IDENTIFICATION OF NOVEL PSYCHOACTIVE SUBSTANCES IN COMPLEX MIXTURES USING A DESIGN OF EXPERIMENT GUIDED APPROACH AND HANDHELD RAMAN SPECTROSCOPY

By

STEVE HAROLD NKWAMO TCHAKOUNTE

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School of Life and Medical Sciences
MSc, (Postgraduate Diploma, Postgraduate Certificate)
Sustainable Planning

University of Hertfordshire

Research Degrees

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Surname (Family Name): NKWAMO TCHAKOUNTE

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ABSTRACT

The screening of NPS in street samples has been proven to be problematic in recent years due to their fast appearance, the unavailability of adequate reference materials and their complexity. The aim of this project focussed on the use of handheld Raman spectroscopic technique in the identification of NPS in samples of street-like complexity. Additionally, it mainly endeavoured at the application of a model in NPS screening and its ability to predict NPS responses in complex mixtures. In fact, chemometrics namely a mixtures' design of experiments approach was used to propose a set of 26 NPS samples of varying amount of NPS (5F-PB-22, N-Me-2-AI and phenibut) and of most common cutting agents/adulterants (benzocaine, caffeine, creatine, and sodium glutamate) to represent maximum variability of a five components mixture. Each mixture was analysed via a Rigaku Progeny handheld Raman spectroscopy device using its in-built algorithms namely wavelet and Rigaku mixture. Matching results obtained from these initial studies were evaluated and were re-implemented in the design of experiment for the generation of a model used to predict NPS responses for proposed test samples. Initial analysis of all 26 vials on a NPS library (99 reference materials) using wavelet displayed good NPS detection notably in samples of high concentrations (above 80 mg) with matching values between 0.59-0.98. Benzocaine and caffeine showed major influence on NPS identification in samples of increased complexity. This was mainly observed in samples of low NPS concentration (10-24 mg) where only 5F-PB-22 displayed detection between 0.10-0.20. However, this was mostly due to the abundant detection of analogues and/or structurally similar substances which were absent within the sample but were similar in Raman spectra to excipients. This was mostly the case with benzocaine derivatives such as dimethocaine and procaine. For comparison purposes, alternative sub-libraries were created containing only five references, an NPS among all three and all cutting agents and adulterants, hence three sub-libraries created. Consequently, analysis of the same 26 samples in similar conditions against sub-libraries exhibited 100% NPS identification for all NPS using wavelet. The influence of analogues was recurrent using Rigaku mixture algorithm against full NPS library. Yet, the algorithm displayed better matching results with an increased matching range between 0.79-0.99 in presence of above 80 mg of NPS. This was due to its ability to match samples spectra to multiples matches for each result. Thus, the values obtained could not be assigned to a match in particular. With wavelet being able to correlate to single matches, each value corresponded to the match obtained hence wavelet results were carried forward for the design of the model. Analysis from the model showed promising results in its capability to predict matching responses for pre-determined sample composition. Screened against the full NPS library, 15% of the test samples (only 5F-PB-22 samples) displayed NPS detection as well as matching values correlating to the predictions. While sub-library analysis showed improvement in detecting NPS in all test vials (100%) as noticed in the initial studies, it mainly highlighted the ability of the model to consistently predict within range of the obtained matching values in 86% of proposed samples. These outcomes

regarding the complexity of samples could be of help to forensic scientists and border control officers in understanding how the use of cutting agents and adulterants happen to hinder detection of NPS by correlating to other substances of similar spectral profile. More importantly, it confirms the use of a model in NPS screening could further show promise in predicting NPS responses depending on the samples composition which would be useful in tackling complexity issue faced in street samples.

ACKNOWLEDGEMENTS

I wish to express my complete gratitude to the School of Life and medical sciences, in particular the department of Pharmacy, Pharmacology and Postgraduate Medicine for providing the required facilities for the completion of this research.

I would like to send my special thank you and my complete appreciation to my supervisors, Dr Jacqueline Stair and Dr Jesus Calvo-Castro for the opportunity to be part of this research as well as the continuing support and the close guidance they have granted me.

I'd like to also show my appreciation to all laboratories technicians, all fellow research students notably Maddie Norbury which has been heavily involved in the acquisition of some samples as well as and the data collection.

To conclude, I voice my profound thank you to my parents for continuously believing in me and supporting me throughout all these years of studies, my family and loved friends for their undying love and encouragements.

Without all of them, none of this would have been possible; deeply thank you.

Steve Harold Nkwamo Tchakounte

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LIST OF ABBREVIATIONS

4Me-N-EnorPent	4-Me-N-Ethylnorpentedrone
AFLO	Afloqualone
ALG.ac	Alginic acid
BEN	Benzocaine
CAF	Caffeine
CREA	Creatine
DIL	Diltiazem
DIM	Dimethocaine
EPHE	Ethylphenidate
FLE	Flephedrone
FLU	Flubromazepam
KET	Ketamine
L-TYR	L-tyrosine
MEPH	Mephedrone
MPHE	Methylphenidate
Na GLU	Sodium glutamate
S FLE	Synthesised FLEPHEDRONE
S MEPH	Synthesised MEPHEDRONE
THEO	Theophylline

1. INTRODUCTION

1.1. Background on NPS

The growth in the number of novel psychoactive substances (NPSs) that are available in the illegal markets has been an increasing challenge in terms of health, security, and monitoring. NPSs also commonly called “designers drugs” are substances synthesised in the sole purpose of reproducing the effect of controlled recreational drugs such as ecstasy, cannabis, and more while bypassing legislation (Tracy, Wood, & Baumeister, 2017). Prior to law changes, structures of existing legal compounds were subjected to small chemical modifications to create new “legal” substances from which comes the unconventional term legal highs (Liechti, 2015).These recreational drugs designate non-medicinal products used to alter one’s general feeling or state of mind. However, they are uncontrolled and are believed to possess serious health risks. In fact, abuse of these substances has been the cause of several cases of violence and aggression (Morrison, 2015) sympathomimetic effects (Wood & Dargan, 2012), acute organ failures (Regunath *et al*., 2012) as well as some fatalities (Corkery *et al*., 2012). These substances, in contrast to illicit drugs are sold on the internet (internet drugs) and head shops and are usually labelled as “bath salt”, “plant food”, “research chemicals”, and others in the aim of avoiding drug regulations. NPSs share a chemical relation to each recreational drug they originate from. In fact, Brandt and co-workers (2010) investigated the analyses of second generation of mephedrone derivatives (Figure 1.1) in the UK. One of the most prominent of those derivatives in the UK has been NRG-1 namely Naphyrone (**d**) due to the naphthalene moiety instead of a substituted benzene ring commonly related to cathinones. This has been the highlight of the fast appearance of these new substances which impact their identification. The term “new” characterise substances recently acquired on the market rather than freshly synthesised drugs (UNODC). In 2013, the World Drug report highlighted to the United Nations Office on Drugs and Crime (UNODC) showed a rise in NPS from 166 in 2009 to 252 by 2012 (Strano Rossi *et al*., 2014). In addition, new NPSs notified through the early warning system (EWS) in Europe climbed from a record 49 drugs since 2005 to 73 new compounds in 2012. These figures have dramatically increased to 560 substances in 2016 with 100 new compounds noted solely in 2015 (Tracy *et al*., 2017). Due to their rapid increasing number as well as the chemical diversity of emerging drugs (Zloh *et al*., 2017), NPS have been categorised into classes and subclasses based on their pharmacological and chemical properties. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has grouped these substances on the basis of 3 main psychotropic effects which are depressants, stimulants and hallucinogenics (Brew, 2016). This arrangement branches into main classes notably phenethylamines, amphetamines, piperidines, cathinones, synthetic cannabinoids, piperazines, aminoindanes, benzofurans and tryptamines which stand as the main classes of NPS and were derived in relation to their chemical affiliations with an additional class of other types of NPS as shown in the Figure 1.2 below. It particularly

highlights the evolution of synthetic cannabinoids and synthetic cathinones which have become the biggest classes of NPSs today. Although a steadily growth, the class of “others” has remained a major group NPS in the NPS classification. For the sake of this review, the synthetic cannabinoid, aminoindane and other class will be highlighted and discussed further.

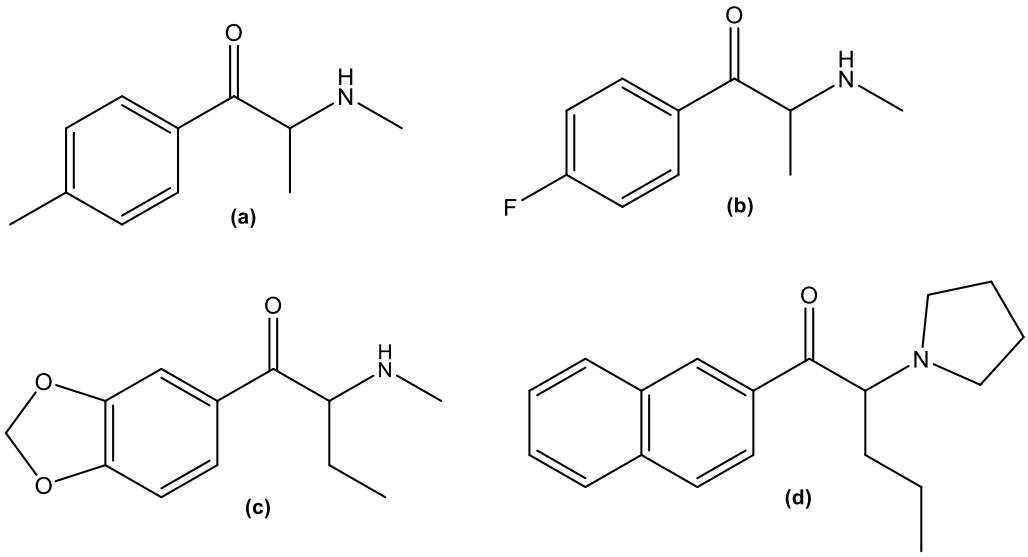


Figure 1.1: Structural similarities in mephedrone derivatives. (a) Mephedrone, (b) Flephedrone, (c) Butylone, (d) Naphyrone

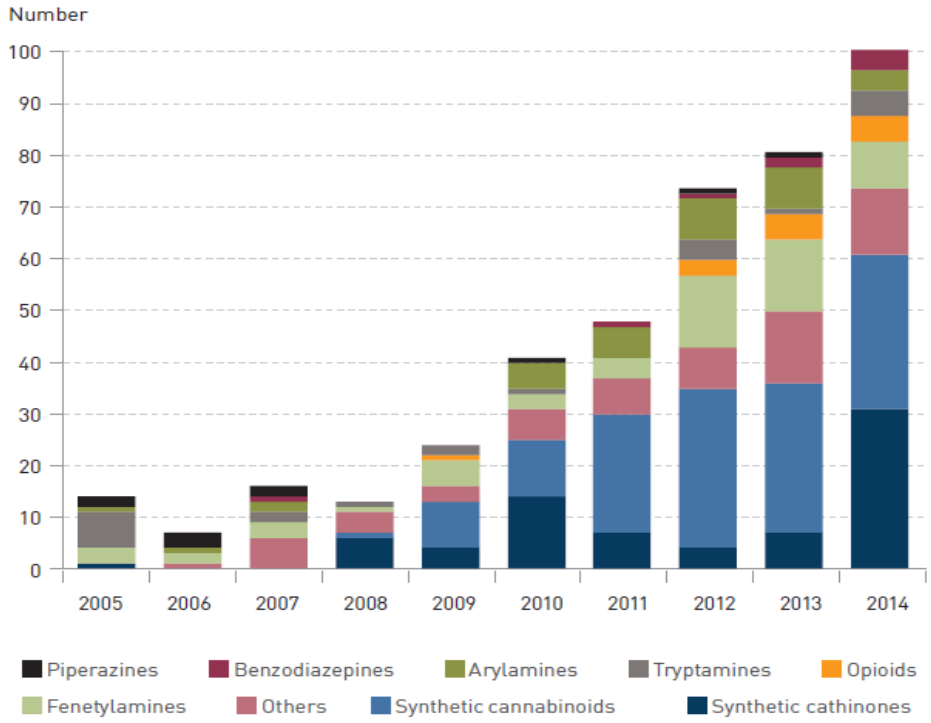
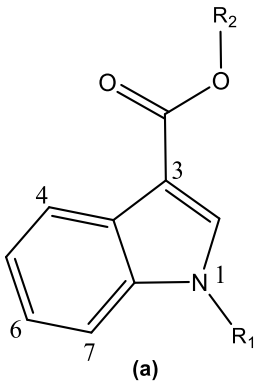


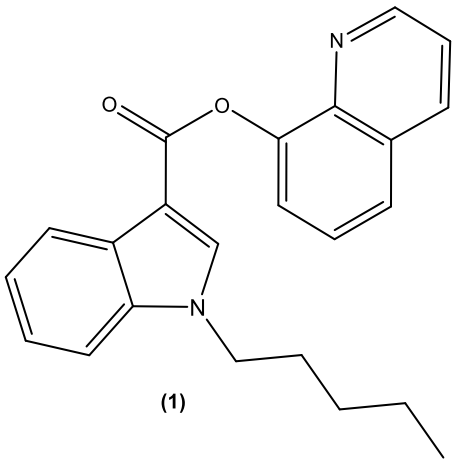
Figure 1.2[1]: Number of different NPS reported each year in Europe (2009-2015)
[1] Source: UNODC, early waring advisory on new psychoactive substances, based on data from 41 European countries

1.2. Synthetic cannabinoids

Synthetic cannabinoids (SCs) represent the biggest and fastest growing class of NPS worldwide. As reported by EMCDDA, approximately 134 new substances were registered in March 2015 since 2008 with 30 SCs in 2014 alone as shown in Figure 1.3 (Banister *et al*., 2015). SCs act on the cannabinoid receptors 1 and 2 (CB1 & CB2) in the brain but their psychoactivity is mainly associated with CB1 receptors. This effect is mainly related to the presence of Δ^9-tetrahydrocannabinol (Δ^9-THC) which is the main bioactive component in cannabis (Pertwee, 2008). The indole (**a**) class within the SCs mainly comprises of the indole-3-carboxylates acid ester derivatives which are the main representatives of this group of substances (Kohyama, *et al*, 2017). First generation of SCs included JWH compounds (JWH-018 and JWH-073) which contained a pentylindole core structure(Wohlfarth *et al*., 2014). A change in the United States legislation from monitoring of specific compounds to the entire structural class led to the appearance of new pentylindole backbone such as UR-144 and XLR-11. However, the latter substances were placed under the Controlled Substance Act by the Drug Enforcement Administration due to the increasing reports of seizures caused by PB-22 and its 5-fluorinated derivative(Drug Enforcement Administration, 2013). Derivatives of indole-3-carboxylates (**a**) such as R_2 substituted by the 8-quinolinyl moiety (**b**) shown in Figure 1.2; such as 5F-PB-22 (also known as 5F-QUPIC) constituted the majority of SCs as well as one of the latest cases of serious drug abuse related to this class (Banister *et al*., 2015). In fact, various cases of fatal intoxications were recorded in the US (2013) to which although investigations, no information was uncovered on their effects. These derivatives are therefore very close in structure particularly PB-22 (**1**) and 5F-PB-22 (**2**) with the only difference of a 5-fluoropentenyl group at position R_1.



(a)



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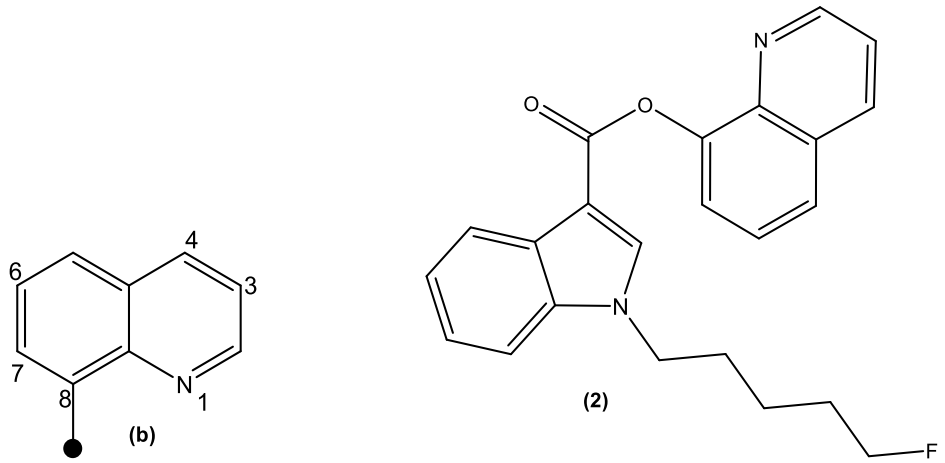


Figure 1.3: Chemical structures of common cannabinoids with an indole core. (1) PB-22, (2) 5F-PB-22, (a) Indole, (b) 8-Quinolynil.

1.3. Aminoindanes

Aminoindanes are one of the most common group of NPS within the miscellaneous class (Others). According to the UNODC, its first psychoactive properties were first noted in the 1970s due to their serotonin re-uptake pharmacological effect. This class of NPS is mostly known for its derivatives (Figure 1.4) such as 2-aminoindane (2-AI: **3**), 5,6-methylenedioxy-2-aminoindane (MDAI: **5**), 5,6-methylenedioxy-*N*-methyl-2-aminoindane (MDMAI: **6**), 5-iodo-2-aminoindane (5-IAI: **7**) and 5-methoxy-6-methyl-2-aminoindane (MMAI: **8**) due to their occurrence on the internet. N-methyl-2-aminoindane (N-Me-2-AI: **4**) is another newly discovered NPS sharing the aminoindanes core structure with the addition of a methyl group at the amine. In fact studies has shown that of 84 NPS detected in the UK out of the purchased 182, N-Me-2-AI appeared to be one of the most common (Brunt *et al*., 2017). However, limited information can be obtained on the use of these substances. These aminoindanes were firstly detected on the drug market from 2006 to 2011 through Early Warning System in Member States of the European Union. In the UK, a Forensic Early Warning System (FEWS) was developed in 2010 in the aim of identifying NPS encountered in the UK. From the samples obtained from various collection plans (internet, head shops, police and other), the presence of aminoindane analogues precisely 2-AI, 5-IAI and MDAI were noted in each plan. However, only 12 samples of a total 1300 showed presence of aminoindanes in 18 months (Brandt *et al.*, 2013).

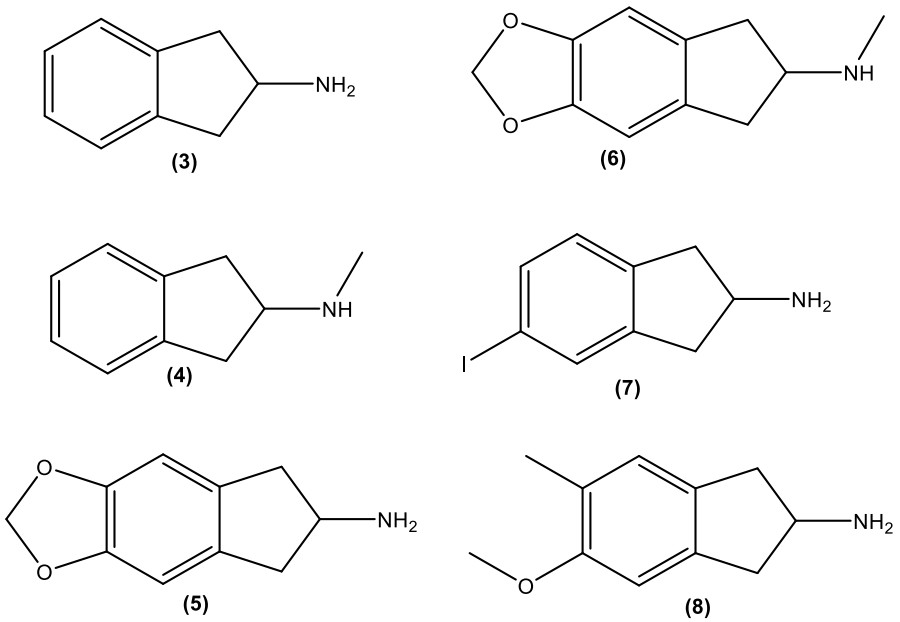


Figure 1.4: Aminoindane derivatives. (3) 2-AI, (4) N-Me-2-AI, (5) MDAI, (6) MDMAI, (7) 5-IAI, (8) MMAI.

1.4. Class of "Others"

This class of NPS comprises of substances of various origins (chemicals, medicinal products) and is ranked among one of the biggest group of NPS. One of the most common case of drug abuse found in this group is phenibut (PB). Indeed, it is not licensed in the European union. Yet case of seizures had been reported to the EMCDDA in 2012 (Owen *et al*., 2016). It's occurrence on the illicit market is mainly noted as "dietary supplements" and "research chemicals" (EMCDDA). Chemically related to Phenethylamines, Phenibut is an anxiolytic and nootropic initially synthesised in Russia in the 1960s. Also known as β-phenyl-γ-amino-butyric acid, PB (**4**) is a GABA mimetic primarily on $GABA_B$ receptors as well as dopamine receptors but antagonises β-phenethylamine receptors (Lapin, 2001). It shares similar structure with GABA (**3**) at the exception of a phenyl ring at the β carbon (Figure 1.5). The presence of this moiety has been found to improve penetration of PB in the blood-brain barrier compared to GABA without increased pharmacological activity. In addition, several cases of addictions have been reported. Samokhvalow (2013) presented a case of Phenibut dependence, purchased as online supplement for anxiety, dysphoria and alcohol cravings. Although Phenibut appeared to have therapeutic effect in attenuating anxiety, mood changes and alcohol cravings, the patient developed dependence towards Phenibut.

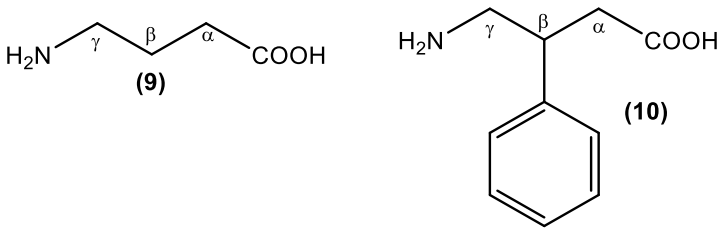


Figure 1.5: Similarity of a GABA backbone with Phenibut. (9) GABA, (10) PB

1.5. Useful analytical techniques in NPS screening

Law enforcing bodies face a real challenge when it comes to the screening of NPSs. This has been linked to the fast pace of appearance of these substances mainly from analogues of recreational drug substances made in unlicensed laboratories (Assi *et al*., 2011). In addition to this, the use of the internet as a source of purchase of NPS has tremendously increased (Davies *et al*., 2010). However, their synthesis in unlicensed laboratories was synonym of their poor quality which was a consequence to unsuitable reference standard libraries in addition to the use of cutting agents and adulterants in NPS samples that happened to interfere with drug identification (Elie *et al*., 2013). Indeed, useful analytical techniques such as thin layer chromatography (TLC) and Fourier transport infrared spectroscopy (FT-IR) appeared to be limited due to inappropriate reference standards (Crean *et al*., 2013, Elie et al., 2013). In fact, with the high number of these substances around the market and the similarities there may be between substances of the same class, the acquisition of pure reference materials from commercial sources is limited and very expensive. Other analytical techniques have been rather successful in the analysis of NPSs in mixtures. In fact, liquid and gas chromatographic techniques (LC and GC) coupled in tandem with Mass spectrometry (MS) were found to be reliable in the screening of NPSs (Smith *et al*., 2014). Although LC-MS appeared to be prevalent in the analysis of biological fluids as blood or urine, GC is more convenient in analysis of tablets, capsules and powders (Bell *et al*., 2011). However, these techniques are qualified as destructive techniques as no samples is recovered after analysis in addition to medium to extreme sample preparations required. GC-MS has been the most widely used analytical technique in forensic analysis of NPS mixtures. However, it faces the potential challenge of regioisomers. Studies conducted by Abdel-Hay *et al* (2014) showed the efficacy of GC-MS in identifying potential regioisomers of diemthoxybenzoyl-N-methylpiperazines (DMBzMPs). Results from the analysis showed all six dimethoxy regioisomers to correlate to a molecular ion mass of 264. Additionally, major fragments from all derivatives shared similar molecular ions mass as *m/z* 165 for the diemthoxybenzoyl and *m/z* 93 for the N-methylpiperazine. Additionally, FT-IR spectra obtained from all six regioisomers highlighted a common absorbance band at 1664 cm^{-1} and denoted the differences that arose from the fingerprint region. Thus GC-MS was used for confirmation of an

isomeric relation with DMBzMPs while FT-IR provided information on the dissimilarity of isomers regarding the NPS. The efficacy of GC-MS has been further proven by Elie *et al* (2013) in the screening of NPS. A fast GC-MS method was used to screen 35 substances purchased from head shops and internet market. Within four minutes, 23 NPS amongst which 5,6-methylenedioxy-2-aminoindane (MDAI) were successfully separated and identified as well as two most common excipients in benzocaine and caffeine. This approach further raised questions regarding the potential composition of these substances. In fact, of all 12 5-iodo-2-aminoindane (5-IAI) samples purchased, only seven were received as 5-IAI samples and yet none of them contained 5-IAI. Although the efficiency of these analytical techniques in rapidly identifying NPS samples, their use and benefits is obstructed by the limitations of sample preparation pre-analysis and the size of instrumentation unsuitable for onsite analysis. However, the application of Raman spectroscopy has shown promise in solving these problems due to its availability for in-field testing with the breakthrough of battery powered portable devices, no samples preparation thus a non-destructive technique and through package analysis. Furthermore, comparative analysis between handheld IR, NIR and Raman has shown Raman to excel in the identification of novel substances in mixtures (Assi *et* al., 2015). IR and NIR techniques appeared to be sensible to carbohydrate cutting agents and adulterants in contrast to Raman.

1.6. Raman Spectroscopy

Raman spectroscopy is a vibrational spectroscopic technique that shares some similarities with Infrared with both being non-destructive first-pass analytical techniques due to little to no sample preparation (Chalmers *et al.*, 2011). It is used in the analysis of vibrational modes that can be used in the elucidation of a molecular structure. Both IR and Raman can be used for identification of molecules with known reference standards. However, they both follow different processes and are selective in terms of molecular electronic properties. Raman focusses on the change in polarizability (electron distribution cloud) that originates from symmetric vibrations of non-polar molecules, at the contrary of IR which is active in the presence of an electric dipole caused by asymmetric vibrations in polar groups (Larkin, 2011). Furthermore, in contrast to IR being an absorption technique, Raman is a scattering phenomenon. When a monochromatic light is directed onto a sample at a frequency (υ), the incident radiation can be scattered at the same frequency as the emergent radiation or at different frequencies ($\Delta\upsilon$). In the case of equality in frequencies of both incident and scattered radiation, the scattering is said to be elastic since there is no energy loss. This is known as the Rayleigh scattering. On the other hand, the scattering can be inelastic from which results a Raman signal (Kudelski, 2008). Inelastic scattering is characterised by Stokes and Anti-stokes lines with Stokes mainly used for the Raman spectra. Energy from the incident radiation is capable of inducing transitions (rotational, vibrational and electronic) which promotes the molecule to an excited energy state. However, in

Raman scattering, this energy state is unstable (virtual state) and the molecule returns to the electronic ground state (Figure 1.6). However, it does not always return to its original energy level. The energy lost in the deactivation transition represents the scattered radiation and the difference from the incident light energy accounts for the change in vibrational mode of the molecule (Pickering, 1971). Molecules initially present in their ground state display Stokes scattering while those present at an excited vibrational state display anti-Stokes. This difference in energy state is due to the temperature of the sample (Larkin, 2011). Indeed, at equilibrium temperature, Stokes lines are more frequent than anti-Stokes (Boltzmann's law) as most molecules are present at the ground state in ambient temperature. Hence, Stokes event is chosen for Raman readings.

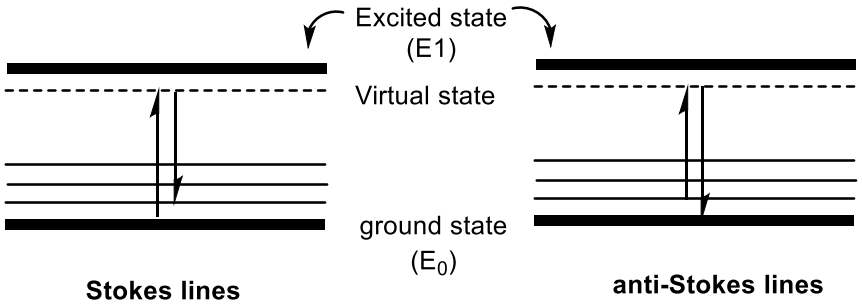


Figure 1.6: Stokes and anti-Stokes event in Raman spectroscopy

One of the main problems faced today with NPSs is the difficulty involved in the monitoring of analogues of controlled drugs. Fluoromethcathinone also called Flephedrone is a mono-substituted cathinone isomer. However, it possesses three regioisomers as the fluorine can potentially be ortho, meta, and para aromatic ring substituted (Figure 1.7). The use of ^{19}F NMR would be beneficial in forensics analysis of such substances, however this technique is rather costly (Archer, 2009). Studies conducted by Christie and associates (2013) investigating the differentiation of cathinone regioisomers by Raman analysis showed that the ortho, meta and para mono-substituted flephedrone and mephedrone analogues could be successfully identified due to the difference in electron distribution caused by the unconjugated benzene ring. In fact, the position of the fluorine substituent can lead to structural changes within the molecule as well as to the redistribution of charges (Wojciechowski *et al*., 2011).

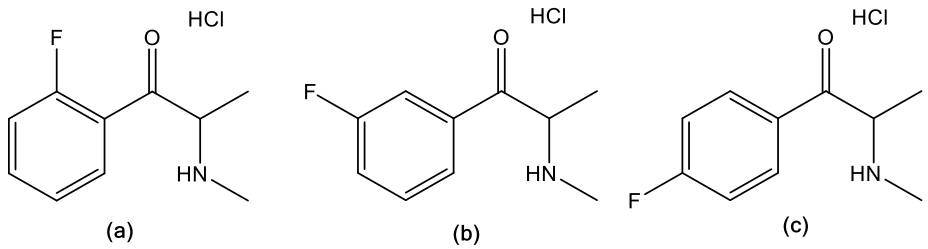


Figure 1.7: Flephedrone regioisomers. (a) ortho-fluoroflephedrone, (b) meta-fluoroflephedrone, (c) para-fluoroflephedrone.

In addition, the use of Raman spectroscopy has been proven to be successful in the analysis of structurally similar β-ketophenethylamines seized samples (Stewart *et al*., 2012). It was further highlighted the variance in peak intensity as a consequence of the change in spot position of irradiation (grid sampling). Raman spectroscopy has been broadly employed in the analysis of drugs of abuse. However, the application of Raman spectroscopy faced some major disadvantages particularly its low efficiency due to scattering cross section of *ca*. 10^{-29} cm^2 per molecule compared to absorption techniques as Ultraviolet and IR of *ca*. 10^{-18} and 10^{-21} cm^2 per molecule respectively (Kudelski, 2008). The Raman cross section is a constant specific to a material which determines how an incident laser intensity is scattered at a particular wavelength. If the incident laser intensity is constant, the intensity of the Raman is proportional to the concentration of the material (Larkin, 2011). In addition, the cost of instrument was expensive in the last decade and they were unsuitable for in-field use.

However, with the advent of technology, low cost and battery powered portable Raman instrument have been manufactured with increased efficiency (2×10^{-14} cm^2 per molecule). Some limitations associated with this technique such as low signal levels were related to low laser power sources, which has restricted its use to specialist laboratories. In addition, fluorescence has been a major issue in Raman analysis due to the high intensity of excitation (short wavelength), good light collection and extremely sensitive detectors (Chalmers *et al*., 2011). A proposed solution to reducing the fluorescence has been the use of surface-enhanced Raman spectroscopy (SERS). In fact, application of SERS in the analysis of amphetamine (XTC tablets) highlights the advantage of SERS in displaying better Raman spectra (Figure 1.8) in comparison to pre-resonance Raman spectroscopy spectra which were overcrowded by a large number of bands which can lead to misleading interpretation (Sägmüller *et al*., 2001). Similarly, Rana and co-workers identified SERS to be appropriate in identification of morphine, codeine and hydrocodone with successful reduction of fluorescence. However, it denoted restrictions with regards to experimental requirements such as time consuming sample preparation and the need for an appropriate substrate (Rana *et al*., 2011). The use of Raman spectroscopy has shown

some promising results in this regard, however studies demonstrating its onsite capabilities in streets and at border control remains to be evaluated.

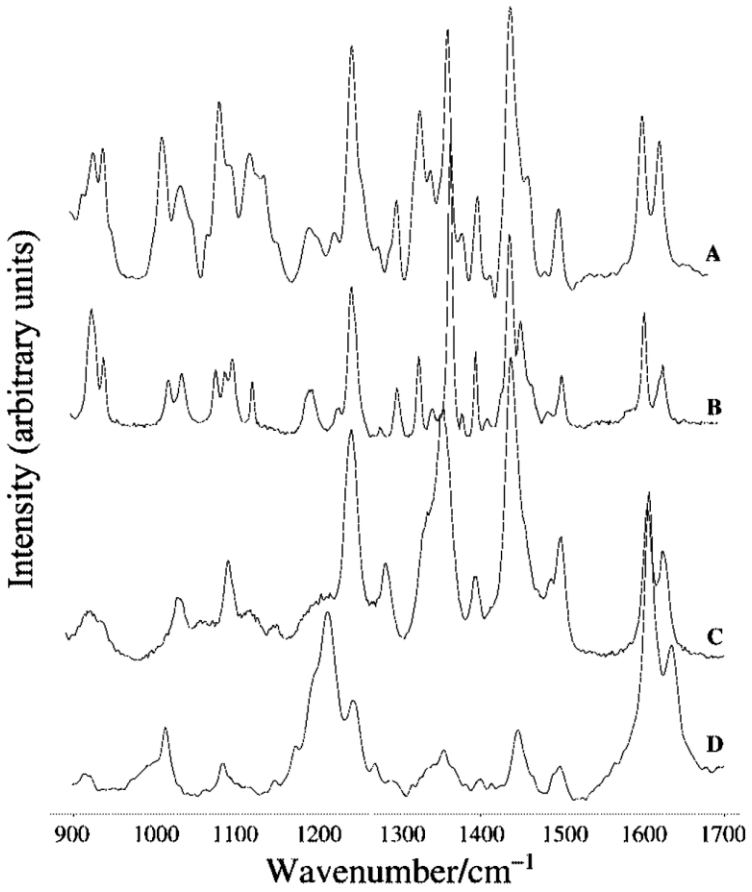


Figure 1.8[1]: Comparison of Raman spectra of MDMA recorded from different types of samples: Trace A: NIR Raman spectrum of an XTC-E tablet, Trace B: Raman spectrum of MDMA.HCl, Trace C: Raman spectrum of the free MDMA base, Trace D: SERS spectrum of the extract of the XTC-E-tablet. λ_{exc} = 1064 nm for trace A, 514.5 nm otherwise.

[1] Note: Obtained from; Sägmüller, B., Schwarze, B., Brehm, G., & Schneider, S. (2001). Application of SERS spectroscopy to the identification of (3, 4-methylenedioxy) amphetamine in forensic samples utilizing matrix stabilized silver halides. *Analyst*, *126*(11), 2066-2071.

Research conducted by Assi (2015) investigated the use of portable Raman instrumentation on 6 NPS products samples purchased from the internet. These samples were available in form of capsules (blueberry, magic beans, and pink champagnes) and power (2-AI, DXM, and NRG-3). Results obtained from a 785nm handheld Raman showed that the label claim did not correlate to matching results. In fact, verification by GC-MS showed that all powders contained caffeine while capsules were made of 2-AI and caffeine. Indeed, Raman spectra for all powders displayed similarities due to the presence of common active ingredients.

Weyermann et al., 2011 investigated the infield use of portable Raman instrumentation via *in situ* (in appropriate place) analysis of illicit drugs; in particular heroin, cocaine and amphetamine at border controls. This study took in consideration the main factors of analysis hence the time of analysis, repeatability and the sensitivity of the method used. Quality of the spectra was shown to increase with the analysis time to a maximum of 30s where additional improvement was insignificant beyond. It further highlighted the precision of the instrument with RSD values of 4% for samples analysed at the same time. This value appeared to increase slightly at different analysis time although attempts to reproduce the same conditions were made. In addition, street samples seized by Swiss police were also analysed. Cocaine samples at 45%, 30% and 16% purity were successfully identified within the mixture. However, heroin analysis showed no detection due to strong fluorescence while the single amphetamine sample (4% purity) showed bands corresponding to caffeine.

Although the useful findings due to the application of a portable Raman *in situ* conditions, limitations regarding fluorescence due to shorter wavelengths (785nm) were still encountered. Furthermore, it highlighted the complexity of samples composition as a vital factor in illicit drugs monitoring. A possible solution to reduce or eliminate fluorescence in Raman analysis was decreasing the excitation energy hence increasing laser's wavelength to the near infrared region. Studies conducted by Guirguis *et al* (2017) showed the efficacy of the handheld Raman in the identification of NPS samples purchased from the internet using 2 wavelengths (785 and 1064nm). The handheld Raman successfully identified 29 NPS out of 60 samples (48%). Analysis at 1064nm showed that 27 out of 28 standards matched to their respective library spectra with 23 standards matching at %HQIs (first pass identification algorithm) between 90 ± 0.9 and 100 ± 0.1%. Analysis of NPS mixtures via handheld Raman instruments showed reduced fluorescence at longer wavelength such as 1064nm but better sensitivity has been found to occur at a shorter wavelength of 785nm (Assi, 2016). The study focused on the analysis of psychoactive substances in mixtures using 3 handheld instruments where 2 operated at a mono-wavelength of 1064 and 785nm respectively, and a dual laser instrument. Results showed that detected ingredients from analysis uncorrelated to samples label claim confirming the presence of impurities in NPS samples capable of influencing detection. Additionally, the use of a 785nm laser provided better signature resolution of active impurities present, but NPS signal was masked due to fluorescence of inactive ingredients. While analysis at 1064nm appeared to resolve fluorescence, overall peak resolution decreased thus influencing sensitivity. In fact, the use of dual laser handheld Raman within near infrared range (700-1100nm) provided both advantages in the increased resolution and low to reduced fluorescence. In addition, it increased spectral range from 2000 cm^{-1} using a mono-laser to 2800 cm^{-1} - 3200 cm^{-1} with a dual laser, which correspond to CH and OH scattering range and can be helpful in further discriminating substances.

Although Raman instrumentation can successfully identify the correct NPS in fairly pure samples, further research needs to be done to evaluate the limitations faced in onsite identification of mixtures. Studies on NPS mixtures by Assi *et al* (2015) showed that detection via handheld Raman instrumentation can be hindered by complications posed by a variety of cutting agents and adulterants and the problem of analogues and structurally close substances. In fact, it further showed common adulterants such as benzocaine, lidocaine, caffeine and procaine in products as well as idled components as talc and cellulose for powder bulk played a major role in obstructing detection. Nonetheless, a proposed solution to dealing with the effect of impurities has been found in the use of dual laser instrumentation or longer wavelength.

1.7. Algorithms of use in the identification of NPS

An algorithm is a set of mathematical and computational rules applied to data input for a desired output (Cormen *et al*, 2014). In order to simplify results from complex analysis, search algorithms are used to process data in relation to a set of criteria. In-built algorithms provide an advantage of being fast in giving answer for test samples. At present, there has only been three studies reporting the use of algorithm in spectroscopic handheld instruments for NPS (Assi, 2015; Assi, 2016; Guirgus, 2017) One of these studies focussed on correlation in wavelength space (CWS) used in NPS screening via NIR handheld instrumentation. It compares dissimilarities between unknown spectra (A) against reference standard (B) by calculating a correlation coefficient *r* (Eq.1). An *r* value of one indicated that the spectra are identical. However due to the level of background noise, an r value of one is difficult to achieve. In fact, studies conducted by Assi and co-workers (2015) investigating the use of handheld instruments in NPS screening and using a microPHAZIR instrument showed that all test products correlated to a reference standard with one value correlating at an *r* ≥0.95. This correlated to the caffeine signature at r=0.9646 which was confirmed by GC-EI-MS analysis.

(Eq.1) $$r = \frac{\sum(A_i - \bar{A})(B_i - \bar{B})}{\sqrt{\sum(A_i - \bar{A})^2 \ \sum(B_i - \bar{B})^2}}$$

Regarding Raman spectroscopy analysis, algorithms used in NPS detection using handheld instruments are designed following a spectral matching criteria. One of the algorithms used to identify NPS that is reported in the literature is %HQI (hit quality index) as a classification measure. HQI is a numerical search algorithm that compares similarities in spectra by calculating HQI values via equation (Eq.2) in order to identify the degree of spectral matching in library reference standards (Lee *et al*., 2013). Although the latter focused on the spectra

matching at different laser wavelengths of analysis, it highlighted some limitations in the use of a first pass identification algorithm better suited for high purity samples mostly faced at border controls. Previous studies by Guirguis *et al* (2017) investigated the use of HQI in the identification of NPS and established HQI to be best fitted for raw materials analysis compared to in mixtures particularly due to interferences posed by adulterants/cutting agents.

(Eq.2) $$HQI=\frac{(library.unknown)^2}{(library.library)(unknown.unknown)}$$

However other algorithms of recent development appear to show promise notably Wavelet algorithms. Wavelet, an in-built search algorithm is a set of mathematical scaling functions used to represent data. It is used to represent transitions in time and frequency of any signal through hierarchical decomposition called wavelet decomposition (Jiang & Adeli, 2004). In terms of matching findings obtained from the instrument, wavelet analysis gives a maximum of five matches (signature readings) with each match correlating to only one reference standard.

Analysis of NPS in mixtures has been a constant challenge in comparison to pure samples thus mixtures algorithms may be an ideal choice. TruScan is a common handheld Raman instrument of use that has shown promising results in the analysis of NPS in mixtures. Its in-built algorithm follows a Bayesian theorem in which a probability value (PVAL) for unknown substance are calculated against reference signatures (Eq.3) (Matthews *et al.*, 2006). A PVAL ≤0.05 indicates that both unknown and reference standard spectra displayed significant differences. In this case, a discovery mode is enabled which works as a mixture algorithm and consist in correlating test spectra to all library signatures (Ahura scientific). Partial least squares regression (PLSR) similarly available with TruScan RM instrument consist in analysing or predicting variables from a set of independent variables or predictors (Abdi, 2010). However, this method is used mainly in the quantification of up to 10 chemicals according to the TruScan's TruTools specification sheet. This differs from PLSDA (Discriminant analysis) which consist in the qualitative classification of groups of chemicals or identify a chemical from a group of up to 10 chemicals and thus can be used in mixture analysis. Although this method could be useful in identification of NPS by handheld Raman instruments, its use in the literature is yet to be reported.

(Eq.3) $$P(A|B) = P(B|A)P(A)/P(B)$$

In this regard, the latter algorithm is similar to Rigaku mixture (RM). RM is a newer in-built identification method supplied along with Progeny portable Raman instruments from Rigaku.

Rigaku mixture works by establishing a correlation coefficient between test spectra against all library signatures. Similarly to wavelet, Rigaku mixture analysis results in a set of five results. However, as a mixture algorithm, the search pattern focusses on all signatures of similar reading to test s. Hence each result corresponds to one or more substances compared to Wavelet where each result only has one matching substance. This is a consequence of having one matching value associated to a combination of 1 to 5 potential hit. However, this algorithm is not described in the literature, thus further research is needed to investigate its utility in NPS screening.

1.8. Design of experiment

One of the main reason behind the difficulty involved in monitoring NPS has been the availability of adequate reference materials. In addition, with over 560 NPS reported, the number of different possibilities in mixtures is countless which makes identification by handheld instrumentation delicate. However, the use of a design of experiment could potentially be beneficial particularly multicomponent systems. In fact, the aim of mixtures experiments focusses on the contribution made by main components or combination of components on the observed response (McConkey *et al*, 2000). According to Scheffe (1963), "the response might be an octane rating of a blend of gasolines". This indicates that the response of a mixture is not dependant on the total amount of the mixture. A mixture of three component is a triangle, for four components a tetrahedron, where the vertices represent single components, an edge represents a combination to two components and a face the combination of components involved (Figure 1.9). The 3D illustration represents the mixture's response in space depending on arrangements known as lattice (Gorman & Hinman, 1962). This is characterised by the equation (Eq.4) where x_i represents the proportions of i^{th} component and *q* the number of component.

(Eq.4) $$x_i \geq 0 \quad (i = 1, 2, 3, .., q), \quad x_1 + x_2 + \ldots + x_q = 1$$

Although McConkey et al (2000) reports the use of the simplex lattice design in the toxicity of polycyclic aromatics hydrocarbons in environmental systems (PAHs), it demonstrated the potential use of a model in mixtures screening. Hence, its application could be beneficial in improving the monitoring and identification of NPS.

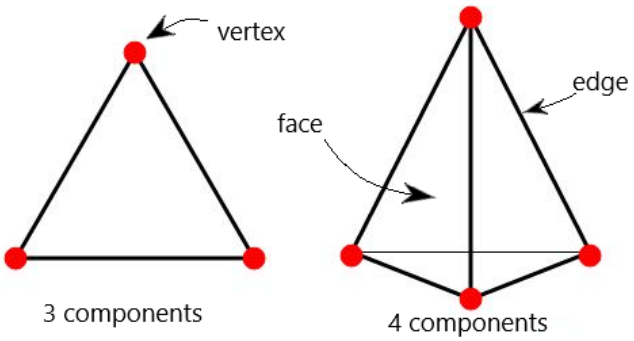


Figure 1.9: Response of mixture in space based on lattice and number of components.

1.9. Aim of research

This research focuses on the rising challenge confronted by handheld Raman instrumentation in the assessment of intricate samples. Studies evaluating the use of Raman have mainly been used on internet samples. No further work has been conducted on the influence of sample complexity in the identification of NPS in street samples. Additionally, the constant need for new and better methods of search highlights the complications behind NPS identification. What role does the complexity of NPS mixtures play in hindering monitoring of these substances? The aim of the project was to investigate the difficulties in NPS screening using a portable Raman device as well as understand how the later works, and explore the use of chemometrics in NPS screening. Achieving this aim required to:

- Evaluate the beneficial aspects of design of experiments approaches in appropriate samples selection.
- Highlight the limitations faced in screening of street-like NPS samples. This includes the effect diverse types of cutting agents and adulterants could have in NPS identification as well as the behaviour of the latter regarding to Raman.
- Understand the working of diverse algorithms in NPS testing, how they differ from each other and the potential advantage they have in NPS studies.
- Assess the use of a model in the prediction of component's response in mixture and how their use could be a potential success in NPS monitoring.

2. EXPERIMENTAL

2.1. Chemicals and Reagents

Annual drug intelligence bulletin from LGC forensics in 2014 showed benzocaine, caffeine and creatine to be present in respectively 40%, 8% and 6% of cocaine samples acquired. These excipients appeared to be mainly present in synthetic cathinones with the addition of monosodium glutamate (Daily, 2014). Thus, as most common adulterants of use in counterfeits substances, these excipients were used in this study. Phenibut, 5F-PB-22, N-Me-2-AI samples of high purity (≥ 97%) were purchased from Chiron Pharmaceuticals Limited (Bristol, UK). Due to the high purity and the purchased price of 100 £ for 10 mg, these NPS were used as reference materials to constitute the NPS library. Additionally, a set of NPS were purchased from UK online suppliers in February 2016 under Home Office licence. Regarding regulations from the Home office, their usage was monitored following Standard Operating Procedure (SOP) guidelines. Previous work was conducted on online purchased samples in order to confirm the presence of the claimed NPS via gas chromatography in tandem with mass spectroscopy (GC-MS) and their purity (90.2 ± 0.9 % for 5F-PB-22, 99.6 ± 0.1 % and 97.3 ± 0.7 % for N-Me-2-AI P067 and P079, and 97 ± 3 % for phenibut) was subsequently determined employing high-performance liquid chromatography (HPLC). It was to note that all investigated samples of phenibut (P041), N-Me-2-AI (P067 and P079) and 5F-PB-22 (P064) acquired from the internet exhibited purities greater than 97%, hence comparable to those of reference standard materials. Cutting agent creatine (CREA) and adulterants of use notably benzocaine (BEN), caffeine (CAF) and sodium glutamate (Na GLU) were purchased from Sigma Aldrich (Dorset, UK) and used without further treatment.

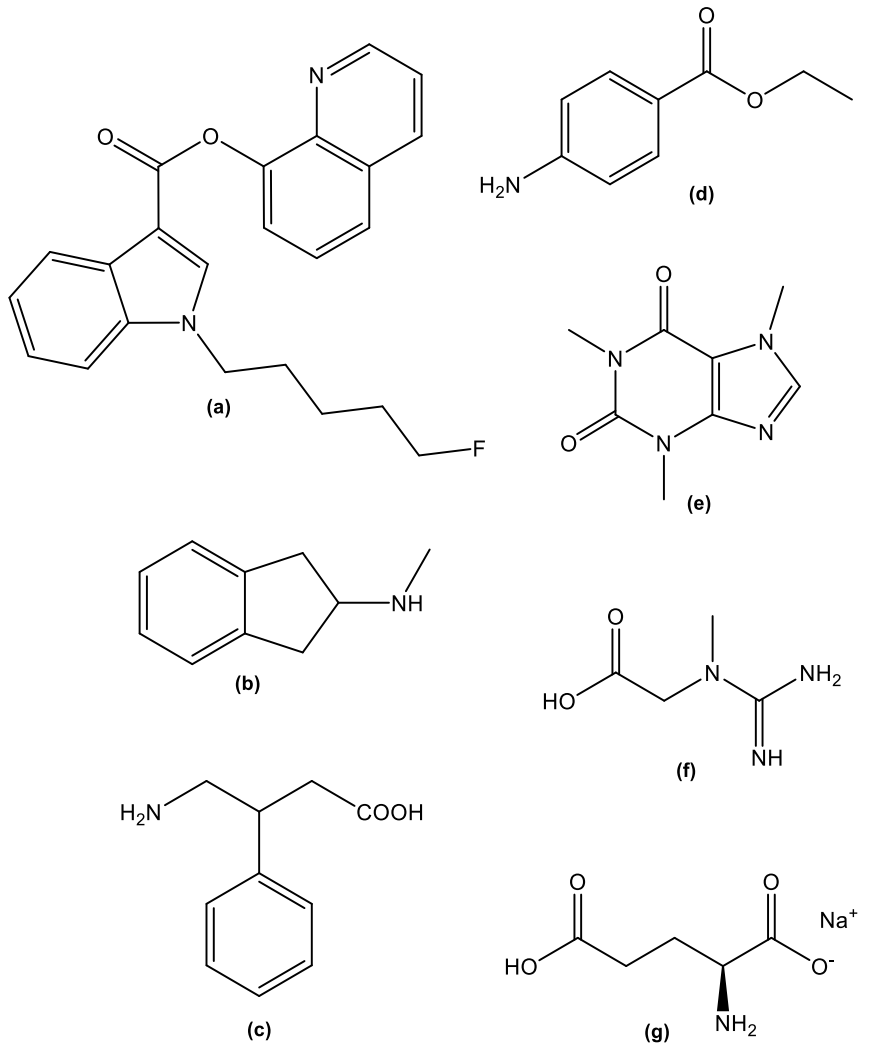


Figure 2.1: Chemical structures of (a) 5F-PB-22, (b) N-Me-2-AI, (c) phenibut, (d) benzocaine, (e) caffeine, (f) creatine, (g) sodium glutamate

2.2. Analysis of NPS using Handheld Raman Spectroscopy

The instrument used in this research is a Progeny handheld Raman (Rigaku, USA). It weights at approximately 1.6 kg with dimensions of 29.9 cm × 8.1 cm × 7.4 cm. With a theoretical battery life of over five hours, the Progeny handheld has optimum operating temperature range of -20 to 50°C. The instrument is equipped with adjustable laser power of 30-490 wavelet, exposure time ranging from 5 ms to 30 s, and a spectral range of 200-2500 cm^{-1} at a resolution of 8-11 cm^{-1}. The excitation wavelength is 1064 nm coupled to a 512 pixels TE cooled InGaAs receptor. With baseline correction settings activated, analysis were conducted using two methods of different specifications; method A (200 mW laser power, 2000 ms exposure time, 10 counts per reading) used for analysis of 5F-PB-22 and N-Me-2-AI, and method B (350 mW laser power, 2000 ms exposure time, 10 counts) for phenibut. Pure reference standard materials of over 97% purity NPSs and adulterants/cutting agents were analysed and their spectra recorded to make

up the NPS search library of analysis called “Terry” composed of 99 spectra. Wavelet and Rigaku mixture were the in-built algorithms of use. Rigaku mixture search settings were set to record a combination of five matches per result out of an available six compared to a constant single match in wavelet. Analysis were conducted through glass vials (Kimble Chase vial screw PTFE cap, China) using the appropriate vial attachment supplied with the instrument. The instrument was calibrated prior to each analysis using a benzonitrile reference standard from Rigaku. The analysis was conducted in quadruplicates for each vial. Vials were vortex mixed using a Vortex-Genie 2 (Scientific industries Inc., US) before the first run for up to a minute, and in-between replicate for 30 seconds to account for any anisotropic effects. Analysis were conducted at a particular set-up, with the instrument inclined at around 45° degrees and a base support accounted for to avoid any slippage.

2.3. Design of experiment and study acquisition

Samples of known composition were made based on five components of which the NPS and adulterants/cutting agents ranging from binary to quinary mixture for each combination with the NPS always present. However, given the high number of possibilities, a design of experiment guided-approach using a simplex algorithm was used resulting in 26 samples (Table 2.1) where the concentration of each components alters in accordance to cover the broad variety of all possible combinations. Vials made in this initial study followed a model created via the Design Expert software (version 10.0.7.0). The model was set for a quadratic fit and the vial total mass limited to 150 mg. With at least 10 mg of NPS of interest always present in every combination investigated and the cutting agents and adulterants ranging between 0 and 140 mg. *A single response was introduced corresponding to the matching value of the NPS. Vial were made up* using aluminium weighting boats and a plastic-coated spatula to avoid errors in weights. Weights were measured by difference while ensuring a percentage error of within 10%.

2.4. Development of the Design of expert model

2.4.1. Initial assessment

In the first part of the experiment, all NPS were analysed using wavelet and then Rigaku mixture at different spot (different occasions) using the methods as described in Section 2.2. Secondly, all generated 26 vials were analysed once more on both Rigaku mixture and wavelet algorithms against full library on the same spot of analysis at each replicate for effective comparison. This meant a change of search algorithm from Rigaku mixture to wavelet without sample vortex for each replicate. These samples were designed in order to evaluate the capabilities of a handheld

Raman instrument in detecting NPSs at various concentrations. On the other hand, it endeavoured in evaluating the influence of complex mixtures in identification of NPS, and the application of both algorithms used.

After interpretation, further work was conducted on all 26 vials originally. They were analysed against newly created libraries of decreased size called sub-libraries. These sub-libraries were restricted to a limited number of five signatures which comprised of one NPS signature of interest specific to the sub-library and the four cutting agents/adulterants common in each NPS vial set. It was important to keep the same reference standards used in the full library for like to like comparison of results. Method A and B were used as previous and samples were run on both wavelet and Rigaku mixture algorithms using the same spot of analysis. The set-up of instrumentation as well as the experimental details were respectively kept the same as previous.

Table 2.1: Sample content of all 26 vials used in the study

Vial	Content (mg)					
	NPS	BEN	CAF	CRE	Na GLU	Total
1	150	0	0	0	0	150
2	10	0	140	0	0	150
3	10	140	0	0	0	150
4	10	0	0	140	0	150
5	10	0	0	0	140	150
6	80	0	70	0	0	150
7	80	70	0	0	0	150
8	80	0	0	70	0	150
9	80	0	0	0	70	150
10	10	70	70	0	0	150
11	10	0	70	70	0	150
12	10	0	70	0	70	150
13	10	70	0	70	0	150
14	10	70	0	0	70	150
15	10	0	0	70	70	150
16	94	14	14	14	14	150
17	24	14	84	14	14	150
18	24	84	14	14	14	150
19	24	14	14	84	14	150
20	24	14	14	14	84	150
21	38	28	28	28	28	150
22	150	0	0	0	0	150
23	10	0	140	0	0	150
24	10	140	0	0	0	150
25	10	0	0	140	0	150
26	10	0	0	0	140	150

2.4.2. Sub-library analysis investigation

Following initial analysis on full library, the 26 vials were analysed on freshly created libraries restricted to a number of five signatures. However, the selected reference standards originated from pure NPS from the sample set (vial 1: 150 mg) and pure cutting agents/adulterants measured at 150 mg and used as reference standards. A consequence to this was inaccurate comparison of full and sub library analysis due to search libraries of different reference standards. The investigations conducted focussed on the timing of analysis as well as the potential impact of library size in matching results. Picked at random, vial 6 was ran against five libraries of different size namely: master library (14472 spectra), full library/NPS library (99 spectra), half (50 spectra), sub-library (5 spectra) and sub sub-library (3 spectra). Taking in account the use of three NPSs, three sub sub-libraries were similarly created. Considering the number of signatures in the NPS library (Appendix: Table 8), the half library was made by selecting 50 reference standards (Appendix: Table 9) from the full library. The reference standards selected were made relevant to the study by selecting analogues of NPS and adulterants/cutting agents. It is key to note that the time taken in consideration was the searching time rather than the sample run time. On the other hand, vial 1, 8, 16, and 22 were analysed on the half library in order to determine the variation in matching values.

2.5. Generation of test vials

Results obtained from the full and sub-libraries of the 26 vials were collected and fed back into the design of experiment. The generated model was optimised on the basics of minimising NPS concentration against maximised cutting agents and adulterants for maximised matching value response in order to replicate street samples conditions. In addition, a maximum importance (+++++) was given to the NPS and the response (matching value), as well as benzocaine, caffeine and creatine. Na glutamate as the weakest scatterer received a medium importance (+++). This resulted in a set of solutions which were a set of samples of new composition for a desired response. Two models were generated per NPS for full and sub-libraries data set. The new samples set (test vials) originating from the model were made in glass vials of same size as in Section 2.4.1. The experimental details of analysis were as previous for effective comparison. These test vials were used as an examination to evaluate the efficiency of the model. The matching values obtained for each vial were directly compared to the model predictions.

2.6. Model validation

In addition to matching values comparison to predictions made by the model, statistical evaluation was conducted. This aimed at evaluating the fit of the model in representing the data. A good model followed a sequential p-value below 0.05 ($p \leq 0.05$). The sequential p-value represented the probability of the model to significantly explain the variation in NPS response. In addition, the model was tested for lack of fit which described how well the model fit the data acquired. An insignificant lack of fit was qualified by p-value greater than 0.10. Finally, the model was assessed via coefficient of determination (r^2) which qualified how well the data was fitted to the regression line. In addition to displaying good regression (r^2 close to a 100%), a good model had to display a difference between adjusted and predicted r^2 of within 0.2. A choice between linear and quadratic was made if both followed the model's requirements. A successful statistical test was synonym of a good model. However, the predictive effectiveness of the latter was also to be assessed. In addition to analysing sub-library and full library test vials with their respected libraries, full libraries vials were analysed against sub-libraries for model validation. The equations of the line for the appropriate sub-library models were used in order to determine the matching value taking in consideration the sample composition. The model's equation followed the format shown below (Eq.4) where A, B, C, D, and E represented the amount in mg of the component and x, y, z, t, u corresponded to the set of real numbers. The predicted values were also compared to the obtained matching values. In this case the uncertainty values were used to determine whether matching was within range or not. In fact, if both predicted and observed values overlap within one or both uncertainty values, they said to agree with each other. In the opposite case, they said to disagree and hence out of range within the uncertainty.

(Eq.4) $$\boldsymbol{P} = \mathrm{xA} + \mathrm{yB} + \mathrm{zC} + \mathrm{tD} + \mathrm{uE} \qquad (x, y, z, t, u)\ \mathrm{U}\ R$$

2.7. Analysis of cutting agents and adulterants

Sub-library analysis was subjective to correlation of sample spectra against the exact required reference standards. However, possibility of good correlation between two reference standards was likely. Hence, pure cutting agents/adulterants samples weighted at 150 mg were made following the method discussed in section 2.2 and analysed against each NPS sub library. Each sample was run against all three sub-libraries in order to establish comparison with all NPS spectra while respecting the parameters for each NPS library: 5F-PB-22 and N-Me-2-AI using method A, phenibut using method B.

3. RESULTS AND DISCUSSION

In order to initially evaluate the identification capabilities of the handheld instrument, the 26 vials (Table 3.1) pertaining to each NPS were subjected to analysis employing two inbuilt algorithms, wavelet algorithm and Rigaku mixture as implemented in the instrument. The identification of NPS and cutting agents/adulterants were noted as the average matching position across all four replicates. Results obtained from the handheld Raman were recorded through a matching value which measures the similarity between an unknown mixture spectrum against a library of reference standards. These findings constituted the data required for the conception of the model. The test vials generated were analysed and compared against model prediction for validation.

3.1. Initial studies

Initial performance of the handheld Raman investigated the efficacy of the instrument in identifying NPS in the presence of mixtures. NPS samples purchased from internet sources displayed high purity greater than 90% (Section 2.1) while library signatures originated from reference materials of above 97% purity. As NPS certified reference material are approximately £100 per 10 mg, it was decided to use the high purity Internet sources for construction of the NPS mixtures. In fact, considering the high purity of the internet NPS sample, it was observed that in the presence of pure NPS (vial 1 and 22), the average matching value ranged between 0.80 and 0.99 as shown in Table 3.1. This mainly applied to 5F-PB-22 and phenibut. N-Me-2-AI (A) was only recorded at 0.58 ± 0.05 and 0.56 ± 0.02 for both respective vials. In fact, the Raman spectra for N-Me-2-AI (A) showed a raised background and considerable presence of fluorescence as shown in Figure 3.1. This could be the reason behind the poor spectral correlation observed to the certified reference standard. In fact, correlation coefficient analysis between their spectra generated an r value of 0.84. Therefore, a different N-Me-2-AI sample (B) originating from a different internet source was instead selected for the study and found to have a purity of 99.6% and was analysed under the same conditions. A comparison of the spectra showed no difference in the peak positions with major signals at 777, 847, and 1211 cm^{-1} respectively corresponding to the N-substituted methyl, the aromatic ring, and the amino group. For N-Me-2-AI (B), results showed a 50% increase in the matching value being detected at 0.90 ± 0.01 and 0.89 ± 0.01 respectively for vials 1 and 22 (150 mg of NPS). This was also noticed in the signal's intensities for sample B which were marginally greater than sample A. Additionally, correlation analysis between both N-Me-2-AI samples showed they matched to 84.3% similar. Though this coefficient is subjective of great similarity between both N-Me-2-AI A and B, it further highlights the difference in matching results. Although, the internet sources

were of high purity (97%), this difference between the certified reference and internet sources in the Raman matching could be due to the presence of small amount of impurities in particular trace metals, most likely in the internet samples. Due to the interferences caused by impurities and the significant difference in matching results, N-Me-2-AI (B) was carried forward for the reminder of the study.

Table 3.1: Wavelet matching results of pure NPS samples on same spot of analysis.

	vial 1	vial 22
5F-PB-22	0.98 ± 0.01[1]	0.98 ± 0.01
N-Me-2-AI (A)	0.58 ± 0.05	0.56 ± 0.02
N-Me-2-AI (B)	0.90 ± 0.01	0.89 ± 0.01
Phenibut	0.88 ± 0.01	0.85 ± 0.01

[1] The (±) indicated standard deviation values

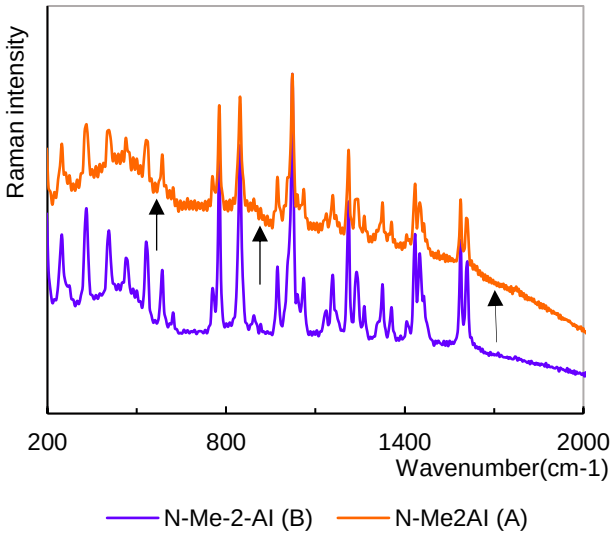


Figure 3.1: Raman Spectra of internet samples N-Me-2-AI sample A and B.

3.2. Same spot analysis of all NPS

The handheld instrument of used possessed two in-built algorithms in Rigaku mixture useful due to its mixture format while wavelet was able to match to only one reference standard at a time. Hence, this section displays same spot analysis results as well as a comparison between wavelet and Rigaku mixture algorithm in the identification of NPS on full library.

3.2.1. Wavelet algorithm

The Table 3.2 showed the findings obtained from same spot analysis of all 26 vials on wavelet algorithm. In the case of binary mixtures (vial 2-9 and 23-26), results showed that all NPS were mostly detected when present at a range of 80-150 mg using the wavelet algorithm. However, it is key to note that no match was observed for N-Me-2-AI and phenibut in the presence of benzocaine (140 mg) with the exception of 5F-PB-22. In fact, vial 7 only registered 0.35 ± 0.35 matching value for 5F-PB-22 although being present at 80 mg compared to 70 mg of

benzocaine. Although detection, the precision value for 5F-PB-22 (0.35) which was detected two out of four replicates was an indication of poor reproducibility with the NPS having a 50% chance of being identified. In addition, the matching value of NPS across vial six to nine appeared to increase as the second component of the vial changed from benzocaine, caffeine, creatine, to sodium glutamate. However, in similar mixtures with lower amount of NPS i.e. 10 mg, no detection of NPS was observed in the presence of benzocaine, caffeine, and creatine for N-Me-2-AI samples and phenibut. 5F-PB-22 results showed consistent matching in the presence of creatine and sodium glutamate as observed in vial 4 and 5 detected at 0.09 ± 0.15 and 0.38 ± 0.25. The duplicate vials in 25 and 26 displayed similar results i.e. 0.07 ±0.13 and 0.28 ± 0.30 while the reverse was observed in the case of caffeine detected only in vial 2 at 0.10 ± 0.16. Although all adulterants and cutting agents have the same amount (140 mg), the matching value, as noticed at the higher end (vial 6-9), appeared to increase in presence of creatine and sodium glutamate compared to benzocaine and caffeine. Hence benzocaine and caffeine can mask the detection of NPS. 5F-PB-22 performed the best being detected in 50% of lower end samples with caffeine (vial 2 and 23). Nonetheless, this matching value increase seemed to be apparent with NPS too, with phenibut displaying the lower values while 5F-PB-22 higher values.

Table 3.2: Same spot results of all 26 vials on wavelet algorithm using full library.

Vial	Content (mg)					Wavelet Average		
	NPS	BEN	CAF	CREA	Na GLU	5F-PB-22	N-Me-2-AI	Phenibut
1	150	0	0	0	0	0.98 ± 0.01[1]	0.90 ± 0.01	0.88 ± 0.01
2	10	0	140	0	0	0.10 ± 0.16	0.00[2]	0.00
3	10	140	0	0	0	0.00	0.00	0.00
4	10	0	0	140	0	0.09 ± 0.15[3]	0.00	0.00
5	10	0	0	0	140	0.38 ± 0.25	0.20 ± 0.20	0.00
6	80	0	70	0	0	0.87 ± 0.04	0.62 ± 0.07	0.59 ± 0.03
7	80	70	0	0	0	0.35 ± 0.35	0.00	0.00
8	80	0	0	70	0	0.95 ± 0.01	0.75 ± 0.07	0.70 ± 0.04
9	80	0	0	0	70	0.95 ± 0.02	0.83 ± 0.02	0.76 ± 0.08
10	10	70	70	0	0	0.00	0.00	0.00
11	10	0	70	70	0	0.11 ± 0.19	0.00	0.00
12	10	0	70	0	70	0.00	0.00	0.00
13	10	70	0	70	0	0.00	0.00	0.00
14	10	70	0	0	70	0.00	0.00	0.00
15	10	0	0	70	70	0.20 ± 0.35	0.19 ± 0.19	0.00
16	94	14	14	14	14	0.93 ± 0.03	0.53 ± 0.31	0.38 ± 0.34
17	24	14	84	14	14	0.19 ± 0.32	0.00	0.00
18	24	84	14	14	14	0.00	0.00	0.00
19	24	14	14	84	14	0.13 ± 0.22	0.00	0.00
20	24	14	14	14	84	0.20 ± 0.34	0.00	0.30 ± 0.27
21	38	28	28	28	28	0.68 ± 0.11	0.00	0.00
22	150	0	0	0	0	0.98 ± 0.01	0.89 ± 0.01	0.85 ± 0.01
23	10	0	140	0	0	0.00	0.00	0.00
24	10	140	0	0	0	0.00	0.00	0.00
25	10	0	0	140	0	0.07 ± 0.13	0.00	0.00
26	10	0	0	0	140	0.28 ± 0.30	0.21 ± 0.21	0.21 ± 0.19

[1] The (±) indicated standard deviation values

[2] Matching value of 0.00 indicated no match

[3] Colour coding: GREEN: 4/4 matches, YELLOW: 3/4 matches, ORANGE: 1 or 2 of 4 matches, RED: unidentified

In the case of ternary mixtures, it was noticed that in the presence of benzocaine, the NPS was not detected. However, 5F-PB-22 and N-Me-2-AI samples were detected in presence of both creatine and sodium glutamate (vial 15). In fact, vial 15 (NPS: 10 mg, creatine: 70 mg, and sodium glutamate: 70 mg) displayed matching values of 0.20 ± 0.35 and 0.19 ± 0.19 respectively for 5F-PB-22 and N-Me-2-AI. Although the proximity in values, 5F-PB-22 was identified one out of four in comparison to two for N-Me-2-AI further explained by the precision values of 0.35 compared to 0.19. In fact, the presence of sodium glutamate and creatine which displayed greater noise level compared to benzocaine and caffeine, could also hinder detection notably in low NPS concentration (10 mg) compared to as in vial 8 and 9. The poor precision value synonym of bad reproducibility was due to low signal to noise ratio hence low matching value observed in complex mixtures compared to in mixtures of higher matching.

The opposite was encountered in the presence of caffeine. Findings showed that of all three ternary mixtures with caffeine included, no NPS was detected at the exception of 5F-PB-22 identified in one out of three (vial 11; NPS: 10 mg, caffeine: 70 mg, and creatine: 70 mg) at 0.11 ± 0.19. As discussed previously regarding the impact of benzocaine, caffeine appeared to also influence to a lesser effect the detection of NPS in mixtures. This confirms that the role of cutting agents and adulterants in NPS detection depends on their spectral properties in relation to the NPS of use. Benzocaine and caffeine appeared to mostly hinder identification of NPS while sodium glutamate and creatine, though weaker in high concentration of NPS (80 mg) displayed more influence in small NPS amount (10 mg). Spectra from NPS, adulterants and cutting agents in Figure 3.2 emphasised on their differences in terms of spectral behaviour. Benzocaine showed distinct peaks at around 1606 cm^{-1} related to the ketone group (C=O) and a moderate peak at 1687 cm^{-1} corresponding to the ester (-COO-) while sodium glutamate presented significant noise levels highlighting its weak scattering properties.

The influence of weaker cutting agents as creatine and sodium glutamate was mostly noticeable with phenibut. Indeed, results from the Table above displayed lower phenibut matching in presence of creatine (0.70 ± 0.04) and sodium glutamate (0.76 ± 0.08) compared to N-Me-2-AI and 5F-PB-22. These findings are justified by low signals within phenibut spectra with a single aromatic reading at 1000 cm^{-1}. Similarly, 5F-PB-22 appeared to be a stronger Raman scatterer compared to N-Me-2-AI. Strong readings at 777, 847, and 1022 cm^{-1} as highlighted in section 3.1 were noticed in N-Me-2-AI spectra. However, it also displayed fluorescence before 700 cm^{-1}. This was the opposite of 5F-PB-22 spectra highlighted by low noise level and a strong peak at 1713 cm^{-1} (-COO-). In addition to results highlighting poor to no detection of NPS in presence of benzocaine and caffeine, it suggested that the choice of cutting agents and adulterants with regards to sample concentration displayed different level of effects in hindering NPS detection depending on their Raman properties. However, it is evident the same observation applies to

NPS. Indeed, detection of 5F-PB-22 observed in vial 11 in addition to findings discussed in binary mixtures, this is an indication that 5F-PB-22 has strong Raman cross-section/scattering properties in comparison to N-Me-2-AI and phenibut.

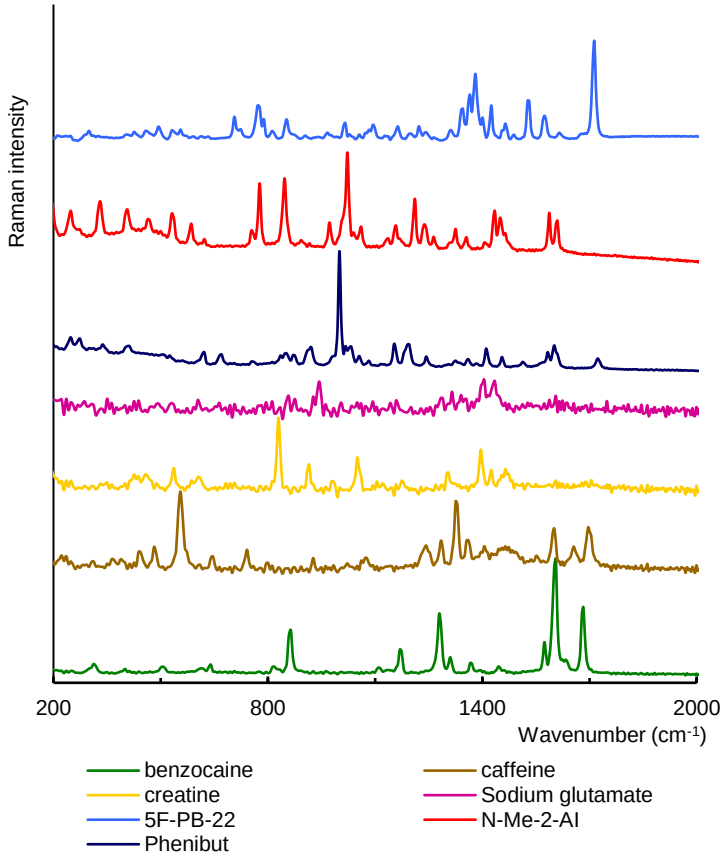


Figure 3.2: Raman spectra of all NPS, cutting agents and adulterants.
In addition to analysis conducted with baseline correction, all spectra above were normalised and spaced out in order to distinguish signals.

Quinary mixtures explored in this study included vial 16 (NPS: 94 mg, BEN: 14 mg, CAF, 14 mg, CREA: 14 mg, Na GLU: 14 mg) where the amount of NPS was 94 mg. 5F-PB-22 results yielded strong matching at 0.93 ± 0.03. On the other hand, N-Me-2-AI and phenibut samples showed weaker detection values at 0.53 ± 31, and 0.38 ± 0.34 respectively for N-Me-2-AI and phenibut. Although the mass of all excipients relatively low at 14 mg, the complexity of mixture was subjective to poor reproducibility demonstrated by undetected NPS in two out of four replicates for phenibut and three of four replicates for N-Me-2-AI.

As the complexity of samples in terms of concentration varied between NPS and excipients, more changes were highlighted in vials of low NPS amounts. Vial 17-21 as shown in the Table 3.2 displayed matching of 5F-PB-22 in presence of caffeine, creatine and sodium glutamate with values of 0.19 ± 0.32, 0.13 ± 0.22, and 0.20 ± 0.34. In presence of benzocaine no match was obtained. Recurrently, this was due to the interaction of analogues. Indeed, matching data

displayed substances non-apparent in samples yet correlating to at least 50% of benzocaine (dimethocaine, mephedrone, procaine). However, the latter had more influence in N-Me-2-AI and phenibut results. In fact, no match was recorded for N-Me-2-AI while phenibut only showed detection in presence of sodium glutamate with a value of 0.30 ± 0.27. As highlighted throughout these results, matching values obtained via wavelet analysis displayed poor precision values with the influence of analogues being the main cause.

3.2.2. Rigaku mixture

Being a mixture's algorithm, Rigaku mixture was considered in order to explore its identification capabilities for NPS. Rigaku mixture algorithm matches a 'sample spectrum' to all potential results thus increasing the chance of detection of NPS particularly in more complex mixtures. In fact, though similar to wavelet in providing only up to five results, findings from Rigaku mixture showed one or more hits per result. Thus, the matching result reports all reference spectra that may contribute to a sample Raman spectrum. Analysis showed some comparable results to wavelet in the identification of NPS, yet the use of Rigaku mixture appeared to be more beneficial in the identification of NPS especially in complex mixtures.

As discussed above, sample 12 (NPS: 10 mg, CAF: 70 mg, Na GLU: 70 mg) showed no detection of 5F-PB-22, N-Me-2-AI, and phenibut using wavelet. However, Rigaku mixture recordings highlight the detection of N-Me-2-AI detected only in the third replicate, and 5F-PB-22 in three out of four replicates. In fact, Table 3.4 shows the presence of 5F-PB-22 analogues in PB-22 in the wavelet results instead of the actual NPS as well as caffeine related compounds such as sucrose, theophylline (in green). While Rigaku mixture displayed identification of 5F-PB-22, its analogues were more abundant with records of NM-2201 and PB-22 (in red) in addition to repetition of caffeine and its structure related compounds. With phenibut weaker scatterer compared to 5F-PB-22 and N-Me-2-AI as demonstrated in Section 3.2.1, it was unidentified using Rigaku mixture. However, these findings were not always replicated. In fact, samples 13 (NPS: 10 mg, BEN: 70 mg, CREA: 70 mg) and 14 (NPS: 10 mg, BEN: 70 mg, Na GLU: 70 mg) both resulted in undetected NPS via wavelet algorithm.

While similar results were noted in Rigaku mixture, the influence of analogues and chemically identical substances was noticeable by their repetition across the results such as dimethocaine and mephedrone, analogues of benzocaine as noticed in vial 13. The scattering profile of benzocaine appeared to display the same effect in Rigaku mixture as previously observed in wavelet with the appearance of its analogues and structurally similar substances. Although there are some similarities between these two algorithms, it was important to note if the spot of analysis had the same effect in Rigaku mixture as in wavelet. Results from vial 17 (NPS: 24 mg, BEN: 14 mg, CAF: 84 mg, CREA: 14 mg, Na GLU: 14 mg) showed identification of 5F-PB-22 using wavelet at an average of 0.19 ± 0.32 with only a 25% reproducibility indicated by the

precision value (0.32). Although the complexity of the mixture, the NPS was identified in three out of four runs using Rigaku mixture. Similarly to wavelet analysis of 5F-PB-22, N-Me-2-AI and phenibut were mostly undetected with the aminoindane registered one out of four replicates. The same pattern was shown in phenibut analysis in the examples of sample 19 (NPS: 24 mg, BEN: 14 mg, CAF: 14 mg, CREA: 84 mg, Na GLU: 14 mg) and 21 (NPS: 38 mg, BEN: 28 mg, CAF: 28 mg, CREA: 28 mg, Na GLU: 28 mg) in which no NPS was identified via wavelet algorithm. However, the NPS was detected at least once of all replicates using Rigaku mixture on the same spot.

This variance in the detection pattern with regard to the algorithm was also associated to the spectral properties of the NPSs of interest explained by phenibut having the weakest detection compared to 5F-PB-22. This is highlighted by Figure 3.3 where vial 19 spectra although abundance of peaks respectively at around 1606 (C=O in benzocaine) and 829 cm^{-1} (C=N in creatine), showed a reading at 1000 cm^{-1} corresponding to the monosubstituted ring in phenibut. While the spectra of 5F-PB-22 sample with the inclusion of benzocaine and creatine showed bands at 1531 and 1713 cm^{-1} respectively corresponding to an aromatic system and the ketone group (C=O) of 5F-PB-22. The study showed Rigaku mixture as the superior algorithm of use for in field testing of unknown sample mixtures compared to wavelet.

Table 3.4: Comparison between wavelet and Rigaku mixture matching data of 5F-PB-22 vial 12

Wavelet		**Rigaku mixture**	
Run 1	**Valid Hit**	**Run 1**	**Valid Hit**
CAFFEINE	0.88	CAFFEINE, 5F-PB-22	0.97
THEOPHYLLINE[1]	0.47	CAFFEINE	0.93
SUCROSE	0.37	CAFFEINE, 5F-PB-22	0.92
PB-22[1]	0.37	CAFFEINE, PB-22	0.92
MEBROQUALONE	0.36	NM-2201, CAFFEINE, THEOPHYLLINE	0.91
Run 2	**Valid Hit**	**Run 2**	**Valid Hit**
CAFFEINE	0.8	CAFFEINE	0.9
THEOPHYLLINE	0.47	CAFFEINE, PB-22	0.88
SUCROSE	0.37	CAFFEINE, 5F-PB-22	0.88
PB-22	0.35	THEOPHYLLINE, NM-2201, CAFFEINE	0.84
MEBROQUALONE	0.33	NM-2201, CAFFEINE, THEOPHYLLINE	0.84
Run 3	**Valid Hit**	**Run 3**	**Valid Hit**
CAFFEINE	0.79	CAFFEINE, L-GLUTAMIC ACID	0.93
THEOPHYLLINE	0.46	CAFFEINE, L-GLUTAMIC ACID	0.9
SUCROSE	0.38	L-GLUTAMIC ACID, PB-22, CAFFEINE	0.89
4-Me-N-ETHYLNORPENTEDRONE	0.34	NM-2201, CAFFEINE, L-GLUTAMIC ACID	0.87
L-GLUTAMIC ACID	0.33	THEOPHYLLINE, L-GLUTAMIC ACID, CAFFEINE	0.85

Run 4	Valid Hit	Run 4	Valid Hit
CAFFEINE	0.89	CAFFEINE	0.95
THEOPHYLLINE	0.45	CAFFEINE, THEOPHYLLINE	0.89
4-Me-N-ETHYLNORPENTEDRONE	0.38	CAFFEINE, PB-22	0.88
SUCROSE	0.37	CAFFEINE, 5F-PB-22	0.86
Synth MEPHEDRONE	0.35	CAFFEINE,4-Me-N-ETHYLNORPENTEDRONE	0.85

[1] Colour coding: GREEN: caffeine analogues, RED: 5F-PB-22 analogues

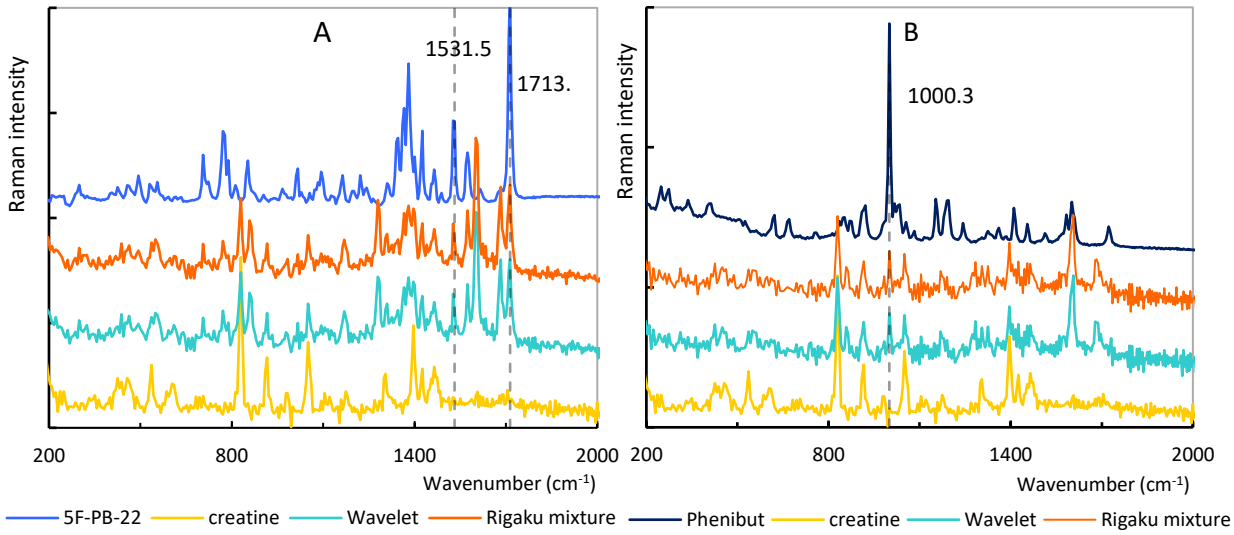


Figure 3.3: Vial 19 spectra of 5F-PB-22 (A) and phenibut (B) on full library using wavelet and Rigaku

3.3. Influence of different spot of analysis in comparison to same spot

In the previous experiments, the wavelet and mixture assessment were carried out on the same spot to ensure the data was 100% comparable. It could be assumed that analysis at different sample spots could lead to bias results in presence of poorly homogeneous mixtures. Thus, a comparison of the two algorithms using different spots was then carried out to evaluate if this may lead to bias. The results showed that a change in the spot of analysis did indeed have different outcomes in the matching results. On one hand, samples of high purity displayed no change in the detection pattern. Indeed, both vial 1 and 22 containing 150 mg of NPS showed identification of the NPS with values relatively close of each other except for phenibut readings for vial 1 detected at 0.79 ± 0.03 compared to 0.88 ± 0.01 in same spot analysis (Table 3.5). However, as sample complexity increased with the number of components, some variation in matching results were observed. Phenibut displayed major differences in vial 20 with a matching value of 0.37 ± 0.20 in the same spot analysis compared to no identification in at a different spot. This was the other way around for vial 5 where phenibut was identified in different spot records

(0.40 ± 0.27) but not the case in same spot analysis. This difference in results lies in the homogeneity of sample. In fact, the standard deviation obtained showed lack of reproducibility apparent in both spot analysis. 5F-PB-22 was detected in one out of four replicates (vial 4) in same spot analysis and only twice in different spot. In a total of 150 mg, the probability of detection is 1/15. However, in addition to the spot size, the minimal amount of sample powder required within the laser's hit spot to generate a reading is low and varies for each NPS.

As discussed previously, light scattering properties of NPS are key in their detection. Same spot analysis of 5F-PB-22 in vial 2 lead to 0.10 ± 0.16 match although poor reproducibility while a change of spot displayed no detection. Although previous hypothesis made on the strong scattering properties of 5F-PB-22 compared to phenibut, the latter matched at a higher value compared to 5F-PB-22 detected at 0.20 ± 0.20 for vial 5 (NPS: 10 mg, Na GLU: 140 mg). The most flagrant case of difference in spot was noticed in vial 16 for phenibut where different displayed four matches correlating to an average of 0.75 ± 0.03 while only two replicates displayed detection indicated by a match of 0.38 ± 0.34. With the NPS present at 94 mg, homogeneity of the sample was expected to be less problematic supported by results obtained in vial of high NPS concentration (≥80 mg). However, this would be predominantly linked to the optimised spot hit by the laser related to the distance at which the sample is compared with regards to the laser source. This could be altered by adjusting the vial adjustment at the correct distance.

Table 3.5: Variation of results in change of spot using wavelet algorithm.

Vial	Content (mg)					5F-PB-22		Phenibut	
	NPS	BEN	CAF	CREA	Na GLU	diff spot	same spot	diff spot	same spot
1	150	0	0	0	0	0.98 ± 0.01[1]	0.98 ± 0.01	0.79 ± 0.04	0.88 ± 0.01
2	10	0	140	0	0	0.00	0.10 ± 0.16	0.00	0.00
3	10	140	0	0	0	0.00[2]	0.00	0.00	0.00
4	10	0	0	140	0	0.21 ± 0.26[3]	0.09 ± 0.15	0.00	0.00
5	10	0	0	0	140	0.20 ± 0.23	0.38 ± 0.25	0.4 ± 0.27	0.00
6	80	0	70	0	0	0.91 ± 0.02	0.87 ± 0.04	0.48 ± 0.03	0.59 ± 0.03
7	80	70	0	0	0	0.13 ± 0.26	0.35 ± 0.35	0.00	0.00
8	80	0	0	70	0	0.91 ± 0.04	0.95 ± 0.01	0.63 ± 0.07	0.70 ± 0.04
9	80	0	0	0	70	0.95 ± 0.02	0.95 ± 0.02	0.79 ± 0.04	0.76 ± 0.08
10	10	70	70	0	0	0.00	0.00	0.00	0.00
11	10	0	70	70	0	0.00	0.11 ± 0.19	0.00	0.00
12	10	0	70	0	70	0.28 ± 0.32	0.00	0.00	0.00
13	10	70	0	70	0	0.00	0.00	0.00	0.00
14	10	70	0	0	70	0.00	0.00	0.00	0.00
15	10	0	0	70	70	0.00	0.20 ± 0.35	0.00	0.00
16	94	14	14	14	14	0.89 ± 0.09	0.93 ± 0.03	0.75 ± 0.03	0.38 ± 0.34
17	24	14	84	14	14	0.00	0.19 ± 0.32	0.00	0.00
18	24	84	14	14	14	0.00	0.00	0.00	0.00
19	24	14	14	84	14	0.40 ± 0.27	0.13 ± 0.22	0.00	0.00
20	24	14	14	14	84	0.14 ± 0.29	0.20 ± 0.34	0.00	0.30 ± 0.27
21	38	28	28	28	28	0.18 ± 0.36	0.68 ± 0.11	0.00	0.00
22	150	0	0	0	0	0.98 ± 0.01	0.98 ± 0.01	0.81 ± 0.03	0.85 ± 0.01
23	10	0	140	0	0	0.00	0.00	0.00	0.00
24	10	140	0	0	0	0.00	0.00	0.00	0.00
25	10	0	0	140	0	0.24 ± 0.28	0.07 ± 0.13	0.00	0.00
26	10	0	0	0	140	0.37 ± 0.04	0.28 ± 0.30	0.12 ± 0.24	0.21 ± 0.19

[1] The (±) indicated standard deviation values

[2] Matching value of 0.00 indicated no match

[3] Colour coding: GREEN: 4/4 matches, YELLOW: 3/4 matches, ORANGE: 1 or 2 of 4 matches, RED: unidentified

This study confirms that there appeared to be no significant difference in spot change. With sample homogeneity assured by mixing in between run, it can be concluded that a change in spot of analysis can result in an improvement in detection of NPS. However, this is at the

disadvantage of poor reproducibility at low concentrations or in complex mixtures. In fact, detailed matching results showed that most cases of poor reproducibility and no identification of NPS was due to the presence of analogues and structurally similar standards presents within the search library. Due to their similarities, these analogues appeared to match highly to the cutting agents and adulterants they are associated with. As an example, benzocaine being the strongest scatterer appeared to strongly influence identification of NPS even when present at low concentrations (14 mg). Indeed, vial 17, 19 and 20 exhibited poor NPS identification particularly for N-Me-2-AI and phenibut. Matching results were dominated by the presence of dimethocaine, procaine, and mephedrone. Table 3.6 shows the matching results of vial 17 (NPS: 24 mg, BEN: 14 mg, CAF: 84 mg, CREA: 14 mg, Na GLU: 14 mg) where the concentration of caffeine was 84 mg.

Table 3.6: Vial 17 matching results for all NPS on wavelet algorithm

Matching results					
5F-PB-22		**N-Me-2-AI**		**Phenibut**	
Run 1	**Hit**	**Run 1**	**Hit**	**Run 1**	**Hit**
CAFFEINE	0.76	CAFFEINE	0.89	CAFFEINE	0.87
DIMETHOCAINE[1]	0.65	4-Me-N-Ethylnorpentedrone	0.46	DIMETHOCAINE	0.62
BENZOCAINE	0.61	Synth MEPHEDRONE	0.43	Synth MEPHEDRONE	0.59
Synth[2] MEPHEDRONE	0.58	MEPHEDRONE	0.42	MEPHEDRONE	0.59
MEPHEDRONE	0.58	Synth FLEPHEDRONE HCl	0.41	4-Me-N-Ethylnorpentedrone	0.57
Run 2	**Hit**	**Run 2**	**Hit**	**Run 2**	**Hit**
CAFFEINE	0.83	CAFFEINE	0.84	DIMETHOCAINE	0.88
DIMETHOCAINE	0.59	DIMETHOCAINE	0.62	BENZOCAINE	0.87
Synth MEPHEDRONE	0.55	Synth MEPHEDRONE	0.6	MEPHEDRONE	0.75
MEPHEDRONE	0.55	MEPHEDRONE	0.6	Synth MEPHEDRONE	0.75
BENZOCAINE	0.54	BENZOCAINE	0.58	PROCAINE	0.66
Run 3	**Hit**	**Run 3**	**Hit**	**Run 3**	**Hit**
DIMETHOCAINE	0.75	DIMETHOCAINE	0.76	CAFFEINE	0.85
BENZOCAINE	0.74	CAFFEINE	0.73	DIMETHOCAINE	0.61
MEPHEDRONE	0.64	BENZOCAINE	0.73	Synth MEPHEDRONE	0.58
Synth MEPHEDRONE	0.63	Synth MEPHEDRONE	0.69	MEPHEDRONE	0.58
CAFFEINE	0.61	MEPHEDRONE	0.69	4-Me-N-Ethylnorpentedrone	0.57
Run 4	**Hit**	**Run 4**	**Hit**	**Run 4**	**Hit**
5F-PB-22	0.75	CAFFEINE	0.83	CAFFEINE	0.78
PB-22	0.73	Synth MEPHEDRONE	0.54	DIMETHOCAINE	0.73
NM-2201	0.64	4-Me-N-Ethylnorpentedrone	0.54	BENZOCAINE	0.69
FDU-PB-22	0.59	MEPHEDRONE	0.53	Synth MEPHEDRONE	0.97
CAFFEINE	0.56	DIMETHOCAINE	0.52	MEPHEDRONE	0.66

[1] Colour coding: RED: benzocaine analogues

[2] Synth: synthesised

Similarly, findings from vial 4 (NPS: 10 mg and CREA: 140 mg) displayed detection of 5F-PB-22 in two and one out of four replicates respectively for different and same spot analysis. Once more, the results showed detection of L-tyrosine (adulterant), and NPSs such as N-ethylamphetamine, and 4-fluoro-α-Pyrrolidinopentiophenone (4F-α-PVP) with their structure shown in the Figure 3.4.

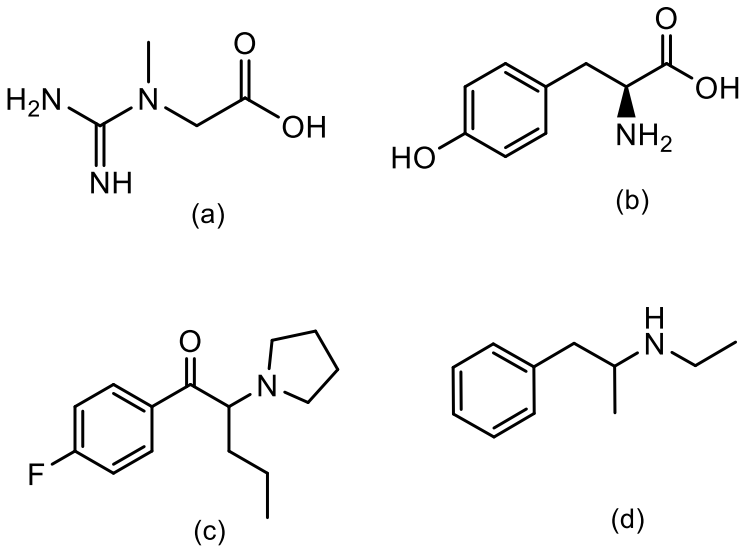


Figure 3.4: Structures of substances wrongly recorded in vial 4 (false positives): (a) creatine, (b) L-tyrosine, (c) 4F- α-PVP, and (d) N-ethylamphetamine.

Comparative results of same spot and different spot between wavelet and Rigaku mixture algorithms displayed no significant improvement in the identification of NPS. Although findings showed detection of NPS in different spot analysis when absent in same spot results, the reverse situation was as frequent. In fact, the spot change was done in the aim of evaluating improvement in NPS search from one algorithm to another rather than actual improvement in results. Wavelet showed promising results in its ability to accurately assign a reference standard to a precise matching value while identifying 69% of 5F-PB-22, 35% of N-Me-2-AI and 31% of phenibut vials. However, with each result assigned to only one signature, the later face some limitations in the detection matrix with the abundance of analogues and structurally similar substances. In case of samples of unknown composition, this issue would be of a greater influence in determining what is present or absent. Thus, further investigation was made in order to evaluate the potential impact of analogues and the size of search libraries on NPS identification.

3.4. Sub-libraries investigations

As mentioned above, the use of wavelet algorithm appeared to be useful in the identification of NPS detecting up of 69% of 5F-PB-22 mixtures. However, the abundance of analogues and structurally similar compounds within the library appeared to impact NPS detection more noticeable in the case of N-Me-2-AI and phenibut (35% and 31% identified respectively). Indeed, annual drug intelligence bulletin from LGC forensics (2014) showed benzocaine to be the most common excipient in NPS mixtures. This was explained by its influence in NPS detection due to its strong Raman properties as well as the analogues and structurally similar substances (dimethocaine, procaine) present within the search library. Similar observations regarding analogues were seen in the case of caffeine, creatine and sodium glutamate. Since these analogues were not present within the 26 vials, they were omitted by creating sub-libraries in the aim to further investigate the wavelet algorithm. Thus, a library containing the only five chemicals present was used. Results showed that all NPS were identified across all 26 samples as opposed to the previously discussed case for full library (FL) analysis. Initial observations showed that the matching values for the NPS appeared to increase even in pure NPS vials. In fact, vial 1 (NPS: 150 mg) still displayed an increase for 5F-PB-22 from 0.98 ± 0.01 in full library to 1.00 ± 0.00 in sub-library readings. The same pattern was noticed for phenibut and N-Me-2-AI with full libraries values increasing from 0.88 ± 0.01 and 0.90 ± 0.01 to 0.99 ± 0.01. In addition, vial 22 which was a copy of vial 1 displayed the same increase. However, matching results from sub-library analysis showed no interference from analogues and all NPS were identified as the highest match. Early conclusions could be drawn to the size of the library being reduced to five. Could the size of search library be a significant factor in the detection of NPS? Hence, further checks were conducted in order to explain the increase in matching values

3.4.1. Potential impact of library size in NPS detection

Primary investigation was focussed on the timing on various search libraries of different sizes. Two Additional libraries were created and made relevant to this study. These were the sub sub-library (SSL) and the half library which were created with reference standards included in the full library. The time recorded presented in Table 3.7 represented the searching time after scanning and processing. Master library possessing the entirety of the reference standards within the instrument was timed at an average of 15 s. On the other hand, SSL was timed at an average 2.87 s while SL was timed approximately a second further at 3.71 s with an additional two reference standards. However, FL and half library runs were timed only approximately half a second adrift, at 4.06 and 4.02 with up to 87 reference standards on top. In fact, N-Me-2-AI which was subjective to a 50% increase in SL analysis did not follow the same trend with an average time for SL and FL of respectively 4.12 and 4.10s

Table 3.7: Searching time in seconds on libraries of various size.

	Time (s)				
	Master library	FL	Half library	SL	SSL
number of spectra	+14000	98	50	5	3
5F-PB-22	15.4	3.73	3.95	3.54	2.8
	14.54	3.99	3.49	3.74	2.86
	14.78	4.09	3.62	3.65	2.84
	15.01	4.16	4.04	4.12	2.83
	15.21	4.22	3.91	3.91	2.81
N-Me-2-AI	15.12	4.54	3.96	4.33	2.85
	14.72	4.2	4.22	4.31	2.83
	15.05	4.16	3.5	3.8	2.74
	14.61	3.98	4.67	4.04	3.07
	15.25	3.54	3.89	4.1	2.85
Phenibut	14.85	4.16	4.25	3.47	2.69
	15.32	3.91	4.27	3.55	2.78
	15.21	4.14	4.29	3.9	2.83
	14.89	3.96	4.2	3.73	3.09
	15.11	4.08	4.03	3.71	2.87
Average	15.00	4.06	4.02	3.71	2.87

For investigation purposes, libraries were created in the interest of searching rather than matching results. Pure adulterants and NPS samples were analysed and their spectra saved as library standard. However, half library was created by the addition of 50 reference signatures (Appendix: Table 9) were copied directly from the master library by identifying their file names. This led to questioning in the difference between the original reference standards and standards run as pure samples. Initially, the NPS and adulterants reference standards presented within the full library displayed a different file name to that of newly created SL. This meant that full library and sub-library contained different reference standards rejecting like for like comparison. Indeed, due to the use of pure standards as reference materials, the similarities with samples spectra is closer thus increasing the correlation between them. In fact, pure N-Me-2-AI saved as a library reference standard correlated to 99% similar as observed in SL results yet only matched to 90% against in-built N-Me-2-AI reference signature. Although this trend was not as apparent for other NPS in particular 5F-PB-22, similar observation was noticed. These investigations confirmed that the size of the library regarding the number of reference standards does not impact in the identification of NPS. However, it emphasised on the importance of possessing pure reference standards materials for optimum identification results. What would be the outcome of a reduced library with the original reference standards as full library analysis?

Hence, sub-library analysis was repeated and the reference standards used were added directly from the full library to ensure like to like analysis.

3.4.2. Sub-libraries findings

As noticed previously in Section 3.4.1, matching results showed detection of NPS across all 26 vials in sub-library. However, the apparent difference in values observed in vials of pure composition as vial 1 and 22 was related to inadequate reference standards introduce within the library. In fact, pure NPS vials (vial 1 and 22) showed newly sub-library matching results similar to those recorded in full library analysis. 5F-PB-22 displayed identical values in vial 1 and 22 with matching value of 0.9, in sub-library as well as full library. Similarly, N-Me-2-AI matching results appeared level in both libraries with readings of 0.90 ± 0.01 for both vial 1 and 22. There seemed to be more of a difference in the case of phenibut where vial 1 decreased from 0.88 ± 0.01 in full library to 0.79 ± 0.02 in sub-library. However, since vial 1 is a duplicate of vial 22, the latter displayed a closer value where 0.80 ± 0.04 was recorded for sub-library analysis compared to 0.85 ± 0.01 in full library.

Binary mixture results included in Table 3.8 showed an average increase in the sub-library matching notably for vials that had the NPS originally detected in full library analysis. In the presence of 80 mg of NPS, sub-library results appeared to match consistently with full library with the exception of benzocaine being present. In fact, 5F-PB-22 detected in vial 6 (NPS: 80 mg, CAF: 70 mg), 8 (NPS: 80 mg, CREA: 70 mg), and 9 (NPS: 80 mg, Na GLU: 70 mg) were very similar to sub-library results with good individual precision values of 0.01-0.02. In the case of sub-library where there is an absence of benzocaine analogues in the library, such as dimethocaine and procaine, match values were 0.45 ± 0.09 for 5F-PB-22 in comparison to an average of 0.35 ± 0.35 in full library. This was due to the average of only two values (0.77 and 0.61) with the NPS detected in two out of four replicates emphasised by the precision value of 0.35. The same pattern was highlighted in N-Me-2-AI and phenibut results. In the case of vial 7, both displayed no detection of the NPS in full library. In addition to benzocaine being the strongest Raman scatterer of use in this study, N-Me-2-AI and phenibut were found to be the weaker scatterers of the three NPS (Section 3.2.1). Figure 3.4 below show the spectra of vial 7 for both N-Me-2-AI and phenibut in both libraries. Although full library results gave no match, spectral correlation between both sub-library and full library confirmed they are almost identical demonstrated by correlation coefficient of 0.94 in N-Me-2-AI and 0.99 in phenibut. In fact, vial 7 spectra obtained from phenibut sample possessed a main peak at 1005 cm^{-1} corresponding to the aromatic moiety in Phenibut, while Figure 3.5 B showed N-Me-2-AI spectral features respectively N-substituted methyl and the aromatic moiety at 777 and 1022 cm^{-1}. This could have indicated that the reading of 0.28 ± 0.08 and 0.28 ± 0.02 obtained respectively for N-Me-

2-AI and phenibut in sub-library could be significantly similar in full library. However, with the presence of analogues, the fifth match corresponding to procaine was detected at an average value of 0.65, significantly higher than values obtained for both NPS, thus the NPS match was not included in the matching list.

Table 3.8: Comparison of matching between full library and sub-library of all 26 vials on wavelet algorithm

	5F-PB-22		N-Me-2-AI		Phenibut	
Vial	FL	SL	FL	SL	FL	SL
1	0.98 ± 0.01[1]	0.98 ± 0.01	0.90 ± 0.01	0.90 ± 0.01	0.88 ± 0.01	0.79 ± 0.02
2	0.10 ± 0.16	0.27 ± 0.04	0.00	0.11 ± 0.01	0.00	0.14 ± 0.02
3	0.00[2]	0.07 ± 0.02	0.00[3]	0.12 ± 0.01	0.00	0.19 ± 0.01
4	0.09 ± 0.15	0.25 ± 0.08	0.00	0.14 ± 0.02	0.00	0.08 ± 0.01
5	0.38 ± 0.25	0.48 ± 0.14	0.20 ± 0.20	0.34 ± 0.03	0.00	0.32 ± 0.04
6	0.87 ± 0.04	0.89 ± 0.02	0.62 ± 0.07	0.54 ± 0.05	0.59 ± 0.03	0.53 ± 0.06
7	0.35 ± 0.35	0.45 ± 0.09	0.00	0.28 ± 0.08	0.00	0.28 ± 0.02
8	0.95 ± 0.01	0.94 ± 0.01	0.75 ± 0.07	0.72 ± 0.07	0.70 ± 0.04	0.66 ± 0.03
9	0.95 ± 0.02	0.95 ± 0.01	0.83 ± 0.02	0.80 ± 0.02	0.76 ± 0.08	0.72± 0.04
10	0.00	0.07 ± 0.01	0.00	0.14 ± 0.01	0.00	0.21 ± 0.01
11	0.11 ± 0.19	0.19 ± 0.05	0.00	0.20 ± 0.05	0.00	0.17 ± 0.05
12	0.00	0.33 ± 0.06	0.00	0.19 ± 0.04	0.00	0.17 ± 0.03
13	0.00	0.09 ± 0.01	0.00	0.14 ± 0.02	0.00	0.20 ± 0.01
14	0.00	0.09 ± 0.01	0.00	0.14 ± 0.02	0.00	0.22 ± 0.02
15	0.20 ± 0.35	0.44 ± 0.16	0.19 ± 0.19	0.34 ± 0.17	0.00	0.31 ± 0.05
16	0.93 ± 0.03	0.95 ± 0.03	0.53 ± 0.31	0.58 ± 0.09	0.38 ± 0.34	0.57 ± 0.07
17	0.19 ± 0.32	0.38 ± 0.09	0.00	0.19 ± 0.03	0.00	0.27 ± 0.05
18	0.00	0.17 ± 0.08	0.00	0.14 ± 0.01	0.00	0.21 ± 0.01
19	0.13 ± 0.22	0.69 ± 0.01	0.00	0.31 ± 0.04	0.00	0.32 ± 0.04
20	0.20 ± 0.34	0.47 ± 0.18	0.00	0.31 ±0.07	0.30 ± 0.27	0.38 ± 0.05
21	0.68 ± 0.11	0.47 ± 0.19	0.00	0.36 ± 0.12	0.00	0.32 ± 0.03
22	0.98 ± 0.01	0.98 ± 0.01	0.89 ± 0.01	0.90 ± 0.01	0.85 ± 0.01	0.80 ± 0.04
23	0.00	0.16 ± 0.02	0.00	0.14 ± 0.03	0.00	0.13 ± 0.01
24	0.00	0.06 ± 0.01	0.00	0.12 ± 0.01	0.00	0.19 ± 0.01
25	0.07 ± 0.13	0.35 ± 0.09	0.00	0.14 ± 0.03	0.00	0.0 7± 0.01
26	0.28 ± 0.30	0.69 ± 0.08	0.21 ± 0.21	0.39 ± 0.06	0.21 ± 0.19	0.46 ± 0.14

[1] The (±) indicated standard deviation values

[2] Matching value of 0.00 indicated no match

[3] Colour coding: GREEN: 4/4 matches, YELLOW: 3/4 matches, ORANGE: 1 or 2 of 4 matches, RED: unidentified

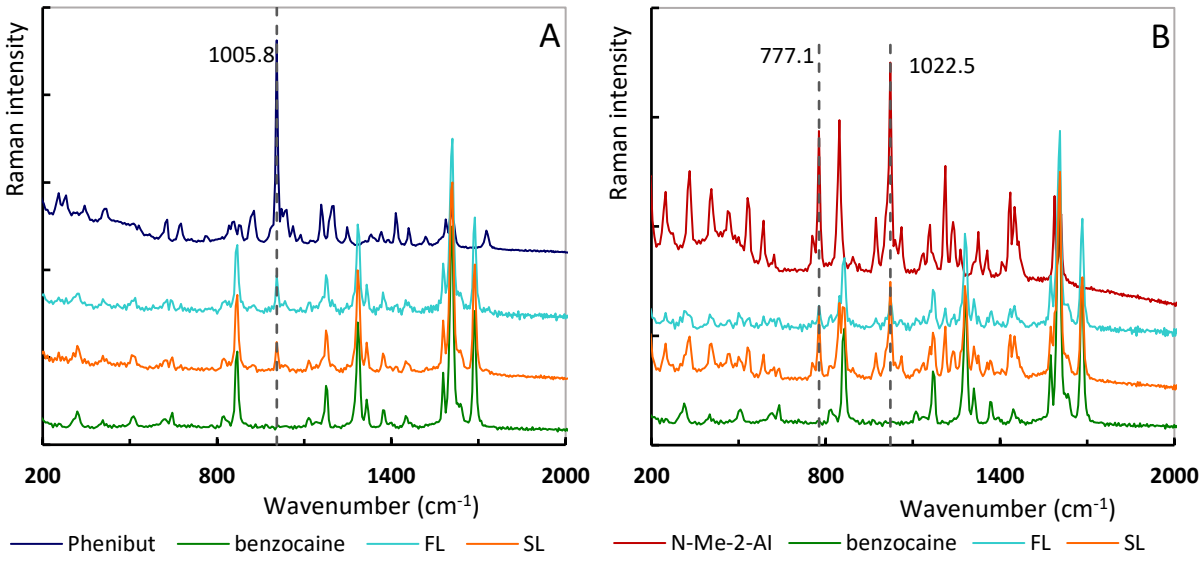


Figure 3.5: Spectral comparison between SL and FL of phenibut (A) and N-Me-2-AI (A) for vial 7 using wavelet.

In the presence of 10 mg of NPS, results displayed more alternations in terms of matching value increase. In the presence of 140 mg of benzocaine results followed the same pattern as described above. In fact, full library results showed no detection of NPS due to analogues while sub-library displayed low matching values of 0.07 ± 0.02, 0.12 ± 0.04, and 0.19 ± 0.03 respectively for 5F-PB-22, N-Me-2-AI, and phenibut respectively. These values were significantly similar to that of the duplicate vial 24: 0.06 ± 0.01, 0.12 ± 0.01, 0.19 ± 0.01. In the presence of caffeine which was confirmed to be a strong scatterer in Section 3.2.1, only 5F-PB-22 showed detection in one out of two vials at 0.10 ± 0.16 in full library. However, though sub-library matching observed, the values obtained appeared to be further apart from each other noticeably in vial 2 with a value of 0.27 ± 0.04 although vials of same composition. However, caffeine vials in N-Me-2-AI and phenibut samples displayed detection with low values of 0.12 ± 0.04 and 0.19 ± 0.03 respectively while full library showed no match. Interestingly, vial 5 (NPS: 10 mg, Na GLU: 140 mg) always displayed detection across all NPS in full library at the exception of phenibut. However, this value was consistently lower than that obtained in sub-library. In fact, 5F-PB-22 and N-Me-2-AI presented matching value increase from 0.38 ± 0.25 and 0.20 ± 0.20 in full library to 0.48 ± 0.14 and 0.34 ± 0.03 while phenibut showed no match in full library analysis but was detected at 0.32 ± 0.04 in sub-library. However, matching values recorded in the duplicate vial 26 specially 5F-PB-22 (0.69 ± 0.08) and phenibut (0.46 ± 0.14) were greater although vials of same composition. This could be due to the reduction of reference standards within the library. Since the sub-library was limited to five standards, it was possible that the instrument would try to strongly match the vial spectra to the library content, potentially increasing the matching value. In fact, vial 2 (NPS: 10 mg, CAF: 140 mg) matching data showed

detection of benzocaine (matching above 20%), creatine and sodium glutamate although absent in the vial composition, as highlighted in the Table 3.9.

Table 3.9: Matching data of vial 2 on wavelet algorithm.

Matching results					
5F-PB-22		**N-Me-2-AI**		**Phenibut**	
Run 1	**Valid Hit**	**Run 1**	**Valid Hit**	**Run 1**	**Valid Hit**
CAFFEINE	0.9	CAFFEINE	0.99	CAFFEINE	0.94
5F-PB-22	0.31	N-Me-2-AI	0.3	BENZOCAINE	0.25
BENZOCAINE	0.24	BENZOCAINE	0.23	PHENIBUT	0.14
CREATINE	0.03	CREATINE	0.16	CREATINE	0.03
L-GLUTAMIC ACID	0.03	L-GLUTAMIC ACID	0.1		
Run 2	**Valid Hit**	**Run 2**	**Valid Hit**	**Run 2**	**Valid Hit**
CAFFEINE	0.95	CAFFEINE	0.97	CAFFEINE	0.95
5F-PB-22	0.26	N-Me-2-AI	0.39	BENZOCAINE	0.25
BENZOCAINE	0.07	BENZOCAINE	0.23	PHENIBUT	0.1
CREATINE	0.03	CREATINE	0.21	CREATINE	0.03
		L-GLUTAMIC ACID	0.17		
Run 3	**Valid Hit**	**Run 3**	**Valid Hit**	**Run 3**	**Valid Hit**
CAFFEINE	0.92	CAFFEINE	0.97	CAFFEINE	0.93
BENZOCAINE	0.26	N-Me-2-AI	0.38	BENZOCAINE	0.26
5F-PB-22	0.2	BENZOCAINE	0.23	PHENIBUT	0.17
CREATINE	0.04	CREATINE	0.2	CREATINE	0.03
L-GLUTAMIC ACID	0.01	L-GLUTAMIC ACID	0.15	L-GLUTAMIC ACID	0.01
Run 4	**Valid Hit**	**Run 4**	**Valid Hit**	**Run 4**	**Valid Hit**
CAFFEINE	0.9	CAFFEINE	0.97	CAFFEINE	0.94
5F-PB-22	0.29	N-Me-2-AI	0.39	BENZOCAINE	0.25
BENZOCAINE	0.24	BENZOCAINE	0.23	PHENIBUT	0.14
CREATINE	0.03	CREATINE	0.19	CREATINE	0.04
L-GLUTAMIC ACID	0.02	L-GLUTAMIC ACID	0.16		

[1] Colour coding: RED: cutting agents/adulterants absent from the vial composition.

In the case of complex mixtures notably quinary in which low detection for the NPS (4/5 for 5F-PB-22, 1/5 for phenibut and none for N-Me-2-AI) was recorded using wavelet algorithm on full library, a matching value was consistently recorded in sub-library analysis. Table 3.10 shows the matching results obtained in quinary mixtures. In presence of 84 mg of benzocaine, 5F-PB-22, N-Me-2-AI, and phenibut was undetected in full library while a match respectively at 0.17 ±

0.08, 0.14 ± 0.01, and 0.21 ± 0.01 in sub-library was found. In the case of N-Me-2-AI, no matching value was obtained in full library. Previous assumptions made in section highlighted N-Me-2-AI to be a better scatterer than phenibut. However, vial 20 (NPS: 14 mg, BEN: 14mg, CAF: 14 mg, CREA: 14 mg, Na GLU: 84mg) showed detection for phenibut and no detection for N-Me-2-AI full library. Sub-library records displayed a matching value of 0.31 ± 0.07 and 0.38 ± 0.05 respectively for N-Me-2-AI and phenibut. As similarly explained previously in Figure 3.3 for vial 19 (NPS: 14 mg, BEN: 14mg, CAF: 14 mg, CREA: 84 mg, Na GLU: 14mg), vial 20 spectra showed characteristics of the NPS in full library and sub-library spectra for 5F-PB-22 and phenibut. Interestingly, vial 20 full library spectra displayed no N-Me-2-AI characteristics compared to sub-library with a moderate peak at 1022 cm^{-1} (aromatic) as shown in Figure 3.5. This could mean in this case that although analogues capable of influencing detection of NPS, failure in identification could also be justified by the spot of analysis.

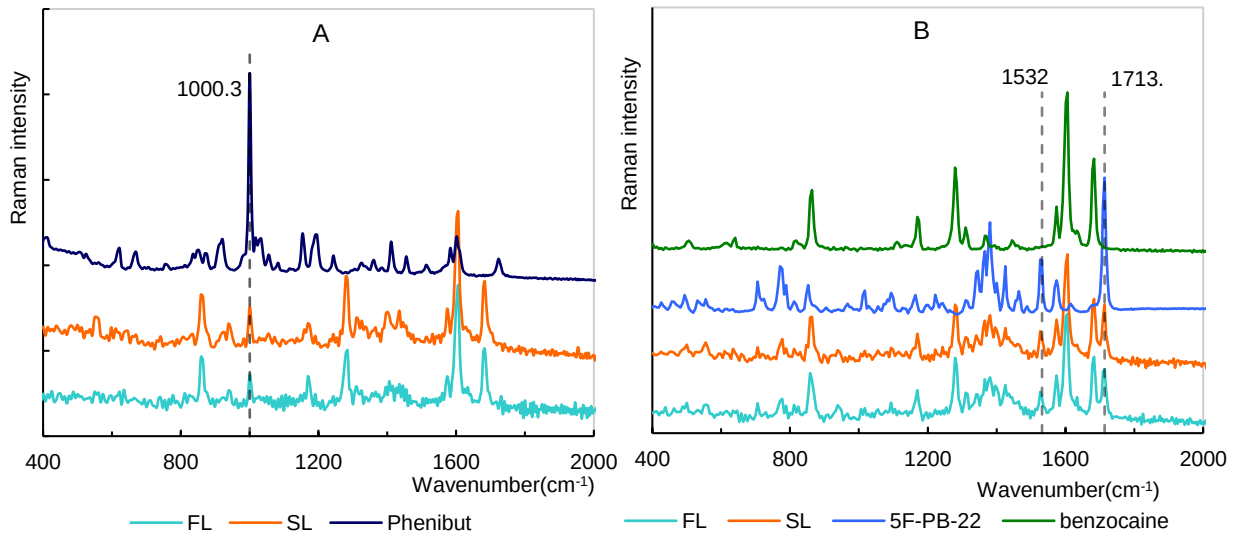
Table 3.10: Full and sub-library comparison of quinary mixtures using wavelet algorithm

Vial	5F-PB-22		N-Me-2-AI		Phenibut	
	FL	SL	FL	SL	FL	SL
17	0.19 ± 0.32[1]	0.38 ± 0.09	0.00[3]	0.19 ± 0.03	0.00	0.27 ± 0.05
18	0.00[2]	0.17 ± 0.08	0.00	0.14 ± 0.01	0.00	0.21 ± 0.01
19	0.13 ± 0.22	0.69 ± 0.01	0.00	0.31 ± 0.04	0.00	0.32 ± 0.04
20	0.20 ± 0.34	0.47 ± 0.18	0.00	0.31 ±0.07	0.30 ± 0.27	0.38 ± 0.05
21	0.68 ± 0.11	0.47 ± 0.19	0.00	0.36 ± 0.12	0.00	0.32 ± 0.03

[1] The (±) indicated standard deviation values

[2] Matching value of 0.00 indicated no match

[3] Colour coding: GREEN: 4/4 matches, YELLOW: 3/4 matches, ORANGE: 1 or 2 of 4 matches, RED: unidentified



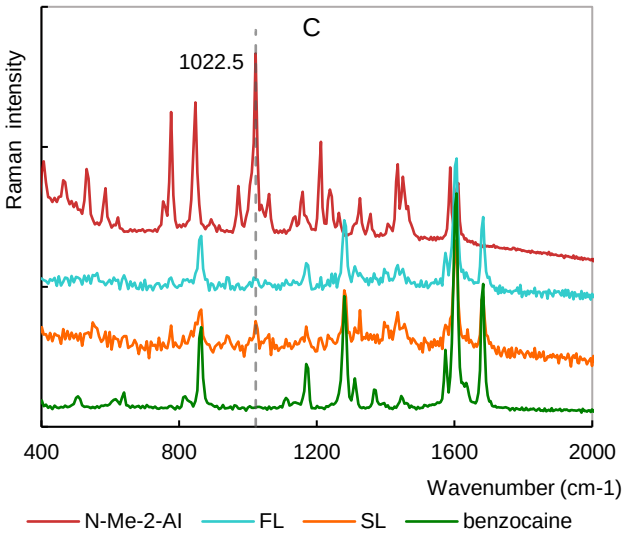


Figure 3.6: Spectral comparison between full and sub-library of vial 20 using wavelet algorithm for phenibut (A), 5F-PB-22 (B), N-Me-2-AI (C).

In conclusion, the use of sub-library confirmed that the presence of analogues within search libraries posed major problems in the identification of NPS. In fact, even with benzocaine present at 14 mg, full library showed abundance of analogues in dimethocaine and procaine in the matching list. In addition, although the close similarity in peak signals from the sample spectra used with full library and sub-library, the latter displayed greater or equal matching values compared to full library findings. This was due to the abundance of analogues in the displayed matching lists.

In presence of unknown samples, search library needs to be as large as possible to cover all eventual possibilities. Hence, it would difficult to attribute a match as a certain component of a sample. Although the potential advantages of using sub-libraries in monitoring NPS, this aspect would be particularly difficult to implement in the case of unknown samples. In fact, mixtures of unknown composition would pose the challenge of assigning a match to a potential component considering the possibility of analogues also being recorded. The use of chemometrics in the design of a model could be beneficial in elucidating the issue behind corresponding a matching result to a certain vial composition. A model can be used to determine the response to a mixture depending on the proportions of the components present rather than on the total amount of mixture. Although this study aimed at its application in real life situations where the composition of samples is unknown, its use in presence of know samples was investigated.

4. MODEL ACQUISITION, EVALUATION AND VALIDATION

Underpinned by the complexity of street samples, further work was conducted in the aim of understanding the variation in matching response in relation to the sample. In this regard, analysis of these samples could be extremely challenging in the case of unknown content as well as the concentration of the various components. Hence, the use of a model could be beneficial in this aspect as it could help predict a matching value for a certain sample composition and shed some light on the matching capabilities of the algorithm of use. Results obtained from the initial study for both full library and sub-library were subjected to in-depth chemometrics analysis re-inserting those results in the design of experiments generating a model for each NPS. For consistency, same spot results were used for both libraries and the analysis was conducted using wavelet algorithm due its single matching profile.

4.1. Quality control data and model application

As discussed throughout Section 3.1, 48.6% of samples displayed good Raman responses notably in those of high NPS concentration (≥80 mg). However, full library results showed some poor reproducibility in complex mixtures noted by standard deviations of up to 0.35 as in 5F-PB-22's vial 7(NPS: 80 mg, BEN: 70 mg). This was caused by the impact of strong Raman light scatterer namely adulterants such as benzocaine and caffeine but most importantly by the influence of analogues and structurally similar compounds of these excipients sharing similar spectral profile and hence recorded although absent within the sample. In addition, these poor precision values also qualified the heterogeneity of samples of low NPS amount as noted in N-Me-2-AI's vial 5 (NPS: 10 mg, Na GLU: 140 mg) with a precision value of 0.20. However, matching values were recorded as averages of four replicates, emphasising on the potential influence of these responses (particularly in the case of one, two, or three matches out of four replicates) on the accuracy of a model. In fact, an average value of 0.10 ± 0.19 recorded in full library analysis of 5F-PB-22's vial 2 (NPS: 10 mg, CAF: 140 mg) qualified the identification of the NPS in only one (0.38) of four replicates. Whereas an average value would imply that analysis of the said vial 2 in similar conditions at any given moment would generate a significantly similar matching value which was not the case. Indeed, the lack of reproducibility proved to be the main problem behind assigning a matching response to a particular vial composition.

For samples with poor reproducibility, a quality control method was applied solely to full library since sub-library results displayed four consistent matches for all samples. This consisted of omitting matching values where the NPS was identified once or twice out of four replicates, thus

making them 0.00. Of the 26 vials, ten samples for 5F-PB-22, three for N-Me-2-AI, and phenibut were converted. Also matching values consistent in three out of four replicates were recorded as the average of three. This aimed at ensuring that the vial's response obtained was representative of the consistent match for the NPS. Table 4.1 presents the quality controlled (QC) and non-quality controlled data (NQC) used in the model.

Table 4.1: Quality controlled and non-quality controlled data from full library analysis using wavelet.

Vial	5F-PB-22		N-Me-2-AI		Phenibut	
	NQC	QC	NQC	QC	NQC	QC
1	0.98 ± 0.01[1]	0.98 ± 0.01	0.90 ± 0.01	0.90 ± 0.01	0.88 ± 0.01	0.88 ± 0.01
2	0.10 ± 0.19	0.00[2]	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00
4	0.09 ± 0.18[3]	0.00	0.00	0.00	0.00	0.00
5	0.38 ± 0.29	0.50 ± 0.17	0.20 ± 0.24	0.00	0.00	0.00
6	0.87 ± 0.04	0.87 ± 0.04	0.62 ± 0.08	0.62 ± 0.08	0.59 ±0.03	0.59 ± 0.03
7	0.35 ± 0.40	0.00	0.00	0.00	0.00	0.00
8	0.95 ± 0.01	0.95 ± 0.01	0.75 ± 0.08	0.75 ± 0.08	0.70 ± 0.06	0.70 ± 0.06
9	0.95 ± 0.02	0.95 ± 0.02	0.83 ± 0.02	0.83 ± 0.02	0.76 ± 0.10	0.76 ± 0.10
10	0.00	0.00	0.00	0.00	0.00	0.00
11	0.11 ± 0.22	0.00	0.00	0.00	0.00	0.00
12	0.00	0.00	0.00	0.00	0.00	0.00
13	0.00	0.00	0.00	0.00	0.00	0.00
14	0.00	0.00	0.00	0.00	0.00	0.00
15	0.20 ± 0.40	0.00	0.19 ± 0.21	0.00	0.00	0.00
16	0.93 ± 0.03	0.93 ± 0.03	0.53 ± 0.36	0.71 ± 0.03	0.38 ± 0.44	0.00
17	0.19 ± 0.38	0.00	0.00	0.00	0.00	0.00
18	0.00	0.00	0.00	0.00	0.00	0.00
19	0.13 ± 0.26	0.00	0.00	0.00	0.00	0.00
20	0.20 ± 0.39	0.00	0.00	0.00	0.30 ± 0.34	0.00
21	0.68 ± 0.13	0.68 ± 0.13	0.00	0.00	0.00	0.00
22	0.98 ± 0.01	0.98 ± 0.01	0.89 ± 0.01	0.89 ± 0.01	0.85 ± 0.01	0.85 ± 0.01
23	0.00	0.00	0.00	0.00	0.00	0.00
24	0.00	0.00	0.00	0.00	0.00	0.00
25	0.07 ± 0.15	0.00	0.00	0.00	0.00	0.00
26	0.28 ± 0.35	0.00	0.21 ± 0.25	0.00	0.21 ± 0.24	0.00

[1] The (±) indicated the standard deviation values

[2] Matching value of 0.00 indicated no match

[3] Colour coding: GREEN: 4/4 matches, YELLOW: 3/4 matches, ORANGE: 1 or 2 of 4 matches, RED: unidentified

Both data sets (i.e., QC and NQC) were evaluated in the model generated after insertion of the data sets into the design of experiment. The accuracy of prediction of the model was evaluated using statistical tests completed by the Design expert software. According to the Design Expert guidelines, a good model followed a sequential p-value below 0.05 ($p \leq 0.05$) describing the probability of the model to significantly explain the variation in NPS response. The p-value had to be greater than 0.10 synonym of an insignificant lack of fit and the difference between adjusted and predicted r^2 values within 0.2 which qualified how well the data is fitted to the

regression line. After development of the models, statistical analysis showed that the model originating from the NQC data followed all three criteria as shown in Table 4.2. In addition, a linear arrangement in space displayed better sequential p-values (3×10^{-07}, 2.27×10^{-07}, and 6.36×10^{-07} respectively for 5F-PB-22, N-Me-2-AI, and phenibut) and lack of fit p-values (0.01, 7.41×10^{-08}, and 0.08 respectively for 5F-PB-22, N-Me-2-AI, and phenibut) compared to the quadratic order. Although displaying lower r^2 values of 0.79 for phenibut and 0.81 for 5F-PB-22 and phenibut, the adjusted and predicted r^2 difference indicated the linear NQC model to be a good choice. Hence, NQC data was carried forward and the appropriate models used for the conception of test vials. It would be assumed that the QC data would generate a better model. However, this would indicate that a matching of 0.00 in vial 7 (NPS: 80 mg, BEN: 70 mg) would represent the consistent matching response although the NPS present at 80 mg. In fact, NQC showed detection of the NPS at 0.35 ± 0.35 although the precision value highlighted the poor reproducibility. Hence, QC was not representative of the true matching value while NQC accounted for the difficulty in achieving sample homogeneity.

Table 4.2: Statistical test obtained from QC and NQC data of initial study.

		5F-PB-22		N-Me-2-AI		Phenibut	
		Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
QC	Sequential p-value	3×10^{-07}	0.01	2.27×10^{-07}	0.02	6.36×10^{-07}	0.08
	Lack of fit p-value	0.01	0.03	7.41×10^{-08}	3.85×10^{-07}	0.08	0.17
	Adjusted r^2	0.77	0.91	0.78	0.91	0.75	0.85
	Predicted r^2	0.72	0.38	0.74	-0.85	0.70	0.08
	r^2	0.81	0.96	0.81	0.96	0.79	0.94
NQC	Sequential p-value	5.47×10^{-06}	0.10	9.93×10^{-07}	0.02	9.84×10^{-07}	0.16
	Lack of fit p-value	0.06	0.11	9.66×10^{-07}	4.63×10^{-07}	0.28	0.43
	Adjusted r^2	0.69	0.81	0.74	0.89	0.74	0.82
	Predicted r^2	0.63	0.05	0.70	-0.24	0.68	0.02
	r^2	0.74	0.92	0.78	0.95	0.78	0.92

4.2. 5F-PB-22

4.2.1. Full library outcomes

This study focussed on evaluating the use of a model in predicting the matching values of samples of different concentration. Findings obtained from full library as presented in Section 3.1 were introduced into the design of experiment and optimized as described in Section 2. The model produced a set of test vials which where samples of various concentrations of NPS and adulterants/cutting with their predicted matching responses to be confirmed by analysis using wavelet algorithm. The 5F-PB-22 full library model (Eq.1) generated the most test vials with concentrations of NPS of up to 40 mg. Table 4.3 below summarises the matching value results from full library analysis of 5F-PB-22 test vials as well as the actual vial content and the expected match predicted by the model. This also provides a clear comparison on whether the measurement agrees with the estimated value. In fact, if both predicted and observed value overlap within the uncertainty, both values are said to be in agreement. Vials were made to ensure a percentage error of within 10% with an expected total mass of 150 mg as will all previous vials for consistency. Full expected and obtained mass distribution data is displayed in the appendix (Table 7)

(Eq.1): $\boldsymbol{P} = 6.65 \times 10^{-3} \times A + 3.33 \times 10^{-4} \times B - 1.33 \times 10^{-3} \times C + 6.38 \times 10^{-4} \times D + 9.23 \times 10^{-4} \times E - 7.5 \times 10^{-5} \times A \times B + 7.76 \times 10^{-5} \times A \times C + 6.62 \times 10^{-5} \times A \times D + 5.99 \times 10^{-5} \times A \times E - 1.2 \times 10^{-5} \times B \times C - 2.4 \times 10^{-5} \times B \times D - 3.9 \times 10^{-4} \times B \times E - 2.9 \times 10^{-5} \times C \times D + 1.24 \times 10^{-5} \times C \times E - 5.6 \times 10^{-5} \times D \times E$

Where P is the prediction.
Amount in mg of A: 5F-PB-22, B: benzocaine, C: caffeine, D: creatine, D: sodium glutamate.

Table 4.3: Full library matching results of 5F-PB-22 test vials on wavelet algorithm

Vial	Content (mg)						Matching value of 5F-PB-22	
	5F-PB-22	BEN	CAF	CREA	Na GLU	TOTAL	Average	Expected match
1	34.95	22.01	40.01	25.8	23.15	145.92	**0.31 ± 0.36**[1]	0.3
2	10.53	16.66	15.84	99.41	7.11	149.55	0.00[2]	0.06
3	10.18	136.14	2.91	3.33	0.71	153.27	0.00	0.002
4	23.38	72.87	15.04	37.62	0	148.91	0.00	0.04
5	40.49	66.87	37.71	1.41	0	146.48	**0.14 ± 0.29**	0.12
6	28.52	86.95	9.26	22.76	0	147.49	0.00	0.03

[1] The (±) indicated standard deviation values, GREEN: measurement agree with predicted match

[2] Matching value of 0.00 indicated no match

The application of a model aimed at evaluating its use in predicting NPS responses in complex mixtures. Initial observations showed that four of the six test samples displayed matching values relatively low (0.002-0.06). In fact, this range in matching was not reached in initial full library

analysis notably due to the presence of analogues sharing similar peaks and Raman cross section. This is the case of procaine detected as the last match correlating to up to 50% to benzocaine. In fact, matching data from vial 2 which possessed the lowest amount of benzocaine (16.66 mg) displayed benzocaine analogues with the last match in procaine detected at an average 0.65. However, although the test vials uncorrelated, the model equation generated from the software could be also be used to predict a NPS matching value from the random composition. On the other hand, vial 1 and 5 showed predicted values of 0.3 and 0.12 which were of potential reach for NPS in complex mixtures as demonstrated in Section 3.1. Coincidently, the average matching value recorded appeared to be narrowly close to the predicted value in both cases. However, the standard deviations of 0.36 and 0.29 were higher than the matching values obtained. These uncertainty values were indicative of poor reproducibility across all 4 replicates. Although agreement between both values, it confirmed that the experimental error was higher than the theoretical error. In fact, vial 1 and 5 were identified respectively in two (0.65 and 0.60) and one (0.57) out of four replicates. This indicated that the average value correlating to the expected match was not consistent across all replicates with the NPS being unidentified. However, the model resulting from initial full library analysis accounted for reproducibility issues which justifies the NQC data.

4.2.2. Sub-library outcomes

Due to the presence of analogues in full library precluding NPS match values from being presented in the matching list, full library test samples were analysed against sub-library employing wavelet algorithm in order to evaluate the accuracy of the model. Table 4.4 presents the predicted matching value obtained from the equation of the sub-library model (Eq.2) as well as the actual matching value. Vial 1, 2, 5, and 6 showed the model was successful in estimating the average match with respective predictions of 0.43, 0.32, 0.30, and 0.21 in agreement with actual measurements. On the other hand, vial 3 and 4 values appeared to disagree with with each other as they fall out of range qualified by the respective model prediction values of -0.01 and 0.22. However, it is key to note that the later was still very close to the matching range of 0.25 ± 0.02. On the contrary, vial 3 detection was estimated at -0.01. In addition to not correlating to predicted, the negative value could be interpreted as a no match since the value recorded from the Raman instrument do not fall below zero. Additionally, considering the model used originates from sub-library data, the presence of 136.1 mg of benzocaine within vial 3 is mainly due to the negative value.

(Eq.2) $\boldsymbol{P} = 7.56 \times 10^{-3} \times \mathrm{A} - 7.17 \times 10^{-4} \times \mathrm{B} + 1.14 \times 10^{-3} \times \mathrm{C} + 2.15 \times 10^{-3} \times \mathrm{D} + 3.43 \times 10^{-3} \times \mathrm{E}$

Where P is the prediction.

Amount in mg of A: 5F-PB-22, B: benzocaine, C: caffeine, D: creatine, D: sodium glutamate.

Table 4.4: 5F-PB-22 model validation of test vials using sub-library on wavelet.

Vial	Content (mg)						Matching results			
	NPS	BEN	CAF	CREA	Na GLU	TOTAL	Average	MIN	MAX	Model prediction
1	34.95	22.01	40.01	25.8	23.15	145.92	0.52 ± 0.11[1]	0.41	0.62	0.43
2	10.53	16.66	15.84	99.41	7.11	149.55	0.26 ± 0.06	0.20	0.32	0.32[2]
3	10.18	136.1	2.91	3.33	0.71	153.27	0.07 ± 0.03	0.04	0.10	-0.01[2]
4	23.38	72.87	15.04	37.62	0	148.91	0.25 ± 0.02	0.23	0.27	0.22
5	40.49	66.87	37.71	1.41	0	146.48	0.27 ± 0.08	0.19	0.35	0.3
6	28.52	86.95	9.26	22.76	0	147.49	0.17 ± 0.04	0.13	0.21	0.21

[1] The (±) indicated the standard deviation values

[2] Colour coding: GREEN: In agreement with predicted value, RED: In disagreement with predicted value

Sub-library model qualified by the equation above, generated only one test vial of composition: 5F-PB-22: 10.64 mg, BEN: 29.57 mg, CAF: 35.04 mg, CREA: 42.78 mg, Na GLU: 33.36 mg. Considering 5F-PB-22 was a strong scatterer, sub-library analysis of this NPS displayed good results across all study vials which could be the results of only one test vial. The test match was expected at 0.30 although a relatively low NPS amount. However, readings showed detection of NPS at 0.14 ± 0.03 which is significantly lower than the prediction. The vial complexity was evident due to the spread in cutting agents and adulterants concentration. The test of only one vial was insufficient to evaluate the performance of the model in sub-library. However, analysis of full library vials on sub-library showed that the model was successful in approximately predicting matching of NPS.

4.3. N-Me-2-AI

4.3.1. Full library outcomes

In contrast to 5F-PB-22, N-Me-2-AI matching results (Table 4.5) showed no detection of NPS across all test samples even in high concentrations as observed in vial 1. In fact, this sample had an expected matching value of 0.16 which was the highest expected matching value. Yet, results were dominated by benzocaine analogues with the last match shared between procaine

and synthesised mephedrone with an average matching value of 0.62. In addition, caffeine which was proven to be a strong adulterant was also not identified even in vial 3 where its composition exceeded 60 mg. Similar findings were observed in vial 2, 3, and 4 with the predominance of analogues in matching results. Additionally, with an expected match of 0.05-0.06, similar results to 5F-PB-22 (sample 2, 3, 4, and 6) were observed. No NPS was identified due to analogues as procaine matching to up to 50% of benzocaine heightened by the results limited to five matches. These findings reiterate the continuous use of adulterants and cutting agents. Beyond their Raman properties, they happen to correlate with other compounds in their Raman cross section that play a major role in bypassing monitoring. In addition, the variation in spectral capabilities between NPS can also pose a problem in detection suggesting that weaker scatterer as N-Me-2-AI would present less chance of identification. This model was qualified by the equation (Eq.3) below.

(Eq.3): $\boldsymbol{P} = 6.15 \times 10^{-3} \times A - 1.34 \times 10^{-3} \times B - 6 \times 10^{-4} \times C - 2.3 \times 10^{-4} \times D + 8.43 \times 10^{-4} \times E$

Where P is the prediction,

Amount in mg of, A: N-Me-2-AI, B: benzocaine, C: caffeine, D: creatine, D: sodium glutamate

Table 4.5: Full library matching results of N-Me-2-AI test vials on wavelet algorithm.

Vial	Content (mg)						Matching results of N-Me-2-AI	
	N-Me-2-AI	BEN	CAF	CREA	Na GLU	TOTAL	Average	Expected match
1	33.28	27.67	30.82	32.51	25.72	150	0.00[1]	0.16
2	10.15	20.1	27.44	34.63	56.81	149.13	0.00	0.06
3	27.94	52.58	61.61	8	0	150.13	0.00	0.06
4	22.19	52.6	4.3	68.81	0	147.9	0.00	0.05

[1] Matching value of 0.00 indicated no match

4.3.2. Sub-library outcomes

Identically to Section 4.1.1, full library test vials were also analysed against sub-library and compared against model predictions obtained from sub-library model equation (Eq.4) as shown in Table 4.6. Initial observations showed that in comparison to full library model, the model predictions were much higher (0.19-0.31). Vial 1 and 3 displayed detection values of respectively 0.31 ± 0.03 and 0.17 ± 0.02 which were very similar to the predicted match of 0.31 and 0.19. These two samples coincidently were made of the two highest amounts of NPS in 33.28 mg

(Vial 1) and 27.94 mg (Vial 3). On the other hand, vial 2 and 4 displayed values (0.18 ± 0.02 and 0.17 ± 0.01) which did not match with the predicted values of 0.22 and 0.20. Vial 2 displayed high sample complexity with an even weight distribution although benzocaine concentration at a lowest 20.1 mg. However, a precision value of 0.02 assured good sample reproducibility and homogeneity. As discussed previously, the main issue of detection of NPS was related to library standards sharing similar Raman readings with the sample spectra. Indeed, absent sodium glutamate was also recorded at 0.13, higher than creatine detected at 0.06 which was present at 68.81 mg (Table 4.7). Additionally, vial 4 contained 4.3 mg of caffeine, yet matching list showed detection of caffeine at an average match of 0.22. The spectra of benzocaine and caffeine (Section 3.1) both present signals at around 1279 and 1512 cm^{-1} of variating intensities. But once again, predicted matching values were very close as noticed in the 5F-PB-22 model validation.

(Eq.4) $$\boldsymbol{P} = 5.97 \times 10^{-3} \times \mathrm{A} + 9.42 \times 10^{-4} \times \mathrm{B} + 4.4 \times 10^{-4} \times \mathrm{C} + 2.48 \times 10^{-4} \times \mathrm{D} + 1.86 \times 10^{-3} \times \mathrm{E} - 5.01 \times 10^{-5} \times \mathrm{A} \times \mathrm{B} + 2.45 \times 10^{-6} \times \mathrm{A} \times \mathrm{C} + 3.72 \times 10^{-5} \times \mathrm{A} \times \mathrm{D} + 3.07 \times 10^{-5} \times \mathrm{A} \times \mathrm{E} + 7.98 \times 10^{-7} \times \mathrm{B} \times \mathrm{C} - 7.06 \times 10^{-7} \times \mathrm{B} \times \mathrm{D} - 2.51 \times 10^{-5} \times \mathrm{B} \times \mathrm{E} + 1.05 \times 10^{-5} \times \mathrm{C} \times \mathrm{D} - 1.44 \times 10^{-5} \times \mathrm{C} \times \mathrm{E} + 1.53 \times 10^{-5} \times \mathrm{D} \times \mathrm{E}$$

Where P is the prediction,

Amount in mg of, A: N-Me-2-AI, B: benzocaine, C: caffeine, D: creatine, D: sodium glutamate

Table 4.7: N-Me-2-AI model validation of full library test vials on wavelet.

Vial	Content (mg)						Matching results			
	NPS	BEN	CAF	CREA	Na GLU	TOTAL	Average	MIN	MAX	Model Prediction
1	33.28	27.67	30.82	32.51	25.72	150	0.31 ± 0.03[1]	0.28	0.34	0.31[2]
2	10.15	20.1	27.44	34.63	56.81	149.13	0.18 ± 0.03	0.16	0.21	0.22[2]
3	27.94	52.58	61.61	8	0	150.13	0.17 ± 0.03	0.15	0.20	0.19
4	22.19	52.6	4.3	68.81	0	147.9	0.17 ± 0.01	0.16	0.18	0.2

[1] the (±) indicated the standard deviation values

[2] Colour coding: GREEN: In range, RED: Out of range

Table 4.8: Matching data from N-Me-2-AI full library analysis of vial 4 on sub-library using wavelet algorithm.

Wavelet	
Run 1	**Valid Hit**
BENZOCAINE	0.95
CAFFEINE[1]	0.22
N-Me-2-AI	0.18
L-GLUTAMIC ACID	0.13
CREATINE	0.08
Run 2	**Valid Hit**
BENZOCAINE	0.96
CAFFEINE	0.21
N-Me-2-AI	0.15
L-GLUTAMIC ACID	0.13
CREATINE	0.02
Run 3	**Valid Hit**
BENZOCAINE	0.96
CAFFEINE	0.24
N-Me-2-AI	0.17
L-GLUTAMIC ACID	0.12
CREATINE	0.05
Run 4	**Valid Hit**
BENZOCAINE	0.95
CAFFEINE	0.22
N-Me-2-AI	0.18
L-GLUTAMIC ACID	0.13
CREATINE	0.1

[1] RED: detection of caffeine (absent from the vial)

Table 4.9 present the matching value of N-Me-2-AI sub-library test vials. Although a more evenly spread of the sample components, the expected value appeared to be significantly within the actual matching range. In fact, vial 1 and vial 3 were detected at 0.31 ± 0.06, 0.21 ± 0.05, and 0.15 ± 0.02 comparable to expected values of respectively 0.28, 0.20 and 0.13. Coupled with previous results obtained in 5F-PB-22 and full library N-Me-2-AI, the use of a model appeared to be successful in the prediction of matching response.

Table 4.9: Sub-library matching results of N-Me-2-AI test vials on wavelet algorithm

Vial	Content (mg)						Matching results	
	N-Me-2-AI	BEN	CAF	CREA	Na GLU	TOTAL	Average	Expected match
1	24.42	24.02	30.56	42.22	27.1	148.32	0.31 ± 0.06[1]	0.28
2	10.68	22.91	30.18	49.07	36.87	149.71	0.21 ± 0.05	0.2
3	14.96	111	15.43	6.67	0	148.03	0.15 ± 0.02	0.13

[1] The (±) indicated the standard deviation values, Colour coding: GREEN: In range

4.4. Phenibut

4.4.1. Full library outcomes

Results obtained from initial studies determined that phenibut was the weakest scatterer of the three NPS of use. The model corresponding from the full library followed the equation (Eq.5). As observed in N-Me-2-AI, no detection of phenibut was observed in all test vials analysed. Vial 1 contained the highest amount of NPS at 35.19 mg while benzocaine as the strongest adulterants was marginally lower. However, there was yet no identification although the expected match of 0.16 (Table 4.10). On the contrary of vial 2 and 3 predictions, they were predicted at the lowest 0.06 for both at which matching results hardly reach due to similar Raman cross section between the predominant excipients and analogues. In this case, the abundance of structurally similar substances to benzocaine such as dimethocaine and procaine was noticed across the matching data. Indeed, due to the full library influence on identification, the sub-library model characterised by the equation (Eq.3) was once more used in order to validate the model.

(Eq.5): $\boldsymbol{P} = 5.77 \times 10^{-3} \times A - 1.19 \times 10^{-3} \times B - 6.2 \times 10^{-4} \times C - 4.5 \times 10^{-4} \times D + 9.78 \times 10^{-4} \times E$

Where P is the prediction,

Amount in mg of, A: phenibut, B: benzocaine, C: caffeine, D: creatine, D: sodium glutamate

Table 4.10: Full library matching results of phenibut test vials on wavelet algorithm.

Vial	Content (mg)						Matching results of phenibut	
	Phenibut	BEN	CAF	CREA	Na GLU	TOTAL	Average	Expected match
1	35.19	27.49	30.78	30.67	27.61	151.74	0.00[1]	0.16
2	10.5	25.88	27.91	28.79	61.52	154.6	0.00	0.06
3	28.28	51.42	58.17	7.79	0	145.66	0.00	0.06

[1] Matching value of 0.00 indicated no match

4.4.2. Sub-library outcomes

Previous model validation conducted in Sections 4.2.2 and 4.3.2 displayed promising results regarding the application of a model in predicting NPS response to handheld Raman analysis. Although questionable outcomes in full library analysis, sub-library findings shown in Table 4.11, have been successful in addressing the flaws observed in full library. That pattern was replicate with phenibut's model validation where all test vials matched to the predicted values. As well as

being a different NPS, these results confirm that the use of chemometrics in NPS identification could be beneficial. The use of a sub-library model appeared to solve the issues posed by analogues in identification. However, its use in presence of unknown mixtures will face the challenge posed by the use of big libraries.

(Eq.3) $$\boldsymbol{P} = 5.30 \times 10^{-3} \times \mathrm{A} + 1.45\mathrm{E} \times 10^{-3} \times \mathrm{B} + 4.89 \times 10^{-4} \times \mathrm{C} - 2.43 \times 10^{-4} \times \mathrm{D} + 2.17 \times 10^{-3} \times \mathrm{E} - 4.59 \times 10^{-5} \times \mathrm{A} \times \mathrm{B} + 1.08 \times 10^{-5}\mathrm{A} \times \mathrm{C} + 4.34 \times 10^{-5} \times \mathrm{A} \times \mathrm{D} + 2.45 \times 10^{-5} \times \mathrm{A} \times \mathrm{E} + 7.87 \times 10^{-6} \times \mathrm{B} \times \mathrm{C} + 1.24 \times 10^{-5} \times \mathrm{B} \times \mathrm{D} - 1.62 \times 10^{-5} \times \mathrm{B} \times \mathrm{E} + 1.30 \times 10^{-5} \times \mathrm{C} \times \mathrm{D} - 1.86 \times 10^{-5} \times \mathrm{C} \times \mathrm{E} + 1.60 \times 10^{-5} \times \mathrm{D} \times \mathrm{E}$$

Where P is the prediction,

Amount in mg of, A: phenibut, B: benzocaine, C: caffeine, D: creatine, D: sodium glutamate

Table 4.11: Phenibut model validation of full library test vials on wavelet.

Vial	Content (mg)						Matching results			
	Phenibut	BEN	CAF	CREA	Na GLU	TOTAL	Average	MIN	MAX	Model Prediction
1	35.19	27.49	30.78	30.67	27.61	151.74	0.32 ± 0.05[1]	0.27	0.37	0.3
2	10.5	25.88	27.91	28.79	61.52	154.6	0.24 ± 0.04	0.20	0.28	0.25[2]
3	28.28	51.42	58.17	7.79	0	145.66	0.29 ± 0.05	0.24	0.34	0.25

[1] The (±) indicated the standard deviation values

[2] Colour coding: GREEN: In range

Regarding sub-library study, only one test vial (NPS: 11.69 mg, BEN: 36.57 mg, CAF: 33.45 mg, CREA: 41.14 mg, Na GLU 26.36 mg) was generated from the phenibut model. Similarly, to the model validation results, the prediction value of 0.22 was identical to the obtained matching value of 0.22 ± 0.02. This result particularly highlighted the low amount of NPS within the vial. In comparison to 5F-PB-22 sub-library, the latter test vial (NPS: 10.64 mg) displayed a matching value significantly different to that of predicted although lower concentration of benzocaine (29.57 mg). With 5F-PB-22 being a stronger scatterer than phenibut, this pose the question of the importance of substances sharing similar readings in their Raman spectra commonly noticed in full library analysis. Having said that, this could mean that the presence substances with similar Raman profile could impact on the detection of one the other, raising their effective matching value. This was mainly the case of benzocaine and caffeine which both presents a peak at 1279 and 1512 cm^{-1}. In fact, full library analysis of N-Me-2-AI vial 4 (NPS: 22.19 mg, BEN: 52.6 mg, CAF: 4.3 mg, CREA: 68.81 mg) showed caffeine detected at 0.22 although present in low amount (4.3 mg). In order to evaluate the influence of this in NPS screening, further analysis was conducted in order to determine the effects of cutting agents/adulterants on NPS matching values.

4.5. Influence of cutting agents and adulterants.

In the aim of determining the impact of spectral similarities between NPS and adulterants/cutting agents, 150 mg of each excipients was analysed against each NPS library following their respective methods (Method A for 5F-PB-22 and N-Me-2-AI sub-library and Method B for phenibut). Table 4.12 presents the average matching value obtained for each NPS from every cutting agent/adulterant run. Considering the absence of the NPSs in these samples, all the values obtained were false positive as the responses were not a representation of the NPS content. Initial observations showed that creatine shared the least similarities with all NPS only matching at 0.02 ± 0.01, 0.09 ± 0.02, and 0.02 ± 0.01 respectively for 5F-PB-22, N-Me-2-AI and phenibut. In addition, creatine appeared to also share the least similarities with all other excipients. This was explained by its position in matching records usually last or non-apparent as shown previously in Table 4.8. Previous studies in Section 3 and 4.4 demonstrated that benzocaine appeared to cause the most problems in hindering NPS detection. NPS matching values obtained were much lower compared to benzocaine analogues such procaine recorded at 0.65 on average. This justifies the difficulty linked to the identification of NPS in presence of benzocaine. The identification of 5F-PB-22 at 0.35 ± 0.35 in vial 7 (NPS: 80 mg, BEN: 70 mg) was mainly associated to the NPS strong Raman properties as well as it concentration (mg) within the sample. Previous results obtained in Section 4.4 showed a 22% caffeine matching in N-Me-2-AI sub-library analysis of Sample 4 (NPS: 22.19 mg, BEN: 52.6 mg, CAF: 4.3 mg, CREA: 68.81 mg) while present at 4.3 mg. Caffeine seemed to display similar results to benzocaine qualified by NPS matches of 0.04 ± 0.02, 0.12 ± 0.01, and 0.10 ± 0.01 respective to 5F-PB-22, N-Me-2-AI and phenibut. However, the latter correlated to up to 23% with benzocaine which explains results described above. On the other hand, all NPS appeared to correlate the most with sodium glutamate demonstrated by 0.16 ± 0.03, 0.23 ± 0.03, and 0.19 ± 0.03 from 5F-PB-22 to phenibut. As a poor Raman light scatterer, sodium glutamate spectra displayed high signal to noise ratio with low peak intensity. No sodium glutamate was recorded in analysis of benzocaine and caffeine at 350 mW compared to an average of 0.12 for benzocaine and 0.07 for caffeine at 200 mW. In fact, at a higher power, benzocaine and caffeine peaks intensify creating more differences between their spectra. These characteristics prove to be the reasons behind detection of sodium glutamate in every excipient particularly at lower power settings (200 mW).

Table 4.12: NPS matching values on sub-library analysis of excipients using wavelet algorithm

	Matching results		
Cutting agents/adulterants (150 mg)	5F-PB-22	N-Me-2-AI	Phenibut
Benzocaine	0.04 ± 0.01[1]	0.11 ± 0.01	0.19 ± 0.01
Caffeine	0.04 ± 0.02	0.12 ± 0.01	0.10 ± 0.01
Creatine	0.02 ± 0.01	0.09 ± 0.02	0.02 ± 0.01
Sodium glutamate	0.16 ± 0.03	0.23 ± 0.03	0.19 ± 0.03

[1] the (±) indicated the standard deviation values

Sub-library results showed that DOE use was beneficial in the objective of predicting a matching value from a sample composition. In fact, in addition to displaying better reproducibility across all 4 replicates, matching scores appeared to be significantly similar to predicted values. Also, the use of sub-library model appeared to show promising results in the prediction of test vials undetected using full library. Indeed, by using the sub-library model's equation, predictive values resulting from the analysis of full library test vials on sub-library could be obtained. Matching results obtained from analysis of the latter in the aim of counteracting the influence of analogues showed the use of a model successful in predicting detection. Although this indicated that the reverse situation where the potential composition of a sample can be approximately identified from a matching result via use of a model, it puts emphasis on the samples being of known concentration and the library reduced to a perfect size. In situation of unknown sample composition, larger library size would be of consideration in order to evaluate all possibilities. Hence, better algorithm of search need to be considered with the objective of dealing with analogues and structurally similar substances. Considering the use of a model, the said algorithm would require a similar approach to wavelet; assigning one matching value to only one match. However, since wavelet is limited to display only five matches, it would be interesting to expand this in order to evaluate the extent of having substances matching well to a component due to spectral similarities. Additionally, sub-library analysis proved that in the absence of analogues, the chances of matching considerably increased notably in complex samples Hence, the algorithm could potentially be refined such as to omit these substances achieved by initially comparing correlations between sample and references spectra after a first run, followed by a second run where the high correlating reference standards (from the first run) are omitted.

5. CONCLUSION/FUTURE WORK:

The monitoring of NPS has faced an increasing challenge in the last decade that has seen the number of NPSs as well as the cases of abuse rise dramatically. Although there appeared to be a few other techniques used in their identification, the use of handheld Raman spectroscopy has proven it can be considered in this regard. Additionally, with the growing use of chemometrics in science, the use of mixtures design of experiments showed potential in the ability to predict NPS responses in complex mixtures. The analysis of 5F-PB-22, N-Me-2-AI and phenibut were carefully selected in this study displayed intriguing results when it comes to the issues behind NPS identification.

Initial studies investigated the detection of NPS in mixtures of various complexity designed via a simplex lattice approach. wavelet investigations of all 26 vials showed high matching of pure NPS samples (vials 1 and 22) at above 80% which was expected considering their high purity. However, matching results alternated considerably in proportion to the complexity of vials in concentration of excipients present and the number of component. Identification of 5F-PB-22 displayed productive results in binary mixture with matching values of up to 0.95 ± 0.01 with an NPS concentration of 80 mg. The same pattern was also noticed for N-Me-2-AI and phenibut respectively matching to up to 0.83 ± 0.02 and 0.76 ± 0.08 in presence of sodium glutamate exhibiting good reproducibility (precision values below 0.1), at the exception of no reading in presence of 70 mg of benzocaine as noted in vial 7. Although detection of 5F-PB-22 in vial 7, the precision value 0.35 highlights poor reproducibility with the NPS identified in two of four replicates.

Benzocaine, is the most commonly used adulterants due to its additional psychoactive effects. However, it happened to play a major role in NPS screening due to its Raman scattering profile known to obstruct detection of other mixture components by detector overload. In fact, the results interpretation showed that detection of NPS in relation to benzocaine depended on the concentration difference between them as well as the spectroscopic properties of the NPS in question. Indeed, N-Me-2-AI and phenibut displayed no detection although present at similar concentration (80 mg). Nonetheless, this varied depending the excipients of use along with the proportions of substances involved. Sodium glutamate and creatine also impacted NPS readings to a lesser effect due to their noisier Raman spectra. However, this applied to lower NPS concentrations demonstrated by vial 15 (NPS: 10, CREA: 70 mg, Na GLU: 70 mg) with identification of only 5F-PB-22 and N-Me-2-AI respectively at 0.20 ± 0.35 and 0.19 ± 0.19. Thus, the relation between concentration and spectral properties of both NPS and cutting agents/adulterants accounts for the complexity in successful identification of Novel Substances. This confirmed that benzocaine was the stronger Raman scatterer in this study compared to creatine and sodium glutamate. However, it was key to note that this classification in spectral properties also applied to NPS.

In fact, matching value appeared to decrease from 5F-PB-22 to phenibut shown by pure vials recordings (vials 1 and 22: 150 mg NPS) of 0.98, 0.90, and 0.88 in vial 1 and 0.98, 0.89, and 0.85 in vial 22 respectively for 5F-PB-22, N-Me-2-AI and phenibut. This explains identification of 5F-PB-22 in even more complex mixture when the remaining NPS fail, confirming its stronger Raman properties with phenibut the weaker of all three. In addition to the effects of adulterants and cutting agents on NPS detection, further problems were encountered notably in analogues and structurally similar substances of adulterants/cutting agents. These substances shared similarities in Raman spectra, thus increasing the correlation coefficient between them. With wavelet limited to display five matches, the detection of analogues was more apparent, detected following the excipients they derived from. Consequently, no NPS could be recorded due to the presence analogues of various correlation with the excipients they derived from. Once more, benzocaine was responsible of most cases of undetected NPS due to strong matching of its analogues such as dimethocaine and procaine. In high concentrations (140 mg) however, a similar situation was observed with caffeine, creatine and sodium glutamate. However, the in-built Rigaku mixture being a mixture algorithm was used for the analysis of the same 26 vials for comparison purposes. Due to Rigaku mixture recognition pattern to match each result with all possibilities, it seemed better suited to cope with the tricky complexity of samples along with the influence of analogues. Whilst Rigaku mixture results still displayed an abundant number of analogues, NPS were more likely to be identified. This was the example of vial 19 where no NPS was detected in wavelet analysis with identification of 5F-PB-22 (PB-22) and caffeine (theophylline, sucrose) analogues. However, the NPS was identified three out of four replicates. Although Rigaku mixture improvement in dealing with analogues and structurally similar substances of notably caffeine, creatine, and sodium glutamate, benzocaine still displayed as much influence. Having vials of known composition helped in the interpretation of matching data as the detection of one analogue could be an indication of its corresponding NPS or cutting agents/adulterants. These findings would be of good use at border controls where the purity of samples encountered is relatively high. However, in the case of samples of unknown composition, analogues would be of a greater influence with search libraries designed to be as large as possible which therefore replicate conditions encountered by forensic scientists in street samples.

Hence, further investigations focussed on the potential improvement in matching results in the absence of derivatives and structurally similar substances. Sub-libraries were created based on five reference standards (NPS and adulterants/cutting agents) and the initial 26 samples were analysed using the same experimental details. Findings showed that all 26 vials were successfully identified for all NPS in comparison to full library. Additionally, there was a matching value increase from full library to sub-library mainly noticed in vials of low NPS amount (10 mg) and quinary mixture (vial 17-20). In the example of 5F-PB-22's vial 12 (NPS: 10 mg, CREA: 70 mg, Na GLU: 70 mg) where no detection of NPS was recorded in full library , as well as in poor

matching value (1 or 2 out of 4 replicates) as 5F-PB-22's vial 11 (NPS: 10 mg, CAF: 70 mg, CREA: 70 mg) detected at 0.11 ± 0.19, increased matching values of 0.33 ± 0.06 and 0.19 ± 0.05 were respectively recorded for vial 12 and 11. Indeed, with the absence of analogues, library components where able to match better to the sample spectra. It confirmed that the size of the library especially with the involvement of analogues was the main obstacle in NPS detection. Although these results would be useful to forensic scientists, its application wouldn't be straightforward due to their need for large library sizes. Yet, this study focussed on the monitoring of NPS in street-like samples usually of high complexity in terms of sample composition.

The use of design of experiment in mixtures has been proven to be a potential technique in determining component responses in mixtures depending on the components involved. However, its use in NPS screening had yet to be established. In order to further investigate the use of chemometrics in NPS monitoring, results obtained from full and sub-libraries were re-inserted into the design of experiments which generated a model for each library. It is key to note that no quality control was applied to the data due to successful statistical test on the non-quality controlled model. The generated test vials analysed on full library using wavelet showed no detection in N-Me-2-AI and phenibut. Additionally, 5F-PB-22 showed detection in only two vials which matched predictions from the model. Vials of undetected NPS displayed low prediction values of below 0.1. However, with the presence of analogues within full library, the latter generally matched to up to 50% of the predominant component within the matching data (usually benzocaine). Whereas, sub-library analysis showed better correlation to model prediction. Cases of matching values out of range compared to model predictions were still encountered notably in 5F-PB-22 and N-Me-2-AI, but these values were significantly closer.

In order to further justify the use of a model by proving its efficiency, model validation was conducted by analysing full library test vials against sub-library. The obtained matching values were compared to predictions drawn from the model's equation. 5F-PB-22 and N-Me-2-AI displayed 66.6 and 50% of samples correlating to matching predictions. Once again, cases of out of range were very narrow as noticed in sub-library results. The matching of analogues and other substances to components of vials notably the excipients have been the main issues throughout the study. Yet it could be assumed that a correlation between NPSs and cutting agents/adulterants of use could also be established. Analysis of pure excipients (150 mg) on all sub-libraries showed that 5F-PB-22 displayed the least similarities with all excipients. Interestingly, phenibut showed the highest correlation of 0.19 with regards to benzocaine which could explain better model validation of phenibut's full library test vials. On the other hand, sodium glutamate exhibited the highest correlation with matching values of up to 0.23 with N-Me-2-AI. This explained the appearance in all excipients analysis at 200 mW. In fact, at increased power of 350 mw, benzocaine and caffeine bands are intensified stimulating more differences between them.

The results obtained in the study would suggest that the use of handheld Raman spectroscopy could be used for on-site testing of NPS samples. However, this would be ideal in samples of high NPS purity mainly encountered in border controls and airports. Further testing will need to be conducted investigating the limits of detection of NPS in complex mixtures such as in street samples. Additionally, the use of a model for the prediction of NPS responses in mixtures displayed promising results. In fact, considering the number of samples that could be possible, the use of a model could be of an advantage in the selection of samples. Furthermore, it could be used to determine outcomes of untested samples. This could further be utilised in exploring the limits of detection of NPS in complex mixtures through model equations. A model development highlighted the importance of appropriate algorithm. Considering the requirement of large library size for the testing of samples of unknown composition, the impact of having analogues or structurally similar compounds present increases the chances of NPS being unidentified. Although Rigaku mixture displayed a better approach in the identification of NPS notably in complex mixtures, wavelet algorithm was important for the model application. However, further investigations will need to be conducted regarding its application. In addition, it would be beneficial to design new methods of search which considers omitting analogues and structurally similar substances or eventually displays more results for better identification. Further in-depth analysis could be conducted notably Principal component analysis (PCA). PCA helps identify the variations in sample outcomes while retaining the patterns. This can be used to further analyse data and classify samples.

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APPENDIX

Table 1: Summary results table of cutting agents/adulterants matching results with 5F-PB-22 using wavelet library on both full library (FL) and sub-library (SL)

Vial	Content / mg					AVERAGE ± SD							
						BEN		CAF		CREA		Na GLU	
	NPS	BEN	CAF	CREA	Na GLU	FL	SL	FL	SL	FL	SL	FL	SL
1	150	0	0	0	0		**0.00**[1]		**0.00**		**0.00**		**0.11 ± 0.01**
2	10	0	140	0	0		**0.25 ± 0.01**	0.90 ± 0.03	0.92 ± 0.02		**0.03± 0.01**		**0.02 ± 0.01**
3	10	140	0	0	0	0.98 ± 0.04[1]	0.97 ± 0.00		**0.22 ± 0.01**		**0.00**		**0.12 ± 0.00**
4	10	0	0	140	0		**0.00**		**0.05 ± 0.02**	0.87 ± 0.04	0.88 ± 0.01		**0.09 ± 0.02**
5	10	0	0	0	140		**0.12 ± 0.02**		**0.05 ± 0.03**		**0.03 ± 0.02**	0.70 ± 0.04	0.65 ± 0.06
6	80	0	70	0	0		**0.09 ± 0.01**	0.13 ± 0.22	0.38 ± 0.04		**0.00**		**0.09 ± 0.01**
7	80	70	0	0	0	0.79 ± 0.12	0.86 ± 0.04		**0.20 ± 0.01**		**0.00**		**0.16 ± 0.01**
8	80	0	0	70	0		**0.00**		**0.02 ± 0.01**	0.00	0.16 ± 0.02		**0.14 ± 0.01**
9	80	0	0	0	70		**0.02 ± 0.02**		**0.02 ± 0.01**		**0.00**	0	0.21 ± 0.03
10	10	70	70	0	0	0.95 ± 0.02	0.96 ± 0.01	0.00	0.39 ± 0.02		**0.00**		**0.11 ± 0.01**
11	10	0	70	70	0		**0.17 ± 0.02**	0.76 ± 0.03	0.81 ± 0.03	0.32 ± 0.18	0.37 ± 0.07		**0.05 ± 0.02**
12	10	0	70	0	70		**0.22 ± 0.01**	0.84 ± 0.05	0.85 ± 0.01		**0.03 ± 0.00**	0.00	0.14 ± 0.03
13	10	70	0	70	0	0.96 ± 0.01	0.96 ± 0.01		**0.23 ± 0.00**	0.00	0.02 ± 0.02		**0.14 ± 0.01**
14	10	70	0	0	70	0.95 ± 0.01	0.97 ± 0.01		**0.22 ± 0.01**		**0.00**	0.00	0.15 ± 0.01
15	10	0	0	70	70		**0.02 ± 0.02**		**0.10 ± 0.03**	0.80 ± 0.04	0.62 ± 0.09	0.28 ± 0.33	0.38 ± 0.10
16	94	14	14	14	14	0.00	0.19 ± 0.11	0.00	0.10 ± 0.03	0.00	0.00	0.00	0.15 ± 0.02
17	24	14	84	14	14	0.47 ± 0.28	0.60 ± 0.09	0.69 ± 0.11	0.75 ± 0.03	0.00	0.04 ± 0.01	0.00	0.09 ± 0.01
18	24	84	14	14	14	0.97 ± 0.01	0.95 ± 0.02	0.00	0.25 ± 0.01	0.00	0.00	0.00	0.13 ± 0.01
19	24	14	14	84	14	0.67 ± 0.07	0.60 ± 0.14	0.00	0.27 ± 0.05	0.00	0.30 ± 0.11	0.00	0.22 ± 0.05
20	24	14	14	14	84	0.77 ± 0.17	0.78 ± 0.13	0.00	0.27 ± 0.02	0.00	0.01 ± 0.01	0.00	0.29 ± 0.05
21	38	28	28	28	28	0.62 ± 0.12	0.80 ± 0.15	0.00	0.33 ± 0.06	0.00	0.02 ± 0.02	0.00	0.16 ± 0.04
22	150	0	0	0	0		**0.00**		**0.00**		**0.00**		**0.11 ± 0.00**
23	10	0	140	0	0		**0.23 ± 0.01**	0.94 ± 0.00	0.93 ± 0.01		**0.04 ± 0.00**		**0.01 ± 0.01**
24	10	140	0	0	0	0.97 ± 0.01	0.97 ± 0.00		**0.22 ± 0.00**		**0.00**		**0.12 ± 0.00**
25	10	0	0	140	0		**0.00**		**0.05± 0.01**	0.89 ± 0.01	0.85± 0.05		**0.09 ± 0.01**
26	10	0	0	0	140		**0.10 ± 0.02**		**0.03 ± 0.02**		**0.01 ± 0.01**	0.67 ± 0.09	0.58 ± 0.06

[1] Colour coding: GREEN: 4/4 matches, YELLOW: 3/4 matches, ORANGE: 1 or 2 of 4 matches, RED: unidentified

Table 2: Summary results table of cutting agents/adulterants matching results with N-Me-2-AI using wavelet library on both FL and SL

Vial	Content / mg					AVERAGE ± SD							
						BEN		CAF		CREA		Na GLU	
	NPS	BEN	CAF	CREA	Na GLU	FL	SL	FL	SL	FL	SL	FL	SL
1	150	0	0	0	0		**0.13 ± 0.02**		**0.06 ± 0.02**		**0.07 ± 0.02**		**0.19 ± 0.01**
2	10	0	140	0	0		**0.25 ± 0.01**	0.93 ± 0.01	0.95 ± 0.01		**0.06 ± 0.01**		**0.00**
3	10	140	0	0	0	0.98 ± 0.00[1]	0.97 ± 0.01		**0.23 ± 0.01**		**0. 00**		**0.12 ± 0.01**
4	10	0	0	140	0		**0.00[1]**		**0.06 ± 0.01**	0.91 ± 0.02	0.93 ± 0.01		**0.10 ± 0.01**
5	10	0	0	0	140		**0.15 ± 0.04**		**0.01 ± 0.01**		**0.07 ± 0.03**	0.80 ± 0.04	0.81 ± 0.02
6	80	0	70	0	0		**0.25 ± 0.01**	0.68 ± 0.07	0.76 ± 0.04		**0.08 ± 0.01**		**0.08 ± 0.02**
7	80	70	0	0	0	0.95 ± 0.01	0.95 ± 0.02		**0.22 ± 0.01**		**0.00**		**0.15 ± 0.01**
8	80	0	0	70	0		**0.10 ± 0.03**		**0.06 ± 0.01**	0.37 ± 0.21	0.51 ± 0.10		**0.18 ± 0.01**
9	80	0	0	0	70		**0.18 ± 0.03**		**0.10 ± 0.01**		**0.01 ± 0.01**	0.00	0.43 ± 0.04
10	10	70	70	0	0	0.95 ± 0.01	0.94 ± 0.02	0.00	0.45 ± 0.09		**0.00**		**0.10 ± 0.01**
11	10	0	70	70	0		**0.20 ± 0.02**	0.82 ± 0.05	0.83 ± 0.03	0.11 ± 0.18	0.35 ± 0.05		**0.06 ± 0.02**
12	10	0	70	0	70		**0.26 ± 0.01**	0.88 ± 0.01	0.92 ± 0.02		**0.04 ± 0.01**	0.00	0.09 ± 0.04
13	10	70	0	70	0	0.96 ± 0.01	0.96 ± 0.01		**0.23 ± 0.01**	0.00	0.06 ± 0.03		**0.13 ± 0.01**
14	10	70	0	0	70	0.97 ± 0.01	0.97 ± 0.01		**0.23 ± 0.01**		**0.00**	0.00	0.16 ± 0.03
15	10	0	0	70	70		**0.05 ± 0.05**		**0.09 ± 0.03**	0.69 ± 0.06	0.67 ± 0.20	0.33 ± 0.19	0.35 ± 0.07
16	94	14	14	14	14	0.51 ± 0.30	0.71 ± 0.14	0.00	0.31 ± 0.04	0.00	0.07 ± 0.05	0.00	0.26 ± 0.09
17	24	14	84	14	14	0.44 ± 0.44	0.60 ± 0.16	0.61 ± 0.36	0.81 ± 0.10	0.00	0.08 ± 0.02	0.00	0.07 ± 0.03
18	24	84	14	14	14	0.97 ± 0.00	0.97 ± 0.00	0.00	0.24 ± 0.01	0.00	0.00	0.00	0.12 ± 0.01
19	24	14	14	84	14	0.67 ± 0.39	0.71 ± 0.11	0.00	0.37 ± 0.06	0.00	0.43 ± 0.08	0.00	0.19 ± 0.03
20	24	14	14	14	84	0.81 ± 0.06	0.89 ± 0.03	0.00	0.32 ± 0.04	0.00	0.02 ± 0.02	0.00	0.31 ± 0.02
21	38	28	28	28	28	0.89 ± 0.03	0.91 ± 0.06	0.00	0.39 ± 0.05	0.00	0.03 ± 0.03	0.00	0.15 ± 0.02
22	150	0	0	0	0		**0.14 ± 0.02**		**0.08 ± 0.01**		**0.02 ± 0.01**		**0.19 ± 0.01**
23	10	0	140	0	0		**0.25 ± 0.00**	0.95 ± 0.01	0.95 ± 0.01		**0.04 ± 0.005**		**0.01 ± 0.01**
24	10	140	0	0	0	0.97 ± 0.01	0.98 ± 0.00		**0.23 ± 0.01**		**0.00**		**0.12 ± 0.00**
25	10	0	0	140	0		**0.00**		**0.08 ± 0.02**	0.90 ± 0.04	0.92 ± 0.02		**0.08 ± 0.01**
26	10	0	0	0	140		**0.19 ± 0.01**		**0.05 ± 0.03**		**0.00**	0.78 ± 0.02	0.78 ± 0.04

[1] Colour coding: GREEN: 4/4 matches, YELLOW: 3/4 matches, ORANGE: 1 or 2 of 4 matches, RED: unidentified

Table 3: Summary results table of cutting agents/adulterants matching results with phenibut using wavelet library on both FL and SL

Vial	Content / mg					AVERAGE ± SD							
						BEN		CAF		CREA		Na GLU	
	NPS	BEN	CAF	CREA	Na GLU	FL	SL	FL	SL	FL	SL	FL	SL
1	150	0	0	0	0		**0.16 ± 0.01**		**0.11 ± 0.01**		**0.00**		**0.15 ± 0.01**
2	10	0	140	0	0		**0.25 ± 0.01**	0.95 ± 0.01	0.94 ± 0.01		**0.03 ± 0.01**		**0.00**
3	10	140	0	0	0	0.97 ± 0.00[1]	0.97 ± 0.01		**0.22 ± 0.00**		**0.00**		**0.11 ± 0.00**
4	10	0	0	140	0		**0.00**[1]		**0.07 ± 0.01**	0.95 ± 0.01	0.94 ± 0.02		**0.07 ± 0.03**
5	10	0	0	0	140		**0.18 ± 0.01**		**0.03 ± 0.02**		**0.00**	0.83 ± 0.01	0.80 ± 0.03
6	80	0	70	0	0		**0.25 ± 0.01**	0.69 ± 0.02	0.72 ± 0.08		**0.01 ± 0.01**		**0.06 ± 0.02**
7	80	70	0	0	0	0.95 ± 0.01	0.96 ± 0.01		**0.22 ± 0.01**		**0.00**		**0.13 ± 0.01**
8	80	0	0	70	0		**0.12 ± 0.01**		**0.12 ± 0.01**	0.00	0.40 ± 0.09		**0.17 ± 0.01**
9	80	0	0	0	70		**0.19 ± 0.01**		**0.09 ± 0.01**		**0.00**	0.13 ± 0.23	0.37 ± 0.11
10	10	70	70	0	0	0.95 ± 0.01	0.96 ± 0.01	0.00	0.38 ± 0.03		**0.00**		**0.10 ± 0.00**
11	10	0	70	70	0		**0.19 ± 0.02**	0.82 ± 0.03	0.81 ± 0.02	0.35 ± 0.2	0.41 ± 0.09		**0.03 ± 0.02**
12	10	0	70	0	70		**0.25 ± 0.01**	0.91 ± 0.02	0.92 ± 0.02		**0.02 ± 0.01**	0.00	0.09 ± 0.04
13	10	70	0	70	0	0.96 ± 0.01	0.96 ± 0.01		0.22 ± 0.01	0.00	0.04 ± 0.05		**0.12 ± 0.01**
14	10	70	0	0	70	0.97 ± 0.01	0.97 ± 0.00		**0.22 ± 0.01**		**0.00**	0.00	0.16 ± 0.01
15	10	0	0	70	70		**0.09 ± 0.02**		**0.07 ± 0.01**	0.75 ± 0.02	0.71 ±0.10	0.29 ± 0.17	0.40 ± 0.1
16	94	14	14	14	14	0.39 ± 0.39	0.74 ± 0.12	0.00	0.28 ± 0.02	0.00	0.01 ±0.01	0.00	0.18 ± 0.01
17	24	14	84	14	14	0.39 ± 0.40	0.69 ± 0.08	0.63 ± 0.36	0.75 ± 0.06	0.00	0.05 ± 0.05	0.00	0.07 ± 0.02
18	24	84	14	14	14	0.96 ± 0.01	0.97 ± 0.01	0.00	0.24 ± 0.01	0.00	0.00	0.00	0.12 ± 0.01
19	24	14	14	84	14	0.29 ± 0.29	0.62 ± 0.20	0.00	0.38 ± 0.06	0.46 ± 0.27	0.54 ± 0.15	0	0.14 ± 0.02
20	24	14	14	14	84	0.54 ± 0.35	0.80 ± 0.10	0.00	0.38 ± 0.07	0.00	0.04 ± 0.04	0.28 ± 0.28	0.38 ± 0.08
21	38	28	28	28	28	0.79 ± 0.08	0.91 ± 0.03	0.00	0.41 ± 0.05	0.00	0.08 ± 0.04	0.00	0.13 ± 0.01
22	150	0	0	0	0		**0.17 ± 0.01**		**0.13 ± 0.04**		**0.00**		**0.14 ± 0.01**
23	10	0	140	0	0		**0.26 ± 0.01**	0.93 ± 0.02	0.94 ± 0.01		**0.05 ± 0.01**		**0.00**
24	10	140	0	0	0	0.97 ± 0.00	0.97 ± 0.00		**0.22 ± 0.00**		**0.00**		**0.11 ± 0.00**
25	10	0	0	140	0		**0.00**		**0.07 ± 0.01**	0.94 ± 0.01	0.95 ± 0.01		**0.05 ± 0.01**
26	10	0	0	0	140		**0.22 ± 0.01**		**0.05 ± 0.02**		**0.00**	0.75 ± 0.03	0.71 ± 0.13

[1] Colour coding: GREEN: 4/4 matches, YELLOW: 3/4 matches, ORANGE: 1 or 2 of 4 matches, RED: unidentified

Table 4: 5F-PB-22 Rigaku mixture results on full library (FL)

						5F-PB-22							
	Content / mg					Replicate 1		Replicate 2		Replicate 3		Replicate 4	
Vial	NPS	BEN	CAF	CREA	Na GLU	Value	Match	Value	Match	Value	Match	Value	Match
1	150	0	0	0	0	0.99	5F-PB-22	0.99	5F-PB-22	0.99	5F-PB-22	0.99	5F-PB-22
2	10	0	140	0	0	0.88	CAF; THEO; 5F-PB-22	0.84	CAF; 5F-PB-22	0.93	CAF; 5F-PB-22	0	—
3	10	140	0	0	0	0	— [1]	0	—	0	—	0	—
4	10	0	0	140	0	0.87	5F-PB-22; CREA	0.96	5F-PB-22; CREA	0	—	0.85	5F-PB-22; CREA; L-TYR
5	10	0	0	0	140	0.84	5F-PB-22; Na GLU	0.86	5F-PB-22; Na GLU	0.87	5F-PB-22; Na GLU	0.85	5F-PB-22; Na GLU
6	80	0	70	0	0	0.98	5F-PB-22; CAF	0.98	5F-PB-22; CAF	0.98	CAF; 5F-PB-22	0.98	CAF; 5F-PB-22
7	80	70	0	0	0	0.98	BEN, 5F-PB-22	0.98	BEN, 5F-PB-22	0.99	BEN, 5F-PB-22	0.98	BEN, 5F-PB-22
8	80	0	0	70	0	0.98	5F-PB-22	0.98	5F-PB-22	0.97	5F-PB-22	0.97	5F-PB-22
9	80	0	0	0	70	0.98	5F-PB-22	0.97	5F-PB-22	0.97	5F-PB-22	0.99	5F-PB-22
10	10	70	70	0	0	0	—	0	—	0	—	0	—
11	10	0	70	70	0	0.95	5F-PB-22; CAF; CREA	0	—	0.9	5F-PB-22; CAF; CREA; gelatine	0.88	5F-PB-22; CAF; CREA
12	10	0	70	0	70	0.97	5F-PB-22; CAF	0.88	5F-PB-22; CAF	0	—	0.86	5F-PB-22; CAF
13	10	70	0	70	0	0	—	0	—	0	—	0	—
14	10	70	0	0	70	0	—	0	—	0	—	0	—
15	10	0	0	70	70	0.85	5F-PB-22; Na GLU; CREA	0	—	0.85	5F-PB-22; CREA	0.8	5F-PB-22; CREA
16	94	14	14	14	14	0.97	5F-PB-22; BEN	0.98	5F-PB-22	0.98	5F-PB-22	0.98	5F-PB-22; DIM
17	24	14	84	14	14	0.97	5F-PB-22; DIM; CAF	0	—	0.97	5F-PB-22; BEN; CAF	0.96	5F-PB-22; CAF
18	24	84	14	14	14	0	—	0	—	0	—	0	—
19	24	14	14	84	14	0.97	5F-PB-22; CREA; BEN	0.94	5F-PB-22; CREA; DIM	0.96	5F-PB-22; CREA; BEN	0.93	5F-PB-22; CREA; BEN
20	24	14	14	14	84	0.94	5F-PB-22; Na GLU; DIM	0.91	5F-PB-22; BEN	0.96	5F-PB-22; BEN	0	—
21	38	28	28	28	28	0.96	5F-PB-22; DIM	0.95	5F-PB-22; BEN	0.98	5F-PB-22; DIM	0.96	5F-PB-22; BEN
22	150	0	0	0	0	0.99	5F-PB-22	0.99	5F-PB-22	0.99	5F-PB-22	0.99	5F-PB-22
23	10	0	140	0	0	0	—	0	—	0	—	0	—
24	10	140	0	0	0	0	—	0	—	0	—	0	—
25	10	0	0	140	0	0.88	5F-PB-22; CREA	0.86	5F-PB-22; CREA	0.87	5F-PB-22; CREA	0.88	5F-PB-22; CREA
26	10	0	0	0	140	0.84	5F-PB-22; Na GLU	0	—	0.82	5F-PB-22; Na GLU	0.87	5F-PB-22; Na GLU

[1] The (—) indicated no match for the NPS

Table 5: N-Me-2-AI Rigaku mixture results on full library (FL)

						N-Me-2-AI							
Vial	Content / mg					Replicate 1		Replicate 2		Replicate 3		Replicate 4	
	NPS	BEN	CAF	CREA	Na GLU	Value	Match	Value	Match	Value	Match	Value	Match
1	150	0	0	0	0	0.95	N-Me-2-AI	0.95	N-Me-2-AI	0.95	N-Me-2-AI	0.91	N-Me-2-AI
2	10	0	140	0	0	0	—	0	—	0	—	0	—
3	10	140	0	0	0	0	—	0	—	0	—	0	—
4	10	0	0	140	0	0	—	0	—	0	—	0	—
5	10	0	0	0	140	0.52	N-Me-2-AI	0	—	0.84	N-Me-2-AI; Na GLU	0	—
6	80	0	70	0	0	0.96	N-Me-2-AI; CAF	0.96	N-Me-2-AI; CAF	0.96	N-Me-2-AI; CAF	0.96	N-Me-2-AI; CAF
7	80	70	0	0	0	0	—	0.98	N-Me-2-AI; BEN	0	—	0.98	N-Me-2-AI; BEN
8	80	0	0	70	0	0.94	N-Me-2-AI; CREA	0.94	N-Me-2-AI; CREA	0.92	N-Me-2-AI	0.94	N-Me-2-AI; CREA
9	80	0	0	0	70	0.79	N-Me-2-AI	0.75	N-Me-2-AI	0.9	N-Me-2-AI	0.83	N-Me-2-AI
10	10	70	70	0	0	0	—	0	—	0	—	0	—
11	10	0	70	70	0	0	—	0	—	0	—	0	—
12	10	0	70	0	70	0	—	0	—	0.89	N-Me-2-AI, CAF	0	—
13	10	70	0	70	0	0	—	0	—	0	—	0	—
14	10	70	0	0	70	0	—	0	—	0	—	0	—
15	10	0	0	70	70	0	—	0	—	0.89	N-Me-2-AI; CREA; Na GLU	0.77	N-Me-2-AI; CREA
16	94	14	14	14	14	0.91	N-Me-2-AI; BEN	0.94	N-Me-2-AI; BEN	0.93	N-Me-2-AI; BEN	0.96	N-Me-2-AI; BEN
17	24	14	84	14	14	0	—	0	—	0	—	0.89	N-Me-2-AI; CAF; FLUBROMAZEPAM
18	24	84	14	14	14	0	—	0	—	0	—	0	—
19	24	14	14	84	14	0	—	0	—	0	—	0	—
20	24	14	14	14	84	0	—	0	—	0.89	N-Me-2-AI; S. Fle; BEN; CAF	0	—
21	38	28	28	28	28	0	—	0	—	0.92	N-Me-2-AI; CAF; S Fle; BEN	0	—
22	150	0	0	0	0	0.94	N-Me-2-AI	0.95	N-Me-2-AI	0.95	N-Me-2-AI	0.96	N-Me-2-AI
23	10	0	140	0	0	0	—	0	—	0	—	0	—
24	10	140	0	0	0	0	—	0	—	0	—	0	—
25	10	0	0	140	0	0	—	0	—	0.87	N-Me-2-AI; CREA	0	—
26	10	0	0	0	140	0.85	N-Me-2-AI; Na GLU	0	—	0	—	0	—

[1] The (—) indicated no match for the NPS

Table 6: N-Me-2-AI Rigaku mixture results on full library (FL)

						phenibut							
	Content / mg					Replicate 1		Replicate 2		Replicate 3		Replicate 4	
Vial	NPS	BEN	CAF	CREA	Na GLU	Value	Match	Value	Match	Value	Match	Value	Match
1	150	0	0	0	0	0.93	PHENIBUT	0.91	PHENIBUT	0.94	PHENIBUT	0.94	PHENIBUT
2	10	0	140	0	0	0	—	0	—	0	—	0	—
3	10	140	0	0	0	0	—	0	—	0	—	0	—
4	10	0	0	140	0	0	—	0	—	0	—	0	—
5	10	0	0	0	140	0	—	0	—	0	—	0	—
6	80	0	70	0	0	0.93	CAF; PHENIBUT	0.92	CAF; PHENIBUT	0.92	CAF; PHENIBUT	0.92	CAF; PHENIBUT
7	80	70	0	0	0	0	—	0	—	0	—	0	—
8	80	0	0	70	0	0.85	PHENIBUT	0.82	PHENIBUT	0.83	PHENIBUT	0.78	PHENIBUT
9	80	0	0	0	70	0.86	PHENIBUT	0.9	PHENIBUT	0.9	PHENIBUT	0.67	PHENIBUT
10	10	70	70	0	0	0	—	0	—	0	—	0	—
11	10	0	70	70	0	0	—	0	—	0	—	0	—
12	10	0	70	0	70	0	—	0	—	0	—	0	—
13	10	70	0	70	0	0	—	0	—	0	—	0	—
14	10	70	0	0	70	0	—	0	—	0	—	0	—
15	10	0	0	70	70	0	—	0	—	0	—	0	—
16	94	14	14	14	14	0.91	PHENIBUT	0.85	PHENIBUT	0.95	BEN; PHENIBUT	0.92	BEN; PHENIBUT
17	24	14	84	14	14	0	—	0	—	0	—	0	—
18	24	84	14	14	14	0	—	0	—	0	—	0	—
19	24	14	14	84	14	0.93	PHENIBUT; DIM; CREA	0	—	0	—	0	—
20	24	14	14	14	84	0	—	0.84	BEN; PHENIBUT	0	—	0.67	PHENIBUT
21	38	28	28	28	28	0.87	PHENIBUT; DIM	0	—	0	—	0	—
22	150	0	0	0	0	0.88	PHENIBUT	0.9	PHENIBUT	0.9	PHENIBUT	0.89	PHENIBUT
23	10	0	140	0	0	0	—	0	—	0	—	0	—
24	10	140	0	0	0	0	—	0	—	0	—	0	—
25	10	0	0	140	0	0	—	0	—	0	—	0	—
26	10	0	0	0	140	0	—	0.83	PHENIBUT; GHB; Na GLU	0	—	0.81	PHENIBUT; GHB; Na GLU

[1] The (—) indicated no match for the NPS

Table 7: Tables of expected and actual weight distribution in test vials generated from the model for full library (FL) and sub-library (SL) analysis

	Vial	Content (mg)																	
		5F-PB-22			BEN			CAF			CREA			Na GLU			TOTAL		
		Actual	Exp[1]	% Err[1]	Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err
FL	1	34.95	36.49	4.21	22.01	23.023	4.40	40.01	38.19	4.76	25.8	28.12	8.25	23.15	24.18	4.26	145.92	150	2.72
	2	10.53	10	5.30	16.66	16.40	1.59	15.84	15.01	5.56	99.41	101.34	1.94	7.11	7.25	1.93	149.55	150	0.3
	3	10.18	10	1.80	136.14	137.15	0.74	2.91	1.29	125.58	3.33	1.15	65.35	0.71	0.40	77.94	153.27	150	2.18
	4	23.38	22.63	3.31	72.87	76.49	4.73	15.04	13.69	9.88	37.62	37.20	1.11	0	0	0	148.91	150	0.73
	5	40.49	39.72	1.94	66.87	69.185	3.35	37.71	39.32	4.09	1.41	1.77	25.53	0	0	0	146.48	150	2.34
	6	28.52	27.92	2.15	86.95	89.21	2.53	9.26	9.57	3.24	22.76	23.29	2.32	0	0	0	147.49	150	1.67
SL	1	10.64	10	6.40	29.57	28.17	4.97	35.04	36.09	2.91	42.78	42.59	0.45	33.36	33.15	0.63	151.39	150	0.93

	Vial	Content (mg)																	
		N-Me-2-AI			BEN			CAF			CREA			Na GLU			TOTAL		
		Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err
FL	1	33.28	33.5	0.66	27.67	27.11	2.07	30.82	30.56	0.85	32.51	33.18	2.02	25.72	25.40	1.26	150	150	0.17
	2	10.15	10	1.5	20.10	20.60	2.43	27.44	28.01	2.03	34.63	34.23	1.17	56.81	57.17	0.63	149.13	150	0.59
	3	27.94	28.16	0.78	52.58	52.73	0.28	61.61	61.51	0.16	8	7.60	5.26	0	0	0	150.13	150	0.09
	4	22.19	23.03	3.65	52.60	53.57	1.81	4.30	4.67	7.92	68.81	68.73	0.12	0	0	0	147.9	150	1.4
SL	1	24.42	24.87	1.81	24.02	24.33	1.27	30.56	31.49	2.95	42.22	42.02	0.48	27.10	27.28	0.66	148.32	150	1.11
	2	10.68	10	6.8	22.91	23.11	0.87	30.18	30.92	2.39	49.07	48.65	0.86	36.87	37.32	1.21	149.71	150	0.19
	3	14.96	15.83	5.5	110.97	112.74	1.57	15.43	15.48	0.32	6.67	5.95	12.1	0	0	0	148.03	150	1.31

	Vial	Content (mg)																	
		Phenibut			BEN			CAF			CREA			Na GLU			TOTAL		
		Actual	Exp	%Err	Actual	Exp[1]	% Err	Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err	Actual	Exp	% Err
FL	1	35.19	33.73	4.33	27.49	27.58	0.31	30.78	29.99	2.63	30.67	31.80	3.54	27.61	26.91	2.62	151.74	150	1.16
	2	10.5	10	5	25.88	21.92	18.07	27.91	27.09	3.03	28.79	30.08	4.27	61.52	60.90	1.02	154.6	150	3.08
	3	28.28	28.16	0.42	51.42	52.73	2.48	58.17	61.51	5.43	7.79	7.60	2.5	0	0	0	145.66	150	2.89
SL	1	11.69	11.41	2.45	36.57	37.34	2.06	33.45	34.54	3.16	41.14	40.49	1.61	26.36	26.22	0.53	149.21	150	0.53

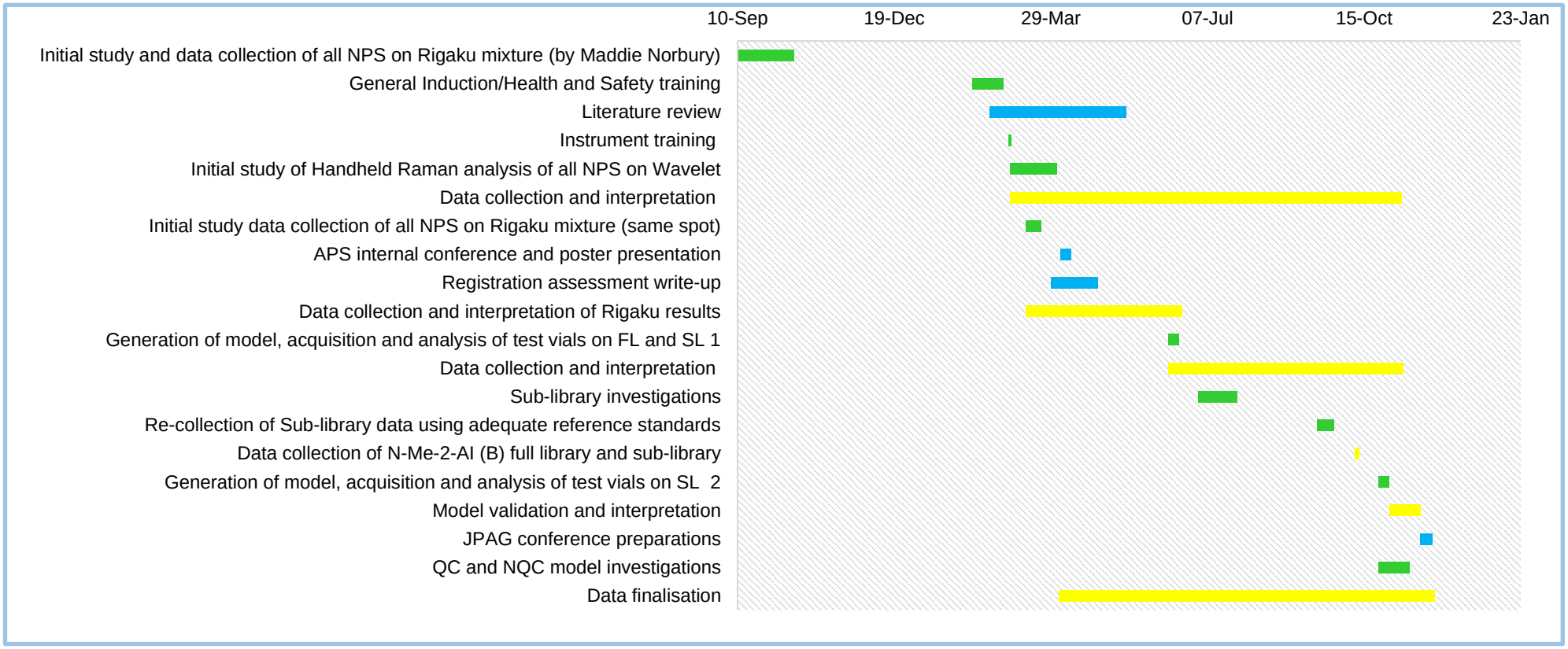
[1] Annotations: Exp: expected % Err: % error

Table 8: Full library reference standards

2,5-dimethoxy-4-methylamphetamine	BK-2C-B	METHOXETAMINE
2-AI	C8-CP-47-497	METHTYLONE
2-MAPB HCl	CAFFEINE	METHYLPHENIDATE
25H-NBOMe HCl	CETYLPYRIDINIUM CHLORIDE	MEXEDRONE
4-ACETYLPSILOCIN FUMARATE	CREATINE	MPA
HYDROXY-DET	CaCO3	MAGNESIUM STEARATE
4-Me-N-ETHYLNOPRPENTTEDRONE	COCAINE	MICROCRYSTALLINE CELLUSOSE
4-MeO-PENCYCLIDINE HCl	D-GLUCOSE	N-METHYL-2C-B
4F-PVP	D-MANNITOL	N-Me-2-AI
5,6-MDAI	DEXTROMETHORPHAN	N-N-DIPROPYLTRYPTAMINE
5-APB	DEXTROSE	N-PB-22
5-EAPB HCl	DILTIAZEM	N-ETHYLAMPHETAMINE
5I-AI	DIMETHOCAINE	NIACINAMIDE
5-MeO-DALT	DL-4662 HCl	NM-2201
5-MeO-MiPT	EHTYPHENIDATE	PARACETAMOL
5F-APINACA	ETIZOLAM	PB-22
5F-APICA	FDU-PB-22	PHENACETIN
5F-PB-22	FLUBROMAZEPAM	PHENAZEPAM
6-APB	FLUBROMAZOLAM	PHENIBUT HCl
6-MAPB HCL	GELATINE	PROCAINE
AB-FUBINACA	GHB	PYRAZOLAM
AB-PINACA	JWH-015	S-CATHINONE
ACETONE	JWH-073	SDB-006
ADRAFINIL	JWH-122	SODIUM BENZOATE
AM-2201	KETAMINE HCl	SUCROSE
AM-679	L-GLUTAMIC ACID	Synth FLEPHEDRONE
AFLOQUALONE	L-TYROSINE	Synth MEPHEDRONE
ALGINIC ACID SODIUM SALT	LIDOCAINE	TALC
α-PBT HCl	MAIZE STARCH	TAURINE
α-PVP HCl	MDMA	THEOPHYLLINE
α-LACTOSE MONOHYDRATE	MEBROQUALONE	THJ-018
BB-22	MEPHEDRONE	UR-144
BENZOCAINE	METHAMPHETAMINE	ZOPICLONE

Table 9: Reference standards used for half library creation

2,5-dimethoxy-4-methylamphetamine	L-TYROSINE
5-MeO-DALT	MEBROQUALONE
5F-APINACA	MEPHEDRONE
5F-APICA	METHAMPHETAMINE
5F-PB-22	METHOXETAMINE
AB-FUBINACA	MEXEDRONE
AB-PINACA	MPA
AM-2201	MAGNESIUM STEARATE
AM-679	N-METHYL-2C-B
AFLOQUALONE	N-Me-2-AI
ALGINIC ACID SODIUM SALT	N-N-DIPROPYLTRYPTAMINE (DPT)
BENZOCAINE	NM-2201
CAFFEINE	PARACETAMOL
CREATINE	PB-22
D-GLUCOSE	PHENACETIN
D-MANNITOL	PHENIBUT HCl
DILTIAZEM	PROCAINE
DIMETHOCAINE	PYRAZOLAM
EHTYPHENIDATE	S-CATHINONE
FDU-PB-22	SDB-006
FLUBROMAZEPAM	SUCROSE
FLUBROMAZOLAM	SYNTHESISED FLEPHEDRONE
GHB	SYNTHESISED MEPHEDRONE
KETAMINE HCl	THEOPHYLLINE
L-GLUTAMIC ACID	THJ-018


10-Sep
19-Dec
29-Mar
07-Jul
15-Oct
23-Jan
Initial study and data collection of all NPS on Rigaku mixture (by Maddie Norbury)
General Induction/Health and Safety training
Literature review
Instrument training
Initial study of Handheld Raman analysis of all NPS on Wavelet
Data collection and interpretation
Initial study data collection of all NPS on Rigaku mixture (same spot)
APS internal conference and poster presentation
Registration assessment write-up
Data collection and interpretation of Rigaku results
Generation of model, acquisition and analysis of test vials on FL and SL 1
Data collection and interpretation
Sub-library investigations
Re-collection of Sub-library data using adequate reference standards
Data collection of N-Me-2-AI (B) full library and sub-library
Generation of model, acquisition and analysis of test vials on SL 2
Model validation and interpretation
JPAG conference preparations
QC and NQC model investigations
Data finalisation