“Using Isotopic Fractionation to Link Precursor to Product in the Synthesis of Diphenidine Hydrochloride: A Tool for Combating New Psychoactive Substances (NPSs)”

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"I declare that none of the work detailed herein has been submitted for any other award at Manchester Metropolitan University or any other Institution."

"I declare that, except where specifically indicated, all the work presented in this report is my own and I am the sole author of all parts. I understand that any evidence of plagiarism and/or the use of unacknowledged third part data will be dealt with as a very serious matter"

Signature F. H. Owen Date: 30-Jan-2017

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List of Abbreviations

Abbreviation	Definition
ACMD	Advisory Council on the Misuse of Drugs
AKB-48	*N*-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide
CAHID	Centre for Anatomy and Human Identifications
CD_2Cl_2	Deuterated dichloromethane
CH_2Cl_2	Dichloromethane
CN	Cyanide Ion
CNS	Central Nervous System
EI	Electron Impact Ionisation Energy
EMCDDA	The European Monitoring Centre for Drugs and Drug Addiction
EWS	EU Early Warning System
GCMS	Gas chromatography-Mass Spectrometry
H_2SO_4	Sulphuric Acid
HCl	Hydrochloric Acid
IR	Infrared Spectroscopy
IRMS	Isotopic Ration Mass Spectrometry
LCMS	Liquid chromatography-mass spectrometry
MDMA	3,4-methylenedioxymethamphetamine
MgBr	Magnesium bromide
$MgSO_4$	Magnesium sulphate
MXE	methoxetamine
MXP	Methoxphenidine
NaOH	Sodium Hydroxide
NH_4Cl	Ammonium Chloride
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NPS	New Psychoactive Substances
NRG-1	Naphyrone
PCA	1-(1-phenylcyclohexyl)amine
PCP	phencyclidine
PMMA	para-methoxymethamphetamine
*p*TSA	p-toluene sulphuric acid
TFA	Trifluoroacetic acid
U.K.	United Kingdom
WHO	World Health Organisation

1. Abstract

In recent years, there has been an unprecedented growth in the emergence of "legal highs", or as they are now commonly known, New Psychoactive Substances (NPS). In most cases, they mimic the effects of illicit substances, however, they circumvent legislation through changes in their chemical structures. NPS are being successfully sold through online venders under the titles "bath salts" and "research chemicals" and along with their low cost, are becoming an increasingly popular, convenient, alternative to controlled substances.

Diphenidine, is an example of a fourth generation NPS that has increased in popularity as a recreational dissociative drug since its appearance in 2013. Diphenidine poses a threat to public health and safety due to lack of legislative control but also through the lack of literature concerning its toxicity, chemical and physical data, with evidence showing diphenidine has attributed to several deaths already. It is becoming apparent that there is a need for a rapid and selective detection method in order to identify and control not only diphenidine, but other legal and illicit substances too. Isotopic Ratio Mass Spectrometry (IRMS) is an analytical technique that has flourished within the forensic community, having the potential to not only link diphenidine samples of the same manufacturer, but also differentiate between sample seizures. IRMS looks beyond chemical composition but instead, at the relative isotopic abundances of the elements composing the starting materials and final product. Trace impurities could be used as a method of source identification, as the impurity will be unique to the drug and its history of origin. This could potentially allow discrimination between synthetic routes used.

The main objective of the study is to determine whether IRMS can be used as tool to link product to precursor in the case of diphenidine hydrochloride, which if successful, would could have a wide variety of forensic applications for further NPS and illicit drugs.

After designing and implementing a new GC-MS method to determine the purity of diphenidine hydrochloride, whilst considering peak area normalisation calculations and percentage yield data, the optimum method for the synthesis of diphenidine

hydrochloride is F. This method uses 30 mmol piperidine and 1 hour stirring time under an inert atmosphere to yield diphenidine hydrochloride in much quantities with minimal trace impurities compared to the other methods employed.

From IRMS data it is possible to distinguish between sources of precursors except when two sources are owned by the same brand, i.e. Sigma Aldrich can be distinguished from Alfa or Acros, but Alfa and Acros products cannot be discriminated. There are also significant changes the isotopic rations for both carbon and nitrogen when the physical parameters for the synthesis are changed, notably when the reaction is performed under argon and the wen the molar equivalents of the precursors are increased, i.e. the mmol ration of piperidine/ Like the source differentiation results, there are substantial different between 10 mmol and 20 mmol ratios, but it is difficult to distinguish between 20 mmol and 30 mmol. In conclusion, it would be possible to link precursor to product and also the synthetic pathway used, but not with 100% certainty.

2. Introduction

2.1. New Psychoactive Substances

New Psychoactive Substances (NPS) is a term now used by the scientific community to identify substances, formally known as "legal highs", which are abused on the recreational drugs market.[1] In most cases, they mimic the effects of illicit and controlled drugs, for example, cathinones (**1**) (which mimic amphetamine substances such as 3,4-methylenedioxymethamphetamine (MDMA)) and synthetic cannabinoids (which mimic the effects of Δ^9-tetrahydrocannabinol, the principle active ingredient in cannabis), yet are lacking definite legislation control.[1]

Drugs of abuse can fall into one of three main categories: natural illicit drugs that are misused, for example cocaine and morphine; synthetic designer drugs specifically made to mimic illicit drugs, for example methoxetamine (MXE, **2**); and drugs that have previously been used for clinical purposes, for example, benzodiazepines.[2]

NPS, or as they were more commonly known as "legal highs", tend to fall into either the designer drugs or the clinical drugs category. Currently on the recreational drugs market, there are many classes of new psychoactive substances, including but not limited to: cathinones (e.g. mephedrone) phenethylamines (**3**, e.g. 4-methoxymethamphetamine, PMMA), piperazines (**4**, e.g. benzylpiperazine), synthetic cannabinoids (e.g. *N*-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide), AKB-48) and dissociative agents (e.g. piperidine (phencyclidine, PCP, **5**) and ketamine (**6**) derivatives), shown in Figure 1.[1, 3-5]

The fundamental concept when initially designing an NPS is to expand upon a previously known structure and by subtly changing it, for example, the addition of functional groups or side-chains, causing the drug to no longer be illegal by circumventing legislation, for example, the Misuse of Drugs Act (1971) in the UK.[1, 6-9] Identifying the psychoactive element of the drug and using the structure as a replicable template, the new psychoactive substance is designed to possess the same pharmacophore and target, consequently exhibiting similar psychoactive properties as

the well-known and studied illicit drugs. By this process, it is possible to create a limitless library of new psychoactive substances.

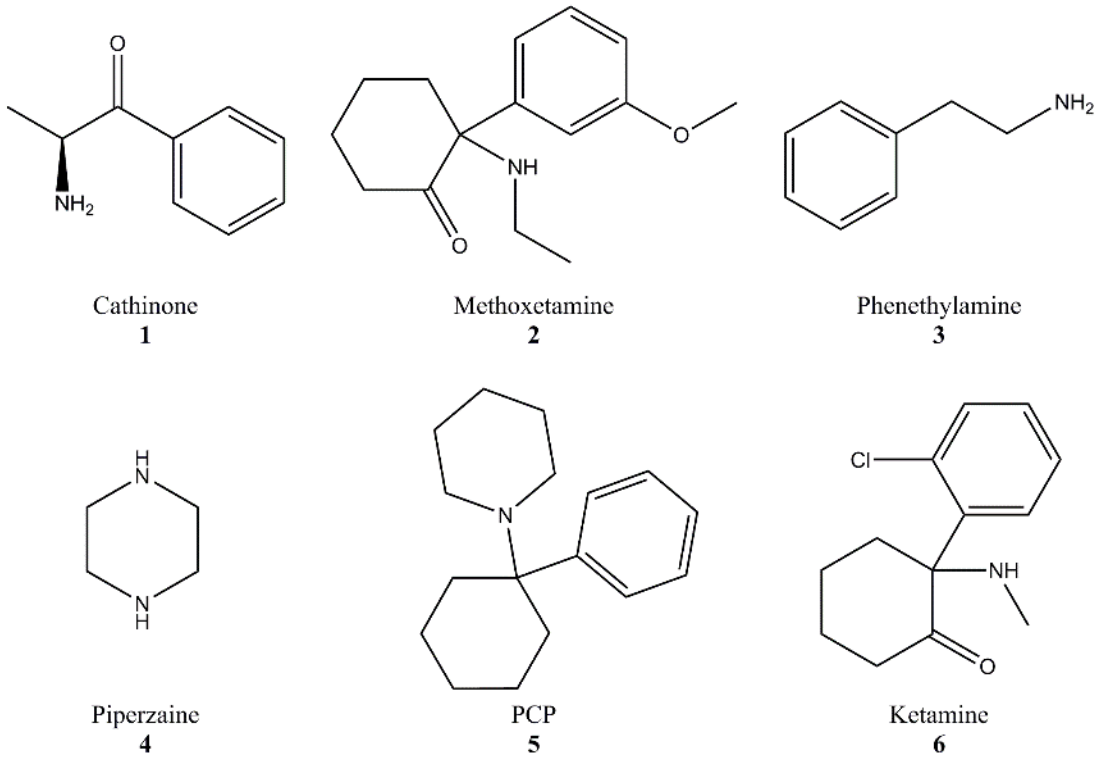

Figure 1. Several of the more common new psychoactive substances on the recreational drugs market.

With marketing playing a crucial role in NPS popularity,[8] they are sold under the titles "bath salts", "plant food", "research chemicals" and "not for human consumption" tactfully avoiding marketing regulations and therefore being legally sold, exploiting laws such as the United Kingdom Medicines Act (1968).[1, 2, 6-14] Because they are branded as "legal highs" and their wide availability through online suppliers and head shops combined with their low cost means they are becoming an increasingly popular and convenient alternative to controlled substances.[1, 2, 6, 7]

The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) through the EU Early Warning System (EWS), have reported a dramatic growth of the amount of legal highs emerging and their availability. With an increase of the amount of online venders now selling NPS, 693 documented in 2012, which is a 300% increase since 2010, it is not surprising that the EMCDDA documented 101 new substances in 2014 and a further 100 in 2015.[8, 9]

Lack of literature concerning their pharmacology, toxicity and analytical characteristics, for example physical and chemical data, means they pose a threat to society and human health.[8, 12-15] In most cases, their mechanisms of action are not fully understood and with evidence showing that they are commonly found in conjunction with other new or even controlled drugs, their potential for harm is increased.[8, 10, 14] Not only does this indicate that the contents are different to those stated, but if higher doses are required to achieve the same psychedelic effects, this could result in adverse effects being exhibited; including cognitive impairment, paranoia and possibly fatalities.[15] With limited analytical data available on these new psychoactive substances, they are not being detected in standard drug screenings.[3, 8] This adds to their appeal and broadens the consumer market, identifying that 8% of 15-24 year old in the U.K. have used "legal highs".[8]

Several recent deaths in the U.K.[11, 13, 16, 17] supported by toxicology reports and autopsy findings,[18, 19] suggest that new psychoactive substances have contributed considerably to the deaths; it is now apparent that legislation needs to adapt to this constantly evolving drug market. Through such evidence, it is now recommended that law enforcement agencies constantly monitor their use and prevalence,[20] prompting more rapid identification to the EWS and in turn their control.[8]

2.2. Drug Classification and Legislation

In the U.K., the first form of legislation began with the Misuse of Drugs Act (1971). Brought around in an attempt to control drugs, it categorises scheduled substances into either Class A, B, or C. Each class comes with varying levels of legal control based on the substances' potential for being misused, whether it is being misused and the harmful effects associated with the substance and whether these would constitute to be a social problem, with the overall aim to limit their distribution and usage.[21]

As of April 2010, many cathinones and synthetic cannabinoids were brought under the Misuse of Drugs Act (1971) in the U.K.[1, 2, 6-11] This change in legislation was expected to decrease the popularity and use of such drugs, however, prompted the emergence of second, (e.g. Naphyrone, NRG-1, **7**), third (e.g. MXE, **2**) and fourth generation (e.g.

diphenidine, **8** and methoxphenidine, **9**) NPS, shown in Figure 2, to flood the recreational drugs scene.

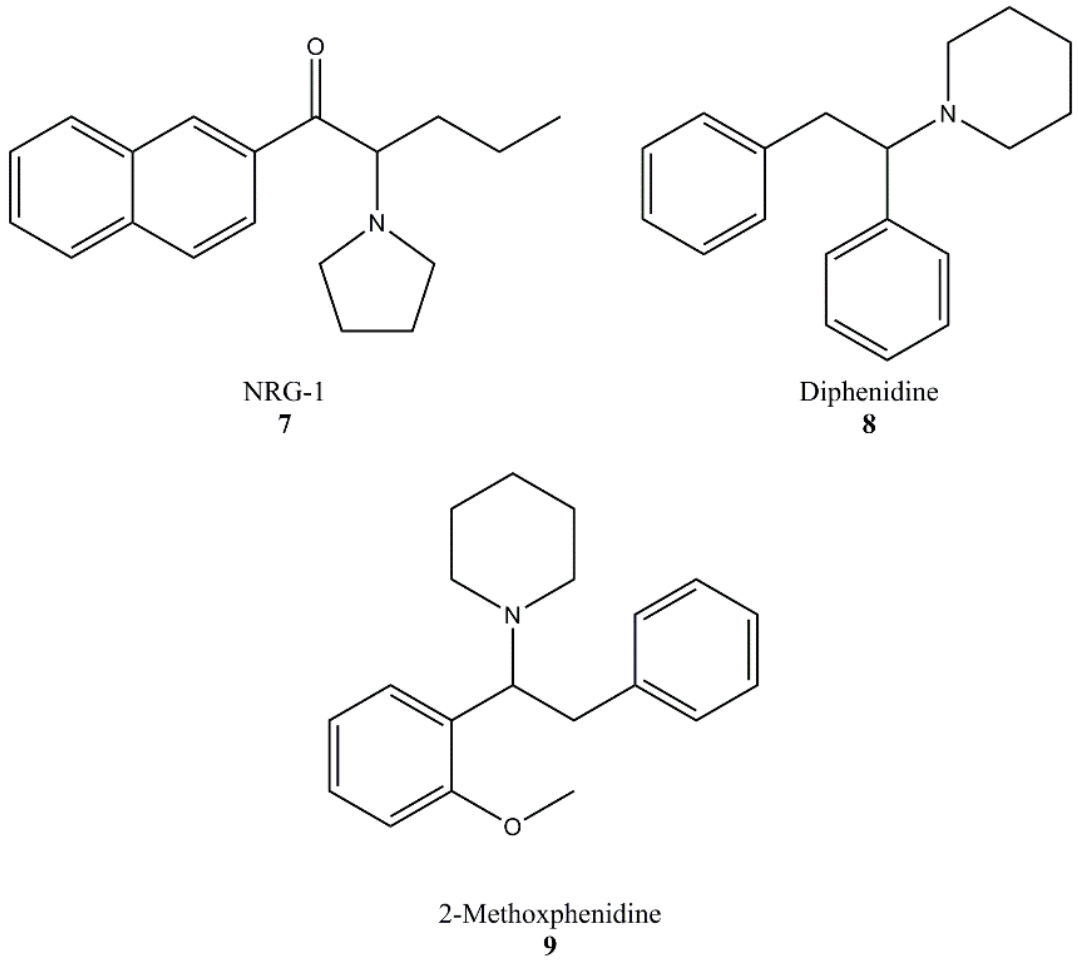

Figure 2. Examples of second (NRG-1, **7**) and fourth generation (diphenidine, **8** and methoxphenidine, **9**) NPS that have begun to populate the recreational drugs scene.

With NPS emerging in great diversity and at speed, it has caused legislation to evolve. Initially managing NPS under existing drug legislation and consumer safety laws allowed temporary drug orders to come into place quickly and can last up to one year, in which time thorough investigation into their harm and threat to society can occur. The U.K. has initiated a "generic legislation system" where a precise definition of a family of substances is created in order to group substances together in terms of their chemical structure. This attempts to control the emergence of newly emerging derivatives and analogues.[8]

In 2013, the U.K. implemented an arylcyclohexylamine ban, causing recreational dissociative agents to become illegal, consequently creating a gap in the market. This

gap prompted a new class of dissociative agent to emerge, diarylethylamines, for example, diphenidine (**8**) and 2-methoxphenidine (**9**), which currently remain legal under U.K. law.

As of January 2016, the U.K. brought in the Psychoactive Substances Act, defining a psychoactive substance as "a substance produces a psychoactive effect in a person if, by stimulating or depressing the person's central nervous system, it affects the person's mental functioning or emotional state". Though the Act doesn't specifically criminalise possession (unless within a custodial setting), it effectively makes it a criminal offence to produce, supply, offer to supply, possess with intent to supply, or import or export psychoactive substances thereby hopefully restricting the circulation of potentially dangerous psychoactive substances. Though the Act has successfully shutdown the sale of NPS through headshops and/or specialist internet sites, it is unclear, at present, how effective the legislative change will be on curtailing the illegal sale (through organised crime) and the variety of substances in circulation.

2.3. Clinically Abused Drugs

Many pharmaceuticals, such as anaesthetics and benzodiazepines, are clinically abused drugs, which have become increasingly popular in recent years. Usually obtained *via* prescription or are readily available over the counter at pharmacists, they are being used as recreational drugs and not for their originally intended use. Clinical drugs that have been previously researched and tested are now emerging under the title "research chemicals" and are contributing heavily to the research chemical market.

One of the more notable classification of clinical drugs to be abused began to emerge in the 1950s, the arylcyclohexylamine family. Structurally composed of a cyclohexamine unit with an aryl unit attached geminally, this group of substances is collectively known as "dissociative anaesthetics", originally employed as pharmaceutical anaesthetics before becoming drugs of abuse.

2.4. Dissociative Agents

2.4.1. NMDA Receptor Activity

The most recognised pharmacological feature associated with dissociative agents is their ability to act as *N*-methyl-D-aspartate receptor antagonists (NMDAR), acting upon the NMDA receptors, shown in Figure 3, inducing anaesthesia. NMDA receptors are fundamental for mediating neurotransmitters within the central nervous system (CNS), in particular glutamate. Here, they can initiate intracellular pathways, consequently affecting neuronal activity.[10, 22]

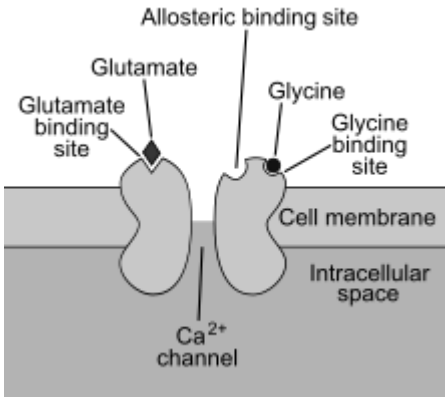

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Figure 3. An example of the activated NMDA receptor (**10**) showing how glutamate is within the glutamate -binding site.

It is well documented that dissociative agents, like PCP and its derivatives, act as competitive inhibitors, binding to these receptors and causing normal neurotransmitter activity to cease.[22]

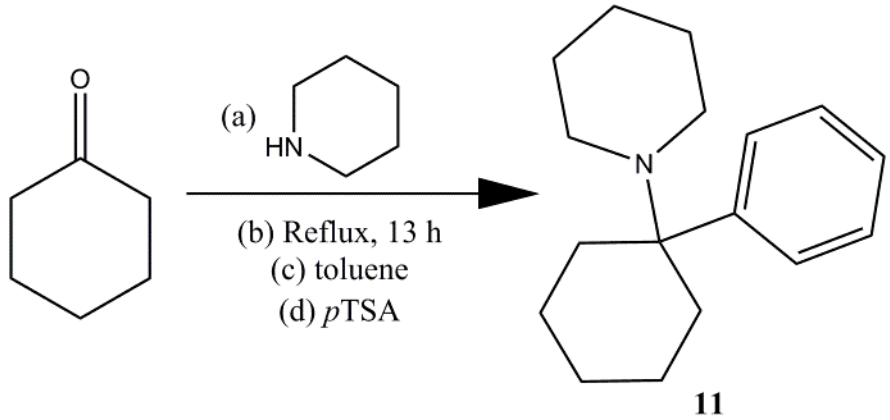
NMDAR activity plays a vital role in physiological processes, for example neurophysiology.[23] It is the NMDAR antagonism *via* open channel blockade[10, 14, 22, 24] that is suspected to be the fundamental pharmacological mechanism contributing towards the "dissociative" and dream like states,[25] however, the degree of dissociation experienced is suspected to be dependant entirely on dose.[14, 16, 22]

At low doses, memory improvement and stimulation occurs, however, the well-known adverse effects include short-term amnesia (memory loss), analgesia (pain relief) and altered cognition are experienced at high doses. It is also documented that perceptual alterations, hallucinations, ataxia (loss of control of bodily movements) and paraesthesia (pins and needles/tingling) occur, regardless of dose.[25] In some cases,

tachycardia and hypertensions have been reported[26] suggesting that the NMDAR action is not limited to the CNS but stretches to the circulatory system too.

2.4.2. Phencyclidine

1-(1-phenylcyclohexyl)piperidine hydrochloride salt (**11**), also known as phencyclidine or PCP, was the first arylcyclohexylamine synthesised in 1956 by Victor Maddox of Parke-Davis, a subsidiary of the pharmaceutical corporation, Pfizer.[25, 26] Maddox unknowingly performed a Bruylants amination, however, literature disclosed only the synthesis of the immonium intermediate, and in order proceed, the intermediate must undergo a substitution reaction with potassium cyanide and phenylmagnesium. The suspected synthetic pathway is shown in Scheme 1.[25, 26]



Scheme 1. A possible synthetic pathway for the original synthesis of PCP in 1956.[26]

The original analogue of (**11**), 1-(1-phenylcyclohexyl)amine (PCA, **12**), shown in Figure 4, was synthesised as early as 1907 where it was established to be a potent sedative, however, studies were discontinued and (**12**) was consequently dismissed as a pharmaceutical agent.

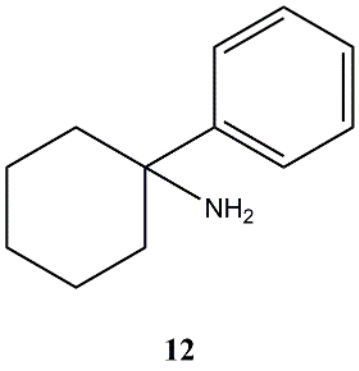


Figure 4. PCA, the original analogue which led to the synthesis of the derivative, PCP **(11)**.

It was not until 1957 when clinical trials for (**11**) started, where the potential of such dissociative agents as effective anaesthetics became apparent. Soon afterwards, (**11**) became a surgical anaesthesia, due to its actions on NMDA receptors. However, in 1965, it was discontinued as a pharmaceutical drug because the majority of patients experienced adverse side effects, including psychosis and agitation.[14, 25]

Since PCP's withdrawal as an anaesthetic, it became regarded as a hallucinogenic drug and has thrived as a drug of abuse since the 1960s, where it is often mixed with other illicit substances and subsequently intensifying the toxicity. With idealistic side effects such as auditory hallucinations, altered perception and euphoria being experienced, its popularity has shown an overall increase.[14]

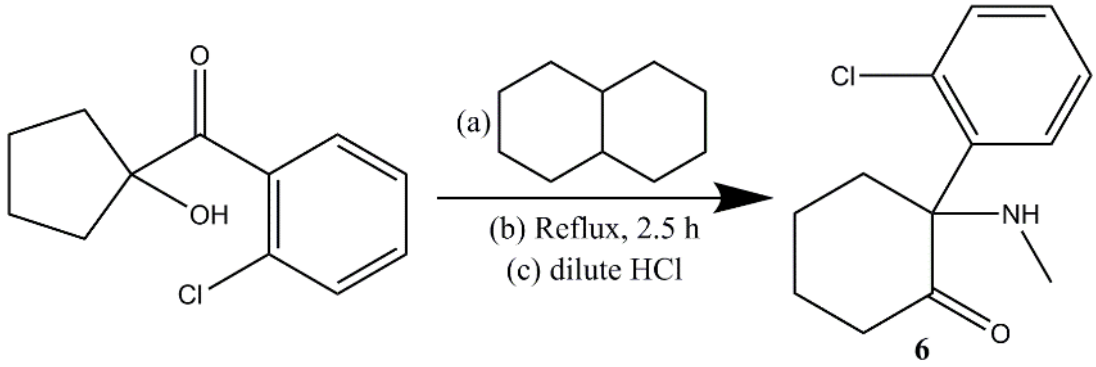
Evidence also reports PCP users experiencing convulsions, delirium, along with serious neurological, cardiovascular and psychotic reactions, ultimately leading to psychotic behaviour.[14, 25] Through this, and in addition to, a vast amount of media coverage, a change in legislation was prompted, with PCP becoming classified as a Class A drug in the U.K, and subsequently, derivatives of this illicit drug, such as ketamine (**6**), has begun to populate the recreational drug scene.[25]

2.4.3. Ketamine

An example of such a PCP derivative is the ketamine hydrochloride salt (2-(2-chlorophenyl)-2-(2-methylamine)cyclohexanone), (**6**), which was first synthesised in 1962 by Calvin Steven's of Parke-Davis, see Scheme 2. Once pharmacologically evaluated, (**6**) was established to be a fast, though short-acting, general anaesthetic to be used for both human, in particular paediatric, and veterinary practises.[25, 27] However, it was not until 1969 that (**6**) became a commercial anaesthetic, sold under the brand name Ketalar.[25]

Categorised in 1985 as an essential drug by the World Health Organisation (WHO), (**6**) is a popular anaesthetic agent and pain relief in developing countries. With having a rounded safety profile,[27] in addition to being easily administered, it is often utilised in the sedation of both adults and children, particularly where medical equipment and conditions are lacking. Ketamine has a wide range of therapeutic applications

including depression and psychiatric treatment, which has led to multiple studies investigating the use of ketamine as a treatment for depression and anxiety.[1, 25, 29]



Scheme 2. 1-hydoxycyclopentyl-(o-chlorophenyl)-ketone-*N*-methylamine (obtained *via* a Grignard reagent) reacting with decalin under acidic conditions to produce **6**.[28]

Despite the apparent positive applications of ketamine, it has become one of the most popular drugs of abuse not only in the U.K., but globally, and has grown in popularity over recent years.[1, 25] Ketamine's primary pharmacological mechanism is *via* the antagonism of the NMDA receptor,[22] similar to that of PCP, which has been a target of exploitation when used as a recreational drug.

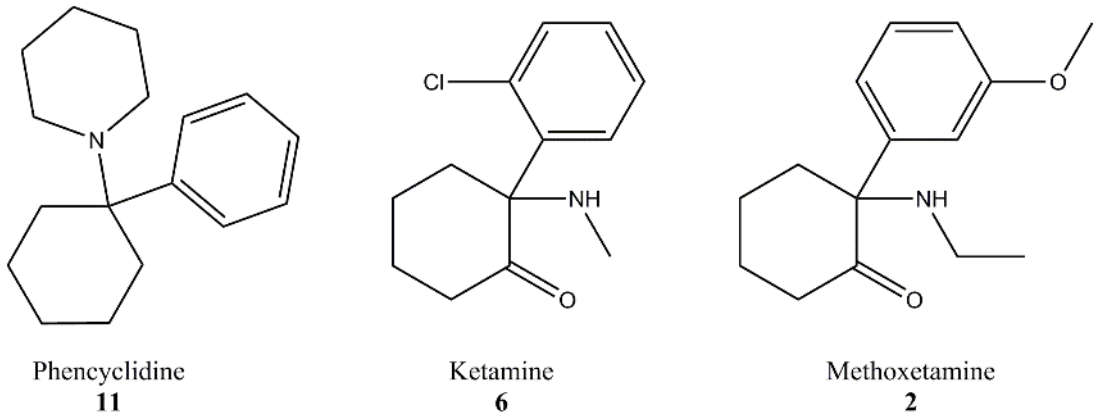
Ketamine users report feelings of "depersonalisation" once consumed, commonly referred to as a "k-hole", often experienced in conjunction with hallucinations and altered perception of realities. These idealistic effects, along with being readily and cheaply available, has caused ketamine's abuse to increase greatly over recent years. Nevertheless, as with all drugs, adverse effects are common. When high doses of ketamine are consumed, adverse effects include; tachycardia, hypertension, and amnesia. Recent literature has linked ketamine use to haematuria, bladder shrinkage and degradation.[1, 25, 27, 29]

Through the discovery of the latest adverse effects and with the Advisory Council on the Misuse of Drugs (ACMD) reporting a recent increase in the illicit use of ketamine, it regained its Class B status in the U.K.[25]

2.4.4. Methoxetamine

The scheduling of ketamine (**6**) caused a third generation of NPS to emerge, 2-(3-methoxyphenyl)-2-(ethylamino)cyclohexanone, more commonly referred to as methoxetamine (MXE, **2**), which is marketed as the new, legal and bladder safe alternative[27] to its predecessor with its appearance in the U.K. first reported in 2010 by the EMCDDA. [25, 30]

In terms of chemical structure, it remains an arylcyclohexylamine class and is considered to be a structural derivative of ketamine, whereby a 3-methoxy group has replaced the 2-chloro attached to the phenyl ring and the *N*-methyl group replaced by an *N*-ethyl group.[10, 27] Although to the eye they are minor modifications, the entire pharmacology is changed, causing methoxetamine (**2**) to be a more potent and longer lasting hallucinogenic drug than the ketamine parent.[10, 25] Figure 5 shows the development of structures from PCP through to ketamine followed by MXE.



Phencyclidine **11** Ketamine **6** Methoxetamine **2**

Figure 5. The development of structures from the original dissociative anaesthetic **11**, followed by derivative **6**, and then to the designer drug **2**.

Initially marketed as a "research chemicals" or "not for human consumption",[10] methoxetamine (**2**) quickly increased in popularity on a global scale through users experiencing similar psychedelic effects produced by the previous recreational dissociatives. For example, hallucinations, depersonalisation and euphoria are commonly reported.[10, 27]

Although designed to have a similar safety profile to that of ketamine by retaining the 2-keto functional group,[25] there have been an increasing amount of scientific

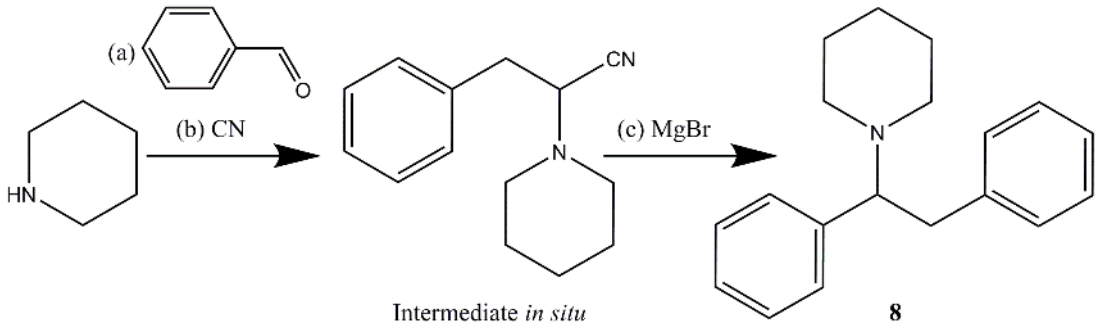
publications documenting evidence on (**2**) intoxication and related fatalities,[16] with 110 non-fatal intoxications reported by the EMCDDA in 2012 alone.[27, 31] Adverse effects associated with higher doses of this designer drug include; anxiety, aggression, ataxia, hypertension, violence and agitation.[10, 27]

A potential, yet significant, concern for public health and safety is that (**2**) has an increased potency and if consumed in the same dose as ketamine, accidental intoxication could occur ultimately resulting in fatalities.

With evidence mounting and the growing need for legislation to control this NPS, it has since been classified as a Class B drug in the U.K. as of February 2013.[25] Illegal across Asia and Europe too, it was predicted that the appearance of more legal highs would start to influx the recreational drugs scene.[10]

2.5. Diphenidine

Change in legislation through the restriction and banning of arylcyclohexylamine derivatives in February 2013 prompted a new dissociative class of diarylethylamines to emerge onto the research chemical (RC) market, as little as 2 weeks later.[25] 1-(1,2-diphenylethyl)piperidine or more commonly known as diphenidine hydrochloride (**8**) and its derivative 1-(1-(2-methoxyphenyl)-2-phenylethyl)piperidine, MXP (**9**, see Figure 2) both fall under this classification and are known as a legal replacement for MXE and other PCP-derived drugs.

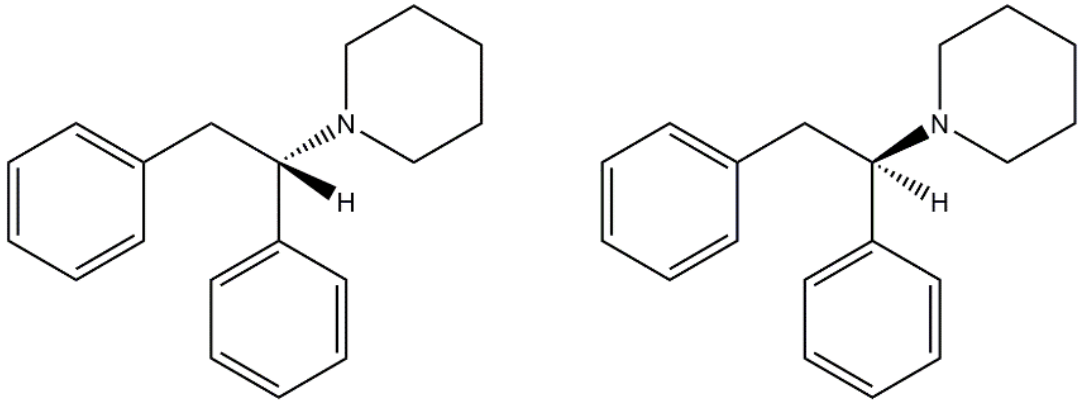


Scheme 3. A predicted scheme of how the diphenidine hydrochloride salt would be synthesised if following the Bruylants amination, showing the predicted experimental conditions and intermediate.

Diphenidine hydrochloride was originally synthesised in 1924 *via* the Bruylants amination, utilising organometallic Grignard reagents, shown in Scheme 3, which is a

similar preparation to that which was later used by Maddox in the synthesis of PCP in 1956, see Scheme 1.[25]

Similar in structure to that of illicit dissociatives, it is not surprising that pharmacological studies have identified NMDAR activity as the primary method of action of (**8**). However, the exact binding has not been fully explored but evidence does suggest that the *S*-enantiomer, shown in Figure 6, is more potent, showing a higher affinity for binding.[32]



(*S*)-Diphenidine
8

(*R*)-Diphenidine
8

Figure 6. The two enantiomers of diphenidine hydrochloride (**8**), *S* and *R*

The subtle differences in terms of structure that cause diphenidine hydrochloride to be classified as a diarylethylamine also allows diphenidine hydrochloride to circumvent legislation, remaining legal to possess and sell. Being sold by online vendors as a legal replacement for MXE, with users reporting similar psychedelic effects that of arylcyclohexylamines, such as strong dissociative effects and "k-hole" like experiences.[25]

Of recent years, since the appearance of diphenidine in 2013,[25] its popularity as a drug of abuse has increased significantly, especially in Japan.[18, 19, 33] When taken in relatively low doses, diphenidine seems to present a low threat to public health and safety with mild effects reported, but when administered in higher doses, severe adverse effects are experienced which could even result in death. This is supported by toxicology and post-mortem results that report diphenidine to be a causative agent in several fatalities.[18, 19]

An example of such an outcome occurred in Japan, where diphenidine was found in a sample of "herbal incense", amongst with several synthetic cannabinoids, including AB-CHMINACA. After a 30-year old male died from inhaling "herbal incense, the super lemon", post-mortem results determined that the cause of death was through diphenidine poisoning. There was a significantly high concentration found in the adipose tissue (~11,000 ng/g mL^{-1}), where the diphenidine remained to be in its unchanged form and in a concentration which was obviously too high for the body to metabolise, especially when in conjunction with other synthetic drugs.[19]

Through such evidence, it is being recommended that law enforcement agencies should make a conscious effort to monitor the usage of diphenidine for harm-reduction purposes, as there is clearly a large risk to public health and safety.[18, 20] With little analytical data available in scientific literature,[1, 6, 25] the trafficking of this legal substance is becoming an increasingly difficult task. To meet demand, there is a need for a rapid and selective detection method in an attempt to identify and control not only diphenidine, but also other derivatives and novel drugs of abuse.

2.6. Isotopic Ratio Mass Spectrometry

One method by which the reduction in trafficking, not only diphenidine, but also illicit substances, can potentially be achieved is through the application of Isotopic Ratio Mass Spectrometry (IRMS) as a method of source identification. Until recently, IRMS is a technique that has been previously overlooked as a tool for the analysis of controlled drugs. However, as this is one of the largest areas of active development, along with a significant increase of publications focusing on IRMS, it has become widely use amongst many disciplines and has particularly flourished within the forensic science community. [34-36]

With the unprecedented growth of illicit drugs and NPS on not only a national, but a global scale, the demand for a technique that would allow seized drugs to be differentiated by source, has significantly increased.[11, 36, 37] The development of drug profiling and intelligence gathering, which are methods used to aid and support crime scene investigations, have the potential to identify samples of the same, and theoretically, different sample seizures.

More advanced than simpler analytical methods, such as Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS), IRMS has opened the possibility of not only discriminating the geographical provenance of the drug but also the precursors used, synthetic route and even product batches, all of which data that before has been unobtainable. A similarity of these applications is that they depend on the exploitation of stable isotopes in order to deduce a trace source.[11, 34-36]

The fundamental concept of IRMS as an analytical technique is that it looks beyond the chemical composition of the compound, but rather at the isotopic composition, in this case, a synthetic drug, which is extremely desirable as a forensic tool.[34, 36]

The relative natural isotopic abundances of the chemical elements in the precursors used to synthesis the compound, for example $^{13}C/^{12}C$ and $^{15}N/^{14}N$ are expected to remain constant throughout the synthesis, until a chemical or physical process alters them. If changes are observed, this would potentially be due to reaction specific isotopic fractionation.[11, 35, 36]

Trace impurities have the potential to play a crucial role in changing the isotopic ratios of the compound and can be created from an array of sources: i.e. in the original precursors, during the synthesis, handling and environmental factors. All of these factors, if they change the relative isotopic abundances, could potentially be traced and are common amongst drugs synthesised in clandestine laboratories, where health and safety and quality control standards are lax in comparison to pharmaceutical companies.[11]

A notable example of such methods was in North-Korea, whereby 20 samples of heroin were seized. The nitrogen isotopic ratios of the samples were calculated then compared to a library of over 200 authentic samples. Despite there being no matches to that on record, it could be determined that the seized samples were likely to have been from a new source.[36, 38]

Another area of investigation using IRMS is linking precursors to product in the synthesis of mephedrone. It was suggested that the natural abundance of the chemical elements in the precursors would be integrated into the intermediate and consequently,

the final product. Samples of mephedrone were synthesised using precursors from two separate suppliers and throughout each synthesis, the $\delta^{13}C$ and $\delta^{2}H$ values were measured for each of the precursor, intermediate and final product. Although there were little differences in the $\delta^{13}C$ (%) between the precursors, there was a notable difference between the $\delta^{2}H$ (%). The changes have possibly given a quantifiable link between precursor and product, providing a foundation for future work in which IRMS could provide more than just the synthetic route, but a potential link to the precursor and/or manufacturer.[11]

Other examples of this methodology in the forensic discipline has already been applied to the geo-location of heroin and cocaine,[39] and the profiling of methamphetamine samples.[40].

3. Aims

Diphenidine (isolated as its corresponding hydrochloride salt) is isolated through a zinc-mediated Barbier-type reaction involving benzyl bromide, benzaldehyde and piperidine. Although very similar to a Grignard reaction, this reaction can, however, be performed in "one pot" with the organometallic intermediate created *in-situ*.

The main objective of the study is to determine whether IRMS can be used as tool to link product to precursor which if successful, would could have a wide variety of forensic applications. Diphenidine hydrochloride will be synthesised *via* the Barbier reaction, using only three different sources of the precursors (batch specific). Changing only these variables whilst keeping the physical paraments controlled could allow for the discrimination between different supplies of the precursors and thereby potentially determine the source of precursors within a seized sample through the application of IRMS. The physical paraments of the synthesis can then also be investigated, example, moisture/oxygen, stirring time and the order of addition of the reagents and the affect these changes have on first the yield then isotopic ratio of the products. This will provide insight into whether the physical parameters used in a chemical synthesis affect the isotopic ratio and whether these changes can be used as a *"fingerprint"* by which we can determine the method by which a sample of diphenidine hydrochloride has been produced. With only two publicised methods to synthesise diphenidine hydrochloride and currently clandestine methods are unknown, the impurities and IRMS produced for both synthetic pathways need to identified which would provide a reference for when seized samples are analysed. Impurity profiling *via* this method would enable the synthetic pathway used by clandestine laboratories to be identified and the intrinsic characteristics associated with each sample.

In each variation of the parameter conditions, the product will be prepared in batches of six replicates, each of the samples will be analysed by GC-MS using an in-house developed method before being sent for IRMS analysis. Though the precursors and products are not controlled under current U.K. legislation, the synthesis will be carried out in strict accordance with Home Office guidelines (under licence) and the samples stored securely in compliance with School of Science and Environment procedures and practices.

4. Experimental

4.1. Materials and Equipment

All chemicals were obtained from either Sigma Aldrich, Acros Organics or Alfa Aesar and were between 98% and 100% pure and were used without purification. NMR spectra, including ^{1}H and ^{13}C spectra, were acquired on a JEOL AS-400 (JEOL, Tokyo, Japan) NMR spectrometer operating at a proton resonance frequency of 400 MHz. Samples were prepared via 10-20 mg of sample in 750 μL of deuterated solvent/ TFA 0.03 %. Infrared spectra were obtained in the range of 400 – 4000 cm^{-1} (8 scans) using a ThermoScientific Nicolet iS10ATR-FTIR instrument (ThermoScientific, Rochester, USA). High-resolution mass spectra were recorded on an Agilent 1260 infinity LC coupled to a 6540 UHV accurate mass Q-TOF mass spectrometer by looped injection using electrospray ionisation (ESI, collision energy: 15 eV). Melting points were acquired using Gallenkamp 5A 6797 apparatus and are uncorrected.

4.2. The synthesis of diphenidine hydrochloride (8)

4.2.1. Exemplar synthesis of diphenidine hydrochloride, Method A

An oven dried 100 mL round-bottom flask was charged with acetonitrile (40 mL) and zinc dust (2.0 g, 30 mmol). Trifluoroacetic acid (TFA, 0.2 mL) and benzyl bromide (0.4 mL, 3 mmol) were added and the resulting solution stirred for 5 mins. Benzyl bromide (3.0 mL, 25 mmol), benzaldehyde (1.2 mL, 11 mmol) and piperidine (0.9 mL, 10 mmol) were introduced to the mixture and the solution stirred at room temperature for 1 h. The resulting solution was then quenched with saturated aqueous ammonium chloride solution (NH_4Cl, 150 mL) and extracted with dichloromethane (CH_2Cl_2, 2 x 100 mL). The organic layers were combined, dried over anhydrous magnesium sulphate ($MgSO_4$) and concentrated *in vacuo*. Diethyl ether (150 mL) was added to the resultant oil and after complete dissolution, concentrated sulphuric acid (H_2SO_4, 0.75 mL) was added dropwise to the stirred solution and allowed to react for 5 min. After decanting the sulphuric acid, the remaining precipitate was then dissolved upon addition of 5% sodium hydroxide aqueous solution (NaOH, 100 mL) before being extracted with dichloromethane (CH_2Cl_2, 2 x 100 mL). The combined organic fractions were dried over magnesium sulphate ($MgSO_4$) and concentrated *in vacuo* to give an amber oil. The resultant yellow oil was then dissolved in diethyl ether (200

mL) before adding hydrochloric acid in dioxane (4 M, 3 mL) dropwise, whilst stirring. Upon repeated trituration with ice cold acetone, a white powder was yielded (0.45 g, 17%).

Mpt: 210 – 213 °C. IR (ATR-FTIR, cm $^{-1}$): 3644 (N-H stretch), 3030 (alkyl C - H stretch), 2933 (C-H stretch), 2365 (NH^+), 1604 (aromatic C=C bend), 1494 – 1453 (aromatic C-C stretch). LC-MS (ESI+, 70 eV: m/z = 266.1909. GC-MS (EI, 70 eV): t_R 5.70 min; m/z = 91, t_R 14.61 min; m/z = 174. ^{1}H NMR (400 MHz, CD_2Cl_2) δ ppm: 12.45 (NH+, br, 1H), 7.02 – 7.39 (Aromatic, CH, m, 10H), 4.10 – 4.23 (CH, d, 1H), 3.8 – 3.9 (CH_2, t, 2H), 3.3 – 3.6 (CH_2, m, 3H), 2.18 – 2.60 (CH_2, m, 4H), 1.80 – 1.88 (CH_2, m, 4H), 1.25 – 1.28 (CH_2, t, 2H). ^{13}C NMR (400 MHz, CD_2Cl_2) δ ppm: 126.91 – 135.90 (Ar), 73.05 (CH), 48.88 (CH_2), 36.88 (CH_2), 22.86 (CH_2), 22.39 (CH_2).

The specific methods for batches (B – H) are provided in the supplementary information. A summary of the modifications is provided in Table 1, below.

4.2.2. Synthesis of N-benzylpiperidine hydrochloride

To an oven dried 50 mL round bottom flask, equipped with stirrer bar, diethyl ether (25 mL) was added before benzylpiperidine (0.5 mL, 11.4 mmol) was introduced, dropwise. The mixture was concentrated *in vacuo* to yield a viscous, colourless oil. The resultant oil was fully dissolved in diethyl ether (10 mL) before adding HCl in dioxane (4 M, 0.5 mL) dropwise, whilst stirring. The resultant mixture filtered to yield a white crystalline powder (0.8 g, 40.1%).

Mpt: 6 - 7 °C. GC-MS (EI, 70 eV): t_R 5.80 min; m/z = 91. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 12.44 (NH+, br, 1H), 7.2 – 7.8 (Aromatic, m, 5H), 4.10 (d, 2H, J = 8 Hz), 3.36 (d, 2H, J = 12 Hz), 2.57 (q, 2H, 12 Hz), 2.16 (t, 2H, J = 16 Hz), 1.80 (t, 3H, J = 20 Hz, J = 16 Hz,), 1.3 (q, 1H, 4 Hz). ^{13}C NMR (400 MHz, CD_2Cl_2) δ ppm: 129.12 – 131.95 (Ar), 60.92 (CH), 52.84 – 54.38 (CH_2), 36.88 (CH_2), 22.86 (CH_2), 22.40 (CH_2).

Table 1. All the parameters that were changed but also kept constant throughout the different methods. For the detailed experimental method, please see the supplementary information.

Method	Physical Parameters
A	Order of addition without argon flush, piperidine added last, stirring time = 60 mins, temp = RT. Changing sources of precursors.
B	Order of addition without argon flush benzaldehyde, stirring time = 60 mins, temp = RT.
C	Order of addition with argon flush, piperidine added last, stirring time = 60 mins, temp = RT
D	Order of addition with argon flush, benzaldehyde added last, stirring time = 60 mins, temp = RT.
E	Order of addition: benzaldehyde last with argon flush, stirring time = 60 mins, temp = RT, mmol piperidine = 20 mmol.
F	Order of addition: benzaldehyde last with argon flush, stirring time = 60 mins, temp = RT, mmol piperidine = 30 mmol.
G	Order of addition: benzaldehyde last with argon flush, stirring time = 120 mins, temp = RT.
H	Order of addition: benzaldehyde last with argon flush, stirring time = 30 mins, temp = RT.

4.2.3. Gas Chromatography Mass Spectroscopy

Gas Chromatography Mass Spectrometry spectra were recorded on an Agilent 6890 gas chromatograph with split injection (sample volume: 1 μL, split ratio 20 mL min^{-1}) and a HP-5MS column (30 m x 0.25 mm, 0.25 μm film thickness). Sample preparation: 1 mg of sample in 1 mL of methanol (spiked with an internal standard, eicosane at 1 mg in 1 mL methanol). Helium was used at the carrier gas at a flow rate of 1 mL min^{-1}. The gas chromatogram was coupled to an Agilent 5973 (El, 70 eV, TIC mode scanning m/z 50-500) and injector port was set at 280 °C and transfer line at 280°C.

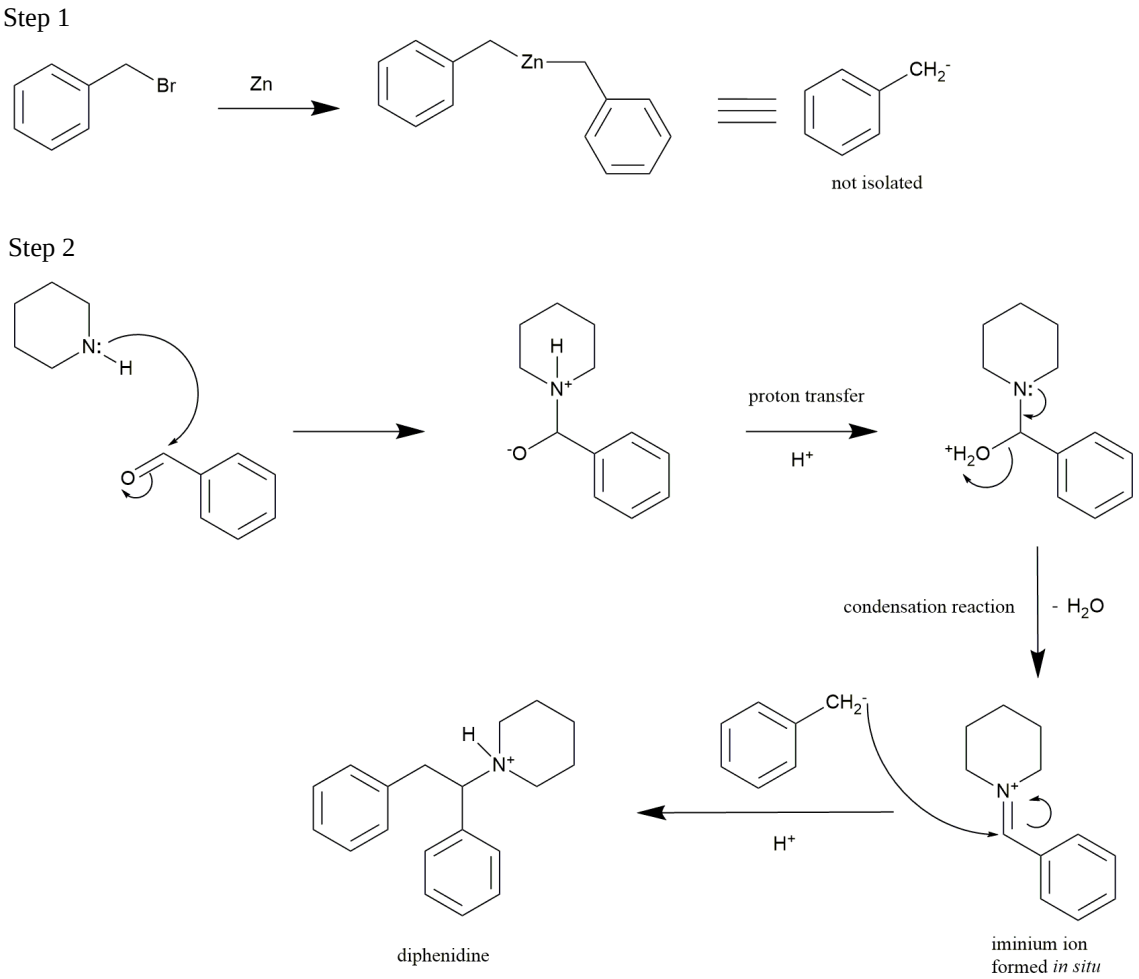
The oven temperature programme was 120 °C for 3 min, 20 °C min^{-1} to 170 °C, 170 °C for 1 min, 10 °C min^{-1} to 210 °C, 210 °C for 1 min, 20 °C min^{-1} to 250 °C, 250 °C for 1 min. A 1 μL aliquot of the samples were manually injected with a split ratio of 20:1. The injector and the GC interface temperatures were both maintained at 280 °C respectively. The MS source and quadrupole temperatures were set at 230 °C and 150 °C respectively. Mass spectra were obtained in full scan mode (50-550 amu).

Sample preparation: 100.00 mg of analytes were weighed accurately into a 100.00 mL clear glass volumetric flask and diluted to volume with methanol to give a soluation containing all components at 1000 μg mL^{-1}. This solution was further diluted with methanol and 5 mL of eicosane (1.0 mg mL^{-1} in methanol).

5. Results and Discussion

5.1. Synthesis

Using a modified version of Le Gall's experimental method,[41] the synthesis is an example of a three-component Barbier-type reaction, shown in Scheme 4, whereby benzyl bromide, benzaldehyde and piperidine are reacted in a single reaction vessel. To create the organozinc Grignard reagent, a small amount of benzyl bromide and trifluoroacetic acid (TFA) are added to the reaction vessel containing zinc powder suspended in acetonitrile, and allowed to stir for 5 minutes. The remaining benzyl bromide, benzaldehyde and piperidine were added, where a condensation reaction occurs generating an iminium ion *in situ*, which subsequently reacts with the organozinc reagent *via* nucleophilic addition, forming the free-based diphenidine complex. The reaction is left to stir for a further hour to ensure reaction completion, leaving no un-reacted precursors in the reaction mixture. To purify the crude diphenidine, an acid-base work up was employed rather than the alternative purification over a neutral alumina column, which yielded a yellow-colourless oil. The oil was then dissolved in diethyl ether before HCl in dioxane (4 M) was added, dropwise, to isolate the hydrochloride salt. Upon the addition of the hydrogen chloride, there were two potential scenarios that followed. Either, a white solid was produced and no further purification was required or an oil was obtained instead. The oil is suspected to form through dioxane becoming trapped within the matrix of the diphenidine complex and in order to yield a powder, the oil was triturated several times with cold acetone and then further purified by recrystallization using a solution of diethyl ether and acetone (3:1) to remove any final impurities. After synthesis, one sample was fully characterised and each subsequent sample was characterised through GC-MS analysis.



Scheme 4. The detailed mechanism for the synthesis of diphenidine hydrochloride.

The infrared spectroscopy (IR) spectrum for diphenidine hydrochloride, shown in Figure 7, illustrates the main components stereotypical of a diarylethylamine compound, summarised in Table 2.

The peaks located between 2900 – 3030 cm^{-1} suggests the presence of the ethyl linker, whilst peak 3644 cm^{-1} identifies that the piperidine molecule has been successfully incorporated. Although the IR spectrum substantiates that diphenidine has been synthesised, it is the peak at 2365 cm^{-1} that indicates the hydrochloride salt rather than the free base.

Table 2. The infrared bands identified in diphenidine hydrochloride.

Peak (cm^{-1})	**Band**
3644	N-H (stretch)
3030	Alkyl C-H (stretch)
2933	C-H (stretch)
2365	NH+ (stretch)
1604	Aromatic C=C (bend)
1494	Aromatic C-C (stretch)

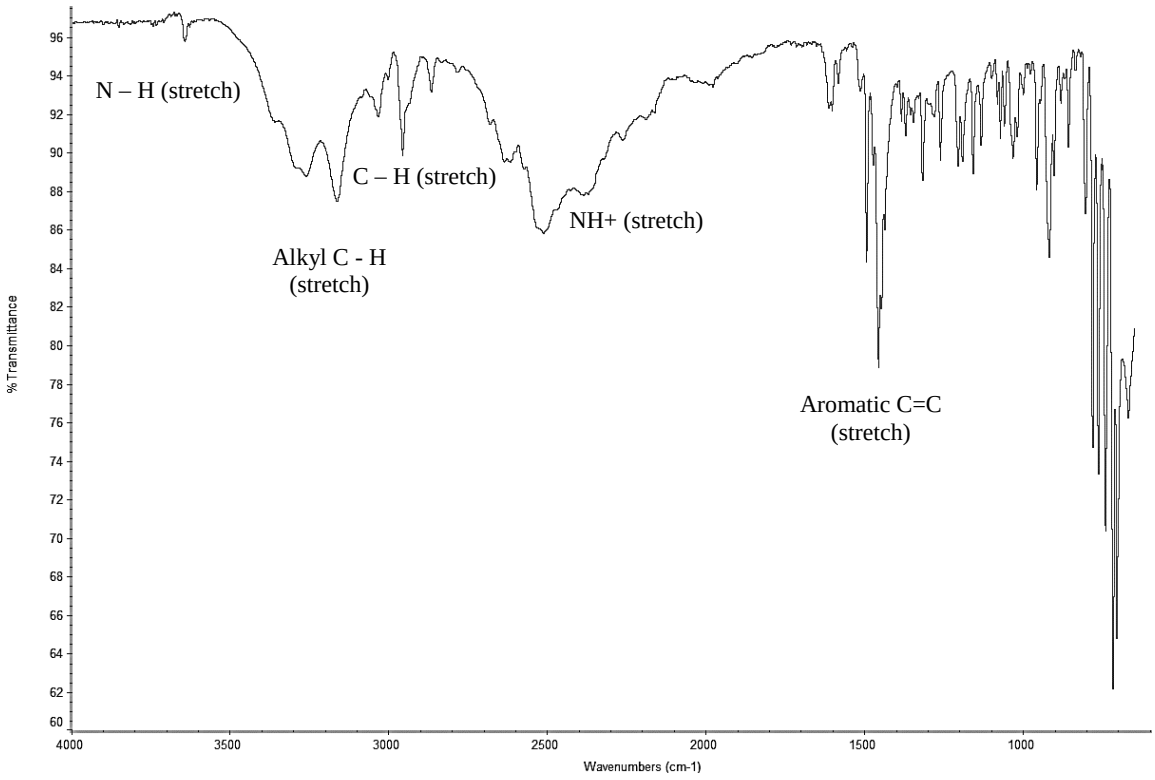


Figure 7. The infrared spectrum for diphenidine hydrochloride.

The ^{1}H-NMR for diphenidine (**8**), shown in Figure 8, shows three identifiable components of the diarylethylamine structure, and are consistent with the literature values reported.[6]

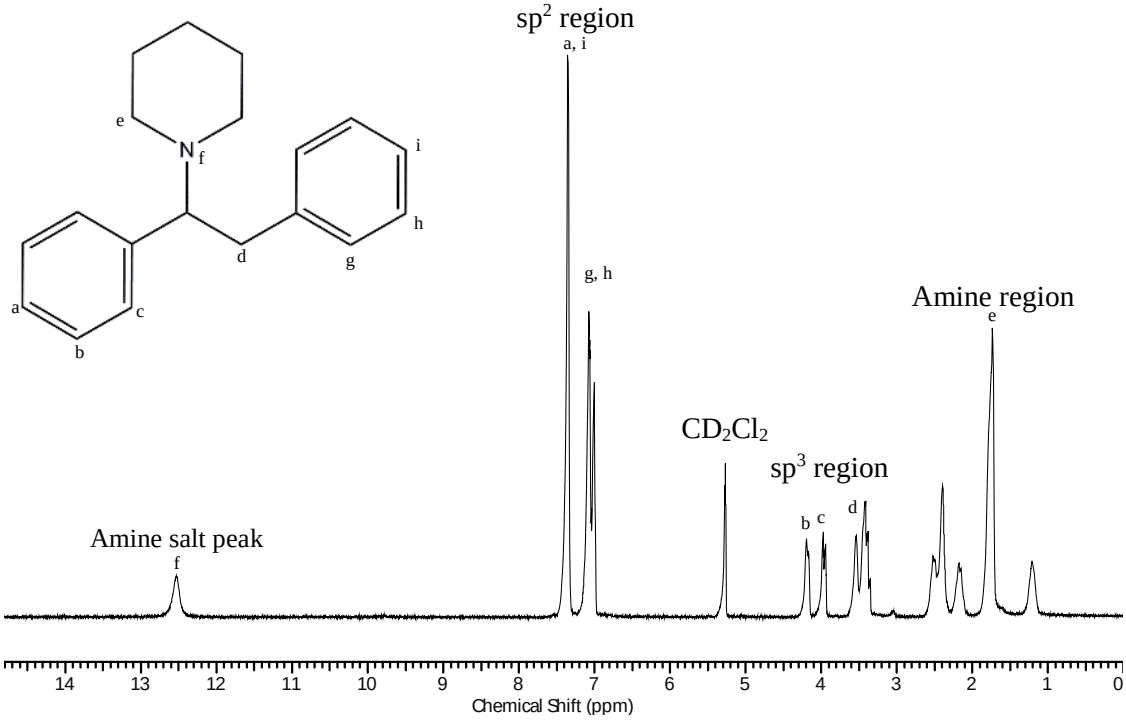


Figure 8. ^{1}H-NMR (400 MHz, CD_2Cl_2) spectrum of diphenidine hydrochloride.

The broad amine salt peak located at 12.5 ppm, confirms that the piperidine precursor has been incorporated successfully and with the aromatic region, located between 6.9 and 7.5 ppm having an integral of 10 protons, confirms that diphenidine has been synthesised. With nitrogen being more electronegative in comparison to carbon, electron density is drawn towards to the nitrogen consequently increasing the chemical shift of the surrounding protons. Despite there being scattered data within the aromatic region making identification of overlapping multiplets difficult, the aromatic protons of the benzyl bromide precursor will be found with a lower chemical shift, 6.9 ppm, whilst the benzaldehyde aromatic protons are located at 7.5 ppm. The alkyl protons located on the ethyl linker appear to be equivalent as they are attached to the same carbon and exhibit geminal/J^2 coupling with each other, but also vicinal/J^3 coupling with the proton attached to the chiral carbon. Being in close proximity to the electronegative nitrogen, the protons are no longer equivalent and therefore produce three separate peaks, shown in Figure 8, at 3.3 ppm, 3.9 ppm and 4.1 ppm. The NMR spectrum also shows two triplets corresponding to the environments within the amine

function. With there being 3 different proton environments, a range of J coupling, i.e. J^3 and J^4, producing the splitting pattern exhibited.

The ^{13}C-NMR, shown in Figure 9, also coincides with the literature values reported and reinforces the evidence in the ^{1}H NMR. In comparison with the ^{1}H NMR, there are now two extra peaks present, from the quaternary carbons on the aromatic rings, whilst there is no peak present for the N-H group.

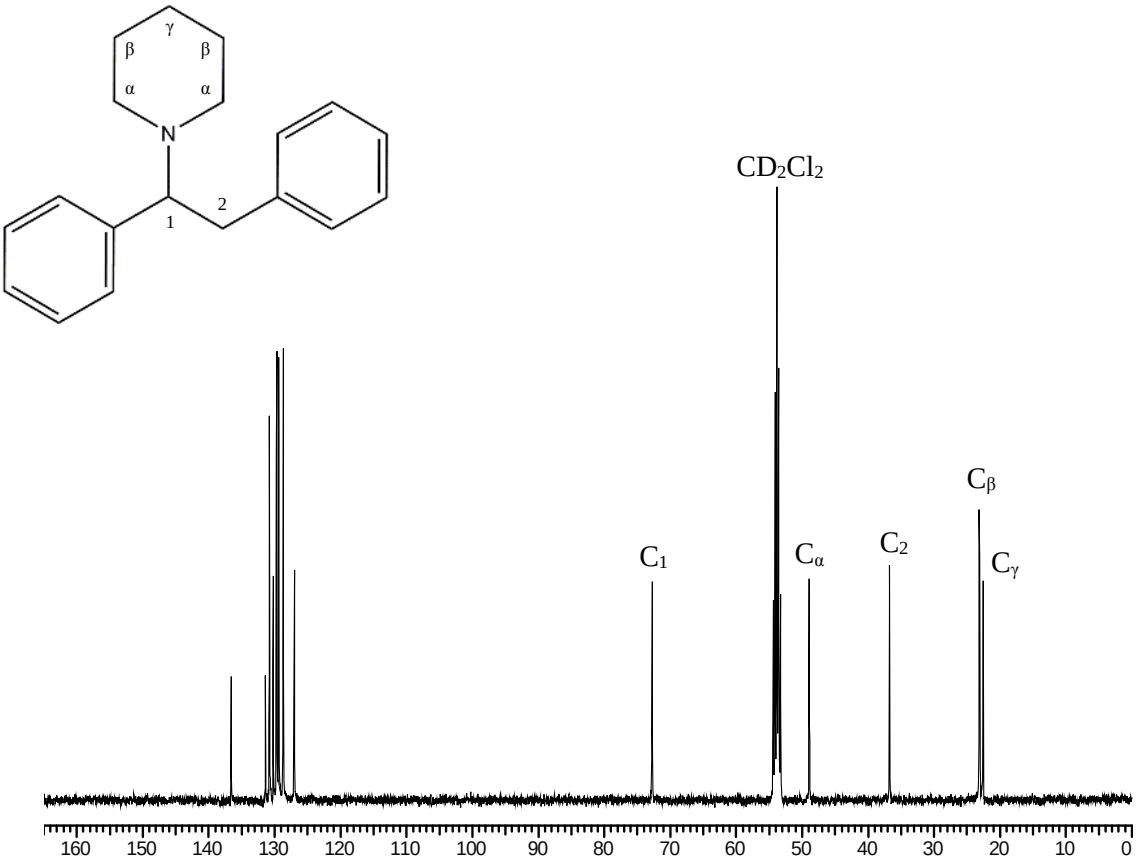


Figure 9. ^{13}C-NMR (100 MHz, CD_2Cl_2) spectrum of diphenidine hydrochloride.

The majority of carbons seen in the spectrum are quaternary carbons, which are shown between 128 ppm and 132 ppm. The clustering of the peaks proves difficult to assign, however, they do confirm that there are several aromatic systems present. The aliphatic carbons within the spectrum are more interesting, revealing more insight into the amine region. The chiral carbon can easily be identified at 73 ppm, again being pushed downfield as it is located in extremely close proximity to the electronegative nitrogen atom. The subsequent carbons on the piperidine ring are found in decreasing chemical shifts the further they are away from the nitrogen atom.

5.2. Gas-Chromatography Mass-Spectrometry (GCMS)

Gas-chromatography is an analytical technique that is usually coupled with mass spectrometry in order to determine the exact composition of a test sample. The dissolved sample, typically in a volatile solvent such as methanol, is injected into the GC column and is carried through by an inert gas, for example helium. Theoretically, depending on the chemical properties of the sample, the compounds elute separately from the column producing a retention time, then allowing the mass spectrometer to subject the compounds to ionization energy and to detect their fragments separately. The use of these two methods in conjunction with each other allows for a finer degree of sample identification and is now an incredibly useful application in a range of disciplines.

Diphenidine is identified at a single peak with a retention time of 14.56 minutes as shown in Figure 10, (a pre-bought standard has been used to produce the spectrum shown in Figure 10). Compound (**8**) undergoes fragmentation initially through the α-cleavage of the ethyl linker, resulting in an iminium ion. This particular fragment has been identified as the base peak within the mass-spectrum with a *m/z* = 174. Upon formation of this iminium species, a secondary fragmentation occurs, cleaving the

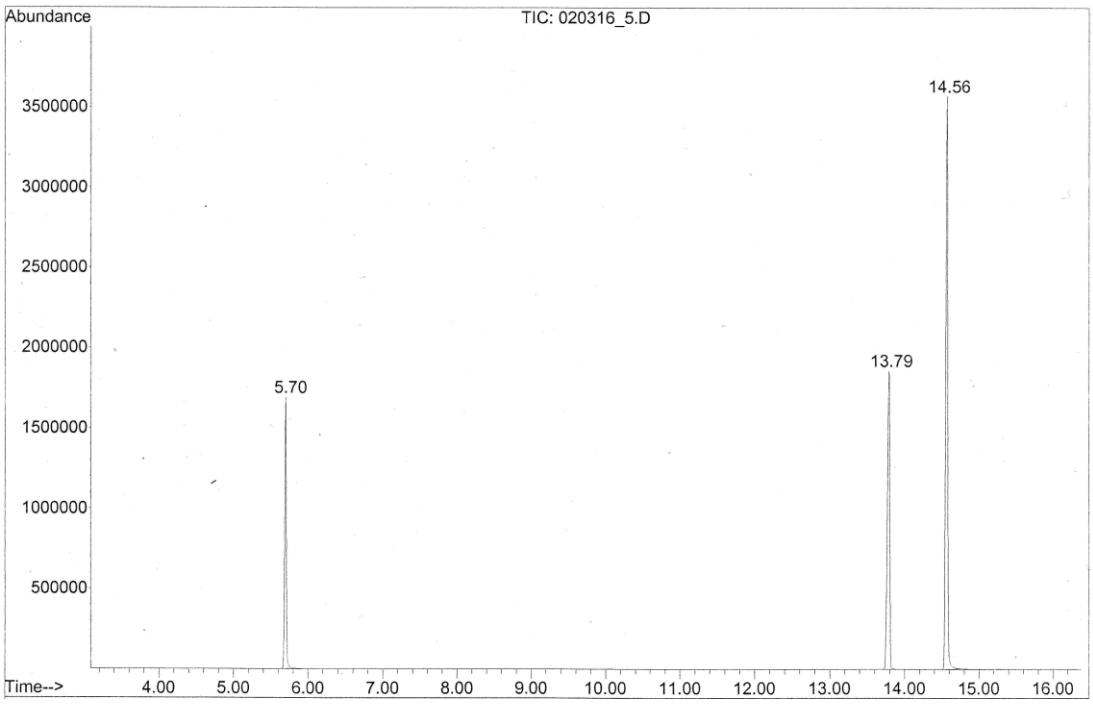


Figure 10. A chromatograph showing the retention times of *N*-benzylpiperidine, eicosane and (**8**).

C=N bond forming a stable aromatic ion, the tropylium cation with a *m/z* = 91, shown in Scheme 5.

With the secondary fragmentation producing a highly stable ion (in comparison to the first iminium species), it is seen in a relatively high abundance in the mass spectrum, which is shown in Figure 11. Despite this stable fragmentation leading to the tropylium cation, there are alternative fragments formed, shown in Figure 11, accounting for peaks at *m/z* = 181, *m/z* = 165 and *m/z* = 55.

However, in several diphenidine samples, particularly in method G, there has been an impurity seen, having a retention time of 5.70 minutes as shown in Figure 10, which has been identified as *N*-benzylpiperidine hydrochloride. The fragmentation that occurs now produces the tropylium ion, *m/z* = 91 in the mass spectrum, as the most stable peak in the fragmentation, followed by the benzylpiperidine ion at the second highest in abundance, *m/z* = 174, shown in Figure 12. The suspected fragmentation pattern of diphenidine is detailed in Scheme 5. It is suspected that this fragment forms as the major impurity due to the chiral carbon being the most susceptible point of attack. As nitrogen is more electronegative than carbon, electron density is donated towards the nitrogen, causing the carbon to be more susceptible to attack, in this instance, by electron impact (EI) ionisation energy.

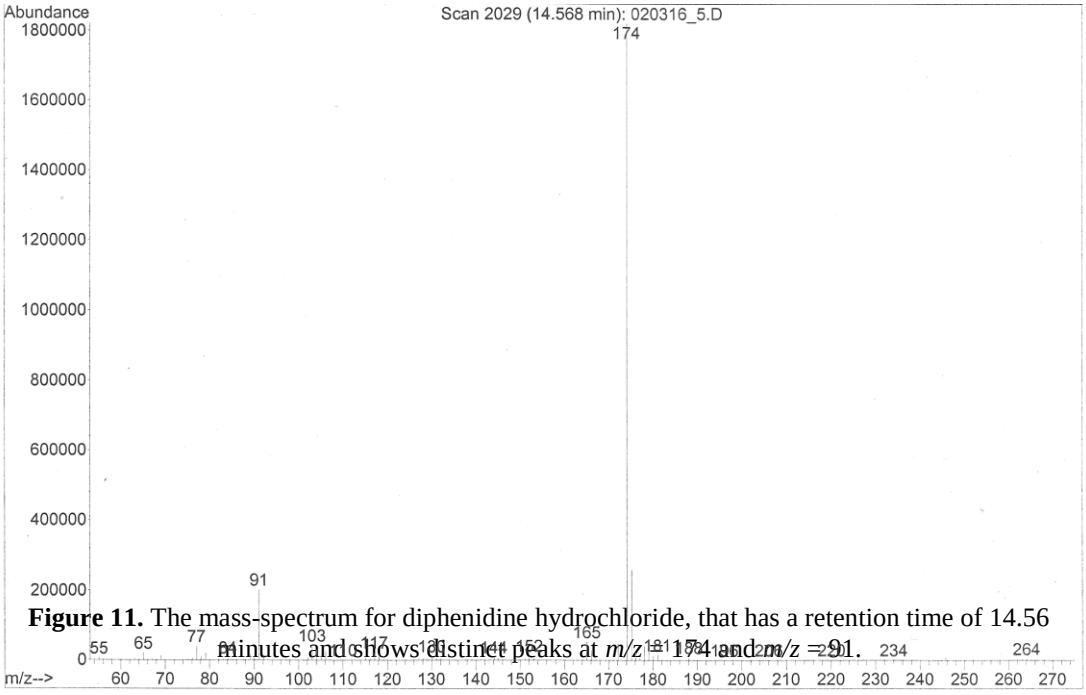


Figure 11. The mass-spectrum for diphenidine hydrochloride, that has a retention time of 14.56 minutes and shows distinct peaks at *m/z* = 174 and *m/z* = 91.

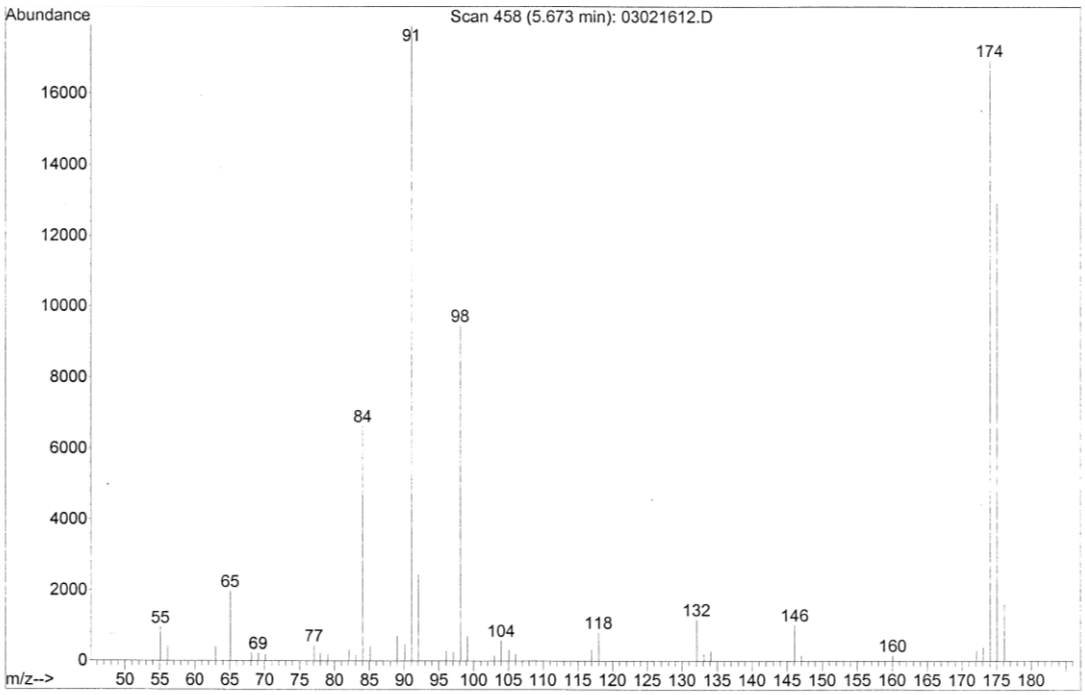
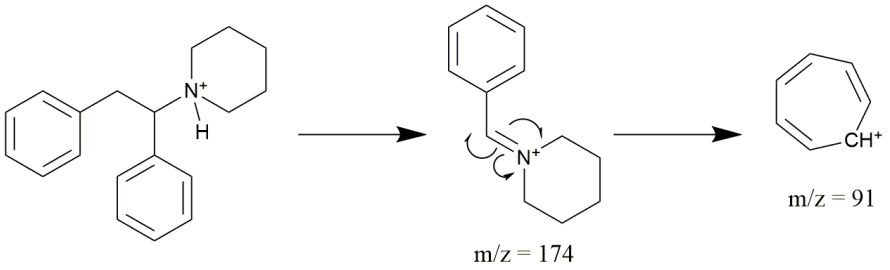
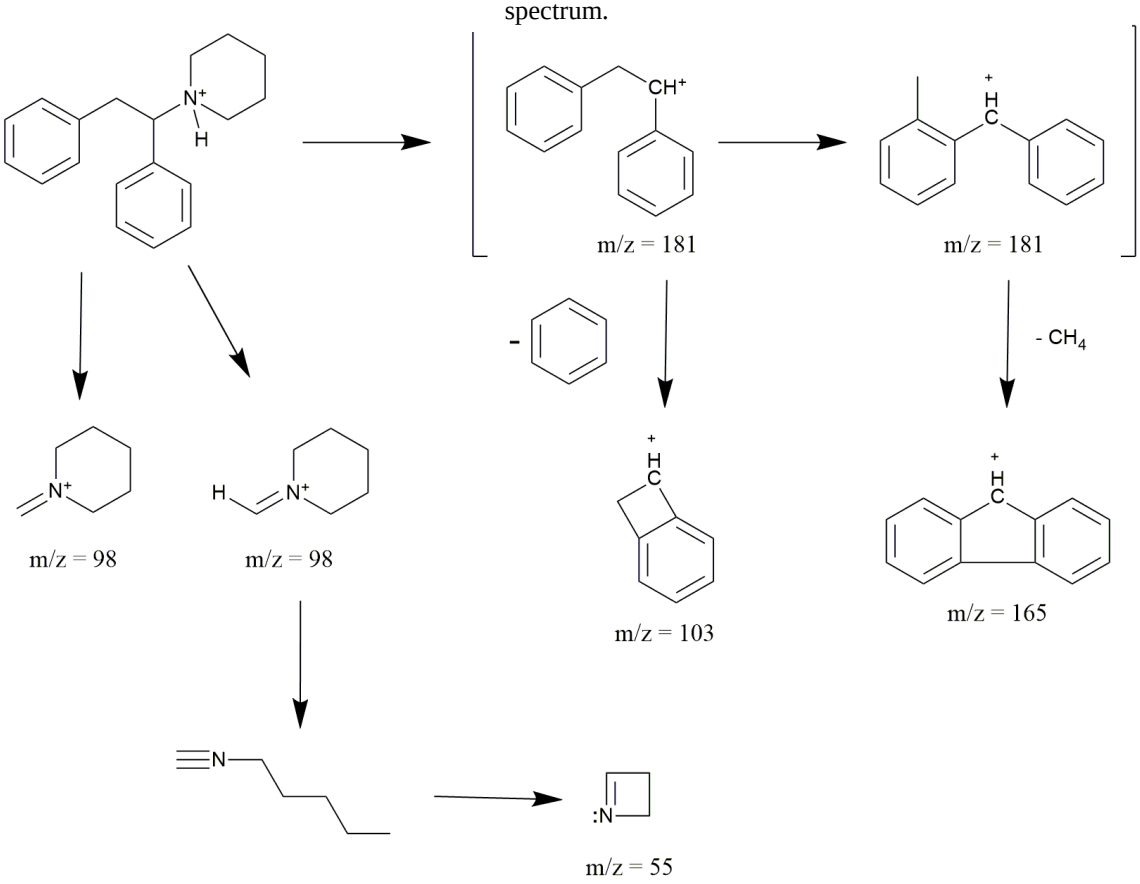


Figure 12. The mass-spectrum for *N*-benzylpiperidine, now showing *m/z* = 91 as the base peak.

A – Formation of the tropylium ion from diphenidine hydrochloride.



B – other possible fragmentation of diphenidine hydrochloride to form prominent ions present in the mass spectrum.



Scheme 5. The suspected fragmentation pathway for diphenidine hydrochloride. The first fragmentation, A, shows how the two most abundant peaks from the diphenidine hydrochloride mass-spectrum is formed. The second fragmentation, B, shows the alternative fragments that are visible within the spectrum for diphenidine hydrochloride but also *N*-benzylpiperidine.

5.3. Quantitative Gas-Chromatography Validation Method

Instrumentation: GC-MS analysis was performed using an Agilent 6850 GC and MS5973 mass selective detector (Agilent Technologies, Wokingham, UK). The mass spectrometer was operated in the electron ionisation mode at 70 eV. Separation was achieved with a capillary column (HP5 MS, 30 m Å~ 0.25 mm i.d. 0.25 μm) with helium as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The oven temperature programme was 120 °C for 3 min, 20°C min^{-1} to 170°C, 170°C for 5 min, 10°C min^{-1} to 210°C, 210°C for 1 min, 20°C min^{-1} to 250°C, 250°C for 1 min. A 2 μL aliquot of the samples were injected (manually) with a split ratio of 100:1. The injector and the GC interface temperatures were both maintained at 280°C respectively. The MS source and quadrupole temperatures were set at 230°C and 150°C respectively. Mass spectra were obtained in full scan mode (50–550 amu). All samples were injected six times.

Sample preparation: 100.0 mg of analytes were weighed accurately into a 100.0 mL clear glass volumetric flask and diluted to volume with methanol to give a solution containing all components at 1000 μg mL^{-1}. This solution was then further diluted with methanol and 5 mL of eicosane (1.0 mg mL^{-1} in methanol) added (in each case) to give calibration standards (100 mL) containing 50.0 μg mL^{-1}, 100.0 μg mL^{-1}, 150 μg mL^{-1}, 200.0 μg mL^{-1} and 250.0 μg mL^{-1} of each analyte and the internal standard at 25 μg mL^{-1}.

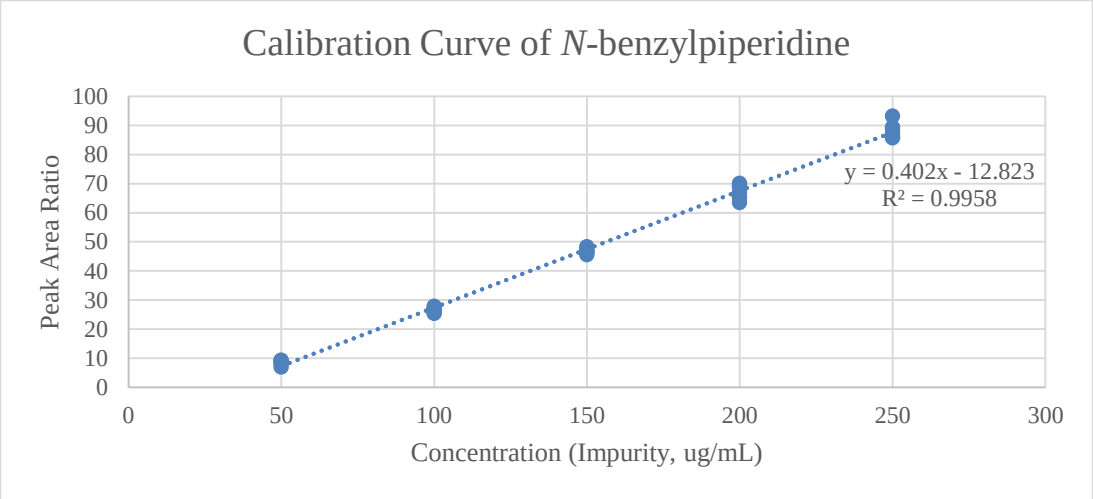


Figure 13. A calibration curve for the impurity, *N*-benzylpiperidine, using standards of 50 ug mL^{-1}, 100 ug mL^{-1}, 150 ug mL^{-1}, 200 ug mL^{-1} and 250 ug mL^{-1} and the internal standard, eicosane, at 25 ug mL^{-1}.

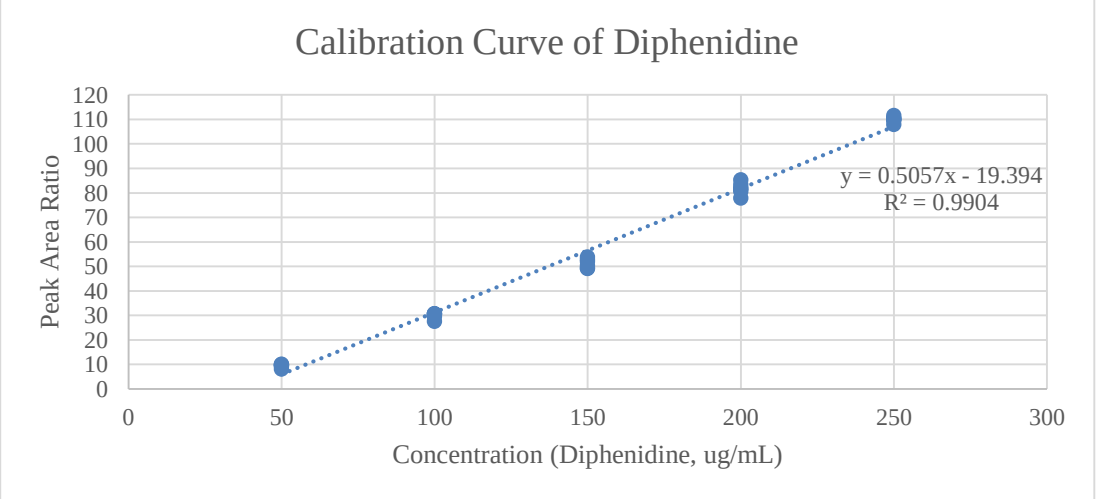


Figure 14. A calibration curve for diphenidine using standards of 50 ug mL^{-1}, 100 ug mL^{-1}, 150 ug mL^{-1}, 200 ug mL^{-1} and 250 ug mL^{-1} and the internal standard, eicosane, at 25 ug mL^{-1}.

Table 3. Summary of validation data for the quantification of diphenidine hydrochloride (100 µg mL^{-1}) and N-benzylpiperidine (100 µg mL^{-1}).

Analyte	Diphenidine	Impurity
t_R(min)	15.49	5.84
RRT[a]	0.95	0.36
RRF[b]	1.16	0.86
LOD[c]	6.74	1.07
LOQ[d]	20.42	30.83
Co-efficient (r^2)	0.9904	0.9958
Precision (RSD n=6)		
50 ug mL^{-1}	7.80	10.26
100 ug mL^{-1}	3.68	3.18
150 ug mL^{-1}	3.47	1.79
200 ug mL^{-1}	3.08	3.50
250 ug mL^{-1}	1.06	3.08

[a] Relative retention time.

[b] Relative response factor.

[c] Limit of detection (based on the standard deviation of the response and the slope).

[d] Limit of quantification (base on the standard deviation of the response and the slope).

5.4. Percentage Yield

For batches A1, A2, and A3, the synthesis followed the experimental procedure outlined in *4.2.1. method A*. It was noted that during the addition of the chemicals, there was little change to the reaction mixture and despite being an exothermic reaction, proceeded to generate little, if any, heat. Once the final compounds were yielded, in very low amounts ~0.46 g, 17%, they were analysed *via* GC-MS and were shown to contain a significant amount of a single impurity, identified at 5.70 mins alongside the diphenidine complex, seen at 14.60 mins.

Table 4. All the parameters that were changed during phase 1 and 2, involving the order of addition and quantities of precursors, stirring time and inert atmospheres.

Batch		Parameter change
A	1. Sigma Aldrich 2. Alfa Aesar 3. Acros Organics	Order of addition, without argon flush and piperidine added last
B	1. Sigma Aldrich 2. Alfa Aesar 3. Acros Organics	Order of addition, without argon flush, benzaldehyde added last
C		Order of addition, with argon flush, piperidine added last
D		Order of addition, with argon flush, benzaldehyde added last
E		20 mmol piperidine, 1 h stir, benzaldehyde added last, with argon flush
F		30 mmol piperidine, 1 h stir, benzaldehyde added last, with argon flush
G		2 h stirring, with argon flush, benzaldehyde added last
H		0.5 h stirring, with argon flush, benzaldehyde added last

Batch		Parameter change
A	1. Sigma Aldrich 2. Alfa Aesar 3. Acros Organics	Order of addition, without argon flush and piperidine added last
B	1. Sigma Aldrich 2. Alfa Aesar 3. Acros Organics	Order of addition, without argon flush, benzaldehyde added last
C		Order of addition, with argon flush, piperidine added last
D		Order of addition, with argon flush, benzaldehyde added last
E		20 mmol piperidine, 1 h stir, benzaldehyde added last, with argon flush
F		30 mmol piperidine, 1 h stir, benzaldehyde added last, with argon flush
G		2 h stirring, with argon flush, benzaldehyde added last
H		0.5 h stirring, with argon flush, benzaldehyde added last

The results from method A prompted the question whether the physical parameters, such as order of addition and the presence of moisture/oxygen, severely affected the yield and contributed to the production of contaminated samples. This led to changing the physical parameters within the experimental procedure, detailed in Table 4. This was not only to investigate the change in yield and to determine an optimised method for the synthesis of diphenidine, but also to investigate whether the isotopic fractionation would indeed change too.

The first physical parameter to change was the order of addition of the precursors. In batches A1, A2, and A3, piperidine was added last as this was suspected to initiate the reaction. However, changing the order of addition and adding the benzaldehyde last increased the yield by 70% on average, over the six repeats (n=6). During the addition, a self-induced reflux (reaction is exothermic) was observed along with a colour change of grey to green-yellow. Upon GC-MS analysis of the samples, there were no impurities seen; only a single peak was detected for the diphenidine sample at 14.56 mins, and through peak area normalisation calculations, the final compound was determined to be 100% diphenidine.

An example on how to calculate the purity using peak area normalisation (PAN):

$$\text{Peak Area Ratio Diphenidine} = \frac{Peak\ Area\ Diphenidine}{Peak\ Area\ Eicosane}$$

$$\text{Peak Area Ratio Impurity} = \frac{Peak\ Area\ Impurity}{Peak\ Area\ Eicosane}$$

$$\%\ \text{Diphenidine} = \frac{Peak\ Area\ Ratio\ Diphenidine}{(Peak\ Area\ Diphenidine + Peak\ Area\ Ratio\ Impurity)}\ x\ 100$$

$$\%\ \text{Impurity} = \frac{Peak\ Area\ Ratio\ Impurity}{(Peak\ Area\ Ratio\ Diphenidine + Peak\ Area\ Ratio\ Impurity)}\ x\ 100$$

Grignard reactions generate better yields when performed under inert conditions and with thoroughly dried glassware. For methods A and B, the reactions were performed in an open reaction vessel and with the Barbier-type reaction being similar to that of a Grignard, it was considered that moisture/oxygen may cause oxidation of the precursors or intermediate therefore further affect the percentage yield and purity of diphenidine. Both methods, C and D, were carried out under argon with C being where

piperidine was added last whilst D was when benzaldehyde was added last. The comparison between the two batches wasn't as substantial as changing the order of addition. There was only a 6% difference in percentage yield between the two, however, comparing methods A and C, there's an increase in percentage yield (by 20%) but more importantly, method C when analysed *via* GC-MS, showed no impurities, whereas A showed a single impurity at ~5.70 mins. This may be crucial when analysing the isotopic fractionation data, see *5.4.2 effect of order of addition in air*. Conversely, methods B and D showed no impurities upon analysis, with both being 100% diphenidine.

As the zinc present is zinc(II), and therefore in a d^{10} configuration, it can coordinate with piperidine up to four times in either square planar or distorted tetrahedral geometry, and thus obey the 18-electron rule. It was suspected that increasing the number of moles of piperidine in the reaction, would have a considerable impact on the percentage yield. This hypothesis resulted in methods E and F containing 20 mmol of piperidine and 30 mmol piperidine respectively. The reactions were performed using the physical parameters that had produced the best results so far in the investigation, see *4.2.5.* and *4.2.6.* method E had a percentage yield of 38% on average over the replicates whilst method F, had a percentage yield of 64% on average over the replicates. Despite the obvious difference in yield, the GC-MS data showed that method E produced pure products in comparison to method F, had a low yet detectable impurity, at 5.77 mins relating to 1.75% of the overall sample.

F. Gyenes *et al* investigated the effect of time on the percentage yield of Barbier-type reactions and concluded that "reaction times greater than 30 min resulted in only a modest increase in the yield and at 60 min the yield decreased considerably". [42] It was determined by prolonging the reaction time that the benzyl bromide is fully consumed but is also accompanied by the decomposition of the reactive imine intermediate. Consequently, this resulted in a decreased yield with an increased reaction time.

From these findings and considering what conditions could be used in clandestine laboratories, the stirring time for the final two batches were changed, from 1 hour to 2 hours and ½ hour respectively for methods G and H. Despite both batches having consistently low yields, 30% and 28% respectively, there were considerable

differences in the texture and appearance of the final compounds. All 6 replicates making up method G were yellow in colour and grainy, indicating impurities within the sample. This was confirmed by GC-MS data showing two peaks, again one at 5.75 mins with m/z = 91 and 13.86 mins with *m/z* = 174. Method H samples were all, however, white crystalline powders and proved to be pure with single peaks being obtained at ~14.68 mins from GC-MS data.

From evaluating all GC-MS data, taking into account of the percentage yield data and peak area normalisation calculations, the most optimised method was found to be method F, see *4.2.6*. Using 30 mmol piperidine rather than 10 mmol or 20 mmol but also keeping with the 1 hour stirring time, diphenidine hydrochloride was produced in much higher quantities, with a percentage yield of 64% with only 1.75% being the impurity, *N*-benzylpiperidine.

Clandestine laboratories could use any of the methodologies in order to synthesise diphenidine but would rely on not only the experience of the chemist, quality of the work environment but also the cost of supplies. The methods mentioned require specialist apparatus i.e. the use of argon/inert gas, which would mean the use of a laboratory environment in order to achieve a high yield and purity in which case, it could be sold as a "premium" product with potentially a higher price. Alternatively, if the standard method publicised were used to simply product the drug on large scale for profit, the product is likely to contain a higher percentage of impurities, produced at a quicker rate with less concern to the final product.

5.5. Isotopic Ration Mass Spectrometry

5.5.1. Natural Abundances and Delta

To establish or identify a "chemical fingerprint" for diphenidine hydrochloride, the ratios of the stable isotopes of both carbon and nitrogen, $^{13}C/^{12}C$ and $^{15}N/^{14}N$ can be measured. On a global scale, the natural isotopic abundances of these elements remain relatively stable, however, subtle variations do occur during biological, chemical and physical processes. In the case of diphenidine hydrochloride (**8**), the variation could occur during the synthesis and by applying IRMS, it is possible to measure any potential changes in the isotopic abundance of both carbon and nitrogen. This variation will be unique to the origin and history of diphenidine, which could help us link the product to precursor. Isotopic ratios, at natural abundance levels, are measured relative to international standards which define the measurements scale for particular isotopes.[42]

Variations in the natural abundance of stable isotopes are expressed using delta notation as shown in the following equations:

$$\text{Ratio } (R) = \frac{abundance\ of\ the\ heavy\ isotope}{abundance\ of\ the\ light\ isotope}$$

$$\delta = \left(\frac{Rsample}{Rstandard} - 1 \right)$$

Delta values are commonly multiplied by 1000 so that they are reported in parts per thousand (‰) or by 1000,000 to give results in parts per million, ppm.

5.5.2. Batches A1, A2 and A3, effect of changing supplier

Based on previous research published involving IRMS analysis on laboratory synthesised mephedrone samples[11], it was theorised that future work could possibly reach to precursor and/or manufacture differentiation. To apply this to diphenidine hydrochloride, the precursors were sourced from 3 different manufactures: Sigma-Aldrich, Alfa-Aesar and Acros Organics, with each batch (A1, A2 and A3) being synthesised using only the precursors from the same supplier. It is predicted that if all three suppliers source their precursors from different manufacturers, then upon IRMS analysis, there would be three separate and distinguishable cluster patterns. The data from the IRMS analysis is shown in Figure 15.

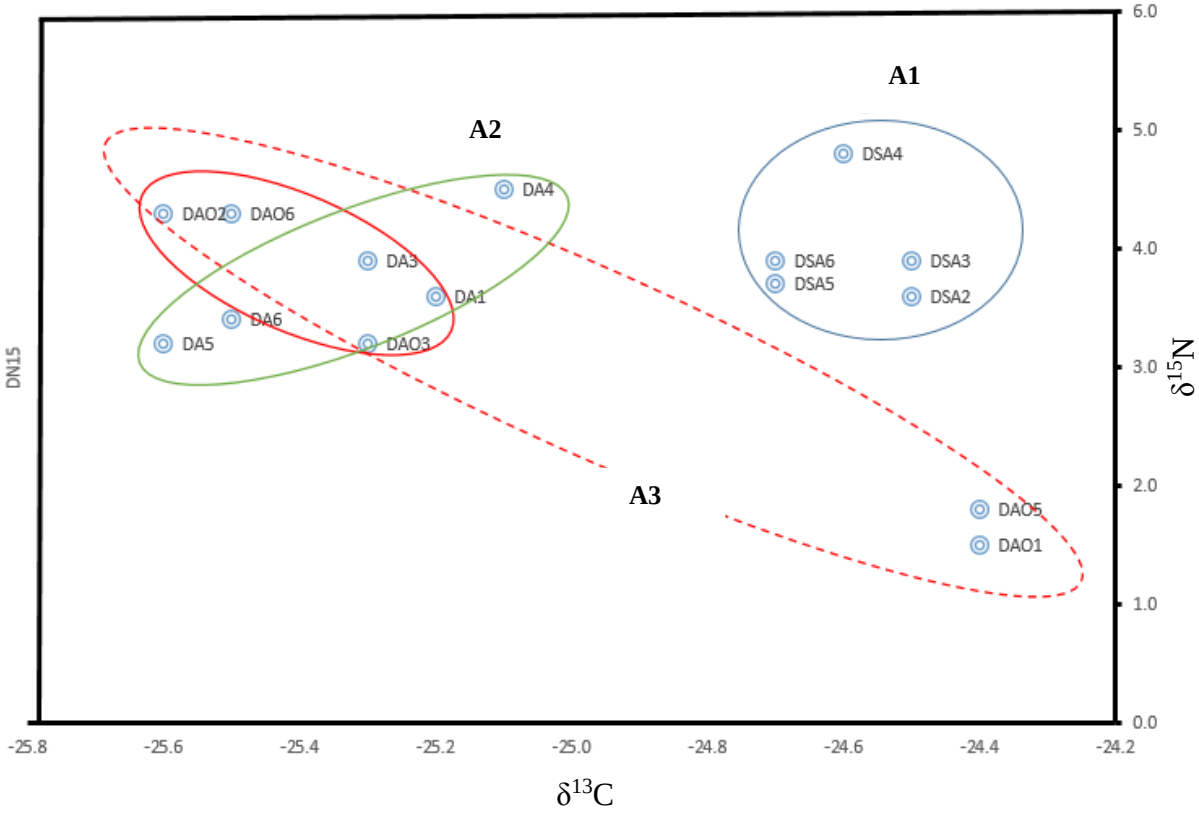


Figure 15. A graphical representation of the clustering patterns of the starting material batches (A1, A2 and A3) after IRMS analysis has been performed. From this, it is apparent that there is a cluster pattern associated with the Sigma-Aldrich starting materials, batch A1.

From looking at the data, there is only one distinguishable pattern of clusters and these are associated with the Sigma-Aldrich batch, A1 (DSA2-DSA6 on Figure 15). From the array of clusters, there seems to be little difference in the $\delta^{15}N$ values, but a more comparable difference in the $\delta^{13}C$ values.

A2 and A3 corresponding to the Alfa-Aesar and Acros Organics batches and have relatively similar values for both $\delta^{15}N$ and $\delta^{13}C$ values, all except two outliers, DA01 and DA05 which are both significantly different from the majority.

It is interesting to note that both Acros Organics and Alfa-Aesar are both part of the Fischer Scientific brand and, therefore, could potentially source their precursors from the same manufacturer. If the precursors are from the same manufacturer, the relative isotopic abundances of the elements would essentially be the same, if not, marginally different. This would account for the similarities in both of the $\delta^{13}C$ and $\delta^{15}N$ values, along with the both the carbon and nitrogen percentages. However, Sigma-Aldrich is a separate brand and could consequently have a different provider of chemicals. This could potentially explain why batch A1 can be distinguished from A2 and A3, but A2 and A3 cannot be distinguished from each other.

Tables 5, 6 and 7 show the actual values for both $\delta^{13}C$ and $\delta^{15}N$ along with the percentages of carbon and nitrogen, for each of the batches. It is a common trend that the nitrogen percentages are consistently low throughout, ranging from only 4.2 – 4.8 %, despite all carbon percentages remaining comparably high, ranging from 66.6 – 76.4 % across all of the starting material samples. The low nitrogen percentages could be due to not only the amount of piperidine used, but also the order of addition.

For the reaction to proceed, the organometallic Barbier reagent needs to be present in abundance and is created through reacting zinc with piperidine, forming a stabilised complex allowing the piperidine reagent to fully react, leaving behind no unreacted starting material. During all three batches, A1, A2 and A3, the order of addition meant piperidine was added last, as it was originally suspected to initiate the reaction. This suggests that the stabilised complex wasn't formed, or not in a large enough amount for the reaction to yield a significant yield, or a clean product, supported by both the percentage yield results, ~17% on average over all the samples in batch A, and the GC-MS data, where *N*-benzylpiperidine was seen in high abundances in addition to diphenidine.

Table 5. The processed data regarding samples DSA02-DSA06, relating to batch A1. All precursors from the same manufacturer, Sigma-Aldrich.

Sample	$\delta^{13}C$	%C	$\delta^{15}N$	%N
DSA02	-24.5	70.6	3.6	4.4
DSA03	-24.5	71.9	3.9	4.5
DSA05	-24.7	72.2	3.7	4.5
DSA06	-24.7	70.5	3.9	4.4

Table 6. The processed data regarding samples DA1-DA6, relating to batch A2. All precursors from the same manufacture, Alfa-Aesar.

Sample	$\delta^{13}C$	%C	$\delta^{15}N$	%N
DA1	-25.2	76.4	3.6	4.8
DA3	-25.3	73.8	3.9	4.6
DA5	-25.6	66.6	3.2	4.2
DA6	-25.5	68.7	3.4	4.3

Table 7. The processed data regarding samples DAO1-DAO6, relating to batch A3. All precursors from the same manufacturer, Acros Organics.

Sample	$\delta^{13}C$	%C	$\delta^{15}N$	%N
DAO1	-24.4	71.2	1.5	4.2
DAO2	-25.6	72.9	4.3	4.7
DAO3	-25.3	73.1	3.2	4.5
DAO5	-24.4	74.1	1.8	4.4
DAO6	-25.5	72.3	4.3	4.7

5.5.3. Methods A and B, effect of order of addition in air

Method A's replicates were all synthesised without the use of argon, but in a reaction vessel, open to air. Method A was also prepared using benzyl bromide, benzaldehyde and piperidine, added individually in that order. Upon the synthesis of method A, it was noted that the reaction proved to be extremely temperamental, rarely reaching completion and producing minimal final product, with the average being only ~0.45 g, or 17%. To increase yield and the purity of (**8**), the order of addition was questioned first. By keeping the supplier, the same throughout methods B-H (Acros Organics), it would be possible to draw conclusions accurately if any valuable IRMS was obtained.

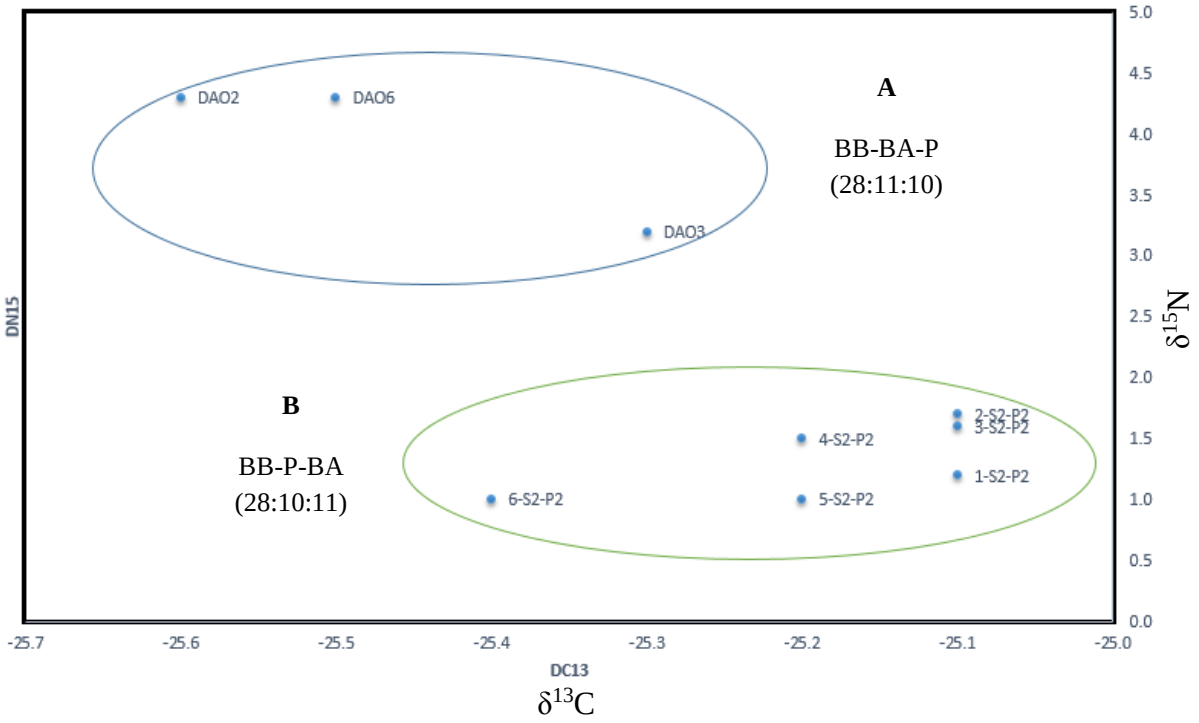


Figure 16. A graphical representation of the clustering patterns of batches A and B after IRMS analysis has been performed. The order of addition has been changed but the reaction is still performed in an open vessel, with the same molar equivalents.

Figure 16 shows the effect of changing the order of addition but keeping the reaction open to air, comparing method A(3) and method B. By adding piperidine, second, rather than third, theoretically there would be a higher probability of forming the stabilised Zn/Br intermediate, and consequently a higher yield, which is confirmed by percentage yield calculations showing an increase of ~70% between methods A and B. By changing the order of addition, it was expected that the only change would be

to the yield, and not to the isotopic ratio. However, changing the order of addition, from the initial benzyl bromide, benzaldehyde and piperidine to benzyl bromide, piperidine and then benzaldehyde, has shown to affect the $\delta^{15}N$ values, more so than the $\delta^{13}C$ values, shown in tables 7 and 8.

The $\delta^{15}N$ are shown to decrease upon changing the order of addition, from 4.3 in batch A, to as little as 1.0 in batch B, however this decrease has minimal effect on the percentage of nitrogen incorporated into the final product, which remains stable at ~4.7 % throughout both methods. The decrease in $\delta^{15}N$ values is a result that was not anticipated, however, performing the reaction under argon, methods C and D, will give more insight into this change. Despite the variance in their δN^{15} values, their $\delta^{13}C$ values stay relatively constant with a range of only 0.5 across all samples, shown by samples DAO3 and 6-S2-P2 in Figure 16. From these results alone, it is possible to distinguish between methods A and B.

Table 8. The processed data regarding samples composing of batch B, whereby the order of addition has changed, consequently decreasing the $\delta^{15}N$ values.

Sample	**$\delta^{13}C$**	**%C**	**$\delta^{15}N$**	**%N**
B1	-25.1	72.4	1.2	4.6
B2	-25.1	73.0	1.7	4.6
B3	-25.1	76.0	1.6	4.8
B4	-25.2	72.3	1.5	4.6
B5	-25.2	74.3	1.0	4.7
B6	-25.4	75.2	1.0	4.8

5.5.4. Methods C and D, effect of order of addition under argon

There has been an increase in $\delta^{13}C$ values methods for methods C and D in comparison to batches A and D, showing an overall increase of 0.6, which can be seen in Figure 17 and Tables 9 and 10. Both methods C and D show a condensed region of clustering on the Y axis but not the X, whereby the majority of samples are located, suggesting replicable data. This overall pattern follows the initial hypothesis that the $\delta^{15}N$ value would increase upon performing the reaction in an inert atmosphere.

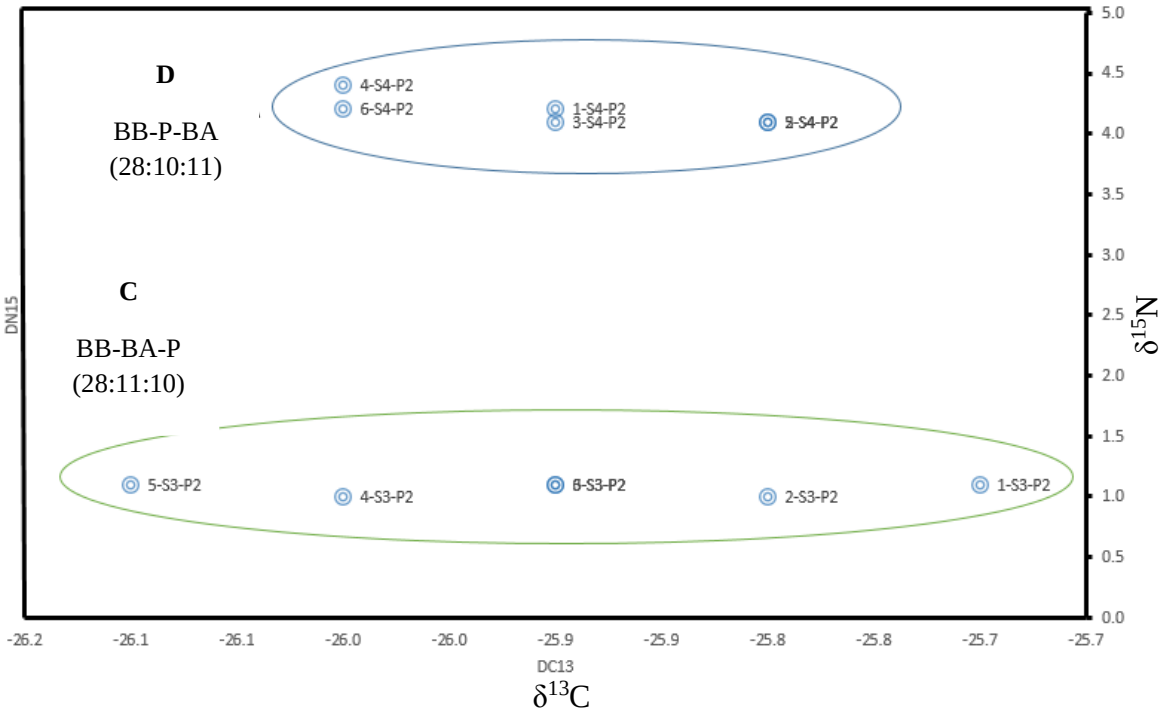


Figure 17. A graphical representation of the clustering patterns of methods A and B after IRMS analysis has been performed. It is possible to distinguish between both batches due to changes in the $\delta^{15}N$ values.

Perhaps the increase observed for both methods, in comparison to A and B, is through the amount of oxidation occurring upon exposure to air, a variable in this instance is now controlled that has not previously. Controlling the exposure to air reduces the amount of oxidation occurring upon the nitrogen element of the piperidine molecule, meaning that there is a higher percentage remaining in the reaction mixture, in comparison to that of the exposed and potentially oxidised piperidine in batches A and B.

Through changing the order of addition so that piperidine is added second, there is additional time for piperidine and benzyl bromide to react, resulting in a higher probability of the stabilised intermediate forming. In terms of percentage yield, there is only a 6% difference between C and D and it is also interesting to note that both batches B and D, whereby the benzaldehyde was added last, show no impurities (*N*-benzylpiperidine) in their GC-MS results but are in fact 100% pure samples of diphenidine. All analytical data concerning the initial batches, A, B, C and D, support the original suspicion that the order of addition effects the progress of the reaction and consequently the yield.

Table 9. Processed data regarding samples composing of batch C, whereby the order of addition is the same as batch A, but is now performed under argon.

Sample	$\delta^{13}C$	%C	$\delta^{15}N$	%N
C1	-25.7	73.1	1.1	4.6
C2	-25.8	74.6	1.0	4.6
C3	-25.9	73.0	1.1	4.5
C4	-26.0	72.1	1.0	4.4
C5	-26.1	72.5	1.1	4.5
C6	-25.9	73.9	1.1	4.6

Table 10. The processed data regarding samples composing of batch D, whereby the order of addition is the same as batch B, but is now performed under argon.

Sample	$\delta^{13}C$	%C	$\delta^{15}N$	%N
D1	-25.9	72.0	4.2	4.5
D2	-25.8	71.4	4.4	4.4
D3	-25.9	70.2	4.4	4.4
D4	-26.0	67.7	4.2	4.2
D5	-25.8	69.1	4.3	4.3
D6	-26.0	69.9	4.3	4.3

5.5.5. Batches E and F, effect of mmol piperidine

During the synthesis of method A, it was suggested that piperidine was the component to initiate the reaction. However, upon performing the synthesis, it was decided that in order for the reaction to proceed piperidine needs to be added second. In addition to this, it was hypothesised that zinc would not be fully coordinated by using only 10 mmol of piperidine, therefore methods E and F investigate the effect of increasing this to initially 20 mmol and then 30 mmol. Although the percentage yield of method E was low, 38%, in comparison to F, 64%, GC-MS data identified the products to be clean samples, with F having only a slightly detectable impurity, *N*-benzylpiperidine which related to just 1.75% of the sample.

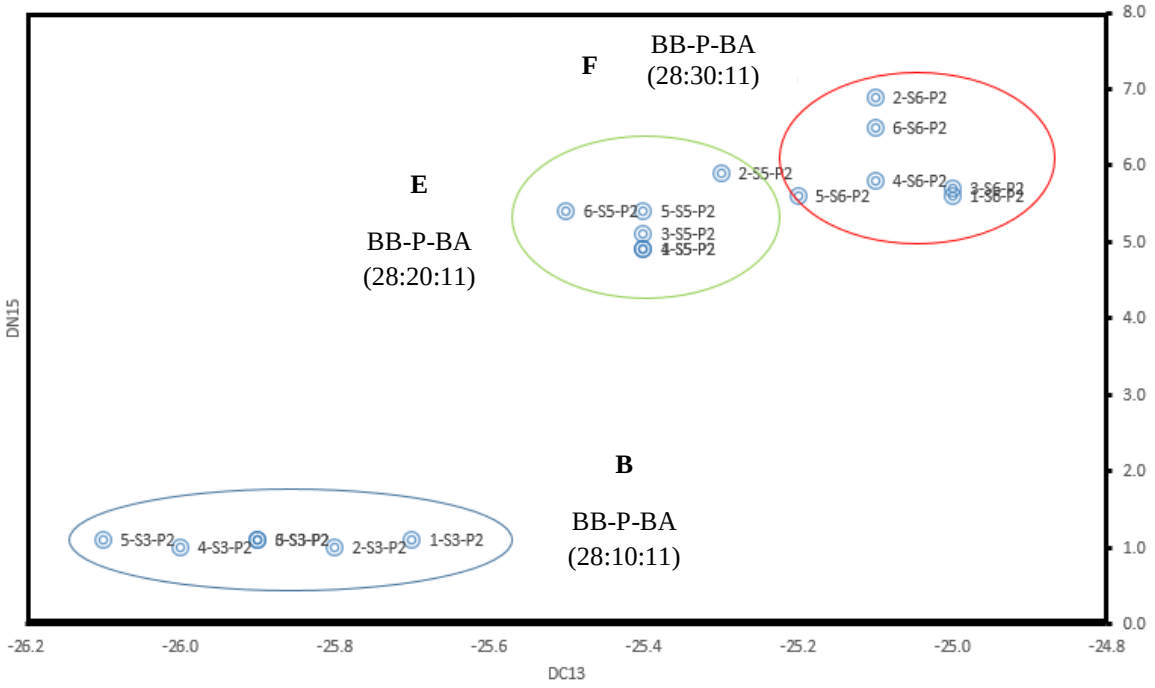


Figure 18. A graphical representation of the clustering patterns of batches B, E and F after IRMS analysis has been performed. Upon doubling the equivalents of piperidine, there is a significant increase in $\boldsymbol{\delta}^{15}$N values followed by a more gradual increasing on increasing 20 mmol to 30 mmol.

Figure 18 shows the IRMS data comparing methods B, E and F. Changing the mmol of piperidine not only effects the $\boldsymbol{\delta}^{15}$N values but also influences the $\boldsymbol{\delta}^{13}$C values. Starting with batch B, where the mmol of piperidine remains only 10 mmol, the δ^{15}N values are significantly lower than that of E and F, with values as low as 1.0, shown in Table 8. The $\boldsymbol{\delta}^{13}$C values stay relatively constant, with an average of -25.9. However, upon doubling the equivalent up to 20 mmol, there is a sudden, noticeable increase in

the $\boldsymbol{\delta}^{15}$N values, ranging from 4.9 – 5.9, with $\boldsymbol{\delta}^{13}$C values now lower, ranging from -25.0 to -25.2, shown in Table 11. This evidence supports the hypothesis that more piperidine is coordinated with zinc, incorporating more nitrogen consequently increasing not only the $\boldsymbol{\delta}^{15}$N values through the nitrogen element, but also the $\boldsymbol{\delta}^{13}$C values from the carbon ring. Increasing the equivalents from 20 mmol to 30 mmol shows a decrease of 0.5 from E to F for δ^{13}C and then an increase of 1.0 for $\boldsymbol{\delta}^{15}$N values, shown in Table 12 and Figure 17, perhaps now there is steric hindrance from the piperidine already coordinated, with a lower probability of more coordinating to create the square planar or distorted tetrahedral geometry.

Sample	$\boldsymbol{\delta}^{13}$C	%C	$\boldsymbol{\delta}^{15}$N	%N
E1	-25.4	76.5	4.9	4.8
E2	-25.3	73.9	5.9	4.5
E3	-25.4	75.1	5.1	4.7
E4	-25.4	75.1	4.9	4.7
E5	-25.4	75.8	5.4	4.7
E6	-25.5	77.1	5.4	4.8

Table 11. The processed data regarding samples composing of batch E, whereby increasing the equivalent of piperidine to 20 mmol has increased the δ^{15}N values by 4.1.

Sample	$\boldsymbol{\delta}^{13}$C	%C	$\boldsymbol{\delta}^{15}$N	%N
F1	-25.0	76.5	5.6	4.8
F2	-25.1	73.9	6.9	4.5
F3	-25.0	75.1	5.7	4.7
F4	-25.1	75.1	5.8	4.7
F5	-25.2	75.8	5.6	4.7
F6	-25.1	77.1	6.5	4.8

Table 12. The processed data regarding samples composing of batch F, whereby increasing the equivalent of piperidine to 30 mmol has increase the δ^{15}N values by 5.9 from batch B and 2.0 from batch F.

5.5.6. Batches G and H, effect of stirring time

Gyenes *et al* concluded that in Barbier-type reactions that "reaction times greater than 30 min resulted in only a modest increase in the yield and at 60 min the yield decreased considerably".[43] By prolonging the reaction time, the reactive imine intermediate decomposes, resulting in a decreased yield and a higher percentage of impurities, *i.e.* side reactions from Wurtz coupling. Both batches G and H, where the reaction times have changed to 2 hours and ½ respectively. G was composed of yellow, grainy samples with GC-MS data confirming the impurity, *N*-benzylpiperidine at 5.75 mins, supporting the hypothesis of increase impurities and a decreased yield. In the case of H, the samples were 100% diphenidine and were white crystalline powders, suggesting that a decreased time gives a purer sample. It was predicted that these results would affect the IRMS data, by decreasing the $\boldsymbol{\delta}^{15}$N and $\boldsymbol{\delta}^{13}$C values with an increase in time. Figure 19 shows the effect of stirring time whilst under argon, between batches B, G and H.

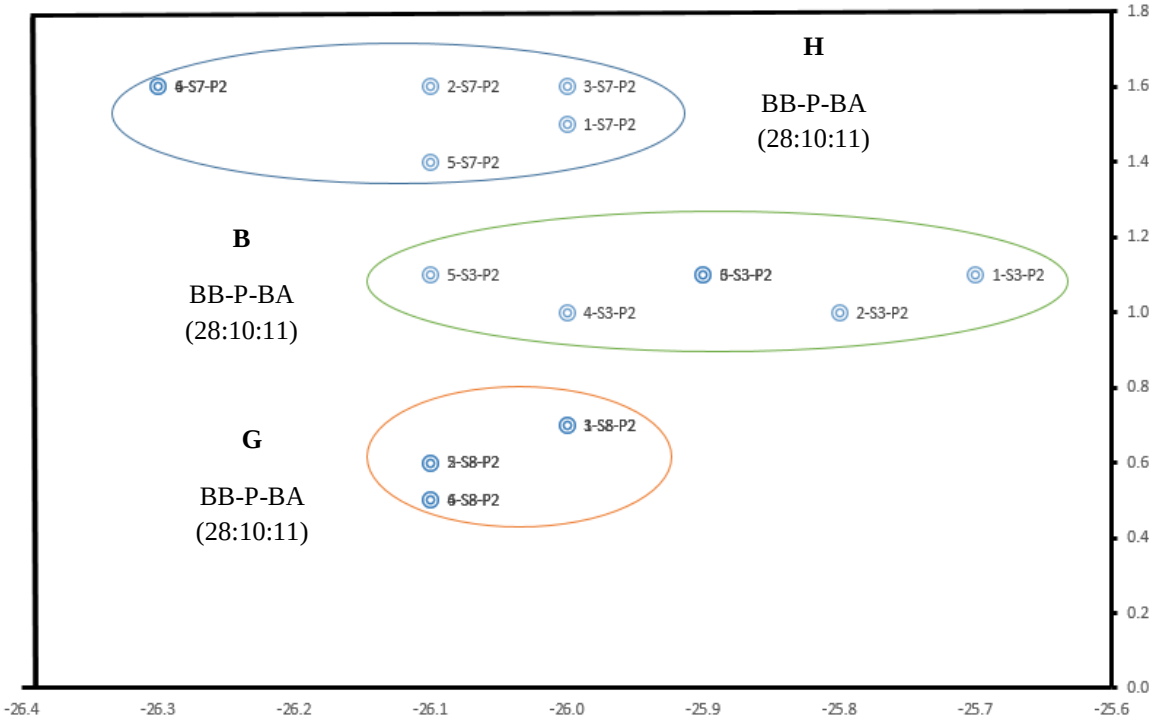


Figure 19. A graphical representation of the clustering patterns of batches B, G and H after IRMS analysis has been performed. Upon halving the time, there is an increase in $\boldsymbol{\delta}^{15}$N values and upon halving, there is a significant decrease in δ^{15}N values.

In comparison to method B, whose stirring time was just one hour, method H is as predicted, with higher $\boldsymbol{\delta}^{15}$N values which now range from 1.4 – 1.6. Although still quite low to values previously seen, for example, in comparison to batch F, it is still higher than both batches B and G. A shorter reaction time reduces the amount of oxidation occurring upon the nitrogen element, if any, but also prevents the imine intermediate being broken down. Comparing this to batch G, the $\boldsymbol{\delta}^{15}$N values have dropped to as little as 0.5, shown in Tables 13 and 14, suggesting that the majority of the reactive intermediate is no longer present during the reaction mixture, reflected by the percentage yield results. With less nitrogen being incorporated throughout the synthesis due to decomposition of the imine intermediate, a necessity for the Barbier reaction to proceed, there is less nitrogen available to successfully react. This data supports the findings from Gyenes and has identified that in this circumstance, it is possible to distinguish between chosen synthetic pathways in this instance.[42]

Table 13. The processed data regarding samples composing of batch G, whereby increasing the stirring time to 2 hours causes a decrease in δ^{15}N values, with the δ^{13}C samples staying relatively constant.

Sample	$\boldsymbol{\delta}^{13}$C	%C	$\boldsymbol{\delta}^{15}$N	%N
G1	-26.0	72.4	1.5	4.6
G2	-26.1	73.6	1.6	4.7
G3	-26.0	73.8	1.6	4.7
G4	-26.3	73.6	1.6	4.7
G5	-26.1	73.5	1.4	4.7
G6	-26.3	73.9	1.6	4.7

Table 14. The processed data regarding samples composing of batch H, whereby decreasing the stirring time to 0.5 hour causes an increase in δ^{15}N values, with the δ^{13}C samples staying relatively constant.

Sample	$\boldsymbol{\delta}^{13}$C	%C	$\boldsymbol{\delta}^{15}$N	%N
H1	-26.0	69.4	0.7	4.3
H2	-26.1	70.7	0.6	4.4
H3	-26.0	72.1	0.7	4.4
H4	-26.1	71.7	0.5	4.4
H5	-26.1	70.5	0.6	4.3
H6	-26.1	71.5	0.5	4.4

6. Conclusion

From evaluating all GC-MS data, taking into account of the percentage yield data and peak area normalisation calculations, the optimum method was found to be method F. Using 30 mmol piperidine rather than 10 mmol or 20 mmol but also keeping with the 1 hour stirring time, diphenidine hydrochloride was produced in much higher quantities, with a percentage yield of 65% with only 1.75% being the impurity, *N*-benzylpiperidine. It is possible for clandestine laboratories to use any of the methodologies mentioned to synthesise diphenidine hydrochloride, however, to produce a product with few impurities whilst maintaining a profitable yield, the gross input is consequently higher. A skilled chemist would be required along with a higher quantity of precursor resulting in a larger initial cost. Alternatively, if the standard method publicised by Le Gall *et al* [41] were used to simply produce the drug on large scale for profit, the product is likely to contain a higher percentage of impurities, produced at a quicker rate with less concern to the final product. Whichever method chosen, would depend entirely on the on the supplier, hence why a library of different physical parameters was created.

From the IRMS data, shown in Figure 15, there is an obvious difference in the $\boldsymbol{\delta}^{13}C$ values between batches A1 compared to A2 and A3, but a minimal difference between A2 and A3 individually. These two batches correspond to Alfa-Aesar and Acros Organics, both of which are owned by the Fischer Scientific brand. If the two obtain their chemicals from the same supplier, this could account for why there is little difference in their IRMS data. If a seized sample of diphenidine was analysed, it would be possible to identify between samples synthesised using Sigma Aldrich precursors but not from that of Alfa or Acros. Through changing the order of addition, and when the synthesis is performed under argon, there is an increase in $\boldsymbol{\delta}^{15}N$ values, in comparison to when synthesised open to air, where the $\boldsymbol{\delta}^{13}C$ values decrease. From this, it would be possible to distinguish whether a diphenidine sample had been synthesised in an inert atmosphere or not. An interesting observation is that through changing the equivalents of piperidine, there is a significant difference on the $\boldsymbol{\delta}^{15}N$ values between batches B and E, relating to 10 mmol and 20 mmol, however, not

between 20 mmol and 30 mmol. It would be possible to determine whether the seized sample was synthesised using 10 mmol or 20 mmol, but not with certainty.

Although some valuable data has been obtained involving IRMS as a form of source identification, it does have its limitations in comparison to other analytical methods. Despite giving more detailed results, it does take longer than methods such as GC-MS and with only a limited library available currently, identification could still be lengthy. Not only that, it hasn't been possible to distinguish between all samples in this case and therefore needs further investigation.

7. Future Work

Impurity profiling could be performed whereby the alternative publicised method is used in order to synthesise diphenidine hydrochloride (**8**). The investigation should be conducted again, by another, or group of individuals to identify whether the data is replicable. In addition, all batches synthesised after batches A were prepared using precursors from Acros Organics. It would be beneficial to conduct the synthesis using Sigma-Aldrich and Alfa-Aesar precursors to draw more accurate conclusions between any significant differences in the IRMS data obtained. Further studies could also focus on the metabolites of diphenidine or by changing to an aldehyde used to create a new library of NPSs, for which IRMS analysis could be used to distinguish between them.

8. References

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