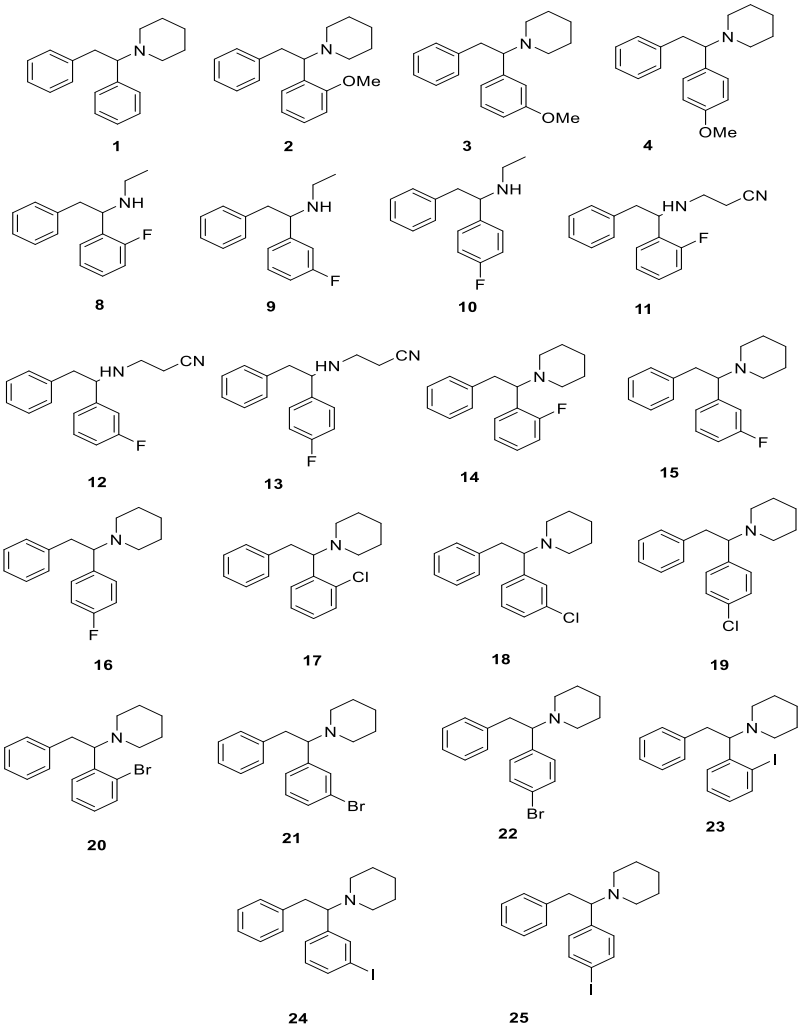


Figure 6. Chemical structures of diphenidine and 21 of its derived compounds tested in this research project

1.5.3 Synthesis of diphenidine

Diphenidine **1** was first synthesised by Christian in 1924 via a reaction of benzylmagnesium bromide with the corresponding α-arylamino nitrile, **1** was first synthesized as an anaesthetic agent, [76] but it was also synthesised in

2015 (Wallach *et al.*), [71] and in 2016 (Geyer *et al.*), [74] using two different synthetic procedures (Figure 7).

In 2016, Geyer performed the synthesis of **1** by reaction of benzyl bromide with piperidine and benzaldehyde in acetonitrile, zinc and trifluoroacetic acid (TFA) at room temperature for one hour. The free-base of **1** was obtained as a yellowish oil, dissolved in ether, treated with hydrogen chloride to give off-white powder of the hydrochloride salt that was then fully structurally characterised by chromatographic techniques infrared and NMR spectroscopy.[74]

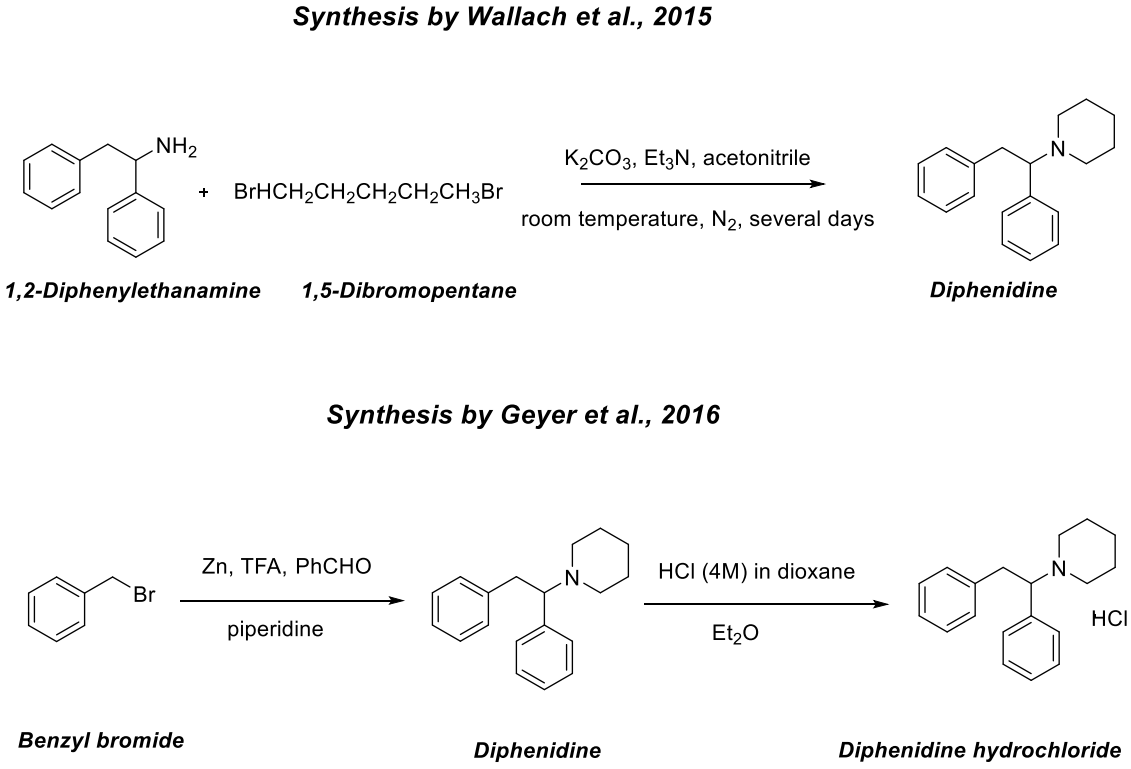


Figure 7. Synthesis of diphenidine according to Wallach *et al.*,[72] and Geyer *et al.*[68]

1.5.4 Pharmacology of diphenidine

In a pharmacological study by Wallach et al.[71], the results suggest that **1**, **2**, **3**, **4** and **15** are selective N-methyl-D-aspartate receptor antagonists (NMDAR).[71] In addition, **1** has dopamine and serotonin reuptake inhibition

activities. It is also an agonist for the μ-opioid receptor, and it possesses an affinity for the σ_1 receptor, σ_2 receptor and dopamine transporter.[71, 77] [56]

The *in vitro* studies of **1** and other diarylethylamines such as the compounds that are used in treating neurotoxic injury revealed that these compounds act as NMDA receptor antagonists.[78-80] In comparison to codeine phosphate, in dogs **1** is more potent as an antitussive agent.[81] The (S)-enantiomer of **1** has affinity of 40 times greater than that of the (R)-enantiomer for blocking the NMDA receptors.[77]

1.5.5 Toxicology of diphenidine

The data about the history of toxicity due to human usage of **1** is very limited. Trying **1** in low or moderate doses produces no negative health effects; this was anecdotal evidence from some the drug users. While using **1** chronically can cause tolerance and addiction, and if the drug was stopped suddenly, the withdrawal effects might happen. The co-administration of **1** with other dissociatives leads to cross-tolerance, consequently lowering the effect of the latter.[69]

Dangerous interactions can be produced as a result of administering stimulants and dissociative agents leading to unwanted psychological effects such as delusions, mania, anxiety and psychosis, these effects are worsened by taking a combination of these substances. While, the co-administration of the dissociatives such as **1** with depressant drugs can cause augmented risk of unconsciousness, vomiting and death because of respiratory depression. The subcutaneous injection of **1** in mice produced lethality in a dose of 325 mg/kg, whereas in humans the toxic dose is still unknown [64], while the exact toxic dose for humans is still unknown. The most frequently revealed effects defined in the literature that can lead to hospitalization in some users, include tachycardia, tachypnea, anxiety, confusion, disorientation, hallucinations, and high body temperature.[66, 67]

1.5.6 Metabolism of diphenidine

There are no comprehensive studies on **1** metabolism in humans due to ethical reasons.[69] In 2015, through the investigation of an autopsy urine sample by means of matrix-assisted laser desorption ionization (MALDI) quadrupole time-of-flight (QTOF)/MS, Minakata *et al.*, confirmed that **1** metabolites occur through oxidation of the piperidine ring followed by the phenyl ring (Figure 8).[82] Again in 2015, Elliot *et al.* investigated the biotransformation of 2-MXP and reported a hydroxylated **1** metabolite.[83]

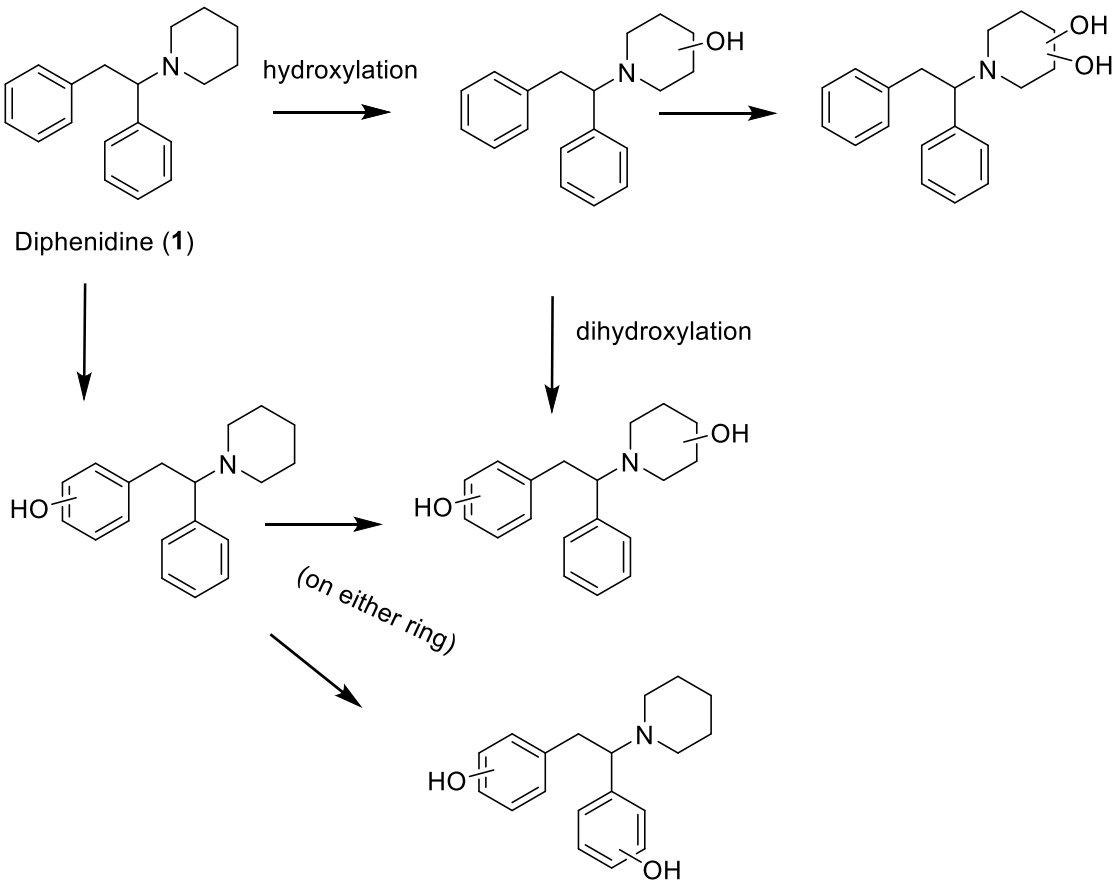


Figure 8. Biotransformation pathways of diphenidine in humans suggested by Minakata *et al.*[82] and Kusano *et al.*[68]

In 2018, Kusano *et al.*, investigated the biotransformation pathways of **1** in humans by testing [68] a post-mortem urine sample utilising the LC–QTOF/MS

technique. The results of this study showed that from the fragmentation of the resulted mono- and dihydroxylated metabolites and ion intensities; the monohydroxylation (and similarly the dihydroxylation) on the piperidine moiety is by and large preferred over the phenyl ring.[68]

Later in 2016, Wink *et al.* used GC-MS, LC-MS(n), and LC-HR-MS (Liquid chromatography–high resolution n mass spectrometry, which is an LC system coupled to the TF LTQ Orbitrap, the Linear Trap Quadropole (LTQ) Orbitrap is a high performance LC-MS and MSn system, combining rapid LTQ ion trap data acquisition with high mass accuracy Orbitrap mass analysis.) to investigate metabolic animal studies in rats. This study suggested that mono- and dihydroxylation at different positions occurred in the metabolism of diphenidine. This metabolic pathway occurs in a similar manner in humans, which was defined by both Minakata *et al.* [82] and Kusano *et al.*[68] LC-MS(n) refers to multi-stage mass (MS^n) fragmentation study, a technique which enables a complete fragmentation pathway of the drug to be established in order to characterize all the degradation products.[84],[85]

Wink *et al.* also concluded that hydroxylation is to some extent followed by dehydrogenation or *N*-dealkylation, or both. This is followed by glucuronidation and/or methylation of the dihydroxyphenyl groups. They also found that the cytochrome-P450 (CYP) isoenzymes CYP1A2, CYP2B6, CYP2C9 and CYP3A4 were involved in the formation of hydroxyaryl, hydroxypiperidine and dihydroxypiperidine metabolites, whereas in the formation the hydroxyphenyl and hydroxypiperidine metabolites, the CYP2D6 isoenzyme was involved.[84]

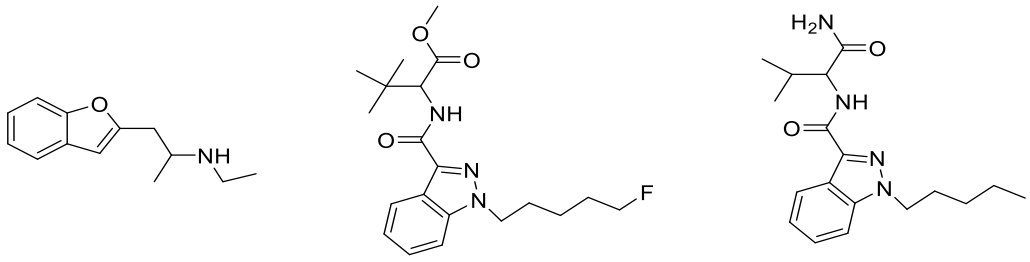
1.5.7 Intoxications by diphenidine

There are many diphenidine-related cases (some fatal) that have been reported in many European countries and Japan.[63, 66, 68, 86] In a recent investigation in Japan, a fatal ingestion of "liquid aroma" and "bath salt" products was confirmed that **1**, three substituted cathinones, three

benzodiazepines, and alcohol were consumed together and all these drugs were associated in this incident.[86]

In a case of fatal poisoning by a product called Super Lemon, **1**, AB-CHMINACA and 5F-AMB were detected (Figure 9).[63] A further toxicological study in Japan by Uchiyama *et al*., detected the presence of **1** and 1-benzylpiperidine in a powdered product called “fragrance powder”, whereas each sample solution was analysed by ultra-performance liquid chromatography–electrospray ionization mass spectrometry (UPLC–ESI-MS) and by gas chromatography–mass spectrometry (GC–MS) and also by NMR. In this study, a benzofuran derivative, 2-(2-ethylaminopropyl) benzofuran (2-EAPB), eight synthetic cannabinoids, five cathinone derivatives, and five other designer drugs were identified in illegal products. Most of the identified substances appeared as alternatives to controlled drugs such as narcotics and designated substances in Japan.[87]

Another report determined a large amount of **1**, coexisting with a synthetic cannabinoid 5-fluoro-AB-PINACA (5F-AMB) found in a suspicious herbal product.[87]



2-EAPB 5F-AMB AB-PINACA

Figure 9. Chemical structures of AB-PINACA, 5F-AMB and 2-EAPB

1.6 Methods of analysis of NPS/diphenidine derivatives

This section provides details of reports that highlight the analytical methods being applied to the detection and quantification of **1** and some of its derivatives.

Recently, in a study by Lowdon and Alkirkit *et al*., the first report of a molecularly imprinted polymer (MIP) for a NPS was reported. The MIP was synthesized with 2-MXP as a template, styrene and methacrylic acid (MAA) as the functional monomers and varying cross linker monomers. Binding capacities of the MXP isomers were in the range of 170–190 μmol g^{-1} as determined by optical batch rebinding and chromatographic methods. The NPS mixtures were studied by HPLC coupled to UV-vis spectroscopy and this technique was also used to validate optical batch rebinding experiments. The developed HPLC system allows for a clear separation between the three MXP regioisomers, which is a new method that has not been reported in the literature previously. It was determined that there is high recovery of all MXP isomers in pure solutions (>90%). Selective extraction of the MXP isomers was possible in mixtures with numerous percentages of the MXP regioisomers, and even in the presence of additional adulterants and other illegal substances. This indicates that molecular imprinting is a powerful technology for the extraction and quantification of (traces) of psychoactive components in complicated samples.[88]

In a study by McLaughlin *et al.*, three diphenidine derived compounds (2-, 3- and 4-MXP) were studied in addition to three powdered samples suspected to contain 2-MXP, were subjected to analytical characterization by GC and HPLC attached to various forms of mass spectrometry (MS). Nuclear magnetic resonance (NMR), infrared spectroscopy (IR) and thin layer chromatography (TLC) were also used, this was supported by carrying out two different synthetic routes for synthesising all three MXP isomers. The analytical data obtained suggested the possibility of differentiation between the isomers.[75]

In a different work by Geyer *et al.*, thirteen diphenidine-derived compounds were resolved from each other, and in the presence of caffeine, benzocaine and procaine as common adulterants, using GC-MS. Calibration standards for these thirteen diphenidine-derivatives indicated a linear response (r^2=0.996–0.998) over a 25.0–250.0 μg mL^{-1} range. The limit of detection (LOD) and limit of quantification (LOQ) for the analytes were as follows: 4.23–5.99 and 12.83–17.51 μg mL^{-1} correspondingly (based on the slope and standard deviation of the response). In this assay, the accuracy (percentage recovery study) was determined from spiked samples prepared in triplicate at three levels over a range of 80–120% of the target concentration (100 μg mL^{-1}). However, the repeatability (%RSD) of this method was considerably less than expected, probably because of the manual injection of the calibration standards, the validated GC-MS method was considered appropriate for analysing two street samples. The assay can be improved if we know the chemical structure of the target compound, which will help in choosing the solvent and decide on the solubility (polar or non-polar) of the target to be used in the next step and perform the extraction. The next step will be the purification of the sample to be ready for analysis and then finding a suitable column that matches with the chemical structure of the target is essential.[74]

In a method of determination of **1** and its metabolites using *MALDI-Q*-TOF/MS technique, which is an ionization quadrupole time-of-flight mass spectrometry. Matrix-assisted laser desorption/ionization (MALDI) is an ionization system, which utilises a laser energy absorbing matrix to produce ions from bulky molecules with slight fragmentation. Minakata *et al.* used this technique (MALDI-Q-TOF/MS) to analyse blood and urine samples containing **1** and its metabolites; the percentage recovery of the extracted analytes ranged between 80-100%. This method was applied to identify and quantify diphenidine in samples of post-mortem urine and blood.[82, 89]

In the target analysis, the use of LC-MS/MS (triple quad) is generally better than using TOF in terms of sensitivity and quantitation. TOF is a perfect

instrument to use for unknown analysis, metabolism and metabolomics study etc. TOF sensitivity if lower in comparison with LC-MS/MS but it depends on what kind of instrument (vendor and series).

In addition to that, sample preparation for mass spectrometry is very important for optimization of a sample for analysis because each ionization method has certain factors that must be considered for the success of the applied method, this includes volume, concentration, sample phase, and composition of the analyte solution. The analyte must be purified in some cases before entering the ion source. Moreover, mass spectrometry results have remained fundamentally dependent on sample preparation and quality, because sample ionization and mass measurements are susceptible to a wide range of interferences, such as buffers, salts, and detergents. These contaminants also impair MS system performance, often demanding time-consuming maintenance or costly repairs to restore function.

In a similar method, LC–Q-TOF/MS and LC–MS/MS techniques were applied to quantify **1** and its metabolites in post-mortem blood and urine samples. The blood samples were pre-treated, precipitated with methanol, centrifuged, and filtered. The results suggested that the method showed adequate linearity.[68] In another method, Kudo *et al.* used samples of whole blood and urine to determine **1** with other drugs and metabolites present. After dilution of blood, **1** was isolated, following centrifugation, and the diluted sample injected into a LC–MS/MS, the percentage recovery of each drug in whole blood and urine was found to be in the range of around 80-90%. All substances were well separated and each chromatogram revealed nearly no impurity peaks. All tested drugs showed a satisfactory precision, accuracy data according to ICH limits and guidelines, linearity with correlation coefficients (r^2) greater than 0.997 for whole blood and urine. [86] In a similar approach, Hasegawa *et al.* detected and determined **1** in solid tissues and biological fluids, the redistributed **1** was also investigated in a post-mortem samples.[63]

In another report, diphenidine metabolites were also determined by Wink *et al.* in rat urine samples using a liquid chromatography–high resolution mass spectrometry (LC–HRMS) method.[84] In an experimental investigation by Gerace *et al.*, [67] **1** was determined in samples of blood and urine of a nonfatal subject applying GC-MS and they also detected **1** in a white powder found at the patient's home. The results of this study suggested that the LOD and LOQ were 20 and 66 ng/mL in blood, and 25 and 82 ng/mL in urine, respectively.

Salomone *et al.* used ultra-performance liquid chromatography-tandem mass spectrometer (*UPLC-MS*/*MS*) to quantify **1** in hair samples among other recreational substances. The UHPLC–MS/MS system technique combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. The results suggested that limit of detection (LOD) and limit of quantification (LOQ) values were 3.4 and 6.8 pg/mg (pictogram = 10^{-12} g), respectively. The analytical method was simple, fast, specific, sensitive and linear (10–1000 pg/mg), and was successfully applied to real hair samples.[90] The disadvantages of hair sampling is the high cost and the longer time to obtain results compared with the time required by other matrices.

In a recent review of screening methods for the rapid determination of NPS by Graziano *et al.*, it was concluded that the colorimetric and immunochemical assays were unsuitable for the rapid and specific detection of these substances. Conversely, chromatographic assays showed to be more appropriate because of high flexibility, selectivity and sensitivity for detection of NPSs and/or their metabolites at low amounts in different biological media. The issue is that some NPSs can produce the same metabolites, making more challenging the identification. The results suggested that LC–MS appears to be the most promising tool for NPS analysis. The HRMS can be utilised due to the advantages of its high resolution and mass accuracy data that enable high selectivity and specificity. [40]

Overall, a review of the literature has shown a number of HPLC and GC systems for the identification and determination of diphenidine derivatives. However, most of the published methods relate to the detection and do not focus on the separation of diphenidine regioisomers by using suitable techniques, in particular HPLC methods.

Because of the existing knowledge gap in NPS research, the research detailed in this thesis will explore the development of a simple, appropriate and consistent HPLC method that can be validated and applied to detect and measure numerous diphenidine-derived NPSs.

1.7 Chromatography

Chromatography is a technique for separating the components, or solutes, of a mixture based on the relative amounts of each solute distributed between a moving fluid stream, called the mobile phase, and a stationary phase. The mobile phase may be either a liquid or a gas, while the stationary phase is either a solid or a liquid.

The main types of chromatography include, e.g. liquid chromatography, gas chromatography, thin layer chromatography and ion-exchange chromatography. HPLC is an example of liquid chromatography, the main components of HPLC are, the pump, injector, column and detector (Figure 10).

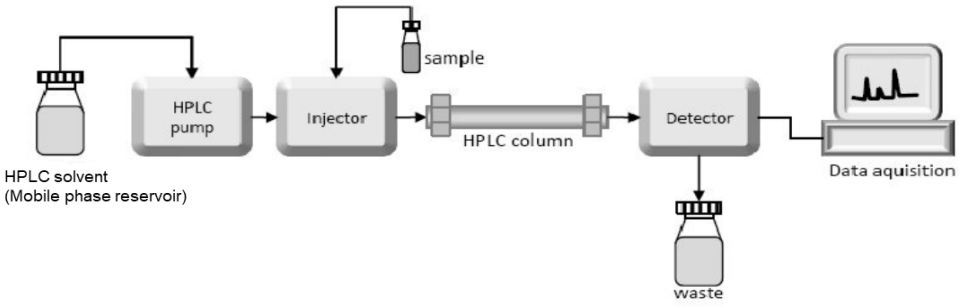


Figure 10. HPLC system

1.7.1 Background on chromatographic theory

Partitioning in chromatography is understood as a method of separation of solutes utilizing the partition of the solutes between two liquid phases. Paper chromatography, HPLC and TLC work on the principle of the same.

Partition chromatography is a method of separation in which the components present in the mixture are distributed into two liquid phases because of differences in partition coefficients. It is based on differences in retention factor as well as distribution coefficient of the analytes. Partition chromatography can be divided into liquid-liquid chromatography and bonded-phase liquid chromatography. There are two theories to explain chromatography:

1.7.1.1 Plate theory

Plate theory is the older (in comparison to rate theory); developed by Martin & Synge in 1941. The plate model supposes that the chromatographic column contains a large number of separate layers, called *theoretical plates*. Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next.

1.7.1.2 Rate theory

This theory is currently in use, proposed by van Deemter in 1956, accounts for the dynamics of the separation.

1.7.2 The hydrophobic-subtraction model of reversed-phase column selectivity

The hydrophobic subtraction model provides a quantitative description of column selectivity in (RP) HPLC columns, it increases our understanding of intermolecular interactions in solution. There are seven specific sample–column interactions that define its selectivity (although five interactions are sufficient for most columns).

This added insight into the basis of sample retention that can guide chromatographers during method development and help them to interpret unexpected results from routine or research experiments.

Different columns can now be compared in terms of selectivity, allowing the selection of alternative columns. Other possible applications include the further development of chromatographic theory, column design and manufacture, and the investigation of practical problems such as column deterioration. [91]

Upon characterization of a given RP stationary phase, the hydrophobic substraction model yields quantitative values for five parameters (**H**, **S***, **A**, **B**, and **C**) that describe the physico-chemical nature of that particular phase. Specifically:

H parameter is a measure of the phase hydrophobicity

S* is a measure of the resistance of the stationary phase to penetration by a solute molecule

A is a measure of the hydrogen-bond acidity of the phase

B is a measure of the hydrogen-bond basicity of the phase

C is a measure of the interaction of the phase with ionized solute molecules

These parameters, along with the companion parameters that describe the same characteristics of a given solute (η, σ, β, α, κ) are related to the retention of that solute (kx) relative to the retention of a reference compound (in this case, ethylbenzene - kEB) by the model. Retention can be described quantitatively by the relationship:

$$\log (kx'/k'EB) = \eta'H - \sigma'S* + \beta'A + \alpha'B + \kappa'C$$

where:

α is a measure of the chromatographic selectivity

η parameter is a measure of the solute hydrophobicity

σ is a measure of the bulkiness of the solute molecule

β is a measure of the hydrogen-bond basicity of the solute

α is a measure of the hydrogen-bond acidity of the solute

κ is a measure of the ionization state of the solute molecule. [92]

1.7.3 The impact of increasing log P of the solute on retention

In the physical sciences, a partition coefficient (*P*) or distribution coefficient (*D*) is the ratio of concentrations of a compound in a mixture of two immiscible solvents at equilibrium. This ratio is therefore a comparison of the solubilities of the solute in these two liquids. The partition coefficient generally refers to the concentration ratio of un-ionized species of compound, whereas the distribution coefficient refers to the concentration ratio of all species of the compound (ionized plus un-ionized). The log P value is a measure of lipophilicity or hydrophilicity. The non-polar phase in such experiments is usually dominated by the un-ionized form of the solute, which is electrically neutral. To measure the partition coefficient of ionisable solutes, the pH of the aqueous phase is adjusted such that the predominant form of the compound in solution is un-ionized. Measurement of the partition coefficient at another pH of interest requires consideration of all species, un-ionized and ionized.

Numerous methods exist to measure or estimate the pK_a (pK_a is the negative log of the acid dissociation constant or K_a) and log P_{ow} values. The shake-flask method and RP-HPLC method are the main experimental methods to determine partition coefficients. The shake-flask procedure is a standard method to determine octanol/water partition coefficients in the range of −2 to 4. There are some theoretical approaches to predict lipophilicity. Most of them add up the log P_{ow} contribution from each fragment and then apply structure-based correction factors. There are at least 20 software packages available at present, which provide convenient and fast prediction of lipophilicity for novel compounds. [93]

Purely chromatographic alternatives to the partition coefficient between octanol and water have emerged as well because of the potential for

automation, higher throughput, and minimising sample preparation efforts. In particular, reversed phase-HPLC (RP-HPLC) has been suggested to provide a suitable means to directly assess the lipophilic property of an investigational compound. As these methods do not involve the shake flask procedure, they have the additional advantage of being independent of the concentration effects.

In this setup, RP-HPLC is performed by using a C18-bonded stationary phase and a polar mobile phase, the latter being a mixture of water and acetonitrile. Chromatographic retention results from the partition of analytes between the two phases and can thus directly relate to the lipophilicity of an analyte. i.e. retention time is increased by increasing the lipophilicity or hydrophobicity of the analyte. For example, high capacity factors are indicative of a strong interaction with the lipophilic stationary phase and, thus, the strong lipophilic character of an analyte.[94]

1.7.4 Introduction to van Deemter Equation

In liquid chromatography, the flow rate of the mobile phase is an important factor that determines the partition efficiency of solutes under a given set of other conditions. The relationship between the partition efficiency and flow rate (the relationship between the efficiency of the column and the mechanism behind band broadening) could be described by an equation known as the van Deemter equation. In a simplified form, the van Deemter equation is:

$$\mathbf{H}\ (\text{or HETP}) = \mathbf{A} + (\mathbf{B} / u) + \mathbf{C}u$$

Where:

- **H** (Height equivalent per theoretical plate) is given as partition efficiency in terms of theoretical plate number divided by the length of the separation column (H = L / N); u, the flow rate of the mobile phase; and A, B, and C are constants.

- **A** is the Eddy-diffusion parameter
- **B** is the longitudinal diffusion coefficient
- **C** is the resistance to mass transfer of the analyte between mobile and stationary phases
- u is the average mobile phase velocity

When H is plotted against u in the coordinate, it forms a characteristic U-shape curve where the flow rate that gives the highest partition efficiency is at the bottom of the curve. A lower flow rate will result in a loss of efficiency due to the longitudinal diffusion caused by increased elution time (due to increased B/u), whereas a higher flow rate will cause loss of efficiency by insufficient time for solute partitioning between the two phases (due to increased Cu). [95], [96]

1.7.5 Introduction to effects of temperature (Van't Hoff equation)

In a study by Edge *et al.*, the use of elevated temperatures led to elution of compounds that would otherwise be retained on the column at low temperatures in reversed-phase chromatography. The reduction in the viscosity of the mobile phase also allowed for higher flow rates, improved the mass transfer within the chromatographic system and increased the rate of diffusion. Therefore, with increasing temperatures, the optimal flow rate for chromatographic efficiency is obtained.

By increasing temperature there is a reduction in the retention factor, this common mechanism can be understood by the use of a Van't Hoff plot, which shows the relationship between the retention factor and the absolute temperature. The linearity of the Van't Hoff plots indicates that the retention mechanism is not changing with increasing temperature. Since the Van't Hoff relationship is based on an equilibrium existing between the entropy and enthalpy within a system. Entropy (ΔS) is a measure of the random activity in a system, whereas enthalpy (ΔH) is a measure of the overall amount of energy in the system.

Binding is a process controlled by thermodynamics, it is evident that the enthalpic optimization of a compound is critical for achieving extremely high affinity. In addition, because the enthalpy and entropy changes reflect different types of interactions, other drug properties, like selectivity, are also affected by the enthalpy/entropy balance of a compound. Enthalpic optimization is difficult but can be facilitated by monitoring the enthalpic and entropic consequences of introducing or modifying different chemical functionalities. [97], [98]

Based on the data produced in a study by Edge *et al.*, a model has been developed that enables the accurate prediction of pressures across a HPLC column at a variety of temperatures and flow rates that would enable rapid method development to be performed. This model demonstrates the relationship between temperature, flow and pressure. The Van't Hoff plots for the test probes run on the Acquity column in this experiment demonstrated the linear relationship that exists between the log of the retention time and reciprocal temperature. In addition, the inset shows the point at which the elution order is reversed for the compounds analysed. The model compounds used in this study were antipyrine, aminohippuric acid, paracetamol, hydroxyantipyrine, aminoantipyrine, atenolol, aminobenzoic acid, theophylline, phenacetin, and caffeine.

From the results, it is worth noting the effect that temperature can have on chromatographic selectivity. In the plot, two of the test probes are highlighted, caffeine and aminoantipyrine, and it can be seen that the plots actually cross, indicating that there was a reversal in the elution order. Thus, whilst separated at low temperature the peaks coalesced at 113°C but as the temperature was raised further they once again start to separate relative to each other, but with a reversed elution order. [98]

1.7.5.1 Modelling the pressure–temperature-flow rate parameter space

For effective utilisation of temperature as a parameter in HPLC and UPLC it was considered to be advantageous to devise a simple, predictive model,

which would allow for characterisation of the chromatographic system. This model is based around two fundamental concepts.

Concept 1: Viscosity is the dominant temperature dependent variable within the system. It is assumed that the temperature only affects the viscosity directly; all other physical parameters are affected indirectly by the change in viscosity.

Concept 2: This concept depends on the flow and pressure drop in the system.

Thus a plot of ln P versus $1/T$ will give a linear plot where the gradient is a term relating to the physical variation of viscosity of the fluid to temperature. If the model is correct then this term will be independent of the column characteristics, thus once calculated for one mobile phase, it can be used for all columns using that mobile phase. [98]

1.7.6 GC theory and Golay equation

Gas chromatography (GC) is used for separating and analyzing compounds that can be vaporized without decomposition. In GC, the sample is injected into the instrument where it is vaporized and mixes with the carrier gas to become a part of the mobile phase. This mobile phase is then carried through to the chromatographic column where it interacts with the stationary phase of the column i.e. the gaseous compounds being analyzed interact with the walls of the column, which is coated with a stationary phase. This causes each compound to elute at a different time, known as the retention time of the compound.

1.7.6.1 Golay equation

The van Deemter equation describes the main factors contributing to column (packed) band broadening. This was described in Section 1.7.4. In 1958, Golay described a similar relationship to deal with capillary gas chromatography columns, which contain no packing material and therefore do not possess an Eddy-diffusion parameter (A term). Thus the Golay equation is defined as:

$$HETP = B / u + (C_s + C_M).u \qquad \textbf{(Golay equation)}$$

Where, C_s = mass transfer in the stationary phase and C_M = mass transfer in the mobile phase [99], [100]

1.8 Aims and objectives

The overall aim of this project is to develop analytical methods for the detection and quantification of diphenidine-derivatives (see Figure 11 for the chemical structures of all the tested compounds in this research project) in the laboratory, which can be applied to a real world setting for testing NPS samples encountered within a forensic framework.

The requirement for this study is because **1** and its derivatives are dissociative NPS that pose a threat for public health and safety, with many reported deaths and toxicities due to their extensive use in addition to the related clinical and forensic toxicology case reports in many countries. In general, improving efforts in the detection and identification of these dangerous substances have become as a global analytical challenge.

In order to achieve this aim, many diphenidine-derived compounds will be screened through the development of new chromatographic methods for the analysis of their positional isomers.

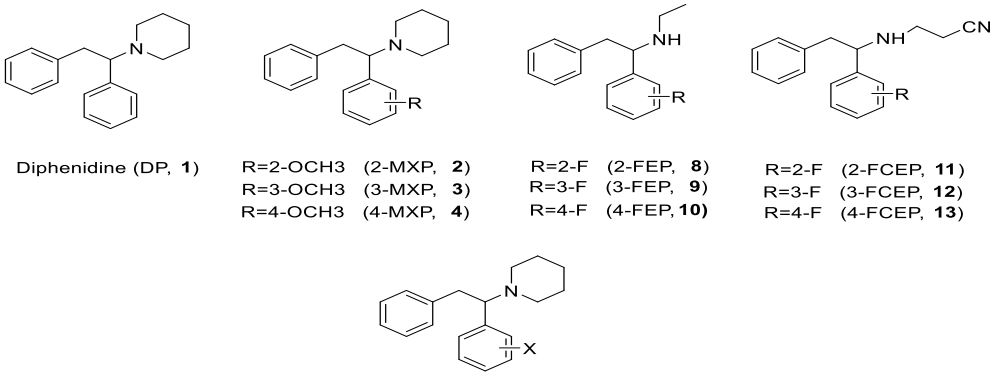


Figure 11. Chemical structures of diphenidine and twenty-one derived compounds tested in this research project

The main objective of the study is to decide whether HPLC can be used to qualitatively and quantitatively test diphenidine-derived compounds, which if achieved, would have a wide utility in forensic applications, particularly in analysing bulk/seized samples suspected to contain these harmful substances.

In this research project, diphenidine (**1**) and twenty-one diphenidine derived compounds will be tested, the first group to be investigated is methoxphenidine, MXP regioisomers (**2**, **3** and **4**, see Figure 11), these will be tested on HPLC and GC-MS in order to be detected, quantified and separated. While, the second group is the fluorinated ephenidine, FEP regioisomers (**8**, **9** and **10**), which will be tested using the developed/validated HPLC method that was applied for methoxphenidine isomers as stated in the first study. The third group is the fluorocyanoephenidine, FCEP regioisomers (**11**, **12** and **13**), which will be investigated by both reverse phase and gradient HPLC methods together with testing on three different GC columns in order to detect, determine and separate them.

The last group are the halogenated diphenidine, Halo DP regioisomers (**14** - **25**). These twelve compounds will be examined using HPLC in four different groups (fluoro, chloro, bromo and iodo isomers) in order to test whether the developed HPLC method in this study is able to detect and separate all these regioisomers with excellent resolution values (equal or greater than 2) i.e. by achieving fully base line separation, this aim is not only required in the case of Halo DP but also for all diphenidine-derived compounds investigated in this research project.

Chapter 2: Materials and Methods

2.1 Separation of the regioisomers of methoxphenidine (MXP) using Reverse Phase HPLC.

2.1.1 General experimental and characterisation of the regioisomeric methoxphenidine (MXP) analytes

All reagents were obtained from Sigma-Aldrich Limited (Gillingham, UK) and were used without further purification. Methoxphenidine (2-MXP, 3-MXP and 4-MXP), were synthesised at Manchester Metropolitan University under UK Home Office Licence. ^{1}H-NMR (10 mg/600µL in DMSO-d_6) and ^{13}C-NMR spectra (20 mg/600µL in DMSO-d_6) were acquired on a JEOL AS-400 (JEOL, Tokyo, Japan) NMR spectrometer operating at a ^{1}H resonance frequency of 400 MHz and referenced to the residual solvent peak (^{1}H-NMR, δ = 5.32; ^{13}C-NMR, δ = 53.84 respectively) [101]. IR spectra were obtained in the range 4000-400 cm^{-1} using a Thermo Scietific Nicolet Is10ATR-FTIR instrument (Thermo Scientific, Rochester, USA) on an IR spectrometer. GC-MS spectra were recorded on an (Agilent 6850 Series GC System, The GC was coupled to an Agilent 5973 Network Mass Selective Detector, USA). Ultraviolet spectra were obtained using a UV spectrometer (Agilent 8453, UV-VIS), the separate MXP regioisomers samples (40 mg/100 mL) were dissolved in mobile phase [acetonitrile: ammonium acetate (55:45% v/v)]. The absorbance and maximum wavelength for each isomer was detected. Thin layer chromatography (TLC) was carried out on aluminum-backed SiO_2 plates (Merck, Germany) and spots were visualized using ultra-violet light (254 nm). The mobile phase used was dichloromethane-methanol (9:1 *v/v*) containing 0.8% ammonia (7 N in methanol). The developed plate was viewed under UV light (254 nm) and any spots noted. The plate was sprayed with modified Dragendorff-Ludy-Tenger reagent.[102] The blood-red spots marked with a pencil and the Retention Factor (R_f) and Relative Retention Factor (RR_f, with respect to diphenidine) calculated for each analyte. The uncorrected melting points were measured

using Stuart SMP10 apparatus, (Bibby Sterilin Ltd, Staffordshire, UK). In all HPLC experiments, all the compounds (**1**-**25**) tested in this project were more soluble in acetonitrile, and less soluble in water and methanol. HPLC data was collected using a Agilent HP series 1100 Liquid Chromatogram. Two versions (2004) are available: one ("online") in connection with the modules of the HPLC chain is designed to control instruments and run experiments, and the other ("offline"), without a connection with the HPLC chain, is designed to analyse data (see Table 2 for more details).

Table 2. The HPLC system parameters for method development and validation of MXP isomer separation

Equipment /Part	**Model/ Description**
HPLC	Agilent HP Series 1100 Liquid Chromatogram
Degasser	In-line degasser
Auto sampler	100 place auto sampler
Pump	Binary Pump
Detector	DAD (Diode-Array Detector)
Software	ChemStation

2.1.2 Synthesis of diphenidine and methoxphenidine

The hydrochloride salts of diphenidine and its methoxy-substituted derivatives were prepared using an adaptation of the method reported by Geyer *et al.* [74] with the following modifications: To a dried round-bottomed flask (100 mL) containing zinc dust (2.0 g, 30 mmol) suspended in acetonitrile (40 mL), was added benzyl bromide (0.4 mL) and trifluoroacetic acid (0.2 mL). The resulting solution was stirred for 5 min and then benzyl bromide (3.0 mL, 25 mmol), piperidine (0.99 mL, 10 mmol) followed by the pre-requisite benzaldehyde (11 mmol), were introduced to the mixture, and the solution was stirred at room temperature for an additional 1 h (CARE! Exothermic). The resulting solution was poured into a saturated aqueous NH_4Cl solution (150 mL) and extracted

with dichloromethane (2×100 mL). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to give a crude yellowish oil. The oil was dissolved in diethyl ether (150 mL) and concentrated sulphuric acid (0.75 mL) was added dropwise, to the vigorously stirred solution. After five minutes, the precipitated ammonium salt was filtered, washed with diethyl ether (2×50 mL) and air-dried for 5 – 10 minutes. The ammonium salt was re-dissolved in aqueous sodium hydroxide (5% w/v, 150 mL) and then extracted with dichloromethane (2×150 mL). The combined organic fractions were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellowish oil. The oil was dissolved in diethyl ether (200 mL), treated with hydrogen chloride (4M in dioxane, 3.0 mL, 12 mmol) and left to stand for 5 minutes. The crystallized products were filtered and washed sequentially with the minimum volume of ice-cold acetone and ice-cold ethyl acetate-diethyl ether (1:5) to afford the corresponding hydrochloride salts as colourless to off-white powders (>99.5% by elemental analysis), which were fully structurally characterized by ^{1}H-NMR, ^{13}C-NMR and FTIR. Yields of products (after purification): diphenidine hydrochloride (29%); 2-methoxphenidine hydrochloride (2-MXP, 35%); 3-methoxphenidine hydrochloride (3-MXP, 21%); 4-methoxphenidine hydrochloride (4-MXP, 25%).

*2-Methoxphenidine hydrochloride (**2**)*: For ^{1}H NMR, ^{13}C NMR, FTIR, GC-EI-MS, melting point and UV data see Table 3.

*3-Methoxphenidine hydrochloride (**3**)* and *4-Methoxphenidine hydrochloride (**4**):* For ^{1}H NMR, ^{13}C NMR, FTIR, GC-EI-MS, melting point and UV data (See Appendix A 1, Appendix A 2).

Table 3. FTIR, GC-EI-MS, melting point and UV* data of **2**

Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	GC-EI-MS (m/z)	Mpt (uncorrected), (˚C)
1	1.15-1.45, m, 2H	22.33	1601.08 2490.24 2935.49 3435.80	**204** 188 121 91 65	135-137
2	1.80, br.s, 2H	35.86			
3	2.05-2.30, m, 2H	44.33			
4	2.31-2.66, m, 2H	52.81			
5	3.45, d, 2H	55.67			
6	3.53-3.81, m, 2H	111.5			
7	3.98, d, 2H	119.68			
8	5.02, br.s, 1H	126.55			
9	6.96-7.03, m, 3H	128.21			
10	6.85, dd, 2H	129.18			
11	6.99-7.47, m,1H	130.27			
12	7.75, br.s, 1H	136.46			
13	12.33, br.s, 1H	158.43			

*^{1}H-NMR (400 MHz, DMSO-d_6); ^{13}C-NMR (100 MHz, DMSO-d_6); GC-EI-MS: m/z **204** (base peak); UV (0.3 mg/mL in acetonitrile: ammonium acetate 55:45 % v/v), λ_{max} = 278 nm, abs.= 1.31, ε_{278}= 1290 L mol^{-1} cm.$^{-1}$

2.1.3 Solutions preparation

2.1.3.1 Preparation of HPLC column test mixture

Biphenyl (60 mg, 0.4 mmol), phenanthrene (40 mg, 0.2 mmol) were transferred to a 100 mL volumetric flask. To this, dimethyl phthalate (310 µL, 1.9 mmol) and toluene (2400 µL) were added. The mixture was then dissolved in methanol:water (85:15% v/v) and then made up to100 mL. To act as an injector marker, uracil (2.5 mg, 0.02 mmol) was dissolved in methanol: water (85:15% v/v, 50 ml).

2.1.3.2 Preparation of 0.1% formic acid

To HPLC grade water (500 mL) was added formic acid (500 µL) to achieve a concentration of 0.1% v/v for the aqueous part of the mobile phase. Similarly, for the organic component, formic acid (500 µL) was added to HPLC grade acetonitrile (500 mL) to again achieve a concentration of 0.1% v/v. The solutions were then filtered and degassed under vacuum using a Nylon membrane filter (Whatman, 0.45 µm, 47 mm diameter). Formic acid 0.1 % is added to the improve the chromatographic peak shape and to provide a source of protons in reverse phase HPLC experiments.

2.1.3.3 Preparation of aqueous ammonium acetate

Ammonium acetate (3.85 g, 50 mmol) was dissolved in HPLC grade water (500 mL). This created a stock solution of 100 mM ammonium acetate solution which would be diluted accordingly for the mobile phase. For the mobile phase composition of acetonitrile:20 mM ammonium acetate in water (55:45% v/v), 100 mL of the stock solution was added to acetonitrile (550 mL) and water (350 mL), to give a dilution factor of one in five for the ammonium acetate in water and a concentration of 20 mM. All mobile phase combinations were filtered and de-gassed for 10 minutes at 25°C using an ultrasonic path under vacuum using a Nylon membrane filter (Whatman, 0.45 µm, 47 mm diameter). Ammonium acetate provides buffering around pH 4.75 (the pK_a of acetic acid) and around pH 9.25 (the pK_a of ammonium). [103] Mobile phase; acetonitrile:ammonium acetate (55:45% v/v) was used without preheating or

cooling because no band broadening was produced in the run, during method development.

2.1.3.4 Sample preparation (stock solution of 2-, 3- and 4-MXPs)

Each of the separate MXP isomers (5 mg, 17 mmol) were dissolved in acetonitrile (25 mL) and 0.1% formic acid in water (25 mL, prepared as described in section 2.1.3.2) to give a total volume of 50 mL. The stock solution (1 mL) was used then diluted with water (10 mL) to produce a final stock solution which had a concentration of 10 μg mL^{-1} of each isomer.

2.1.4 Method development (HPLC separation of MXP isomers)

The HPLC method conditions: column selection and size, temp, mobile phase and run as an isocratic (adapted from McLaughlin's HPLC method).[75] The UV max of MXP isomers is 278 nm (**2**), 277 nm (**3**) and 273 nm (**4**), and the 278 nm was applied for all 3 isomers (McLaughlin *et al*. used 210 nm for all MXP isomers).HPLC conditions utilised are outlined in the following table:

Table 4. The HPLC conditions used in the injection of the linearity samples of methoxphenidine

Parameter	**Information/Value/Percentage**
Mobile Phase	Acetonitrile:20 mM ammonium acetate in water (55:45% v/v)
Temperature	50°C (adapted from McLaughlin's method)
Injection Volume	20 μL
Flow Rate	1.0 mL min^{-1}
Detection (UV) Wavelength	278 nm (from method development data)

2.1.5 HPLC method validation of MXP isomers

The HPLC method was validated in accordance with the ICH guidelines, Q 2 (R1) Validation of Analytical Procedures, 2005), using the following

parameters: linearity, accuracy, precision, specificity, limit of detection (LOD), limit of quantification (LOQ) and system suitability [resolution (R_s), column efficiency (N), peak asymmetry (A_s)]. *Linearity, precision and system suitability tests:* six replicate injections of the calibration standards (*vide supra*) were performed and the data analysed under the same conditions. The %RSD was calculated for each replicate sample. *Specificity:* six replicate injections of the specificity standards were performed and the data analysed under the same conditions. *Limits of detection and quantification:* six replicate injections of the calibration standards were performed and the data analysed under the same conditions. The limits of detection and quantification were calculated based on the standard deviation of the response and the slope.

2.1.6 Calibration standards (for linearity studies)

2-, 3- or 4-MXP (8 mg, 0.027 mmol) was dissolved in formic acid in acetonitrile (50 mL) and formic acid in water (50 ml), (these solutions/solvents were made as mentioned in Section 2.1.6.2) to create a stock solution of 80 µg mL^{-1}. A dilution scheme was carried using this stock solution to create calibration standards in the range of 2.5, 5, 10, 20, 40 µg mL^{-1}. All dilutions were performed using HPLC grade water. The standards were injected using the system described in Table 4 and the column ACE 5 C18-AR (150 x 4.6 mm, 5 µm particle size). They were injected in order of increasing concentration; two injections of the blank at the beginning of the calibration series, followed by six injections of each concentration. Injecting samples in an increased order to avoid any possible trace amounts of the previous sample left in the injector.

2.1.7 Calibration Standards (for precision and accuracy studies)

A calibration range of 2.5, 5, 8, 10, 12, 20, 40 µg mL^{-1} was used in the determination of the accuracy and precision samples. As with the linearity standards, the solutions 8, 10, 12 µg mL^{-1} were injected in order of increasing concentration with two blank injections at the beginning of the calibration series. The HPLC conditions used are the same as described in table 2.

2.1.8 Preparation of samples for accuracy and precision studies

To a 100 mL flask, MXP isomers (5 mg, 0.017 mmol) were dissolved in formic acid in acetonitrile (50 mL) and formic acid in water was added (to make a 100 mL solution), to create a stock containing 100 µg mL^{-1} of 2-, 3- or 4-MXP. The dilution scheme 2.5, 5, 8, 10, 12, 20, 40 µg mL^{-1} was carried out from the stock solution.

Each dilution was repeated six times and all were made into HPLC grade water. The calibration standards were injected six times each and used in method development and validation. While, the concentrations of 8, 10, 12 µg mL^{-1} were used in determination of accuracy. Each solution (six of each concentration, totalling 30 samples) was injected in replicates using the system detailed in Table 2 and a C18-AR column. Blank injections (2x) were carried out at the beginning of the calibration series. Peak area, %recovery and %RSD were determined.

2.1.9 Robustness studies

In this study, the following parameters were investigated (the working concentration of MXP isomers is 10 µg mL^{-1}): *Temperature:* 48, 50, 52˚C (2 blanks were injected prior to injecting MXP isomers 10 times each); *Flow Rate:* 0.9, 1.0, 1.1 mL min^{-1} (2 blanks were injected prior to injecting MXP isomers 10 times each); *Mobile Phase:* 54:46, 55:45, 56:44 % v/v (2 blanks were injected prior to injecting MXP isomers 10 times each); *Intra-day* (am and pm) and *Intra-day* (am and pm) and *inter-day* (pm plus am and pm) precision was also carried out (2 blank injections were carried out prior to injecting MXP isomers 10 times each). Peak area, retention time and relative retention time were determined. Retention factor (k) can also be used as a parameter in HPLC calculations instead of using retention time (t_R).

2.1.10 Injection of adulterants/diluents for Specificity (MXP isomers)

In order to assess the specificity of the method, standards of the following adulterants: caffeine, paracetamol and benzocaine were prepared at concentrations of 100 µg mL^{-1}, in addition to preparing solutions of 4-MMC,

diphenidine at concentrations of 100 µg mL^{-1} and a solution of uracil at a concentration of 10 µg mL^{-1}.

The specificity of the method was performed using two replicate injections of the specificity standards and the data analysed under the same conditions. The results showed that the strongly UV-absorbing components (benzocaine, caffeine and paracetamol) demonstrated baseline separation from the target analytes (methoxphenidine isomers, 4-MMC and diphenidine).

2.1.11 Forensic application

Two street samples were obtained from independent Internet vendor (BRC Fine Chemicals Limited, https://www.brc-finechemicals.com) as white crystalline powders in clear zip-lock bags, prior to the legislative change (20th May 2016). The samples were weighed accurately (2 times each) and dissolved in acetonitrile:ammonium acetate 55:45% v/v in a 50 mL volumetric flask, and then diluted with HPLC grade water in a 10 mL volumetric flask to a working concentration of 10 µg mL^{-1}. Three injections of each weighing were injected into the system.

2.1.12 Application of the HPLC standard method for separation of some diphenidine derivatives present in mixtures

In this experiment, the following compounds; 2,3 and 4-methoxphenidine isomers (MXP), 3,4,5-trimethoxyphenidine (3,4,5-TMXP), 2,3 and 4-trifluoromethoxphenidine isomers (TFMXP), 1- and 2-naphenidine, methylenedioxydiphenidine derivatives, (2,3-MDDP), (3,4-MDDP), IAS 013 and uracil (as a void marker) were tested for detection (determination of retention time) and separation by applying the HPLC validated standard method (Table 5). The compounds were synthesised in-house, by the MANchester DRug Analysis & Knowledge Exchange (MANDRAKE) team, using the procedures reported by Geyer *et al*. [74]

The λ_{max} for each of the above listed compounds was measured prior to commencing HPLC experiments. The experimental parameters were as follows: mobile phase is acetonitrile:ammonium 55:45% v/v, temperature 50˚C,

flow rate 1 mL min^{-1}, sample concentration is 10 µg mL^{-1}. Uracil concentration is 1 µg mL^{-1} (dissolved in MP). Two blank (MP) injections and two injections of the mixture were run, respectively.

Table 5. Application of the HPLC standard method in the separation of two mixtures (A and B) containing 12 diphenidine derivatives

Mixture A	**Mixture B**
Uracil	Uracil
1-Naphenidine	2-MXP
2-Naphenidine	3-MXP
2,3-MDDP	4-MXP
3,4-MDDP	2-TFMXP
3,4,5-TMXP	3-TFMXP
IAS-013	4-TFMXP

2.2 Fluephenidines (FEP)

The general experimental details for the FEP study were analogous to those used in the MXP study (Section 2.1.1.1) with the following modifications:

The ^{19}F-NMR spectra (20 mg/600 µL in DMSO containing 0.03% *v/v* trifluoroacetic acid, TFA) for FEP compounds were acquired on the same instrument and referenced to TFA (^{19}F-NMR, δ = -76.55 ppm).

*2-Fluephenidine (**8**), 3-Fluephenidine (**9**)* and *4-Fluephenidine (**10**):* For ^{1}H NMR, ^{13}C NMR, FTIR, melting point and UV data (See Appendix A 3, Appendix A 4 and Appendix A 5).

2.2.1 Forensic Application

Three bulk forensic samples of FEP were obtained from Greater Manchester Police via the MANchester DRug Analysis and Knowledge Exchange

(MANDRAKE) partnership. The three samples were analysed using the developed HPLC method in this study i.e. using acetonitrile:ammonium acetate 25:75% v/v as the mobile phase.

The samples were tested in a working concentration of 50 μg mL^{-1}, each sample was injected three times into the HPLC system. For comparison with street samples, three samples of the mix of 2-, 3- and 4-FEP isomers were injected separately into the system in concentrations of 50 μg mL^{-1}, these were taken from the stock solutions of the calibration standards series of FEP isomers.

2.3 Fluorocyanoephenidines (FCEP)

The general experimental details for the FCEP study were analogous to those used in the MXP study (Section 2.1.1) with the following modifications:

2.3.1 Synthesis of FCEP isomers

To a dried round-bottomed flask (250 mL) was added a mixture of zinc dust (2.0 g, 30 mmol) suspended in acetonitrile (40 mL), to this benzyl bromide (0.4 mL), and trifluoroacetic acid (0.2 mL) was added. The resulting solution was stirred for an additional 5 min and then benzyl bromide (3.0 mL, 25 mmol), aminopropionitrile (0.99 mL, 10 mmol), followed by benzaldehyde (1.17 g, 1.12 mL, 11 mmol) were introduced to the mixture, and the solution was stirred at room temperature for 1h (CARE! Exothermic). Saturated aqueous NH_4Cl solution (150 mL) was poured into the resulting solution and extracted with dichloromethane (2×100 mL). The combined organic layers were dried (Na_2SO_4) and concentrated *in vacuo* to give a crude yellowish oil. The oil was dissolved in diethyl ether (150 mL) and concentrated sulphuric acid (0.75 mL) was added dropwise, cautiously to the vigorously stirred solution. After five minutes, the precipitated ammonium salt was filtered, re-dissolved in aqueous sodium hydroxide (5% w/v, 150 mL) and then extracted with dichloromethane (2×150 mL). The combined organic fractions were dried (Na_2SO_4) and concentrated *in vacuo* to give a yellowish oil. The oil was dissolved in diethyl ether (5 mL), treated with hydrogen chloride (4M in dioxane, 5.0 mL, 20 mmol)

and stirred for 30 minutes. The volatiles were removed *in vacuo* and the products recrystallized from acetone to afford the corresponding hydrochloride salts as colourless to off-white powders.

*2-Fluorocyanoephenidine hydrochloride (**11**), 3-Fluorocyanoephenidine hydrochloride (**12**)* and *4-Fluorocyanoephenidine hydrochloride (**13**):* For ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, FTIR, melting point and UV data see Appendix A 6, Appendix A 7 and Appendix A 8.

2.4 The halogenated diphenidine isomers

The general experimental details for the halogenated diphenidine isomers study were analogous to those used in the MXP study (Section 2.1.1.1) with the following modifications:

2.4.1 Reagents and Solvents

All reagents and solvents were obtained as mentioned in Section 2.1.1.1 and were used without further purification. The halogenated diphenidine (2-, 3- and 4-regioisomers); fluorodiphenidine (FDP), chlorodiphenidine (CLDP), bromodiphenidine (BrDP) and iododiphenidine (IDP), were synthesised at Manchester Metropolitan University under UK Home Office Licence.

2.4.2 Chromatographic Conditions

These conditions were analogous to MXP study with the following modifications: Acetonitrile:ammonium acetate (67:33% v/v), flow rate (1.5 mL min^{-1}) and detection wavelength (220 nm). Two blank injections (30 mins each) followed by two injections of each halogenated diphenidine isomer (10 min) and one injection of washing solution, acetonitrile:ammonium acetate (50:50% v/v, 20 min).

2.4.3 Standard Solutions

The standard linearity stock solutions of 2-, 3- and 4-halogenated diphenidine isomers were made in 80 µg mL^{-1} concentration followed by the following serial dilution 1.25, 2.5, 5, 10 and 20 µg mL^{-1} which was injected in the HPLC system

mentioned above (see Section 2.4.1.2). Whereas the concentrations 8, 10 and 12 μg mL^{-1} were used to determine the method accuracy.

2.4.4 Forensic application

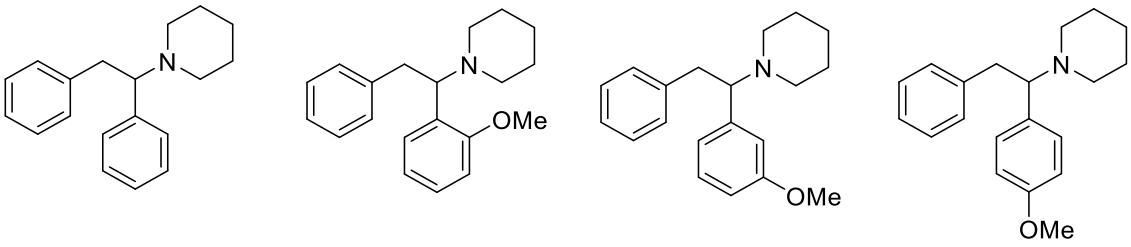
Two bulk forensic samples (SS1H and SS2H) were obtained from Greater Manchester Police as a white crystalline powder. The samples were tested in a working concentration of 10 μg mL^{-1} (in replicate). Each sample was injected three times into the HPLC system. For comparison with street samples, three samples of the mix of 2-, 3- and 4-halogenated diphenidine isomers were injected separately into the system in concentrations of 10 μg mL^{-1}, these were taken from the stock solutions of the calibration standards series of the three groups of the halogenated diphenidine isomers.

Chapter 3: Development and validation for the separation of the regioisomers of methoxphenidine using Reversed Phase HPLC.

3.1 Methoxphenidine

Methoxphenidine is a diphenidine (1) derivative; known by the following names (methoxydiphenidine, 2-MeO-diphenidine, 2-MXP, (2) and it is also a dissociative of the diarylethylamine class that has been sold online as a designer drug (see Figure 12 for chemical structures of diphenidine and MXP isomers).[105]

Methoxphenidine was first reported in a 1989 patent where it was tested as a treatment for neurotoxic injury. In 2013, the arylcyclohexylamines including diphenidine and the related methoxy-substituted compounds, methoxphenidine, became available on the black market, where they are encountered in both a powder and tablet form. Diphenidine [106] has greater affinity for the *N*-methyl-D-aspartate receptor (NMDA receptor or NMDAR) than methoxphenidine. The receptor is a glutamate receptor and ion channel protein found in neurons (Figure 13). It is activated when glutamate binds to it, and when activated it allows positively charged ions to flow through the cell membrane. The NMDAR is very important for controlling synaptic plasticity and memory function.[107] Many reports reveal methoxphenidine possesses higher oral potency.[78] These drugs mediate their dissociative-psychoactive effects via potent *N*-methyl-D-aspartate receptor antagonism. Monoamine transporter inhibition could, however, contribute to their psychoactive properties. Methoxphenidine was also studied as it stimulates the tendency of the abusers to sabotage less than that caused by diphenidine with more enjoyable effects.[108]



Diphenidine, (**1**) 2-MXP, (**2**) 3-MXP, (**3**) 4-MXP, (**4**)

Figure 12. Dissociative Anaesthetics, diphenidine, 2-, 3- and 4-methoxphenidine (MXP)

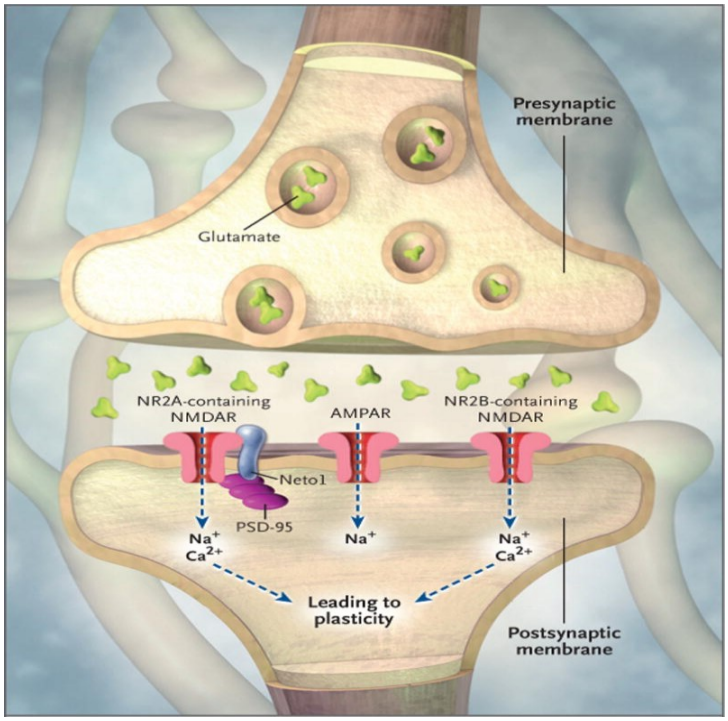


Figure 13. Neuron showing glutamate receptors and synaptic plasticity.[109]

The NMDAR is very important for controlling synaptic plasticity and memory function.[107] Methoxphenidine (2) is marketed as a legal replacement for methoxetamine (6), diphenidine (1) and ketamine (7). Figure 12 shows examples of some dissociative anaesthetics.

Methoxetamine (6) is a dissociative anaesthetic showing pharmacodynamic similarities with its analogue ketamine (7), a medication with demonstrated rapid-acting antidepressant effects. Like ketamine and other arylcyclohexylamine compounds, MXE is thought to be both a non-competitive NMDA antagonist and a dopamine reuptake inhibitor.[55] The receptor (NMDAR), is a glutamate receptor and ion channel protein found in neurons (Figure 13). It is activated when glutamate binds to it, and when activated it allows positively charged ions to flow through the cell membrane.

There is an urgent requirement to study and analyse these NPSs in more depth and detail i.e. diphenidine derivatives such as MXP isomers. This goal can be achieved by applying various analytical approaches such as the development of HPLC methods to detect, quantify and separate these regioisomers, which exist in a given sample(s). The urgent requirement is because of the speed and variety of drugs entering the market posing a new complex challenge for forensic toxicology as it is considered a threat to public health, especially when the content of NPS being sold is not reported on the label or is misleading.[61, 110] Furthermore, the detection of these substances in biological matrices can be difficult as the exact compounds of interest may not be known. Many NPSs are sold under the same brand name and therefore users themselves may not know what substances they have ingested.

Besides, concentration variability among the NPS samples and the presence of multiple psychoactive substances in single products represent risk factors for the users. In some NPS products there is no information on active ingredients, all these issues can lead to severe or toxic adverse effects produced by NPS especially knowing that only little is known about NPS metabolism and interaction with other medicines (drug-drug interaction).[111] Therefore, educating the public and raising their awareness about the use of these drugs, and also intervention by policy makers particularly in schools and universities, is an urgent requirement. [112, 113]

Perhaps more importantly is the continued monitoring of new trends in NPS (non-prescribed drugs) within the Internet and crypto markets. In addition, monitoring the rising market in prescribed drugs, such as benzodiazepine and non-benzodiazepine hypnotics (Z-hypnotic drugs), is a further area of concern.[114] As mentioned previously, longitudinal integrated monitoring systems incorporating international data from emergency departments, treatment services, toxicology services, police, sentinel groups and the internet are required continuously.[42]

3.2 Methods of analysis of MXP and other diphenidine derivatives

In a study by Baron *et al*., a number of NPS products were analysed by FTIR followed by GC-MS (as methanol extracts). The obtained spectra were compared to reference standards and the findings have confirmed the absence of the active ingredient in 6 out of 7 NPS products in question.[115]

In a different study by Hofer *et al*., the acute toxicity produced after the recreational use of the NPS methoxphenidine by using LC-MS toxicological screening method (plasma and urine samples) was studied. The outcome of this study revealed that methoxphenidine has similar toxic effects that are seen after administration of arylcyclohexylamine drugs, such as phencyclidine (PCP). These effects include hypertension, tachycardia and confusion.[116]

In forensic casework by Elliot *et al.*, three death cases were related to the detection of 2-MXP in post-mortem blood and urine. The 2-, 3- and 4-MXP isomers where synthesised and tested to confirm the identity and concentration of 2-MXP, in addition to the diphenidine that was also present. Analysis of these biofluids allowed the detection and characterisation of various metabolites including the suggested presence of hydroxyl 2-MXP using UHPLC and LC-MS-MS analysis The involvement of 2-MXP in the results of the current case studies are the first published fatalities and it also provides analytical information which will assist analytical toxicologists with future forensic samples.[83]

In Sweden, Helander *et al.* have reported an observational case series in an emergency room. The adverse effects noted have confirmed cases of intoxication associated with diphenidine or MXP. Nevertheless, these results suggest the probability of polysubstance use. NPS analysis was performed by multi-component LC-MS methods. The adverse effects noted in analytically confirmed cases of NPS intoxication involving diphenidine or MXP were similar to those reported for other dissociative substances such as ketamine and methoxetamine.[66]

In a different study, an autopsy case in which the cause of death was judged as poisoning by multiple new psychoactive substances, including AB-CHMINACA, 5-fluoro-AMB and diphenidine. In this study, LC-MS was used to test these compounds.[117]

In another study, a case of driving under the influence of MXP was studied and involved liquid-liquid extraction of MXP. Methoxphenidine serum samples, calibration or quality control standards were mixed with ketamine as the internal standard. MXP was extracted with ethyl acetate. The organic phase was dried under nitrogen, reconstituted in ammonium acetate (pH 3.2)/methanol/acetonitrile (50:10:40% v/v). Three microliters were injected into (LCMS/ MS) system. A five-point calibration curve covering a concentration range of 20–100 ng MXP/mL serum was prepared from drug-free serum by spiking. Imprecision and accuracy were tested at 20 and 50 ng MXP/mL serum (n=5, respectively). Further validation parameters could not be established due to the limited size of the powder specimen. Finally, MXP was detected and reported using LC-MS/MS.[118]

3.2.1 Instruments and methods utilised in the detection and separation of MXP isomers

Analysis of MXP was performed by Stachel *et al.*, on a mass spectrometer, interfaced to a HPLC pump and an auto sampler. Separation was achieved on a Luna C18 column (150×2.0 mm; 5 µm particle size), with ammonium acetate (pH 3.2)/methanol/acetonitrile (50:10:40 %v/v) as the mobile phase.[118]

From the results of the study performed by Stachel *et al*., amphetamine, MDMA, and MDA were detected in serum samples at a concentration of 111, 28, and 3 ng/mL, respectively. The concentration of MXP was found to be 57 ng/mL serum.[118] The concentration of MXP in this study was significantly lower than those in fatalities attributed to MXP. So far, the MXP toxicity in human has not been studied, but by comparison in structure to PCP or ketamine; for example, it is likely that MXP has severe psychotropic action in humans.[118]

In a different work by McLaughlin *et al*., the preparation of 2-, 3-, and 4-MXP isomers (**2**, **3** and **4**) was carried out by using a synthetic route which was first published by Le Gall *et al.* who employed an approach utilising one-step three-component coupling-reactions between an aromatic organozinc reagent, a secondary amine, and an aromatic aldehyde for the preparation of diarylmethylamines.[75, 119] The application of this one-step procedure provided a convenient route of synthesis of the desired isomers. An alternative synthesis method (Figure 14) was also explored for its applicability to the synthesis of 2-, 3-, and 4-MXP. This procedure involves the reaction of triethylamine (Et_3N) with a mixture of 2-methoxybenzoyl chloride and dimethyl hydroxylamine plus 4-dimethylaminopyridine (DAMP) to produce the Weinreb amide, prior to the addition of benzylmagnesium chloride ($PhCH_2MgCl$), ammonium acetate and sodium cyanoborohyrdide ($Na(CN)BH_3$). The last step involves the addition of dibromopentane $Br(CH_2)_5Br$ and potassium carbonate to yield the MXP isomers. In this method, piperidine is not required as a reagent. All three isomers could be distinguished by ^{1}H and ^{13}C NMR.

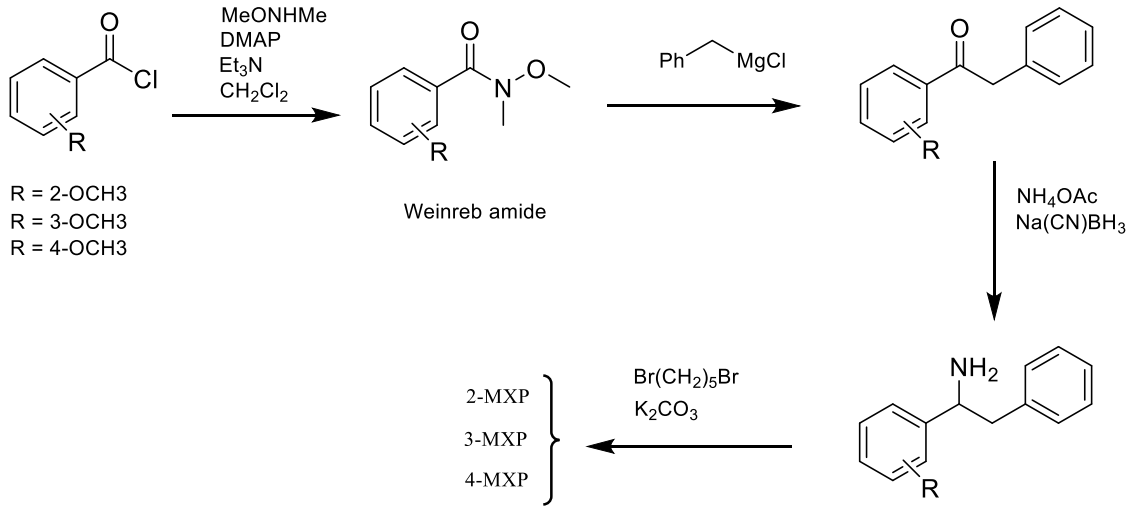


Figure 14. Synthesis of three MXP isomers

A further experimental approach focused on three samples believed to contain 2-MXP, which were obtained from three different Internet providers and analysed by GC-MS. A comparison with the synthesized reference material confirmed that all test purchase samples were consistent with the identity of the 2-MXP isomer as indicated on the product label. The employed GC method did allow for the separation of all three isomers as well. The samples labelled to contain 2-MXP and the MXP standards were also analysed using an alternative GC-EI quadrupole MS method. The GC method used was able to distinguish between all three isomers and baseline separation was achieved between each isomer. The retention times were recorded at 19.15 min, 19.54 min and 19.86 min for 2-MXP (**2**), 3-MXP (**3**) and 4-MXP (**4**) isomers, respectively. HPLC-MS was also utilized for the discrimination of the MXP isomers. Early attempts using an Allure® PFP Propyl column failed to resolve the isomers. However, switching to a phenyl hexyl column successfully permitted differentiation between isomers. The HPLC method achieved baseline separation for the **2** isomer and the **3** and **4** isomers were partially separated, although this appeared suitable for identification purposes.[74, 75, 88, 120]

In a study by Geyer *et al*, thirteen diphenidine derivatives were synthesised and then analysed by GC-MS using other rapid screening methods such as presumptive (colour) tests and TLC. Taken together, the results of this study have shown the ability of the developed GC–MS method to provide a screening protocol, which facilitates the separation and identification of these 13 substances.[74] In a recent research by Boateng *et al*, the focus was on studying the chromatographic retention behaviour, modelling and optimization of a UHPLC-UV separation of the regioisomers of methoxphenidine (**2**, **3** and **4**).[120]

In another recent study Lowdon *et al.*, the first developed Molecularly Imprinted Polymers (MIPs) for the specific detection of methoxphenidine (MXP) and its regioisomers was reported. Selectivity of the MIP towards MXP was studied by analysing mixtures and an acquired street sample with HPLC coupled to UV detection. The study demonstrates that the engineered polymers selectively extract MXP from heterogeneous samples, which makes for a very powerful diagnostic tool that can detect traces of MXP in complicated NPS samples.[88]

3.3 Recent studies and research on the positional MXP regioisomers with some comparisons with the developed HPLC method in this study

In 2015, Elliot *et al*., have studied HPLC-UV retention time (elution) and UV spectrum on MXP isomers, using Phenomenex Synergi column, mobile phase (the mobile phase consists of acetonitrile and triethylammonium phosphate (TEAP) as the buffer solution, at 30°C.[121] The findings from this study on HPLC-UV were as the following: the retention time of **2**, t_R =8.04 mins, at 278 nm; **3**, t_R =8.06 mins, at 276 nm and **4**, t_R =8.08 mins, at 229 and 272 nm. In addition, limit of detection, LOD of 0.05 mg/L and a limit of quantification, LOQ of 0.10 mg/L.[121] The results of the developed study that the total run time is less by more than two minutes in comparison with Elliot's findings. It is also clear that Elliot's work has not dealt with the separation of MXP isomers i.e. just with identification or detection. In 2016, McLaughlin *et al.*, have

investigated the RP-LC separation of the MXP regioisomers (**2**, **3** and **4**), which has been reported using a superficially porous phenyl hexyl material (i.e. 2.6 µm Kinetex) coupled with acetonitrile/formic acid gradient at 30 °C. While the 2-isomer was well resolved from the other two isomers, only partial separation of the 3- and 4-isomers was observed (the elution order was reported to be firstly **3**, then **4** and finally **2**). In contrast, the results of the developed HPLC method have shown a well-resolved baseline separation between all the three isomers in question (Table 7).

In 2018, Boateng *et al.,* studied 2D modelling on Agilent 1290 Infinity UHPLC (e.g. gradient time versus temperature) to optimize the gradient separation of the MXP isomers using a gradient and temperature design space.[120] This study has reported many findings: The retention/separation of MXP isomers is controlled by electrostatic/hydrophobic mechanisms. The stationary phase chemistry is not a major selectivity parameter. There was a synergistic effect between the electrostatic and partitioning mechanisms. Enhanced retention and separation of all MXP isomers was obtained at intermediate pH (6.8). The elution order at low pH (3) and at intermediate pH (6.8) was **2**, **3** and **4**, respectively. But at pH 10.7, MXP isomers were separated in the following order **4**, **3** and **2** at 278 nm (7 mins). In this project, the elution order in the developed HPLC method is **2**, **4** and **3**.[120] In 2018, a study by Lowdon *et al.*, selectivity of the MIP towards MXP is studied by analysing mixtures and an acquired street sample with HPLC-UV detection. The study demonstrates that the engineered polymers selectively extract MXP from heterogeneous samples, which makes for a very powerful diagnostic tool that can detect traces of MXP in complicated NPS samples. The developed HPLC method is superior to the previously reported GC-MS [74] and HPLC [75, 83] methods in terms of overall run time and resolution of the three regioisomers.

To summarise, and after analysing the prior discussed studies, there is an urgent requirement to study and analyse these NPSs (in more depth and detail). That is because of the health problems resulting from NPS use and the

lack of a quick, HPLC method in this study were superior to Elliot's method, the retention times and maximum wavelengths for methoxphenidine isomers were as follows: **2**, t_R =3.70 mins, at 278 nm; **3**, t_R = 5.82 mins, at 277 nm and **4**, t_R =4.33 mins, at 273 nm. LOD of 0.04-0.15 µg $mL^{-1}$and a LOQ of 0.38-0.47 µg mL^{-}1. Furthermore, it can be concluded from the findings of the present study that the new validated HPLC method is suitable and robust analytical method for MXP separation. This goal can be achieved by applying various analytical approaches such as the development of HPLC methods to detect, quantify and separate these regioisomers that exist in a given sample(s).

3.4 Synthesis and analysis of MXP

The three MXP derivatives were prepared by using the synthetic scheme (Figure 15), which shows the reaction of benzyl bromide with piperidine and the pre-requisite anisaldehydes (methoxybenzaldehydes) in the presence of zinc dust, acetonitrile and TFA. The three compounds were isolated as their corresponding hydrochloride salts. The samples were fully characterised and gave physical and spectroscopic data that were consistent with their proposed structures and the literature. The obtained data were on par with the analytical data obtained by McLaughlin *et al*, where the three MXP isomers were subjected to analytical characterization by gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled to various forms of mass spectrometry (MS).[75] Nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy and thin layer chromatography (TLC) were also employed. This was supported by the synthesis of all three isomers (**2**, **3** and **4**) by two different synthetic routes. The obtained results for the three purchased samples were consistent with the synthesized 2-MXP standard and differentiation between the isomers was possible.[75] Once the provenance of the samples had been confirmed the development of a suitable chromatographic method could be carried out in order to identify and separate these isomers.

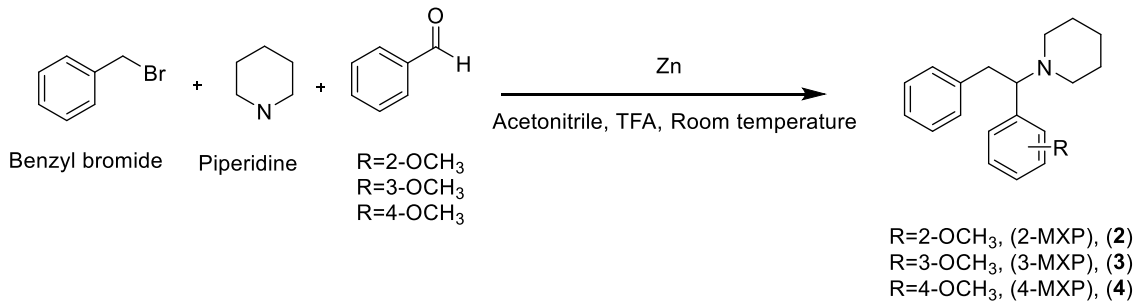


Figure 15. Synthesis of three MXP isomers.[78]

3.5 Results and discussion

3.5.1 Synthesis

Samples of the three methoxphenidine regioisomers were prepared as their corresponding hydrochloride salts. The hydrochloride salts of diphenidine and its methoxy-substituted derivatives were prepared using an adaptation of the method reported by McLaughlin *et al.*[75]

The actual yield of 2-methoxphenidine hydrochloride (2-MXP, 35%); 3-methoxphenidine hydrochloride (3-MXP, 21%); 4-methoxphenidine hydrochloride (4-MXP, 25%). This yield can be increased or improved in several ways such as rinsing glassware three times with reaction solvent, adding reagents dropwise if necessary, quenching the reaction exactly when it is complete and by storing compounds at low temperature if possible.

To ensure the authenticity of the materials utilised in this study, the synthesised samples (**2**–**4**) were fully structurally characterised by ^{1}H-NMR, ^{13}C-NMR and FTIR.

3.5.2 Infrared spectroscopy

In the present study, the IR spectra of the three MXP regioisomers were obtained. The IR spectra of all three MXP isomers are shown in Figure 16, (**2**), Appendix A 9, (**3**) and Appendix A 10, (**4**). The C-O stretch can be seen at around 1100 cm^{-1}. From the results of the IR, these C-O stretch vibrations were: 1114 cm^{-1} (**2**), 1154 cm^{-1} – (**3**) and finally 1100 cm^{-1}(**4**).

The other stretch is C-H, in which the values were as follows: 2935 cm^{-1} (**2**), 2941 cm^{-1} (3) and 2940 cm^{-1} (**4**). The C=C stretch of the aromatic rings is shown as follows: 1492 and 1601 cm^{-1} (**2**), 1493 and 1592 cm^{-1} (**3**) and 1500 and 1600 cm^{-1} (**4**). The N-H stretch region is at about 3400 cm^{-1} to 3500 cm^{-1}, from the results, firstly, there are two sharp and weak peaks at 3435 and 3511 cm^{-1} (**2**), while in the case of (**3**) there were no peaks in this region, this could be due to reversible hydrogen bonding between the hydrogen in HCl and the nitrogen in NH group, as a result, giving rise to a very weak IR signal. Finally, there is a broad weak over tone in the region of 3000 to 3500 cm^{-1} for (**4**).

In the fingerprint region, which is the region to the right-hand side of the diagram (from about 1500 to 500 cm^{-1}) usually contains a very complicated series of absorptions. The significance of the fingerprint region is that each different compound produces a different configuration of troughs in this part of the spectrum. These are mainly due to all manner of bending vibrations within the molecule. (1500-500 cm^{-1}), the bending signals can be noted, the *ortho* isomer has a peak at exactly 780 cm^{-1} (**2**), in the *meta* regioisomer, there are two peaks below 800 cm^{-1}, at 707 and 762 cm^{-1}, in addition to the presence of one peak at 873 cm^{-1} (**3**). The last observation for the *para* isomer, in this region, there is one peak above 800 cm^{-1}, at approximately 900 cm^{-1} (**4**). The IR findings for MXP isomers have shown the following: The C-O stretch for the three isomers located all in the region of about 1100 cm^{-1}. However, there is a difference of 40 cm^{-1} between **2** and **3** and 44 cm^{-1} between **3** and **4**.

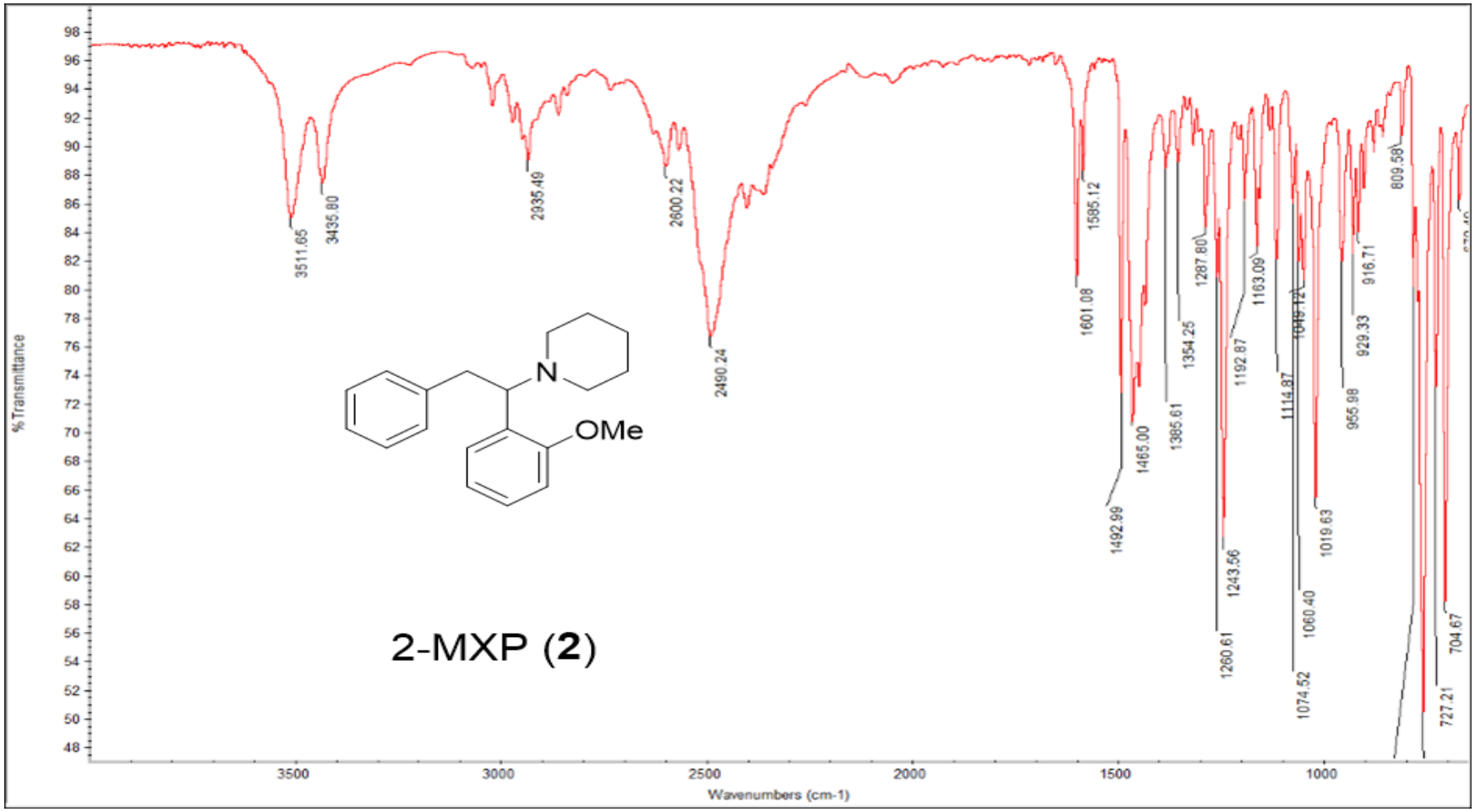


Figure 16. The ATR-FTIR spectrum of **2**

This could be due to the variation in the position of methoxy group and its electronic effect among these positional isomers. The band at 2935 cm^{-1} refers to C-H stretches (**2**, **3** and **4**). Moreover, the bands at 2940 (strong) and 2840 cm^{-1} (weak) are typical for the asymmetric and the symmetric (C-H) vibrations of CH_2 groups, respectively. The C-H wag (strong) at (770-735 cm^{-1}) is typical for *ortho*-substituted rings, this peak can be clearly seen in the fingerprint region for **2**, while it is not present in **3** and **4**.[122] However, the C-H stretches here are less important for differentiation because they exist in the vast majority of the organic compounds. It can be concluded that the IR spectrum gives an idea about the functional groups present in the chemical compound, which therefore, facilitates its identification.

3.5.3 Nuclear magnetic resonance

The NMR spectral data of the three MXP isomers were obtained (^{1}H and ^{13}C NMR spectra) using DMSO as a solvent. In the ^{1}H NMR spectrum of 2-MXP, there is a broad peak at δ 10.92; this is assigned to the NH group which is present as the compound was isolated as a hydrochloride salt i.e. this is possibly because of the formation of reversible hydrogen bond in the hydrochloride group with the nitrogen atom in the piperidine ring. In the aromatic region, there is a broad singlet that integrates to a single proton, a doublet of doublets of doublets (ddd) centred at δ 7.34 that again integrates to one proton and a multiplet at 7.16-6.97 ppm that integrates to seven protons. The ddd arises from one of the proton nuclei in the methoxybenzene ring having similar $^{3}J_{HH}$ couplings to two unique proton neighbours, and a fourth, smaller $^{4}J_{HH}$ coupling to a third proton nucleus through a “W” coupling. Two couplings were identified, one of 7.52 Hz which is a $^{3}J_{HH}$ coupling and the other being 1.46 Hz, which is of the right magnitude for a $^{4}J_{HH}$ coupling. In the ^{1}H-^{1}H COSY (Correlation Spectroscopy) spectrum, this peak possesses three cross-peaks, which reinforces the observations made by analysing the ^{1}H NMR spectrum.

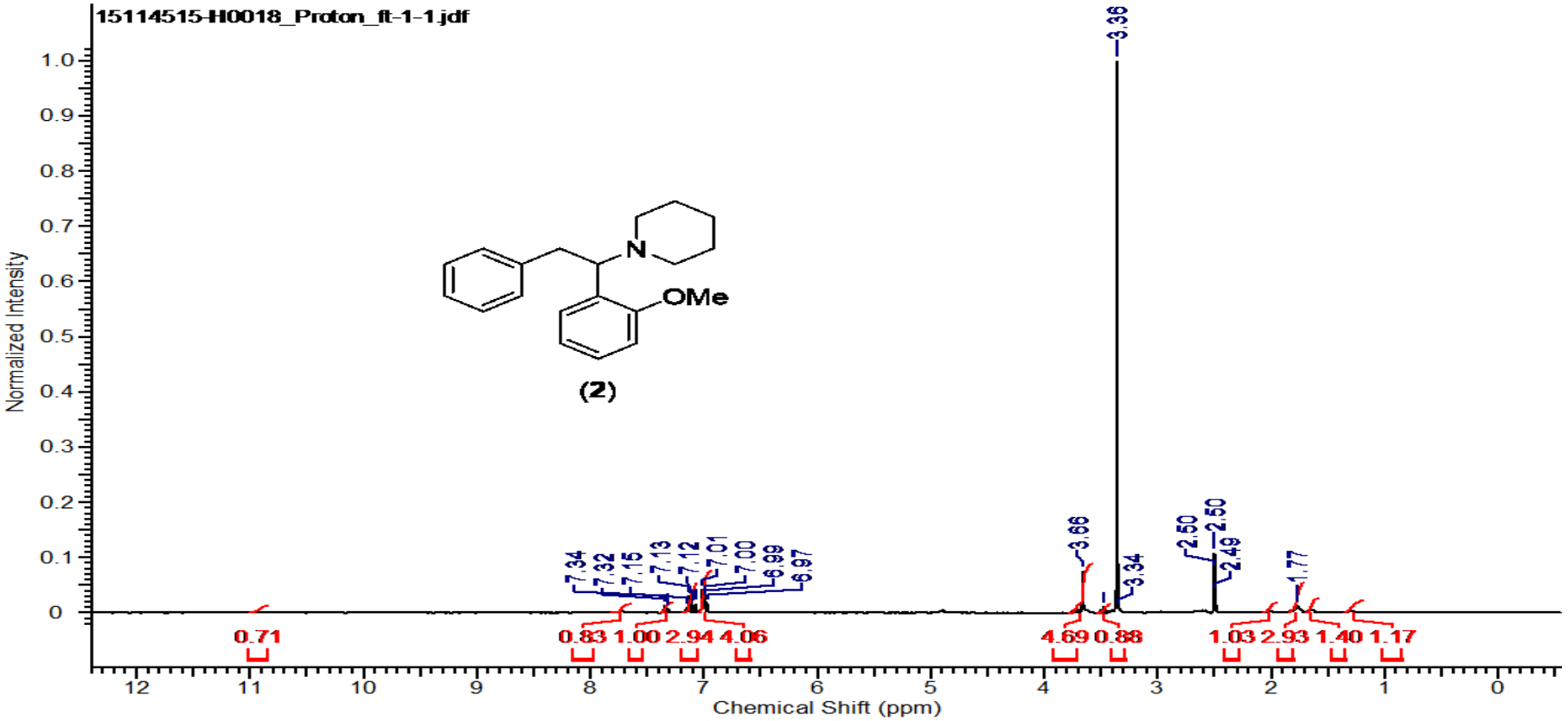


Figure 17. ^{1}H NMR spectrum of **2**

The chiral centre present in 2-MXP **(2)** leads to the CH_2 protons becoming inequivalent (Figure 17). In the ^{1}H-^{1}H COSY NMR spectrum, the doublet located at 4.91 ppm ($^{3}J_{HH}$ = 12.3 Hz) shows two cross peaks; one cross peak is to the doublet of doublets at 3.70 ppm ($^{3}J_{HH}$ coupling = 12.19 Hz, $^{2}J_{HH}$ coupling = 3.19 Hz) whilst the other is to the triplet ($^{3}J_{HH}$ = 12.8 Hz) at 4.91 ppm. All of these environments integrate to one proton each. Further evidence for the positioning of these peaks was obtained from the ^{1}H-^{13}C HMBC spectrum. The two peaks located at δ 3.70 and 3.19 both show a cross peak to two aromatic carbons located at δ 128.9 and 136.6. The former is a CH, as identified through DEPT analysis whereas the latter is a quaternary carbon. These two peaks are thus located on the benzene ring to which the CH_2 is attached to as part of the aliphatic backbone of the molecule.

The piperidine ring consists of 10 proton nuclei. Due to the hindered rotation of the bonds, the signals for these environments are not observed as simple splitting patterns due to the range of couplings that are present. However, the integration of these regions does equal ten, thus matching the proposed structure.

The ^{13}C NMR spectrum (Figure 18) of **2** possesses 16 peaks which corresponds to the number of carbon environments in **2**. There are 10 aromatic peaks, which reduces to 7 in the ^{13}C[120] DEPT-135 spectrum. The reason why there is a reduced number of peaks in the $^{13}C\{^{1}H\}$ DEPT-135 spectrum is because there are three quaternaries in the structure. These are assigned to the peaks at 158.1, 136.6 and 119.4 ppm in the ^{13}C NMR spectrum, with the peak at 158.1 ppm being the quaternary carbon to which the methoxy group is attached to the benzene ring – this was rationalised from the ^{1}H-^{13}C HMBC spectrum as it was the only signal that showed this interaction. The remaining peaks are all positive in the $^{13}C\{^{1}H\}$ DEPT-135 spectrum indicative of either a CH or CH_3; given their chemical shifts, these are most likely to be aromatic CHs. In addition to the aromatic peaks, there are 6 aliphatic peaks, again matching that of the proposed structure. The peak at 55.60 ppm is positive in

the $^{13}C\{^{1}H\}$ DEPT-135 spectrum, suggestive of a CH or CH_3. Given the deshielded nature of this peak, this is assigned to the methoxy carbon. The methoxy protons are observed as a singlet at δ 3.66 in the ^{1}H NMR that integrate to three protons. In the ^{1}H-^{13}C HMQC this peak possesses a cross-peak to a peak at 55.60, thus confirming the proposed assignment.

HMQC data for the *meta* and *para* MXP isomers were as follows: (3.69, 3H, s) this proton has a cross-peak to a peak at 55.73 ppm (3-MXP (**3**)) whereas the proton (3.68, 3H, s) possesses a cross-peak to a peak at 55.42 ppm (4-MXP (**4**)).

Similarly, HMBC data for the two isomers have shown the following: the proton at 3.69 ppm has a cross-peak to 159.7 ppm (**3**) and finally, the proton at 3.68 ppm possesses a cross-peak to a peak at 160.3 ppm (**4**). These findings from both HMQC and HMBC confirm the position of the methoxy group in **3** and **4** as well.

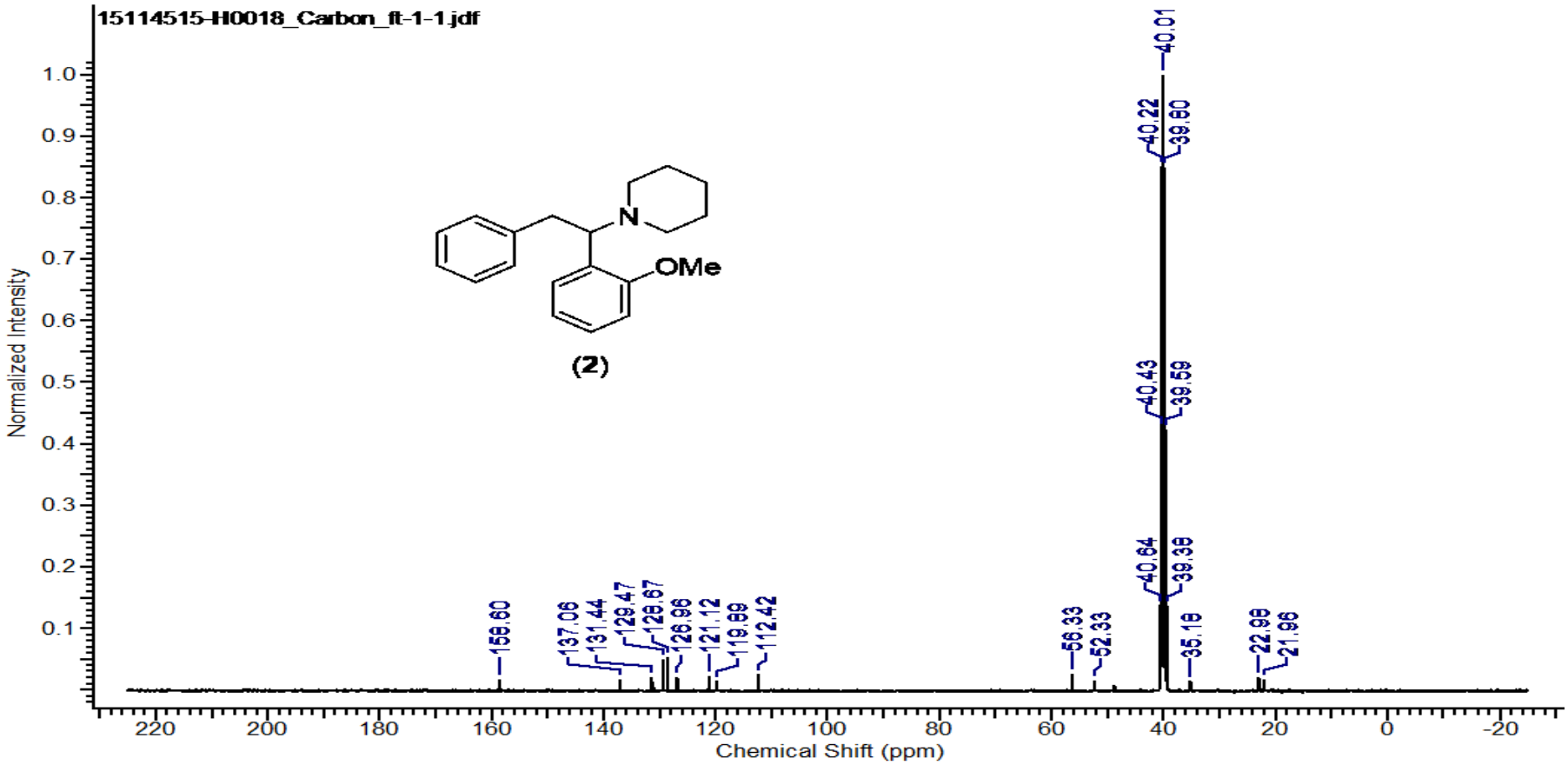


Figure 18. $^{13}C\{^{1}H\}$ NMR spectrum of **2**

Compared with **2**, **3** and **4** have similar ^{1}H NMR spectra (Appendix A 11 and Appendix A 12). For example, the CH_2 protons in the piperidine ring have similar chemical shifts in that they are observed as multiplets over the regions of 1.0-1.4 ppm and 1.4-1.6 ppm. The methoxy group is also observed in the same region, although this peak does show some variation in its chemical shift dependent on the regioisomer under review. The ^{1}H NMR chemical shifts for the methoxy protons are as follows: **2** = 3.66 ppm, **3** = 3.69 ppm and **4** = 3.68

The meta-substituted MXP: 4 chemical shifts: 2 doublets and one singlet.(7.1 – 7.6 ppm). 6.85 (dd, J=8.08, 3.16, 4H). The characteristic J constant for meta coupling is between 2 and 3 Hz. The hydrogens are magnetically equivalent due to the symmetry in 4-MXP molecule. (Appendix A 12).

In **4**, the aromatic region has 2 sets of protons that are equivalent and produce two chemical shifts. The spectrum is a pair of doublets that show second order effects. This pair of doublets is readily observed for *para*-disubstituted benzene due to second splitting and less so for *ortho*- or *meta*-disubstituted benzenes. The latter display much more complex patterns.

From the multiplicity of the ^{1}H NMR signals for the aromatic protons in the range of 6.50-7.50 ppm, the three isomers can be distinguished from each other. In the ^{1}H NMR spectra shown in Figure 17, Appendix A 9 and Appendix A 10, 7.34-6.97 ppm (**2**), 7.27-6.91 ppm (**3**), 7.32-6.90 ppm (**4**) each multiplet integrates to the number of aromatic protons in the corresponding MXP isomer e.g. 9 protons in (**2**). The signal produced by the *meta*-substituted MXP has a similar shape to the signal produced by *ortho* substituted isomer, with the exception of the presence of a singlet at 7.07 ppm (due to the proton in *ortho* position). The signal due to *para*-substituted MXP shows two symmetrical doublets due to the two protons on both sides of the ring i.e. the multiplets "lean" towards each other (second order effect, sometimes referred to as the "*roof*" *effect*). The effect is due to the difference in chemical shift being of the same order as the chemical shift, the characteristic *J* constant for *para* coupling is between 0 and 1 Hz.[123]

The spectral data collected for (**2**) isomer have shown that the methoxy protons are observed as singlet at δ 3.66 in the ^{1}H NMR that integrate to three protons. This is in agreement with the data reported in the literature (in comparison with the McLaughlin *et al*. study, 3.71 (s; 3 H; OCH_3) i.e. ^{1}H and ^{13}C NMR data (HCl salt).[75]

3.5.4 Ultraviolet spectroscopy

The three isomers were run on a UV spectrophotometer; the UV-visible spectrum showed that all three MXP isomers exhibit a λ_{max}. For **2**, this centred at 278 nm (See Appendix A 15), whilst for **3** and **4** they were centred at 277 nm (See Appendix A 16) and 273 nm respectively. There is, therefore, no real significant difference in terms of the absorption properties of the three regioisomers investigated.

From the results, the molar absorptivity (ε) for each isomer was given from the Beer's Law Equation (Absorbance = ε.L.c) i.e.

$$\varepsilon = A/L.c,$$

ε = molar absorptivity, A = absorbance, L = path length, c = concentration

The values of molar absorptivity for MXP isomers are given below:

In the case of 2-MXP **(2)**; λ_{max} = 278 nm, abs. = 1.31, ε_{278}= 1290 L mol^{-1} cm^{-1}, while for 3-MXP **(3)**; λ_{max} = 277 nm, abs. = 1.117, ε_{277}= 1100 L mol^{-1} cm^{-1} and finally for 4-MXP **(4)**; λ_{max} = 273 nm, abs. = 0.673, ε_{273}= 660 L mol^{-1} cm^{-1}

It is clear from the results that **2** has greater molar absorptivity (ε) than the other two isomers. Molar absorptivity is a term used in chemistry to measure how a particular chemical absorbs light at a particular wavelength. It is also known as molar extinction coefficient denoted by 'ε'. The absorbance values for all three MXP isomers were within the range of (0.5-1.5) which is acceptable according to Beer-Lambert's law and ICH guidelines. This can be explained by the possibility of obtaining an absorbance value of greater than one (if the

solution is concentrated). Nevertheless, it is recommended to use diluted solution whose absorbance is below one.

3.5.5 Gas chromatography-mass spectroscopy

The three MXP regioisomers were studied using GC-MS in order to detect and separate the three MXP isomers qualitatively in a mixture and compared to the work by Geyer *et al*.[74] The base peaks observed for **2**, **3** and **4** in the MS is *m/z*=204, which is consistent with the literature.[74] The retention times and relative retention times of **2**, **3** and **4** were determined and found to be consistent with Geyer's results (Table 6).

Methoxphenidine isomers with some common adulterants were also investigated using GC-MS and their retention times (t_R) and relative retention times (RRT) recorded (Table 6). The adulterants were caffeine, benzocaine and procaine. This experiment was performed in order to measure the purity of MXP derivatives. The adulterants/diluents are commonly blended to the drugs (NPSs) to increase drug volume and therefore drug trafficking profits.[124]

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Table 6. GC-MS Retention times (t_R) and Relative Retention Times (RRT) of **2**, **3** and **4** with some common adulterants using Eicosane as a reference.

	GC-MS (this study)		GC-MS (Geyer et al.)	
Analyte	t_R (min)	RRT	t_R (min)	RRT
2	32.02	1.24	28.06	1.28
3	34.69	1.35	29.94	1.37
4	36.81	1.43	31.40	1.43
Caffeine	19.12	0.74	15.68	0.72
Benzocaine	12.37	0.48	10.98	0.50
Eicosane	25.73	1.00	21.85	1.00
Procaine	27.15	1.06	24.25	1.10

3.5.6 High performance liquid chromatography

3.5.6.1 HPLC Method Development (MXP isomers)

The next step after characterisation is developing HPLC method [125, 126] for MXP isomers separation. The method was developed by changing different HPLC experimental parameters in order to obtain optimum resolution and retention time. The flow rate, temperature and mobile phase composition, were selected for testing as the parameters for modification. HPLC separation conditions were optimised during method development; the most important factor is the resolution (Rs) which should be > 1.5 (desirable for quantitative analysis), but in this study, the value of Rs > 2.0 was the goal for separation of MXP sample mixture. Calculation of resolution in HPLC is shown in (Figure 19), where t_R is the retention time, t_W is obtained is from the intersection of the tangents (which are drawn at 0.6 times the peak height) with the baseline. Resolution in HPLC is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks.[127]

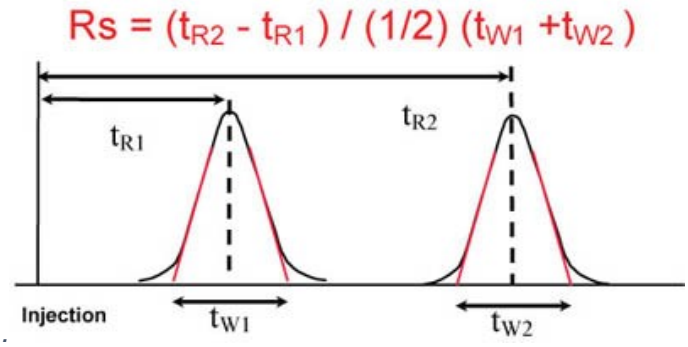


Figure 19. Calculation of resolution in HPLC

During method development, the HPLC conditions used were as follows: mobile phase, acetonitrile:ammonium acetate 55:45% v/v, column ACE 5 C18-AR (150 x 4.6 mm, 5 µm particle size), flow rate (1.0 mL min^{-1}), temperature (50°C), injection volume (20 µL) and detection wavelength (278 nm). Two blank injections (30 min each) followed by two injections of MXP mixture (10 min).

3.5.6.2 Method Validation (MXP isomers)

The optimised method was validated in accordance with the ICH guidelines.[128] The following parameters: linearity, accuracy, precision, specificity, limit of detection (LOD), limit of quantification (LOQ) and system suitability [resolution (Rs), column efficiency (N), peak asymmetry (As)]. Linearity, precision and system suitability tests were carried out using six replicate injections of the calibration standards were performed and the data analysed (Table 7). The %RSD was calculated for each replicate sample. Moreover, symmetry, relative retention time (RRT), number of theoretical plates (N), equation of the straight line, capacity factor (k) which is a measure of the retention of a peak that is independent of column geometry or mobile phase flow rate. The capacity factor is calculated as: $k = (t_R - t_0)/t_0$, the height equivalent to a theoretical plate H(m); or HETP = L / N, where, L is the column length and N is the plate number. R-squared (r^2) = a statistical measure of how close the data are to the fitted regression line. Theoretical plate number (N) is

an index that indicates column efficiency. It describes the number of plates as defined according to plate theory, and can be used to determine column efficiency based on calculation in which the larger the theoretical plate number the sharper the peaks. These parameters and data are reported in Table 7. In this experiment, calibration standards of methoxphenidine regioisomers were prepared and tested on HPLC (The results are shown in Table 7).

Table 7. Representative validation data for MXP isomers obtained using an ACE 5 (150 x 4.6 mm i.d, 5µm) column, mobile phase: acetonitrile:ammonium acetate 55:45% v/v, flow rate: 1.0 mL min^{-1}; Detector: UV-DAD.

	2	**3**	**4**
t_R (min) (t_0=1.50min)[a]	3.70	5.82	4.33
RRT	1.58	1	1.34
Capacity Factor (k')	2.66	4.81	3.32
Resolution (R_s)	-	7.41	3.88
Plates (N)	7164	9053	7417
Asymmetry Factor (As)	0.75	0.97	0.86
LOD (µg mL^{-1})[b]	0.12	0.04	0.15
LOQ (µg mL^{-1})[c]	0.38	0.47	0.45
Co-efficient of Regression(r^2)	0.999	0.999	0.999
Precision (%RSD) (n = 5)			
2.5 µg mL^{-1}	1.75	2.17	1.79
5 µg mL^{-1}	0.43	0.92	0.69
10 µg mL^{-1}	0.09	0.28	0.49
20 µg mL^{-1}	0.28	0.48	0.62
40 µg mL^{-1}	0.31	0.39	0.59

Key: (a) Determined from the retention time of a solution of uracil (1 µg mL^{-1}) eluting from the column; (b) limit of detection (based on the standard deviation of the response and the slope); (c) limit of quantification (based on the standard deviation of the response and the slope). (d) y = 5.7588x + 0.2432; (e) y = 4.6324x + 0.4973; (f) y = 3.1087x - 0.0844.

Method validation is important to be carried out after the completion of method development. The HPLC method was validated using an adaptation of the method reported by Geyer *et al*.[74] The MXP isomers were tested in this study to ensure the HPLC: DAD system (or the detector) produced a linear response for the repeated injections of the three MXP isomers by testing a series of five

MXP concentrations using the standard developed method, from which both LOD and LOQ were calculated.

The concentrations were calculated using the equation of the calibration line:

$$y = mx + c$$

Where, y = Peak Area; x = Concentration of analyte (µg mL^{-1}); m is the gradient of the line and c is the y-intercept (where the graph crosses the y-axis). The following diagram (Figure 20) shows HPLC separation of MXP regioisomers. The order of elution was 2-MXP (**2**) followed by 4-MXP (**4**) and then finally 3-MXP (**3**). The peak of **2** shows a bit of tailing which might be due to the fact that compounds possessing amine and other basic functional groups interact strongly with residual silanol groups on the silica producing tailing peaks.

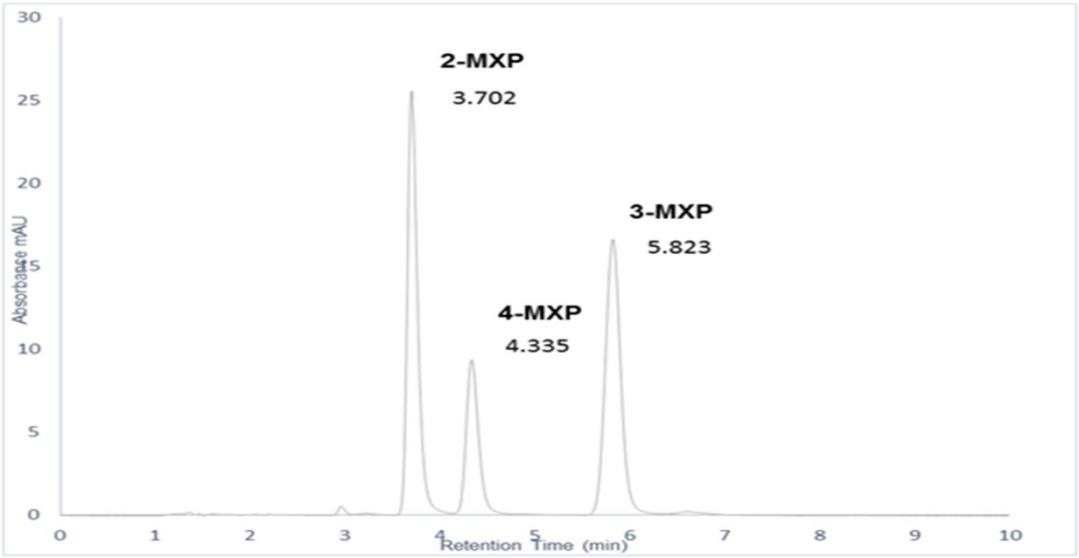


Figure 20, HPLC separation of MXP isomers (concentration = 40 µg mL^{-1}); **2** t_R= 3.703 min; **4** t_R = 4.336 min and **3** t_R = 5.823 min, respectively.

The method shows excellent linearity i.e. all three substituted methoxphenidines demonstrated a linear response (r^2 = 0.999) over the 2.5 - 40 µg mL^{-1} range with good repeatability in each case (%RSD = 0.09 – 2.17 %, n = 5). The system suitability tests were within the specifications of the ICH.

The peak area and relative retention times of the analytes were measured and the %RSD calculated to determine the precision. The repeatability (%RSD) of the method was high and complies with ICH guidelines (0.09-0.92) for all concentrations except for the lowest concentration (i.e. 2.5 µg mL^{-1}, 1.75-2.17).[128]

3.5.6.3 Method Robustness (MXP isomers)

In this experiment the following variations were tested: influence of variations temperature; influence of variations in mobile phase composition; flow rate and the effect of intra- and inter-day variation as the method should be robust in routine operation and usable by all laboratories. Robustness can be assessed by measuring some experiment parameters such as temperature, flow rate, the percentage of mobile phase composition and the intra- and inter-day precision.

The evaluation of robustness should be considered during the development phase. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure.

The peak area and relative retention times of the analytes were measured and the %RSD calculated to determine the precision. In all cases (except the lowest conc. 2.5 which was >1) both the peak area and the relative retention time gave %RSDs which are <1% and within the guidelines stipulated by the ICH. The data indicate that the method is robust and should be suitable for use in the routine analysis of seized samples (see Table 8, Table 9, Table 10, Appendix A 17 and Appendix A 18).

Table 8. Robustness testing (temperature = 48, 50 or 52 °C) for the three MXP analytes using a mobile phase consisting of acetonitrile:ammonium acetate 55:45% v/v, concentration of MXP = 10 µg mL^{-1}

	t_R (min) using temp. 48 °C			**t_R (min) using temp. 50 °C**			**t_R (min) using temp. 52 °C**		
Injection	**2**	**3**	**4**	**2**	**3**	**4**	**2**	**3**	**4**
1	3.904	5.944	4.493	3.911	5.980	4.515	3.915	5.995	4.526
2	3.913	5.943	4.469	3.911	5.978	4.514	3.917	5.999	4.529
3	3.913	5.944	4.496	3.915	5.981	4.518	3.918	5.997	4.527
4	3.912	5.945	4.497	3.912	5.977	4.513	3.916	5.993	4.524
5	3.909	5.949	4.497	3.914	5.979	4.516	3.920	5.996	4.528
6	3.916	5.946	4.499	3.914	5.984	4.518	3.920	5.995	4.528
7	3.914	5.947	4.498	3.912	5.984	4.517	3.918	5.990	4.524
8	3.917	5.950	4.501	3.921	5.979	4.519	3.923	5.994	4.528
9	3.917	5.953	4.503	3.916	5.978	4.515	3.921	5.991	4.526
10	3.919	5.954	4.504	3.921	5.978	4.518	3.922	5.995	4.527
Average	**3.913**	**5.947**	**4.495**	**3.914**	**5.979**	**4.516**	**3.919**	**5.994**	**4.526**
STD	**0.004**	**0.003**	**0.009**	**0.003**	**0.002**	**0.002**	**0.002**	**0.002**	**0.001**
%RSD	**0.112**	**0.064**	**0.221**	**0.094**	**0.041**	**0.044**	**0.066**	**0.044**	**0.037**

Table 9. Robustness testing (flow rate = 0.9, 1.0 or 1.1 mL min^{-1}) for the three MXP analytes using a mobile phase consisting of acetonitrile:ammonium acetate 55:45% v/v, concentration of MXP = 10 µg mL^{-1}

Injection	t_R (min) using flow rate **0.9 mL min^{-1}**			t_R (min) using flow rate **1.0 mL min^{-1}**			t_R (min) using flow rate **1.1 mL min^{-1}**		
1	**2**	**3**	**4**	**2**	**3**	**4**	**2**	**3**	**4**
2	4.366	6.669	5.037	4.366	6.669	5.037	4.366	6.669	5.037
3	4.370	6.663	5.035	4.370	6.663	5.035	4.370	6.663	5.035
4	4.368	6.664	5.035	4.368	6.664	5.035	4.368	6.664	5.035
5	4.372	6.678	5.045	4.372	6.678	5.045	4.372	6.678	5.045
6	4.373	6.667	5.040	4.373	6.667	5.040	4.373	6.667	5.040
7	4.374	6.665	5.039	4.374	6.665	5.039	4.374	6.665	5.039
8	4.376	6.666	5.040	4.376	6.666	5.040	4.376	6.666	5.040
9	4.378	6.672	5.044	4.378	6.672	5.044	4.378	6.672	5.044
10	4.377	6.670	5.042	4.377	6.670	5.042	4.377	6.670	5.042
Average	**4.378**	**6.671**	**5.043**	**4.378**	**6.671**	**5.043**	**4.378**	**6.671**	**5.043**
STD	**4.373**	**6.668**	**5.040**	**4.373**	**6.668**	**5.040**	**4.373**	**6.668**	**5.040**
%RSD	**0.004**	**0.004**	**0.003**	**0.004**	**0.004**	**0.003**	**0.004**	**0.004**	**0.003**

Table 10. Robustness testing for MXP isomers (The effect of modifying flow rate)

Flow Rate 0.9 mL min^{-1}	**2**	**3**	**4**
t_R (min)(t_0=1.50min)	4.37	6.66	5.04
RRT	1.52	1.00	1.32
Plates (N)	7250	9400	7550
Resolution (R_s)	-	6.45	3.06
Flow Rate 1.0 mL min^{-1}	**2**	**3**	**4**
t_R (min)(t_0=1.50min)	3.94	6.00	4.54
RRT	1.52	1.00	1.32
Resolution (R_s)	-	6.30	2.97
Plates (N)	7120	9150	7340
Flow Rate 1.1 mL min^{-1}	**2**	**3**	**4**
t_R (min)(t_0=1.50min)	3.59	5.46	4.13
RRT	1.52	1.00	1.32
Resolution (R_s)	-	6.22	2.94
Plates (N)	6790	9030	7250

3.5.6.3.1 Robustness testing (temperature and the application of Van't Hoff equation)

In this HPLC method robustness testing, three temperatures (48, 50 and 52°C) were investigated; the results revealed that by increasing the temperature (see Table 8). The retention times (t_R) of the three MXP analytes were just slightly changed or remained constant e.g. in the case of 2-MXP (**2**), at 48°C t_R was 3.913 min, at 50°C (3.914 min) and at 52°C (3.319 min).

By plotting ln k (retention factor) versus 1/T (absolute temperature in kelvins, K^{-1}), a linear Van't Hoff plot is obtained, which suggests that the retention mechanism for MXP analytes is the same or constant (parallel trend lines, see Figure 21); that is, the values for ΔH and ΔS for the analytes are constant over the temperature range under consideration. These findings are in line with the literature, Boateng *et al.* concluded that as the temperature was increased the retention time should decrease (i.e. van't Hoff relationship) and that the mechanism controlling the retention and separation of the MXP regioisomers was attributed to an electrostatic interaction which facilitated hydrophobic interactions.[120], [129]

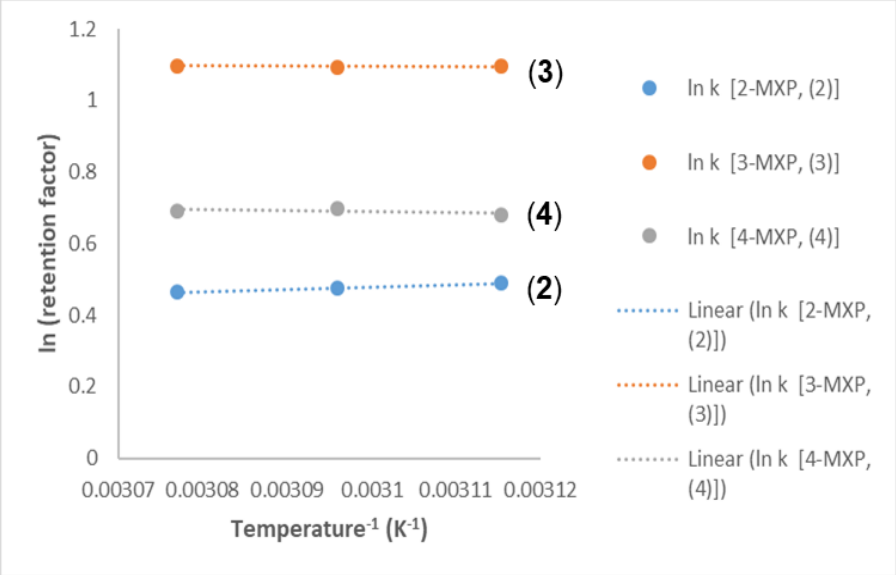


Figure 21. Van't Hoff plot of ln k versus 1/T for methoxphenidine isomers (**2**, **3** and **4**)

In thermodynamics, this means that when the temperature of a system increases, the kinetic and potential energies of the atoms and molecules in the system increase. Accordingly, the internal energy or the enthalpy of the system increases; this is true under constant pressure or constant volume. Furthermore, when the temperature increases, this will increase entropy causing more energy put into the system, which excites the molecules and the amount of random activity. A reaction can be predicted whether if it will occur spontaneously by combining the entropy, enthalpy, and temperature of a system in a Gibbs free energy (G), or (Gibbs equation)

The change in free energy (ΔG) is the difference between the heat released during a process and the heat released for the same process occurring in a reversible manner.

The thermodynamic relation: $\Delta G = \Delta H - T\,\Delta S$ (Gibbs equation)

Where, (G, Gibbs free energy, T = absolute temperature in kelvins), (ΔH) = enthalpy change or internal energy (total heat content) in the thermodynamic system. While, (ΔS) = entropy change is the measure of the randomness (disorder) of molecules in the system. The ΔH can be calculated from the slope of van't Hoff plot and ΔS is calculated from the intercept of van't Hoff plots.

The thermodynamic quantity that governs retention is the free energy (ΔG) which has an entropy component (ΔS). At lower temperatures, where the mobile phase is hydrogen bonded, there is a favourable entropy change upon retention. This is commonly referred to as "hydrophobic effect." However, at high temperatures, where there is little or no hydrogen bonding, the entropy change would be expected to be much less. As a result, although the enthalpy (ΔH) of retention is more favourable at high temperature, it is outweighed by the entropic (ΔS) contribution. [130]

3.5.6.3.2 Robustness testing (flow rate and the van Deemter equation)

By increasing the flow rate from 0.9 to 1.0 mL min^{-1} and then to 1.1 mL min^{-1}. In Table 9, the t_R was decreased e.g. t_R of **4** was 5.00 min (at 0.9 mL min^{-1}), 4.50 min (1.0 mL min^{-1}) and 4.10 min (1.1 mL min^{-1}), and similarly, the resolution was decreased. For example, the resolution decreased from 6.45 to 6.30 and then to 6.22 between **2** and **3** and from 3.06 to 2.97 and to 2.94 between **4** and **3** peaks (Table 9). A lower flow rate will result in a loss of efficiency due to the longitudinal diffusion caused by increased retention factor (k) and elution time (due to increased B/u) (see van Deemter Equation in chapter 1). As a general rule of thumb regarding the van Deemter Equation and flow rate:

I. The smaller the plate height H, the more efficient the column. However, at the optimum flow rate (with the lowest H), the analysis time will in most cases be unacceptably long.
II. Below the optimum flow rate the analysis time is too long and the quality of the separation suffers because of longitudinal diffusion (contribution of the B-term, see introduction chapter).
III. At extremely high flow rates, both the separation quality and the pressure across the column will become unacceptable.
IV. The practical flow is often two or three times the optimum velocity. At these values, the minor loss of efficiency is still acceptable.

For MXP isomers (see Table 9) the optimum flow rate is 1 mL min^{-1}, because the resolution, peak height and number of plates started to decrease when flow rate was increased from 1 mL min^{-1} to 1.1 mL min^{-1}. The ultimate aim in any separation is to obtain resolution or optimum distance between peaks of the desired compounds. So flow rate is a secondary choice, resolution is the first choice.

3.5.6.3.3 Robustness testing for MXP isomers (mobile phase composition and impact of log P)

By deliberately modifying mobile phase proportion, there were slight changes/reductions in retention times of MXP regioisomers i.e. by increasing the organic part (acetonitrile) of the mobile phase (acetonitrile:ammonium acetate) from 54 to 55% and then to 56%, the retention time was decreased. e.g. t_R of 3 from 6.28 min (54:46% v/v) to 6.04 min (55:45% v/v) and then to 5.77 min (56:44% v/v), in addition, there was also only minor changes in the %RSD (Relative standard deviation), which measures the precision of the average (t_R) of the results, for **3** %RSD was 0.05 (54:46% v/v), 0.10 (55:45% v/v) and 0.11 (56:44% v/v), (See Appendix A 17).

The solutes of MXP isomers (**2**, **3** and **4**) are adsorbed on to the stationary phase and by increasing the percentage of the organic modifier (acetonitrile) from 54 to 55% and then to 56% the retention time of these solutes decreases. The ChemDraw software was used to predict log P of these isomers and was found to be 4.58, which is a positive value for log P, this indicates a higher concentration in the lipid phase (i.e., the compound is more lipophilic). Again, this confirms that MXP isomers possess a hydrophobic effect as a mechanism of retention in reverse phase chromatography.

The data in Appendix A 17 and Appendix A 18 for all the robustness experiments indicate that the method is robust and should be suitable for use in the routine analysis of MXP samples.

Overall, from the results of the robustness of the developed HPLC method, in all cases, the measured retention time showed no significant differences, this again indicates that the method is robust and should be suitable for use in the routine analysis of any samples that contain MXP isomers and can be easily transferred for use in another laboratory if necessary.

3.5.6.4 Method Accuracy (MXP isomers)

According to the ICH guidelines, Q2 (R1) Validation of Analytical Procedures, 2005), accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample. Accuracy is a measure of closeness of a calculated result to the known, true value (i.e. 100% of known concentration, a minimum of nine determinations over a minimum of three concentrations and results should be 98-102% of the true content). An average of 6 repeated injections was performed in this experiment (Appendix A 19). Additionally, the accuracy (percentage recovery study) of the assay was determined from spiked samples prepared at three concentration levels over a range of 80–120 % (8, 10 and 12 μg mL^{-1}) of the target concentration (10 μg mL^{-1}). From the results, the percentage recovery (% assay) and %RSD calculated for each of the three replicate samples demonstrated excellent recoveries for all 3 analytes within the desired concentration range (100 ± 2 %, see Table 8). The percentage recovery for **4** was about 98% in all three concentrations tested in this experiment; while for **3** (99-100.5%) and (98.5-100.5%) in the case of **2**.

3.5.6.5 Method specificity (MXP isomers)

The specificity of the method was performed using three replicate injections of the specificity standards and the data analysed under the same conditions (Table 11). The concentrations of **2**, **4** and **3** were 10 μg mL^{-1}, diphenidine, 4-MMC, paracetamol, caffeine and benzocaine 10 μg mL^{-1} and common adulterants and a seized sample of [10 μg mL^{-1}] using an ACE 5 C18-AR (150 x 4.6 mm, 5 μm particle size) column. Mobile Phase: acetonitrile:ammonium acetate 55:45% v/v, flow rate: 1 mL min^{-1}; Detector: UV-DAD. The tested compounds were injected individually prior to testing them in a mixture (to facilitate comparison).

The results (Table 11) showed that the developed HPLC method is suitable for the detection and quantification of the three common adulterants (benzocaine, caffeine and paracetamol).

Table 11. Representative data obtained for solutions containing regioisomeric MXPs in a seized sample and some common adulterants using the concentration of 10 µg mL^{-1} and mobile phase (acetonitrile:ammonium acetate 55:45% v/v).

	t_R (min)							
Injection	**Benzocaine**	**Caffeine**	**4-MMC**	**Paracetamol**	**2-MXP**	**3-MXP**	**4-MXP**	**Diphenidine**
1	2.63	1.70	2.18	1.63	3.85	6.16	4.56	6.13
2	2.62	1.70	2.18	1.63	3.85	6.16	4.56	6.13
3	2.62	1.70	2.18	1.63	3.85	6.16	4.56	6.13
Average t_R (min)	**2.62**	**1.70**	**2.18**	**1.63**	**3.85**	**6.16**	**4.56**	**6.13**
RRT	**0.68**	**0.44**	**0.56**	**0.42**	**1.00**	**1.60**	**1.18**	**1.59**

The results of specificity/adulterants testing revealed that the strongly UV-absorbing components (benzocaine, caffeine and paracetamol) demonstrated baseline separation from the target analytes, with benzocaine being distinguishable from other controlled psychoactive substance such as mephedrone (4-MMC) or diphenidine. The three MXP isomers were completely separated with excellent RRT and resolution; consequently, they can be easily distinguished from the adulterants and/or NPSs in a mixture. This indicates that the developed HPLC method can be used in detection and quantification of the seized samples of MXPs isomers either as individual samples or in a mixture that contains adulterants.

3.6 Forensic application (MXP isomers)

The street samples supplied were analysed using the GC-MS and HPLC methods developed. The first investigation was conducted using GC-MS to identify the retention times for the components in the two street samples. The results were then compared with Geyer's GC-MS results of the 13 diphenidine derivatives.[74]

This qualitative GC-MS analysis confirmed and indicated that the samples contained 2-methoxphenidine (SS-1: t_R = 32.58 min, m/z (base peak) = 204 $[M+H]^+$, **2**; Figure 22) and 2-methoxphenidine (SS-2: t_R = 32.58 min, m/z (base peak) = 204 $[M+H]^+$, **2**) and 3-methoxphenidine (SS-2: t_R = 35.22 min, m/z (base peak) = 204 $[M+H]^+$, **3**; Figure 23), respectively. In addition to the base peak ions, fragmentation ions were also observed. The peak at *m/z*=65 represents $C_5H_5^+$ and the fragment at *m/z*=91 represents C_6H_5-CH_2^+ or the tropylium $C_7H_7^+$; these two fragments are present in the structure of all the three MXPs which confirms that these isomers have commonality in terms of chemical structure. Eicosane was utilised as an internal standard for both street samples and possessed a retention time of 25.95 min (RRT=0.90). There is no apparent adulteration in comparison with standards of **2** and **3**, implying that the samples were essentially pure (Figure 22 and Figure 23).

To conclude, this finding indicates that **2** and **3** (present in the street sample), can be detected and separated using this GC-MS method in the presence of both diphenidine and eicosane. Furthermore, the MS fragmentation and GC data obtained were consistent with the data reported by Geyer *et al.*[74]

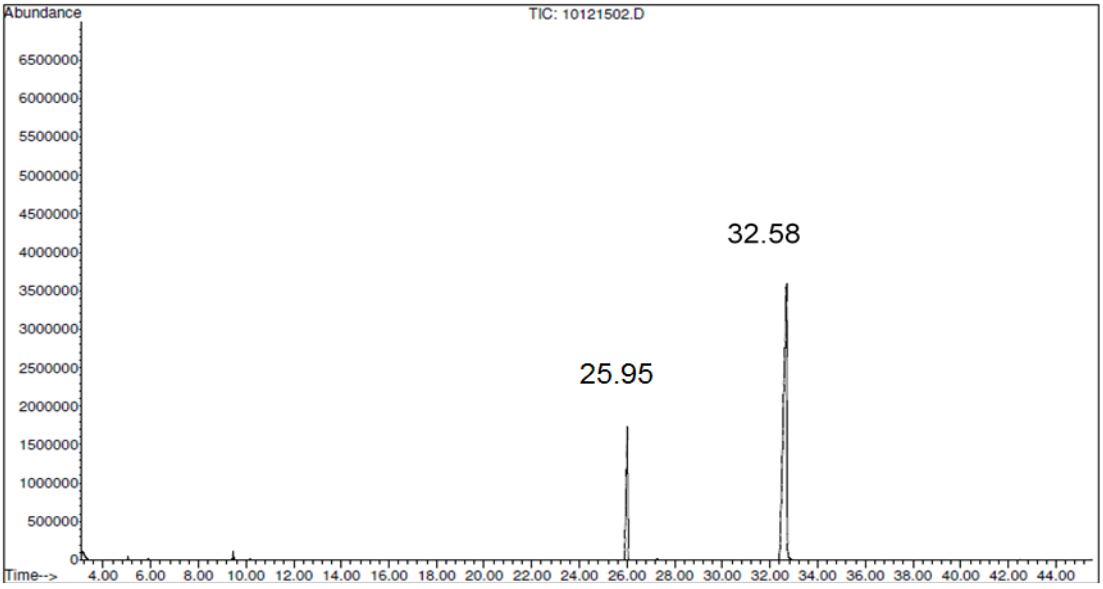


Figure 22. Chromatograph of St S1 (the sample contains **2** at 32.58 mins and Eicosane at 25.95 mins)

From the results of GC-MS spectra, St S1 contains **2**; St S2 contains both **2** and **3** and it is clear that the two isomers are separated from each other with good resolution (Figure 22).

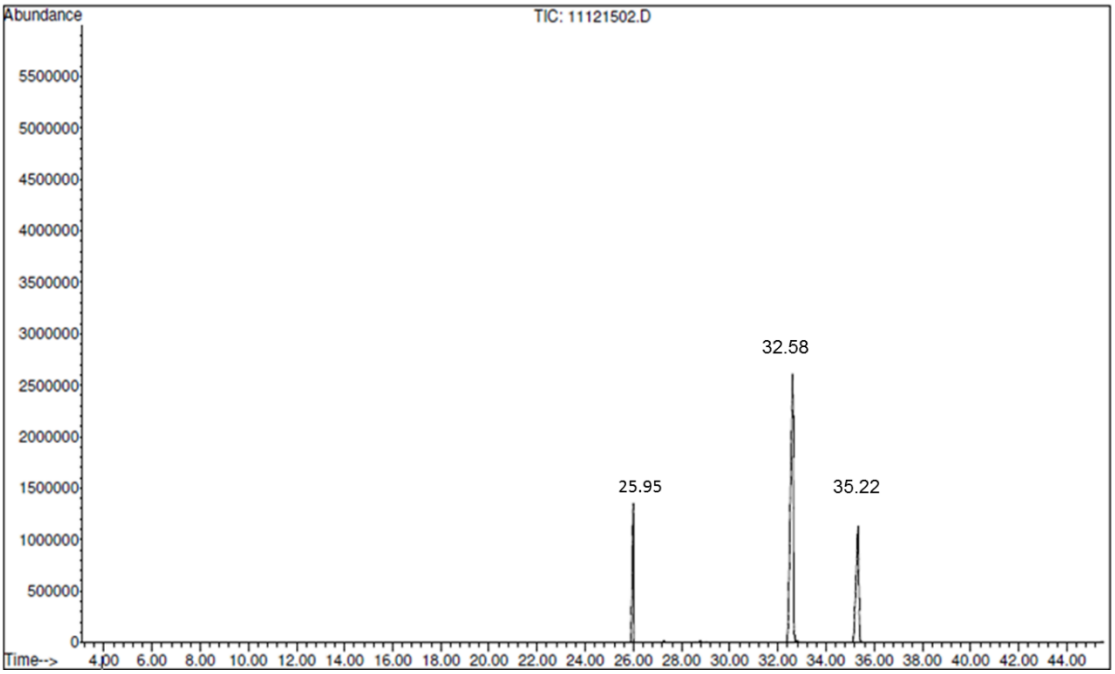


Figure 23. Chromatograph St S2 on GC-MS (the sample contains **2** at 32.58 mins, **3** at 35.22 and Eicosane at 25.95 mins)

Another investigation was performed using HPLC to evaluate two street samples (St S1 and St S2) obtained from independent Internet vendor BRC Fine Chemicals Limited (https://www.brc-finechemicals.com) which presented as white crystalline powders in clear zip-lock bags. These samples were obtained prior to the legislative change (20th May 2016), the samples were both purported to be >99% pure.

This HPLC analysis of the two street samples indicated that the first sample contained one component (St S1, t_R = 3.88 min) and the second sample contained two components (St S2, peak 1, t_R = 3.89 min; **2** and St S2, peak 2, t_R = 6.18 min; **3**). Comparison of the samples with the reference materials (**2**, **3** and **4** mix) confirmed the presence of **2** and **3** correspondingly (see Table 12, Figure 24 and Figure 25).

This indicates that the validated HPLC method is quicker (10 min run time, see the chromatograms in Figure 24, Figure 25) than Geyer's GC-MS method (45 mins).[74] Therefore, this shows that this HPLC method can be utilised to identify and separate MXP positional isomers, which are present in any street sample (see Figure 24 and Figure 25).

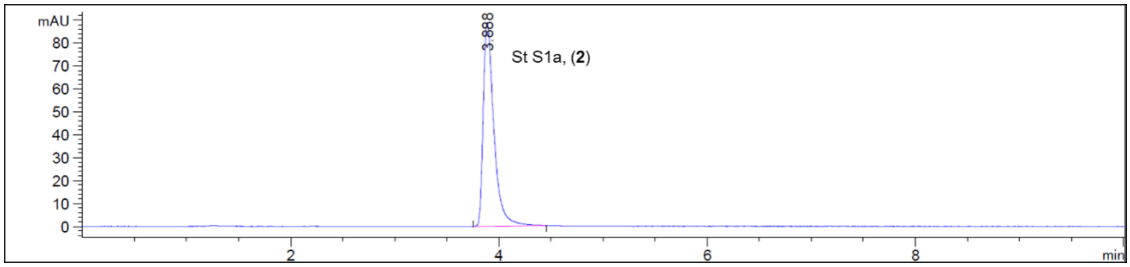


Figure 24. St S1 measured on HPLC (St S1a peak)

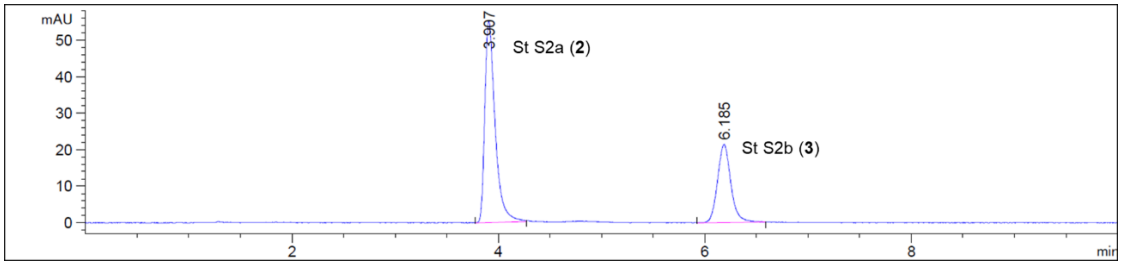


Figure 25. St S2 measured on HPLC (St S2 peak a and peak b)

The developed/validated HPLC method was applied to test street samples suspected to contain MXP isomers, these samples were quantitatively determined and the results are shown in Table 12). From the findings, these samples contain **2** and **3**. In addition, it is clear that the total run time for these compounds using the validated HPLC method is 10 minutes (Figure 24, Figure 25*)*, while the total run time was greater than 44 minutes when these compounds were tested using the GC-MS method (Figure 22, Figure 23).

Table 12. Bulk samples tested on HPLC (MXP isomers). Key: t_R = Retention time, St S1 = Street Sample 1

Street Sample	Qualitative Analysis, t_R (min)	Peak Area	Actual conc. (μg mL^{-1})	Found conc. (μg mL^{-1})	Quantitative Analysis (% w/w)
St S1	**2**, t_R = 3.88 min	58.12	10.20	10.05	98.50
	2, t_R = 3.89 min	64.62	10.20	11.18	109.60
St S2	**2**, t_R = 3.90 min	35.31	10.20	6.09	59.70
	3, t_R = 6.18 min	17.95	3.03	3.77	124.40
	2, t_R = 3.90 min	40.93	10.20	7.07	75.50
	3, t_R = 6.18 min	18.63	3.92	3.92	100.00

One of the advantages of the HPLC method in this study is that it has shown the ability to completely separate **2**, **3** and **4** in a mixture in a 10 minute run with higher resolution (Rs >3, see Table 12, [128]), and more sensitive detection and quantification. LOD and LOQ were determined as being 0.04–0.15 μg mL^{-1} and 0.38–0.47 μg mL^{-1}correspondingly (Table 7). While, in Geyer's method the three MXP regioisomers were partially baseline resolved or separated with lower sensitivity (LOD was between 4.58 and 5.71 μg mL^{-1} whereas LOQ was between 13.88 and 17.30 μg mL^{-1}).[74]

In conclusion, after applying the developed HPLC method to test bulk samples that were suspected to contain **2**, **3** and **4**, it has proven its ability to analyse these samples qualitatively and quantitatively. i.e. their detection (different retention times) and quantification (by determination of their concentration).

3.7 Conclusion

This chapter details the analysis of three methoxphenidine isomers encountered in NPS forensic samples using HPLC and GC-MS. The validated HPLC method provides both a general screening method and quantification of the active ingredients for seized solid samples, both in their pure form and in the presence of common adulterants.

All analyses were undertaken on reference standards prepared in-house and then cross-validated with two bulk samples obtained from an internet vendor. One of the main findings is that the validated method has the advantage of a rapid single-step detection and separation of these street samples in just a 10 minute run time.

The method provided excellent LOD (0.04-0.15 µg mL^{-1}) and LOQ (0.38-0.47 µg mL^{-1}); it acts as an ideal method for both the qualitative and quantitative analysis of MXP isomers, when compared to the previous approaches in the literature.

The unique result, in comparison to the previous published chromatographic studies on MXP isomers, which is that **3** and **4** were fully baseline-separated with a good resolution (Rs= 3.88, see validation data in Table 7 and Figure 20).

From the results, the various spectroscopic information provided herein acts as an important source of characterisation data (^{1}H-NMR, ^{13}C-NMR, COSY, DEPT, HMQC, HMBC, UV and ATR-FTIR) for the reference materials utilised in this work and serves as an additional resource to the previously published data regarding routine analysis of MXP isomers in the laboratory.

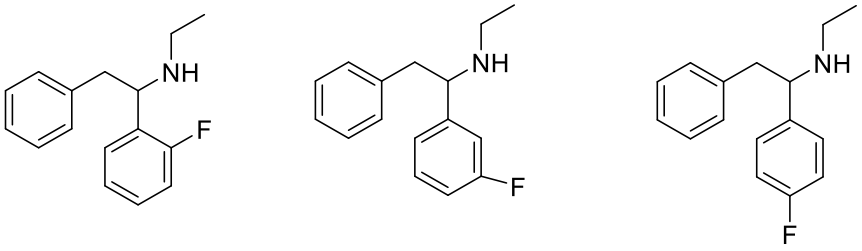
To conclude, the developed HPLC method is suitable for the rapid, specific and sensitive detection, quantification and control of methoxphenidines present within bulk forensic samples. Finally, further research on the relationship between all chromatographic parameters in depth to achieve a faster run time is recommended.

Chapter 4: Development and validation for the separation of the regioisomers of fluephenidine using Reversed Phase HPLC

4.1 Fluephenidine

Fluephenidines (FEP) are fluorinated diphenidine derivatives and, as such, they are new psychoactive substances (NPS).[131-134] NPS are a challenge to forensic toxicologists as a large number of drugs are emerging each year and they are nowadays repeatedly encountered in clinical and post-mortem toxicology investigations and there is a requirement for sensitive and reliable techniques to detect and identify these substances in a variety of different samples.

This chapter discusses the experimental investigation of FEP isomers, **8-10** and the aim of this study is to identify, separate and quantify these substances both as separate components or in a mixture in any forensic framework by carrying out a full characterisation using a number of analytical instrumentation such as HPLC, IR and NMR. This will add to the existing literature of diphenidine derivatives. The chemical structures of the fluephenidines, **8-10**, are shown in Figure 26.



2-FEP (**8**) 3-FEP (**9**) 4-FEP (**10**)

Figure 26. The chemical structures of 2-FEP (**8**), 3-FEP (**9**) and 4-FEP (**10**)

4.2 Results and Discussion

4.2.1 Synthesis of FEP isomers

The three FEP compounds, **8-10**, were prepared in-house using an adaption of the method reported by Geyer *et al.*,[135] (see Chapter 3) and isolated as their corresponding hydrochloride salts. [MC Hulme, PhD thesis: “"New Psychoactive Substances - New Analytical Challenges", Manchester Metropolitan University (2019)], [136].

In comparison with MXP (**2**), there is an ethyl group instead of the piperidine ring in these compounds (FEP) and a fluorine instead of a methoxy group attached to the benzene ring. However, the FEP derivatives possess some structural similarities to MXP, such as the molecular backbone. To confirm the authenticity of the materials used in this study, these synthesised samples (**8-10)** were fully structurally characterised by using ^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR, UV and FTIR.

4.2.2 Ultraviolet spectroscopy

The UV-Vis spectra of **8-10** were collected on a UV-Vis spectrophotometer; the UV-visible spectrum showed that all three FEP isomers exhibit a λ_{max} at 270 nm. Therefore, there is no real significant difference in the absorption properties of the three regioisomers investigated (See Appendix A 3, Appendix A 4 and Appendix A 5). The absorbance wavelength is higher in energy than what was observed for the MXP isomers (e.g. for 2-MXP = 278 nm, see Chapter 3). From these data, the molar absorptivity (ε) for each isomer was calculated. For **8**, $\varepsilon_{270\ nm}$= 501 L mol^{-1} cm^{-1}, whereas for **9** and **10** $\varepsilon_{270\ nm}$= 727 L mol^{-1} cm^{-1} and 552 L mol^{-1} cm^{-1}, respectively. Thus, **8** and **10** have similar molar absorptivities whereas **9** has the highest molar absorptivity. As **8** and **10** are *ortho*- and *para*-substituted respectively, with respect to fluorine, the electronic effects around the ring would be similar, in contrast to when the ring is *meta*-substituted. These data contrast with the methoxphenidine isomers (see Chapter 3) as the molar absorptivities for **2-4** are 1290 L mol^{-1} cm^{-1}, 1100 L mol^{-1} cm^{-1} and 660 L mol^{-1} cm^{-1} respectively. Thus, exchanging the methoxy

group for fluorine substituent acts to reduce the molar absorptivity observed. i.e. the intensity of absorption of UV light by methyl group in (2-MXP) at a given wavelength (λ max = 278 nm) is greater than the intensity of absorption of fluorine in (2-FEP) at a given wavelength (λ max = 270 nm).

The absorbance for all three isomers at $\lambda_{270\ nm}$ were within the range of around 0.5 and just below 1.5, which is acceptable because the linear absorbance range of most spectrometers is between 0.1 and 1. For any measured absorbance that is at or above 1.0, this means that the sample needs to be diluted. In this experiment, the maximum wavelength at 270 nm was applied as one of the parameters used in HPLC method development and validation of FEP regioisomers.

4.2.3 Infrared spectroscopy

In this study, the ATR-FTIR spectra of the three FEP regioisomers were obtained, see Figure 27 (**8**), Appendix A 20 (**9**) and Appendix A 21 (**10**). By consideration of the data, the C-H stretches of the three FEP isomers appear at the following wave numbers: 2974 cm^{-1} (**8**), 2969 cm^{-1}(**9**) and 2971 cm^{-1} (**10**), respectively. The corresponding aromatic C=C stretches were observed at 1580 and 1452 cm^{-1}, 1589 and 1454 cm^{-1}, and 1580 and 1454 cm^{-1}, for **8-10**. The C-H wag (strong) is located at 756 cm^{-1} (Figure 27) with a transmittance of approximately 45%. In comparison, for the other two FEP isomers, the same peak is observed at 790 and 753 cm^{-1} for **9** and **10** respectively, although these peaks are not strong as they have a transmittance of about 75%.

Peaks in the fingerprint region (1500-400 cm^{-1}) arise from complex deformations of the molecule. They may be characteristic of molecular symmetry, or combination bands arising from multiple bonds deforming simultaneously.

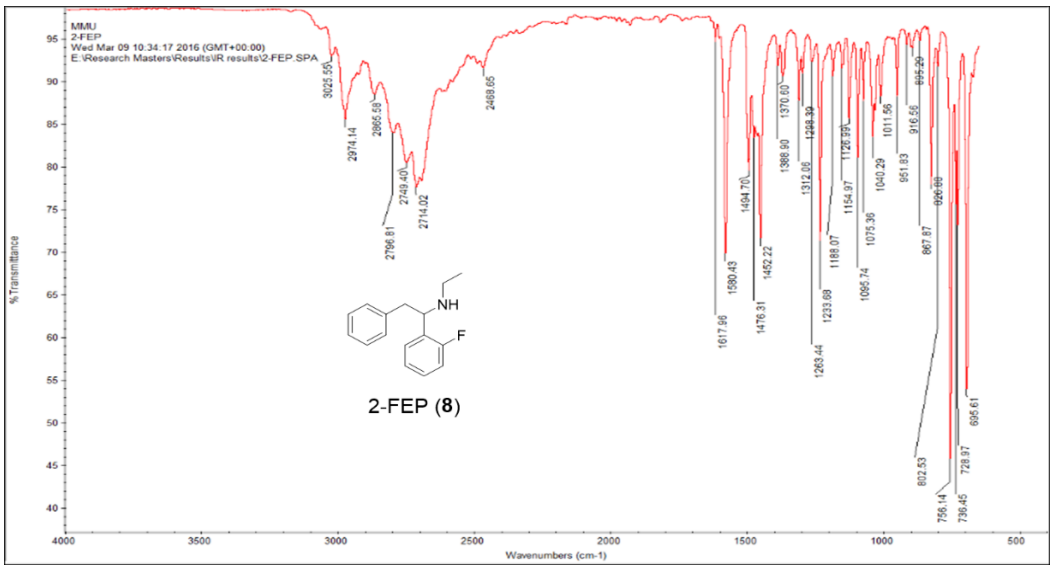


Figure 27. The ATR-FTIR spectrum of **8**

The carbon-fluorine stretching region is 785–540 cm^{-1}. In the spectrum of **8** there is a strong peak at 695 cm^{-1} which is attributed to the C-F stretch. Similarly, stretching frequencies are seen for **9** and **10** (696 and 695 cm^{-1}, respectively). The similarity in these stretching frequencies means that the compounds cannot be differentiated using this individual stretching mode. The bands at about 2860 cm^{-1} (weak) are possibly due to the symmetric (C-H) vibrations of CH_2 groups. Taken together, these results suggest that the change of position of the fluorine of the phenyl group gives different IR spectra depending on the FEP compound under investigation. This facilitates the identification of each compound from other FEP isomers.

4.2.4 Nuclear Magnetic Resonance (NMR)

The ^{1}H and ^{13}C NMR data of the three FEP isomers (as their hydrochloride salts) were obtained using DMSO as a solvent (^{1}H peak at 2.50 ppm, ^{13}C peak at 39.5 ppm).

In the ^{1}H NMR spectrum of **8** (Figure 28), there are two broad peaks at δ 10.22 and 9.69 which are both assigned to NH protons. The observation of two different signals entails that the NH protons are inequivalent, which could be

brought about by the chiral centre adjacent to this environment. Similar chemical shifts for the NH protons are seen for **9** and **10**.

The aromatic region of **8** (Figure 28) is complex due to the seven different aromatic ^{1}H NMR environments that it possesses. Despite the presence of fluorine, the ^{1}H-^{19}F splittings are not readily visible. In fact, it is difficult to discern any notable peaks in this region that would aid structural elucidation. It is, however, readily apparent which regioisomer is *para*-substituted when contrasting **8** with **9** and **10**; in the case of **10** (Appendix A 24), the aromatic region simplifies due to the increased symmetry of the fluorinated ring. However, only the ^{1}H nuclei beta to the C-F site possess splitting that can be measured ($^{3}J_{HH}$ = 8.8 Hz, $^{4}J_{HF}$ = 5.5 Hz); the ^{1}H nuclei alpha to the C-F site appear as a multiplet.

The aliphatic region of the ^{1}H NMR spectrum is more readily interpreted, in that the chiral CH of **8** is observed at δ 4.7 and the diastereotopic protons that this environment couples to are located at δ 3.19 and 3.66. However, when compared with **9** and **10**, the values for the diastereotopic protons do not show significant chemical shift whereas that of the chiral CH are almost identical being 4.54 and 4.51 ppm respectively (Appendix A 22 and Appendix A 24). The backbone can therefore not be used to discriminate these regioisomers from a ^{1}H NMR viewpoint.

The $^{13}C\{^{1}H\}$ NMR spectrum of **8** possesses ten peaks in the aromatic region. The peak at 161.3 ppm is the quaternary carbon to which the fluorine substituent is attached; it possesses a $^{1}J_{CF}$ of 245 Hz. Three further peaks, located at δ 116.4, 123.3 and 132.5, also possess J_{CF} coupling of 22, 14 and 8 Hz respectively. The signals at δ 116.4 and 123.3 is the CH and quaternary carbon *ortho* to C-F respectively whereas the peak at δ 132.5 is the CH *meta* to C-F and *para* to the quaternary ring carbon. The six remaining signals in the aromatic region display no C-F coupling.

The aromatic region of **9** also possesses ten peaks, just like **8**. However, due to the fluorine substituent now being positioned *meta* to the quaternary ring

carbon, there are more peaks present that possess C-F splitting. The C-F carbon is observed at 163.0 ppm and possesses a $^{1}J_{CF}$ coupling of 243 Hz. The two carbons that are alpha to this position are found at 116.8 and 116.5 ppm and display $^{2}J_{CF}$ coupling of 21 and 22 Hz respectively. Two further peaks, located at 131.7 and 138.5 ppm, show $^{3}J_{CF}$ coupling of 8 and 7.5 Hz respectively.

In contrast to **8** and **9**, **10** only possesses eight peaks in the aromatic region. This is because the fluorine is now positioned *para* to the quaternary ring carbon, thus leading to a symmetric system. The peak for the carbon directly attached to fluorine is observed at δ 163.1 and possesses J_{CF} coupling of 244 Hz. This means that this signal would overlap with the same signal of **9** if present in the same sample. The three remaining signals for the fluorinated ring are located at 116.5, 132.1 and 132.0 ppm. These signals correspond to the CH *ortho* to the C-F, the C-H *meta* to the C-F and the quaternary ring carbon respectively. The value of J_{CF} decreases in the order 21.3, 8.4 and 2.9 Hz as the carbon-fluorine spin-spin interaction decreases (See Figure 29, Appendix A 23 and Appendix A 25).

Four aliphatic signals are present in the $^{13}C\{^{1}H\}$ NMR spectrum of **8** and this matches the expected number. The most shielded carbon environment is that of the CH_3 group of the ethyl chain (δ 12.0), whereas the other three signals at δ 39.3, 41.4 and 55.8 are deshielded. The peak at δ 41.4 corresponds to the CH2 of the ethyl chain whereas the peaks at δ 39.3 and 55.8 are the remaining CH_2 and CH respectively. When compared to **9** and **10**, there is very little change in this part of the spectrum with the exception of the chiral CH environment; in **9** this shifts to 62.6 ppm whereas in **10** it shifts to 62.4 ppm.

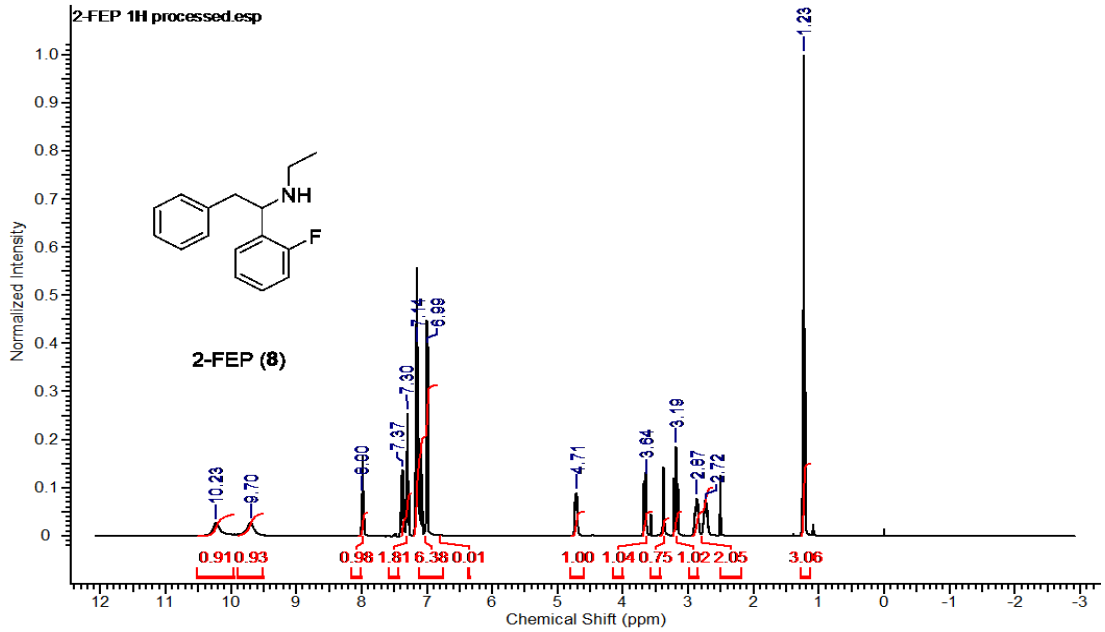


Figure 28. ^{1}H NMR spectrum of **8** collected in d_6-DMSO

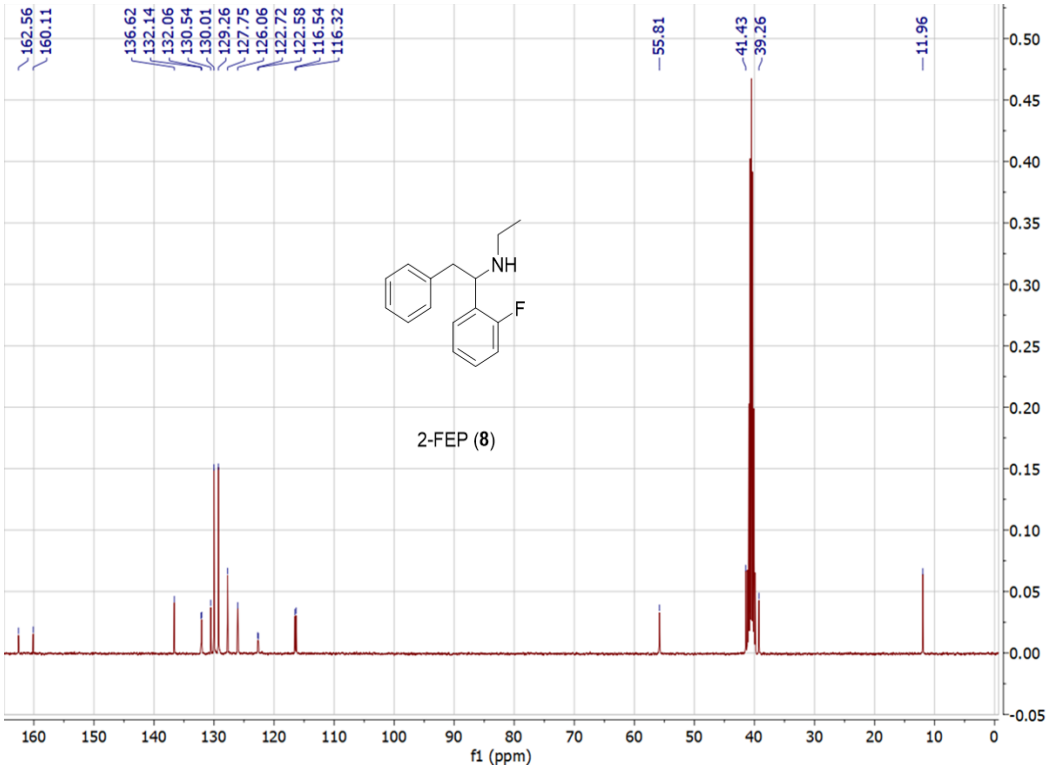


Figure 29. ^{13}C{^{1}H} NMR spectrum of **8** collected in d_6-DMSO

4.2.5 High performance liquid chromatography (HPLC)

A HPLC method was developed to separate the FEP isomers (and then validated in accordance with the ICH guidelines)[137] using the same parameters applied during the method development for MXP isomers; these parameters include the mobile phase, temperature, flow rate and the column etc. as was shown in Chapter 3. In method development, modelling can be applied such as by using computer-aided method-development programs that require accurate models to describe retention and to make predictions based on a limited number of factors or experiment parameters. No modelling was applied to optimisation of the developed HPLC method in this research project.

4.2.5.1 HPLC Method Optimised (FEP isomers)

This HPLC method for testing FEP compounds was the HPLC method used for testing MXP isomers, by applying the same conditions using mobile phase acetonitrile:ammonium acetate 55:45% v/v, flow rate 1.0 mL min^{-1}, temperature 50°C and the column ACE 5 C18-AR (150 x 4.6 mm, 5 µm particle size). The total run time in this experiment was 10 minutes.

Due to the poor resolution (0.60), the proportion of the organic modifier was reduced to improve the resolution (2.11) between the peaks. In addtion, the flow-rate was investigated to improve the run time; the final optimised flow rate was 1.5 mL min^{-1}. The UV wavelength of maximum absorbance of FEP isomers at 270 nm was applied to detect these substances via HPLC. (See

Table *13*).

Table 13. Representative data for the HPLC method development by modifying the flow rate and mobile phase percentage for the separation of the FEPs 8-10 using acetonitrile:ammonium acetate as the mobile phase. Key: Rs (8 and 9) = Resolution between 8 and 9 peaks

% Acetonitrile:ammonium acetate	**Flow rate (mL min^{-1})**	**Rs (8 and 9)**	**Total Run time (min)**
55:45% v/v	1.0	0.60	10
40:60% v/v	1.0	1.28	10
30:70% v/v	1.2	1.87	20
	1.3	1.95	20
	1.5	1.91	20
	1.7	1.84	15
25:75% v/v	1.5	2.11	25

As can be seen from the data, the resolution was improved significantly by reducing the percentage of acetonitrile in the mobile phase, for example: (1.84-1.95) in all four flow rates using mobile phase percentage of 30:70% v/v. However, after applying the flow rate of 1.5 mL min^{-1} the resolution decreased again to about 1.9, this value also dropped to almost 1.8 when the flow rate of 1.7 mL min^{-1} was employed. Lastly, by applying acetonitrile:ammonium acetate 25:75% v/v proportion using the flow rate 1.5 mL min^{-1}, the resulting resolution was 2.11.

In conclusion, from these four experiments, in which the mobile phase organic

component was varied along with the flow rate, an optimised mobile phase and flow rate (acetonitrile:ammonium acetate 25:75% v/v and 1.5 mL min^{-1}, respectively) were identified. These parameters will be applied to the HPLC method validation of the FEP isomers, this is despite the total run time being long (25 minutes); this is 2.5 times longer than that utilised for the MXP isomers (see Chapter 3). The improvement in resolution is because the retention factor k is increased by decreasing the percentage of organic modifier in the mobile phase.

Although HPLC method development will continue to be based on chromatographer's experience, software and mathematical models in method prediction may save a lot of the laboratory budget for organic solvents, not to mention the greener chemistry that will be achieved. For example using the statistical Design of Experiment (DOE) to develop a simple and robust reversed-phase HPLC technique. [138]

4.2.5.2 Method Validation of FEP isomers

This validation was performed according to the optimised method for MXP regioisomers (chapter 3), using the same column. This method was also validated in accordance with the ICH guidelines [128] using the same parameters as detailed previously. The percentage RSD was calculated for each replicate sample. The method shows excellent linearity (R^2 = 0.999) over the 10-60 µg mL^{-1} range with good repeatability in each case (% RSD = 0.03 – 1.41%, n = 6).

Table 14. Representative validation data for FEP isomers obtained using an ACE 5 (150 x 4.6 mm i.d, 5 µm) column. Mobile phase: acetonitrile: ammonium acetate 25:75% v/v, flow-rate: 1.5 mL min^{-1}, measured at 270 nm; detector: UV-DAD

Sample	**10**	**8**	**9**
t_R (min)(t_0=1.29min)[a]	17.47	21.45	23.35
RRT	1.22	1.00	0.91
Capacity Factor (*K'*)	16.47	20.45	22.35
Resolution (R_s)	-	5.26	2.16
Asymmetry (As)	0.75	0.97	0.84
LOD (µg mL^{-1})[b]	0.47	0.23	0.60
LOQ (µg mL^{-1})[c]	1.43	0.70	1.84
Regression coefficient (r^2)	0.9996	0.9999	0.9993
Equation	Y = 0.7497X + 0.0088	Y = 2.1963X - 0.4057	Y = 1.871X - 0.8858
Precision (%RSD)			
10 µg mL^{-1}	0.03	1.41	1.38
20 µg mL^{-1}	0.93	1.38	0.77
30 µg mL^{-1}	1.38	0.57	0.73
40 µg mL^{-1}	0.68	0.58	0.27
50 µg mL^{-1}	0.95	0.21	0.61
60 µg mL^{-1}	0.32	0.17	0.55

Key: (a) Determined from the retention time of a solution of uracil (0.5 µg mL^{-1}) eluting from the column; (b) limit of detection (based on the standard deviation of the response and the slope); (c) limit of quantification (based on the standard deviation of the response and the slope).(d) Y = 0.7497X + 0.0088; (e) Y = 2.1963X - 0.4057; (f) Y = 1.871X - 0.8858.

Table 14 shows the data for the HPLC method validation of FEP regioisomers using an ACE 5 column, acetonitrile:ammonium acetate 25:75 % v/v as the mobile phase and a flow rate of 1.5 mL min^{-1}. Six different concentrations ranging from 10 to 60 µg mL^{-1} were tested. Uracil provided a single sharp peak at about 1.3 minutes, this is known as t_0, which is the time for the unretained compound (uracil) to travel through the column or to reach the detector. As is observed from the validation table, excellent peak shapes and asymmetry factors were obtained (0.75, 0.84 and 0.97) with no fronting or tailing. The limit of detection or LOD (0.23-0.60 µg mL^{-1}) represents the lowest detectable concentrations and the limit of quantification, whereas LOQ (0.70-1.84 µg mL^{-1}) is the lowest amount that can be quantified. The resolution was within the required range (5.26-2.16), while the retention time ranged from just over 17 minutes to just below 24 minutes.

As can be seen in the table, the precision (% RSD) was calculated for all the 6 concentrations and was just below 1 except for the lowest concentrations, such as 10 µg mL^{-1}, where it was approximately 1.40; this is perhaps due to low precision with respect to low concentrations. The highest concentrations (30-60 µg mL^{-1}) gave good %RSD with values less than 1.

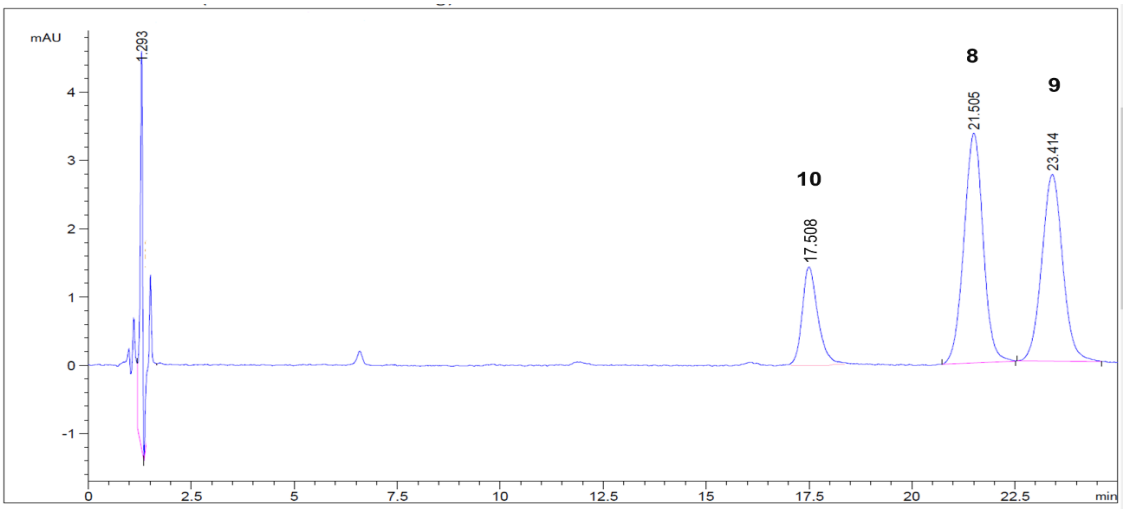


Figure 30. HPLC separation of FEP isomers (concentration = 50 µg mL^{-1}). Key: **10** (17.5 min), **8** (21.5 min) and **9** (23.4 min), respectively.

The chromatogram (Figure 30) demonstrates that the three isomers are fully baseline separated (R_s > 2) in under 25 minutes run time. The order of elution was as follows; 10, 8 and 9, respectively. Among the separated compounds, **10** was detected at 17.5 minutes, which is about 4 minutes before the appearance of **8** at 21.5 minutes and around 2 minutes later, the last peak of **9** at 23.4 minutes was detected. The obtained resolution values (ranged from over 2 to just over 5) are acceptable for separating FEP isomers. Lastly, as can be noted from the chromatogram the shape of the peaks was excellent. **10** shows a bit of tailing which could be due to various mechanisms such as nonspecific hydrophobic interactions with the stationary phase. However, polar interactions with any ionized residual silanol groups on the silica support surface are also common. Compounds possessing amine and other basic functional groups interact strongly with such ionised silanol groups. i.e. tailing occurs when some sites on the stationary phase retain the solute more strongly than other sites. Capacity factors of **8** and **9** (greater than 20) mean that elution takes a very long time. Ideally, the capacity factor for an analyte is between 1 to 5, capacity factors of all three MXP isomers were less than 5 (see Chapter 3).

In summary, it can be proposed that the applied HPLC method was able to detect and separate the fluephenidine isomers in a reasonable time interval, and with good resolution. Gradient method or GC could be possible solutions to obtain shorter retention times compared to this isocratic method.

4.2.5.3 Robustness of the Method (FEP isomers)

The robustness of the method for separating the FEP isomers was assessed by measuring the intra- and inter-day precision, temperature, flow rate and the percentage of mobile phase composition, by following the same procedures as previously mentioned for the MXP isomers (see Chapter 3), a concentration of 50 µg mL^{-1} was used and 10 injections utilised. Firstly, both inter-day and intra-day precession were tested. Secondly, the used temperatures (48, 50 and 52°C) were the same as for MXP isomers. Thirdly, the flow rates were as

follows: 1.4, 1.5 and 1.6 mL min^{-1}. Finally, the percentages of mobile phase (acetonitrile:ammonium acetate) were modified in the following proportions: 24:76, 25:75 and 26:74% v/v (Table 16).

4.2.5.3.1 Robustness testing (intra- and inter-day precision)

The table in Appendix A 26 shows data on the intra- and inter-day precision measurements for the three fluephenidine isomers using mobile phase acetonitrile:ammonium acetate 25:75% v/v, flow rate 1.5 mL min^{-1} and at a working concentration of 50 µg mL^{-1} and ten injections in the HPLC. The inter-day refers to that the measurement is occurring within or between two days, while intra-day means that they are occurring within one day. As is observed in the table, for both intra- and inter-day measurements, the retention time remained almost constant for the three FEP isomers, as relative t_R (RRT) for 10, 8 and 9 were 1.23, 1.00 and 1.08, respectively. The percentage RSD is on average from about 0.25-0.30 for intraday measurements and about 0.10-0.15 for inter-day precision, which refers to a very slight difference in the detection precision without affecting the retention times of these isomers.

In summary, these results indicate that the optimised HPLC method used here has a good precision after testing the FEP sample at different times throughout the 24 hours. This also means that this method is valid/robust to use at various times.

4.2.5.3.2 Robustness testing (Flow Rate and van Deemter equation)

The table in Appendix A 27 compares data on the effect of testing three different flow rates on the retention times of FEP isomers, **8**, **9** and **10**, using ten injections and a concentration of 50 µg mL^{-1}. It is clear from the results that an overall trend is that by increasing the flow rate from 1.4-1.6 mL min^{-1} the t_R is decreased. The resolution between **8** and **9** peaks was on average 2.1 for all three flow rates utilised, whereas, the value of the resolution between the peaks of **10** and **8** was on average 5.1 for all three runs. The optimum flow rate in this experiment is 1.5 mL min^{-1} because the resolution was the highest (5.01)

compared to the flow rates 1.4 and 1.6 mL min^{-1}, and because obtaining the highest resolution value is the main goal for optimum separation (see van Deemter equation in the introduction chapter).

4.2.5.3.3 Robustness testing (Temperature 48, 50 and 52°C) and the Van't Hoff plot of FEP isomers

Table 15 and Figure 31 show information relating to robustness testing (temperatures used: 48, 50 and 52°C) and the Van't Hoff plot for the three FEP

Table 15. Robustness testing (temperature = 48, 50 or 52°C) for the three FEP analytes using a mobile phase consisting of acetonitrile:ammonium acetate 25:75% v/v, concentration of FEP = 50 µg mL^{-1}

Injection	t_R (min) using temp. 48 °C			t_R (min) using temp. 50 °C			t_R (min) using temp. 52 °C		
	10	**8**	**9**	**10**	**8**	**9**	**10**	**8**	**9**
1	16.76	20.56	22.38	16.92	20.56	22.39	16.69	20.64	22.44
2	16.76	20.56	22.38	16.93	20.56	22.38	16.70	20.65	22.45
3	16.77	20.57	22.39	16.92	20.56	22.39	16.70	20.66	22.45
4	16.77	20.57	22.39	16.94	20.57	22.39	16.71	20.65	22.45
5	16.78	20.57	22.38	16.94	20.57	22.39	16.72	20.66	22.46
6	16.79	20.58	22.40	16.95	20.58	22.41	16.73	20.67	22.47
7	16.80	20.59	22.41	16.94	20.57	22.40	16.73	20.67	22.47
8	16.80	20.59	22.41	16.95	20.57	22.40	16.74	20.68	22.48
9	16.81	20.60	22.42	16.95	20.58	22.40	16.75	20.69	22.49
10	16.82	20.61	22.43	16.97	20.59	22.41	16.76	20.69	22.50
Average	**16.79**	**20.58**	**22.40**	**16.94**	**20.57**	**22.40**	**16.72**	**20.67**	**22.47**
STD	**0.022**	**0.015**	**0.018**	**0.015**	**0.008**	**0.008**	**0.023**	**0.016**	**0.019**
%RSD	**0.136**	**0.073**	**0.081**	**0.088**	**0.038**	**0.039**	**0.139**	**0.078**	**0.086**

analytes using mobile phase (acetonitrile: ammonium acetate 25:75% v/v) and concentration of FEP of 50 µg mL^{-1}. It is notable that t_R was almost constant despite minor changes in the temperatures used in this experiment with an average of 16.7, 20.5 and 22.4 minutes, and relative retention times of 1.22, 1 and 0.92 for **10**, **8** and **9** respectively. The resolution values as an average is 5.1 between the two peaks of **10** and **8**, whereas in the case of resolution between the peaks of **8** and **9**, it was 2.1 by taking the average of the three temperature observations. It is interesting to note that the retention time of the three compounds **8, 9** and **10** was almost the same (Appendix A 28). The overall trend for changing temperature by 2°C (i.e. 50±2°C) did not have any significant effect on the retention factor and resolution values. There is difficulty associated with the discussion of the effect of temperature and also the enthalpy and entropy changes in this narrow temperature range for **8-10**.

As is shown in Figure 31, the three FEP isomers show a linear Van't Hoff plot suggesting that the retention mechanism is the same, which is similar to MXP isomers, and Boateng findings (see chapter 3); it can be noted that the trend lines of **8, 9** and **10** are almost parallel indicating that these isomers behave in the same way chromatographically. i.e. in their binding with the silanol groups in the stationary phase (hydrophobic interaction).

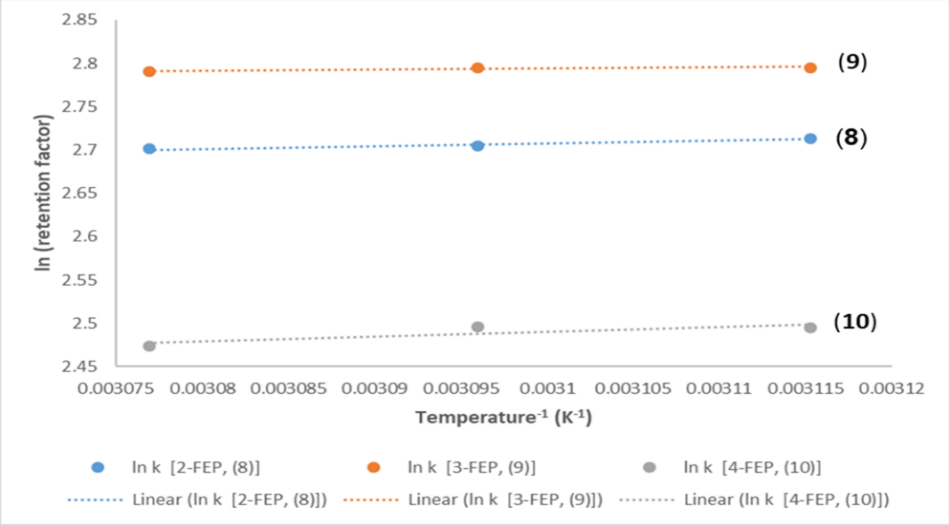


Figure 31. Van't Hoff plot of ln k versus 1/T for fluephenidine isomers (**8, 9** and **10**)

4.2.5.3.4 Effect of minor modifications to mobile phase composition.

The data presented in Table 16 shows the results of the experimental investigation in which the proportion of mobile phase, acetonitrile:ammonium acetate, was modified and how this effected the robustness of the HPLC method applied to separate **8**, **9** and **10**. As is observed from the obtained data, increasing the percentage of the organic part (acetonitrile) of the mobile phase had far better resolution than using less organic solvent. According to the data in Table 16, the used percentage of 25:75% v/v (acetonitrile:ammonium acetate) gave better retention times and resolutions in this study, for example if 25:75% v/v was compared to 26:74% v/v. Nonetheless, the former ratio is still better than the latter; when considering the obtained resolution of 2.11 (relative to 2.04) between the peaks of **8** and **9**. Again, the 25:75% v/v proportion was far better than the other two proportions with regard to the resolution values obtained from the three different experiments. The resolution value between the first two eluted peaks (**10** and **8**) was 4.92 using the percentage 24:76% v/v, while between the last eluted two peaks (**8** and **9**), the resolution was 5.01 when 25:75% v/v was applied (see Appendix A 28). The solutes of FEP isomers (**8**-**10**) are retained in the column; by increasing the percentage of the organic modifier (acetonitrile) from 24-26% v/v, the retention time of these compounds is decreasing, in agreement with linear Van't Hoff plots.

By increasing the percentage of the organic modifier (acetonitrile) from 24 to 25% and then to 26% the retention time of the FEP isomers (**8**, **9** and **10**) are decreasing. Similar to MXP isomers, ChemDraw software was used to predict log P of FEP isomers, in this case it equals 4.09, which is slightly less than MXP isomers (4.58). Again, this confirms that FEP isomers possess a hydrophobic effect as a mechanism of retention.

In conclusion, in all the experiments of robustness testing on HPLC, by applying minor changes in experimental parameters such as flow rate, temperature and mobile phase composition there were minor changes in t_R

and resolution, while both RRT and peak area remain constant. The resolution values obtained for FEP isomers during the three robustness experiments are shown in Appendix A 28, the results displayed in this table represent the average of 10 injections in all robustness experiments.

Table 16. Robustness testing (Mobile phase percentage) for the three FEP analytes using a mobile phase consisting of acetonitrile:ammonium acetate 24:76% - 26:74% v/v, concentration of FEP = 50 µg mL^{-1}

Injection	t_R (min) using acetonitrile: ammonium acetate (24:76 % v/v)			t_R (min) using acetonitrile: ammonium acetate (25:75 % v/v)			t_R (min) using acetonitrile: ammonium acetate (26:74 % v/v)		
	10	**8**	**9**	**10**	**8**	**9**	**10**	**8**	**9**
1	18.58	22.70	24.75	17.19	20.99	22.85	15.63	19.08	20.72
2	18.66	22.72	24.77	17.18	20.98	22.84	15.48	18.92	20.56
3	18.65	22.72	24.76	17.19	20.98	22.83	15.59	19.00	20.61
4	18.66	22.73	24.76	17.19	20.97	22.83	15.49	19.15	20.49
5	18.68	22.73	24.77	17.18	20.97	22.82	15.62	19.03	20.71
6	18.68	22.73	24.77	17.19	20.96	22.82	15.58	19.10	20.56
7	18.67	22.74	24.78	17.18	20.96	22.82	15.57	19.20	20.63
8	18.69	22.74	24.79	17.18	20.96	22.82	15.61	19.02	20.57
9	18.69	22.75	24.79	17.19	20.97	22.82	15.48	19.10	20.75
10	18.70	22.75	24.79	17.20	20.97	22.83	15.63	18.98	20.56
Average	**18.67**	**22.73**	**24.77**	**17.19**	**20.97**	**22.83**	**15.57**	**19.06**	**20.62**
STD	**0.032**	**0.015**	**0.014**	**0.006**	**0.008**	**0.009**	**0.060**	**0.082**	**0.084**
%RSD	**0.176**	**0.066**	**0.060**	**0.037**	**0.040**	**0.041**	**0.390**	**0.431**	**0.408**

4.2.5.4 Accuracy of the method and determination of percentage recovery (FEP isomers)

Table 17. Determination of % Recovery for the FEP regioisomers

	Theoretical conc. = 40 µg mL^{-1}			**Theoretical conc. = 50 µg mL^{-1}**			**Theoretical conc. = 60 µg mL^{-1}**		
Injection	**10**	**8**	**9**	**10**	**8**	**9**	**10**	**8**	**9**
1	98.7	98.7	99.2	99.7	98.5	99.8	99.4	98.03	99.32
2	100.2	98.4	98.9	101.1	98.4	99.8	99.6	98.23	98.7
3	99.2	98.5	98.9	100.9	98.2	99.8	99.9	98.34	98.7
4	99.3	97.4	98.5	100.7	98.6	98.7	99.8	98.14	98.1
5	99.2	98.4	98.8	98.6	98.1	98.8	99.8	98.32	97.7
6	99.2	97.5	98.9	100.9	98.6	98.7	100.4	98.54	98.55
Average	**99.3**	**98.1**	**98.8**	**100.3**	**98.4**	**99.2**	**99.8**	**98.2**	**98.5**
STD	**0.489**	**0.554**	**0.231**	**0.976**	**0.209**	**0.585**	**0.337**	**0.176**	**0.557**
%RSD	**0.493**	**0.564**	**0.233**	**0.973**	**0.213**	**0.589**	**0.337**	**0.179**	**0.565**

Table 17 presents data regarding the determination of accuracy of HPLC method and percentage recovery of three FEP isomers, using three different concentrations (40, 50 and 60 µg mL^{-1}) with an average of six injections each. From the results of the three concentrations investigated in this experiment, the RSD (0.2 – 0.8%), indicated excellent injection repeatability according to ICH guidelines.[137] In conclusion, according to these findings, it is obvious that the results of percentage recovery were within the ICH limit i.e. 98-102% and the percentage RSD was less than one, indicating excellent precision. Additionally, this confirms that the applied method for analysis of fluephenidine isomers is accurate and suitable for routine analysis of any sample that might contain these analytes.

4.3 Forensic Application

Three bulk forensic samples of FEP were obtained from Greater Manchester Police via the MANchester DRug Analysis and Knowledge Exchange (MANDRAKE) partnership. The three samples were analysed using the developed HPLC method i.e. using acetonitrile:ammonium acetate 25:75% v/v as the mobile phase (Table 18, Figure 32, Figure 33 and Figure 34).

Table 18. Bulk samples tested on HPLC (FEP isomers). Key: t_R = Retention time, St S1-3 = Street Sample 1-3

Street Sample	Qualitative Analysis; t_R (min)	Peak Area	Actual conc. (μg mL^{-1})	Found conc. (μg mL^{-1})	Quantitative Analysis (% w/w)
St S1	**8**, t_R = 21.1	94.43	50.16	43.18	86.09
St S2	**8**, t_R = 21.1	29.37	49.65	13.56	27.31
	9, t_R = 22.1	59.69	49.65	32.37	65.19
St S3	**8**, t_R = 21.1	48.76	50.50	20.36	40.31
	9, t_R = 23.0	43.46	50.50	23.70	46.93

Table 18 displays qualitative and quantitative data for the three bulk samples using the developed HPLC method in this study. The obtained retention times (t_R), peak areas, actual and found concentrations are presented in units of microgram per millilitre, and the percentage of the detected FEP analyte in these sample are shown in the table. A representative chromatogram of a street sample is shown in (Figure 32). The peak at 1.28 mins is the uracil peak. All the three street samples were run three times each; the results show that St S1 contains only one FEP compound (**8**), while St S2 contains two FEP compounds (**8** and **9**); which is similar to St S3.

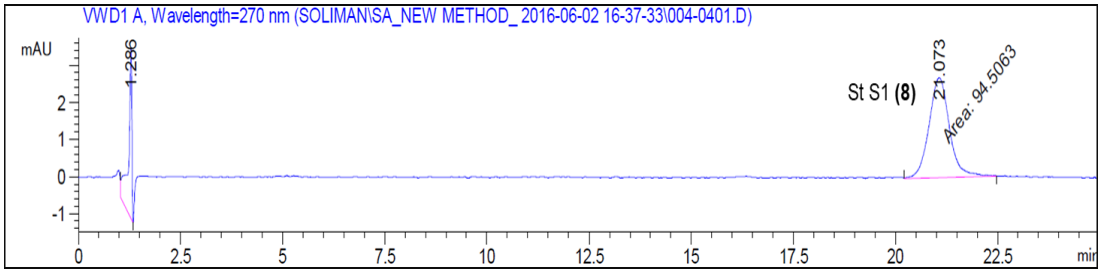


Figure 32, Representative HPLC chromatogram of Sample 1 (St S1) containing: uracil (peak at 1.28 min) and **8** at 21.07 minutes

As is seen in Table 18, **8** was detected in all the three samples (St S1, 2 and 3) whereas **9** was only found in St S2 and 3. **10** was not identified in any of the street samples. The presence of two FEP isomers in one bulk sample could be due to mixing of many substances in one sample. This observation is similar to the findings in a study by Cumba, *et al.*, who analysed "*Synthacaine*" street samples by an electroanalytical sensing technique and validated with HPLC, and found that it contained a mixture of methiopropamine (MPA) and 2-aminoindane (2-AI).[139]

The St S1 had the highest percentage content of **8** (86.09% w/w) among the three tested samples with a peak area of 94.5, the percentage of FEP in St S3 is just below half of that found in St S1 (40.3%), but represents around two thirds of its quantity in St S2 (65.19%). A possible explanation might be because of the different degrees of purity of these street samples. The chromatogram shows no peaks of other additives/adulterants, this is probably because they cannot be detected by using the current HPLC method.

As can be seen in the two chromatograms below, the first (Figure 33) shows two peaks that were assigned to 8 and 9 in bulk sample 2 (St S2), the resolution between the two detected/separated peaks is 2.03, whereas in sample 3 (St S3), 8 and 9 were detected and the resolution between their peaks was 2.13 (Figure 34).

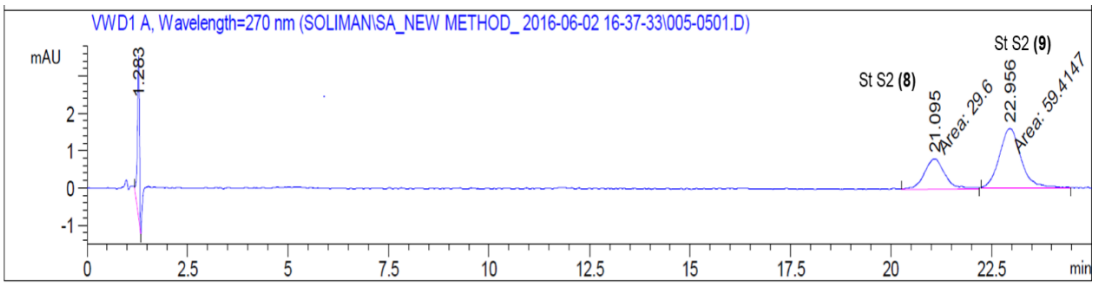


Figure 33. Representative HPLC chromatogram of Sample 2 (St S2) containing: uracil (peak at 1.28 min), **8** and **9** at 21.09 and 22.95 minutes, respectively

These values of resolution are acceptable because they are both over 2 (fully resolved peaks). Comparatively, it is clear that the values of peak areas (See the third column of the table), were consistent with the calculated percentages of FEP in the bulk sample (in the last column in Table 18).

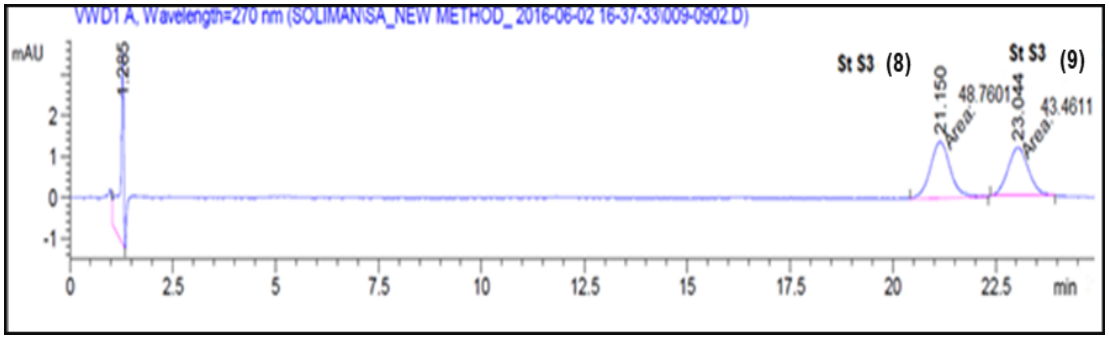


Figure 34. Representative HPLC chromatogram of Sample 3 (St S3) containing: uracil (peak at 1.28 min), **8** and **9** at 21.15 and 22.04 minutes, respectively

In summary, both **8** and **9** were found in the investigated bulk samples and were separated by HPLC with good resolution and peak shape. This confirms the suitability of the optimised/developed HPLC method in this study for routine analysis of these illicit NPS (fluephenidine isomers) either in a mixture or as separate components.

4.4 Conclusion

The previously developed and validated HPLC method was applied for qualitative and quantitative analysis of fluephenidine isomers (FEP), which are

new diphenidine derived compounds and belong to NPS. The numerous spectroscopic data provided in this experimental investigation can be considered as an essential foundation of characterisation data (^{1}H-NMR, ^{13}C-NMR, UV and ATR-FTIR) for the reference materials exploited in this work.

This study detailed the analysis of samples of the three fluephenidine isomers, which can be encountered in NPS forensic samples using HPLC. Specifically, the validated HPLC method which was used to analyse MXP isomers (see Chapter 3) was applied with some modifications in experimental parameters, to analyse these fluorinated diphenidine isomers, for both qualitative and quantitative analysis of these substances as either seized solid samples, as a pure form or when mixed with common additives or adulterants.

All analyses were carried out on reference standards prepared in-house and then cross-validated with three bulk samples. One of the main findings is that the validated method has the advantage of a rapid single-step detection and separation of these street samples in just under 25 minutes run time.

Additionally, the method provided excellent LOD (0.23-.0.60 μg mL^{-1}) and LOQ (0.70-1.84 μg mL^{-1}) for all three isomers; in other words, it acts as an ideal method for both the qualitative and quantitative analysis of FEP isomers, when compared to the previous approaches in the literature.

There is no previous published chromatographic studies on FEP isomers, as these are new substances synthesised by MANDRAKE at MMU. Therefore, the samples were tested and compared to reference standards prepared in-house at MMU. Consequently, bulk samples containing FEP isomers were not encountered by forensic services.

In conclusion, the developed HPLC method was applied to test three bulk samples (St S1, St S2 and St S3), the results confirmed that they all contain 2-FEP (**8**) with t_R of 21 mins, while 3-FEP (**9**) was only present in sample 2 and 3 with a t_R of 23 mins. The total run time was 25 mins, which is lengthy, but did yield good resolution between the peaks observed.

Chapter 5: Analysis of fluorocyanoephenidine regioisomers using Reversed Phase HPLC and three types of GC columns

5.1 Fluorocyanoephenidine

Fluorocyanoephenidine (FCEP) belongs to NPS group and they are derivatives of diphenidine. The chemical structure of FCEP is similar to fenproporex (See Figure 35, Figure 36 and Figure 37), which is utilised in the treatment of obesity by reducing the appetite; however, due to substance abuse potential, it is an illicit substance in many countries. Fenproporex has been shown to produce amphetamine in the urine of users. Previous studies show that the parent compound can be detected in a few hours after administration, while the amphetamine can be detected for several days.

The therapeutic benefits of fenproporex in obesity and losing weight is still not supported with the presence of just limited amount of information in the literature, and this use poses various potential health issues, the risks that may cause is also another concern as an amphetamine derived compound (see chemical structure of amphetamine in Figure 38).[140]

Despite the fact that data on fenproporex is limited, suicide attempts have been linked to it in some case reports,[141] addiction,[141, 142] subarachnoid hemorrhage (surrounding the brain) [143] and morphea (discoloured patches on the skin).[144]

Given the lack of monitoring of fenproporex use, it is certainly possible that other life-threatening events are currently occurring but unrecognized. So, one of the motivations of conducting this experimental investigation on FCEP is to help gain more information on this class of drugs. FCEP compounds are similar to fenproporex and amphetamine in chemical structure and can produce amphetamine as a metabolite.

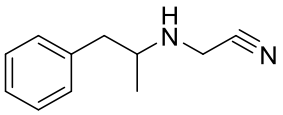


Figure 35. Chemical structure of fenproporex.

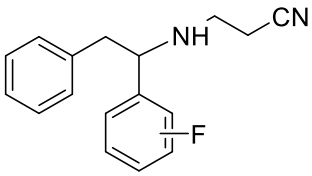
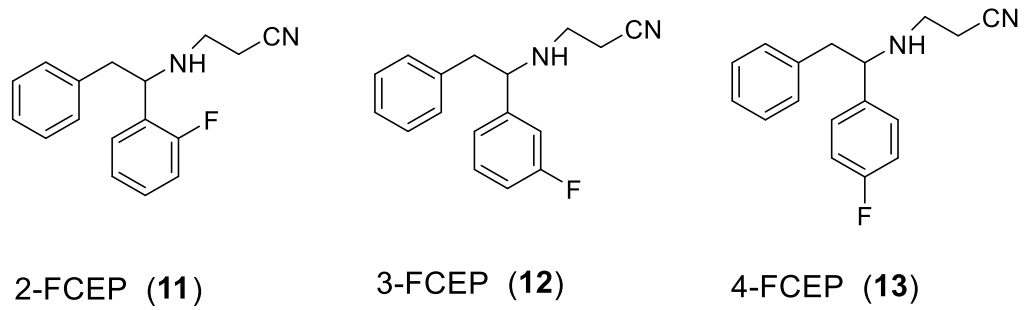


Figure 36. General chemical formula of FCEP isomers.



2-FCEP (**11**) 3-FCEP (**12**) 4-FCEP (**13**)

Figure 37. Chemical structures of FCEP isomers

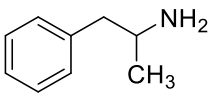
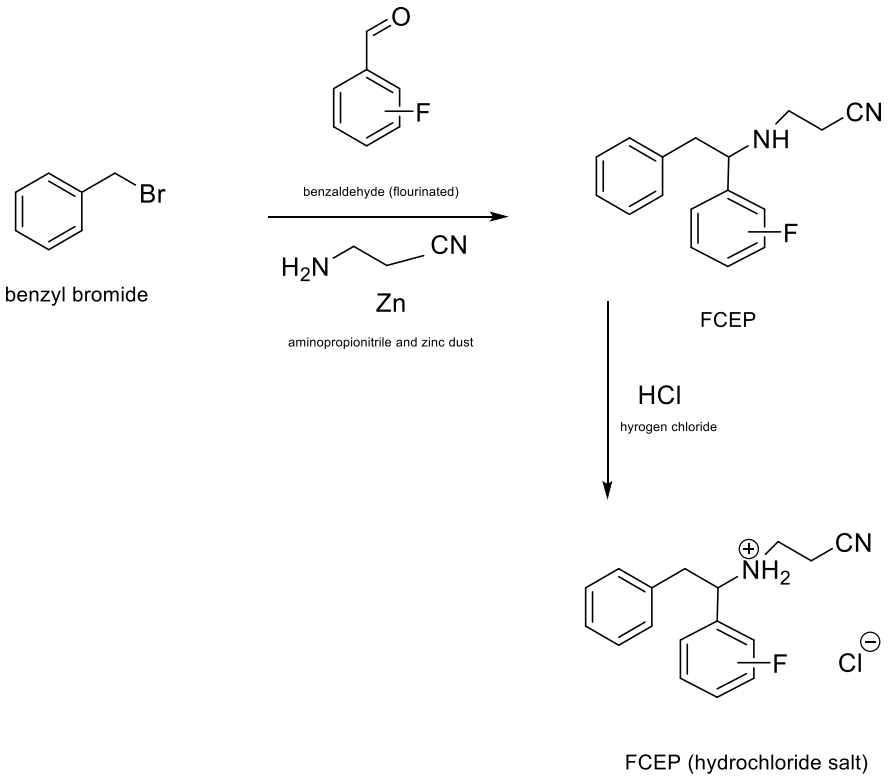


Figure 38. Chemical structure of amphetamine.

5.2 Results and discussion

5.2.1 Synthesis of fluorocyanoephenidine isomers (FCEP)

The FCEP isomers (or FCEP hydrochloride salts) were synthesised as shown in Figure 39, via reaction of benzyl bromide, benzaldehyde (fluorinated), zinc dust and aminopropionnitrile (see chapter 2).



benzyl bromide

Figure 39. Synthesis of fluorocyanoephenidine isomers (FCEP)

5.2.2 Ultraviolet spectroscopy

The results of testing the FCEP isomers via UV spectroscopy are shown in Table 19); which summarises the wavelengths of maximum absorption, absorbance and the concentrations used.

Table 19. The UV max measurement of FCEP isomers

FCEP isomer	**UV max (nm)**	**Absorbance**	**Concentration (mg mL^{-1})**	**Molar absorptivity (L mol^{-1} cm^{-1})**
11	263	0.54	0.20	954
12	263	0.70	0.21	1178
13	258	0.58	0.40	512

Table 19 shows data on UV max measurement of FCEP isomers. As is presented in the illustration, the highest absorbance was for 3-FCEP. As is presented in the UV spectra, a λ_{max} of 263 nm was observed for both 2-FCEP (**11**) and 3-FCEP (**12**) and this value was decreased to 258 nm for 4-FCEP (**13**) (Figure 40 and Figure 41). Furthermore, the absorbance for 4-FCEP was around 0.6 with some fluctuations.

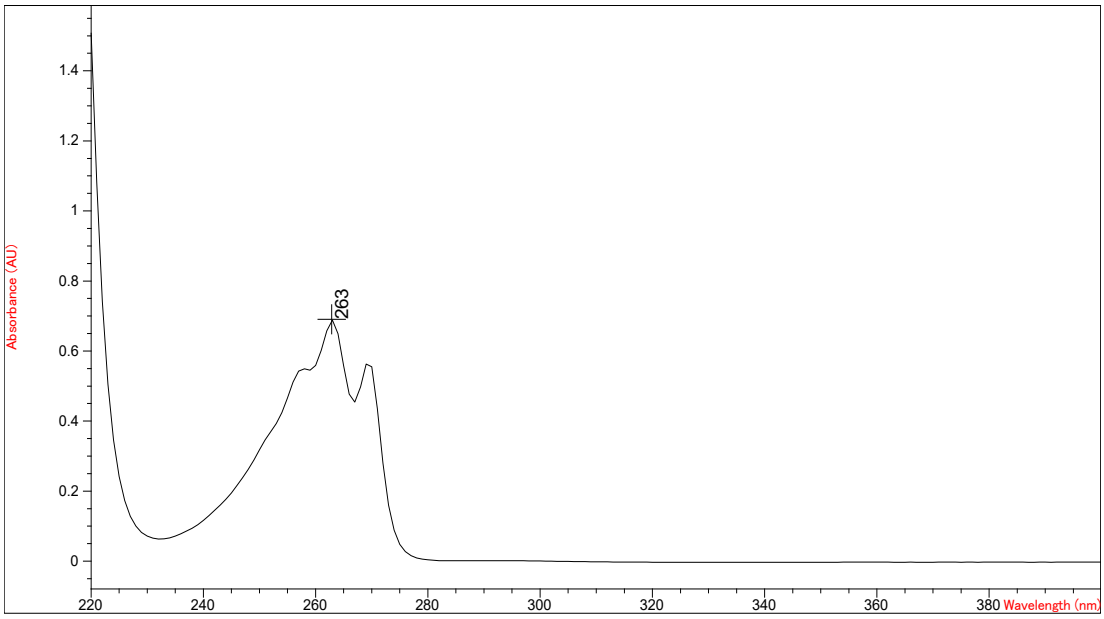


Figure 40. UV spectrum of **11**

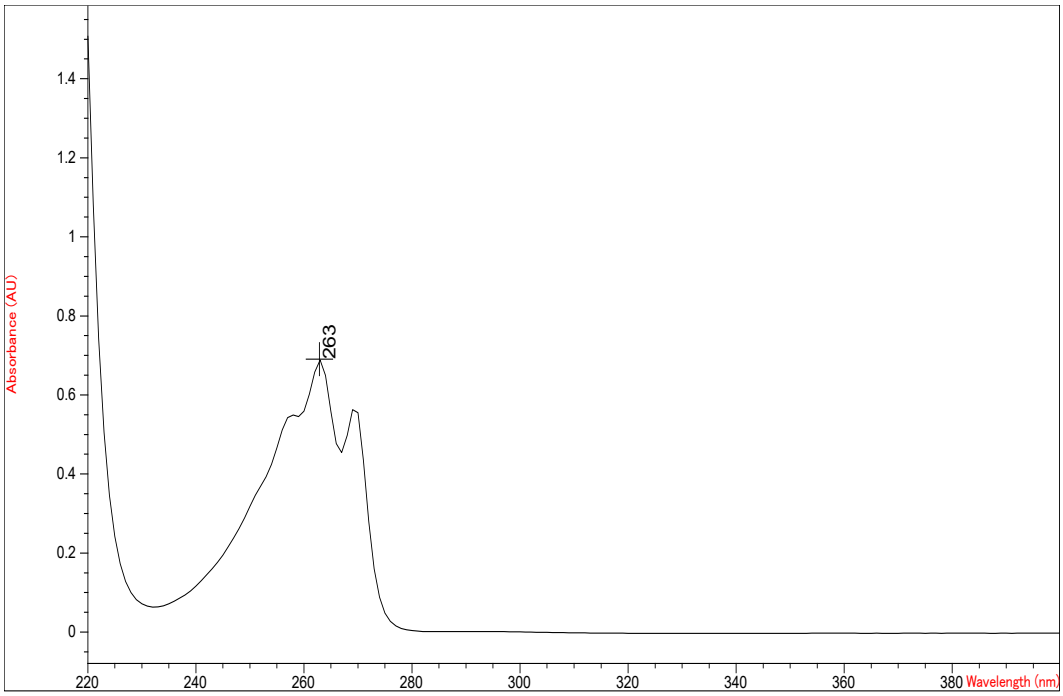


Figure 41. UV spectrum of **12**

5.2.3 Infrared spectroscopy

In this study, the ATR-FTIR spectra of the three FCEP isomers were acquired and the results are shown below (See Figure 42, Appendix A 29 and Appendix A 30).

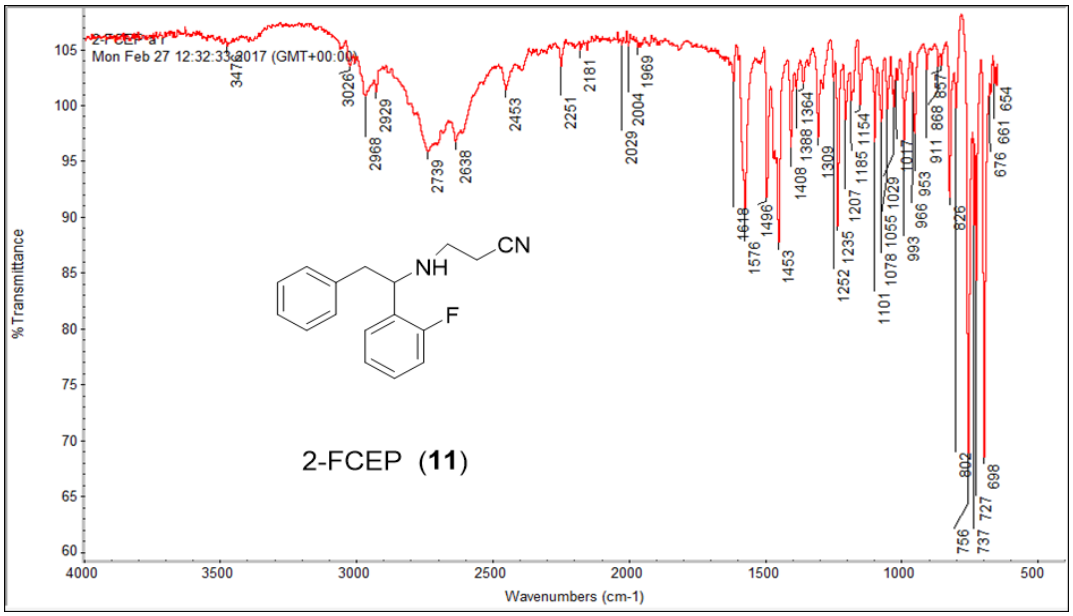


Figure 42. The ATR-FTIR spectrum of **11**

The most important result that can be noted in the IR spectra of FCEP isomers is the presence of the characteristic functional group, cyano group (C≡N) with stretches of intermediate intensity at the 2251 cm^{-1} (See Figure 42, Appendix A 29 and Appendix A 30).

The IR spectra for the three fluorocyanoephenidines show that the C-H stretches of the three FCEP isomers appear at the range of 2960-2970 cm^{-1}. The aromatic C=C stretches are seen in the range (1600 and 1450 cm^{-1}).

The carbon-halogen (C-F) stretching region (785 – 540 cm^{-1}) in the spectra of both **11** and **12**, both possess a strong peak at 698 cm^{-1}. For **13** this signal was at 700 cm^{-1} (strong). This finding suggests that the peaks of C-F bonds can be found easily from the graphs of the IR spectra.

Taken together, these results suggest that the change of position of the fluoride in the phenyl group from *ortho*, *meta* or *para* gives different types of IR spectra depending on the FCEP compound under investigation. This facilitates the identification of each isomer from other FCEP isomers.

These results suggest the requirement of testing these samples on more than one technique i.e. apart from IR, for instance, by investigating these compounds using NMR, GC and UV can give more detailed characterisation data to assist in the confirmation of identity of these isomers.

5.2.4 Nuclear magnetic resonance

The samples of FCEP isomers were run on high and low field NMR instruments. In this study, the results of NMR experiments (^{1}H and ^{13}C NMR) obtained from these two instruments were consistent for all FCEP compounds.

In the ^{1}H NMR spectra of the three FCEP isomers (Figure 43, Appendix A 31 and Appendix A 32), there are two broad peaks at around δ 11.00 and 10.00; this is assigned to the NH group which is present in FCEP compounds. In the aromatic region, there is a triplet that integrates to a single proton, centred at δ 7.89 (**11**, 1 H), 7.40 (**12**, 2 H) and 7.52 (**13**, 2 H) and a multiplet at the region between 7.25-7.00 ppm that integrates to seven protons for all three FCEP compounds. By looking at the ^{1}H NMR spectrum, the value of *J* coupling for the triplet at δ 7.89 (**11**) equals 12 Hz, and in **12** at δ 7.40 this value was 8 Hz and finally, at δ 7.53 in **13** it equals 0-1 Hz. The difference in this value is because of the position of the fluoro group in the benzene ring being *ortho*, *meta* or *para*.

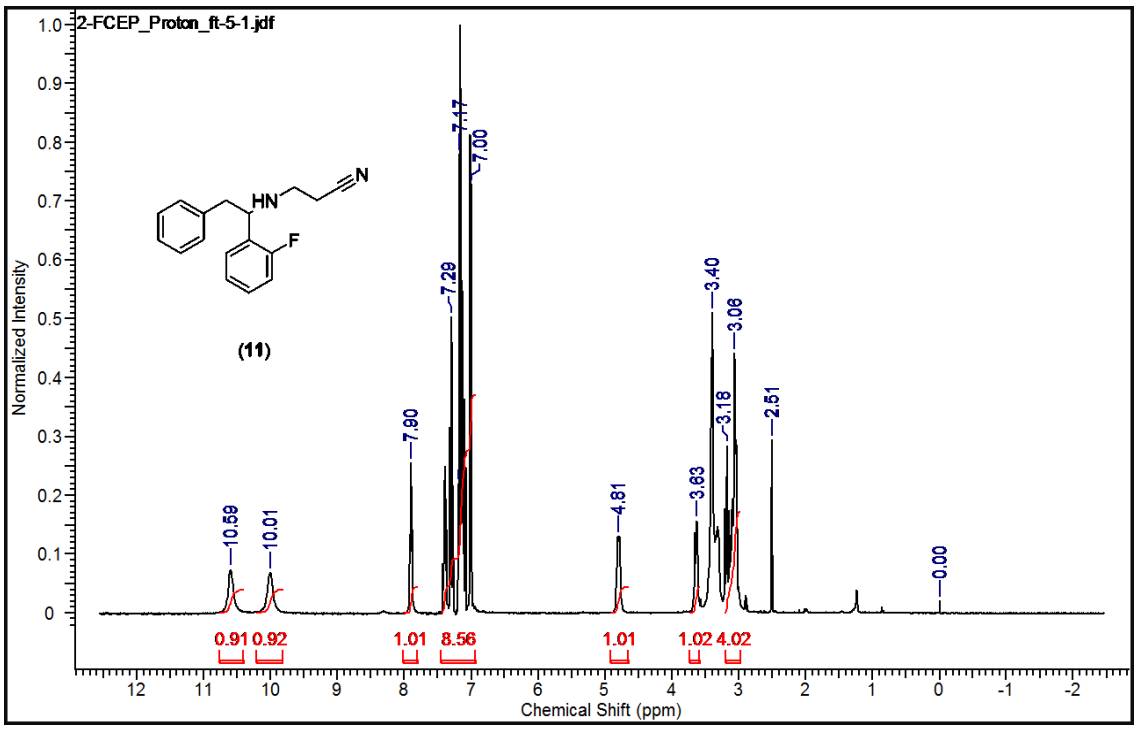


Figure 43. ^{1}H NMR spectrum of **11**

The aliphatic region in **11** consists of 5 proton nuclei, the signals for these environments are observed as simple splitting patterns, the integration of these regions does equal five, thus matching the proposed structure.

The ^{13}C NMR spectrum possesses 17 peaks, which correspond to the number of carbon environments in FCEP isomers. There are nine aromatic peaks, this number decreases to 8 in the ^{13}C {^{1}H} DEPT-135 spectrum due to the presence of three quaternaries in the structure of FCEP (See ^{13}C NMR spectra Appendix A 33, Appendix A 35 and Appendix A 37), and DEPT spectra in Appendix A 34, Appendix A 36 and Appendix A 38).

In **12**, the peaks of the quaternary carbons are assigned to the peaks at 163.7, 136.2 and 118.2 ppm (present in on the benzene ring), these peaks are not present in the DEPT spectrum. In the ^{13}C{^{1}H} NMR spectrum, the peak at 118.2 ppm is the quaternary carbon to which the fluoro group is attached to the benzene ring – this was rationalised from the ^{1}H-^{13}C HMQC spectrum (Heteronuclear Multiple-Quantum Correlation) as it was the only signal that

showed this interaction (i.e. the peaks of FCEP isomers (7.4-7.9 ppm), all have a cross peak with the peak at 118.2 ppm). The advantage of using 2D HMQC is that it permits the collection of a 2D heteronuclear chemical shift correlation map between directly-bonded ^{1}H and ^{13}C nuclei.

In **12**, the peak at 62.7 ppm is positive in the $^{13}C\{^{1}H\}$ DEPT-135 spectrum, which suggests a CH or CH_3 group. Similarly, this peak is shown for **11** at 56.1 ppm and at 62.5 ppm in the case of **13**. The ^{1}H-^{13}C HMQC data for FCEP isomers showed the following cross peaks: in the HMQC spectrum of **11** (See Appendix A 40), the cross peak is between peaks at 4.79 and 56.1 ppm, whereas for **12** and **13** the cross peaks are between peaks at 4.56 and 62.7 ppm, and 4.60 and 62.5 ppm respectively.

In the ^{1}H-^{1}H COSY spectrum, **11** possesses two cross peaks. The first is between peaks at 4.79 and 10.9 ppm and another between peaks at 2.45 and 7.89 ppm (Appendix A 39), while in **12**, there are 3 cross peaks (between peaks at 4.5 and 4.2 ppm, 7.45 and 1.60 ppm, and 10.99 and 9.95 ppm). For **13**, cross-peaks were observed between peaks at 4.2 and 4.55 ppm and 7.1- 7.9 ppm.

Finally, in the ^{19}F NMR (fluorine NMR) of these compounds, the fluorine is observed at δ = -118.67 (**11**), at δ = -113.93 (**12**) and at δ -114.56 (**13**), this shows that these three fluorinated diphenidine isomers can be differentiated by running ^{19}F NMR as each FCEP isomer has a unique peak. Overall, the chemical structures of the three FCEP can be recognized from the NMR data collected in these experiments using trifluoroacetic acid (TFA) as a reference with a peak at δ – 76.55 (Figure 44, Appendix A 41 and Appendix A 42).

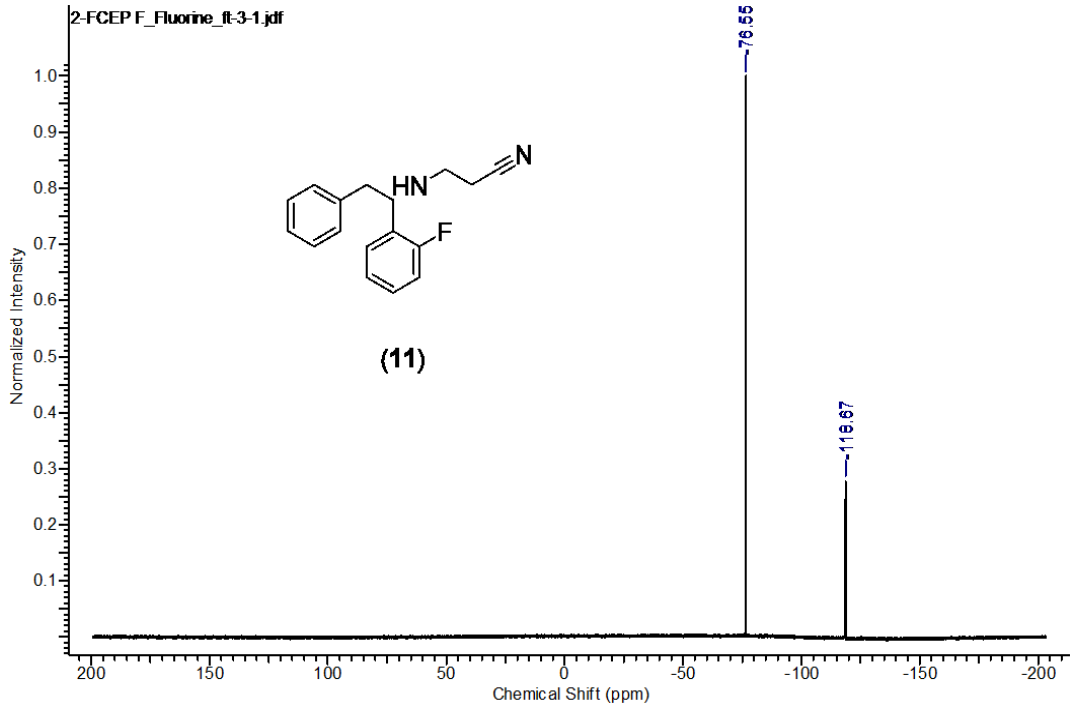


Figure 44. ^{19}F NMR spectrum of 2-FCEP **(11)** isomer

5.2.5 High performance liquid chromatography (HPLC)

5.2.5.1 Detection and separation of FCEP isomers on HPLC

These experiments were carried out after testing the column (ACE 5 C18-AR, 15 cm) prior to applying the HPLC method and reversed phase chromatography in an analogous way as for MXP and FEP (see chapters 3 and 4).

5.2.5.2 Column test

The column was tested and the retention times (t_R) and relative retention time (RRT) obtained for test samples in this experiment were consistent with the standard data of the manufacturer (see Table 20).

Table 20. Column mix test results on HPLC

Peak	Compound in column mix	Standard t_R (min)	RTT	Test t_R (min.)	RTT
1	Dimethyl Phthalate	2.29	0.45	2.27	0.45
2	Toluene	3.18	0.62	3.13	0.62
3	Biphenyl	5.09	1.00	5.00	1.00
4	Phenanthrene	7.18	1.41	6.97	1.40

5.2.5.3 HPLC testing of FCEP isomers using two different percentages of mobile phase

In this study, FCEP isomers were assessed in two experiments using acetonitrile: ammonium acetate by reverse phase method. In the first experiment, the mobile phase percentage was 55:45 % v/v and in the second was 65:35 % v/v, (see Table 21 below).

Table 21. HPLC results of FCEP isomers using two different percentages of mobile phase (acetonitrile:20Mm ammonium acetate)

	MP 55:45% v/v		**MP 65:35%v/v**	
FCEP isomer	t_R (min)	Resolution	t_R (min)	Resolution
11	4.283	-	4.133	-
12	4.512	1.06	4.337	0.99
13	4.512	1.06	4.337	0.99

As can be seen from Table 21, the results of this investigation using two different percentages of mobile phase (55:45 and 65:35% v/v) have shown that

11 was fully separated from the other two isomers (**12** and **13**) at about 4.28 mins in the first experiment using mobile phase, acetonitrile:ammonium acetate 55:45% v/v. **12** and **13** co-elute at about the same time (4.5 minutes) in the first experiment. The results of the second experiment using the same mobile phase with a ratio of 65:35% v/v revealed similar findings with slight fall in retention time and resolution.

In spite of the excellent/short retention time obtained in the two experiments, however, the resolution was almost 1.00 in both cases, which suggests that the current reverse phase method used in these experiments is not suitable for separation of samples containing FCEP isomers. As a result, one of the possible solutions to solve the issue of low resolution is to try the HPLC gradient method.

5.2.5.4 HPLC gradient method (FCEP isomers)

Table 22 gives data on HPLC gradient method applied for FCEP isomers using aqueous formic acid 0.1%, A (%) and acetonitrile (100% MeCN, B (%)) as the mobile phase, each in separate mobile phase bottles. The table also shows the time spent (in minutes) by both solvents in a total run time of 36 minutes, using flow rate of 1 mL min^{-1} and the maximum pressure in the column is 400 bar. The percentages of mixing the two solvents A (%) and B (%) is listed in two columns.

In the gradient method, it is important to use the terms A and B to refer to the aqueous and organic solvents respectively. In this experiment, the A solvent is HPLC grade water with 0.1% formic acid. The B solvent is HPLC grade acetonitrile (organic solvent).

Table 22. HPLC gradient method using aqueous formic acid 0.1% and acetonitrile as the mobile phase

Time (min)	A (%) (Formic Acid 0.1%)	B (%) (100% Acetonitrile)	Flow Rate (mL min^{-1})	Max. pressure limit (bar)
0.0	95	5	1	400
30.0	5	95	1	400
30.1	1	99	1	400
33.0	1	99	1	400
33.1	95	5	1	400
36.0	95	5	1	400

Table 23 shows data on HPLC detection and separation of FCEP isomers in a mixture and as individual components using acetonitrile (MeCN):0.1% aqueous formic acid as the mobile phase.

Table 23. HPLC detection and separation of FCEP isomers in a mixture and as individual components using acetonitrile:0.1% aqueous formic acid as the mobile phase.

	(FCEP mixture)		(individual FCEP isomers)
FCEP isomer	**t_R (min)**	**Resolution**	**t_R (min)**
11	11.34	-	11.39
12	11.47	0.48	11.73
13	11.47	0.48	11.58

These results are also shown below in the following chromatogram (Figure 45), which shows HPLC testing of the FCEP mixture by gradient method with detection at 263 nm. The first peak was for **11**, which eluted at 11.34 minutes, It is also clear that resolution between **11** and the other two isomers was too low (0.48) with no base line separation, while, both **12** and **13** co-eluted at 11.47 minutes and appeared as one peak. This last finding suggests the method applied herein is not suitable for separation of the FCEP isomers in a mixture.

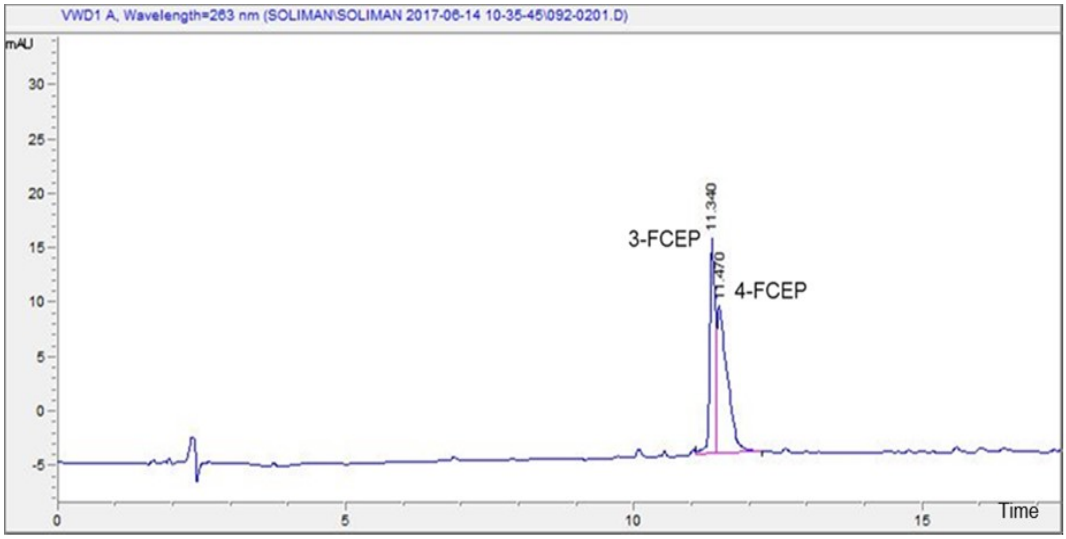


Figure 45. Testing a mixture of FCEP isomers by gradient HPLC method at 263 nm using formic acid:acetonitrile as the mobile phase (volume of injection = 40 µL)

The following table compares results of HPLC detection and separation of FCEP isomers in a mixture and as individual components using acetonitrile:100 mM ammonium acetate as the mobile phase (See Table 24 and Figure 46).

Table 24. HPLC detection and separation of FCEP isomers in a mixture and as individual components using acetonitrile:100mM ammonium acetate as the mobile phase.

	(FCEP mixture)		(individual FCEP isomers)
FCEP isomer	**t_R (min)**	**Resolution**	**t_R (min)**
11	20.04	-	20.05
12	20.26	1.32	20.25
13	20.26	1.32	20.25

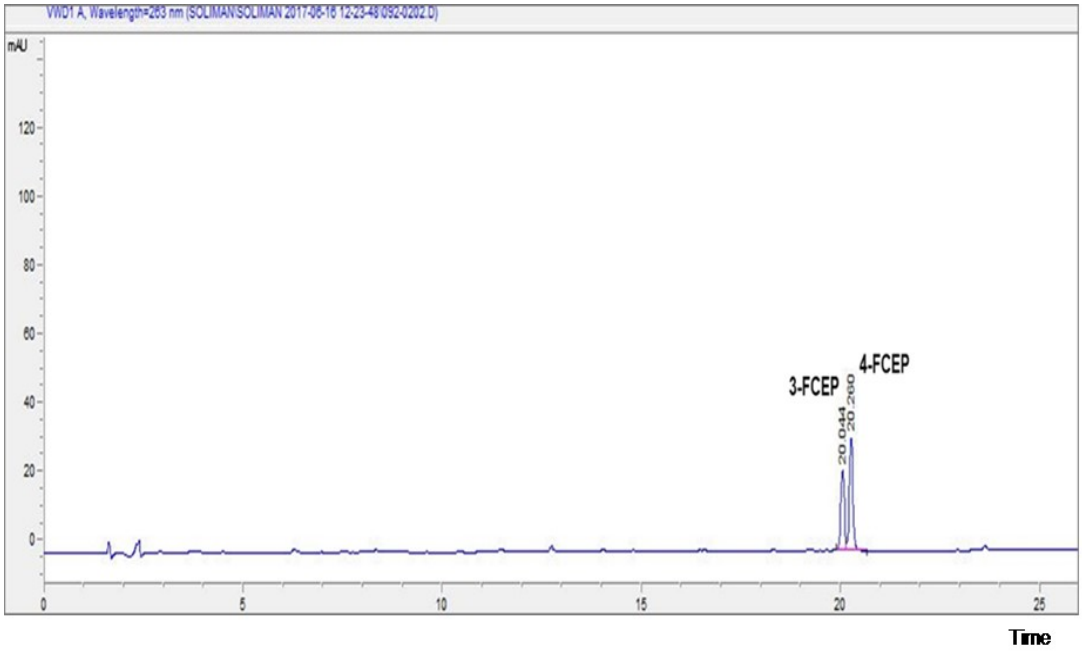


Figure 46. Testing a mixture of FCEP isomers by gradient HPLC method at 263 nm using 100 mM ammonium acetate:acetonitrile as the mobile phase (Volume of injection = 40 µL)

The table and figure below show HPLC detection and separation of FCEP isomers in a mixture and as individual components using acetonitrile: 100 mM ammonium formate as the mobile phase (See Table 25 and Figure 47).

Table 25. HPLC detection and separation of FCEP isomers in a mixture and as individual components using acetonitrile:100mM ammonium formate as the mobile phase.

	(FCEP mixture)		**(individual FCEP isomers)**
FCEP isomer	**t_R (min)**	**Resolution**	**t_R (min)**
11	19.81	-	19.99
12	20.00	1.08	20.30
13	20.00	1.08	20.16

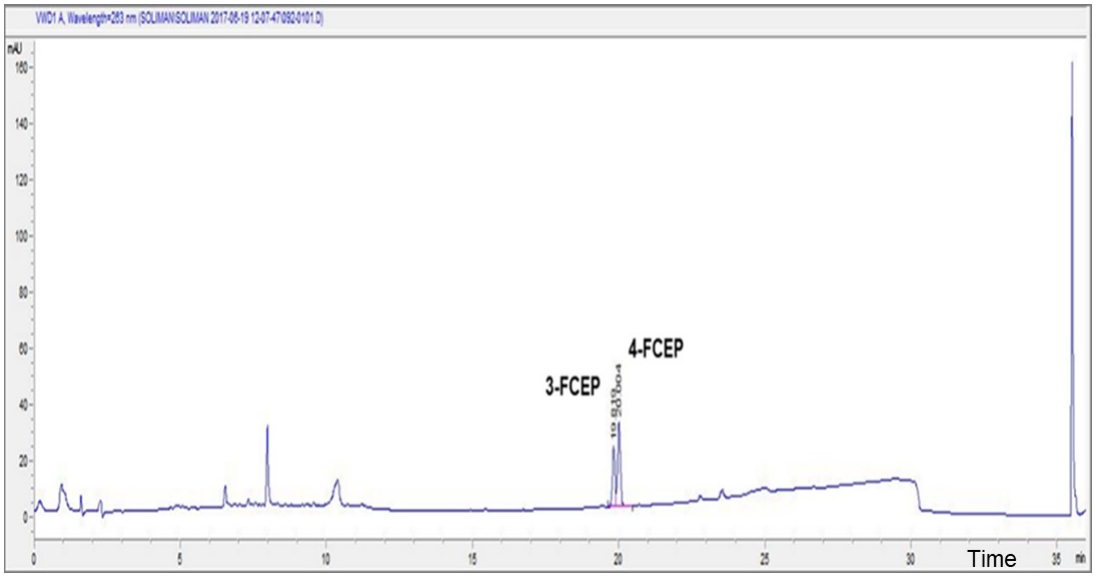


Figure 47. Testing a mixture of FCEP isomers by gradient HPLC method at 263 nm using 100 mM ammonium formate:acetonitrile as the mobile phase (Volume of injection = 40 µL)

Overall, Table 26 below compares HPLC results obtained from testing a mixture of FCEP isomers in three different experiments, applying the gradient method; using 100% acetonitrile (MeCN) which is mixed during the run in the HPLC instrument with either formic acid, ammonium acetate or ammonium formate as the mobile phase.

Formic acid is used in this HPLC experiment to improve the chromatographic peak shape and to provide a source of protons in reverse phase HPLC. As the concentration of the formic acid in the eluent increases, the retention time of the analytes decreases.[145]

Table 26. HPLC results of FCEP isomers (mixture) applying the gradient method; using 100% acetonitrile with formic acid or ammonium acetate or ammonium formate as the mobile phase.

	Acetonitrile :0.1% Aqueous Formic acid		**Acetonitrile :100 mM Ammonium Acetate**		**Acetonitrile :100 mM Ammonium Formate**	
	t_R (min)	Rs	t_R (min)	Rs	t_R (min)	Rs
11	11.34	-	20.04	-	19.81	-
12	11.47	0.48	20.26	1.32	20.00	1.08
13	11.47	0.48	20.26	1.32	20.00	1.08

It is clear from the results that the combination of acetonitrile with formic acid gave the shortest retention time was almost 11.50 mins with a resolution of just below 0.50, in the next experiment by using acetonitrile/ammonium acetate combination, the highest resolution was obtained (1.32), nevertheless, the retention time was a bit lengthy with values of just over 20 minutes.

In the third experiment (acetonitrile:ammonium formate), the retention time was similar to that in the (acetonitrile:ammonium acetate run) with almost 20 minutes, but, the resolution fell to slightly over than one.

5.2.5.5 Conclusion (FCEP on HPLC)

In the first study on HPLC, the FCEP compounds were tested by applying the reverse phase method for separation of these isomers using two different percentages of mobile phase. However, because of both low resolution and incomplete separation obtained, one of the possible solutions to be applied was the application of HPLC gradient method.

Overall, as was seen in the results, changing the gradient method parameters such as the temperature or flow rate might improve the resolution and give rapid analysis time. Another recommendation is by possibly testing these isomers using different instrumental techniques such as using gas chromatography (GC). The study of FCEP isomers on three kinds of GC columns is detailed in the following sections.

5.2.6 Gas chromatography

After using HPLC methods discussed earlier, GC is the next technique that will be used for the characterisation/analysis of FCEP isomers using different temperature programmes on 3 types of GC columns;

1. Nonpolar GC column, capillary tubing is made of fused silica, (1909IS-433E, 8890 GC system, length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm, Agilent Technologies, Inc.).

2. Semi-polar GC column, (35%-phenyl)-methylpolysiloxane, (7890A GC system, 30 m, 0.25 mm, 0.50 µm, Agilent Technologies, Inc.).

3. Polar GC column, this is a polyethylene glycol (PEG) stationary phase that features high polarity, (1909IN-133E, HP-INNOWax S N US86964, 30 m, 0.25 mm, 0.25 µm, Agilent Technologies, Inc.).

The methods used herein are starting at 180°C and increase in different rates up to 300°C (for instance using the rate of 1, 2, 3, 5°C min^{-1} or isothermally at 180°C.

5.2.6.1 Detection of FCEP isomers on non-polar, semi-polar and polar GC column

In this study, both a mixture of fluorocyanoephenidine isomers and the internal standard eicosane (FCEP + E) and (separate FCEP isomers + E) were tested on three different polarities of GC columns in order to detect and separate these compounds. In other words, the aim is to find best column, therefore, method or temperature programme for separating these regioisomers. The first method was applied as a starting point for FCEPs was on the non-polar GC-MS at 100°C and the temperature was increased up to 300°C in a rate of 10°C min^{-1}, flow rate of 1 mL min^{-1}. The elution of the isomers has shown that 180°C is the ideal starting temperature for these experiments. The results of this method have revealed the following retention times: E (18.19 min), **11** (22.67 min), **12** (23.96 min) and **13** (24.20 min), (see Figure 48 below).

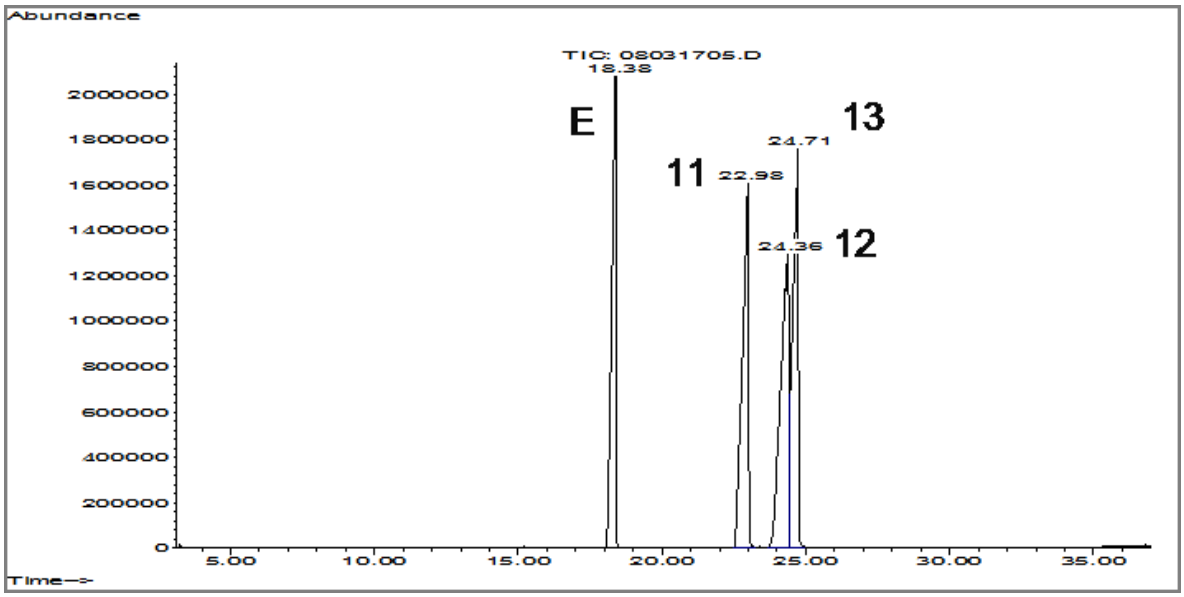


Figure 48. Chromatogram of FCEP isomers and eicosane on non-polar GC column

5.2.6.1.1 Detection and separation of FCEP isomers on non-polar GC column

This experimental investigation was carried out by testing the FCEP isomers on a non-polar GC column (GC-MS).The purpose was to begin with testing FCEP isomer on nonpolar GC column and then followed by the use of semi- or polar GC columns.

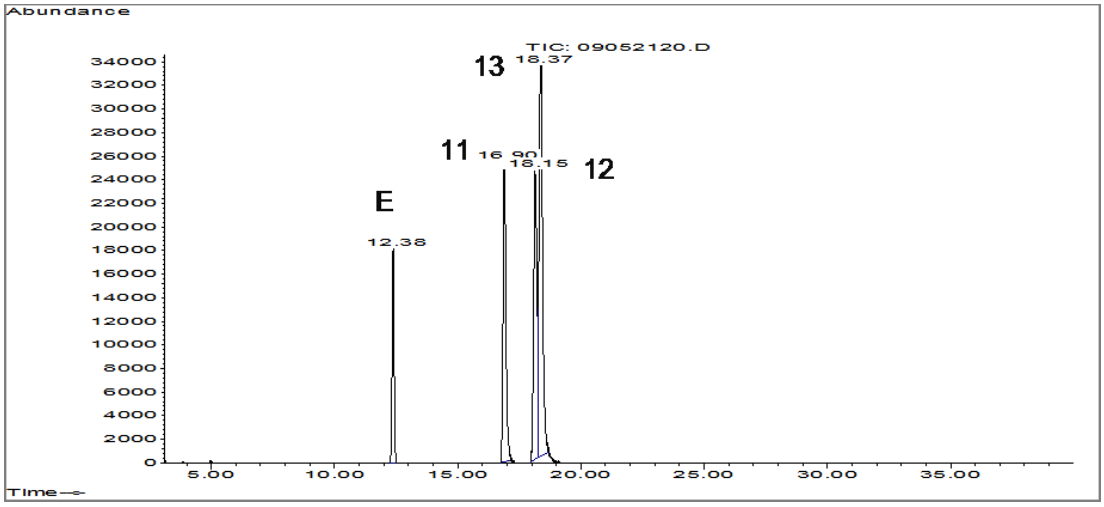


Figure 49. Chromatogram of FCEP mixture+ E on GC-MS (non-polar column) using the isothermal method (180°C)

The chromatogram (Figure 49) above displays data on the retention times obtained by applying the isothermal GC-MS method (at 180°C) on a mix of three FCEP isomers with eicosane (E) as a reference.

It is clear from the results on the chromatogram that after using the isothermal GC-MS method (at 180°C), there was a better separation and resolution when compared to the other methods used in this study (5, 3, 2 and 1°C min^{-1} temperature programmes). The retention times of these substances were: E (12.38 min), **11** (16.90 min), **12** (18.15 min) and **13** (18.37 min), respectively.

In summary, despite the fact that the isothermal method at 180 °C was superior to the other applied methods in terms of the separation obtained, the degree

of this separation is still not acceptable because it is not a fully base line separation particularly between **12** and **13**.

The chromatogram above presents data on the retention times obtained by applying a 1°C min^{-1} temperature programme on a mixture of three FCEP regioisomers using eicosane (E) as a reference (Appendix A 43).

By looking at the results from this run, the separation of the three FCEP compounds was not achieved with an overlap between the detected peaks.

The produced peaks represent the retention times of the following substances: E (10.18 mins), **11** (13.17 mins), **12** (13.88 mins) and **13** (14.2 mins), correspondingly. Overall, this 1°C min^{-1} method was unable to separate the FCEP isomers with an acceptable resolution between their peaks.

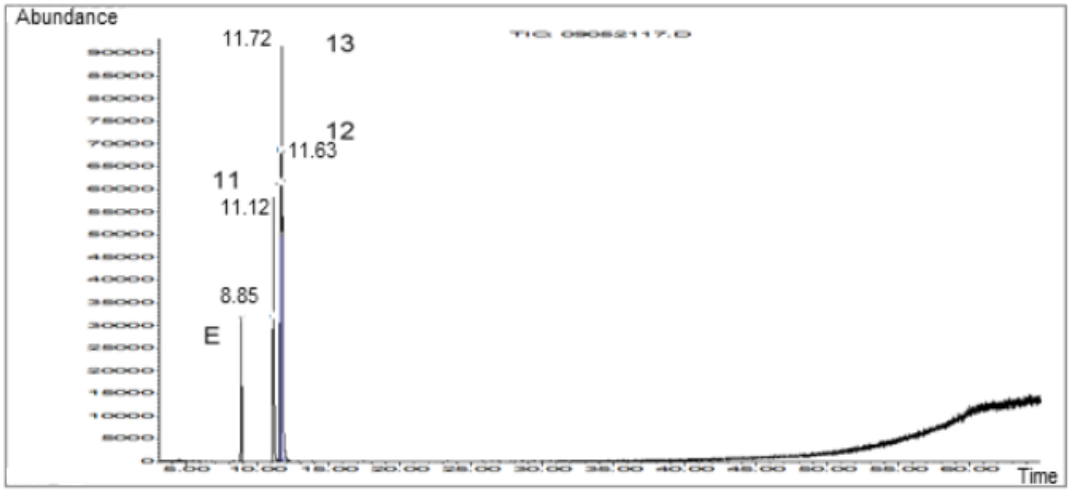


Figure 50. Chromatogram of FCEP mixture+ E on GC-MS (non-polar column) using 2°C min^{-1} method

The chromatogram (Figure 50) displays data on the retention times of FCEP mixture, in the presence of E as a reference, on GC-MS (non-polar column) using 2°C min^{-1} method.

As can be seen from the results, eicosane and all three FCEP were detected by applying 2°C min^{-1} GC-MS method. However, the separation of the three

isomers was partially achieved which is similar to what was observed in the 3°C min^{-1}method results. These findings suggest that this method is not suitable for separation but it can be used for identification purposes of the individual samples of FCEP substances in any forensic framework cases. The order of elution was as the following: E (8.85), **11** (11.12 mins), **12** (11.63 mins) and **13** (11.72 mins), respectively.

In conclusion, this method still needs more changes in the experimental conditions in order to obtain a suitable, rapid and robust GC-MS method for separation of FCEP analytes.

The chromatogram (Appendix A 44) exhibits data on the retention times obtained by applying a 3°C min^{-1} temperature programme on a mix of three FCEP isomers and the corresponding abundances using eicosane (E) as a reference.

By looking at the graph (See Appendix A 44), even though it is obvious that the separation of the three FCEP compounds was achieved, the resolution between the three peaks was low. The retention times (in minutes) of the signals associated with the following compounds were: E (7.93), **11** (9.78), **12** (10.16) and **13** (10.24). In summary, this method requires some extra modifications in its parameters to achieve complete separation of the substances under investigation.

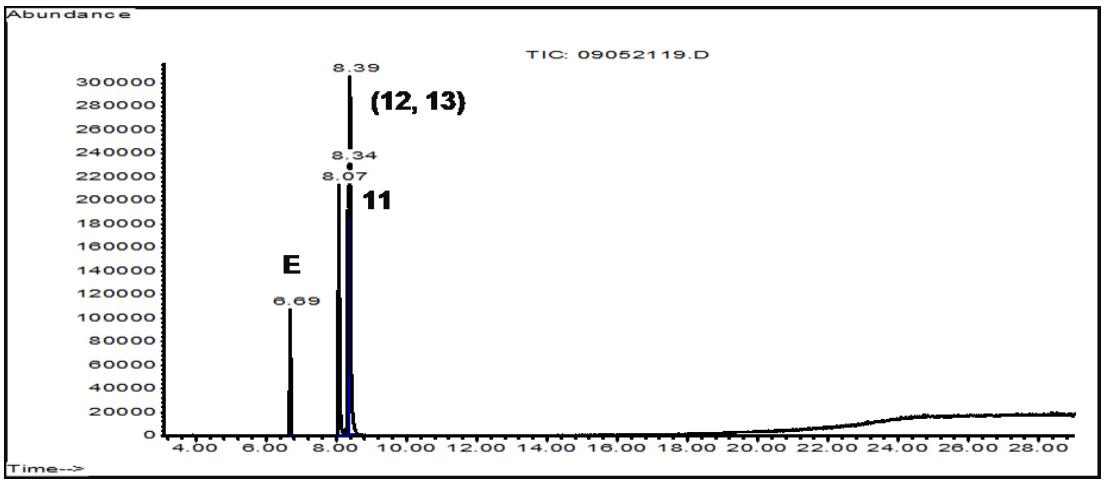


Figure 51. FCEP mixture + E on GC-MS (non-polar column) using 5°C min^{-1} method

The graph above (Figure 51) shows the relationship between the time in minutes and abundance obtained after injecting a mixture of **11**-**13** plus E in to a GC-MS with a non-polar column and by applying a temperature programme of 5°Cmin^{-1}.

As can be clearly seen from the diagram, the main trend is represented by the slight separation of the **11** (8.07 mins) which follows the eicosane peak at (6.69 mins). The other two fluorocyanoephenidine isomers (**12** and **13**) had retention times of 8.34 and 8.39 mins respectively.

In conclusion, although this GC-MS method was able to detect the three FCEP regioisomers, the complete separation of these compounds was not possible by using this protocol.

The chromatogram (Appendix A 45) shows the retention times produced from GC-MS (non-polar column) after the injection of **13** plus eicosane as a reference in a concentration of 1 mg mL^{-1}, and using a 3°C min^{-1} temperature procedure.

As can be seen from the diagram, the two compounds were completely base-line separated with excellent resolution, the retention time of **13** was 10.21 mins and E was 7.88 mins.

In the same way as for **11** and **13**, **12** and eicosane were tested via GC-MS (non-polar column) by applying the temperature programme of 3°C min^{-1}. From the results of this run, the retention time of **12** was 10.17 mins and for E was 7.93 mins.

The chromatogram (Appendix A 46) gives data on the retention times of **12** and eicosane as a reference, using GC-MS (non-polar column) and the temperature programme of 3°C min^{-1}.

In general, it can be noted from the diagram that the retention time of **11** equals 9.78 mins, while that of eicosane was 7.92 mins. Similarly, both compounds were fully base line separated with significant resolution.

The diagram (Appendix A 47) gives data on the retention time of E which is used as a reference material on GC-MS (non-polar column) and the temperature programme of 3°C min^{-1}. As can be seen from the chart, the retention time of E is 7.93 mins.

Table 27. FCEP isomers on GC-MS (non-polar column) using 3°C min^{-1} method

FCEP isomer	Retention Time, t_R (mins)	Eicosane t_R (E)
11	9.78	7.92
12	10.17	7.93
13	10.21	7.88

Table 27 summarises and compares data on the different retention times of the three FCEP isomers acquired on a GC-MS (non-polar column) using eicosane as a reference and a temperature programme of 3°C min^{-1}.

From the table, the retention time of E is on average of approximately 7.9 mins, whereas the retention times of the fluorocyanoephenidines were as follows: **11** = 9.78; both **12** and **13** have retention times of around 10.2 mins.

To conclude, on one hand, this data suggests the possibility of separating **11** when it is present in a mixture/sample containing all three FCEP regioisomers using the above GC-MS method. On the other hand, it is not possible to separate **12** and **13** by applying the same method.

Table 28. FCEP mix and eicosane on non-polar column (GC-MS)

Method	**Run Time** t_R(min)	**Eicosane** (E) t_R(min)	**11** t_R(min)	**12** t_R(min)	**13** t_R(min)
5 °C min^{-1}	29	6.69	8.07	8.34	8.39
3 °C min^{-1}	45	7.93	9.78	10.16	10.24
2 °C min^{-1}	65	8.85	11.12	11.63	11.72
1 °C min^{-1}	125	10.18	13.17	13.88	14.02
Isothermal (180°C)	40	12.38	16.90	18.15	18.37

Table 28 compares the retention times of fluorocyanoephenidines measured in minutes; in several temperature procedures used to detect and quantify these compounds. The study was carried out to investigate different temperature systems/categories.

In general, the results obtained from the isothermal method was the best in all observed methods due to the best resolution between **12** and **13**, followed by the 5°C min^{-1} method and then the 3°C min^{-1} method.

Looking at the details, the 1°C min^{-1} method accounted for the highest run time. The 5°C min^{-1} method and isothermal (180 °C) were faster than the other methods. Using the isothermal method, if compared to **11**, **12** and **13** eluted later, possessing retention times of 18.15 and 18.37 mins respectively. In contrast, the 1°C min^{-1} method had the longest run time at 125 mins. The order

of elution remained the same in that **11** was eluted first followed by **12** and lastly by **13**.

In the non-polar GC column study, the isothermal method at 180 °C min^{-1} was the first procedure applied for testing the FCEP regioisomers, followed by 1, 2, 3 and finally 5 °C min^{-1} , respectively. It is worth noting that the 4 °C min^{-1} method was not used in the whole study/analysis of FCEP isomers on three different GC columns due to insufficient time.

By looking back to the results, in one hand, after applying all these five methods, **11** was separated from **12** and **13**. On the other hand, the peaks of **12** and **13** appeared almost as one peak, which shows the overlap between the two peaks of these two compounds.

The most important finding is that the 5 °C min^{-1} was the quickest method for detection and separation of FCEP isomers, with just below 8.5 mins required for the appearance of the last separated peak. As opposed to the 5 °C min^{-1} method, the isothermal programme at 180 °C min^{-1} was the slowest method for resolving all the FCEP signals with retention time of just below 18.5 mins. Finally, the other three methods remaining in this study, as shown in Table 28, the last peak in the remaining three methods (3, 2 and 1 °C min^{-1}) appeared at exactly (10.2, 11.7, 14.0 mins, respectively). However, the disadvantage of the last mentioned three methods is that the total run time is very long (45-125 mins).

5.2.6.1.2 Detection and separation of FCEP isomers on semi-polar GC column

The FCEP isomers were analysed on the semi-polar GC column (phenyl methyl siloxane, 0.25 µm, 30 m) by using nitrogen as the carrier gas in the first set of experiments and then nitrogen was replaced by helium in the next collection of experiments.

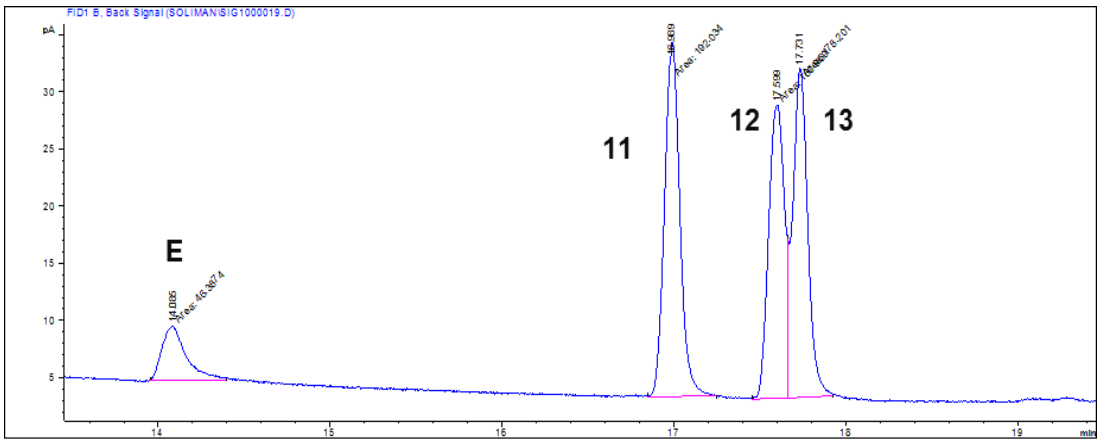


Figure 52. FCEP mixture plus eicosane at 2 °C min^{-1} on semi-polar GC using nitrogen as a carrier gas

Figure 52 shows data on the retention times (in minutes) of FCEP mixture plus eicosane and the peak area (pA) using 2°C min^{-1} method on semi-polar GC using nitrogen as a carrier gas. The most striking result to emerge from the data is that **11** (at 16.98 mins) was separated from the other isomers, **12** (at 17.59 mins) and **13** (at 17.73 mins), while the eicosane peak was (at 14.0 mins). The order of elution was **11**, then **12** and finally **13**. Additionally, the last two peaks of **12** and **13** are not fully base line resolved. Peak areas were approximately 192.0 (**11**), 160.0 (**12**) and 178.0 (**13**), correspondingly.

The chromatogram (Appendix A 48) gives information on the retention times (mins) of FCEP mixture plus eicosane at 3 °C min^{-1}on semi-polar GC using nitrogen as a carrier gas. As can be seen from the diagram, the retention times were eicosane (12.15 mins), **11** (14.4 mins), **12** (14.9 mins) and **13** (15 mins). Even though **11** was separated from the rest of the substances in this experiment, the peaks of **12** and **13** were only partially resolved as was observed in the previous experiment using 2°C min^{-1} method on semi-polar GC using nitrogen as a carrier gas.

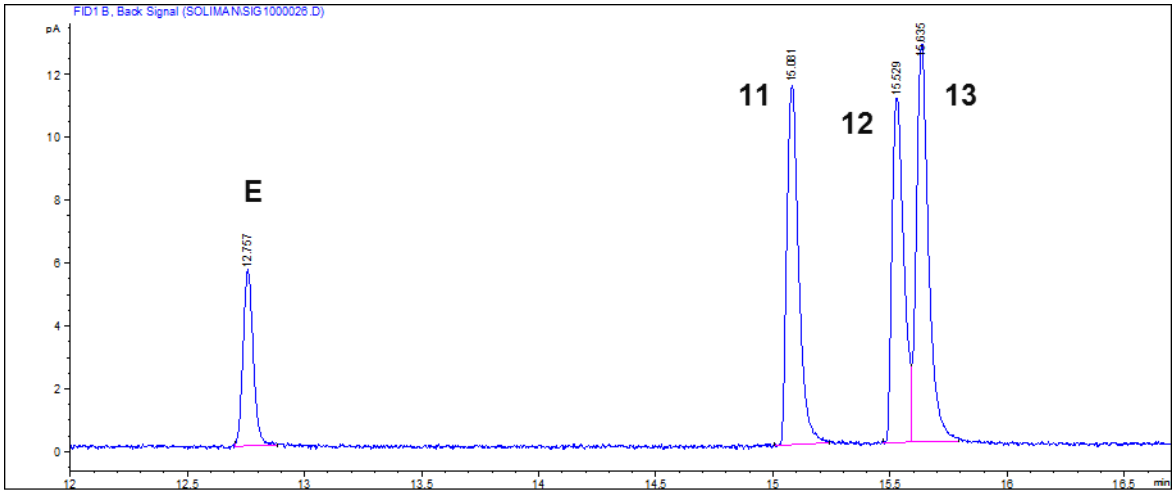


Figure 53. FCEP mixture plus eicosane (0.1 mg mL^{-1}) at 3 °C min^{-1} on semi-polar GC using helium as a carrier gas

Figure 53 shows the retention times of three FCEP isomers and eicosane (0.1 mg mL^{-1}) at 3 °C min^{-1}on semi-polar GC using helium as a carrier gas. The most important finding is the improvement in the shape of the peaks (See Figure 53) and the resolution/separation of the **12** and **13** by using concentrations of 0.1 mg mL^{-1} (i.e. 10 times dilution) and helium as a carrier gas, instead of nitrogen. As can be noted from the graph, the retention times were as follows: eicosane (12.7 mins), **11** (at 15 mins), **12** (15.5 mins) and **13** (15.6 mins).

It is also clear that only **11** was completely separated, while, the peaks of the isomers **12** and **13** were not fully base-line resolved; these results are similar to the findings of the previous experiments using 5 °C min^{-1} and 3 °C min^{-1} techniques on semi-polar GC utilising helium as a carrier gas.

The chromatogram (Appendix A 49) gives data on the retention times (mins) of FCEP mixture and eicosane at 5 °C min^{-1} on semi-polar GC using nitrogen as a carrier gas. As can be seen from the data on the chart, the retention times were as the following: eicosane (9.9 mins), **11** (11.5 mins), **12** (11.8 mins) and **13** (11.9 mins).

Moreover, in one hand, both total run time and retention time declined by increasing the temperature up to 5 °C min^{-1}, on the other hand, **12** and **13** were

just partially separated as was seen in the previous experiments using 2 and 3 °C min^{-1} protocols on semi-polar GC using nitrogen as a carrier gas.

Table 29. Individual FCEP isomers with eicosane on GC (semi-polar column) using 5 °C min^{-1} method

FCEP isomer	FCEP t_R(min)	Eicosane (E) t_R(min)
11	11.49	9.86
12	11.80	9.87
13	11.85	9.88

Table 29 above exhibits the retention times (mins) of the individual FCEP isomers with eicosane, acquired on GC (semi-polar column) using 5°C min^{-1} method.

The key finding from this experiment is that all the 3 isomers in addition to eicosane eluted in a time range of about 10 minutes (E) to 12 minutes (FCEP isomers). The order of elution is as follows: eicosane, **11**, **12** and **13**, in sequence. As far as the retention times of FCEP isomers is concerned, it can be seen clearly that there is a similarity/overlap in the values of t_R(min), therefore, a different method or some modifications in the current method's parameters is required to perform the separation of the isomers in question.

Table 30. FCEP isomers mixture with eicosane on GC (semi-polar column) using 5°C min-1method

FCEP isomer	FCEP t_R(min)	Eicosane (E) t_R(min)
11	11.50	9.86
12	11.79	9.86
13	11.86	9.86

Table 30 shows the retention times (mins) of the mixture of FCEP isomers and eicosane, obtained from GC (semi-polar column) using the temperature programme, 5°C min^{-1}. In comparison of the results of this experiment (See Table 12) with the findings of the individual FCEP compounds (see Table 11), it can be clearly noticed that the retention times are almost the same in the two experiments; by taking the average for eicosane (9.8 mins), **11** (11.5 mins), **12** and **13** (11.8 mins), respectively.

To summarise, these results are consistent for both cases (separate FCEP substances or a mix of FCEP isomers) provided that the same GC method was used (5 °C min^{-1} method).

Table 31. FCEP isomers mixture with eicosane on GC (semi -polar column) using different temperature-programmed methods (Nitrogen as the carrier gas)

Method	Run Time (min)	**Eicosane** t_R(min)	**11** t_R(min)	**12** t_R(min)	**13** t_R(min)	Rs between (**12**) and (**13**)
5 °C min^{-1}	29	9.880	11.507	11.797	11.866	0.73
3 °C min^{-1}	45	12.156	14.450	14.898	14.999	0.70
2 °C min^{-1}	65	14.083	16.990	17.599	17.732	0.69
1 °C min^{-1}	125	17.180	21.372	22.491	22.569	0.67
Isothermal (180°C)	40	26.814	34.074	36.487	37.023	0.69

Table 31 summarises and compares data on the retention times of a mixture of FCEP isomers with eicosane tested on GC (semi-polar column) using four different temperature-programmed methods and nitrogen as a carrier gas.

Looking back at the details of this study, apart from the isothermal method (at 180°C), the main trend is by increasing the temperature the retention time and

the total run time are decreasing. For instance, using the 5 °C min^{-1} temperature-programmed method, the retention times were as the following: **11** (11.5 mins) this figure was almost 10 minutes less using 1°C min^{-1} protocol (21.3 mins). While for both **12** and **13**, the retention time was ca. 11.8 mins; this figure is nearly 25 minutes quicker than using the isothermal procedure (about 37.0 mins).

What is more, the retention times obtained from the use of the isothermal method were: **11** (34 mins), **12** (36 mins) and **13** (37 mins); these times are two and a half times higher than those observed while using the 3 °C min^{-1} GC method.

From the most important findings in this study is that all the applied four methods have all given almost an average of approximately 0.7 as the resolution between the **12** and **13**, i.e. increasing the temperature rate does not affect resolution. To improve the resolution of earlier eluting peak, it is possible to decrease the initial temperature or increase the initial hold time. Decreasing the initial temperature usually results in the largest resolution improvement, but analysis times are substantially increased.

Table 32.FCEP isomers mixture with eicosane on GC (semi-polar column) using different temperature-programmed methods (Comparison between nitrogen and helium as the carrier gases)

Carrier Gas	Method	Run Time (min)	t_R(min)					
			Eicosane	11	12	13	R_s (11:12)	R_s (12:13)
Nitrogen	3 °C min^{-1}	45	12.15	14.45	14.89	14.99	3.08	0.70
	2 °C min^{-1}	65	14.08	16.99	17.59	17.73	3.26	0.69
	1 °C min^{-1}	125	17.18	21.37	22.49	22.56	5.16	0.67
	Isothermal (180°C)	40	26.81	34.07	36.48	37.02	3.82	0.69
Helium	3 °C min^{-1}	45	12.75	15.08	15.52	15.63	4.80	1.14
	2 °C min^{-1}	65	14.80	17.79	18.42	18.56	5.26	1.15
	1 °C min^{-1}	125	18.26	22.60	23.60	23.83	5.75	1.28
	Isothermal (180°C)	40	26.09	35.05	37.66	38.22	6.72	1.40

Table 32 summarises and compares the use of nitrogen or helium as the carrier gases on FCEP isomers mix with eicosane on semi-polar GC column, by applying different temperature-programmed methods. The abbreviation, R_s (2:3) shown in the table above refers to resolution between **11** and **12**, while R_s (3:4) represents the resolution between **12** and **13**.

In semi-polar GC column experiments, the four applied methods (1, 2, 3 °C min^{-1} methods and isothermal procedure at 180 °C were run on a total run time from 40 to 125 mins).

These experiments were conducted by using nitrogen as a carrier gas which was then replaced by helium in order to obtain faster retention times and better resolution particularly between **12** and **13** (R_s refers to resolution between **12** and **13**).

From the results, it is clear that by using nitrogen as a carrier gas, the fastest method was the isothermal at 180 °C with just 40 minutes total run time, while the slowest method was the 1°C $min^{-1}$with 125 minutes.

The main finding is that **11** was fully separated with excellent resolution with just over 3 to just over 5, but the issue here is that the peaks of **12** and **13** were partially base line resolved with a resolution of about 0.7 in all four applied temperature programmes.

The second set of experiments used helium as a carrier gas, the results revealed a significant improvement in resolution especially between the two peaks of **12** and **13**. The highest resolution obtained between **13** and **14** was 1.40 by using the isothermal method at 180 °C.

Taken together, by comparing nitrogen and helium experiments, the retention times were slightly decreased in case of using helium, however, the resolution was doubled from just about 0.70 t0 1.40. These results suggest that helium is superior to nitrogen as the carrier gas (in this study), the resolution of 1.40 is acceptable for separating FCEP isomers, but it is preferred to be above 1.5 i.e. around 2 or more according to ICH guidelines.

In summary, it is recommended to re-run these experiments by using helium as the carrier gas in addition to increasing the temperature rate greater than 5 °C min^{-1}, in order to obtain a suitable and rapid separation method for FCEP isomers.

5.2.6.1.3 Detection and separation of FCEP isomers on polar GC column

The FCEP isomers were investigated on the polar GC column (polyethylene glycol, 0.25 µm, 30 m) by both detection of the individual isomers separately and in a mixture, using many temperature programmed protocols. These are 1, 2, 3, 5 °C $min^{-1}$and the isothermal method at 180 °C. The results of this experimental study are detailed below.

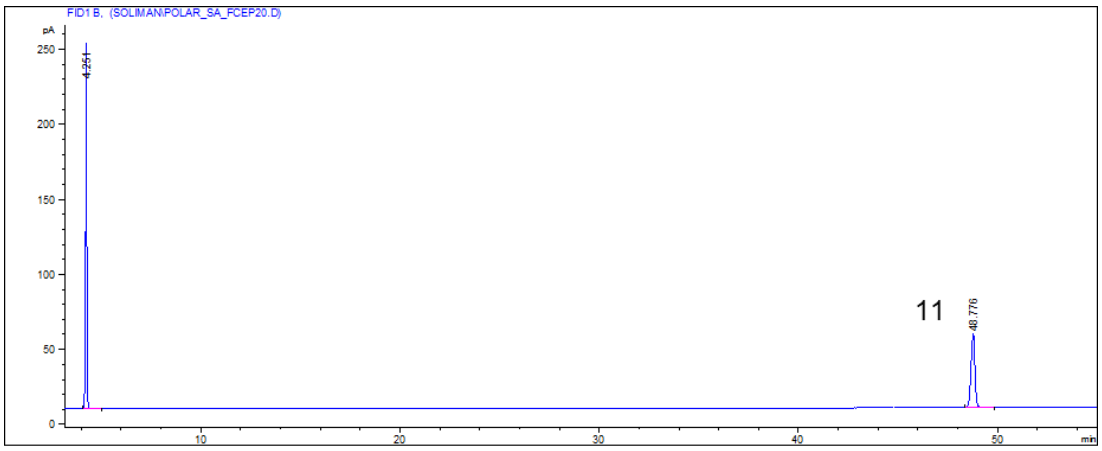


Figure 54. 2-FCEP (**11**), (1 mg mL^{-1}) on polar GC at 1°C min^{-1}

The chromatogram (See Figure 54) provides data on the retention time of **11** and eicosane (1 mg mL^{-1}) versus peak area (pA) on polar GC at 1°C min^{-1}. From the results, t_R of **11** = 48.7 mins and t_R of E = 4.2 mins.

The chromatogram (Appendix A 50) shows data on the retention time of **12** and eicosane (1 mg mL^{-1}) versus peak area (pA) on polar GC at 1°C min^{-1}. From the chromatogram, t_R of **12** = 52.9 mins, t_R of E = 4.2 mins. The total run time was 65 mins.

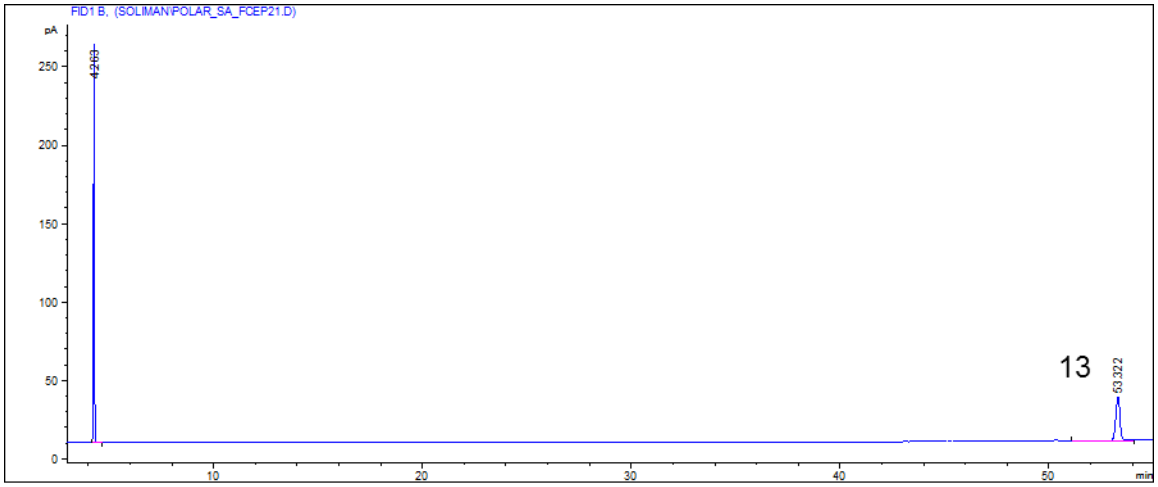


Figure 55. 4-FCEP (**13**), (1 mg mL^{-1}) on polar GC at 1°C min^{-1}

Figure 55 gives information on the retention time of **13** and eicosane (1 mg mL^{-1}) versus peak area (pA) on polar GC at 1°C min^{-1}. From the chart, t_R of **13** = 53.3 mins and t_R of E = 4.2 mins.

The chromatogram (Appendix A 51) gives data on the retention times (mins, x-axis) of FCEP mix (1 mg mL^{-1}) versus peak area (pA) on polar GC at 1°C min^{-1}; with a total run time of over than an hour. It is clear from the chromatogram that the retention times were as follows: eicosane (4.2 mins), **11** (48.7 mins), **12** (53.0 mins) and 13 (53.3 mins). As can be noted from the data, the highest peak area was for eicosane with almost four folds of the peak area of each FCEP isomer. However, the same issue still exists because only **11** was separated from the rest of the isomers in FCEP mixture, with just a partial separation of **12** and **13**, as was observed in the previous experiments on both nonpolar and semi-polar GC.

Furthermore, the retention times obtained in this run (FCEP mix) is consistent with the retention times measured for the individual FCEP isomers.

In conclusion, these results suggest that the use of polar GC is not suitable to separate the three FCEP isomers with a high resolution especially between **12**

and **13**. What is more, the lengthy run time of 65 mins is one of the disadvantages of this method.

Table 33. Individual FCEP isomers with eicosane on GC (polar column) using 1°C min^{-1} method

FCEP isomer	FCEP t_R(min)	Eicosane (E) t_R(min)
11	48.776	4.259
12	52.991	4.244
13	53.322	4.222

Table 34. FCEP isomers mixture with eicosane on GC (polar column) using 1°C min^{-1} method

FCEP isomer	FCEP t_R(min)	Eicosane (E) t_R(min)
11	48.769	4.244
12	53.005	4.244
13	53.332	4.244

Table 33 and Table 34 summarise and compare data on the retention times obtained from different runs for the individual versus the mixture of FCEP in the presence of E as a reference. The findings in these tables suggest that retention times of FCEP compounds and eicosane (E) are consistent in both cases.

Table 35. FCEP isomers mixture with eicosane on GC (polar column) using different temperature-programmed methods

Method	Total Run Time(min)	Eicosane t_R(min)	**11** t_R(min)	**12** t_R(min)	**13** t_R(min)	R_s (12:13)
5 °C min^{-1}	17	3.617		14.599		-
3 °C min^{-1}	25	3.899		-		-
2 °C min^{-1}	35	4.038		32.760		-
1 °C min^{-1}	65	4.244	48.769	53.005	53.332	0.84
Isothermal (180°C)	40	4.418		-		-

Table 35 lists the total run time of FCEP isomers mixture with eicosane on GC (polar column) using different temperature-programmed techniques.

It is obvious that the fastest method was the 5 °C min^{-1} with just 17 minutes and the 1 °C min^{-1} protocol with over than an hour. While, 3 °C min^{-1}, 2 °C min^{-1} and isothermal method at 180 °C were carried out within 25, 35 and 40 minutes, respectively. Apart from the isothermal method, the possible explanation of these findings might be because of the greater the temperature the less the retention time.

With reference to Table 35, there was only one peak at 4.4 min after applying the isothermal method at 180°C. Whereas, after using the 5°C min^{-1}method, there were only two peaks representing the three tested compounds plus eicosane as a reference at 3.6 and 14.5 mins (See Table 35). While, when the 3°C min^{-1} method was applied, only one peak appeared at 3.8 mins (See Table 35).

From the results, in the 2°C min^{-1} method, there were two peaks at 4.0 and 32.7 mins. The last method was the 1°C min^{-1}method, the separate FCEP isomers in presence of eicosane were injected on the polar GC system using 1°C min^{-1} method. The order of elution was in the following sequence; **11**, **12** and **13**. Whereas, the retention times were **11** (48.7 min), **12** (52,9 min), **13** (53.3 min) and eicosane (4.2 min).

In the polar GC column experiments, the 1°C min^{-1} method was the only procedure that gave all the peaks of three FCEP mixture and the eicosane peak, whereas, there were no peaks detected for the FCEP isomers when using both the isothermal (180°C) and 3°C min^{-1} methods. While, for the 2°C min^{-1} and 5°C min^{-1} methods there were only one peak detected for each method (other than the eicosane peak at 4.2 and 3.6 mins) at 32.7 mins and 14.5 mins, respectively.

5.2.6.1.4 Summary of FCEP experiments on 3 different GC systems

Firstly, in nonpolar GC column study, the fastest method was the 5 °C min^{-1} with just 29 minutes, Secondly, in semi-polar column study; the fastest method was the isothermal method at 180 °C with just 40 minutes. Finally, in polar column study, the fastest method was the 5 °C min^{-1} with just 17 minutes.

Secondly, in semi-polar GC column, one of the key findings in this investigation was the improvement noted in resolution after switching from nitrogen to helium as a carrier gas using the isothermal method at 180 °C (from just under 0.70 to 1.40 between 3- and 4-FCEP) which means that the resolution is doubled.

Thirdly, in polar GC column, It is clear from the outcome of the experimental scrutiny that using this type of GC column is not suitable for analysis/separation of FCEP isomers due to either lengthy run time or absence of the peaks of FCEP analytes.

5.2.6.1.5 The impact of helium and nitrogen as carrier gases with reference to Golay Equation

The Golay equation relates plate height to linear velocity of the mobile phase flowing through the GC capillary column. The height equivalent of a theoretical plate is one measure of column efficiency (see Golay equation in chapter 1). HETP depends on the nature of the carrier gas and its linear velocity (not volume flow rate). The smaller the HETP, the more efficient the separation. It is possible to obtain the highest efficiency – best separating power – from GC when the carrier gas linear velocity is set at the value where HETP is the lowest.

The three most important carrier gases used in GC are nitrogen, helium and hydrogen. Nitrogen is the poorest of the three gases as a carrier gas for temperature-programmed chromatography because slight changes in the linear velocity during a run can lead to significant degradation of efficiency (coalescing of the two peaks).

The minimum HETP with hydrogen is insensitive to large changes in the linear velocity. The linear velocity can be set at any value between about 30 cm/sec and 60 cm/sec without losing separation efficiency during the run. On balance, hydrogen is the preferred carrier gas for capillary columns.

The optimum gas velocity is inversely proportional to the column diameter, narrow-bore columns are faster than medium or wide-bore columns. A further advantage of capillary columns over packed columns is that the gas velocity and/or the column temperature can be increased quite easily if the resolution allows. This leads to an even greater increase in analysis speed. In addition, this relationship shows that a lighter carrier gas such as hydrogen or helium provides quicker analysis time without a substantial loss in resolution. Lastly, carrier gas viscosity is a temperature dependent parameter. As temperature increases, the viscosity of the gas increases. When using a constant pressure mode for carrier gas and temperature programming, the viscosity of the gas will increase and the average linear velocity will decrease. [146]

5.3 Conclusion

In the first study on HPLC, the FCEP compounds were tested by applying the reverse phase method for separation of FCEP isomers. Because of both low resolution and incomplete separation, the gradient method was applied to find out the optimum percentage for both acetonitrile and the buffer. In this HPLC study, three different buffers (formic acid, ammonium acetate and ammonium formate) were tested in a combination with the organic solvent (acetonitrile) by carrying out three different experiments; the aim was to examine the effect of changing the buffer solution on the HPLC separation process.

The results of the gradient method suggested that the best resolution between 2-FCEP (**11**) and the other two isomers (3-FCEP (**12**) and 4-FCEP (**13**) was (1.32) obtained using acetonitrile/ammonium acetate combination, the highest resolution was obtained (1.32), yet, the retention time was a bit lengthy with values of just below 21 minutes.

After using HPLC techniques, the next study was by using three GC methods, applying 3 different column polarities. The most important finding was when helium was used as a carrier gas utilising the isothermal method at 180 °C in semi-polar GC column (the resolution between the peaks of **12** and **13** went from just under 0.70 to 1.40). However, the aim in the FCEP isomers study was to achieve a resolution value of around 2.

From the GC results, although **11** was fully resolved by using GC methods, the separation of **12** and **13** was difficult using five different methods on three different GC columns.

In conclusion, HPLC and GC approaches used in this study were not suitable for separation of FCEP isomers using the above-detailed programmes. Although, the scope of this study was limited in terms of the tested parameters, however, the current findings add to a growing body of literature on fluorocyanoephenidine isomers, especially that this study has gone some way towards enhancing our understanding of the behaviour of these isomers on many devices such as GC and HPLC.

As a result, more research is required to analyse/separate the fluorocyanoephenidine isomers. Finally, it is suggested that the association of the parameters tested in this study is investigated in future studies.

Chapter 6: Development and validation for the separation of the regioisomers of the halogenated diphenidine using Reversed Phase HPLC.

As discussed in previous chapters, NPS have become a significant threat to public health that requires a detailed and focussed analytical investigation to help tackle all the problems arising from these drugs. This chapter discusses in detail the application of the validated HPLC method on 12 halogenated diphenidine (DP) regioisomers, which belong to the NPS category. The terms 'halogenated diphenidine (halo-DP) isomers', 'halophenidines and halodiphenidines' are also used interchangeably to refer to these compounds. The utility of HPLC to separate, detect and quantify a number of other diphenidine derivatives such as MXP, FEP and FCEP has been demonstrated (See chapters 3, 4 and 5 for details) and as such this approach was applied to the halogenated DP derivatives. The twelve halogenated DP regioisomers investigated in this chapter are 2-, 3- and 4-fluorodiphenidine (FDP), 2-, 3- and 4-chlorodiphenidine (CLDP), 2-, 3- and 4-bromodiphenidine (BrDP) and 2-, 3- and 4-iododiphenidine (IDP) (Figure 56).

In spite of a considerable amount of literature on diphenidine and its derivatives, there are only few studies/research so far that have been conducted on the halogenated derivatives and primarily focus on the *ortho*-isomer of chlorodiphenidine (2-chlorodiphenidine, 2-CLDP (**15**)).[71] In 2016, Wallach and co-workers investigated (pharmacologically) the binding affinities of the dissociative 'legal highs' diphenidine, MXP isomers and **15** as antagonists for NMDAR.[71] In this pharmacological investigation (on the forebrain homogenate of male Sprague–Dawley rats), the binding affinity reported by Wallach *et al*. [71] was substantially lower than the affinity constant reported by Gray and Cheng, and Berger *et al*., in previous studies.[147] The binding affinity can be defined as the maximum amount of drug or radio ligand, usually expressed as picomoles (pM) per mg protein, which can bind

specifically to the receptors in a membrane preparation.[148, 149] Furthermore, these experimental data are rather controversial, because Gray and Cheng reported 2-CLPH to have higher affinity binding than other known NMDAR antagonists. This reported finding for **15** prompted Wallach *et al.* to reinvestigate this compound. In Wallach's study, **15** was found to have potent low affinity for NMDAR which was substantially less than the affinity reported previously by Gray and Cheng. One of the probable explanations is due to the discrepancies in affinities of these compounds for different NMDAR subunit combinations. Apart from this single pharmacological study on **15**, there have been no studies concerned with the chemical analysis (spectroscopic and chromatographic) of the isomers of chlorodiphenidine or any other halophenidines. The lack of suitable spectroscopic and chromatographic studies into these compounds highlights the need to develop methods for the analysis of such drugs to be prepared to detect and quantify the substances should they be present in any seized sample.

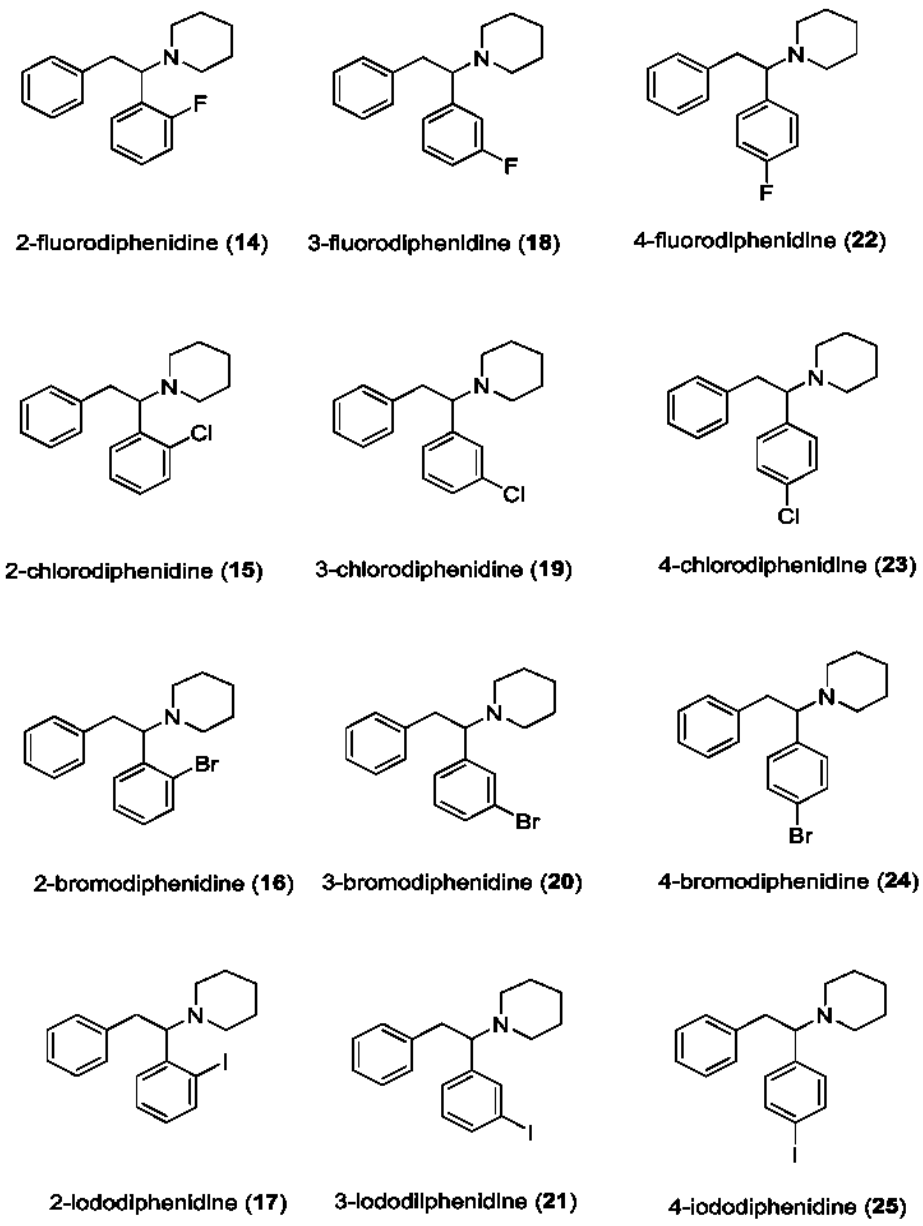


Figure 56. The chemical structures of the halogenated diphenidine compounds

The aim of this study is to examine the detection and separation of the 12 possible halogenated diphenidine derivatives, with the key research question being whether HPLC is a suitable method for the separation of these compounds and whether the method can be extended to allow us to quantify the materials in seized samples.

6.1 Characterisation of halophenidines

The halogenated diphenidine isomers were synthesised in-house at MMU by the MANchester DRug Analysis & Knowledge Exchange (MANDRAKE) unit and were fully structurally characterised [J Ainsworth-Mcmillan, “Guilty by dissociation – synthesis of halogenated diphenidine derivatives”, MChem Thesis (2018)], and then these compounds were investigated/tested on HPLC in this study.

6.1.1 The UV measurement of halophenidines

The UV spectra for the halodiphenidine isomers were extracted using the diode-array detector (DAD) within the range of 190-400 nm. The DAD scanned four selected wavelengths (270, 258, 254 and 220 nm), which have been demonstrated in chapters (3, 4 and 5) of this thesis to represent the maximum absorbance of many diphenidine derived compounds for example, MXP isomers (278 nm) and FEP isomers (270 nm). In addition, benzene rings absorb strongly at 254 nm. In this study, the mobile phase acetonitrile:ammonium acetate 55:45% v/v (blank/solvent) was tested via UV-vis spectroscopy and the result showed the acetonitrile cut-off wavelength at 190 nm (Figure 57), and then it was used in HPLC as a solvent for the halogenated diphenidine compounds in the range (190-400 nm). By using the HPLC-DAD, the data showed that the halophenidine isomers absorb UV light at 220 nm more than at the other three selected points. The optimum wavelength was therefore determined to be 220 nm; this wavelength was applied in the HPLC method development. At lower wavelengths, the decreased UV absorbance of acetonitrile provided better signal-to-noise and larger sensitivity for quantifying analytes. [150]

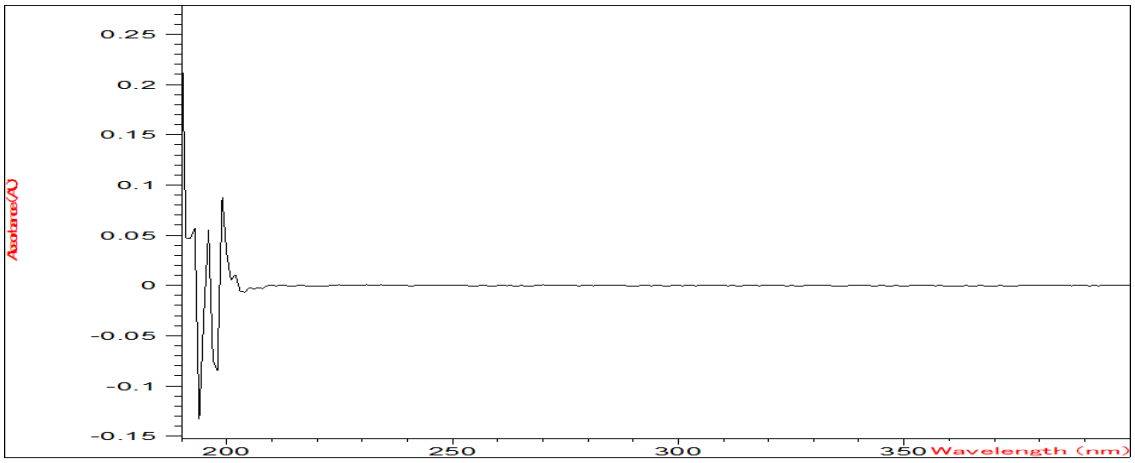


Figure 57. The UV spectrum of acetonitrile:ammonium acetate 55:45% v/v

6.2 HPLC method development

In order to develop an HPLC method to analyse these 12 isomers, a number of flow rates were studied using a similar mobile phase (acetonitrile:ammonium acetate 55:45% v/v) used to separate the three MXP isomers (Chapter 3). The twelve compounds were split into as either *ortho* (four examples), *meta* (four examples) and *para*-halogenated (four examples) derivatives. Figure 58 exemplifies the separation and detection of the 2-halophenidines using HPLC-DAD. Prior to starting the HPLC analysis, the analyst should consider the cut-off point of the solvent and make sure that there is no impurity peaks near the solvent peak, which means that any peaks that appear after the solvent peak belong to the analytes.

In Figure 58, the UV-DAD detector is an analyte specific property detector, therefore, responding to analytes that absorb UV light at a particular wavelength (i.e. specific), in this case 2-halogenated diphenidine isomers at 220 nm.

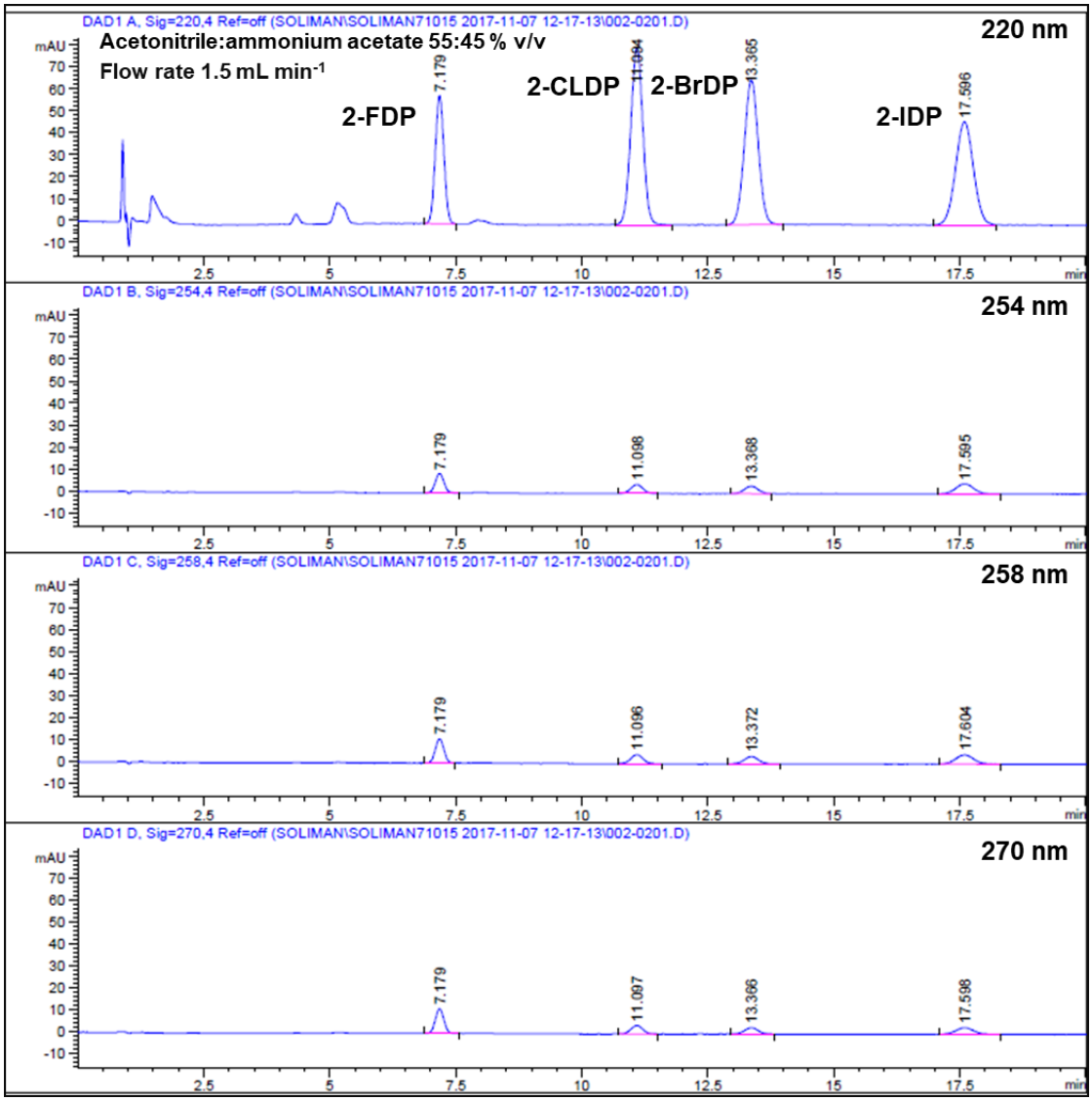


Figure 58. Detection and separation of the 2-halophenidines at a flow rate of 1.5 mL min^{-1} and at four different wavelengths using HPLC-DAD

From the results, the order of elution (which is governed by polarity) was as follows: **14**, **15**, **16** and **17**. The order of elution is related to partitioning of the analytes between the solid phase (C18) and the mobile phase, therefore in this experiment, the most strongly retained halogenated compound is the most non-polar (or lipophilic) of the diphenidine isomers investigated. Under reverse-phase conditions, **17** eluted last because this isomer is the most lipophilic and as such it is this derivative, which is retained more strongly, relative to the other halogenated derivatives. The resolution between each of

the derivatives was determined to be greater than 2 in all cases, which indicates that this method is able to satisfactorily resolve the four compounds. However, the run time is far too long for routine analysis and as such this might be possible by changing the flow rates to decrease the run time and maintain a satisfactory resolution. In Figure 58, there are three tiny peaks at approximately 1 min, 4.5 min and 5 min, the first peak at 1 min is assigned to uracil, the other two peaks were eluted as impurities accompanied with 2-BrDP before recrystallization. The following chromatogram (Figure 59) shows the isomer after recrystallisation at a flow rate of 1.5 mL min^{-1} (at 220 nm), using acetonitrile:ammonium acetate 67:33% v/v, in this chromatogram, there are no impurity peaks detected after recrystallization. When the mixture of these 2-halogenated diphenidine isomers was injected in to the HPLC after recrystallization there were no impurities detected.

Table 36. Retention times and resolution of the 2-, 3- and 4-halodiphenidine isomers using four different flow rates. Mobile phase (acetonitrile:ammonium acetate, 55:45% v/v)

		Flow rate 1.5 mL min^{-1}		Flow rate 1.7 mL min^{-1}		Flow rate 1.9 mL min^{-1}		Flow rate 2.0 mL min^{-1}	
		t_R (min)	**Rs**	**t_R** (min)	**Rs**	**t_R** (min)	**Rs**	**t_R** (min)	**Rs**
2-halo	**14**	7.13	-	6.30	-	5.65	-	5.26	-
	15	11.06	8.55	9.75	8.50	8.74	8.42	8.11	8.33
	16	13.33	4.15	11.75	4.10	10.53	4.08	9.75	4.01
	17	17.56	6.63	15.48	6.53	13.86	6.46	12.79	6.38
		t_R (min)	**Rs**	**t_R** (min)	**Rs**	**t_R** (min)	**Rs**	**t_R** (min)	**Rs**
3-halo	**18**	7.25	-	6.41	-	5.63	-	5.36	-
	19	11.01	7.88	9.73	7.80	8.49	7.48	8.09	7.58
	20	12.54	2.82	11.08	2.78	9.66	2.66	9.19	2.70
	21	14.56	3.50	12.85	3.43	11.20	3.31	10.66	3.34
		t_R (min)	**Rs**	**t_R** (min)	**Rs**	**t_R** (min)	**Rs**	**t_R** (min)	**Rs**
4-halo	**22**	5.59	-	4.89	-	4.32	-	4.11	-
	23	9.73	13.71	8.55	10.23	7.50	9.89	7.13	9.88
	24	11.34	4.13	9.97	3.28	8.73	3.18	8.31	3.18
	25	13.76	5.25	12.11	4.47	10.59	4.35	10.07	4.33

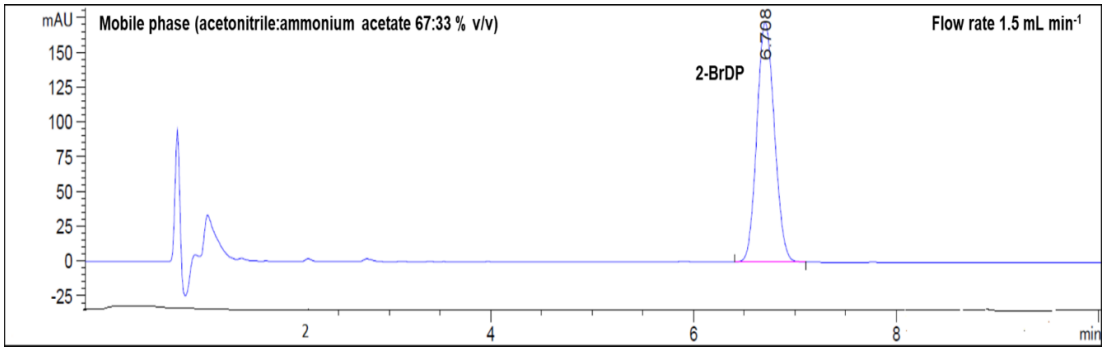


Figure 59. Chromatogram of 2-BrDP acquired after recrystallization

6.2.1 HPLC method development (changing the flow rate)

The flow rate experiments were carried out by employing four different flow rates (1.5, 1.7, 1.9 and 2.0 mL min^{1}). The findings of this study showed completely resolved peaks between the tested isomers with good resolution (Table 36).

Table 36, Figure 60, Figure 61 and Figure 62 present the experimental data on retention times and resolution of the mixture of the 2-, 3- and 4-halogenated diphenidine isomers (halo DP isomers) by HPLC, using the flow rates; 1.5, 1.7, 1.9 and 2.0 mL min^{1}, mobile phase (acetonitrile:ammonium acetate, 55:45% v/v).

By comparing the data of the three groups of the halo DP isomers (12 compounds), for example, in the case of the retention times of the fluoro isomers and the resolution between the peaks of the fluoro and the chloro isomers i.e. for **14**, the t_R dropped from 7.13 (1.5 mL min^{1}) to 5.26 mins (2 mL min^{1}). Whereas, the resolution fluctuated between 8.55 and 8.33 for the first two peaks of **14** and **15** (See Figure 60).

Figure 60 shows the retention times (t_R in minutes) of the 2-halodiphenidine isomers versus the absorbance using acetonitrile:ammonium acetate, 55:45% v/v (flow rate 1.5 mL min^{-1}). From the results the retention times where as follows: **14** = 7.13, **15** = 11.06, **16** = 13.33 and **17** =17.56 mins, respectively.

In summary, the greater the flow rate the less the retention time and resolution. In addition, the elution order is the same in all three tested groups was as follows (FDP, CLDP, BrDP and IDP, respectively), (See Figure 60,Figure 61 and Figure 62).

To conclude, the t_R and resolution decreased significantly by increasing the flow rate from 1.5 to 2.0 mL min^1. The optimum flow rate is 1.5 mL min^{-1} because the highest values of resolution were obtained by using this flow rate (Table 36).

The following chromatograms (Figure 60, Figure 61 and Figure 62) show the retention times of the three groups of the 2-, 3- and 4-halodiphenidines (4 isomers each), using mobile phase acetonitrile:ammonium acetate 55:45% v/v (flow rate 1.5 mL min^{-1}).

Taken together, by comparing the method development of the three distinct groups, it can be noticed that the retention times for the 2-isomers were the longest among the other two groups with an average range of about 7.0-17.0 min. In contrast, the 4-isomers eluted the quickest as they had retention times ranging from 5.5 to 13.5 min, while the third group (3-isomers) had intermediary retention times in the range of 7.3-14.3 min. Overall, the trend in the order of elution and t_R is due to the different degrees of lipophilicity and polarity among these isomers. Overall, by using the flow rate 1.5 mL min^1, the retention times and resolution were decreased compared to the flow rates 1.7, 1.9 and 2.0 mL min^1.

Provided that these are new compounds with no previous HPLC experiments conducted on them, the obtained data is acceptable. However, the overall run times of these experiments were generally a bit lengthy (20 minutes). The next set of experiments were carried out by increasing the percentage of the organic part (acetonitrile) in the mobile phase to be greater than 55% in order to reduce the total run time below twenty minutes.

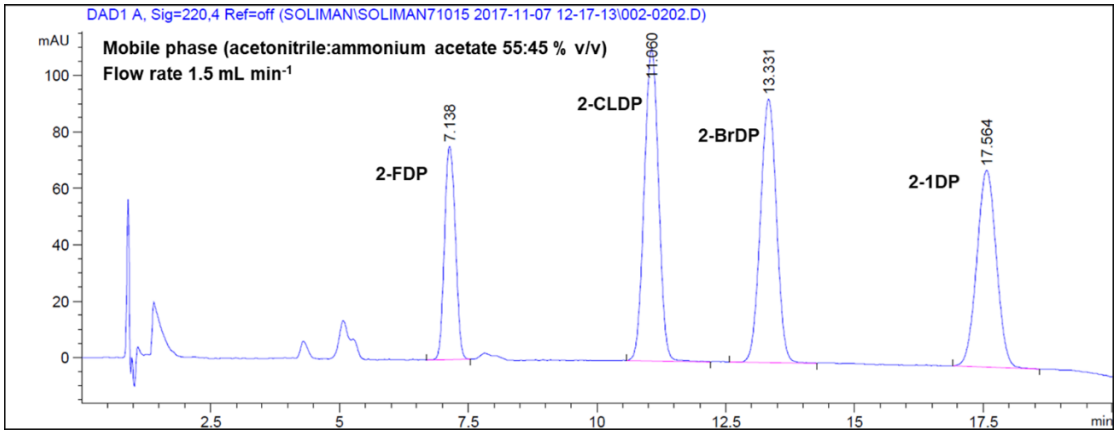


Figure 60. HPLC separation of the 2-halo diphenidine isomers using a mobile phase consisting of acetonitrile:ammonium acetate, 55:45% v/v, and a non-polar stationary phase

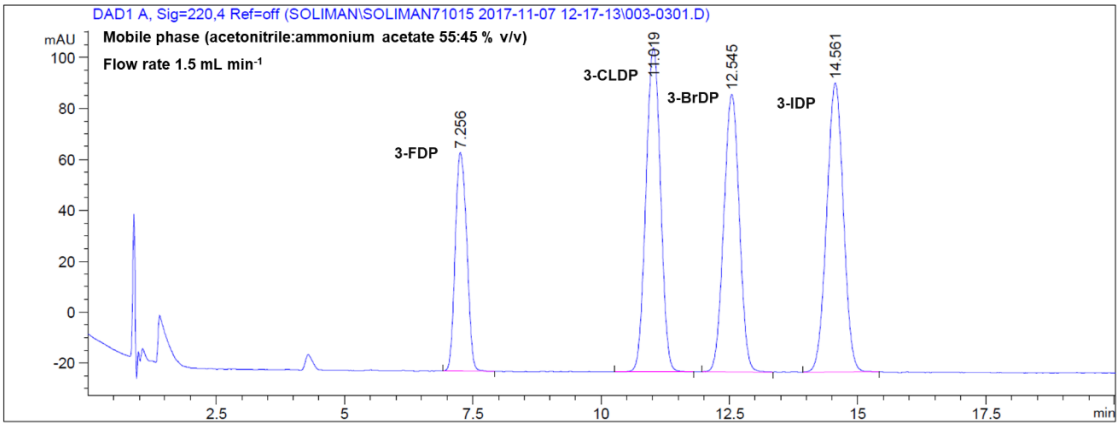


Figure 61. HPLC separation of the 3-halo diphenidine isomers using a mobile phase consisting of acetonitrile:ammonium acetate, 55:45% v/v, and a non-polar stationary phase

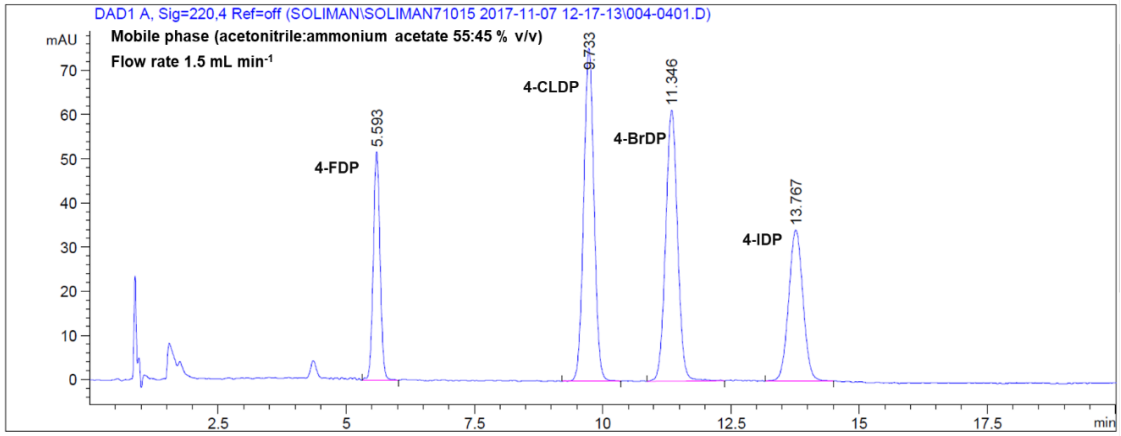


Figure 62.HPLC separation of the 4-halo diphenidine isomers using a mobile phase consisting of acetonitrile:ammonium acetate, 55:45% v/v, and a non-polar stationary phase

6.2.2 HPLC method development (changing the percentage of mobile phase)

The purpose of this new experimental investigation focused on obtaining the optimum mobile phase composition. The percentages of mobile phase that were used in this study were as follows: acetonitrile:ammonium acetate 60:40% v/v, 65:35% v/v, 70:30% v/v and 75:25% v/v, using a flow rate of 1.5 mL min^{-1} as a constant variable in all studies. This flow rate was chosen because it led to less pressure in the instrument, therefore leading to lower load on the column in comparison with using higher flow rates. The average pressure during the different flow rate experiments of the HPLC method development for these compounds was as the following: 75, 90, 100, 110 bar for 1.5 mL min^{-1}, 1.7 mL min^{-1}, 1.9 mL min^{-1} and 2.0 mL min^{-1}, respectively.[151]

The role of pressure in HPLC is to push the mobile phase through the small particles filled into the column. Normally, moderate to high pressure is used to flow the solvent through the chromatographic column, while low pressure is usually because of a leak in the system, and high back pressure is an unexpected increase in the pressure during normal HPLC operation that approach or exceed the maximum pressure ability of the system.

The results of these four experiments can be summarised in the following tables and figures. The data for the 2-haloDP isomers is shown below in a detailed way, and because of the similarities in the experiments, the 3- and 4-isomers data are discussed in less detail in order to facilitate the comparison between all the three groups of halo DP isomers:

Figure 63 below shows the effect of changing the percentage of mobile phase on detection and separation of 2-haloDP isomers, using a flow rate of 1.5 mL min^{-1} and concentrations of 100 µg mL^{-1}. Firstly, using 55:45 and 60:40% v/v gave a total run time of 20 and 15 minutes, respectively. Therefore, these two percentages were not chosen as the optimum proportions of mobile phase to be used in halophenidines detection and separation. Whereas, by applying 65:35, 70:30 and 75:25% v/v, the run time was only 10 minutes. The resolution in these last three percentages was very good, the minimum resolution value was 1.72 between **16** and **15** using mobile phase 75:25%, 2.08 using mobile phase 70:30% and 2.52 applying mobile phase 65:35%.

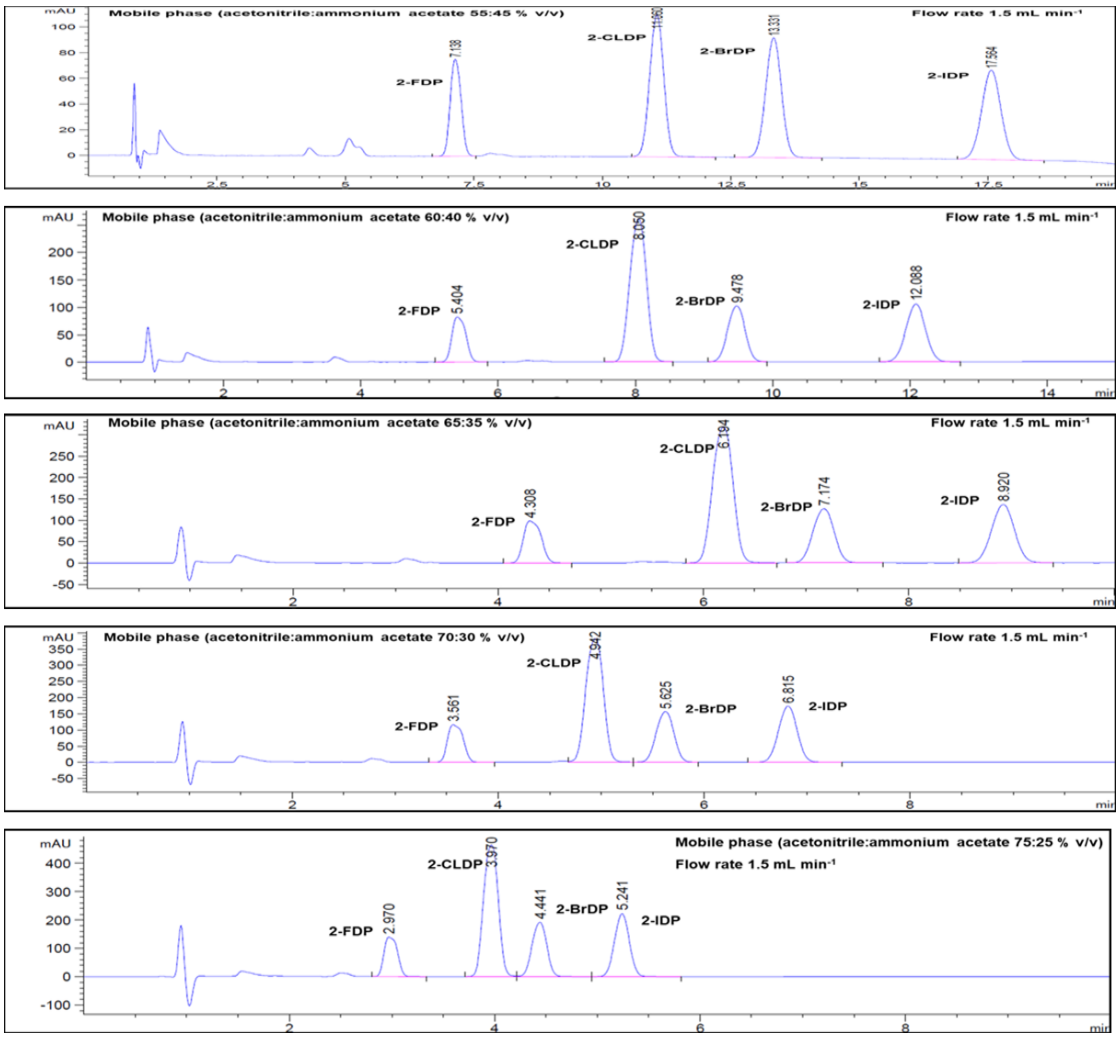


Figure 63. Chromatograms showing the effect of changing the percentage of mobile phase on the detection and separation of 2-haloDP isomers

The goal of this experiment was to obtain a resolution value of about 2 or just above 2 using a quick method. From these results, the 10 minutes total run time meant of the goals was achieved, but in order to improve the resolution mentioned above, it was decided in this experiment to try a mobile phase percentage of 67:33% v/v, which is between 65 and 70% v/v. The 12 halodiphenidine isomers were tested by applying the mobile phase, acetonitrile:ammonium acetate 67:33% v/v; the results of these experiments are shown in Figure 64.

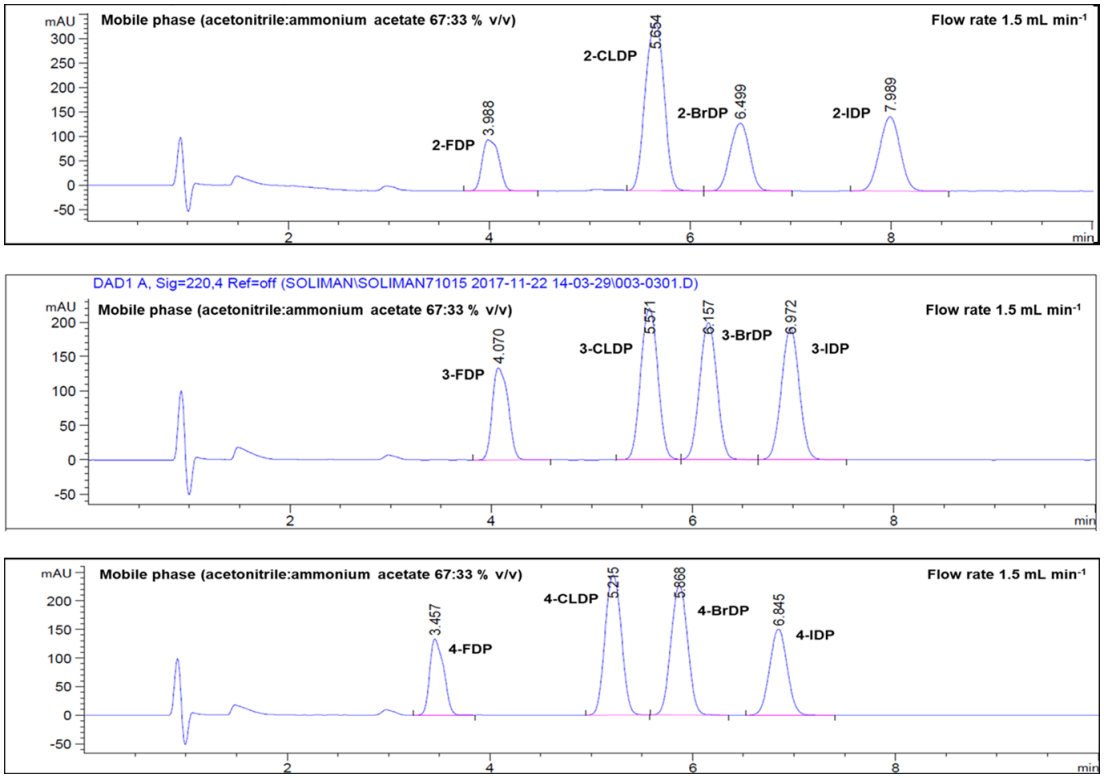


Figure 64. Chromatograms detailing the comparison of using a flow rate of 1.5 mL min^{-1} and a mobile phase consisting of acetonitrile:ammonium acetate, 67:33% v/v, on the separation of 2-, 3- and 4-halo DP isomers

As can be seen in Figure 64, all the peaks in this investigation on 2-, 3- and 4-halo DP isomers were completely base line resolved and the resolution value between **16** and **15** was 2.35, while the resolution value between **20** and **19** was 1.81 and lastly between **24** and **23** it was 2.11. As a result, the second

aim of this investigation was achieved because the resolution was over 2 for the 2- and 4-isomers; the resolution of 1.81 in the case of 3-isomers is still acceptable.

6.2.3 Summary of the three experiments (wavelength, flow rate and mobile phase composition):

The developed HPLC method; using the combination of mobile phase acetonitrile:ammonium acetate, 67:33% v/v, flow rate of 1.5 mL min^{-1} and concentration range of 1.25-20 µg mL^{-1} was used in the analysis/validation of this method on 2-, 3- and 4-halo DP isomers due to the following reasons:

Wavelength: All the 12 halophenidine isomers have the maximum UV at 220 nm in comparison with their absorption at the other tested wavelengths (254, 258 and 270 nm).

Flow rate: despite the 1.5 mL min^{-1} being the slowest flow rate, this flow rate produced the highest resolutions in comparison with the other flow rates (1.7, 1.9 and 2.0 mL min^{-1}) examined in these experiments.

Mobile phase composition: by increasing the percentage of acetonitrile in the mobile phase, both the retention time and the total run time were decreased. The percentage of mobile phase acetonitrile:ammonium acetate 67:33% v/v (between 65 and 70% v/v) was decided to be used in the method validation in a combination with using the flow rate 1.5 mL min^{-1} and wavelength 220 nm.

6.3 HPLC method validation of 2-, 3- and 4-halogenated diphenidine isomers

The table in Appendix A 52 outlines the validation data of the 12 halogenated diphenidine isomers. To prove/confirm that the method is suitable for its intended use, is the purpose of validating analytical protocols,[152] therefore, the current HPLC method was validated according to the ICH guidelines.[135] Parameters such as retention time, resolution, limit of detection (LOD), limit of quantification (LOQ) and percentage relative standard deviation (%RSD) were assessed in this experiment.

As can be noted in in the table, the most important finding is that the limit of detection (LOD) for the 2-isomers was just below 0.08 μg mL^{-1}, whereas for the 3- and 4-isomers this value was between 0.55-070 and 0.60-0.90 μg mL^{-1} respectively. The limit of quantification, LOQ for the 2-isomers was just below 0.24 μg mL^{-1}, 0.16-0.21 μg mL^{-1} for the 3-isomers and lastly, it was 0.17-0.26 μg mL^{-1} for the 4-isomers. These values can be considered as good values/measures for these new compounds in this new HPLC method/study because very small amounts in micrograms of these substances (around 0.10 and 0.25 μg mL^{-1}) can be detected and determined. In addition, there is no specific detection threshold limit for these new substances.

It is clear that the retention times of these halogenated diphenidine derivatives were ranging between just over 3.5 minutes in the case of **22**, up to approximately 8.5 minutes in the case of **17**. The resolution was also good, with a minimum of 2.6 between the peaks of **20** and **19**.

It can be concluded that there are different degrees of interaction between the isomers and the column employed, most likely due to the variance in both the type of halogen atom present in the diphenidine derivative, and its location on the ring.

In summary, the overall run time required to identify and separate the four NPS in question, was just 10 minutes which represents an excellent period of time for analysis. It is also clear that all the peaks were fully resolved with excellent peak symmetry/shape and one of the advantages of this method is the detection of halogenated diphenidine isomers at concentrations of as low as 1.25 μg mL^{-1}.

The equations of the lines are included in the table, showing the relationship between peak area (y) and the concentration (x). On average, all of the given four compounds had an average r^2 of 0.9999.

There were hardly any differences between the values of theoretical plates for all the 12 isomers, which was on average 9000-11500, this reflects that the

peaks are sharp with no fronting or tailing. In a separation process, a theoretical/hypothetical plate produced from two phases refers to a state of equilibrium between two levels.[153]

With regard to the %RSD, generally it is noticeable that it accounted for almost below 1.000 for all the concentrations under investigation (1.25-20 µg mL^{-1}). This is because they shared nearly the same patterns of percentage injection precision in all five categories, which explains that deviation or the variance from the mean is acceptable as it is below one in all cases/concentrations; this is an indicator of good injection precision values in this experiment.

The validation chromatograms of 2-, 3- and 4-halogenated diphenidine isomers (in concentrations of 10 µg mL^{-1}) are shown in Figure 65, Figure 66 and Figure 67.

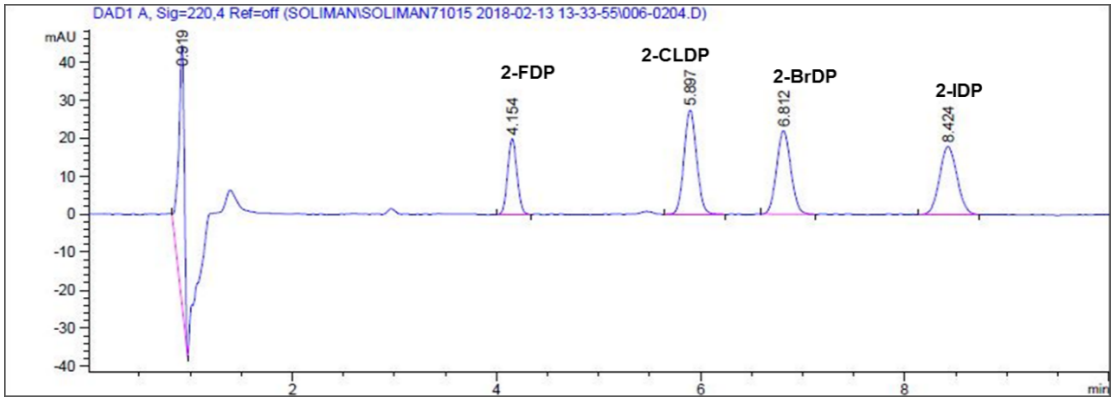


Figure 65. Validation chromatogram of the 2-halogenated diphenidine isomers (concentration=10 µg mL^{-1})

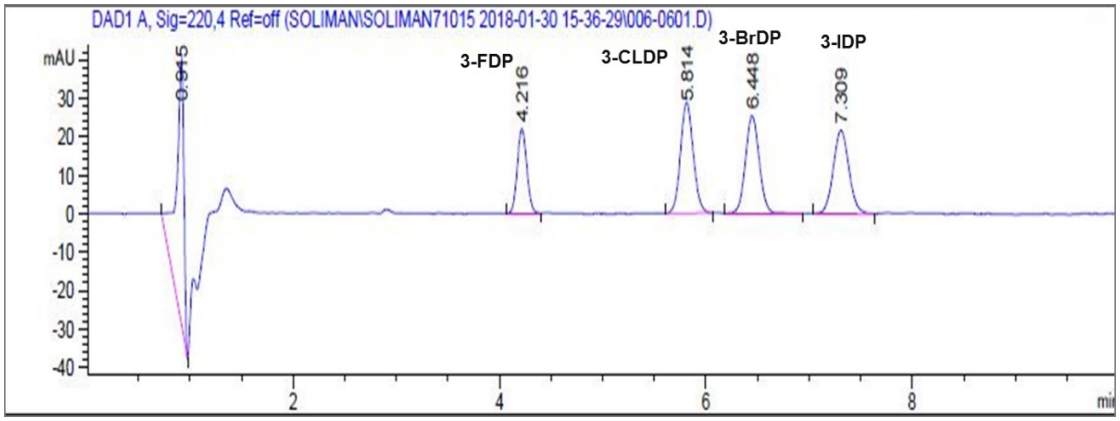


Figure 66. Validation chromatogram of the 3-halogenated diphenidine isomers (concentration=10 µg mL^{-1})

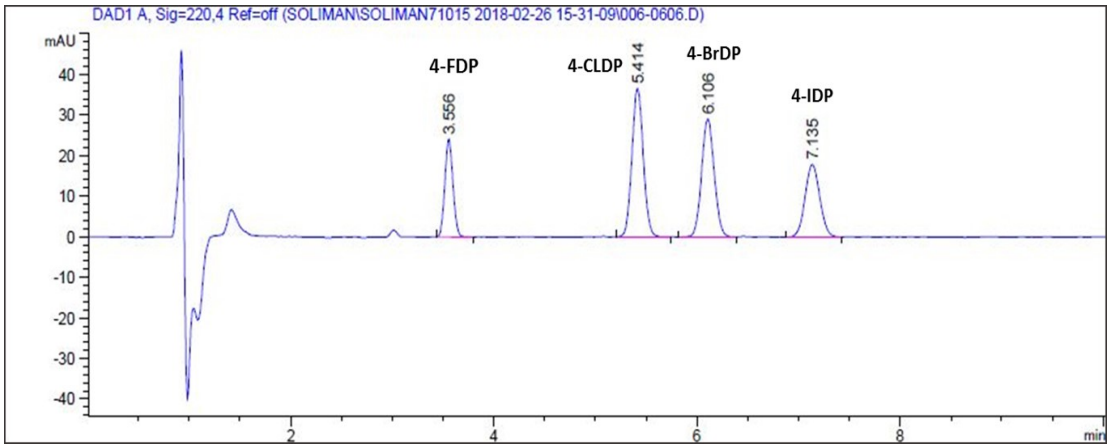


Figure 67. Validation chromatogram of the 4-halogenated diphenidine isomers (concentration=10 µg mL^{-1})

6.4 Robustness of HPLC Method (2-halo DP isomers)

The robustness parameters tested in this study are the intra-day and inter-day precision, flow rate, temperature and the composition/percentage of mobile phase.

6.4.1 Intra- and inter-day precision

In this experiment, two blanks were injected prior to injecting halogenated diphenidine isomers 10 times; Intra-day (am and pm) and Inter-day (the average of pm plus am and pm). The table in (Appendix A 53) shows the

intraday and inter-day precision measurements for the 2-halogenated diphenidine analytes using the mobile phase acetonitrile:ammonium acetate 67:33% v/v, a flow rate of 1.5 mL min^{-1} and a concentration of 10 µg mL^{-1} (see

The retention time remained almost constant for the isomers of all four groups with just a slight/insignificant difference between the inter- and intra-day precision of the iodo-isomers. According to the table in (Appendix A 53), the retention times of these compounds ranged from just over 4 mins (**14)** to just over 8 mins (**17)**. The %RSD is on average of almost 0.11 for intraday measurements and about 0.14 for inter-day precision, which refers to a slight difference in the injection precision without affecting the retention times of these isomers. To conclude, changing the testing time during the day does not have any substantial effect on the robustness of the HPLC applied for analysis of 2-halogenated diphenidine derived isomers.

6.4.2 Flow rate

The flow rates 1.4, 1.5 and 1.6 mL min^{-1} were assessed in this investigation (See Table 37).These findings are in agreement with the linear van Deemter equation, by increasing the flow rate the retention time is decreased slightly for all the halodiphenidine isomers (**14**, **15**, **16** and **17**) in the flow range examined in this experiment.

Table 37. Robustness testing (flow rate 1.4, 1.5 or 1.6 mL min-1) for the 2-halogenated diphenidine isomers using a mobile phase consisting of acetonitrile:ammonium acetate, 67:33 % v/v. Key: tR = Retention time (min).

Injection	t_R (min) using flow rate 1.4 mL min^{-1}				t_R (min) using flow rate 1.5 mL min^{-1}				t_R (min) using flow rate 1.6 mL min^{-1}			
	14	**15**	**16**	**17**	**14**	**15**	**16**	**17**	**14**	**15**	**16**	**17**
1	4.47	6.34	7.32	9.04	4.17	5.91	6.81	8.41	3.91	5.54	6.39	7.89
2	4.47	6.34	7.32	9.05	4.16	5.90	6.81	8.41	3.92	5.55	6.40	7.90
3	4.47	6.34	7.32	9.05	4.17	5.90	6.80	8.40	3.91	5.54	6.39	7.90
4	4.47	6.34	7.32	9.04	4.17	5.91	6.82	8.42	3.91	5.54	6.40	7.90
5	4.47	6.34	7.32	9.05	4.16	5.90	6.81	8.41	3.92	5.55	6.40	7.91
6	4.47	6.34	7.32	9.05	4.17	5.91	6.81	8.41	3.91	5.54	6.40	7.90
7	4.47	6.34	7.31	9.04	4.16	5.90	6.80	8.40	3.92	5.55	6.40	7.91
8	4.48	6.35	7.33	9.06	4.17	5.91	6.82	8.42	3.92	5.55	6.40	7.91
9	4.48	6.35	7.32	9.04	4.17	5.91	6.82	8.41	3.91	5.54	6.40	7.90
10	4.48	6.35	7.33	9.05	4.16	5.88	6.78	8.36	3.92	5.55	6.40	7.91
Average	**4.47**	**6.34**	**7.32**	**9.05**	**4.17**	**5.90**	**6.81**	**8.40**	**3.91**	**5.55**	**6.40**	**7.90**
STD	**0.004**	**0.004**	**0.004**	**0.005**	**0.004**	**0.007**	**0.010**	**0.015**	**0.004**	**0.004**	**0.004**	**0.005**
%RSD	**0.093**	**0.073**	**0.061**	**0.057**	**0.110**	**0.129**	**0.157**	**0.188**	**0.110**	**0.087**	**0.077**	**0.069**

Table 37 presents robustness testing (flow rate) for the 2-halogenated diphenidine analytes in four different groups over the course of a ten minute run. According to the table, as flow rate increases, the retention time decreases. As can be seen in the tables, by increasing the flow rate from 1.4 to 1.5 and then to 1.6 mL min^{-1}, the retention time drops dramatically for **14**, **15**, **16** and **17** by about (0.5, 0.8, 0.9 and 1.1 min), respectively.

Overall, separation time varies relatively across the four compounds, which contain different halogens in their chemical structures; therefore, they have different ways of separation i.e. this could be because of the interaction of the halogenated structure with the column. The order of elution is as follows: (F, Cl, Br and I); this might be because the degree of the hydrophobicity for these halogens follows the following order: fluoro <chloro < bromo < iodo. This means that the iodo group has the highest hydrophobicity. As a result, a hydrophobic molecule (e.g. iodo) in the polar mobile phase (acetonitrile:ammonium acetate) will adsorb to the hydrophobic stationary phase, and thus will pass through the column slower than the other halogenated compounds - therefore the iododiphenidine elutes last. In contrast, the fluorinated diphenidine elutes first because it possesses the lowest hydrophobicity among the four halogenated substances in this study.

All things considered, the flow rate of 1.5 mL min^{-1} has been chosen to separate these isomers, as the retention time and resolution are slightly improved when compared to the other two rates in this experiment.

6.4.3 Temperature effect and Van't Hoff equation

Three different temperatures, 48, 50 and 52°C, were examined in this experiment (see Appendix A 54). applying slight changes in the temperature caused slight changes in the t_R (and Rs). Overall, the 50°C programme was selected for the validation of the HPLC method of analysis for these compounds. This temperature was applied in the HPLC method validation of MXP and FEP (See chapters 3, 4).

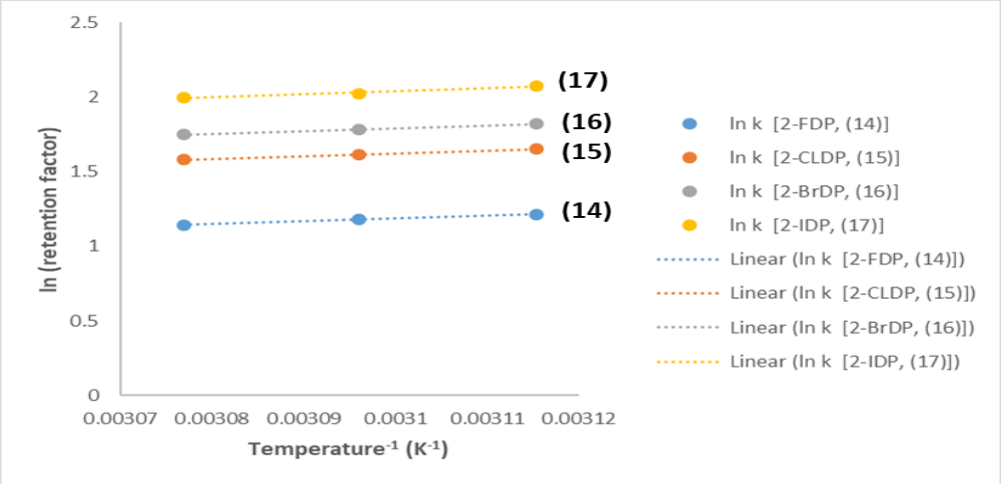


Figure 68. Van't Hoff plot of ln k versus 1/T for the 2-halogenated diphenidine isomers (17, 18, 19 and 20)

As is observed from the information in (Appendix A 54), by increasing the temperature by 2°C, the retention time decreased slightly. The resolution was also slightly increased by employing a temperature of 50°C, whilst a decrease was observed when the temperature was increased to 52°C. However, the change in resolution was not significant. These results are on par with the Van't Hoff behaviour (see Figure 68), the retention mechanism is constant with increasing the temperature, which is based on the equilibrium between enthalpy and entropy changes in the thermodynamic system for the 2-halodiphenidine isomers under investigation (**14**, **15**, **16** and **17**). For the Van't Hoff plots of ln k versus 1/T for the 3- and 4-halogenated diphenidine isomers see Appendix A 55 and Appendix A 56.

Finally, all three isomers were plotted for each halogen (ortho, meta and para) to see if the position of the halogen has an influence on the Van't Hoff relationship using temperatures (48, 50 and 52°C). Figure 69 shows the effect of changing temperature on retention factor of the fluorinated diphenidine isomers (**14**, **18** and **22**).

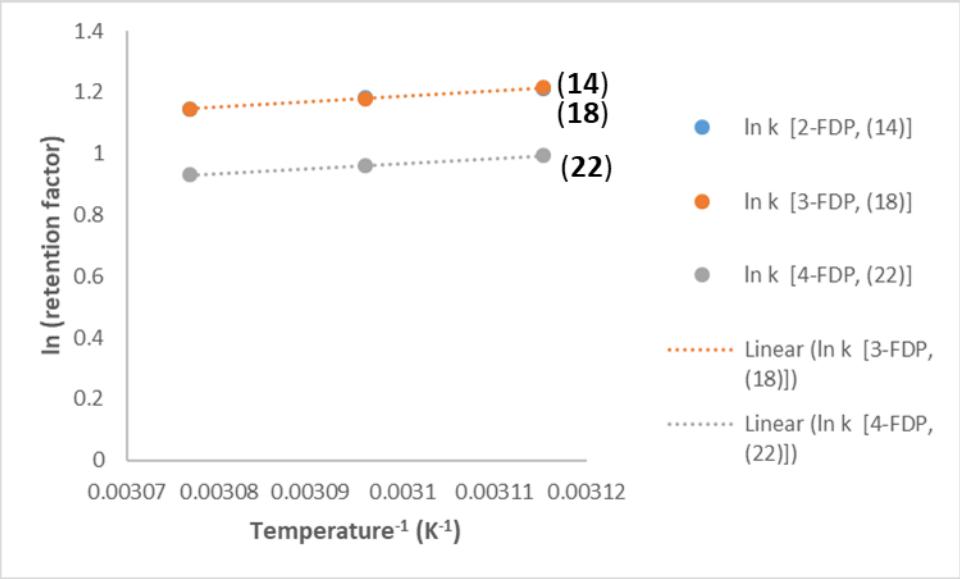


Figure 69. Van't Hoff plot of ln k versus 1/T for the fluorinated diphenidine isomers (**14**, **18** and **22**)

It can be noted that the para-substituted isomer possesses the lowest value of retention factor. In addition, the parallel lines suggest similar retention mechanism for these compounds. The ortho and meta isomers appear as a one line due to similar retention times. Similarly, see plots of the chlorinated (Appendix A 57), brominated (Appendix A 58) and iodinated (Appendix A 59) diphenidine isomers. The general trend with all the 12 halogenated diphenidine isomers investigated in this work is that the order of elution is as follows: *para*, *meta* and *ortho,* respectively.

6.4.4 Robustness of the method relative to mobile phase composition

The following mobile phase compositions: (66:34, 67:33 and 68:32% v/v) were used in this study. Table 38 lists the data of robustness testing (by varying mobile phase compositions) for the four 2-halogenated diphenidine isomers. The tested mobile phase proportions were sorted into different categories; 66:34, 67:33 and 68:32% v/v, respectively.

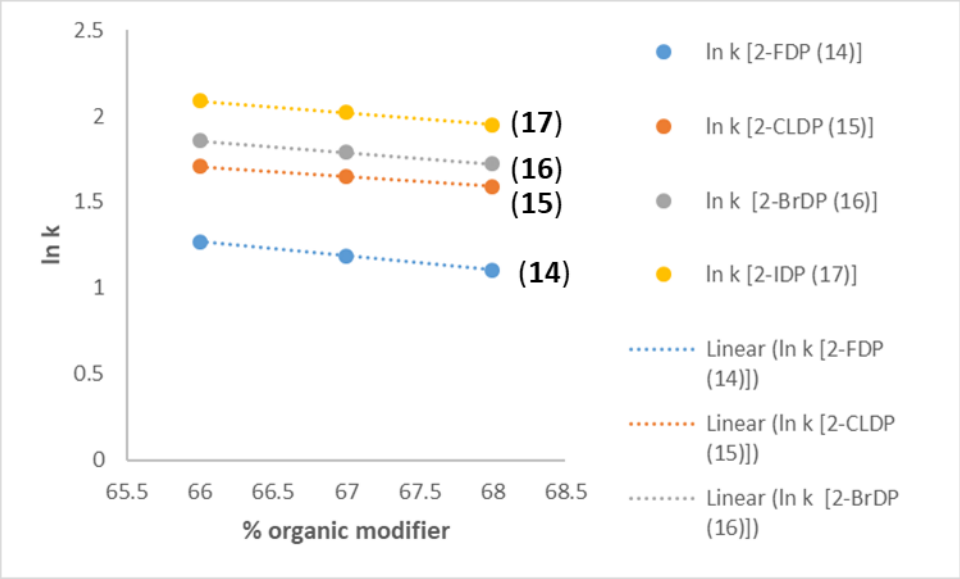


Figure 70. ln k versus % organic modifier (acetonitrile) for 2-halodiphenidine isomers

The plot of ln retention factor (k) versus the percentage of organic modifier (acetonitrile) for 2-halodiphenidine isomers is shown in Figure 70, it is a useful measure to understand the effect of the nature of the halogen on retention mechanisms. It is clear that the retention time decreases when the percentage of acetonitrile increases.

By increasing the percentage of the organic modifier (acetonitrile) from 66 - 68% the retention time of the 2-halogenated diphenidine isomers (**14, 15, 16** and **17**) are decreasing. Log P values of these compounds were calculated using ChemDraw software. Log P values were as follows: 4.86 (**14**), 4.86 (**15**), 4.86 (**16**) and 5.26 (**17**), which is slightly higher than FEP isomers (4.09) and MXP isomers (4.58). Similarly, this indicates these isomers possess a hydrophobic effect as a mechanism of retention. It is obvious that the iodo isomer (2-iododiphenidine, **17**) has higher Log P (5.26), and therefore it is more hydrophobic with greater retention time than **14, 15** and **16.**

All in all**,** in spite of the change in the proportions of mobile phase components, there was not much difference in the obtained values of t_R. A mobile phase of 67:33% v/v was selected to be used for detection and separation of these

analytes, as this percentage of mobile phase produced excellent average resolution values for the ten injections of the mixture of the 2-halogenated diphenidine isomers. The average value of t_R for **14** was 4.1, for **15** was 5.9, for **16** was 6.8 and lastly for **17** was 8.4 minutes.

Table 38. Robustness testing (% mobile phase (acetonitrile:ammonium acetate) = 66:34, 67:33 or 68:32% v/v) for the 2-halogenated diphenidine isomers

	t_R (min) using acetonitrile: ammonium acetate (66:34% v/v)				**t_R (min) using acetonitrile: ammonium acetate (67:33% v/v)**				**t_R (min) using acetonitrile: ammonium acetate (68:32% v/v)**			
Injection	**14**	**15**	**16**	**17**	**14**	**15**	**16**	**17**	**14**	**15**	**16**	**17**
1	4.35	6.24	7.25	9.04	4.17	5.91	6.81	8.41	4.05	5.69	6.54	8.04
2	4.35	6.24	7.24	9.02	4.16	5.90	6.81	8.41	4.05	5.66	6.55	8.04
3	4.35	6.24	7.24	9.02	4.17	5.90	6.80	8.40	4.04	5.66	6.54	8.04
4	4.35	6.24	7.24	9.03	4.17	5.91	6.82	8.42	4.05	5.69	6.54	8.04
5	4.35	6.23	7.24	9.03	4.16	5.90	6.81	8.41	4.04	5.69	6.55	8.05
6	4.35	6.23	7.23	9.01	4.17	5.91	6.81	8.41	4.04	5.68	6.53	8.04
7	4.35	6.24	7.25	9.03	4.16	5.90	6.80	8.40	4.04	5.68	6.53	8.02
8	4.36	6.25	7.25	9.02	4.17	5.91	6.82	8.42	4.05	5.71	6.57	8.08
9	4.36	6.24	7.25	9.03	4.17	5.91	6.82	8.41	4.05	5.71	6.56	8.07
10	4.35	6.24	7.25	9.02	4.16	5.88	6.78	8.36	4.04	5.69	6.55	8.06
Average	**4.35**	**6.24**	**7.24**	**9.02**	**4.17**	**5.90**	**6.81**	**8.40**	**4.04**	**5.69**	**6.55**	**8.05**
STD	**0.004**	**0.005**	**0.005**	**0.006**	**0.004**	**0.007**	**0.010**	**0.015**	**0.005**	**0.016**	**0.011**	**0.017**
%RSD	**0.092**	**0.086**	**0.081**	**0.076**	**0.110**	**0.129**	**0.157**	**0.188**	**0.125**	**0.286**	**0.175**	**0.211**

6.4.5 Robustness of the Method (3- and 4-halo DP isomers)

In this experiment, the same parameters employed for the 2-isomers were utilised. The average t_R, standard deviation (STD) and %RSD were displayed just to simplify the tables and make the comparison much easier between the 12 isomers in this study; the results are shown in Table 39, Table 40, Table 41 and Appendix A 60. The plots of ln k versus the percentage of organic modifier for 3- and 4-halo diphenidine isomers are shown in (Appendix A 61 and Appendix A 62). The calculated/predicted log P values for 3-halo DP isomers were as follows: 18, 19 (5.26) and 20, 21 (5.53). While, log P for 3-halo DP isomers were (5.53) for 22 and (6.06) for 23, 24 and 25. This indicates similar

hydrophobic effects for these compounds in comparison with 2-halo DP isomers.

6.4.6 Conclusion of robustness testing

The HPLC method used in this study is robust because the minor deliberate changes in the chromatographic conditions have little effect on the method. For instance, increasing flow rate resulted in a decrease in retention time (faster elution of analytes), this is because the component molecules have little time to interact with the stationary phase as they are quickly pushed through the column.

Similarly, increasing the temperature caused a decrease in retention time (the elevated temperature increases the solubility of compounds in the eluting solvent and decreases solvent viscosity, which leads to lower back pressure). Lastly, increasing the percentage of organic modifier causes a reduction in retention time because the interaction of polar analytes with nonpolar (more hydrophobic) column is weaker than that of the nonpolar compounds, as a result, polar (less hydrophobic) components spend less time travelling into the column and therefore elute first.

The robustness of the developed RP-HPLC method was evaluated based on the %RSD values obtained after introducing deliberate changes in the flow rate (±0.1 mL min^{-1}), column temperature (±2°C) and mobile phase composition (±2%). The results of robustness data are represented in Table 37, Appendix A 54 and Table 38 for 2-halogenated diphenidine isomers and in Table 39, Table 40, Table 41 and Appendix A 60 for 3- and 4-isomers. It was observed that the %RSD values (<1) remained unaffected and was well within the acceptance criteria. In addition, the relative retention time of the 12 halogenated diphenidine compounds is constant, which confirms the robustness of the developed method and provides an indication of its reliability during the normal usage.

Table 39. Intra- and inter-day precision measurements for 3- and 4-halogenated diphenidine analytes (using 10 µg mL^{-1} concentration and 10 injections). Key: t_R = Retention time (average of 10 injections)

Intraday precision	**18**	**19**	**20**	**21**		**Intraday precision**	**22**	**23**	**24**	**25**
t_R (min)	4.22	5.81	6.44	7.30		t_R (min)	3.55	5.41	6.10	7.13
STD	0.011	0.009	0.015	0.018		STD	0.002	0.003	0.003	0.003
% RSD	0.262	0.157	0.242	0.256		% RSD	0.080	0.058	0.052	0.051
Inter-day precision	**18**	**19**	**20**	**21**		**Inter-day precision**	**22**	**23**	**24**	**25**
t_R (min)	4.23	5.81	6.43	7.27		t_R (min)	3.54	5.39	6.09	7.12
STD	0.003	0.004	0.005	0.006		STD	0.002	0.004	0.005	0.006
% RSD	0.092	0.076	0.080	0.086		% RSD	0.078	0.074	0.084	0.089

Table 40. Summary of robustness testing (Flow Rate 1.4, 1.5 and 1.6 mL min^{-1}) for the 3- and 4-halogenated diphenidine isomers (using 10 µg mL^{-1} concentration and 10 injections). Key: FR = Flow rate, t_R = Retention Time (min), RRT =Relative retention time

FR (1.4 mL min^{-1})	**18**	**19**	**20**	**21**		**FR (1.4 mL min^{-1})**	**22**	**23**	**24**	**25**
t_R (min)	4.549	6.234	6.904	7.820		t_R (min)	3.804	5.800	6.546	7.653
RRT	0.729	1.000	1.107	1.254		RRT	0.655	1.000	1.128	1.319
Resolution (Rs)	-	8.200	2.743	3.409		Resolution (Rs)	-	10.283	3.102	4.036
FR (1.5 mL min^{-1})	**18**	**19**	**20**	**21**		**FR (1.5 mL min^{-1})**	**22**	**23**	**24**	**25**
t_R (min)	4.245	5.811	6.434	7.287		t_R (min)	3.543	5.399	6.092	7.123
RRT	0.730	1.000	1.107	1.254		RRT	0.656	1.000	1.128	1.319
Resolution (Rs)	-	8.115	2.724	3.39		Resolution (Rs)	-	10.334	3.134	4.105
FR (1.6 mL min^{-1})	**18**	**19**	**20**	**21**		**FR (1.6 mL min^{-1})**	**22**	**23**	**24**	**25**
t_R (min)	3.983	5.452	6.035	6.835		t_R (min)	3.327	5.068	5.717	6.681
RRT	0.730	1.000	1.106	1.253		RRT	0.656	1.000	1.128	1.318
Resolution (Rs)	-	7.921	2.660	3.318		Resolution (Rs)	-	10.556	3.113	4.038

Table 41. Summary of robustness testing (temperature; 48, 50 or 52°C) for the 3-halogenated diphenidine isomers (using 10 µg mL^{-1} concentration and 10 injections). Key: t_R = Retention Time (min), RRT =Relative retention time

Temperature 48 °C	**18**	**19**	**20**	**21**		**Temperature 48 °C**	**22**	**23**	**24**	**25**
t_R (min)	4.196	5.782	6.412	7.258		t_R (min)	3.556	5.436	6.140	7.184
RRT	0.725	1	1.109	1.255		RRT	0.654	1	1.129	1.321
Resolution	-	8.588	2.832	3.373		Resolution	-	10.336	3.132	4.082
Temperature 50 °C	**18**	**19**	**20**	**21**		**Temperature 50 °C**	**22**	**23**	**24**	**25**
t_R (min)	4.189	5.747	6.367	7.209		t_R (min)	3.543	5.399	6.092	7.123
RRT	0.728	1	1.107	1.254		RRT	0.656	1	1.128	1.319
Resolution	-	7.937	2.66	3.296		Resolution	-	10.334	3.134	4.105
Temperature 52 °C	**18**	**19**	**20**	**21**		**Temperature 52 °C**	**22**	**23**	**24**	**25**
t_R (min)	4.147	5.670	6.273	7.096		t_R (min)	3.538	5.375	6.060	7.079
RRT	0.731	1	1.106	1.251		RRT	0.658	1	1.127	1.317
Resolution	-	7.920	2.647	3.265		Resolution	-	10.482	3.103	4.016

6.5 Method specificity

The HPLC method specificity was carried out by testing the adulterant (caffeine, benzocaine and paracetamol) in the presence of diphenidine. The table below displays the adulterant testing of the halophenidines (using the concentration of 10 µg mL^{-1} and mobile phase acetonitrile:ammonium acetate, 67:33% v/v).

Table 42. Adulterant testing of the halophenidines (Using the concentration of 10 µg mL^{-1} and mobile phase (acetonitrile:ammonium acetate, 67:33% v/v).

	t_R (min)			
Injection	**Diphenidine**	**Caffeine**	**Benzocaine**	**Paracetamol**
1	3.03	1.46	1.46	1.45
2	3.05	1.44	1.45	1.46
3	3.05	1.44	1.46	1.45
Average	**3.04**	**1.45**	**1.46**	**1.45**
RRT	**2.09**	**1.00**	**1.01**	**1.01**

Moreover, uracil peaks were seen at around 1.032 and the injection peak at the range of approximately 0.92-1.30 in the case of all three mixtures of the halophenidines. The mobile phase 67:33% v/v and flow rate 1.5 mL min^{-1} were utilised for this study as well as the conditions/parameters applied in the validation experiments of these substances.

In Table 42 above, the retention times of the three adulterants are overlapping with the injection peak and uracil peak in this region as well. Although all the halophenidines were separated using this HPLC method, this finding is considered as a limitation of this study. One possible solution to this issue

might be by changing/reducing both the percentage of mobile phase and flow rate or by modifying the rest of experimental parameters.

6.6 Forensic application

In order to test the developed method, it was applied to analyse two bulk forensic samples that were obtained as a white crystalline powder in a clear zip-lock bag from Greater Manchester Police via the MANDRAKE programme. The two samples, which were purported to contain 2-chlorodiphenidine (**15**), were weighed and diluted to a working concentration of 10 μg mL^{-1} (in replicate).

Table 43. Bulk samples measured on HPLC (Halo DP isomers). Key: SS = Street sample, 15 = 2-CLDP

Street Sample	Qualitative Analysis, t_R (min)	Peak Area	Actual conc. ($\mu g\ mL^{-1}$)	Found conc. ($\mu g\ mL^{-1}$)	Quantitative Analysis (% w/w)
SS1H	**15**, t_R = 5.96 min	209.032	9.8	8.911	90.93
		230.694	10.2	9.835	96.42
SS2H	**15**, t_R = 5.96 min	76.009	11	3.236	29.42
		58.983	10.4	2.51	24.13

Firstly, by comparing actual and found concentration, these results suggest that the purity of these two street samples is very different; SS1H with an average of around 93% w/w, whilst for SS2H about the purity was found to be 27% w/w. Secondly, by comparing the retention times of the resulted peaks to the results of the retention times of all 12 halophenidine isomers in this study, and from the retention times obtained (5.96 mins) for the individual isomers, it can be confirmed that both bulk forensic samples (SS1H and SS2H) contain **15**, (see Figure 71).

Figure 71 shows the chromatogram obtained by testing the street ample (SS1H), which was confirmed from its retention time (5.96 mins) to be **15**, in addition to the presence of other peaks; one with retention time of 0.92 min which could be uracil, and another peak at 1.47 min which could be one of the following known additives/adulterants (caffeine, benzocaine or paracetamol), this is an indicator to that this bulk sample contains some impurities. The purity of **15** in this bulk sample (SS1H) was calculated as shown in the table above to be between 90 and 97% w/w.

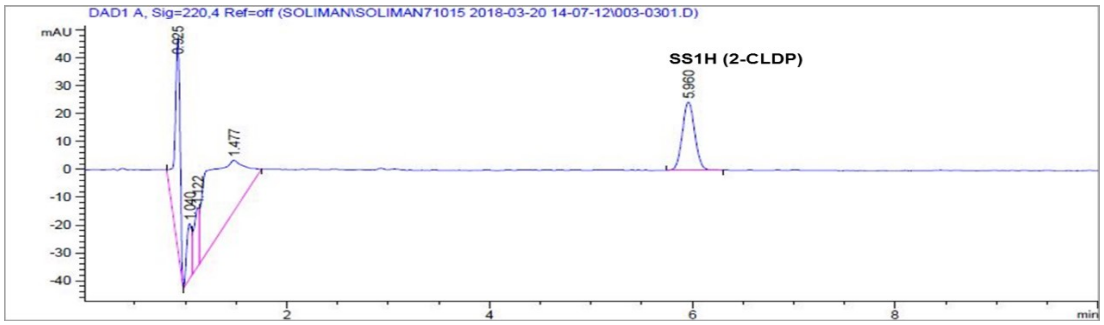


Figure 71. Chromatogram of street sample (SS1H) obtained using HPLC (Halo DPH isomers)

The second bulk sample contains **15**, and its peak eluted at (5.96 mins) which is the same as for the first street sample (SS1H). However, SS2H was less pure than SS1H, as the content of **15** in the sample was found to be between 24 and 30% v/v. It is also found that there are some impurity peaks (at 1.5 mins), which could be because of the presence of adulterants such as caffeine, benzocaine and/or paracetamol (See method specificity results in Table 43).

6.7 Conclusion

The current experimental investigation focuses on the HPLC detection and separation of halogenated diphenidine compounds, by applying the developed HPLC method to analyse these isomers either as individual compounds or in bulk samples. By comparing the retention times obtained in the validation data for all three groups of halophenidines, it can be concluded that the 4-halogenated diphenidine isomers had the lowest retention values as **22** was retained for just 3.5 minutes, while the longest was for the 2-halo DP compounds (**17** eluted in 8.5 mins). The total run time was 10 minutes to elute all the compounds (**14**-**25**).

After applying the method, the most remarkable result to emerge from the data is not only the rapid total run time (less than 10 minutes) but also the excellent LOD (0.05 -0.09 μg mL^{-1}) and LOQ (0.16-0.26 μg mL^{-1}) as an average for all twelve halophenidines. The evidence from this study suggests that the 12 isomers were fully baseline-separated with a resolution values (Rs) of greater than 2 in all three groups of halogenated DP in question, this confirms that the results are in accordance with the ICH standards and it should be suitable for the rapid detection, quantification and control of halophenidines.

The developed HPLC method in this study was applied to analyse two street samples (SS1H) and (SS2H), it was confirmed that they contain the illicit substance **15** in a content of around 95 and 30% w/w, respectively. Some adulterants were also detected in these two street samples by using the same technique, which suggests that this method can be applied to real world samples.

Another possible area of future research would be to investigate the halophenidine isomers by carrying out a full analytical characterisation such as FTIR, GCMS and NMR. Generally, this might involve plans to enhance the detection and separation of these new psychoactive substances. In summary, these results hold significance for potential focus on applying new methods for analysis of these new emerging class of psychoactive substances.

Conclusion

This research project aimed to develop novel validated methods for the detection and quantification of diphenidine derived new psychoactive substances. These compounds were fully characterised using different techniques such as infrared, NMR spectroscopy and gas chromatography, then an HPLC method was developed to analyse four groups of new psychoactive substances (NPS); methoxphenidine (MXP), fluephenidine (FEP), fluorocyanoephenidine (FCEP) and halogenated diphenidine regioisomers (Halo DP), respectively, in four separate studies.

In these analyses, reverse phase HPLC method (RP-HPLC) was developed/optimised by modifying many experimental conditions/variables such as: mobile phase, acetonitrile:ammonium acetate and the other experimental parameters such as flow rate (mL min^{-1}), temperature, injection volume (µL) and detection wavelength (nm) were optimized according to each group of these compounds, the column ACE 5 C18-AR (150 x 4.6 mm, 5 µm particle size) was used in this research.

The developed HPLC method is able to detect, separate diphenidine derivatives (MXP, FEP and halogenated diphenidine isomers, this is because the obtained resolution between the separated peaks was greater than 2 in the case of MXP, FEP and Halo DP isomers and also the rapid overall analysis time obtained in the case of MXP and twelve Halo DP regioisomers was just 10 minutes. In addition, this method was successful in both qualitative and quantitative analyses of street samples that contain diphenidine derivatives.

In the MXP study, the limit of detection, LOD was (0.04-0.15 µg mL^{-1}) and limit of quantification, LOQ was(0.38-0.47 µg mL^{-1}); in other words, the developed

HPLC procedure acts as an ideal method for both the qualitative and quantitative analysis of MXP isomers, when compared to the previous approaches in the literature. In the halogenated diphenidine isomers study, the LOD was (0.05 -0.09 µg mL^{-1}) and LOQ was (0.16-0.26 µg mL^{-1}) as an average for all twelve halophenidines; again which is similar to the findings of MXP experiments. For the FEP study, the forensic application of the developed HPLC method on three bulk samples confirmed that **8** was present in all three samples (SS1, 2 and 3), and **9** was only detected in samples 2 and 3.

For fluorocyanoephenidine, FCEP, both an isocratic and gradient HPLC method was investigated to separate these isomers, with limited success. Though gas chromatographic techniques were investigated using a variety of stationary phases (polar, semi-polar and non-polar) the separation of these compounds proved challenging. Despite being unable to fully resolve the isomers, there was an improvement noted in resolution by employing the semi-polar GC column, after switching from nitrogen to helium as a carrier gas using the isothermal method at 180°C (from just under 0.70 to 1.40 between **12** and **13**). However, the obtained resolution was still below 2, which indicates that both HPLC and GC were not suitable to analyse FCEP isomers.

Three validated chromatographic methods have been developed for a range of diphenidine derivatives, increased our understanding, and add to the existing research in the field of chromatography in general and HPLC methods in particular. The novelty in this research is that the new developed HPLC was superior to the previously reported methods in the literature, in addition to that is the fact that FEP, FCEP and halogenated DP isomers are all new substances investigated for the first time in this research project. The results of this project also confirm the importance of applying the developed method

to real life setup such as in toxicology and forensic framework to analyse any samples that are suspected to contain diphenidine-derived substances.

If the debate is to be moved forward, it is important to continue research in this area of emerging NPSs, so a better understanding of HPLC analysis needs to be developed by changing various experimental parameters in this method. Moreover, these findings provide a good starting point for discussion and further research, which should aim to replicate results of these experiments and shed more light on detection and separation of a broader range of diphenidine derivatives and NPS classes.

Further studies could also focus on the study of the metabolites of diphenidine and its derivatives using the current method, for which HPLC analysis could be used to distinguish between different metabolites/regioisomers of these substances in terms of their retention times and resolution values. These values can be reported/recorded by creating a new library for this class of NPS, which is readily expanded to other classes. Thus, the collected data can be grouped together and used as a database and source of information for any future studies in the field of HPLC and forensic science. Another recommendation could be by attaching HPLC to other techniques such as electrochemistry or various forms of mass spectrometry.

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Appendix A

Appendix A 1. ^{1}H NMR, ^{13}C NMR, IR, melting point and UV* data of **3**

Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	GC-EI-MS (m/z)	Mpt (uncorrected), ($^{\circ}$C)
1	1.27, d, 2H	22.32	1592.98	**258**	151-152
2	1.66-1.98, m, 2H	22.77	2400.02	175	
3	2.05-2.30, m, 2H	36.51	2600.01	91	
4	2.32-2.67, m, 2H	49.14	2900.14	69	
5	3.34-3.51, dd, 2H	55.54			
6	3.62, d, 2H	72.50			
7	3.96, dd, 2H	115.98			
8	4.06,m, 1H	122.50			
9	6.86-6.96, m, 2H	126.73			
10	7.01-7.19, m, 2H	129.97			
11	7.27, t, 2H	132.95			
12	7.35-7.43, m, 1H	136.30			
13	12.45-12.71, m, 1H	160.07			

*^{1}H-NMR (400 MHz, DMSO-d_6); ^{13}C-NMR (100 MHz, DMSO-d_6); GC-EI-MS: m/z **258** (base peak); UV (0.3 mg/mL in acetonitrile:ammonium acetate 55:45% v/v), λ_{max} = 277 nm, abs. = 1.117, ε_{277}= 1100 L mol^{-1} cm.$^{-1}$

Appendix A 2. ^{1}H NMR, ^{13}C NMR, IR, melting point and UV* data of **4**

Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	GC-EI-MS (m/z)	Mpt (uncorrected), (˚C)
1	1.08-1.29, m, 2H	11.96	1600.01	**204**	141-142
2	1.66, m, 2H	39.86	2475.02	188	
3	1.82, d, 2H	40.91	2600.01	121	
4	2.15-2.31, m, 2H	41.31	2900.03	91	
5	3.78, s, 3H	62.59		65	
6	4.00, d, 2H	116.39			
7	4.10-4.27, m, 1H	126.13			
8	7.25-7.32, m, 1H	129.26			
9	6.82-6.95, m, 2H	136.80			
10	6.98-7.20, m, 2H	138.47			
11	7.35-7.48, m, 1H	161.76			
12	12.31-12.64, m, 1H	164.19			

*^{1}H-NMR (400 MHz, DMSO-d_6); ^{13}C-NMR (100 MHz, DMSO-d_6); GC-EI-MS: m/z **204** (base peak); UV (0.3 mg/mL in acetonitrile:ammonium acetate 55:45% v/v) , λ_{max} = 273 nm, abs. = 0.673, ε_{273}= 660 L mol^{-1} cm.$^{-1}$

Appendix A 3. ^{1}H NMR, ^{13}C NMR, IR, melting point and UV* data of **8**

Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	Mpt (uncorrected), (˚C)
1	1.23, t,4H	11.95	1617.96	220-222
2	2.66-2.79, m, 1H	39.24	2468.65	
3	2.80-2.97, m, 1H	39.87	2714.02	
4	3.19, t, 1H	40.08	2974.14	
5	3.38, br.s, 1H	40.91		
6	3.66, dd, 1H	41.12		
7	4.51-4.56, m, 1H	116.30		
8	4.72,d, 1H	126.04		
9	6.96-7.03, m, 3H	127.73		
10	7.05-7.20, m, 5H	129.25		
11	7.26-7.33, m,1H	136.60		
12	7.34-7.42, m, 1H	160.09		
13	7.98, t, 1H	162.54		

*UV (0.5 mg mL^{-1} in acetonitrile:ammonium acetate 55:45% v/v), λ_{max} = 270 nm, abs.= 0.894, ε_{270} = 501 L mol^{-1} $cm.^{-1}$

Appendix A 4. ^{1}H NMR, ^{13}C NMR, IR, melting point and UV* data of **9**

Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	Mpt (uncorrected), ($^{\circ}C$)
1	2.64, br.s, 1H	11.96	1618.82	232-234
2	2.83, br.s, 1H	39.86	2475.42	
3	3.17, t, 1H	40.91	2708.57	
4	3.64, dd, 1H	41.31	2969.94	
5	4.54, br.s, 1H	62.59		
6	7.02, d, 2H	116.39		
7	7.09-7.22, m, 4H	126.13		
8	7.25-7.32, m, 1H	129.26		
9	7.33-7.42, m, 1H	136.80		
10	7.44, d, 1H	138.47		
11	9.73, br.s, 1H	161.76		
12	10.14, br.s, 1H	164.19		

*UV (0.5 mg mL^{-1} in acetonitrile:ammonium acetate 55:45% v/v), λ_{max} = 270 nm, absorbance = 1.297, ε_{270} = 727 L mol^{-1} $cm.^{-1}$

Appendix A 5. ^{1}H NMR, ^{13}C NMR, IR, melting point and UV* data of **10**

Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	Mpt (uncorrected), (°C)
1	1.22, t, 1H	11.95	1610.76	232-234
2	2.54-2.69, m, 5H	39.87	2479.66	
3	2.82, dtd, 1H	40.91	2710.79	
4	3.16, dd, 1H	62.59	2971.06	
5	3.64, dd, 1H	116.39		
6	3.43-4.60, m, 1H	127.62		
7	6.95-7.06, m, 4H	129.26		
8	7.09-7.25, m, 1H	130.11		
9	7.50-7.61, m, 1H	138.47		
10	9.66, d, 1H	161.76		

*UV (0.5 mg mL^{-1} in acetonitrile:ammonium acetate 55:45% v/v), λ_{max} = 270 nm, abs.= 0.984, ε_{270} = 552 L mol^{-1} $cm.^{-1}$

Appendix A 6. ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, IR, melting point and UV* data of **11**

Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	^{19}F NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	Mpt (uncorrected), ($^{\circ}C$)
1	3.58, br.s, 6H	15.12	-118.67	1576	217-220
2	4.74, br.s, 5H	39.40		1618	
3	6.97, s, 2H	41.13		2251	
4	7.03-7.06, m, 1H	56.16		2739	
5	7.06-7.11, m, 2H	115.94		2968	
6	7.13-7.14, m, 1H	116.16			
7	7.31-7.38, m, 1H	118.16			
8	7.86, t, 1H	125.55			
9	10.06, br.s, 1H	128.79			
10	10.55, br.s, 1H	130.07			
11	-	135.92			
12	-	159.58			
13	-	162.03			

*UV (0.20 mg mL^{-1} in acetonitrile:ammonium acetate 55:45% v/v), λ_{max} = 263 nm, abs.= 0.54, ε_{263} = 954 L mol^{-1} $cm.^{-1}$

Appendix A 7. ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, IR, melting point and UV* data of **12**

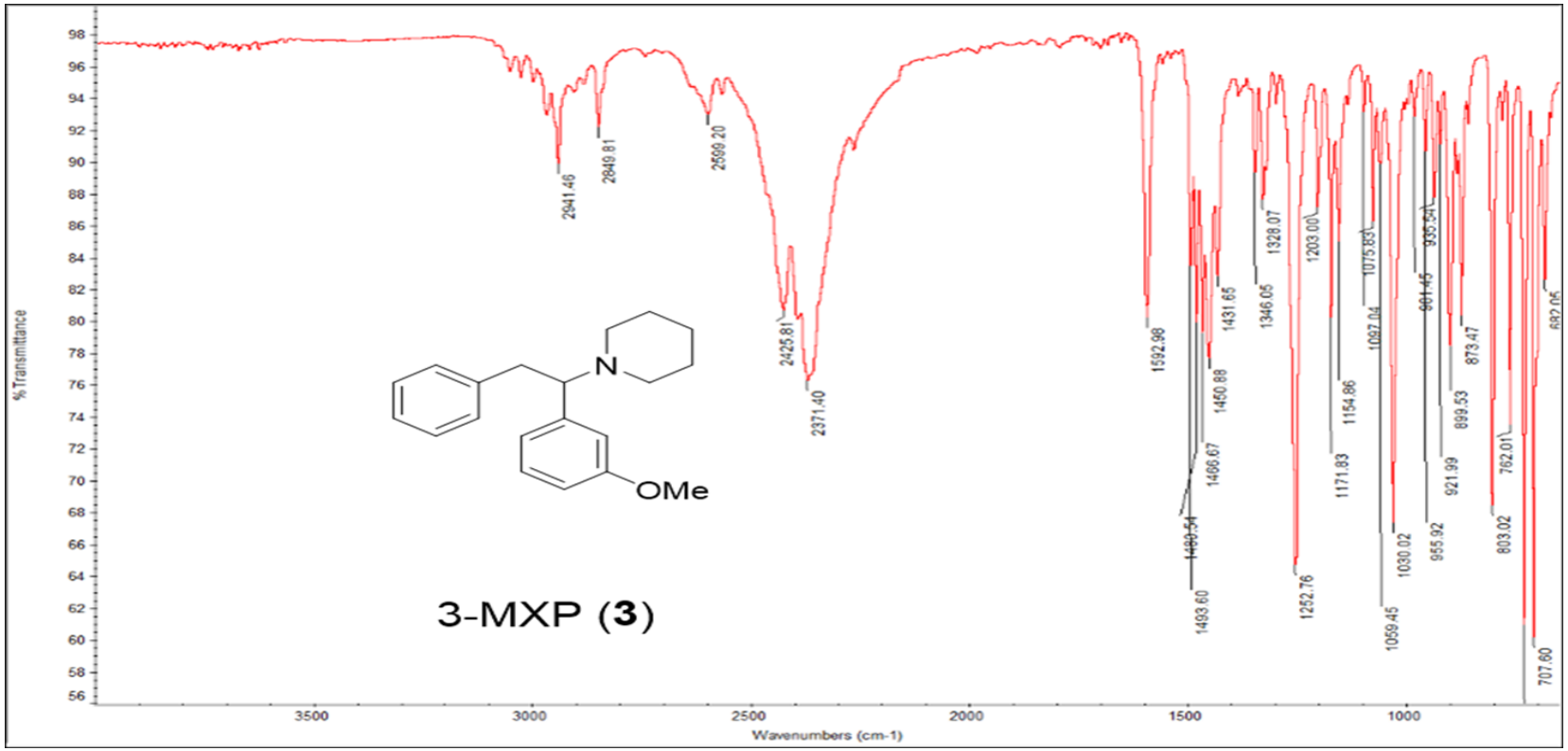
Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	^{19}F NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	Mpt (uncorrected), ($^{\circ}C$)
1	3.07-3.26, m, 6H	15.07	-113.93	1575	217-220
2	3.16, s, 6H	39.01		1618	
3	3.44-3.70, m, 1H	40.64		2251	
4	4.36-4.69, m, 11H	62.70		2738	
5	6.97, d, 1H	116.16		2968	
6	7.04-7.30, m, 1H	118.23			
7	7.27-7.47, m, 1H	125.71			
8	7.34-7.54, m, 1H	128.82			
9	7.86, t, 1H	129.66			
10	10.04, br.s, 1H	131.21			
11	10.54, br.s, 1H	136.25			
12	-	161.31			
13	-	163.74			

*UV (0.21 mg mL^{-1} in acetonitrile:ammonium acetate 55:45% v/v), λ_{max} = 263 nm, abs.= 0.70, ε_{263} = 1178 L mol^{-1} $cm.^{-1}$

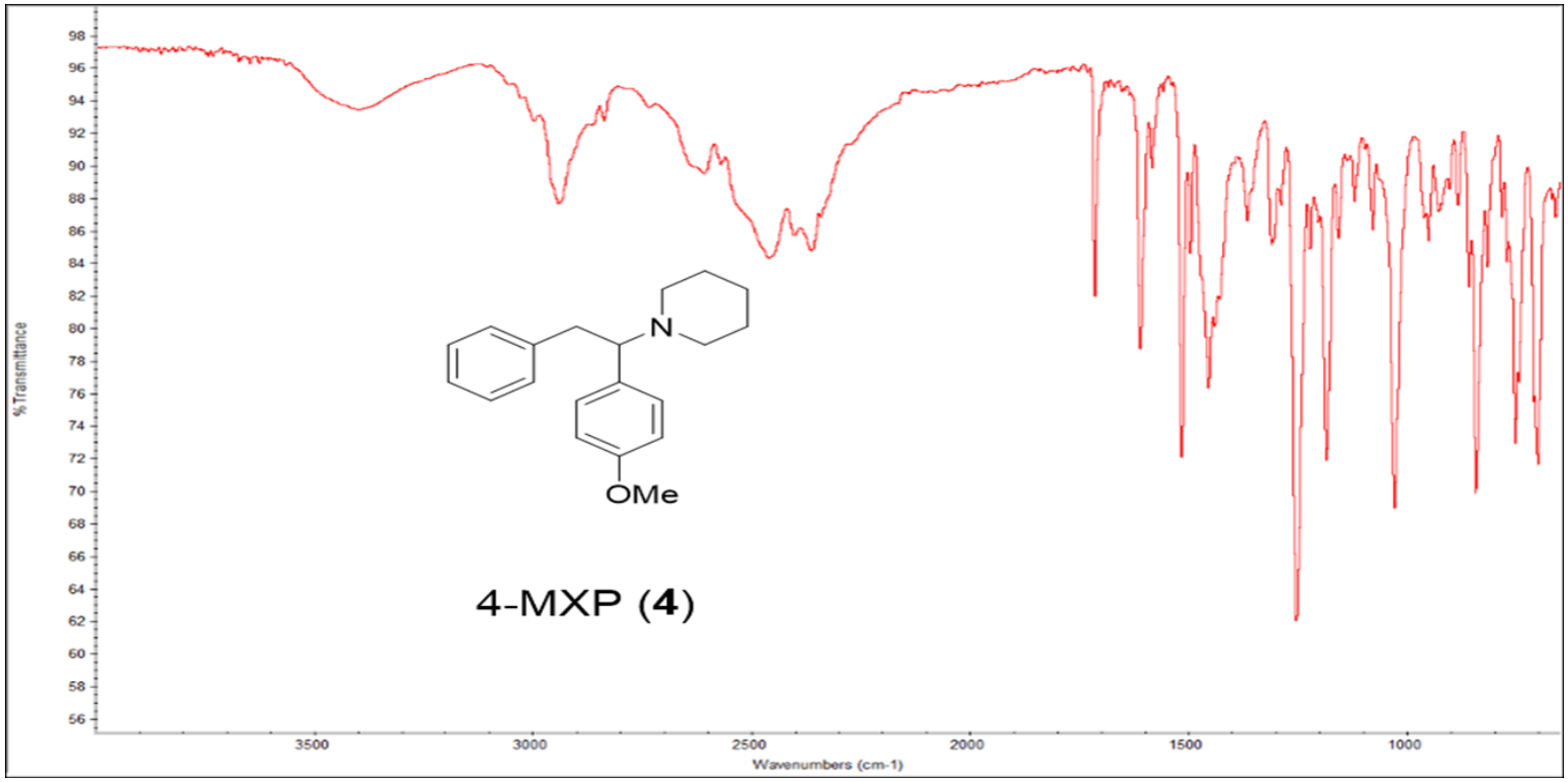
Appendix A 8. ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, IR, melting point and UV* data of **13**

Peak	^{1}H NMR chemical shift (ppm)	^{13}C NMR chemical shift (ppm)	^{19}F NMR chemical shift (ppm)	FTIR frequency (cm^{-1})	Mpt (uncorrected), ($^{\circ}C$)
1	2.93, m, 1H	14.98	-114.56	1568	194-197
2	3.59, m, 1H	35.34		1611	
3	4.55-4.57, d, 1H	40.44		2251	
4	6.97, d, 1H	62.50		2739	
5	7.01-7.16, m, 1H	116.24		2964	
6	7.18-7.20, m, 1H	118.21			
7	7.53-7.55, m, 1H	127.16			
8	7.86, t, 1H	128.81			
9	10.05, br.s, 1H	129.67			
10	10.55, br.s, 1H	131.61			
11	-	136.36			
12	-	161.53			
13	-	163.97			

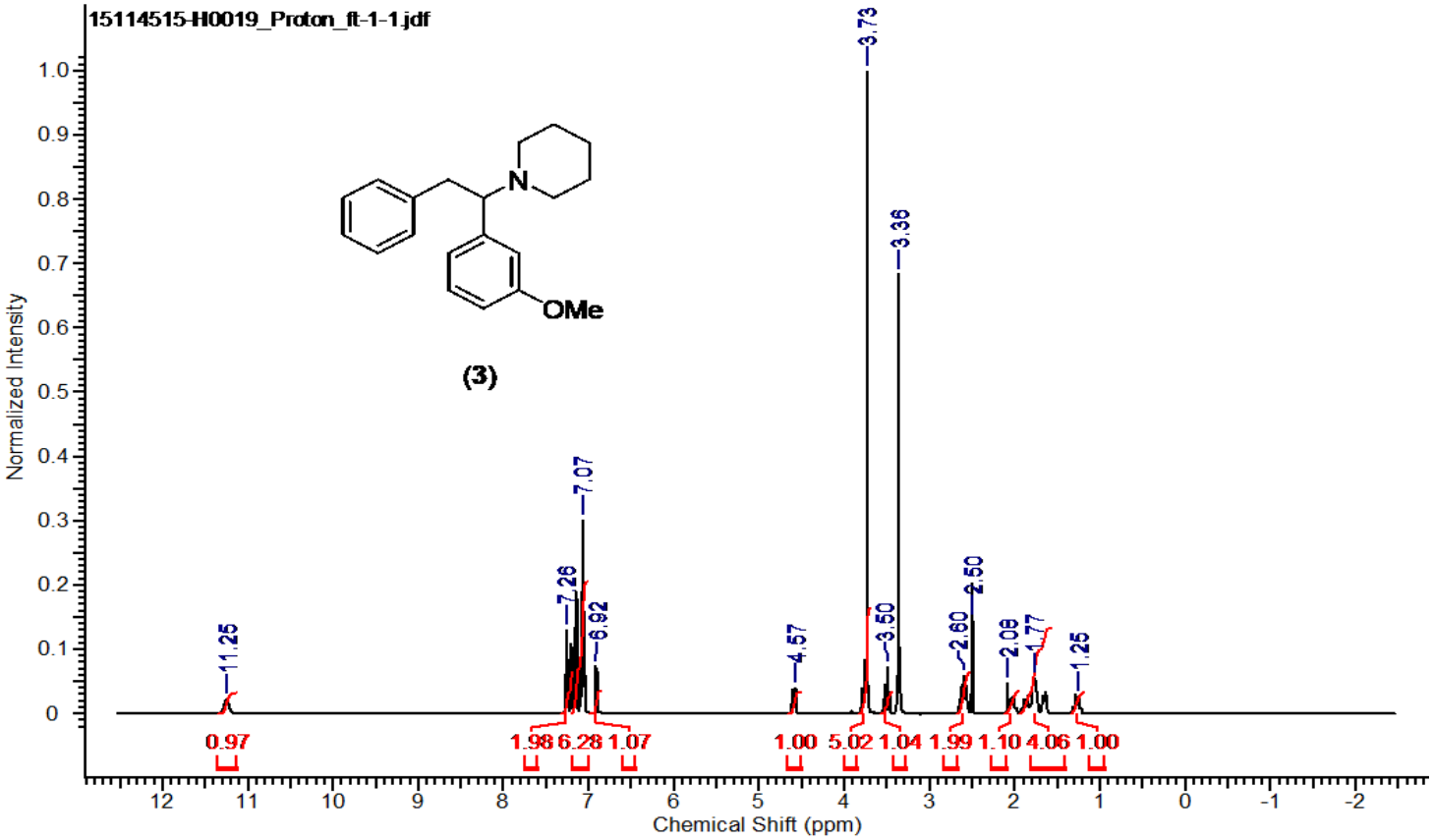
*UV (0.40 mg mL^{-1} in acetonitrile:ammonium acetate 55:45% v/v), λ_{max} = 258 nm, abs.= 0.58, ε_{258} = 512 L mol^{-1} $cm.^{-1}$



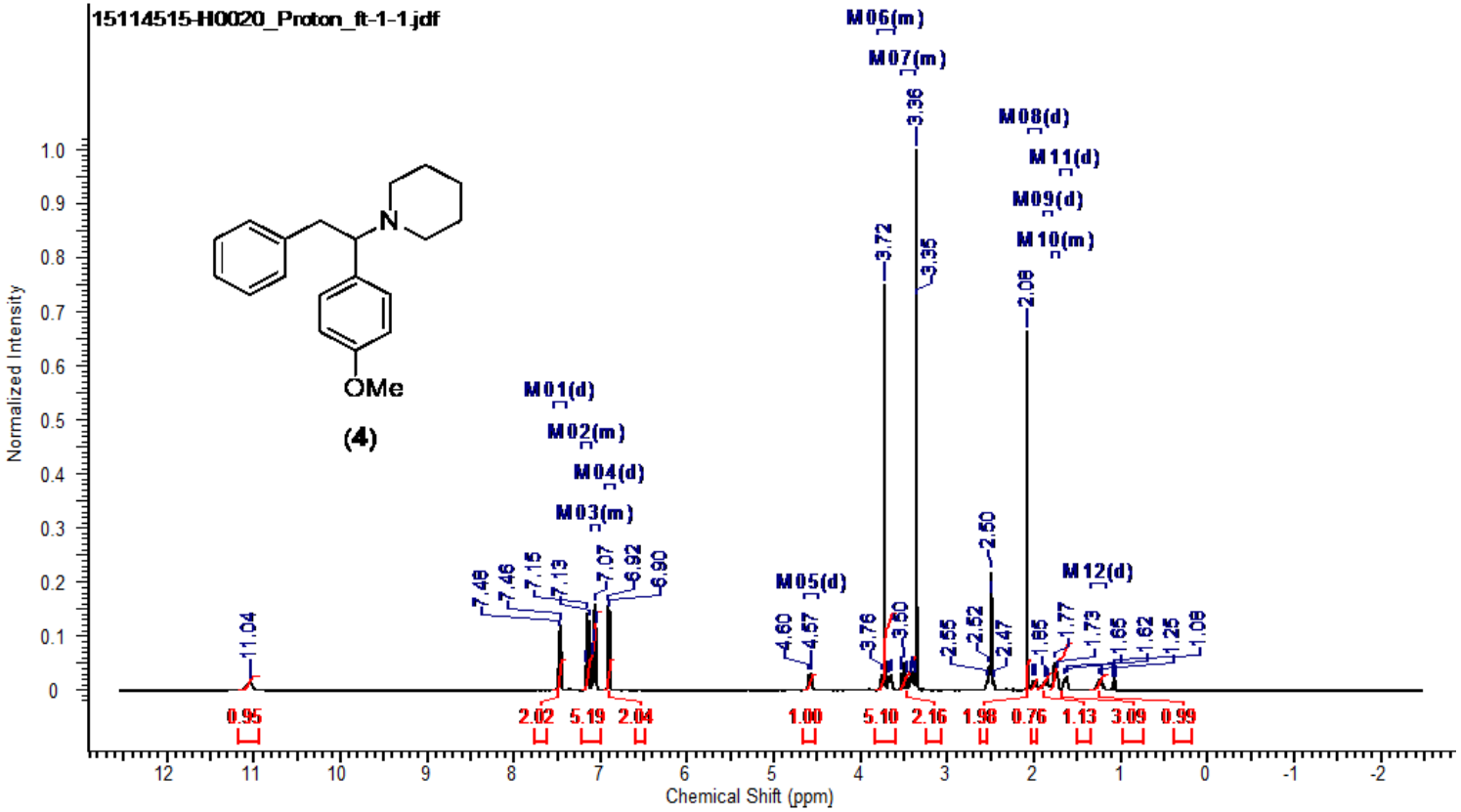
Appendix A 9. ATR-FTIR spectrum of **3**



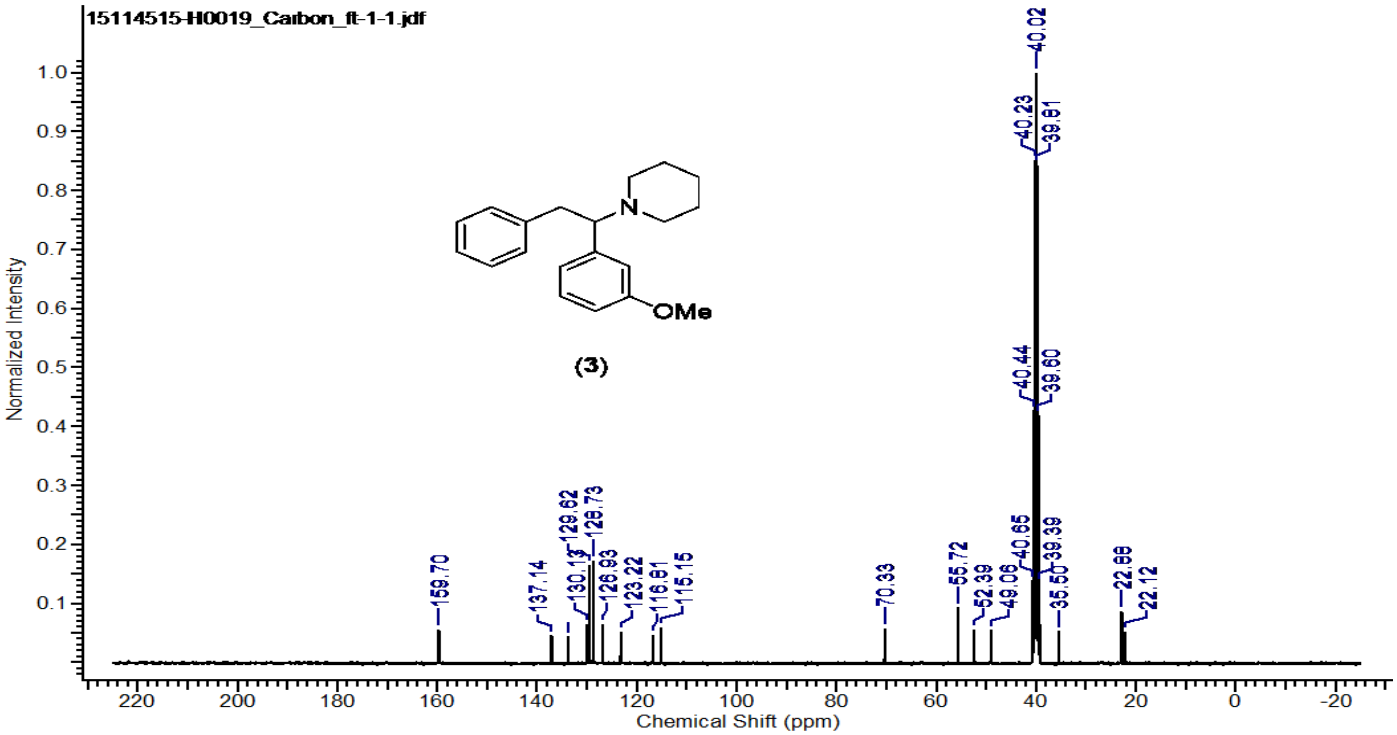
Appendix A 10. ATR-FTIR spectrum of **4**



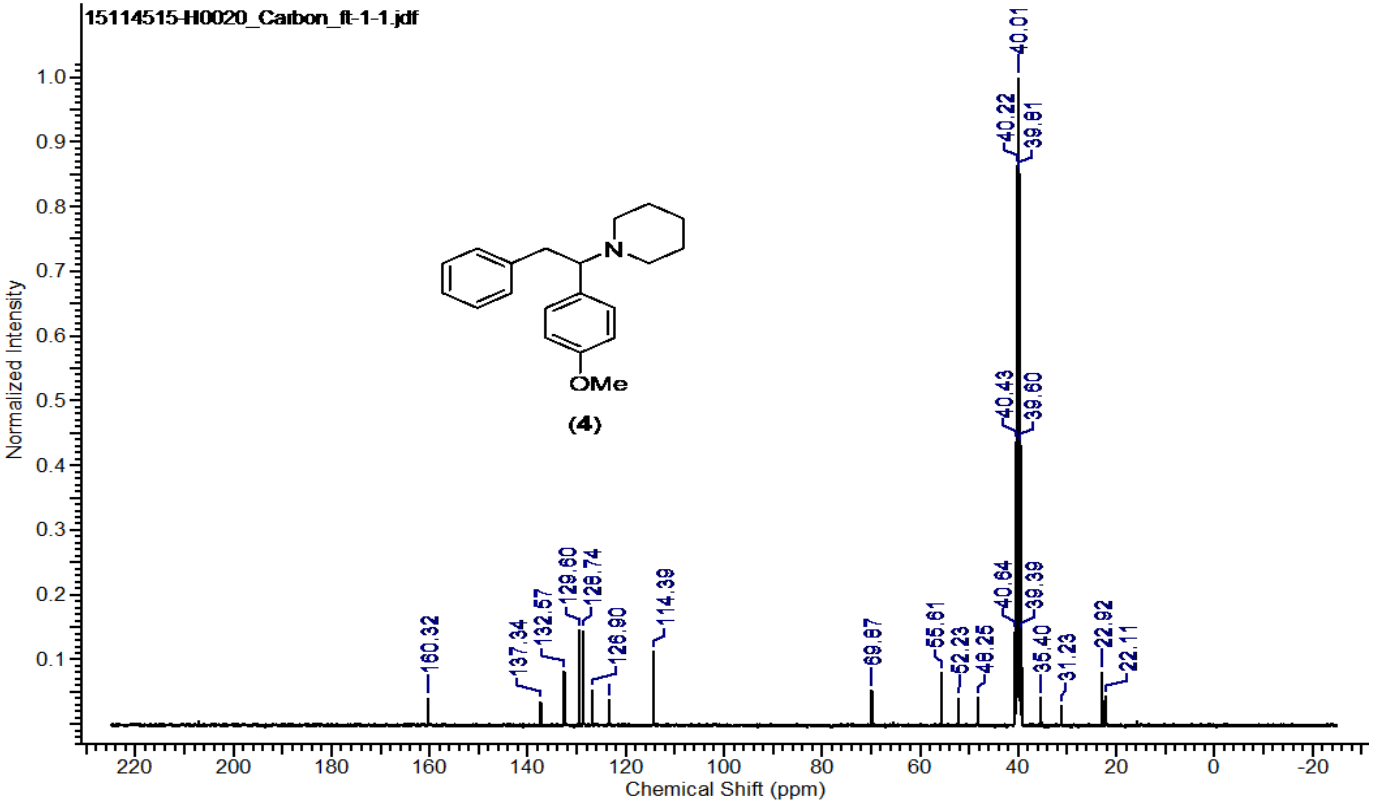
Appendix A 11. ^{1}H NMR spectrum of **3**



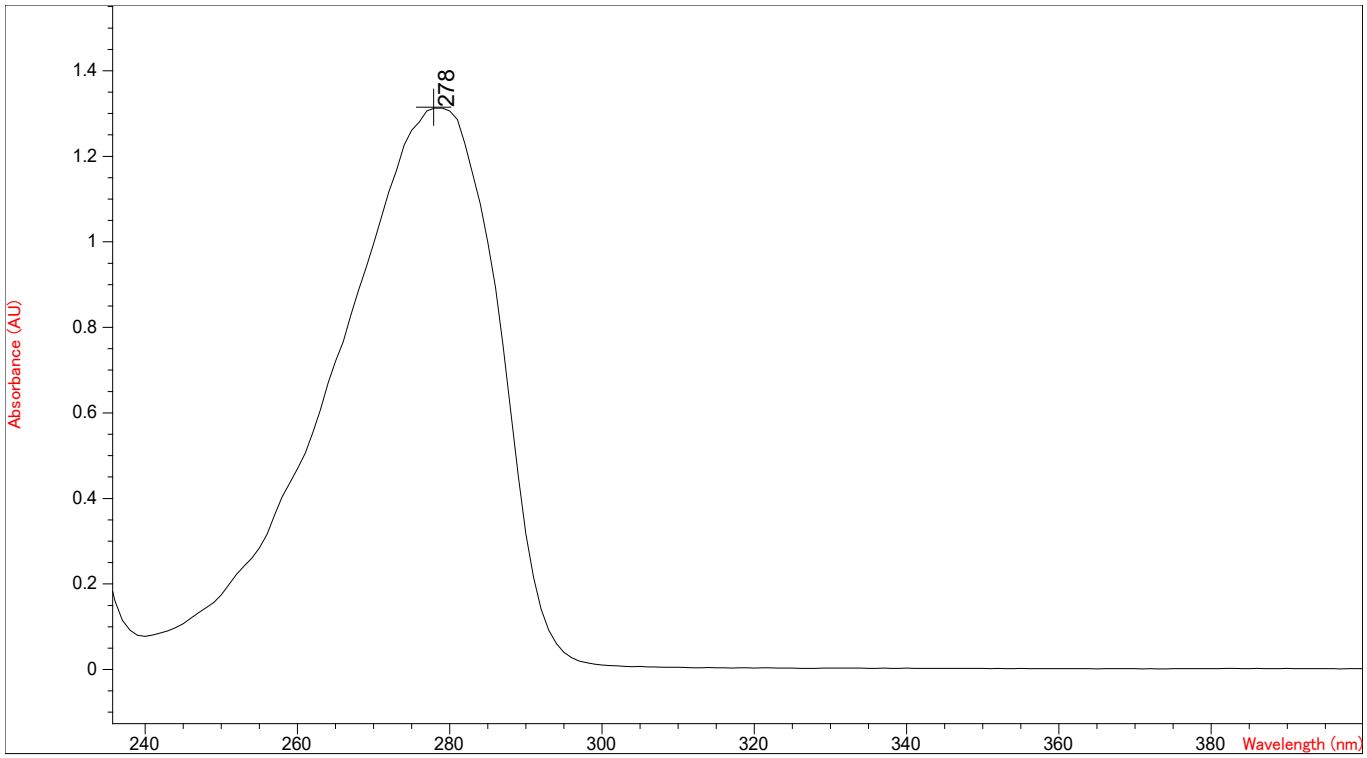
Appendix A 12. ^{1}H NMR spectrum of **4**



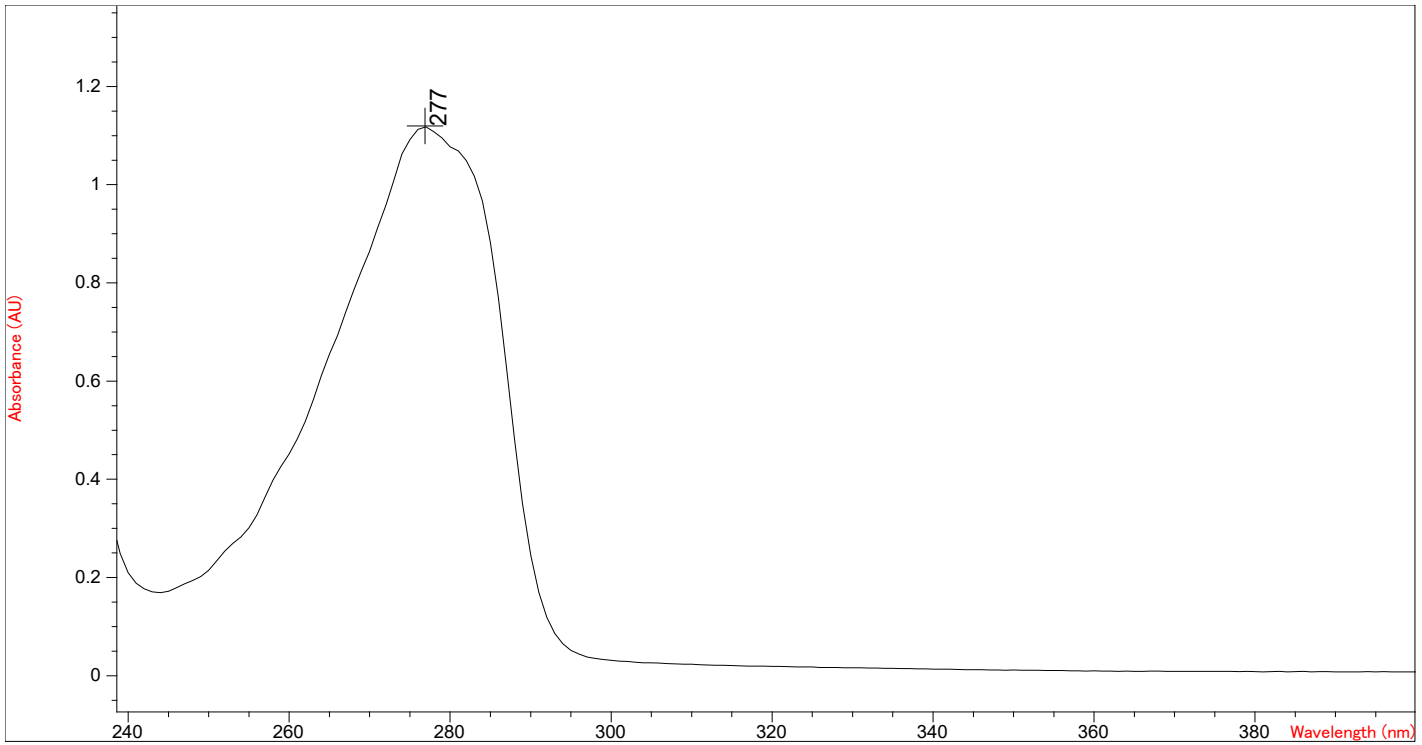
Appendix A 13. $^{13}C\{^{1}H\}$ NMR spectrum of **3**



Appendix A 14. $^{13}C\{^{1}H\}$ NMR spectrum of **4**



Appendix A 15. UV-vis spectrum of **2**



Appendix A 16. UV-vis spectrum of **3**

Appendix A 17. Robustness testing (Mobile phase percentage) for the three MXP analytes using a mobile phase consisting of acetonitrile:ammonium acetate 54:46% - 56:44% v/v, concentration of MXP = 10 µg mL^{-1}

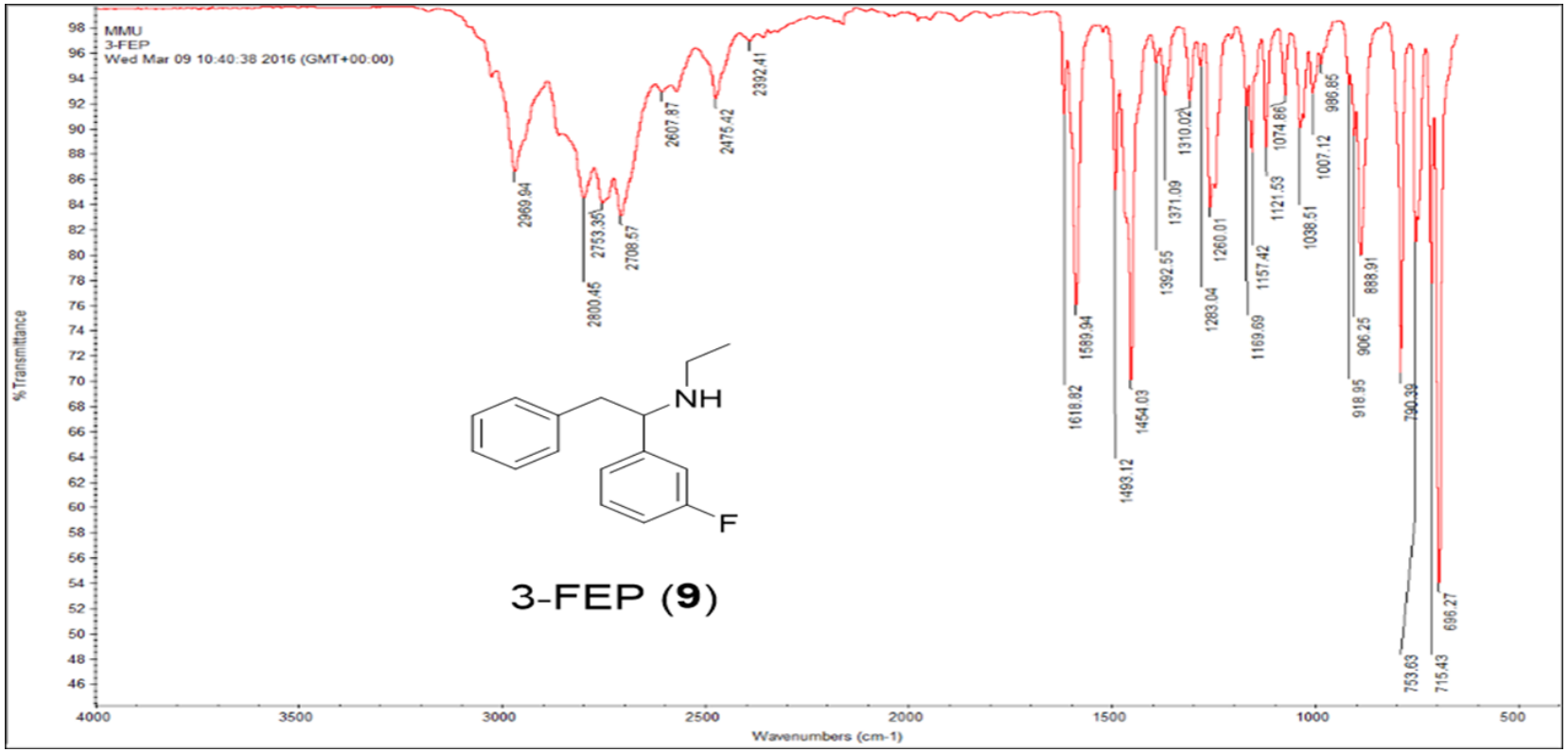
Injection	t_R (min) using acetonitrile: ammonium acetate (54:46% v/v)			t_R (min) using acetonitrile: ammonium acetate (55:45% v/v)			t_R (min) using acetonitrile: ammonium acetate (56:44% v/v)		
	2	3	4	2	3	4	2	3	4
1	4.095	6.279	4.721	3.959	6.042	4.568	3.806	5.768	4.387
2	4.099	6.281	4.724	3.961	6.041	4.567	3.812	5.791	4.391
3	4.099	6.288	4.728	3.961	6.037	4.566	3.811	5.769	4.389
4	4.105	6.284	4.728	3.963	6.039	4.567	3.811	5.773	4.390
5	4.105	6.286	4.729	3.967	6.035	4.567	3.814	5.771	4.391
6	4.106	6.288	4.731	3.966	6.045	4.572	3.818	5.777	4.396
7	4.105	6.284	4.728	3.965	6.047	4.572	3.816	5.776	4.394
8	4.106	6.285	4.729	3.962	6.047	4.569	3.818	5.779	4.397
9	4.109	6.285	4.730	3.964	6.056	4.576	3.819	5.779	4.397
10	4.109	6.290	4.733	3.967	6.052	4.576	3.822	5.774	4.396
Average	**4.103**	**6.285**	**4.728**	**3.963**	**6.044**	**4.570**	**3.814**	**5.775**	**4.392**
STD	**0.004**	**0.003**	**0.003**	**0.002**	**0.006**	**0.003**	**0.004**	**0.006**	**0.003**
%RSD	**0.112**	**0.052**	**0.072**	**0.069**	**0.109**	**0.082**	**0.125**	**0.114**	**0.082**

Appendix A 18. Intraday and inter-day precision measurements for the methoxphenidine analytes using mobile phase acetonitrile:ammonium acetate 55:45% v/v and flow rate 1.0 mL min^{-1}

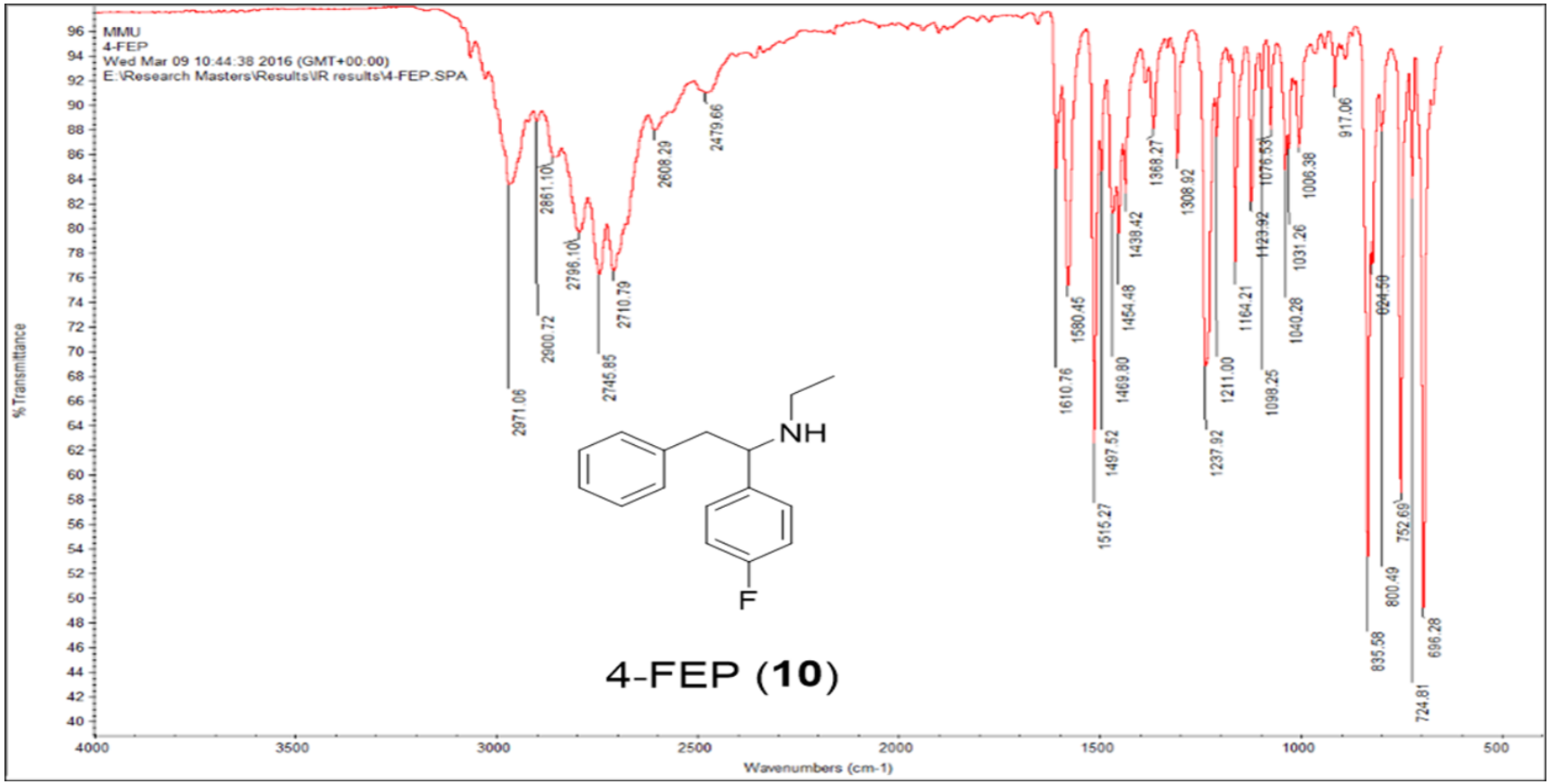
Injection	Intraday Precision t_R (min)			Interday Precision t_R (min)		
	2	**3**	**4**	**2**	**3**	**4**
1	3.72	5.83	4.35	3.70	5.82	4.34
2	3.72	5.84	4.36	3.70	5.82	4.34
3	3.72	5.83	4.35	3.70	5.83	4.34
4	3.72	5.83	4.35	3.71	5.83	4.35
5	3.72	5.83	4.35	3.71	5.83	4.35
6	3.72	5.83	4.35	3.71	5.83	4.35
7	3.72	5.83	4.35	3.71	5.83	4.35
8	3.72	5.83	4.35	3.72	5.83	4.35
9	3.71	5.84	4.35	3.72	5.84	4.35
10	3.71	5.83	4.34	3.72	5.83	4.35
Average	**3.71**	**5.83**	**4.35**	**3.71**	**5.82**	**4.34**
STD	**0.004**	**0.004**	**0.004**	**0.008**	**0.005**	**0.004**
%RSD	**0.11**	**0.072**	**0.108**	**0.220**	**0.097**	**0.111**

Appendix A 19. Determination of % Recovery for the MXP regioisomers

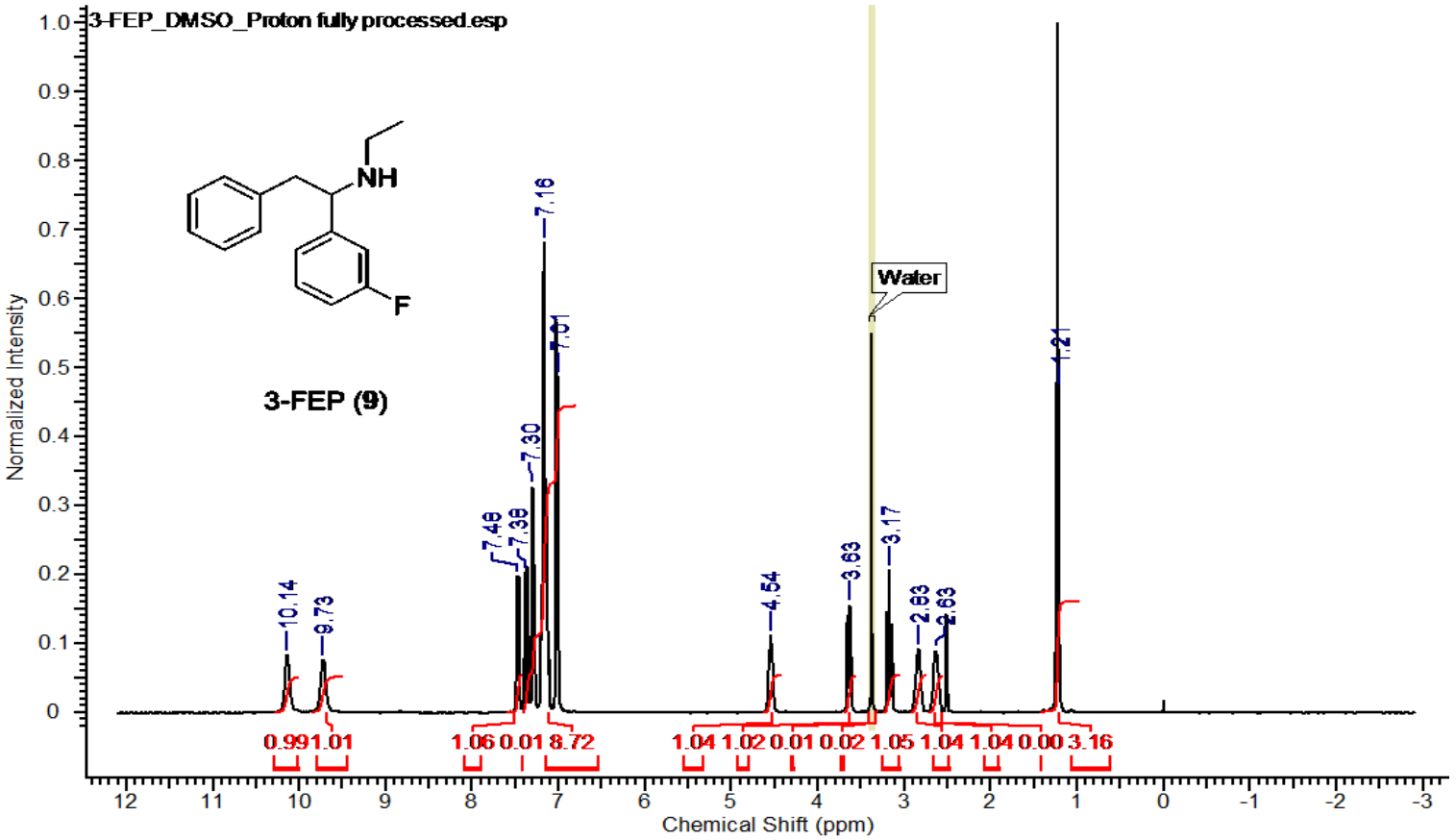
	Theoretical conc. = 8 µg mL^{-1}			Theoretical conc. = 10 µg mL^{-1}			Theoretical conc. = 12 µg mL^{-1}		
Injection	**2**	**3**	**4**	**2**	**3**	**4**	**2**	**3**	**4**
1	100.6	98.4	97.6	100.2	99.6	96.9	98.3	100.8	98.9
2	100.7	99.1	98.3	101.1	99.9	97.0	98.1	99.7	98.1
3	101.1	98.6	97.6	97.8	99.7	96.9	98.6	100.4	98.7
4	100.9	99.4	98.5	97.6	99.6	98.3	97.3	100.2	98.8
5	100.5	99.7	98.5	97.6	100.3	99.1	99.0	99.5	97.6
6	99.6	100.0	97.8	97.8	99.6	99.7	99.3	101.8	96.5
Average	**100.5**	**99.2**	**98.1**	**98.7**	**99.8**	**98.0**	**98.4**	**100.4**	**98.1**
STD	**0.494**	**0.611**	**0.417**	**1.568**	**0.306**	**1.233**	**0.696**	**0.829**	**0.926**
%RSD	**0.491**	**0.615**	**0.425**	**1.588**	**0.306**	**1.258**	**0.707**	**0.826**	**0.944**



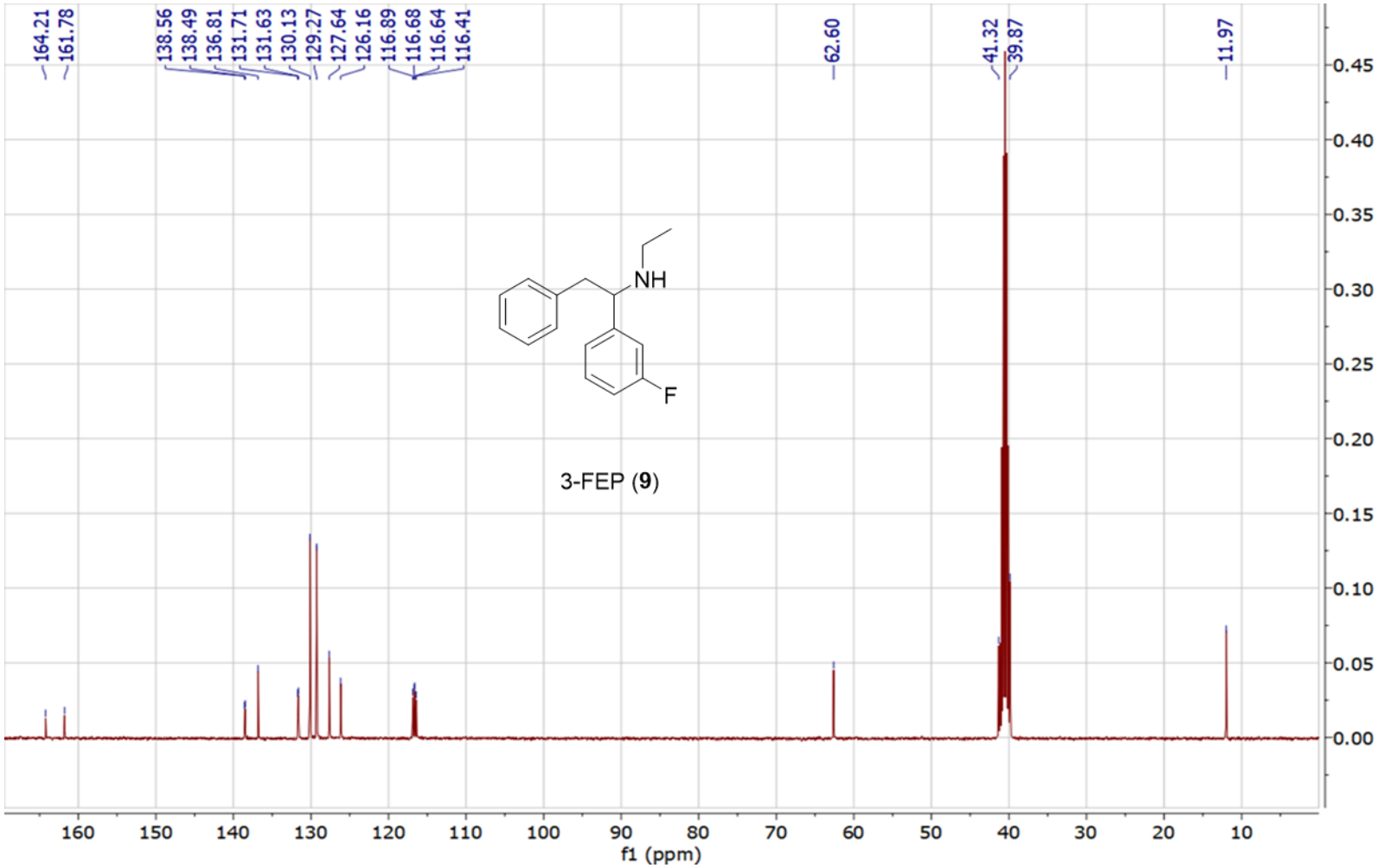
Appendix A 20. ATR-FTIR spectrum of **9**



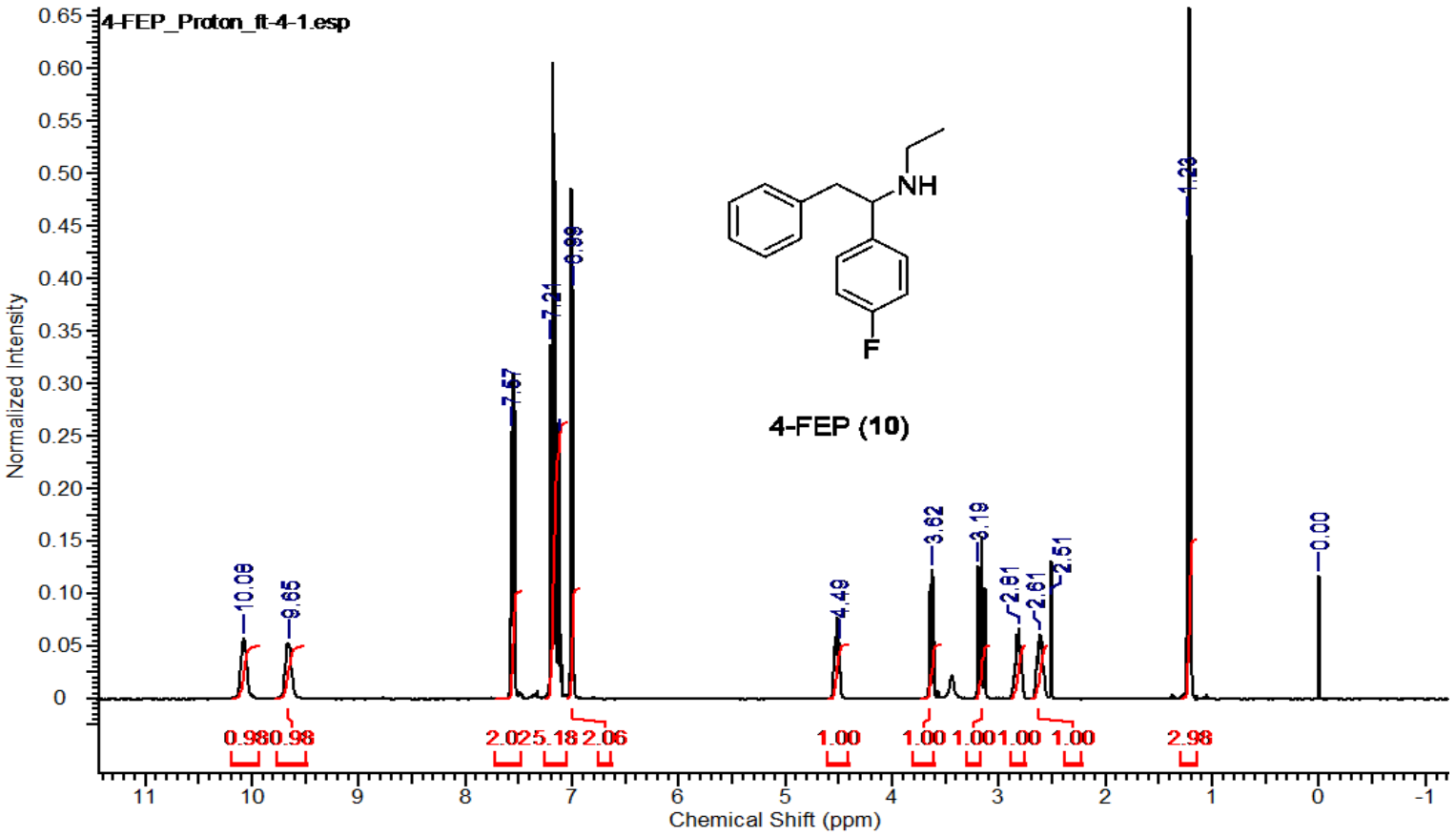
Appendix A 21. ATR-FTIR spectrum of **10**



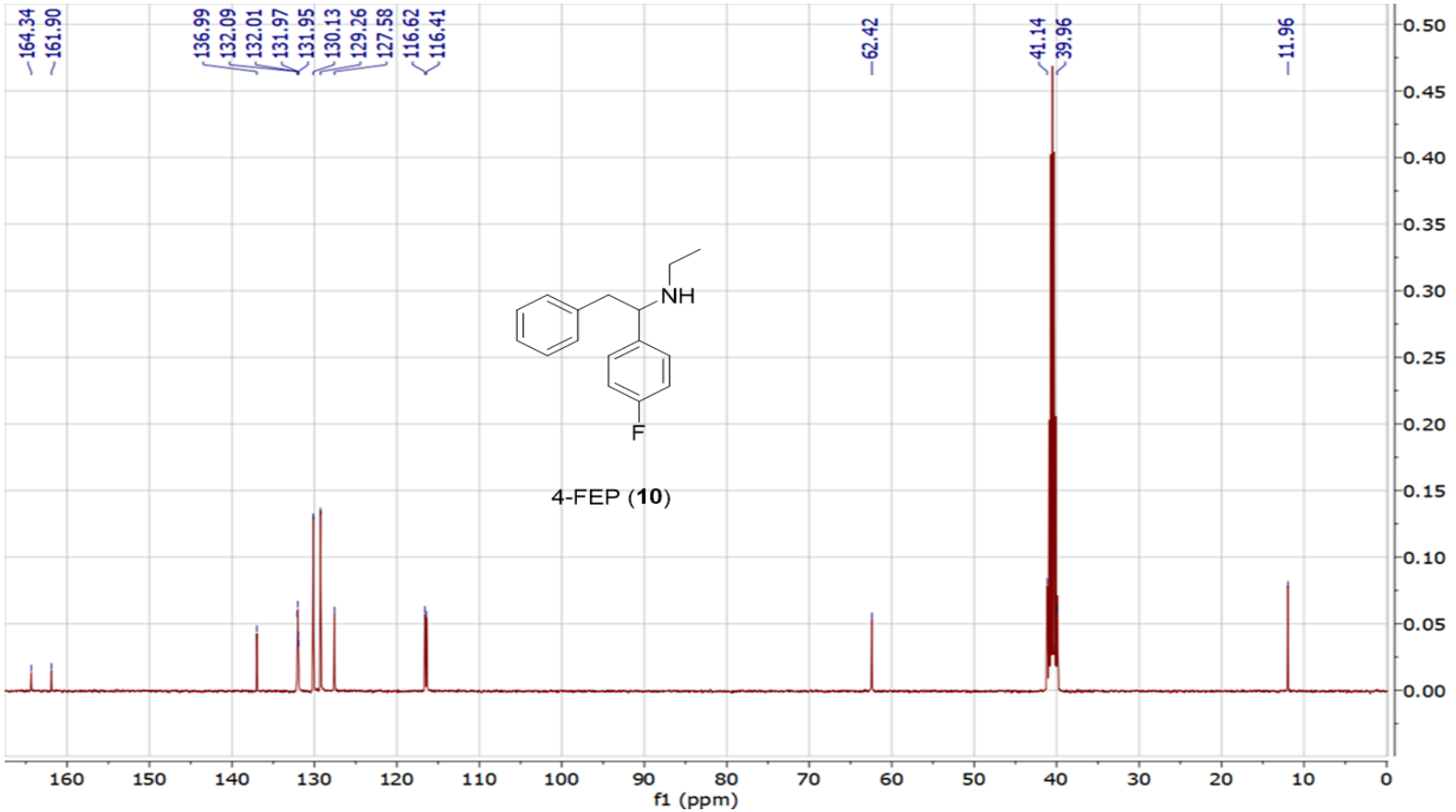
Appendix A 22. ^{1}H NMR spectrum of **9** collected in d_6-DMSO



Appendix A 23. $^{13}C\{^{1}H\}$ NMR spectrum of **9** collected in d6-DMSO



Appendix A 24. ^{1}H NMR spectrum of **10** collected in d_6-DMSO



Appendix A 25. $^{13}C\{^{1}H\}$ NMR spectrum of **10** collected in d6-DMSO

Appendix A 26. Intraday and inter-day precision measurements for the fluephenidine analytes using mobile phase acetonitrile: ammonium acetate 25:75% v/v and flow rate 1.5 mL min^{-1}

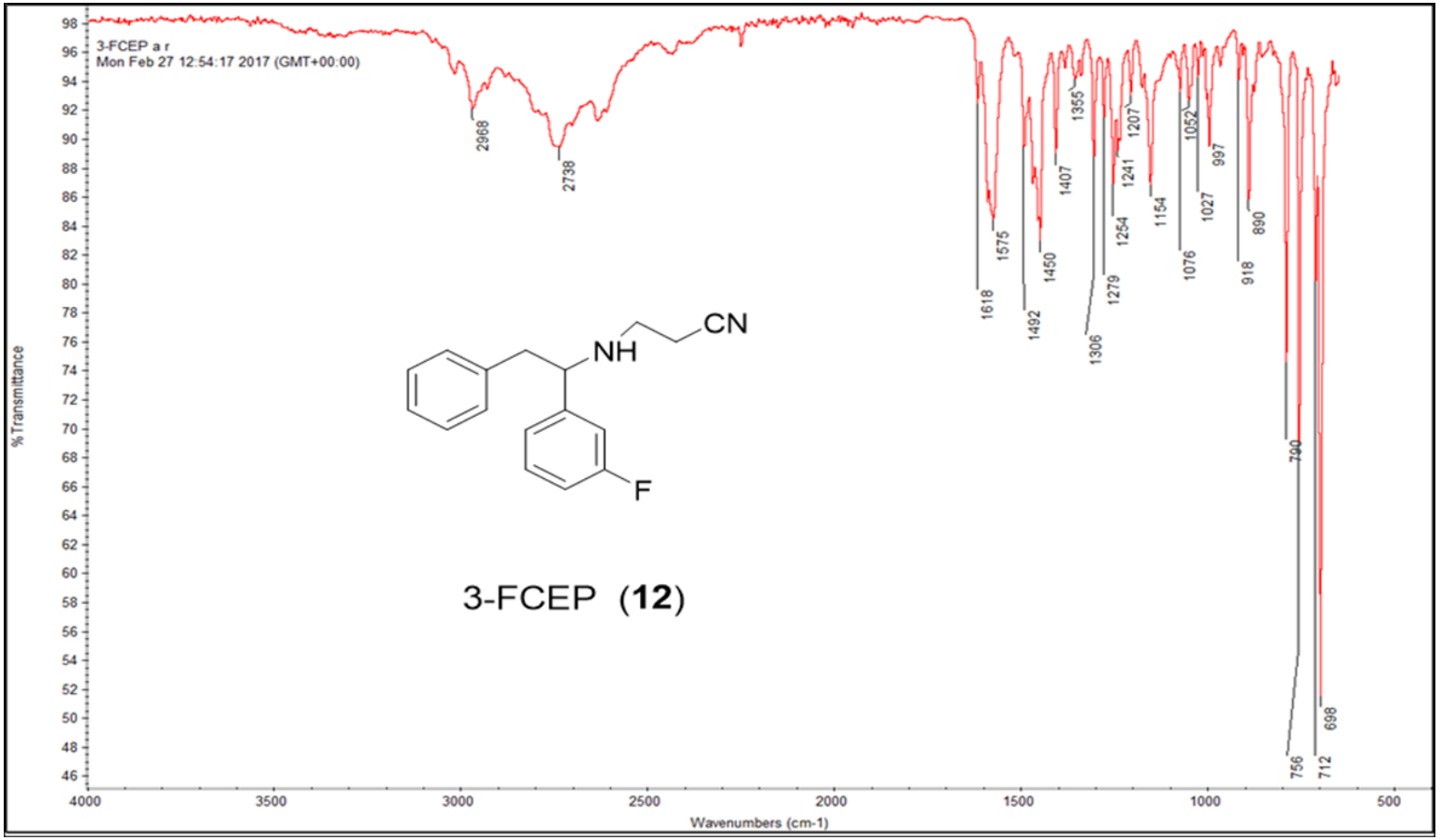
	Intraday Precision t_R (min)			**Interday Precision t_R (min)**		
Injection	**10**	**8**	**9**	**10**	**8**	**9**
1	17.04	20.97	22.81	16.95	20.87	22.70
2	17.06	20.98	22.83	16.96	20.88	22.71
3	17.06	20.99	22.84	16.98	20.88	22.72
4	17.06	20.99	22.84	16.99	20.89	22.73
5	17.08	20.99	22.84	16.99	20.90	22.74
6	17.08	21.00	22.85	17.00	20.91	22.75
7	17.10	21.01	22.86	17.01	20.91	22.76
8	17.11	21.01	22.87	17.03	20.91	22.76
9	17.11	21.01	22.87	17.03	20.92	22.78
10	17.12	21.03	22.88	17.01	20.94	22.78
Average	**17.03**	**20.95**	**22.80**	**17.00**	**20.90**	**22.74**
STD	**0.051**	**0.052**	**0.059**	**0.025**	**0.020**	**0.025**
%RSD	**0.302**	**0.252**	**0.262**	**0.152**	**0.098**	**0.112**

Appendix A 27. Robustness testing (flow rate = 1.4, 1.5 or 1.6 mL min-1) for the three FEP analytes using a mobile phase consisting of acetonitrile:ammonium acetate 25:75% v/v, concentration of FEP = 50 µg mL^{-1}

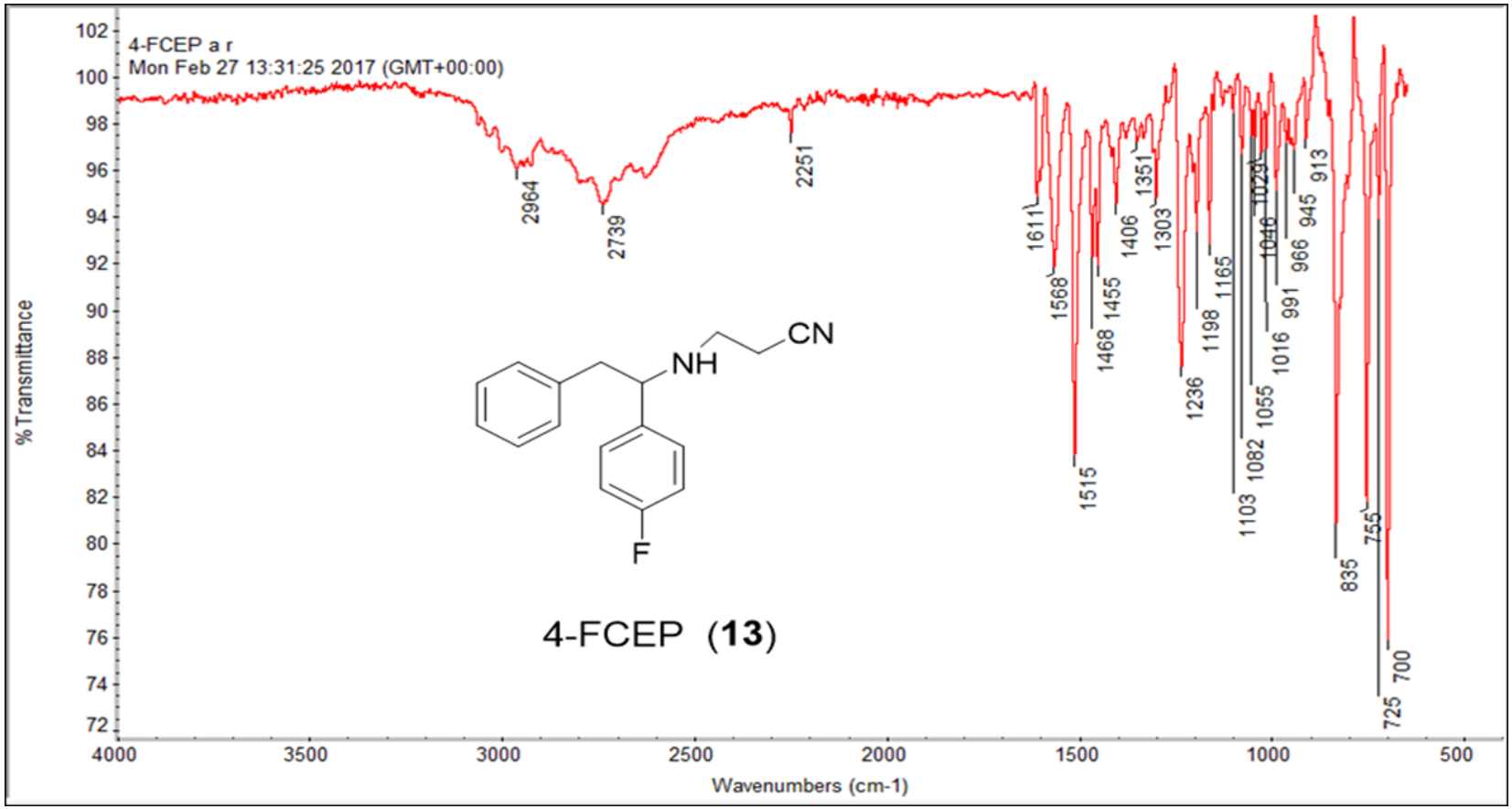
Injection	t_R (min) using flow rate 1.4 mL min^{-1}			t_R (min) using flow rate 1.5 mL min^{-1}			t_R (min) using flow rate 1.6 mL min^{-1}		
	10	**8**	**9**	**10**	**8**	**9**	**10**	**8**	**9**
1	17.769	21.820	23.734	16.955	20.580	22.413	15.636	19.179	20.842
2	17.787	21.843	23.762	16.948	20.537	22.405	15.613	19.169	20.846
3	17.802	21.853	23.773	16.951	20.578	22.403	15.622	19.171	20.848
4	17.813	21.869	23.786	16.951	20.580	22.405	15.618	19.166	20.850
5	17.832	21.880	23.806	16.971	20.591	22.413	15.622	19.175	20.853
6	17.847	21.895	23.814	16.961	20.581	22.411	15.633	19.177	20.859
7	17.839	21.891	23.813	16.954	20.578	22.409	15.623	19.170	20.847
8	17.836	21.885	23.806	16.949	20.582	22.407	15.621	19.170	20.844
9	17.852	21.909	23.826	16.952	20.583	22.405	15.619	19.162	20.842
10	17.851	21.903	23.821	16.947	20.579	22.410	15.622	19.174	20.851
Average	**17.822**	**21.874**	**23.794**	**16.953**	**20.576**	**22.408**	**15.622**	**19.171**	**20.848**
STD	**0.028**	**0.028**	**0.029**	**0.007**	**0.014**	**0.003**	**0.006**	**0.005**	**0.005**
%RSD	**0.161**	**0.130**	**0.124**	**0.042**	**0.070**	**0.016**	**0.043**	**0.026**	**0.025**

Appendix A 28. Comparison of the resolution values obtained for the FEP regioisomers for the three robustness experiments performed

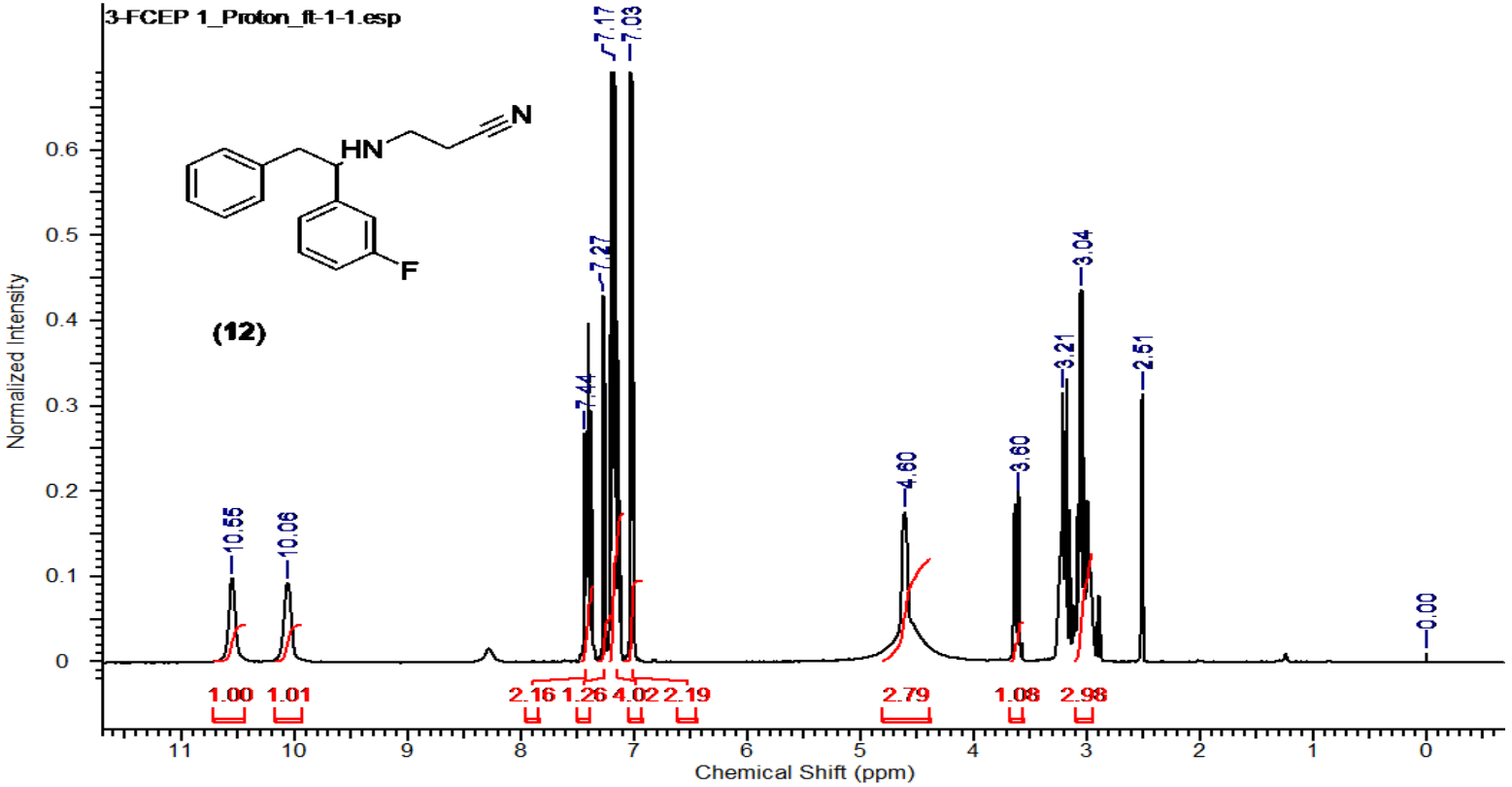
	Resolution using Flow Rate (mL min^{-1})			Resolution using Temperature (°C)			Resolution using % Mobile Phase Acetonitrile:ammonium acetate (% v/v)		
FEP isomer	**1.4**	**1.5**	**1.6**	**48**	**50**	**52**	**24:76**	**25:75**	**26:74**
10	-	-	-	-	-	-	-	-	-
8	5.22	4.81	5.14	5.08	4.81	5.38	4.92	5.01	5.00
9	2.12	2.09	2.09	2.10	2.09	2.11	2.13	2.11	2.04



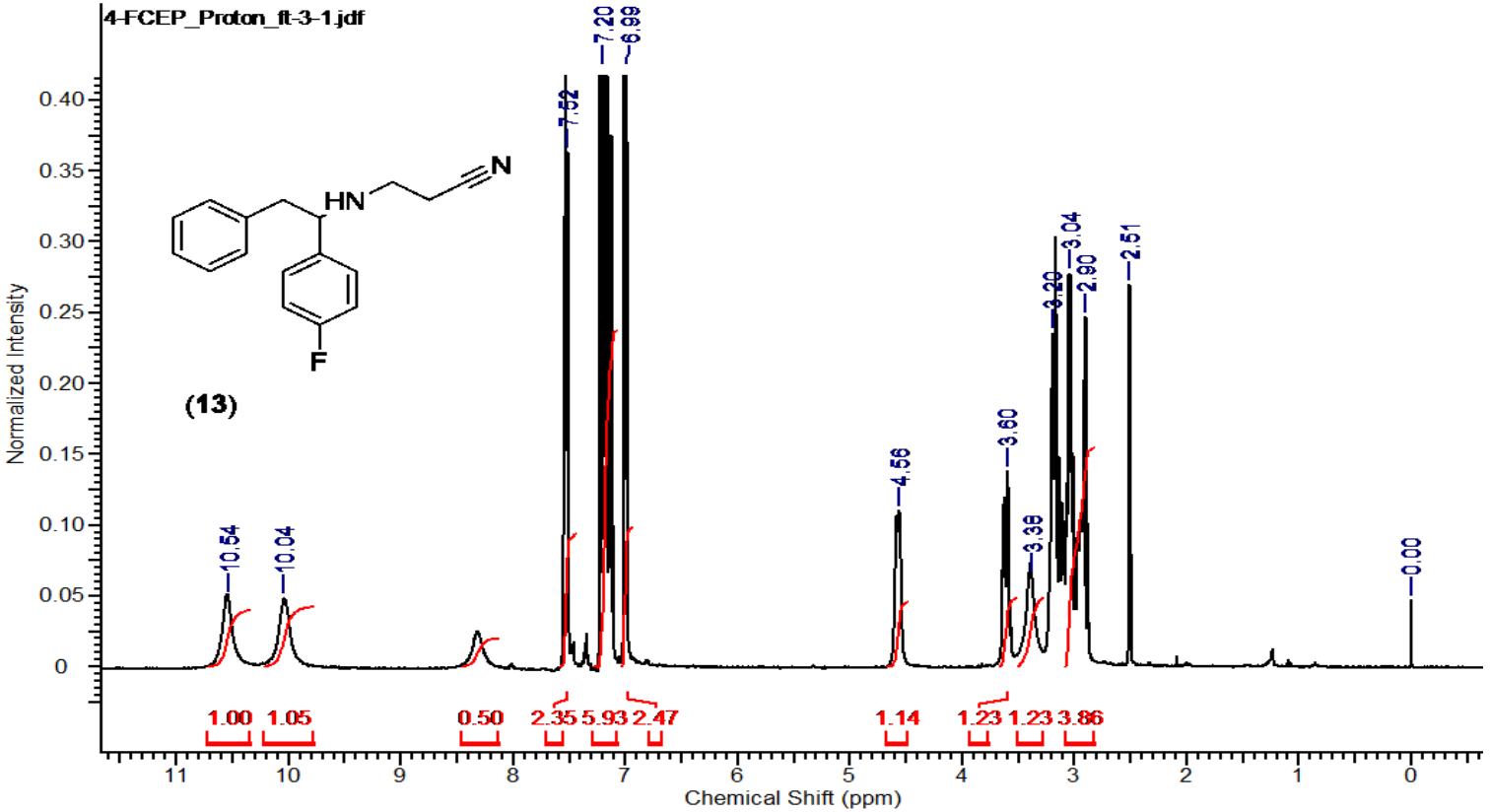
Appendix A 29. ATR-FTIR spectrum of **12**



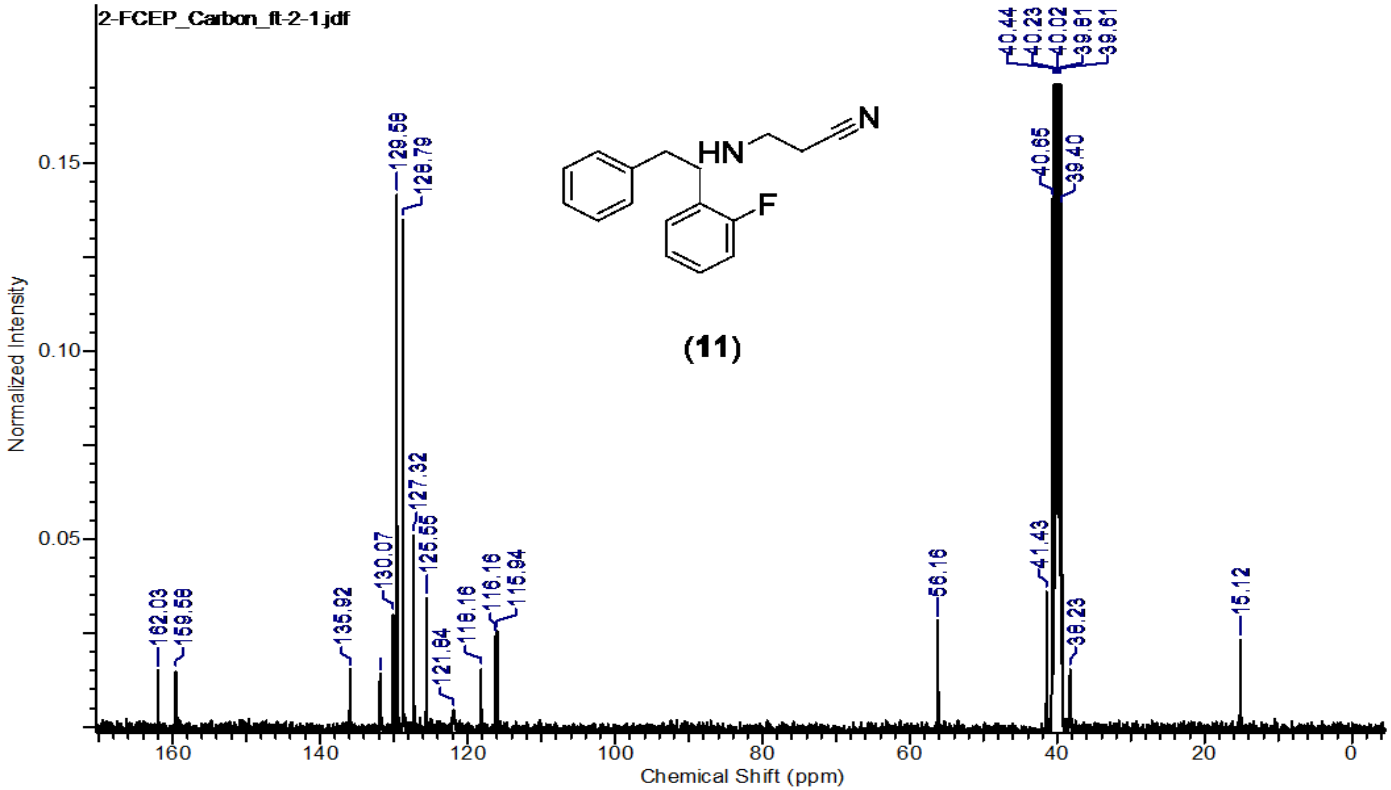
Appendix A 30. ATR-FTIR spectrum of **13**



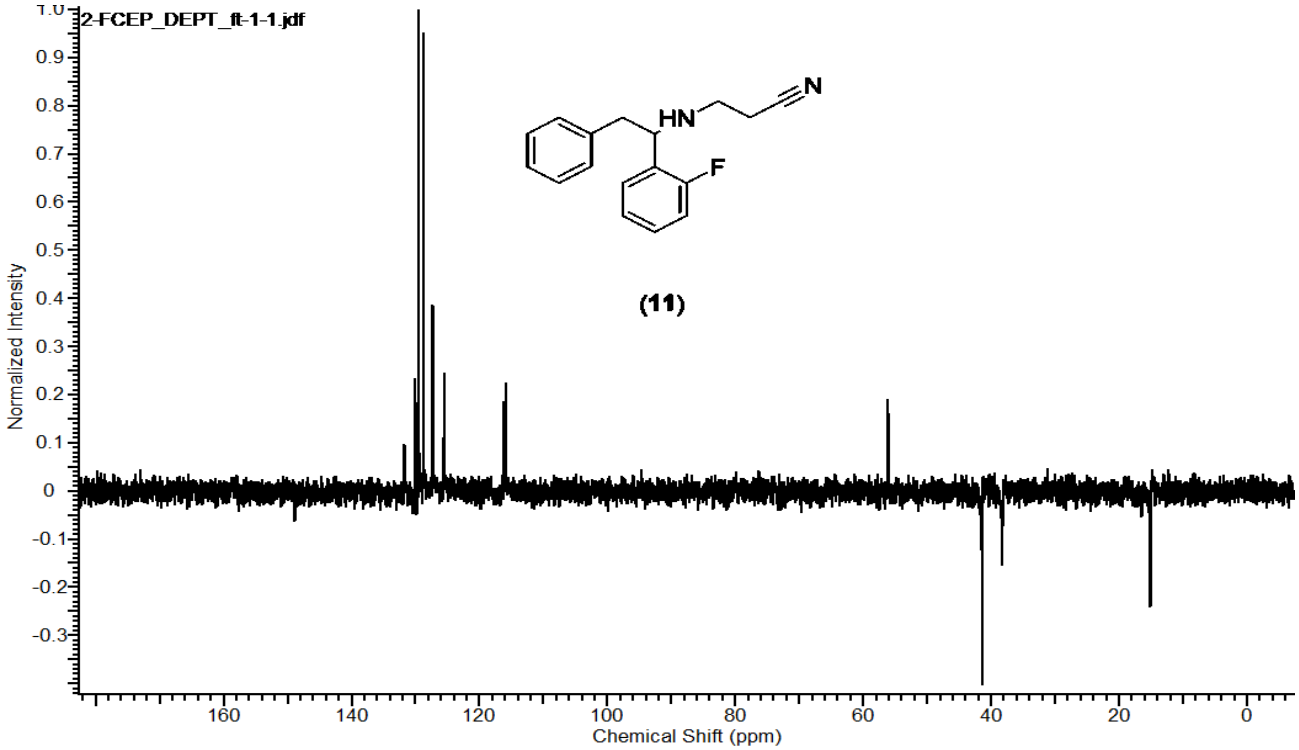
Appendix A 31. ^{1}H NMR spectra of **12**



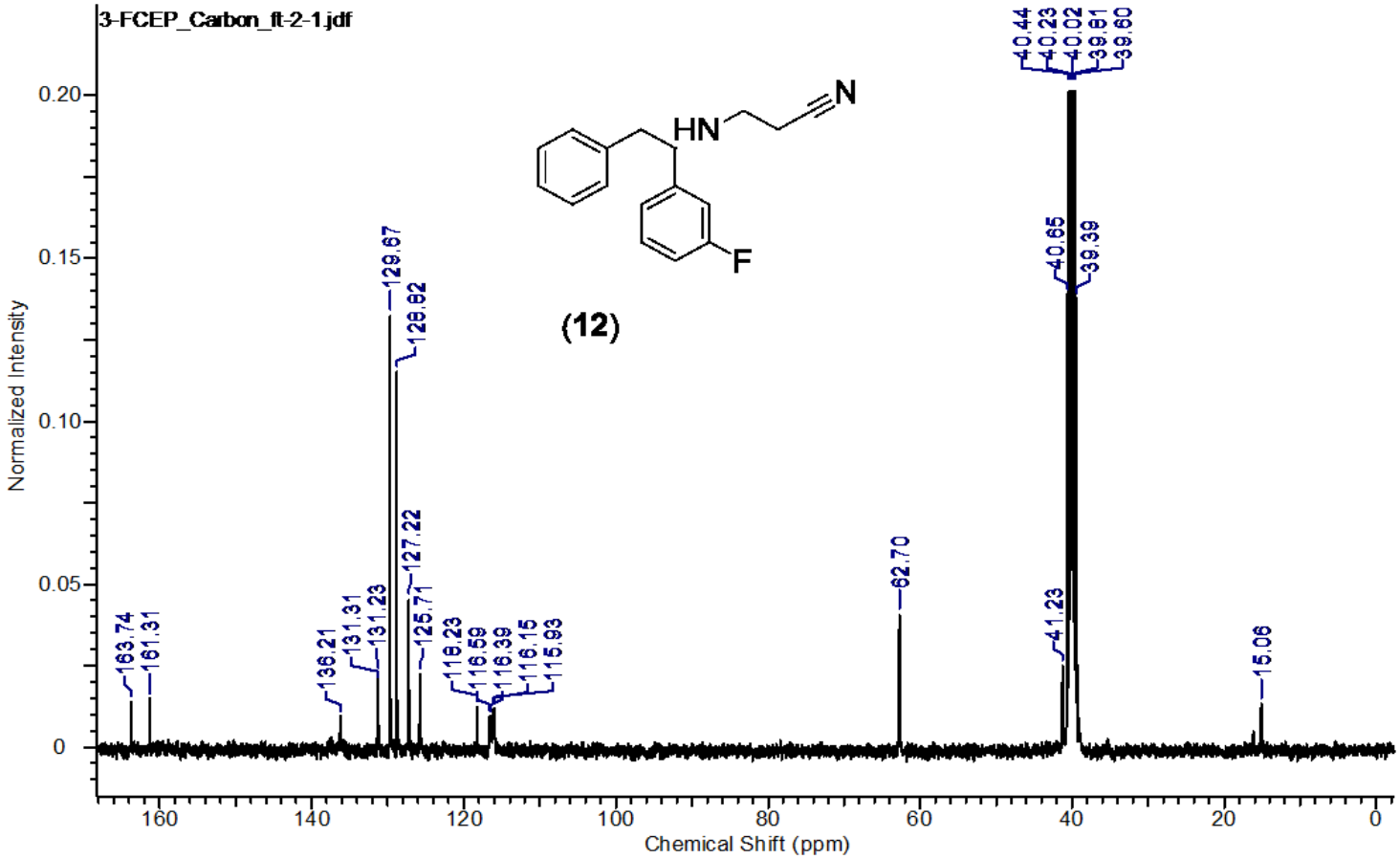
Appendix A 32. ^{1}H NMR spectra of **13**



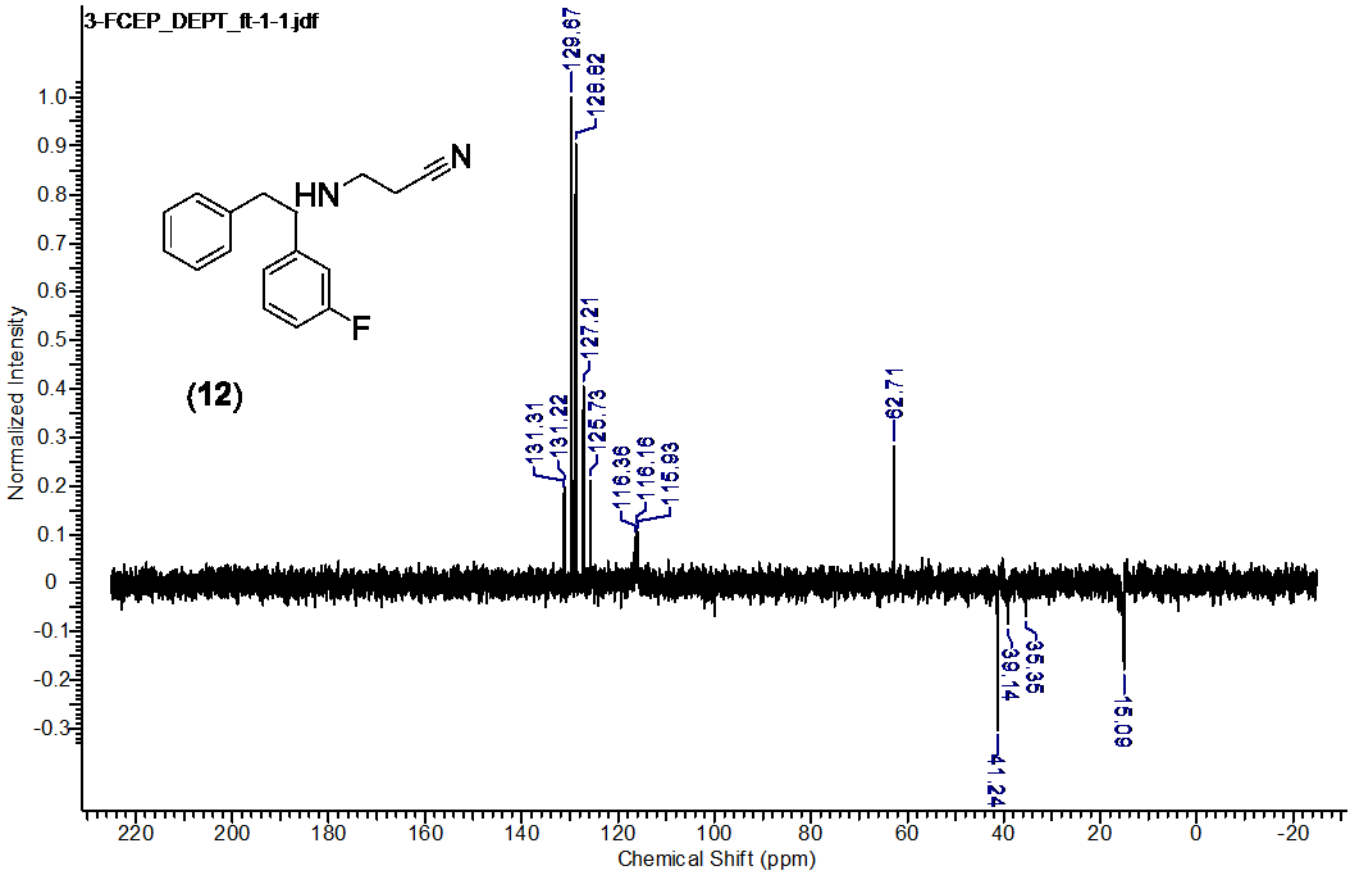
Appendix A 33. $^{13}C\{^{1}H\}$ NMR spectrum of **11**



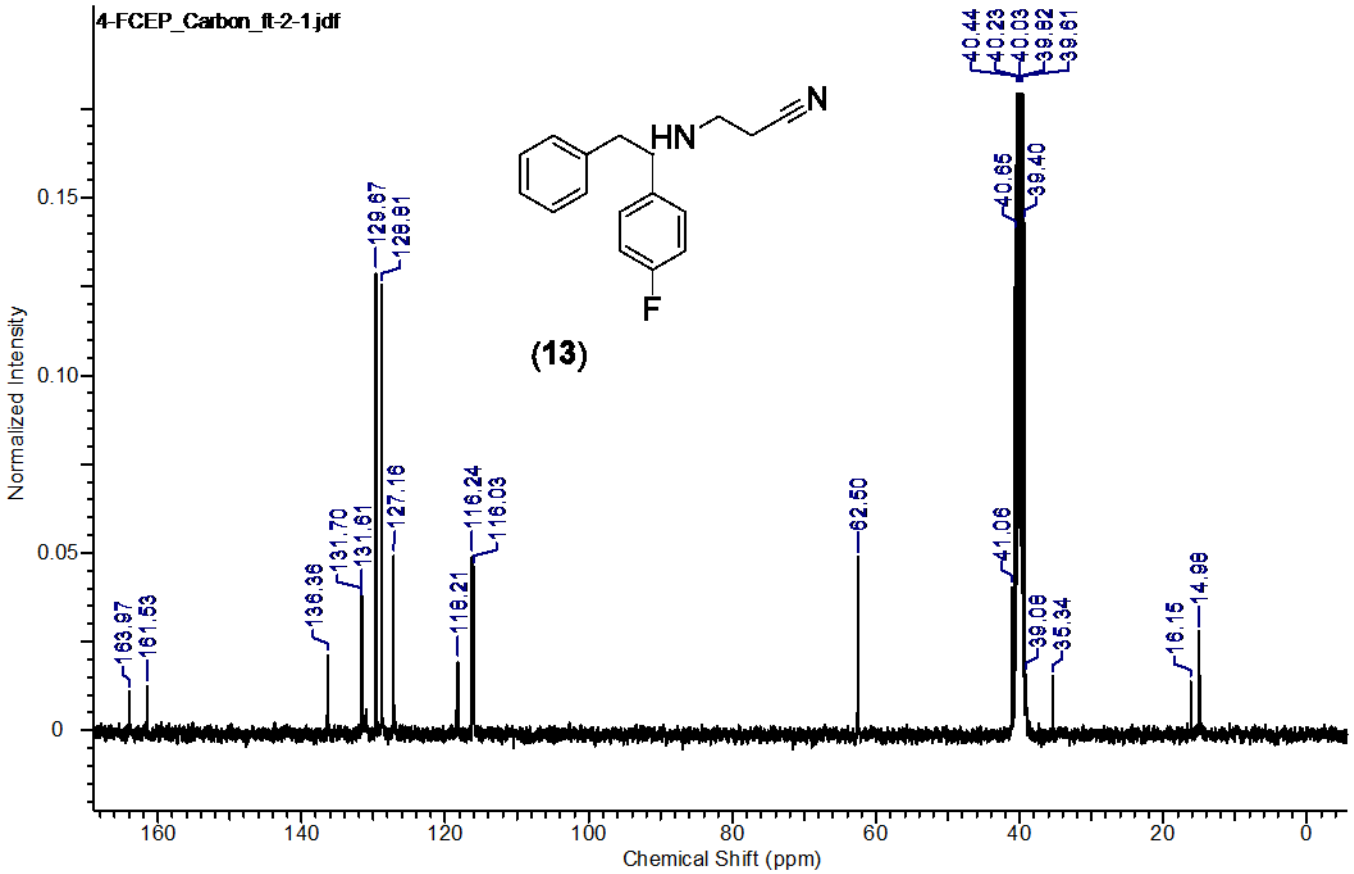
Appendix A 34. DEPT-135 spectrum of **11**



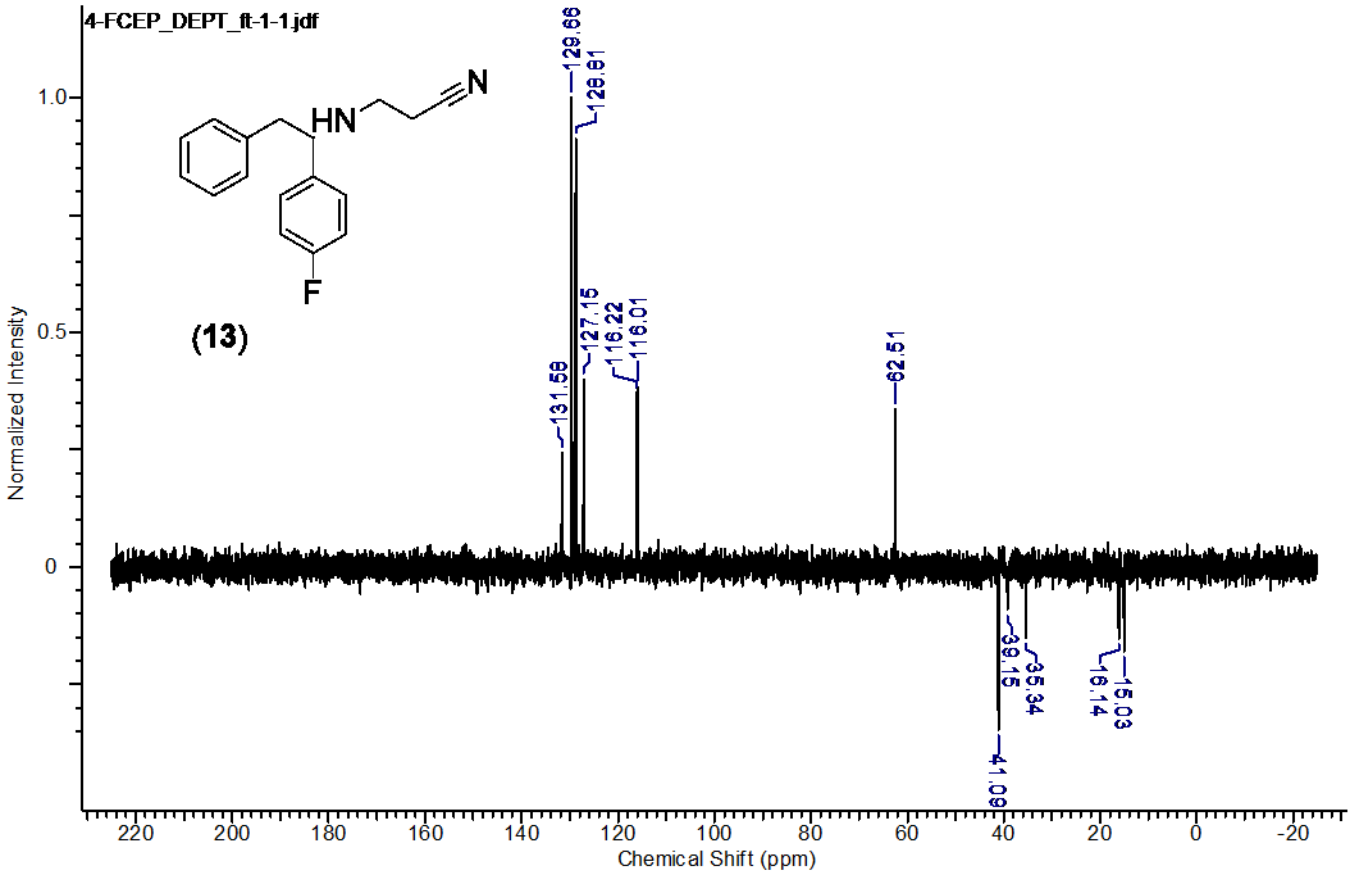
Appendix A 35. $^{13}C\{^{1}H\}$ NMR spectrum of **12**



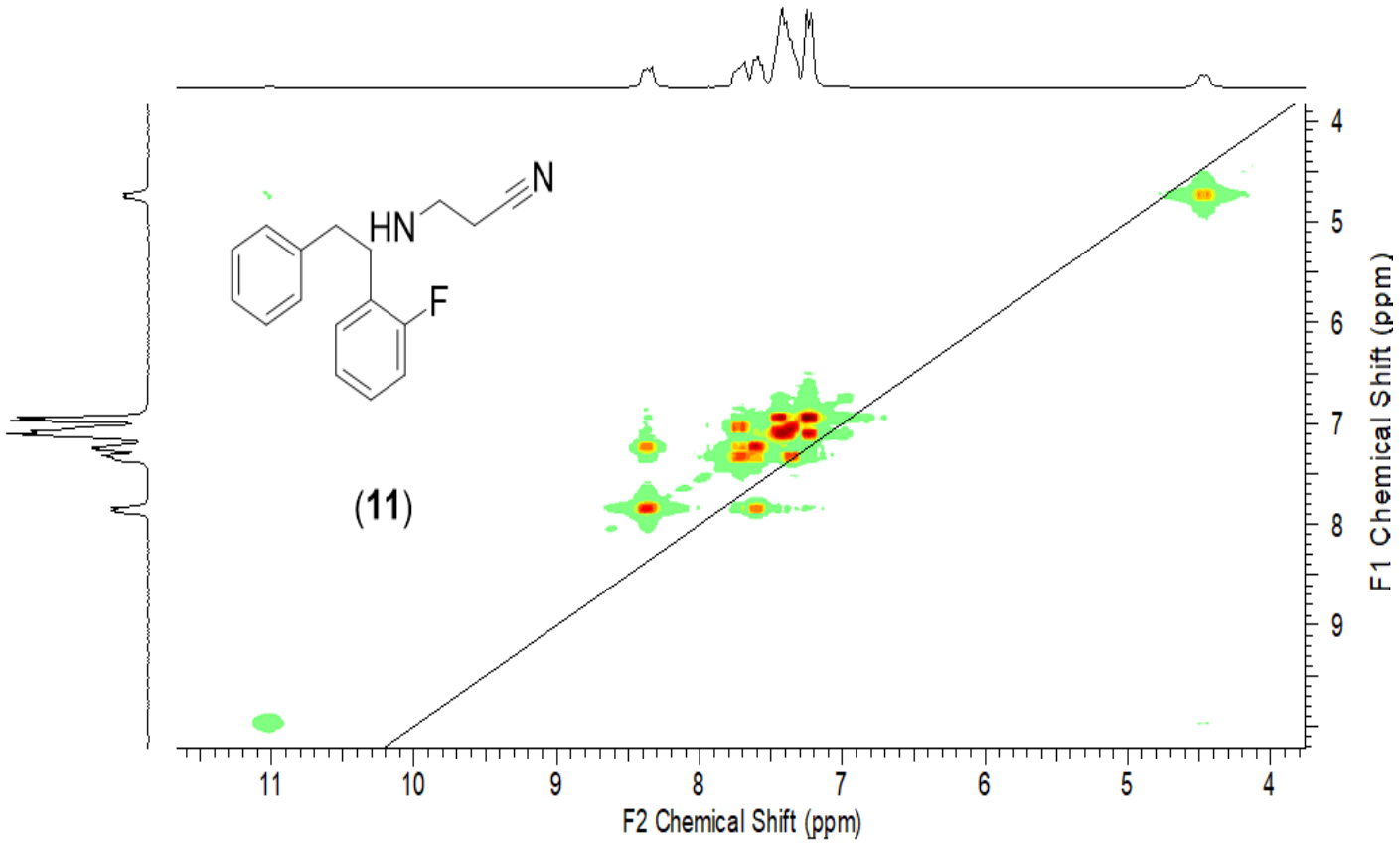
Appendix A 36. DEPT-135 spectrum of **12**



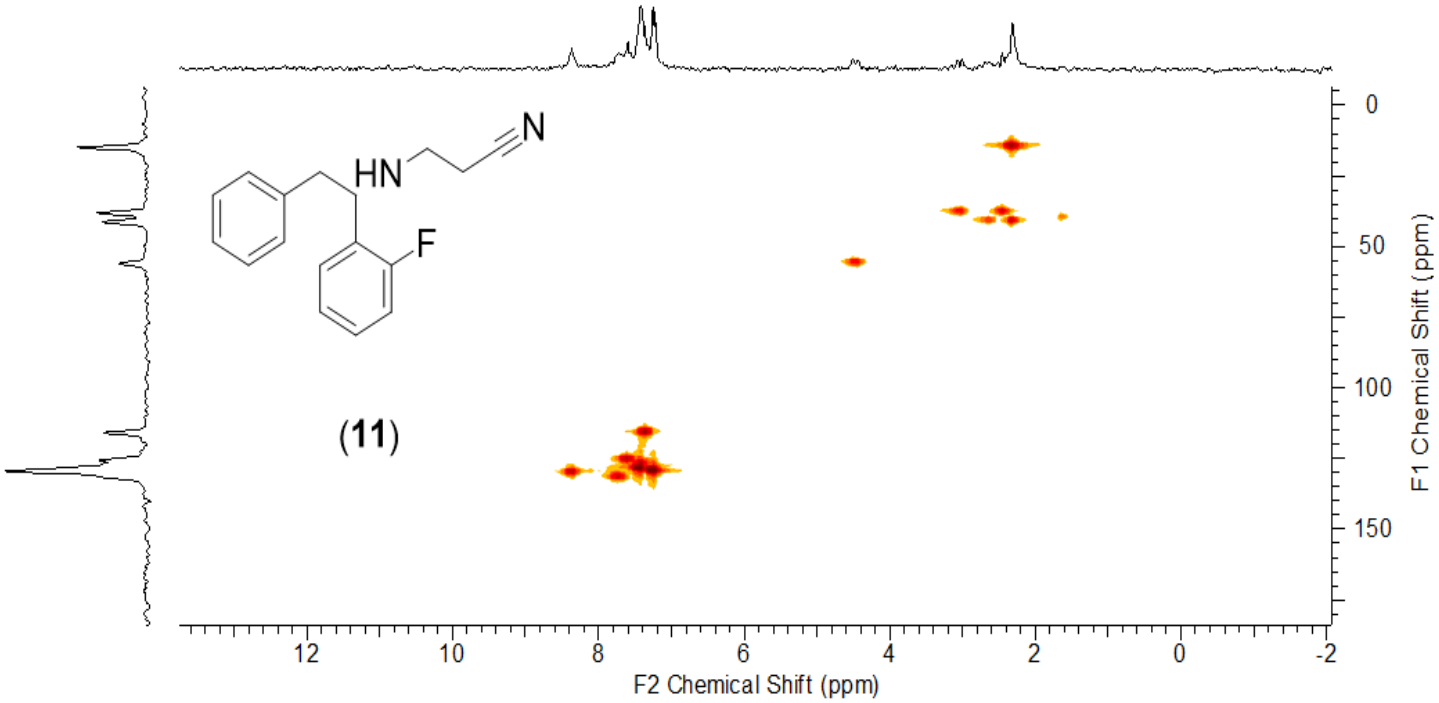
Appendix A 37. $^{13}C\{^{1}H\}$ NMR spectrum of **13**



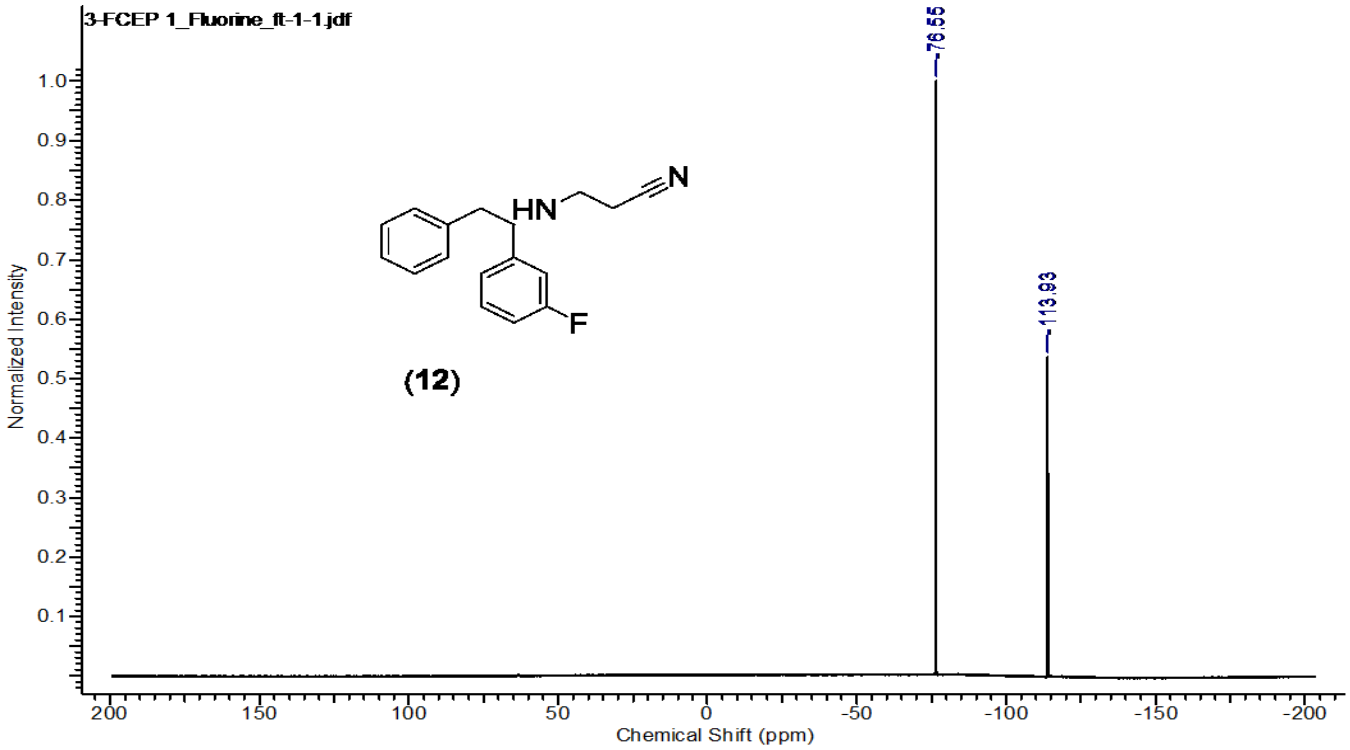
Appendix A 38. DEPT-135 spectrum of **13**



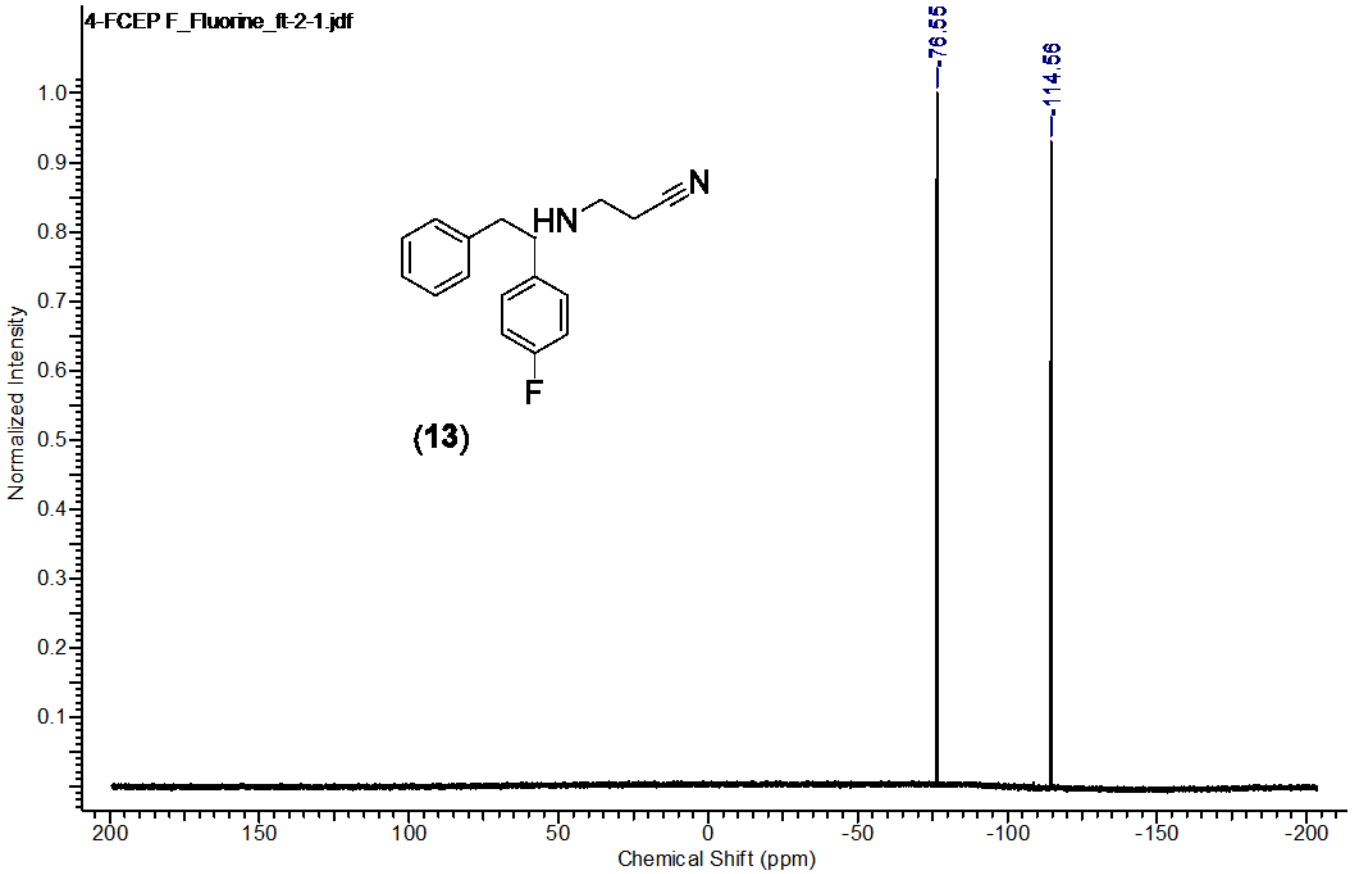
Appendix A 39. 2D ^{1}H-^{1}H COSY NMR spectrum of **11**



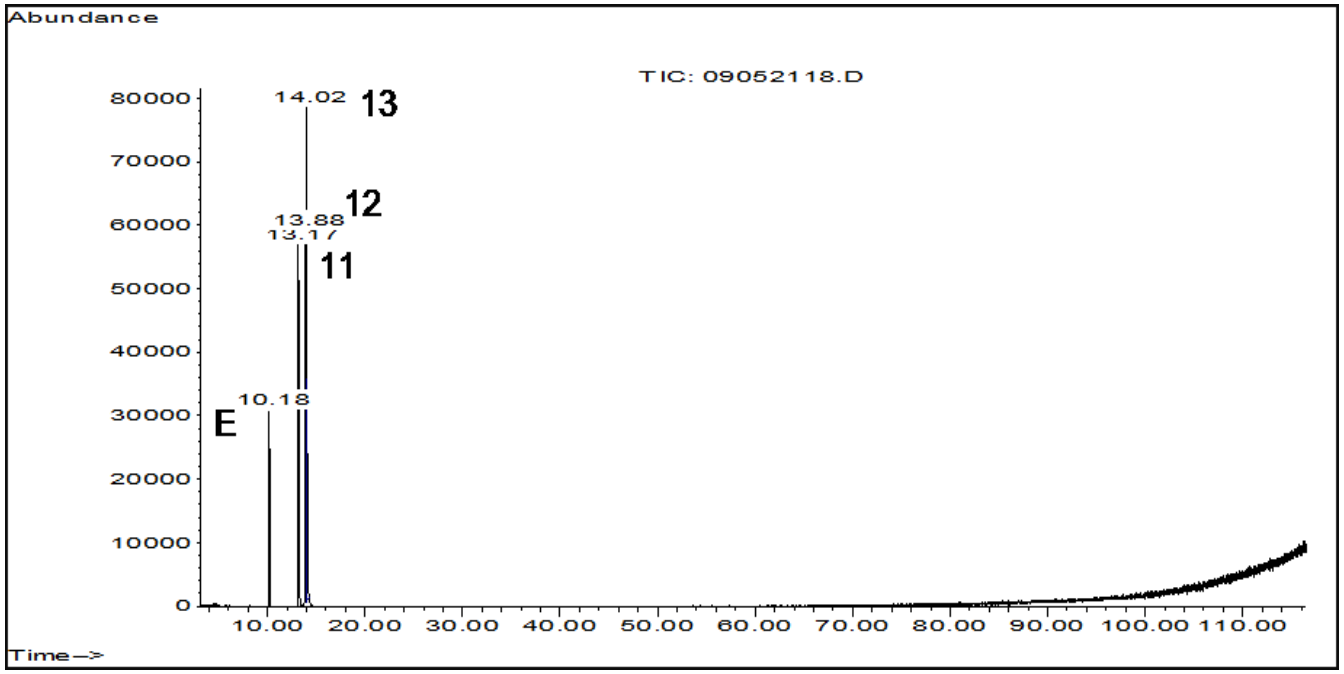
Appendix A 40. 2D ^{13}C-^{1}H HMQC NMR spectrum of **11**



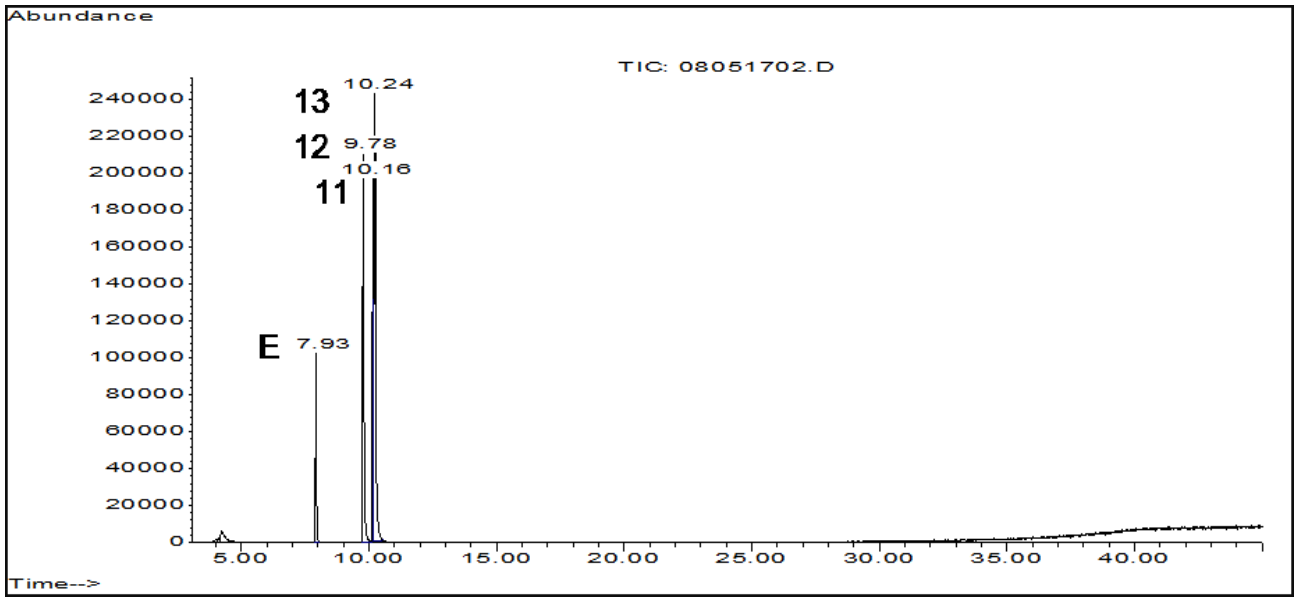
Appendix A 41. ^{19}F NMR spectrum of 3-FCEP (**12**)



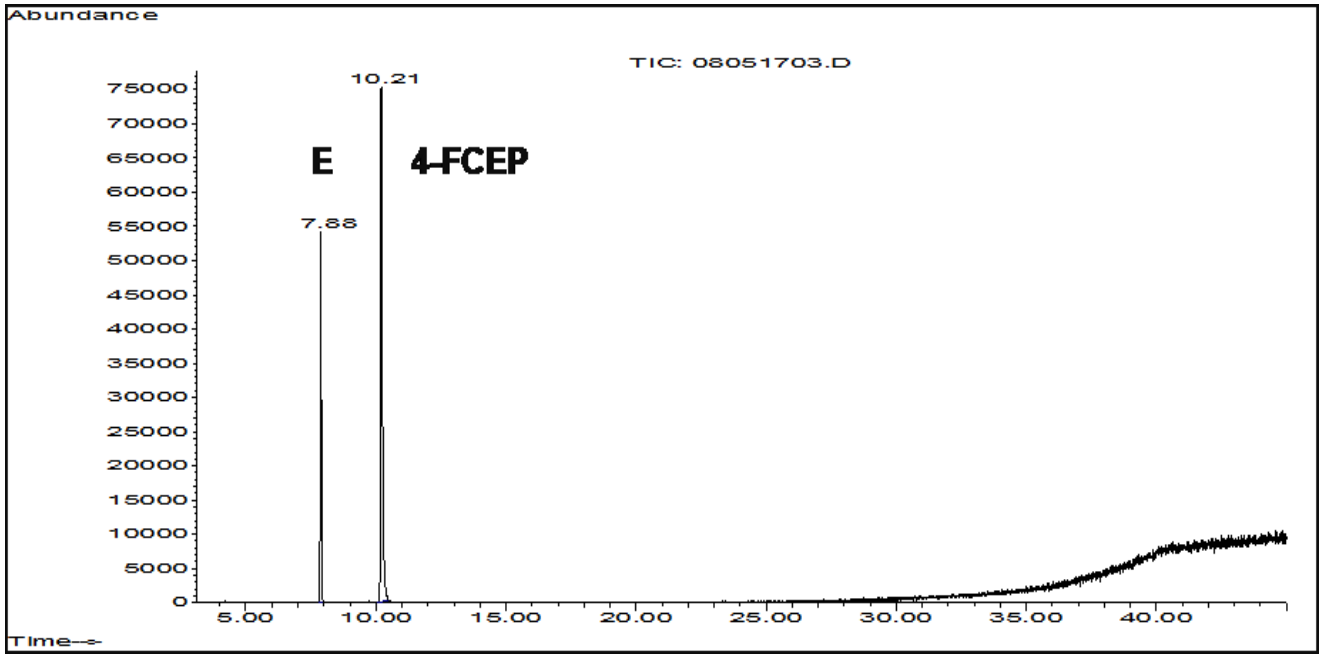
Appendix A 42. ^{19}F NMR spectrum of 4-FCEP (**13**)



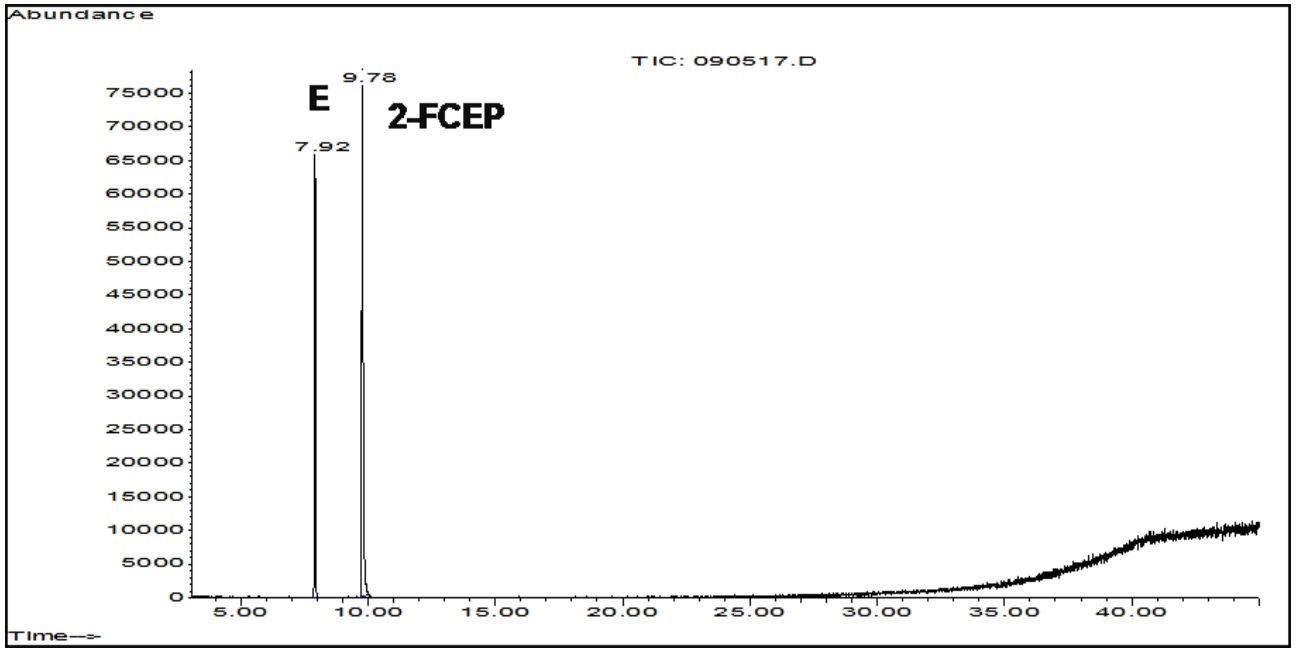
Appendix A 43. FCEP mixture+ E on GC-MS (non-polar column) using 1°C min^{-1} method



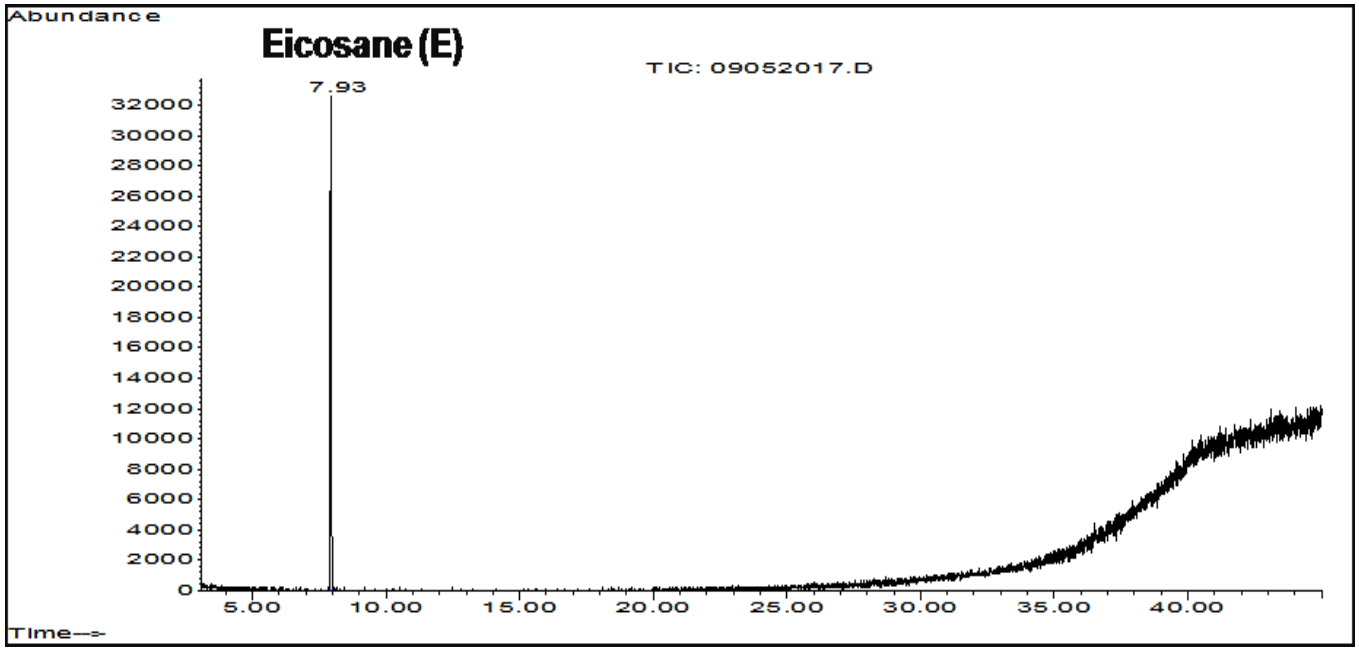
Appendix A 44. FCEP mixture + E on GC-MS (non-polar column) using 3°C min^{-1} method



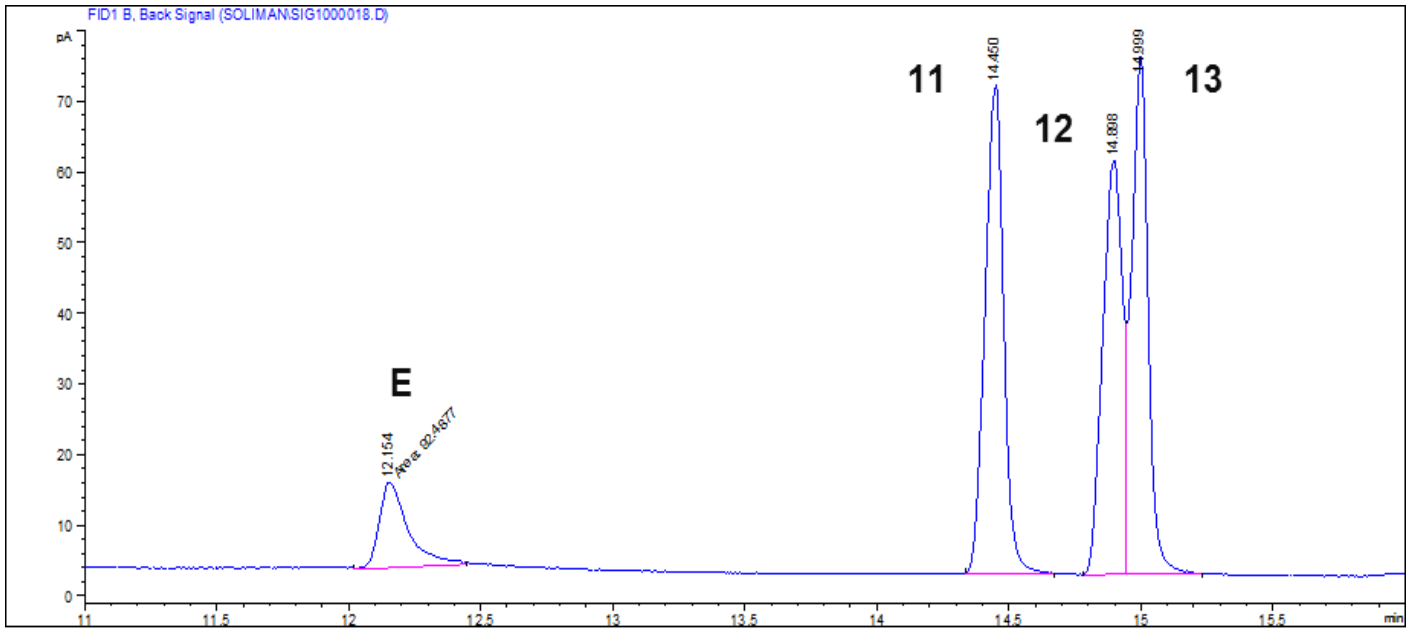
Appendix A 45. 4-FCEP (**13**) + E on GC-MS (non-polar column) using 3°C min^{-1} method



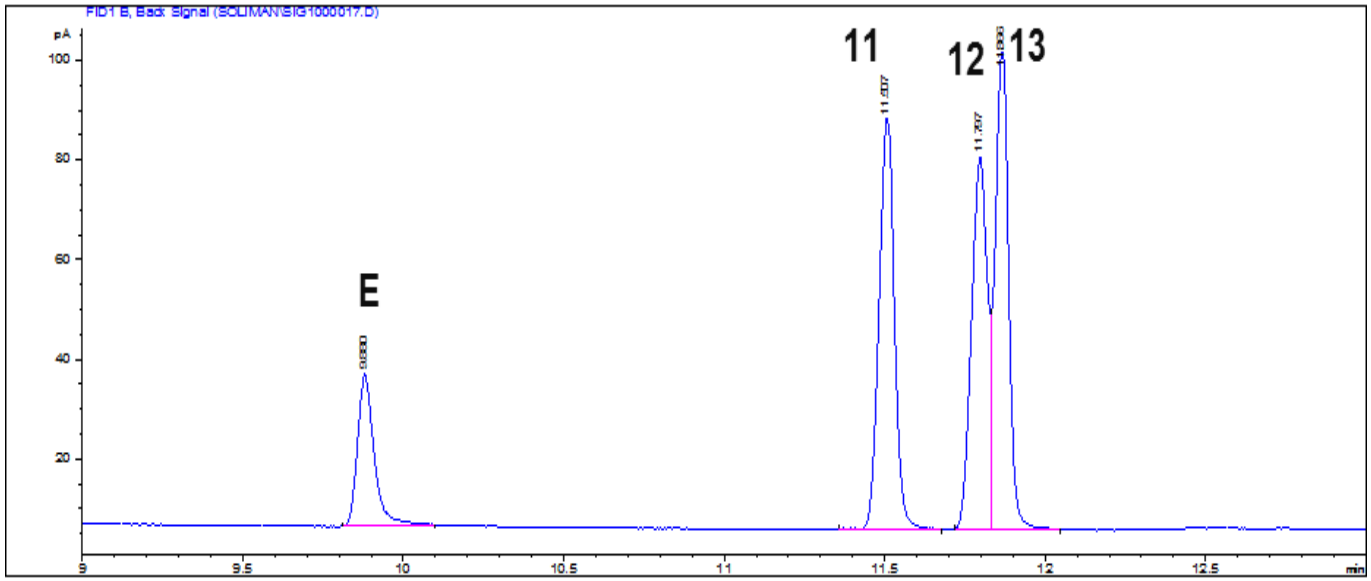
Appendix A 46. 2-FCEP (**11**) + E on GC-MS (non-polar column) using 3°C min^{-1} method



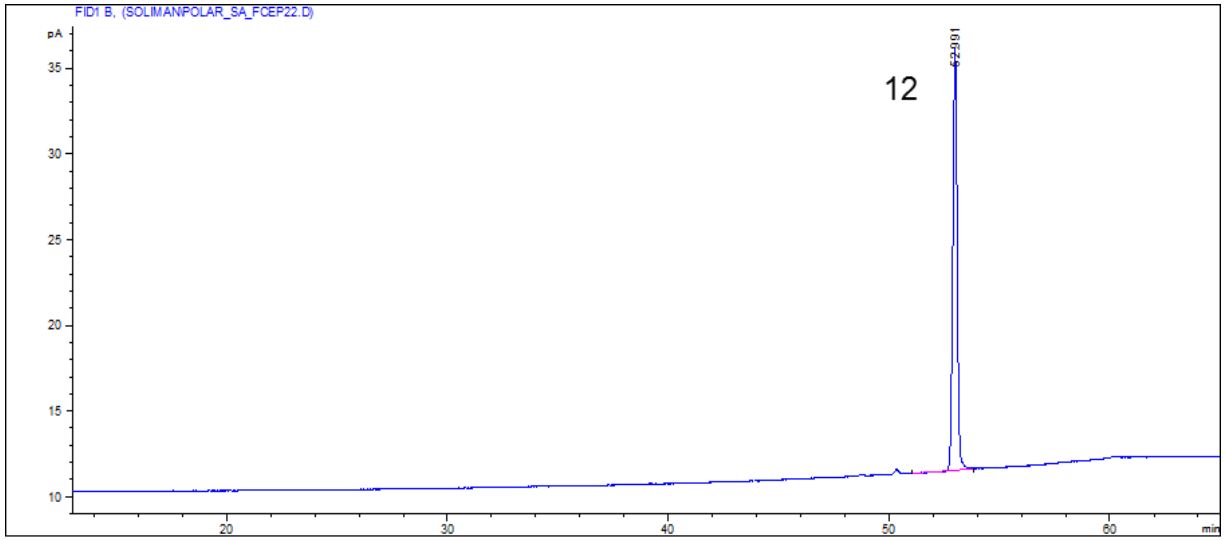
Appendix A 47. Eicosane (**E**) on GC-MS (non-polar column) using 3°C min^{-1} method



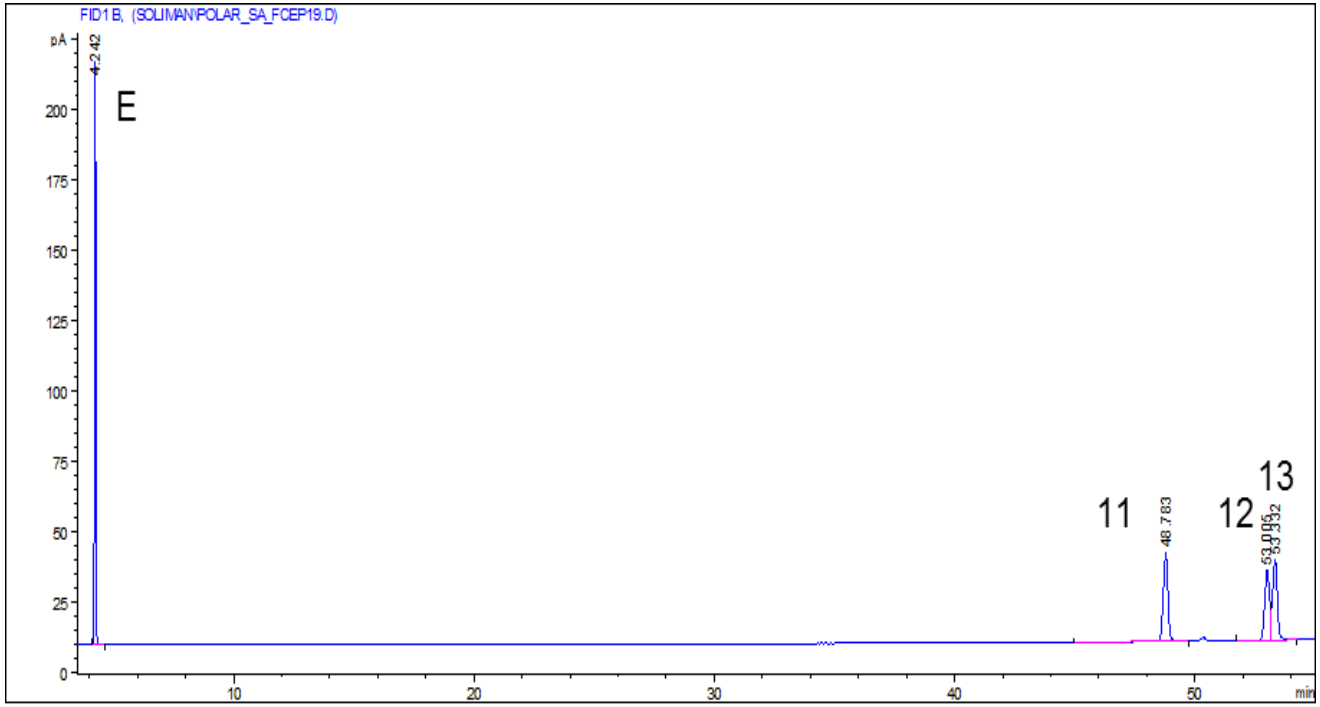
Appendix A 48. FCEP mixture plus eicosane at 3 °C min^{-1} on semi-polar GC using nitrogen as a carrier gas



Appendix A 49. FCEP mixture plus eicosane at 5 °C min^{-1} on semi-polar GC using nitrogen as a carrier gas



Appendix A 50. 3-FCEP (**12**), (1 mg mL^{-1}) on polar GC at 1°C min^{-1}



Appendix A 51. FCEP mix (1 mg mL^{-1}) on polar GC at 1°C min^{-1}

Appendix A 52. Validation of 2-, 3- and 4-halogenated diphenidine isomers

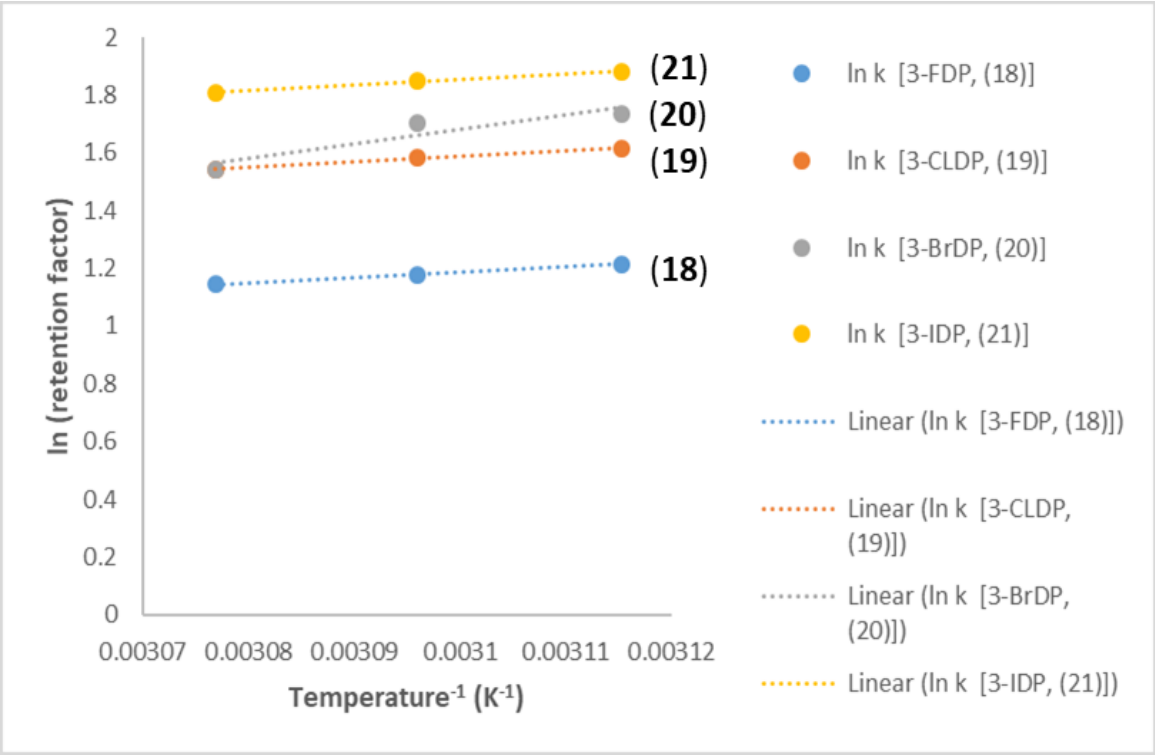
	14	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
tR (min)	4.150	5.891	6.804	8.414	4.217	5.815	6.451	7.309	3.551	5.406	6.098	7.126
RRT	0.704	1	1.154	1.428	0.725	1	1.109	1.257	0.656`	1	1.127	1.318
Capacity factor	3.150	4.891	5.804	7.414	3.217	4.815	5.451	6.309	2.551	4.406	5.098	6.126
N (plates)	9655.0	10972.5	11314.5	11479.9	9742.7	10413.9	10796.0	10975.8	9044.8	10933.2	11177.8	11578.2
H(m) ($x10^{-5}$)	1.55 $x10^{-5}$	1.37 $x10^{-5}$	1.33 $x10^{-5}$	1.31 $x10^{-5}$	1.54 $x10^{-5}$	1.44 $x10^{-5}$	1.39 $x10^{-5}$	1.37 $x10^{-5}$	1.66 $x10^{-5}$	1.37 $x10^{-5}$	1.34 $x10^{-5}$	1.30 $x10^{-5}$
Resolution	-	8.822	3.790	5.636	-	7.994	2.663	3.249	-	10.395	3.155	4.140
Symmetry	0.888	0.895	0.910	0.921	0.876	0.866	0.896	0.903	0.902	0.902	0.909	0.897
LOQ	0.237	0.226	0.222	0.234	0.180	0.166	0.212	0.183	0.262	0.223	0.178	0.264
LOD	0.078	0.074	0.073	0.077	0.059	0.055	0.069	0.060	0.086	0.073	0.059	0.087
Coefficient	0.9999	0.9999	0.9999	0.9999	R^2 = 0.9999	R^2 = 0.9999	R^2 = 0.9999	R^2 = 0.9999	0.9998	0.9999	0.9999	0.9998
Equation	y = 12.79 x + 0.7256	y = 23.44 x + 0.1481	y = 21.52x - 1.5042	y = 21.55 x + 0.921	y = 14.24 x - 0.0476	y = 24.86 x + 0.0177	y = 23.78 x - 2.0693	y = 22.67 x - 0.1191	y = 13.54 x - 0.2874	y = 28.47 x - 0.5569	y = 25.26 x - 1.2813	y = 18.42 x - 1.7425
Precision (%RSD)					**Precision (%RSD)**				**Precision (%RSD)**			
1.25 µg mL^{-1}	0.969	0.913	0.461	0.780	0.782	0.735	0.999	0.522	0.536	0.730	0.545	0.657
2.5 µg mL^{-1}	0.987	0.918	0.948	0.938	0.629	0.982	0.887	0.633	0.993	0.746	0.765	0.819
5 µg mL^{-1}	0.977	0.495	0.593	0.934	0.629	0.982	0.887	0.633	0.905	0.618	0.825	0.601
10 µg mL^{-1}	0.987	0.755	0.488	0.441	0.814	0.814	0.842	0.298	0.743	0.744	0.290	0.576
20 µg mL^{-1}	0.645	0.838	0.827	0.595	0.366	0.289	0.515	0.113	0.353	0.237	0.360	0.246

Appendix A 53. Intraday and inter-day precision measurements for the 2-halogenated diphenidine analytes using a mobile phase consisting of acetonitrile:ammonium acetate, 67:33% v/v, a flow rate of 1.5 mL min^{-1} and a concentration of 10 µg mL^{-1}

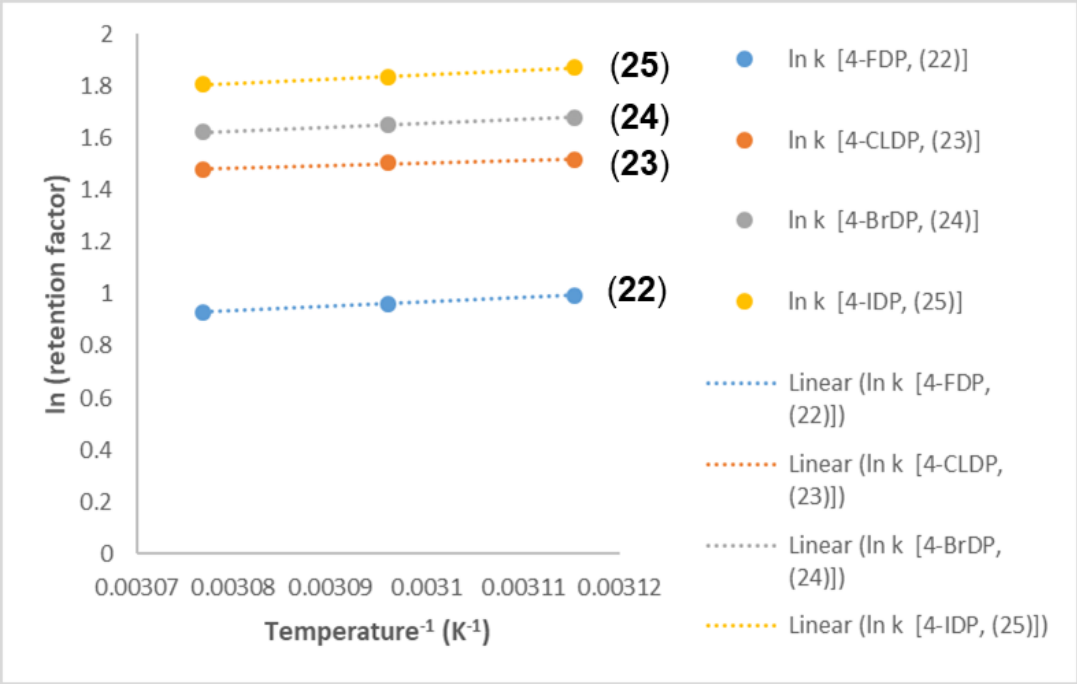
	Intraday Precision t_R (min)				Interday Precision t_R (min)			
Injection	**14**	**15**	**16**	**17**	**14**	**15**	**16**	**17**
1	4.16	5.90	6.81	8.42	4.17	5.91	6.81	8.41
2	4.17	5.91	6.82	8.43	4.16	5.90	6.81	8.41
3	4.17	5.91	6.82	8.42	4.17	5.90	6.80	8.41
4	4.17	5.92	6.83	8.43	4.17	5.91	6.82	8.42
5	4.17	5.91	6.82	8.42	4.16	5.90	6.81	8.41
6	4.17	5.92	6.83	8.43	4.17	5.91	6.81	8.41
7	4.17	5.91	6.82	8.42	4.16	5.90	6.80	8.40
8	4.16	5.90	6.81	8.42	4.17	5.91	6.82	8.42
9	4.16	5.90	6.81	8.41	4.17	5.91	6.82	8.41
10	4.17	5.91	6.82	8.42	4.16	5.88	6.78	8.36
Average	**4.17**	**5.91**	**6.82**	**8.42**	**4.17**	**5.90**	**6.81**	**8.41**
STD	**0.004**	**0.006**	**0.006**	**0.005**	**0.004**	**0.007**	**0.010**	**0.015**
%RSD	**0.099**	**0.105**	**0.100**	**0.067**	**0.110**	**0.129**	**0.157**	**0.188**

Appendix A 54. Robustness testing (temperature 48, 50 and 52°C) for the 2-halogenated diphenidine isomers using a mobile phase consisting of acetonitrile:ammonium acetate, 67:33 % v/v. Key: t_R = Retention time (min).

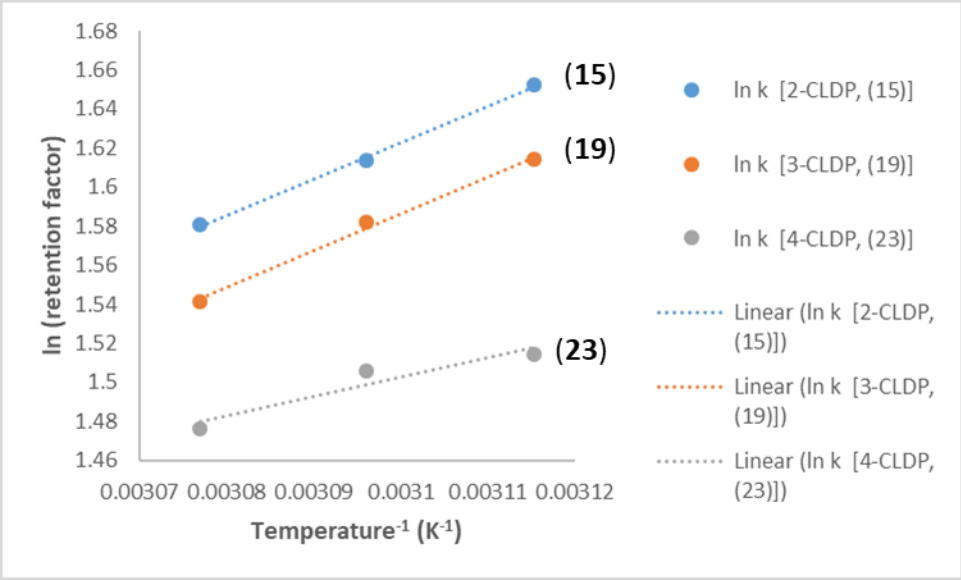
Injection	**t_R (min) using temperature 48°C**				**t_R (min) using temperature 50°C**				**t_R (min) using temperature 52°C**			
	14	**15**	**16**	**17**	**14**	**15**	**16**	**17**	**14**	**15**	**16**	**17**
1	4.19	5.96	6.90	8.56	4.17	5.91	6.81	8.41	4.13	5.86	6.75	8.33
2	4.19	5.97	6.91	8.57	4.16	5.90	6.81	8.41	4.13	5.85	6.75	8.32
3	4.18	5.96	6.90	8.56	4.17	5.90	6.80	8.40	4.13	5.86	6.76	8.34
4	4.18	5.96	6.90	8.56	4.17	5.91	6.82	8.42	4.14	5.87	6.77	8.34
5	4.18	5.97	6.91	8.57	4.16	5.90	6.81	8.41	4.14	5.87	6.77	8.36
6	4.19	5.98	6.92	8.58	4.17	5.91	6.81	8.41	4.13	5.86	6.75	8.34
7	4.17	5.96	6.90	8.56	4.16	5.90	6.80	8.40	4.14	5.86	6.76	8.34
8	4.19	5.97	6.91	8.57	4.17	5.91	6.82	8.42	4.14	5.86	6.76	8.34
9	4.18	5.97	6.91	8.58	4.17	5.91	6.82	8.41	4.14	5.86	6.76	8.34
10	4.19	5.97	6.71	8.59	4.16	5.88	6.78	8.36	4.14	5.86	6.76	8.34
Average	**4.19**	**5.97**	**6.89**	**8.57**	**4.17**	**5.90**	**6.81**	**8.40**	**4.14**	**5.86**	**6.76**	**8.34**
STD	**0.006**	**0.006**	**0.061**	**0.010**	**0.004**	**0.007**	**0.010**	**0.015**	**0.004**	**0.005**	**0.007**	**0.008**
%RSD	**0.149**	**0.112**	**0.887**	**0.120**	**0.110**	**0.129**	**0.157**	**0.188**	**0.107**	**0.094**	**0.105**	**0.102**



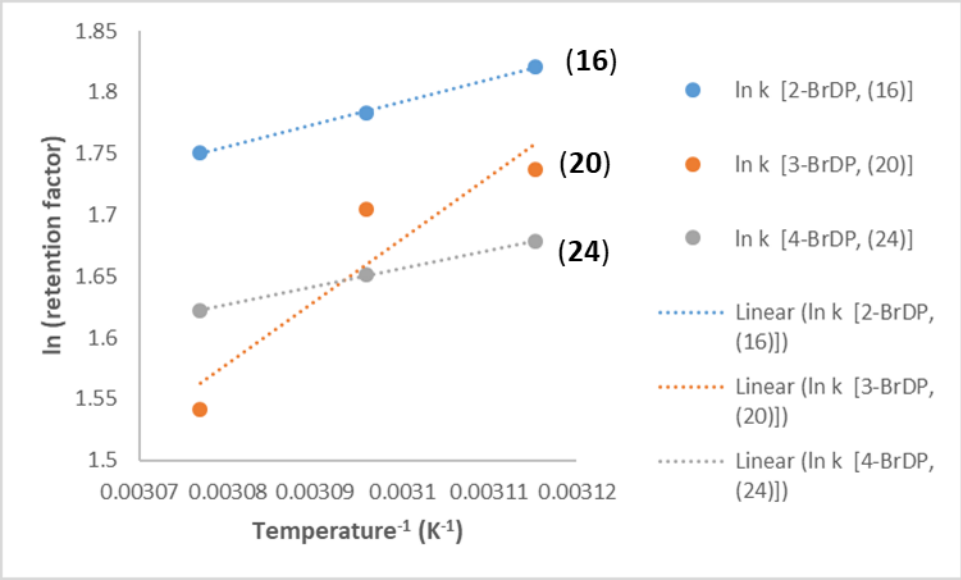
Appendix A 55. Van't Hoff plot of ln k versus 1/T for the 3-halogenated diphenidine isomers (**18, 19, 20** and **21**)



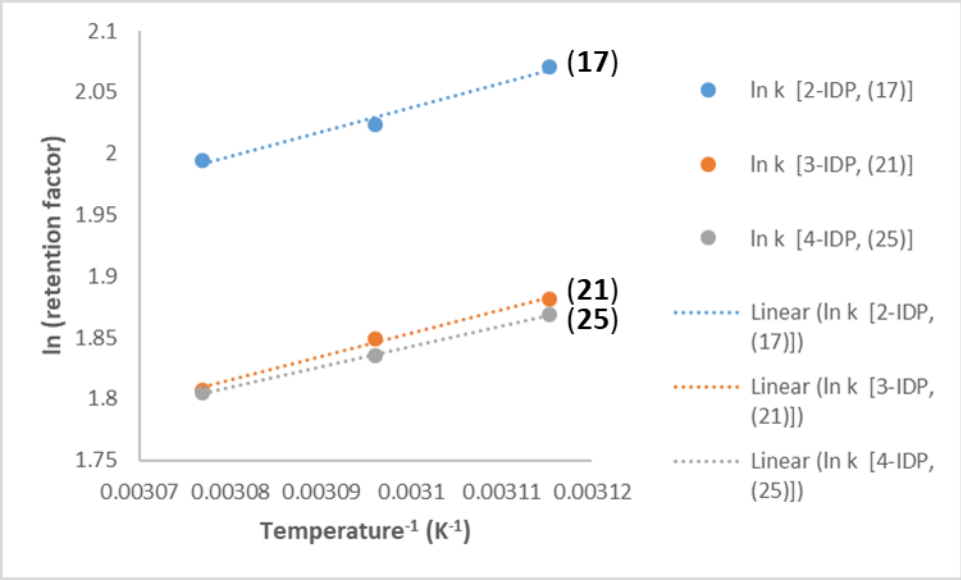
Appendix A 56. Van't Hoff plot of ln k versus 1/T for the 4-halogenated diphenidine isomers (**22**, **23**, **24** and **25**)



Appendix A 57. Van't Hoff plot of ln k versus 1/T for the chlorinated diphenidine isomers (**15**, **19** and **23**)



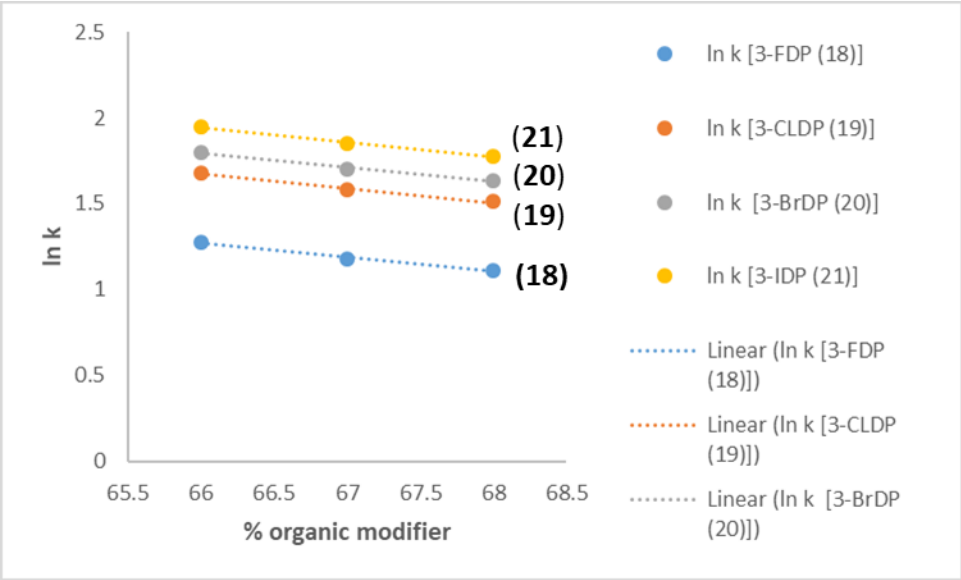
Appendix A 58. Van't Hoff plot of ln k versus 1/T for the brominated diphenidine isomers (**16**, **20** and **24**)



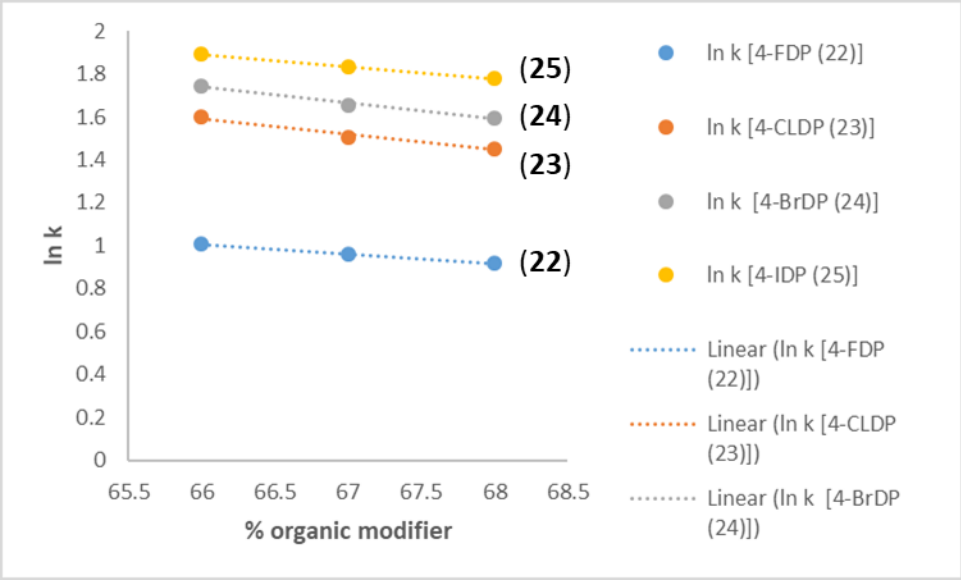
Appendix A 59. Van't Hoff plot of ln k versus 1/T for the iodinated diphenidine isomers (**17**, **21** and **25**)

Appendix A 60. Summary of robustness testing (% mobile phase (acetonitrile:ammonium acetate) = 66:34, 67:33 or 68:32% v/v) for the 3-halogenated diphenidine isomers (using 10 µg mL^{-1} concentration and 10 injections). Key: t_R = Retention time (min), RRT =Relative retention time.

Mobile phase (66:34% v/v)	**18**	**19**	**20**	**21**		**Mobile phase (66:34% v/v)**	**22**	**23**	**24**	**25**
t_R (min)	4.401	6.100	6.777	7.691		t_R (min)	3.697	5.711	6.465	7.582
RRT	0.721	1.000	1.110	1.260		RRT	0.647	1.000	1.131	1.327
Resolution	0	8.192	2.723	3.320		Resolution	0	10.765	3.260	4.228
Mobile phase (67:33% v/v)	**18**	**19**	**20**	**21**		**Mobile phase (67:33% v/v)**	**22**	**23**	**24**	**25**
t_R (min)	4.189	5.747	6.367	7.209		t_R (min)	3.543	5.399	6.092	7.123
RRT	0.728	1.000	1.107	1.254		RRT	0.656	1.000	1.128	1.319
Resolution	0	7.937	2.660	3.296		Resolution	0	10.334	3.134	4.105
Mobile phase (68:32% v/v)	**18**	**19**	**20**	**21**		**Mobile phase (68:32% v/v)**	**22**	**23**	**24**	**25**
t_R (min)	4.054	5.530	6.116	6.916		t_R (min)	3.501	5.273	5.934	6.917
RRT	0.733	1.000	1.105	1.250		RRT	0.663	1.000	1.125	1.311
Resolution	0	7.743	2.606	3.228		Resolution	0	10.061	3.053	4.062



Appendix A 61. ln k versus % organic modifier (acetonitrile) for 3-halodiphenidine isomers (**18**, **19**, **20** and **21**)



Appendix A 62. ln k versus % organic modifier (acetonitrile) for 4-halodiphenidine isomers (**22**, **23**, **24** and **25**)