NOVEL PSYCHOACTIVE SUBSTANCES – METHODS FOR IDENTIFICATION, PREDICTIVE MODELLING SOFTWARE AND AN EXPERIMENTAL DESIGN

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Declaration

I declare that this thesis does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due reference has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Signed: Benjamin Rutherford
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Part One

LITERATURE REVIEW

ASSESSMENT AND DEVELOPMENT OF ALGORITHM FOR THE IDENTIFICATION AND CHARACTERISATION OF UNKNOWN NPS DRUGS BASED ON KNOWN MASS SPECTROMETRY DATA
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List of Abbreviations

α-ET = α-ethyltryptamine
CB = Cannabinoid
CLen = Customs Laboratories European Network
DET = N,N-diethyltryptamine
DMT = N,N-dimethyltryptamine
EMCDDA = European Monitoring Centre for Drugs and Drug Addiction
EWA = Early Warning Advisory
GC-MS = Gas Chromatography-Mass Spectrometry
HR-MS = High Resolution Mass Spectrometry
JRC = Joint Research Centre
MDVP = 3,4-methylenedioxypyrovalerone
NMR = Nuclear Magnetic Resonance
NPS = Novel Psychoactive Substance/s
SWGDRUG = Scientific Working Group for the Analysis of Seized Drugs
THC = Tetrahydrocannabinol
TOF = Time-Of-Flight
UNODC = United Nations Office on Drugs and Crime
US = United States
WHO = World Health Organisation
Abstract
In recent years novel psychoactive substances (NPS) have begun to pose a serious threat to public health and safety. Not only because of their dramatic increase in production and the reported increase of their abuse, but because of their ability to circumvent legislation and the difficulties involved in attempting to identify them. This literature review discusses NPS, the more prevalent classes of NPS, and current methods used for identifying them. The potential for algorithm based predictive modelling software as a technique for identifying NPS will also be investigated.
1.0 Introduction

Novel psychoactive substances (NPS) have experienced a rapid emergence in the last 10 years. As defined by the World Health Organisation (WHO), psychoactive substances are known as substances that when taken or administered affect mental processes. The novel aspect of their name is synonymous with new; however, this does not always refer to them as being newly developed. Although some of these substances have been invented in recent years, some are labelled as novel because abuse of them is only a new phenomenon.

Other names for NPS include “legal highs”, “designer drugs” and “internet drugs”. Their more well-known names highlight the problems that come with these drugs. Through careful design, many of these NPS are actually not illegal in countries around the world. This illegality will be discussed soon. In addition to this they are readily available for purchase on the internet whilst, at a lengthy delay, the relevant authorities are legislating to make them illegal.

The prime causal factor for this lag in performance on the part of the lawmakers is for their requirement through science to deal with the breadth of classes and variation within them. As of 2014, over 450 NPS were being monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Another difficulty involved in trying to control these substances is that they are difficult to detect by standard toxicology screens. When talking about classes of NPS drugs, the reference is to either the chemical family they belong to or their psychotropic effects. The most common NPS abused in Australia are led by phenethylamines, synthetic cannabinoids, synthetic cathinones and tryptamines. Concurring with this, the United States (US) and some European countries also find these four NPS drug classes to be amongst those most commonly abused.
These drugs need to be made illegal as soon as possible after their emergence due to them posing similar health risks to that of classic illicit substances. Due to the variation between different NPS classes, and further variations within those classes, their potential for addiction and negative effects on health are diverse. Abuse of NPS is still a new occurrence and research is scarce on addiction and negative health effects, in both short term and long term scenarios. However, what research has been completed agrees that adverse health effects that NPS drugs are capable of causing can be just as serious as that of classical illicit drugs, with some reported as being much more potent than the classic drugs they are being produced to replace.

Rapid, effective and reliable methods of identification and characterisation need to be utilised when an authority receives what is believed to be a NPS. The problem then arises that there are no genuine standards for screening and identifying NPS drugs. Ultimately it will come down to the authorities governing such practices to determine the process and specific methods utilised when new samples arrive. The more common methods utilised are infrared spectroscopy and gas chromatography-mass spectrometry. The results these methods return can then be compared to available spectroscopic and spectrometric libraries that contain data of known substances. If the substance being investigated is known, then the search of the library will return with its identity. However, if the substance is a NPS drug that has not previously been identified, or who’s data are not available on accessible libraries or databases, different methods then need to be used to identify the chemical structure of the substance to characterise and categorise it. These are nuclear magnetic resonance spectroscopy (NMR) and high resolution mass spectrometry (HR-MS). Even with these highly sophisticated analytical techniques, further experiments may need to be done to gather more spectroscopic information in finalising the composition of the
The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) recommends the use of multiple techniques mentioned above when identifying a seized drug.  

In June of 2013 the first international monitoring system on NPS was launched by the United Nations Office on Drugs and Crime (UNODC) and was titled the Early Warning Advisory (EWA). The EWA has improved the understanding of the NPS market and served as a knowledge hub for customs and forensic laboratories around the world. With their main aim being to enhance the ability of countries to anticipate NPS threats and reduce public health risk. They achieve this through the sharing of information on control measures, best methods for analysis of NPS and legislation that is being applied around the world in the war against NPS.

In 2015, the Commonwealth Government of Australia introduced new offences to the Criminal Code Act 1995 to ban NPS from being imported into the country. The NPS are banned on the basis of their appearance and their psychoactive effects if they are known. Even though they are banned and consequently seized when coming into the country or already in the country, they still need to be processed and identified so it can be determined what is actually being seized. As this is proving to be a difficult task, custom and crime laboratories are being left with a backlog of unknown substances.

Another organisation that is actively looking for ways to speed up the process of identifying and determining the chemical structures of NPS drugs is the Joint Research Centre (JRC), which is the European Commission’s science and knowledge service. In a 2014 report from the JRC, they explore potential ways of developing faster and simpler ways of completing this challenging task with advanced analytical methods that will be discussed shortly. In the
report’s conclusions it is suggested computer aided modelling systems should be developed to assist in the classification of chemical substances. Earlier in the report it is stated that isotopic profiles obtained from HR-MS instruments are helpful when determining the main features of a chemical substance. A solution to what they are trying to achieve could potentially be achieved by the coupling of HR-MS data and predictive modelling software. This literature review will be looking into the more prominent NPS drugs, how they are circumventing legislation, and examples of what authorities have done and are currently doing to make them illegal. Methodologies utilised when identifying seized NPS drugs will also be reviewed. Lastly, how algorithm based software could be potentially used to speed up the process of identifying known and unknown NPS will be investigated.

2.0 Discussion

2.1 Phenethylamines

Perhaps the oldest class of synthetic drugs found in the world are phenethylamines, with amphetamine and methamphetamine first being produced towards the end of the nineteenth century. This large class of drug is well known for its ability to increase monoaminergic transmissions and stimulation of activity in the central nervous system with its abuse recognised as a widespread problem. Evidence of the severity of this problem can be found in Europe during the past few decades where approximately 100 novel phenethylamines have been identified. In fact, among the 256 NPS drugs identified in Europe from 2012 to 2014, 80 of them were phenethylamine or synthetic cathinones. Their use has also been on the rise with one Australian study finding their abuse increase
from 8% in 2010 to 18.6% in 2015. This was the largest increase of use of any NPS drug in the study during the same time frame. The same study states that their findings mirror that of several international studies; however, they clarify it is difficult to make direct comparisons from their results to these other studies due to differences in time frames, samples and categorisation of NPS drugs. Since their inception, different phenethylamines have been used for medical purposes; however, many have become obsolete or discovered to have adverse side effects and have since stopped being used, which leaves few examples as licensed medicines. This in turn led to the pharmaceutical industry having less interest in ring-substituted phenethylamines. Phenethylamines commonly come in the form of pills and are taken orally through ingestion. Use of phenethylamines has been found to cause a wide range of symptoms both psychological and physiological. Some psychological symptoms include anxiety and hallucinations, while some physiological symptoms include tachycardia and hypertension. However, symptoms associated with phenethylamine poisoning have been found to be much more concerning and dangerous, with cases reporting seizures, cardiac arrest and even death. It is important to note that when phenethylamine substances are ingested with other substances such as alcohol, cannabis or other drugs, the risk of poisoning and severe symptoms are more likely to occur.

This class of drug is given its name due to the basic phenethylamine core which all variants are based around (Figure 1). The basic phenethylamine core is made up of an aromatic ring with two carbons ended by an amine group as a side chain. The core and side-chain can be modified in many different ways leading to hundreds of slightly different phenethylamine drugs. One example of this includes amphetamine and its derivatives, which are given rise to by substitution of the alpha carbon by a methyl group. Amphetamine and its derivatives have the optimal structure for psychostimulant activity. Another example is
the 2C and D series which are obtained through substitutions of the benzene cycle at positions 2 and 5 by methoxy groups and at position 4 by a variable substituent. Phenethylamines often interact as agonists of dopamine receptors, resulting in their psychostimulant properties. However, ring substitutions have been found to increase affinity for serotonin receptors, such as 5HT2a receptors, resulting in hallucinogenic properties. Coupled with further substitution, derivatives that express both psychostimulant and hallucinogenic properties can be manufactured. What helps make phenethylamines such a dangerous class of drug is how slight modifications to the phenethylamine skeleton can significantly alter the drug's pharmacological activity.

Figure 1. Chemical structures of phenethylamine and some popular derivatives, including cathinone derivatives.
Another danger that comes with phenethylamine abuse is that by modifying the core or side chain to form a novel phenethylamine, the new drug is not considered illicit until legally defined by the authority of that state. In the United Kingdom Misuse of Drugs Act, some phenethylamines are listed by name while some are still covered by the Act through generic definitions. In the United States of America, a bromine substituted phenethylamine was designated as a Schedule 1 material, which made it illegal to be in possession of or use. However, once that substance became illegal numerous analogs then entered the market, leaving crime laboratories backlogged with unknown substances and consequently resulting in law enforcement struggling to prosecute. This is another example of legislation being one step behind the production of these NPS drugs.

2.2 Synthetic Cannabinoids

Synthetic cannabinoids are one of the more commonly found and reported NPS classes worldwide. As well as being found around the world, its abuse appears to be on the rise with an emergency department having synthetic cannabinoid related visits double within the year of 2010 to 2011. Its abuse also looks to be popular among young adults as recent surveys have indicated that up to 11% of high school seniors in the US have tried synthetic cannabinoids. Although the abuse of synthetic cannabinoids has been on the rise in the past decade, particular ones that will be discussed have been around since the late 1980s. Inhalation is a common method of taking synthetic cannabinoids; however, after chemical synthesis they can also be ingested. Perhaps most often, synthetic cannabinoids are dissolved in volatile solvents and sprayed onto or mixed with a range of plant leaves which are then smoked. In recent times, hundreds of these synthetic cannabinoid mixtures have
become commercially available whilst being quasi-legal, unregulated and labelled as herbal incense. The most common mixes are marketed as K2 and Spice. 18

A popular misconception regarding synthetic cannabinoids is that they are synthetic marijuana/cannabis. Synthetic cannabinoids are not derived from cannabis; however, they are functionally similar to tetrahydrocannabinol (THC), which is the active component of cannabis. They are similar to THC in that they are both agonists of cannabinoid (CB) receptors. They differ as THC is only a partial agonist of CB receptors whereas synthetic cannabinoids are strong agonists. This binding and agonistic effect on CB receptors, which are located throughout the body, is what causes the well documented psychological effects of THC, which include difficulty in concentrating, memory failure and changes in perception of external stimuli, among other symptoms. 19,21 An issue that arises from abusing synthetic cannabinoids is that because they are strong agonists of CB receptors, they consequently produce stronger psychological effects in a shorter amount of time than that of THC. 19 Another health hazard to taking these drugs is that when they are produced they are never thoroughly tested for negative side effects or approved for human use. In fact, the majority of the production practices of these products are often unknown, including the composition, purity and concentration of their active components. 17 As well as psychological effects, synthetic cannabinoids can also elicit a range of physiological effects if ingested. Evidence has suggested that natural cannabis can counterbalance negative effects of THC, which are “softened” due to the presence of phytocannabinoids and terpenoids. 18 According to this study, these two cannabinoids have not been detected in any seized synthetic cannabis products and their
absence potentially leads to the high levels of CB receptor activation that produce severe psychological and physiological symptoms observed in users. 18

Some more prevalent and well documented synthetic cannabinoids include CP-47,497, JWH-018 and HU-210 (Figure 2). These synthetic cannabinoids have been reported as strong agonists of CB receptors and previously been found on supposedly all-natural herbal incense products with the real intention of being abused as cannabis substitutes. 18 This is how these NPS drugs were initially entering countries and being distributed into populations, which provided people a legal alternative to cannabis in the early 2000s. 19 The packaging of these synthetic cannabinoid mixes managed to avoid legislation in some countries simply by having “Not for human consumption” written on them. 18 Over time, many countries have banned these more common synthetic cannabinoids as well as closely related variants. 17 However, when legislation is updated to ban these substances, herbal incense manufacturers replace the banned synthetic cannabinoids with new, unregulated variants. 18 This then leads to having to identify the new variants through monitoring authorities and eventual updating of legislation. 17 Understandably when the abuse of synthetic cannabinoids began to emerge the pharmacology of their active components were not as well documented as they are now. This led to scientists swiftly monitoring, identifying and quantifying with mass spectrometry the persistently changing contents of these synthetic cannabinoids, which continues to this day. 18
It is incredibly difficult to create and implement legislation regarding the use of synthetic cannabinoids due to the large volume of them as well as how quickly new variants of them can be produced. For example, in the United States in 2011 five compounds, including the aforementioned CP-47,497 and JWH-018, were temporarily categorised as Schedule 1 substances by the DEA. However, by 2012, the five synthetic cannabinoids were already replaced by five new compounds with similar structures. Later in 2012, President Obama signed into law the Synthetic Drug Abuse Prevention Act of 2012. This Act specifically named 15 synthetic cannabinoids which permanently categorised them and several chemical structural classes of cannabinoids as Schedule 1 substances. As mentioned before there are hundreds of synthetic cannabinoid mixtures, and the development of new compounds does not seem to be difficult for those that produce and supply them due to new variants continually being synthesised. Identifying known synthetic cannabinoids is not as much of an issue nowadays as more published data have been released over time for comparison. It
is the unknown synthetic cannabinoids that need faster and more reliable characterisation methods developed. An interesting discovery in a study by Ernst was that a sample they obtained was determined to be JWH-018, which had not only been previously prohibited by German regulations, but is regulated in most countries that normally regulate synthetic cannabinoids. This observation in the study demonstrates that even though a synthetic substance may be made illegal it can still appear and be used by the public similarly to classical illicit substances.

Even with the increase in published literature and studies on synthetic cannabinoids, there are still some aspects that need further study. As mentioned it is known that synthetic cannabinoids interact with CB receptors; however, the direct and indirect pharmacology activity they have with non-CB receptor systems needs further research, such as the serotonergic and dopaminergic systems. The pharmacokinetics of synthetic cannabinoids should also be investigated further in order to better understand the effects of parent synthetic cannabinoids and their active metabolites.

2.3 Synthetic Cathinones

Synthetic cathinones are another class of NPS drugs that have experienced a concerning emergence in recent years. 30 new synthetic cathinones were reported to the EMCDDA between 2005 and 2012. They, along with synthetic cannabinoids, represent more than two thirds of NPS drugs notified since 2005. In 2010 mephedrone, a synthetic derivative of cathinone, was found to be the third most commonly abused drug in the UK. Then in 2013, seven synthetic cathinones were reported to the European Union Early Warning System for the first time. The large majority of synthetic cathinones have been reported to be produced in China and South East Asian countries. In an Australian study, synthetic cathinones were
the most prevalent NPS being used by participants in the year 2010 at 18.5%. However, by 2015 there had been a substantial decrease in reports of its use, declining to 7.7%. Although on the surface this looks to be a positive trend, phenethylamines and synthetic tryptamines both made significant increases in their reported use in the same time frame. The same study found that recent synthetic cathinone use was significantly associated with daily tobacco use and poly drug use. The study concluded poly-NPS users to be a high risk group as they were likely to have been involved in criminal activity and to have overdosed on any drug leading up to the study. ¹

Cathinone occurs naturally and is found in the plant Catha edulis (Khat) which has been used for its pharmacological effects for centuries. ²²,²⁴ Cathinones and its synthetic derivatives are closely related to the phenethylamines class of drugs with the main difference between them being the presence of a β-keto group in the aliphatic chain (figure 3).²²,²³ Its chemical structure in modern history has since been subject to different modifications resulting in dozens of synthetic derivatives (figure 4). This structural similarity results in synthetic cathinones exhibiting similar effects to phenethylamine derivatives such as amphetamine and methamphetamine. These effects mainly being stimulation of the central and sympathetic nervous systems.²² Methcathinone is an N-methyl derivative of cathinone developed in the 1980s, the synthesis of which is completed by oxidising pseudoephedrine and ephedrine which are readily available pharmaceuticals. Other approaches to synthesising methcathinone and other derivatives of cathinone include oxidation of the benzylic hydroxyl group to a ketone and substituting methods involving the aromatic ring or amine group.²⁴
Some more well-known synthetic cathinones include mephedrone, methylone and the pyrovalerone types (e.g. 3,4- methylenedioxypyrovalerone (MDVP) (Figure 5)). Synthetic cathinones are commonly sold online and sometimes, similarly to synthetic cannabinoids,
labelled as not for human consumption. They have also been found to come with labels such as “not tested for hazards or toxicity” as well as being sold under slang terms such as bath salts, plant food and research chemicals. Synthetic cathinones commonly come in the form of white powder or crystalline mixture, commonly known as bath salts. In these two forms they are either snorted or the substance is rolled in cigarette paper and smoked. They are also known to come in tablet form in which they are taken orally. A less common, but more concerning, method of administering the drug is through intravenous injection.

![Figure 5. Synthesis of MDVP.](image)

As mentioned above synthetic cathinones are structurally similar to phenethylamine and its derivatives, thus consequently producing similar effects. However, there is a lack of literature and studies investigating synthetic cathinones’ mechanisms of actions and their toxicity. Case reports and experimental studies that have been done still do not provide definitive conclusions. Factors that affect these studies include patients that have consumed several drugs simultaneously, and potential symptoms attributed to synthetic cathinone use
being caused by underlying psychiatric and medical diseases. The dose-effect relationship has also been noted to be absent from human volunteer studies. 23

Synthetic cathinones are known to be potent norepinephrine reuptake inhibitors. 23 However, there have been noted differences between derivatives in their ability to release monoamines as well as inhibit reuptake of dopamine and serotonin. The varying of abilities between derivatives could attribute to clinical differences in their effects and toxicities being reported. 23 Recent reports have shown MDVP to have locomotor-stimulant effects. 25 One study found MDVP to be at least 10 times more potent than cocaine when it was tested in assays measuring motor hyperactivity and cardiovascular stimulation in vivo. 25 This was concluded to be caused by a potent blockade of dopamine uptake. 25 Patients admitted to emergency departments with confirmed MDPV consumption have been reported to display symptoms such as agitation, psychosis, violent behaviour and tachycardia amongst others. 25 Symptoms produced by high-doses of MDPV were reported to resemble those seen in acute cocaine toxicity. 25 This is just one derivative of many which can cause such serious symptoms. This study concluded that to remediate symptoms of overdoses on MDPV or other “bath salts”, efforts to manage excessive dopaminergic and noradrenergic stimulation should be taken. 25

Even though MDPV, mephedrone and methylone are listed as Schedule 1 drugs in the US, new legal synthetic cathinones are rapidly produced and marketed to replace them. 25 Cathinone and its synthetic derivatives are constantly being modified in clandestine laboratories. Coupled with being marketed on the internet and anonymised illegal networks, it is incredibly difficult for the legal process to keep up with their production to control them. 23 It is important that the synthetic cathinones being produced to replace controlled
derivatives are investigated as soon as they emerge. Determining their pharmacology will help treat overdoses and poisonings, as well as assist legislation in identifying and controlling the substances. 25

2.4 Tryptamines
Another class of NPS drugs that have risen in popularity in recent times are synthetic tryptamines. They entered the designer drug market in the late 1990s and were known as psychedelic recreational drugs. 26 Tryptamine is a naturally occurring chemical structure; however, it can be synthesised by the decarboxylation of the amino acid tryptophan to create substituted tryptamines, also known as synthetic tryptamines. Synthetic tryptamines, like some phenethylamines, are agonists of 5HT2a receptors and therefore act as hallucinogens. 27 Synthetic tryptamines have been reported to effect perception, mood and thought with just minor doses. 27 The United Nations Office on Drugs and Crime World Drug Report 28 detailed that up until half way through 2012, 10% of identified NPS drugs were synthetic tryptamines. Three other classes of NPS drugs finished with higher percentages than synthetic tryptamines: phenethylamines, synthetic cannabinoids and synthetic cathinones. 26 The same United Nations body lists 25 different tryptamine molecules as unregulated as of 2013. 27 An Australian study found that synthetic tryptamine use rose from 8% to 10.9% between 2010 and 2015, making it one of the two most commonly used NPS drug classes in those years along-side phenethylamines. 1

As mentioned tryptamine is naturally occurring and is found in both the plant and animal kingdoms. It is a primary amine alkaloid based on a heterocycle indole. Serotonin, a well-known neurotransmitter and local hormone in humans, is an example of a natural occurring tryptamine, with other naturally occurring tryptamines including melatonin and bufotenin
The tryptamine structure has been found in naturally occurring hallucinogens since the 1950s. Due to chemical research over the years, tryptamines have been synthesised into the molecular structures of other natural products. This research has led to some synthetic analogues of tryptamine being produced that have legitimate use as medicine. One such example is sumatriptan which is used to treat migraines. Other tryptamines have been synthesised to exploit their pharmacological effects for use as recreational drugs. Like phenethylamines, synthetic tryptamine derivatives’ pharmacological properties differ from other derivatives due to differences amongst their molecules. Alpha methylation of tryptamine derivatives results in stimulant properties compared to unmethylated derivatives, while substitution in position 5 of the indole ring has been found to make the analogue more potent when compared to the parent drug.

Figure 6. Tryptamine and related compounds.
Similarly to other NPS drug classes that have been reviewed, synthesising new analogues of the drug class is not difficult. There are several different approaches to synthesising tryptamine, mentioned above already is the methylation and ring substitution approach. N,N-diethyltryptamine (DET), N,N-dimethyltryptamine (DMT) and α-ethyltryptamine (α-ET) are three well known synthetic tryptamines. Unfortunately, these three are the only synthetic tryptamines internationally controlled under the 1971 International Drug Control Convention. Under this convention they are listed as Schedule 1 compounds.

Mentioned earlier, there are still 25 unregulated tryptamines, the effects of which are expected to be similar to the three controlled synthetic tryptamines; however, they are available as non-illegal alternatives. Another factor that tempts people into using these drugs is that they are not routinely detected in emergency departments or in routine analysis. This creates a more serious situation though, as symptoms can be misinterpreted and not as much information is gathered about the drug. In line with this, some recent surveys did not explore areas of interest such as the epidemiology of different tryptamines, their prevalence, or differentiate between regulated and unregulated tryptamines. Therefore, there is a lack of information when it comes to the use and distribution of synthetic tryptamines both locally and internationally. This has led to the internet being the main source of information for clinicians and researchers interested in synthetic tryptamines.

Energy Control is a harm-reduction service that allows an anonymous drug-checking service to Spanish nationals. From 2006 to 2015 they carried out a study to better understand synthetic tryptamines, which involved a total of 25,296 samples. During the study, the submission of synthetic tryptamines was found to be higher than that of regulated
tryptamines. This trend suggests people are taking more risks as there is less literature available on the effects of these newer substances. However, regulated tryptamines experienced an increasing trend over the course of the study. Since 2013 there was a decreasing trend in the submission of unregulated tryptamines. An interesting result from the study was that the second most prevalent synthetic tryptamine, α-MT, was initially used as an antidepressant before it was discontinued as it was discovered to be toxic. Therefore, the potential of some users trying to self-medicate must be considered when recreational use is discussed. 27

The pharmacodynamics and pharmacokinetics and clinical effects of synthetic tryptamines have been identified as areas that need further study. 27 Additional receptor subtypes also need further investigation as the 5HT1a and 5HT2a receptors are the only two well documented receptors that have been identified as playing important roles in the binding profiles of synthetic tryptamines. 26

2.5 Methods Used to Identify NPS

The major challenge involved with controlling and regulating NPS products is the rapid screening and identification of said products as there are no uniform standards. 18 The more common methods of identifying NPS include gas chromatography-mass spectrometry (GC-MS) and/or infrared spectroscopy and comparing them to known published data. Raman spectroscopy is another method that has been used in recent times to achieve this. If the NPS has previously been characterised it will be found in the available databases and libraries. 7,9,17 If the NPS has not been previously characterised then the task of identifying
the unknown NPS becomes more time consuming and involves more advanced analytical methods such as NMR spectroscopy and HR-MS.

GC-MS is a method that allows the separation of organic molecules that can be made volatile and not thermally labile. When the analytes are separated a chromatogram is generated (Figure 7) and retention times of the eluting compounds are measured. The mass spectrum of the eluting compounds is generated by passing the eluting stream into a mass spectrometer. If the NPS has previously been characterised the retention time and mass spectrum can be searched against available libraries and databases to find a match. If a positive match is found an analyst can then confirm the identity of the NPS being investigated. If the NPS has not been characterised previously, this method can still provide some important data on unknown NPS such as molecular weight of the compound as well as some of its constituent fragments. This information can be useful in hypothesising the structure of the NPS. Only hypothesis can be made though, as the analytical data obtained does not allow full confirmation of an unknown NPS chemical structure as the instrument may not be of sufficient resolution or the ionisation technique may not yield a molecular ion. This method is widely used and is suitable for routine control as analyses can be performed in anywhere from a few minutes to an hour while being cost effective. This method can also be used to perform quantitative determinations given suitable conditions.
Infrared spectroscopy and Raman spectroscopy are methods commonly used as controls for recognition of drugs and illicit substances. They are used as controls as both can be applied directly to NPS samples when they are in solutions or powder form, whilst being fast and easy to operate. These methods are based on molecular vibrations and generate a spectrum of the NPS being investigated. Patterns produced by these methods can be characteristic of functional chemical groups, which provide information on the chemical structure of the investigated NPS. However like GC-MS, these two methods do not provide enough information for previously unknown NPS. 

NMR spectroscopy is named after the phenomenon it relies, that is nuclear magnetic resonance. The phenomenon occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. This causes nuclei located near one another to exert an influence on each other’s magnetic field, the results of which can be viewed on a NMR spectrum (Figure 8). NMR can be used to determine content and purity of a sample as well as its molecular structure, which is important in characterising unknown NPS. NMR can infer the basic structure directly of unknown NPS, and
consequently be used to determine molecular conformation in solution. Results produced from NMR methods are imported into special software that assigns peaks with corresponding atoms of the structure belonging to the substance being investigated. The coupling constants and types of multiplicity of peaks produced are also determined.

Figure 8. NMR spectroscopy results for two samples in a study by Vincente.

High-resolution time-of-flight (TOF) electrospray mass spectroscopy, shortened to HR-MS, is an advanced analytical method that can utilise several modes to provide different information on known and unknown substances. As the name suggests, the TOF mode is used to determine an ions mass-to-charge ratio with a time measurement. A mass spectrometry spectrum is produced for the substance, from which a substance’s molecular mass and elemental composition can be determined. Electrospray tandem mass spectrometry experiments are additional methods that can be utilised to provide further information contributing to determining the structure of the substance under investigation. HR-MS systems are operated by software that have their results generated into Raw files
(*.RAW) which can then be converted into *.CDF files. Once in *.CDF format, they can be imported into specially designed software that allows for prediction of the fragmentation of a substance as well as displaying spectral ion trees (Figure 9). HR-MS methodologies can be coupled with NMR analysis to further confirm characterisation of unknown substances under investigation.

Figure 9. HR-MS spectra that has been enhanced by Mass Frontier™ software for a sample from a study by Vicente.

In a study by Ernst an unknown NPS believed to be a synthetic cannabinoid was isolated by silica gel column chromatography and had its structure characterised through NMR methods. It was then further characterised by GC-MS, electrospray tandem mass spectrometry and infrared spectroscopy. After the application of these methods the NPS was identified as JWH-307, a synthetic cannabinoid unregulated in Germany where the study was done. A study by Vicente underwent the task of identifying two unknown samples that had been seized by Belgium Customs. The samples were successfully characterised and identified by using a combination of NMR, HR-MS and Raman
spectroscopy. The analytical strategies followed in this study were based on the scientific experience of the JRC. The analytical strategy allowing the characterisation of unknown compounds penned by the JRC has been applied successfully to approximately 100 samples between 2013 and 2015 by the Customs Laboratories European Network (CLEN) as well as in the study mentioned above. Unfortunately the resources at the disposal of the JRC and CLEN are not available to every laboratory around the world that deals with the identification and characterisation of NPS, which makes it more difficult for some governing bodies to keep up with the continuously emerging NPS.

2.6 Predictive Modelling Software

The sophisticated analytical techniques mentioned above have successfully been applied to identifying and characterising NPS by the JRC and CLEN over the past few years. With their successful applications, governing bodies can then take action against the NPS that have been identified. Also mentioned is that the JRC and CLEN being large organisations have a large amount of resources that include technology and facilities but also manpower. Customs and forensic laboratories in many countries around the world may not have access to the equipment and facilities at the disposal of these larger organisations, consequently leaving these laboratories unable to process samples as quickly and accurately. This in turn leads to a backlog of samples in these laboratories. A solution presented by the JRC is the introduction of computer-aided modelling software that is based on predicting the chemical structures of tested substances which can facilitate in the classification of said investigated substances. Data that could be used in achieving the task of predicting chemical structures of NPS could be by isotopic profiles produced by mass spectrometry. With that
information then available, appropriate actions can be taken regarding control and regulation of that drug.

Software that already exists that is used in interpreting mass spectrometry data is Mass Frontier™ created by Thermo Scientific™. This software was used in the study performed by Vicente⁹ discussed earlier. This software predicts and returns mass spectrometry fragmentation pathways and can also be used for identifying specific compound classes.²⁹

3.0 EXPERIMENTAL DESIGN

In Australia NPS are banned and can be seized on appearance alone. Murdoch University does not have access to samples of NPS, instead it has access to mass spectrometry data from databases and libraries available online. Mass spectrometry data pertaining to the classes of NPS covered in this review will be collated from different online sources as that is ideally the only information that should be needed as input for this software to produce information determining NPS class and chemical structure. Understandably, if the data of these NPS are already on databases and libraries online they have been identified previously. As there is only access to online sources only known NPS will be tested with the software being developed. However, this will be beneficial in that data will be available to confirm if the software has correctly identified a NPS.

The specific mass spectrometry data that will be gathered will include average and monoisotopic weights of NPS as well as mass spectra and chemical structure. Other information that will be collated includes NPS name, class and chemical formula.
When the necessary information has been collated, the development of algorithm for the software can begin. This will be done in conjunction with Doctor Giles Oatley, who is the senior lecturer in Information and Technology at Murdoch University. R studio is most likely to be used; however, there is the potential for other software to be utilised. Ways of testing the software will be evaluated concurrently with the development of the algorithm.

4.0 PROJECT AIMS

It is evident from the literature covered in this review that there is significant and ongoing issue regarding the identification, regulation and abuse of NPS around the world. The difficulty in identifying these continuously emerging NPS leads to the lag in governing bodies being able to regulate these substances all while people continue to abuse them. Therefore, the aim of this project is to create a cost and time effective method that can be used to identify which class of NPS a substance may belong to in the form of software that utilises mass spectrometry data. Once mass spectrometry is completed on an investigated substance, the data acquired (isotopic profiles) can be put into this software that will then predict the chemical structure of the substance, or at the least determine the basic core of the substance. For cases of phenethylamines it will at least identify the basic phenethylamine core. For synthetic cathinones and synthetic tryptamines it will identify the cathinone and tryptamine structures respectively. As synthetic cannabinoids do not all share a similar core to each other and are instead characterised by functionality, familiar structures that have been previously been linked to synthetic cannabinoids like the JWH family will try to be identified. In doing so it can determine what class of NPS the substance belongs to and potentially identify the entire chemical structure. With a laboratory able to
successfully use this software it could speed up the process of identifying NPS with only needing to carry out HR-MS and then use this software. Appropriate actions can then be taken regarding the control and regulation of that drug. The software could be seen as an extension of Mass Frontier™ specific for the identification and categorisation of NPS.

5.0 CONCLUSION

NPS are a problem effecting countries around the world. This is not only because of their significant potential hazard to health, but also because of the difficulty involved in regulating them. This difficulty arises because in order for governing bodies to regulate NPS, they need to know what it is exactly they are regulating. Organisations such as the JRC and CLEN have successfully developed advanced analytical methods to identify both known and unknown NPS. However, many customs and forensic laboratories around the world do not have the equipment, techniques and manpower that these organisations possess. Therefore, a method that is both time and cost effective must be developed in order to help customs and forensic laboratories identify these NPS as quickly as unknown NPS derivatives are emerging. A solution could be the development of predictive modelling software that can determine the chemical structure of a NPS based on acquired mass spectrometry data. This could lead to the identification of the NPS and its consequential regulation, and solving the issue of customs and forensic laboratories having backlogs of unidentified substances.
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Part Two

Manuscript

METHODS FOR IDENTIFICATION OF NOVEL PSYCHOACTIVE SUBSTANCES AND AN EXPERIMENTAL DESIGN: A REVIEW
METHODS FOR IDENTIFICATION OF NOVEL PSYCHOACTIVE SUBSTANCES AND AN EXPERIMENTAL DESIGN: A REVIEW

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Abstract

The rapidly emerging and evolving market for new psychoactive substances (NPS) has been recognised as a phenomenon that poses a serious risk to public health and safety. As they continue to circumvent existing legislation around the world, the identification of NPS by forensic laboratories is crucial in controlling them. Techniques such as gas chromatography-mass spectrometry (GC-MS), Fourier transform-infrared spectroscopy (FT-IR), Raman spectroscopy, nuclear magnetic resonance spectroscopy (NMR) and high resolution-mass spectrometry (HR-MS) are known to assist in the identification and characterisation of NPS. However, there are no published or widely accepted standards or workflow to follow when it comes to completing this task. This review looks at a proposed experimental design in which the aforementioned techniques are successfully used in identifying and characterising two unknown NPS acquired by Belgian Customs. Other literature that use these techniques with NPS are also reviewed. The use of these techniques in this workflow design appear to produce promising results.

Key Words: Novel psychoactive substances, new psychoactive substances, analytical methods, GC-MS, FT-IR, Raman, NMR, HR-MS, identification, characterisation, workflow, design.
Introduction

Over the last decade novel psychoactive substance (NPS) abuse has been on the rise around the world and has drawn concerns amongst the international community. As well as the increase in reports of abuse; concerns are drawn from their availability, the similar and sometimes more potent effects they cause as internationally controlled/scheduled illicit substances, and their ease of synthesis. As the relevant governing bodies endeavour to implement legislation to control NPS in their jurisdictions, the issue arises that these substances need to be unequivocally identified in relation to their chemical structure in order to do so. In 2015, the Commonwealth Government of Australia introduced new offences to the Criminal Code Act 1995 to ban NPS from being imported into the country. NPS are banned on the basis of their appearance and their psychoactive effects, if they are known. Even though they are banned and consequently seized when coming into the country or already in the country, they still need to be processed and identified so it can be determined what is actually being seized. As this is proving to be a difficult task, customs and crime laboratories are being left with a backlog of unknown substances.

The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) have recommended minimum standards for forensic identification of illicit substances which involves the utilisation of multiple uncorrelated techniques. Based on their maximum potential discriminating power, analytical methods are placed into three categories (Table 1). SWGDRUG recognises that due to certain circumstances that may arise in a case, some methods may not be as effective as they would be in others.
Table 1: Categories of analytical methods.  

<table>
<thead>
<tr>
<th>Category A</th>
<th>Category B</th>
<th>Category C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infrared Spectroscopy</td>
<td>Capillary Electrophoresis</td>
<td>Colour Tests</td>
</tr>
<tr>
<td>Mass Spectrometry</td>
<td>Gas Chromatography</td>
<td>Fluorescence Spectroscopy</td>
</tr>
<tr>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
<td>Ion Mobility Spectrometry</td>
<td>Immunoassay</td>
</tr>
<tr>
<td>Raman Spectroscopy</td>
<td>Liquid Chromatography</td>
<td>Melting Point</td>
</tr>
<tr>
<td>X-ray Diffractometry</td>
<td>Microcrystalline Tests</td>
<td>Ultraviolet Spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Pharmaceutical Identifiers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thin Layer Chromatography</td>
<td></td>
</tr>
<tr>
<td>Cannabis Only:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macroscopic Examination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscopic Examination</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

When a method from Category A is used, at least one other method must also be used from either Category A, B or C. This is what is seen in many studies involving the identification of NPS, though often three or four methods are used as well as the use of hyphenated methods such as gas chromatography-mass spectrometry (GC-MS). A comment SWGDRUG make is that their recommendations for the identification of drugs in this document only apply to the identification of commonly seized drugs and not NPS. They acknowledge that their minimum standard recommendations coupled with these methods may not be sufficient in the identification of “all drugs in all circumstances”.  

In 2008, the United Nations Office on Drugs and Crime (UNODC) launched the Synthetics Monitoring: Analyses, Reporting and Trends (SMART) program in an attempt to enhance its member states capacity to combat the rapid emergence of NPS. This includes being able to
apply scientific evidence-based knowledge to design protocols and standards for identifying NPS. By 2013, 60 countries and territories had provided information regarding methods they had utilised in the identification of NPS. When it came to chemical analysis methods, most respondents reported using GC-MS. Other methods reported included high resolution-mass spectrometry (HR-MS) techniques, Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance spectroscopy (NMR). Following the use of chemical analytical methods, many countries and territories then utilise mass spectral libraries as reference standards for verification of their results. 1 When laboratories are employing these methods, there are no genuine standards to follow apart from SWGDRUG and some independent accrediting bodies, leading to difficulties in identifying NPS. 1,5,6 Although UNODC have documents containing recommended methods for the identification of particular NPS, they just list and define methods instead of recommending a structure to follow. 1 Therefore, a design of workflow and methodology needs to be implemented that ensures NPS are successfully characterised and identified through the use of a combination of analytical techniques in an effective way that forensic and custom laboratories can follow. 1,7,8,9 This review will look at a particular study that has developed such a design as well as citing other studies that have successfully employed these techniques for identification and characterisation of NPS.

An Experimental Design

In 2015, a meeting was held by the European Commission regarding the Customs Laboratories European Network (CLEN) project on designer drugs and other illicit products which heavily revolved around the identification of NPS and the sharing of data regarding
NPS. A key objective of CLEN2SAND is the creation of an electronic data repository collection which contains both spectral and chemical data produced from samples and is available to other members. This will create a flow of information for each sample encountered and the ability to access information electronically as reference material. Networking and the sharing of data was noted as being highly important when it came to dealing with the widespread dangers of NPS.

A study by Lobo Vicente that was discussed in the report from the 2015 meeting introduced an experimental design in which a combination of advanced analytical methods and chemoinformatic tools were utilised to successfully identify samples that had been seized by Belgian Customs (Figure 1). This study concluded that GC-MS and FT-IR can be used for confirmatory purposes if the initial structure has been previously documented and is successfully matched on spectral libraries and databases. When these methods could not confirm the identity of a sample, NMR, HR-MS and Raman spectroscopy methods were utilised for the NPS' full analytical confirmation. The study recognised the collaborative work between European Customs laboratories and the JRC as being successful as well as noting the importance of sharing data and the wide range of tools outlined in the study.
Figure 1: Experimental design of workflow resulting in the successful identification of two synthetic cannabinoids.  

Chemoinformatic Tools used by Vicente et al:

- ACD/Spectrus Platform Database
- Mass Frontier Software from Thermo Scientific™
- SWGDRUG Website
- European Drug Network Database

Other chemoinformatic tools found to be of use:

- European Customs Inventory of Chemical Substances Database
- Exchange of Unknown Chemical Substances Information Database
- UNDOC Global SMART Programme
- mzCloud.org
- Forendex
- Chemspider
Gas Chromatography-Mass spectrometry (GC-MS)

GC-MS is a method that can be used as a basis for identification of investigated substances by producing a spectrum. This spectrum provides a characteristic fragmentation pattern and the molecular weight of a substance; thus, making this a useful method in the routine identification of NPS.

In a study by Geyer, GC-MS was used to analyse a total of 13 synthesised diphenidine derivatives in an attempt to develop a general screening method and to also quantify the active components of the samples (Figure 2). The methods utilised in this study were validated in accordance with the International Conference on Harmonisation Guidelines and all results from the study were within statistically acceptable limits. The GC-MS method was determined to be suitable for the analysis of diphenidine street seizure samples by providing a screening protocol that facilitated the separation and identification of all 13 diphenidine derivatives in the study. The method was found to be suitable for both the qualitative and quantitative analysis of the psychoactive ingredients present while additionally being a method that involved a rapid single-step extraction that did not have the need for derivatisation.
A study by Shevyrin from 2015 used GC-MS to identify synthetic cannabinoids through investigating the peculiarities of their mass-spectral fragmentation patterns. They concluded their findings of analytical characteristics would enable the identification of the compounds in future materials seized by authorities. A more recent study was able to achieve structural elucidation of 2,2’-difluorofentanyl by coupling GC-MS with other analytical methods including FT-IR and NMR. Characteristic fragmentation routes were also investigated to facilitate forensic laboratories in the identification of similar substances in future cases.

In the Lobo Vincente’s 2016 study, GC-MS was successfully used in the confirmation of one of two NPS apprehended by Belgian Customs. The GC-MS spectrum produced from their analysis of one of the seized NPS corresponded well with analytical data of 5F-AMB, which was available on the SWGDRUG database. For the second seized NPS, a GC-MS spectrum was produced; however, no match could be found with existing spectra on available databases. This meant additional and more advanced methods such as NMR and HR-MS needed to be used in identifying the second NPS.
GC-MS is already a standard method in forensic laboratories and is suitable for routine analysis as the method is quick and affordable.\textsuperscript{8,10,16} However, this method is not sufficient for meeting the identification criteria when the substance being investigated has not been previously identified. As the data produced from GC-MS are searched against available databases and libraries, issue arises when the reference NPS data is not available.\textsuperscript{10} In order to assist laboratories around the world by having more accessible data, two large databases have been created by international organisations. These are the European Network of Forensic Science Institutes (ENFSI) Drugs Working Group MS library and the SWGDRUG library.\textsuperscript{9} With access to these libraries, routine analysis via GC-MS will be made easier. When unknown NPS are encountered GC-MS still provides important data on the unknown substance; however, those more advanced techniques mentioned will be needed for the unequivocal identification of such NPS.\textsuperscript{8,16}

**FT-IR (Fourier Transform Infrared Spectroscopy)**

FT-IR relies on molecular vibrations to produce a spectrum of an investigated substance. Commonly used for routine analysis of drugs, it is a rapid and easy to use method. The spectra produced from FT-IR have the potential to be characteristic of functional chemical groups and consequently can offer information regarding chemical structure of a substance.\textsuperscript{8}

In Vicente’s\textsuperscript{10} 2016 study, FT-IR was used as an initial technique to be utilised in their experimental design for identifying NPS. When the two seized NPS were brought to the laboratory, FT-IR was used to produce a spectrum of them (Figure 3). The spectra were then compared with reference material, one of which corresponded well to the analytical data of
5F-AMB available on SWGDRUG’s website.\textsuperscript{10} The other spectrum was unable to be matched against any reference data available, meaning the more advanced analytical techniques in the next phase of their design needed to be utilised in identifying it.\textsuperscript{10} However, FT-IR was determined to be suitable for confirmatory purposes in casework when the NPS being investigated has previously been documented and whose data are available on spectral libraries such as SWGDRUGs.\textsuperscript{10} In a study investigating the development of a GC-MS screening protocol for GC-MS, FT-IR was used in conjunction with NMR to structurally characterise the NPS to verify the authenticity of the identification in this study.\textsuperscript{12}

![Figure 3: An example of a FT-IR spectrum belonging to the NPS PX3.\textsuperscript{10}](image)

FT-IR has been used successfully in structural elucidation of NPS when coupled with other analytical methods consistently.\textsuperscript{10,12,15,17} It is a technique that has been determined to have high discriminating power and has been shown to be suitable for routine analysis.\textsuperscript{5,8} Vicente’s\textsuperscript{10} 2016 study concluded that FT-IR can confirm the identity of previously reported NPS when coupled with GC-MS results and matched against spectral libraries. When FT-IR spectra do not return an acceptable match against any spectral libraries, the data are still
valuable in full characterisation and identification of the investigated NPS. The results being published and shared is also valuable as it can be useful to future caseworks that may have similar FT-IR spectra returned for NPS under investigation. 10

**Raman Spectroscopy**

Raman spectroscopy techniques utilise molecular vibrations to differentiate between similar unknown chemicals. 8 A fast and easy to operate method, it can be applied directly to substances either in solution or powder forms, which is ideal for being used as an analytical tool for identification of illicit drugs. 8 Its application in successfully characterising and identifying NPS when used in combination with other analytical methods such as NMR is promising. 8,10

In Vicente’s 10 2016 study, Raman was one of the techniques used in unequivocally characterising and identifying two NPS under investigation. Results retrieved from the method were shown as spectra graphs (Figure 4) and peak tables (Table 2), and when coupled with NMR results confirmed the proposed structure and identity of the NPS. 10 The Raman results were also complementary to previously published data on one of the investigated NPS, further confirming the NPS successful identification. 14 An important note is that when it comes to determining the chemical structure of a previously unknown NPS, Raman spectroscopy as a technique on its own will not be sufficient to complete this task. 8
Figure 4: Raman spectra for 5F-AMB. 10

Table 2: Raman spectroscopic data for 5F-AMB. 10

<table>
<thead>
<tr>
<th>Position</th>
<th>Intensity</th>
<th>Possible functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>763.44</td>
<td>37.148</td>
<td>–C–halogens</td>
</tr>
<tr>
<td>779.81</td>
<td>63.751</td>
<td></td>
</tr>
<tr>
<td>882.74</td>
<td>22.351</td>
<td></td>
</tr>
<tr>
<td>908.95</td>
<td>32.149</td>
<td></td>
</tr>
<tr>
<td>1005.61</td>
<td>59.671</td>
<td>–C=C=–</td>
</tr>
<tr>
<td>1136.23</td>
<td>26.042</td>
<td>C–O–C esters</td>
</tr>
<tr>
<td>1178.95</td>
<td>23.548</td>
<td>C–N in aromatic compound</td>
</tr>
<tr>
<td>1214.77</td>
<td>25.593</td>
<td>Branched chain</td>
</tr>
<tr>
<td>1240.12</td>
<td>36.770</td>
<td>in hydrocarbons</td>
</tr>
<tr>
<td>1273.01</td>
<td>28.264</td>
<td>C–N in aromatic amines</td>
</tr>
<tr>
<td>1324.16</td>
<td>33.368</td>
<td>–C–F in aliphatic compounds</td>
</tr>
<tr>
<td>1361.24</td>
<td>51.548</td>
<td>Isopropyl group</td>
</tr>
<tr>
<td>1376.13</td>
<td>38.125</td>
<td>–CH₃</td>
</tr>
<tr>
<td>1405.57</td>
<td>110.571</td>
<td>C–N in primary amides</td>
</tr>
<tr>
<td>1441.51</td>
<td>35.976</td>
<td>–CH₂CH₃</td>
</tr>
<tr>
<td>1472.66</td>
<td>60.965</td>
<td>N aromatic</td>
</tr>
<tr>
<td>1575.51</td>
<td>64.968</td>
<td>Possible N=N aliphatic</td>
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<tr>
<td>1653.89</td>
<td>50.390</td>
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<td>1738.41</td>
<td>17.001</td>
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<td>2915.07</td>
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<td>2959.30</td>
<td>71.425</td>
<td>Aliphatic chain</td>
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<td>2981.67</td>
<td>60.548</td>
<td>Aliphatic chain</td>
</tr>
<tr>
<td>3070.33</td>
<td>72.038</td>
<td>Aromatic ring–possibly substituted</td>
</tr>
</tbody>
</table>
A 2012 study by Stewart 18 investigated the potential use of Raman spectroscopic techniques for forensic examination of seized β-ketophenethylamines. The technique was found to be effective at rapid identification of nine of these NPS due to the differing substitutions on the aromatic ring affecting the Raman spectra in predictable ways. 18 As well as being able to distinguish between the nine NPS, Raman spectra results also showed bands for bulking agents allowing for composition profiles. Reference material was needed in confirming the identity of the NPS, as necessary for the technique; however, the Raman spectroscopic results still provided good indication to which general types of compounds were present without the reference material. 18 A more recent study by Guirguis 19 in 2017 looked at potential use of handheld Raman spectroscopy for NPS identification in the field. The study used a handheld Raman device with a 1064nm source that successfully identified 29 NPS samples out of a total of 60. 19 Although this was less than half of the NPS in the sample size, the study claims the use of handheld Raman spectroscopy in the field for identification of NPS shows promise. The study states future investigations will focus on improving the technique by investigating effects of NPS concentration, mixture algorithms, and improvement of spectral libraries. 19

A technique that is rapid, non-contact and easy to operate, Raman spectroscopy based techniques make effective and efficient screening methods of NPS. 8,18,19 When used in conjunction with NMR, as suggested in the workflow design by Lobo Vicente 10 (Figure 1), the technique assists in the full analytical characterisation and identification of NPS. 10
**Nuclear Magnetic Resonance (NMR)**

NMR is an analytical method used in inferring the basic structure of NPS and can be consequently used to determine molecular confirmation of the investigated substance. As it can determine molecular structure, as well as a sample’s contents and purity, it can be utilised for the investigation of both known and unknown NPS. 8

An Italian paper 20 published in 2017 describes the analytical procedure utilised by a research unit in identifying unknown NPS. 20 The procedure involves initial analysis of the substance by gas chromatography with flame ionisation detector followed by GC-MS and liquid chromatography-mass spectrometry (LC-MS/MS). The last stage involved NMR characterisation, which successfully identified the three unknown substances in the study when other techniques could not. 20

NMR was one of the advanced analytical methods used in the experimental design by Lobo Vicente. 10 The NMR analysis allowed for the structural elucidation of the two NPS being investigated and successful confirmation of both. One of the substances had previously been identified through GC-MS and FTIR, but they were unable to identify the second NPS. The NMR method further confirmed the identity of the previously identified NPS and also lead to the identification of the unknown NPS. 10 ACD/Spectrus 2014® software was used with the NMR data to determine the types of multiplicity and coupling constants of the peaks as well as the assignment of the peaks with corresponding atoms of the structures (Figure 5). This assisted in the full characterisation of the two NPS. 10
Although NMR has been incredibly helpful in the successful identification and unequivocal characterisation of unknown NPS, many customs laboratories around the world do not have access to the technique, hence rely on other techniques that have been mentioned in this review. This can be attributed to how advanced the methodology for NMR is, requiring a technically proficient person to operate the instrumentation and interpret the results, as well as the high cost to purchase and maintain the instrument. This is another reason as to why large NPS databases like the SWGDRUG library and ENFSI MS library are needed. In the JRC’s 2014 report on the characterisation of NPS, they provide detailed descriptions regarding NMR analysis of NPS as well as different methodologies that can be utilised.

**High Resolution-Mass Spectrometry (HR-MS)**

An advanced analytical technique, HR-MS methods allows the accurate determination of the monoisotopic mass of a compound. Information regarding elemental composition and molecular structure of a compound can also be elucidated using particular HR-MS methodologies. When coupled with NMR, characterisation of NPS is achievable.
A 2017 study by Liu\textsuperscript{15} utilised HR-MS methods in the identification and analytical characterisation of a new fentanyl derivative 2,2'-difluorofentanyl. The electrospray ionisation and quadruple time-of-flight mass spectrometry (ESI-QTOF-MS) method was able to indicate the unknown compound being investigated as a fentanyl derivative with two fluorine atoms, with one on each phenyl ring.\textsuperscript{15} This was able to be determined when its chemical formula and fragment ions were compared to that of fentanyl (Figure 6). NMR analysis further confirmed the two substituted fluorine atoms and their positions.\textsuperscript{15} Fragmentation pathways of the investigated substance were obtained via collision-induced dissociation (CID). With the characteristic fragmentation routes of the NPS determined, forensic laboratories could ideally use the data to identify it and similar NPS in future casework.\textsuperscript{15}

Figure 6: Mass spectra of (A) 2,2'-difluorofentanyl and (B) fentanyl obtained via EI-MS.\textsuperscript{15}
HR-MS methods played an important role in the identification and characterisation of five new synthetic cannabinoids in Vadim’s 2015 study. Exact molecular masses, elemental compositions, retention times and fragmentation pathways for all five compounds were determined using the methods and instruments detailed in the study. With the CID spectra of the five compounds, a diagram of the formation of the main characteristic ions was able to be produced (Figure 7). It is important to note that GC-MS and NMR were also needed in completing the difficult task of identifying these new NPS and determining their high cannabimimetic activity.

In Lobo Vicente’s 2016 study, HRMS methods were successfully used in their experimental design for structural confirmation of two NPS. For structural elucidation and fragment prediction, Mass Frontier software by Thermo Fisher Scientific® was used. As well as predicting fragmentation, this software compares the experimental data with theoretical
data that has been previously reported and can generate MS spectral ion trees (Figure 8). Parent ion peaks obtained by the quadruple time-of-flight method suitably matched the results produced by NMR. Coupling results from the earlier methods carried out in Vicente’s experimental design to the HRMS methods allowed for confirmation of molecules by fragmentation matching.

![Figure 8: MS spectral ion tree generated from HRMS results using Mass Frontier software.](image)

HR-MS systems are one of the top choices of methods to use when it comes to structural investigation of NPS as having the ability to measure the exact mass and examine fragment residues is crucial in full characterisation of such substances. The results from studies like those mentioned above, that document fragmentation of compounds, are important as they will assist in elucidation of known and unknown NPS structures in future casework.
Conclusion

This review has investigated an experimental design developed for the identification and characterisation of NPS, as well as the methods utilised within it. The study that first proposed the design used it successfully to identify two NPS they received from Belgian customs. Several other studies that utilised the same methods listed in the design successfully have also been cited to not only demonstrate the techniques’ effectiveness but to supply further literature the reader may be interested in and may find useful. UNODC have manuals that recommend techniques for identification of particular NPS; however do not provide a design to follow such as Lobo Vicente’s 2016 study that was done in conjunction with the JRC and European Commission.

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Disclaimer

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