High performance liquid chromatography method development and chemometric analysis of ecstasy and cocaine

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Submitted to Higher Education and Training Awards Council (HETAC)
in fulfilment for the requirements for the degree of Doctor of Philosophy

Declaration

I hereby declare that the work herein, submitted for Ph.D. in Analytical Science at Letterkenny Institute of Technology, is the result of my own investigation, except where reference is made to published literature. I also certify that the material submitted in this thesis has not been previously submitted for any other qualification.

Kim McFadden

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Abstract

Consumption of illegal drugs of abuse remains a major social issue aligned with a global law-enforcement priority. Forensic analysts are faced with the challenge of continually developing sophisticated methods of analysis to combat the increasing variability that occurs in illicit drug samples. Research work for this thesis has focused on the development of High Performance Liquid Chromatography (HPLC) methods for the analysis of major drug constituents associated with ecstasy and cocaine illicit drug samples. Emphasis has been placed on method development with strategies of univariate or multivariate experimental approaches used in the selection and optimisation of procedures. Considerations with regard to the choice of chromatographic factors, solutes under investigation and the provision of quality assurance data throughout the research work have been the main criteria in methods developed. Two HPLC methods were developed to qualitatively and quantitatively assay for the major drug components and analogue derivatives found in ecstasy and cocaine. Methods developed have undergone validation studies including intra- and inter- reproducibility, accuracy, and linearity of calibration, limit of detection (LOD) and limit of quantification (LOQ) and the use of internal standards. Applications of methods to ecstasy and cocaine samples seized in Ireland ensured their suitability for routine analysis of illicit drug samples.

As part of this study, chemical profiling of 183 ecstasy tablets seized in Ireland during 2002-2004 were recorded as discrete data sets. Chemical data sets include both the quantification and occurrence in individual tablets of the major amphetamine components (i.e. MDA, MDMA, MDEA, MBDB methamphetamine and amphetamine), adulterant components (i.e. caffeine, phenacetin, acetaminophen and acetylsalicylic acid), excipients components (i.e. sucrose, glucose, lactose, fructose, mannitol, sorbitol and inositol) and inorganic components (i.e. Al, Zn, Fe, Mg, Ca, Cr, Pb, Na and K). Chemometrics, including unsupervised methods of principal component analysis (PCA), hierarchical cluster analysis (HCA) and Pearson's correlation coefficient, as well as supervised methods of linear discriminant analysis (LDA) and artificial neural networks (ANN) was applied to the chemical data sets to demonstrate the ability of the statistical approach to linking sample seizures. HCA and ANN were the numerical methods that most efficiently distinguished between

linked and unlinked seizures. Eleven groups were identified from the chemical data sets with group classification dependant on the main amphetamine, adulterant and excipient components present. The benefits from this study can provide strategic intelligence and an understanding of the operational level on the Irish ecstasy market and help evaluate the changing profile or dynamics associated with this illegal market supply.

List of Abbreviations

2C-B	4-bromo-2, 5-dimethoxyphenethylamine
A	Amphetamine
AA	Atomic absorption
ACN	Acetonitrile
ADRU	Alcohol and drug research unit
ANN	Artificial neural networks
ASA	Acetylsalicylic acid
ATS	Amphetamine – type – stimulants
BZP	1- Benzylpiperazine
CE	Capillary electrophoresis
CV	Coefficient of variation
DAD	Diode array detection
DAP	Drug awareness programmme
DEA	Drug enforcement agency
DIMS	Drugs information monitoring system
DOB	4-bromo-2, 5-dimethoxyamphetamine
DOM	4-Methyl-2, 5-dimethoxyamphetamine
ECD	Electron capture detector
ELSD	Evaporative light scattering detection
EMCDDA	European monitoring centre for drugs and drug addiction
ENFSI	European network of forensic science institutes
FID	Flame ionization detector
GBL	Gamma – butyrolactone
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
GHB	Gamma hyrdoxybutyric acid
HCA	Hierarchal cluster analysis
HPLC	High performance liquid chromatography
HETP	Height equivalent to theoretical plate
HRB	Health research board
ICH	International conference on harmonisation
IEC	Ion-exchange chromatography

INCB	International narcotics control board
INEF	Irish needle exchange forum
IR	Infra red
k'	Retention factor
$k'_{ave,}$	Average retention factor
KNN	K-nearest neighbour
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
LDA	Linear discriminant analysis
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Lysergic acid diethylamide
MAOC-N	Maritime analysis and operations centre – narcotics
MDA	3, 4-Methylenedioxyamphetamine
MDEA	3, 4-Methylenedioxy-N-ethylamphetamine
MDMA	3, 4-Methylenedioxymethamphetamine
MBDB	N-methyl-1-1-(1, 3-benzdioxol-5-yl)-2-butanamine
mCPP	(1-(3-chlorophenyl) piperazine)
MEKC	Micellar electrokinetic chromatography
MLP	Multilayer perceptron
MS	Mass spectrometer
N	Efficiency
N_{ave}	Average efficiency
NACD	National advisory committee on drugs
NIR	Near Infra Red
NDST	National drugs strategy team
NPD	Nitrogen-phosphorus detector
NMR	Nuclear magnetic resonance
ODS	Octadecyl-bonded silica
PCA	Principal component analysis
PCP	Phenylcyclohexylpiperidine
PLS	Partial least squares

PMMA	*p*-methoxymethamphetamine
R	Resolution
R_{ave}	Average resolution
RBF	Radial base functions
RI	Refractive index
RP	Reversed phase
R_t	Retention time
RSD	Relative standard deviation
SEC	Size exclusion chromatography
SPE	Solid-phase extraction
SD	Standard deviation
SSRI	Selective serotonin reuptake inhibitors
THC	Tetrahydrocannabinol
TLC	Thin-layer chromatography
UN	United Nations
UNODC	United Nations Office of Drugs and Crime
UV	Ultraviolet
WCO	World Customs Organization
WHO	World Health Organisation

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List of Presentations and Publications

Publications

McFadden K., Gillespie E. (2011). The identification of abused counterfeit drugs. (*In prep*).

McFadden, K., Carney, B., O'Driscoll, D. (2011). An optimised method using experimental design and response surfaces for the chemical analysis of cocaine using high-performance liquid chromatography. Journal of Chromatography A (*In prep*).

McFadden, K., Carney, B., O'Driscoll, D. (2011). The statistical analysis of ecstasy tablets. Forensic Science International (*In prep*).

McFadden, K., Carney B. (2010). The use of monolithic columns for the analysis of drugs of abuse. In: *Monolithic chromatography and its modern applications*. (Wang P., ed). ILM publications, UK, pp 521 - 537.

McFadden, K., Gillespie, J., Carney, B., O'Driscoll, D. (2006). Development and application of a High Performance Liquid Chromatography method using monolithic columns for the analysis of Ecstasy tablets. Journal of Chromatography A, 1120 (2006) 54–60

Conference Presentations

McFadden, K. (2011). The use of monolithic columns in the analysis of drugs of abuse. Presented at Pittcon International Analytical Chemistry Conference 2011, 13th-18th March 2011, Georgia World Congress Center, Atlanta, Georgia, USA.

McFadden, K., Gillespie, J., Carney, B., O'Driscoll, D. (2005). The analysis of ecstasy tablets in Ireland by HPLC-DAD. The 29th International Symposium on High Performance Liquid Phase Separations and Related Techniques (June 2005), Stockholm, Sweden.

McFadden, K. (2004). Forensic analysis of ecstasy using liquid chromatography. Presented at the Institutes of Technology Science and Computing Research Colloquium, Waterford Institute of Technology, 26th – 28th May 2004.

McFadden, K. (2004). Forensic analysis of ecstasy using liquid chromatography: Investigation into the variation of MDMA tablets. Presented at the Irish Research Council for Science, Engineering and Technology symposium, Dublin November 2004.

McFadden, K. (2003). An investigation into the variation of MDMA in ecstasy tablets. Presented at the Irish Research Council for Science, Engineering and Technology symposium, Dublin November 2003.

Acknowledgements

I would like to express my sincere gratitude to my supervisors Dr. Brian Carney, Science Department, Letterkenny Institute of Technology and Dr. Dan O'Driscoll, Drug Section, Forensic Science Laboratory, Dublin, for their advice and their instruction over the course of study. I would like to express my gratitude to the Garda Forensic Science Laboratory, Dublin, for the provision of ecstasy and cocaine samples used in this study.

I would like to thank everyone at Letterkenny Institute of Technology for their help and friendliness throughout the research and in particular Mr. John Gillespie, for his guidance and assistance with all matters chromatography related and beyond. Thanks to Mr. Ken McIntyre for the cups of coffee, banter and unlimited bottles of methanol, and of course his invaluable technical support. A special thanks to my travel partner, Dr. Eoin Gillespie, your assistance in the final stages of my research is gratefully acknowledged. To all my friends and colleagues at CAMBio that I have had the pleasure of getting to know since starting this project.

Thanks to Dr. David Kennedy and Dr. Jonathan Campbell (RIP) at Letterkenny Institute of Technology and Dr. Niamh Nic Daeid, University of Strathclyde, Glasgow for all their assistance with statistics

I would also like to acknowledge the financial support for this project that was provided by the Irish Research Council for Science, Engineering and Technology (IRCSET) funded under the National Development Plan 2000 – 2006.

Special thanks to my family for their encouragement and support. Finally to Rory, Daniel and Niall, thank you for everything.

Chapter 1: Literature review

1.1. Introduction.

Illegal drugs remain a dominant social issue. Drug related deaths are on the rise, crime levels are escalating and seizures of drugs are at record levels. It is a major concern globally for all law enforcement agencies. To conquer this ever growing drug problem, efforts have been made at government level, both nationally and internationally, to curtail the production, trafficking and distribution of illegal drugs. Tighter controls at ports and borders, reduction of cultivation fields in Columbia and Afghanistan and establishing international drug intelligence agencies are a few measures made to control the drug situation.

One key area identified was the importance of background intelligence concerning drug seizures. Drug characterisation studies have shown that it is possible to link samples, to classify material from different seizures into groups of related samples and to identify the origin of such samples (United Nations International Drug Control Programme, 2001). In 1996, the Commission on Narcotic Drugs (United Nations) acknowledged the need for united international policy in the field of drug characterisation and impurity profiling. At European level, the European council launched a strategic plan, the EU Drugs Strategy (2005-2012) on how to 'solve' the drug problem (Council for the European Union, 2004). Within this, they recognised that the reduction of the supply of drugs was a key factor. Central to this was the proposal to "adopt and implement and EU wide system for the forensic profiling of drugs". In Ireland, policy makers had a more general view on this and it is documented in the 'National Drugs Strategy 2009 – 2016' (Department of Community, Rural and Gaeltacht Affairs, 2009). In this, one of the five strategic aims is "To create a safer society through the reduction of the supply and availability of drugs for illicit use", in which an objective is "To disrupt the activities of organised criminal networks involved in the illicit drugs trade in Ireland and internationally and to undermine the structures supporting such networks". One other strategic aim is "To ensure the availability of accurate, timely, relevant and comparable data on the extent and nature of problem substance use in Ireland" and within this, an objective is "To ensure the availability of data to accurately inform decisions on initiatives to tackle problem substance use". In order to meet these objectives, research on the drug situation in this country is essential.

1.2. Drugs of abuse.

The variety of illegal drugs currently available in the *'market place'* can be categorized into four major pharmacological groupings. Narcotics include opiates such as opium, morphine and heroin. Depressants are sedatives that provide relaxation from anxiety and include barbiturates and benzodiazepines. Stimulants such as cocaine and amphetamines increase the actions within the central nervous system resulting in intoxication and often aggressive behaviour. Finally, hallucinogenic agents provide a sense of euphoria to users and include LSD (lysergic acid diethylamide), amphetamines (ecstasy) and cannabis. Regardless, all categories are considered illegal when sold on the *'street market'* and are totally unregulated and have limited medical benefit. Forensic analysts are therefore faced with the challenge of continually developing sophisticated methods of analysis to struggle with the increasing variability that occurs in '*street samples'*.

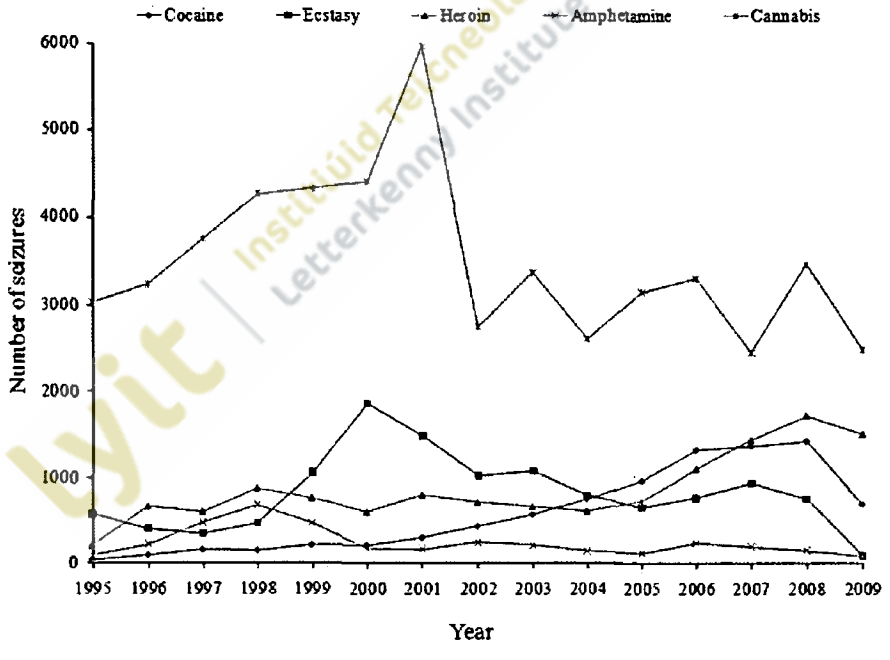


Figure 1.1. Number of seizures of cocaine, ecstasy, heroin, amphetamine and cannabis in Ireland, years 1995 – 2009. (Source: EMCDDA Annual Report, 2010).

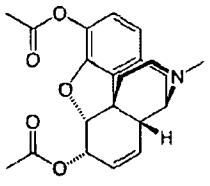
Cannabis has been long established as the most popular drug of abuse among young adults. Until recently amphetamine and its relative designer drugs, such as ecstasy, were the most widespread after cannabis in European and Irish illegal market (Figure 1.1). Since 2000, cocaine and heroin have overtaken ecstasy as the second most abused illicit drug which is a trend confirmed by the European Monitoring Center for Drugs and Drugs Addiction (EMCDDA Annual Report, 2008). National surveys by the National Advisory Committee on Drugs (NACD Annual Report, 2007) indicate that drug use is on the increase in Ireland. Illegal drug use during this time period increased from 18.5% to 24%. The prevalence of cocaine has risen from 3% to 5.3% and ecstasy from 3.7% to 5.4%. These figures are higher that the European average, with 3.6% for cocaine and 2.8% for ecstasy abuse.

1.2.1. Narcotics.

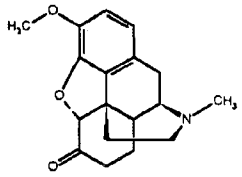
A narcotic is a term that describes a drug that produces narcosis, unconsciousness or sleep. Included in this group of drugs are opiates or any opiate derivative. The opiates are a large group of compounds that are produced from opium, which is found in the poppy *Papaver somniferum*. Opium itself contains morphine, thebaine, codeine and papaverine, from which heroin and hydrocodone can be synthesised.

First synthesised in 1874, heroin is the 3,6-diacetyl ester of morphine, and is chemically known as 'Diacetylmorphine' (Figure 1.2). Most commonly injected, heroin is a painkiller and produces euphoria. Street heroin is typically a brown powder, less commonly white, and likely to be produced in Afghanistan. In 2006, purity levels ranged from 15% to 25% for the brown powder and 45% to 70% for the white powder (EMCDDA Annual Report, 2008). Hydrocodone (Figure 1.2) was first produced in Germany in 1920 from codeine and thebaine. It is a narcotic analgesic and has a high abuse risk. It is usually found in combination with acetaminophen or ibuprofen in tablet or syrup form. Commercially it has many trade names, eg. 'Vicodin'. Oxycodone is another semi-synthetic opioid with high abuse potential, which is derived from codeine.

Narcotics

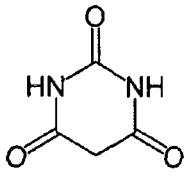


Heroin

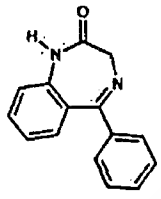


Hydrocodone

Depressants

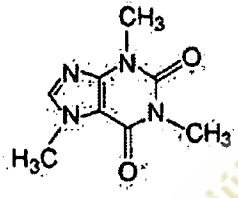


Barbituric Acid

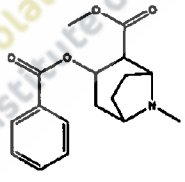


Benzodiazepine

Stimulants

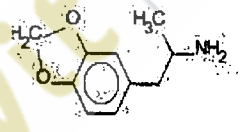


Caffeine

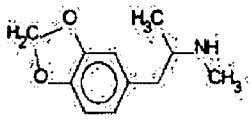


Cocaine

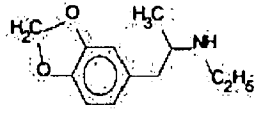
Hallucinogens



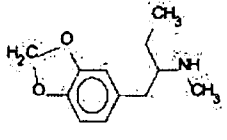
MDA



MDMA



MDEA



MBDB

Figure 1.2. Chemical structures of narcotics, depressants, stimulants and hallucinogens.

1.2.2. Depressants.

Depressants are drugs that act as a sedative on the central nervous system. They reduce an individual's capabilities by increasing the activity of gamma-aminobutyric acid (GABA). The most common legal depressant is alcohol. Alcohol is one of the most common drugs of abuse. Ethanol is the active drug, which is classified as "psychoactive", meaning it acts on the central nervous system, altering brain function. The category of alcohol can be divided into beers, wines and spirits. Alcohol content ranges from 4% to upwards of 40% and production of alcohol is not limited by geography.

Common illegal depressants include barbiturates and benzodiazepines (Figure 1.2). Barbiturates are a group of depressants derived from barbituric acid. They are mostly used as a hypnotic and an anticonvulsant. Recently they have been largely replaced by benzodiazepines owing to the less potential of a lethal overdose. Benzodiazepines are a group of drugs that are similar to barbiturates in medical use (hypnotic, anticonvulsant, sedative) and they have also been used for withdrawal from alcohol and other drugs. They are produced most commonly as tablets and capsules. The most popular benzodiazepine is diazepam or 'Valium'. More recently, sexual assaults have been reported to use flunitrazepam or 'Rohypnol' as a sedative before the assaults. Fluoxetine hydrochloride, or 'Prozac' is the third most commonly prescribed antidepressant in the United States, after sertraline or 'Zoloft' and escitalopram or 'Lexapro'. These are known as 'selective serotonin reuptake inhibitors' (SSRIs) and are the standard drugs prescribed for depression. Because of their effects, these antidepressants are commonly abused.

1.2.3. Stimulants.

Stimulants increase activity in the central nervous system. They boost consciousness, produce euphoria, and raise alertness. The more common legal stimulants include nicotine and caffeine. Nicotine is a chemical found in tobacco, the average cigarette contains 1 mg nicotine. This highly addictive drug is thought to combat anxiety and stress, help concentration and reduce appetite. Caffeine is a psychoactive drug and chemically is a bitter white crystalline xanthine alkaloid (Figure 1.2). It is naturally

occurring in many plants, but primarily found in the cocoa bean. It is both legal and unregulated.

Cocaine is a semi-synthetic drug derived from the leaves of the cocoa plant and is the more popular illegal stimulant. Its chemical name is benzoylmethyl ecgonine (Figure 1.2) and it is a crystalline tropane alkaloid. It is categorised as a stimulant; more specifically it is a dopamine reuptake inhibitor, a noradrenalin reuptake inhibitor and a serotonin reuptake inhibitor. Due to its addictive nature, cocaine has always been a popular street drug and has overtaken ecstasy as the second most popular drug of abuse in Europe (EMCDDA Annual Report, 2008).

1.2.4. Hallucinogens.

Hallucinogens are a group of drugs that change the state of mind of the user to such an extent that reality is altered. Common members include cannabis and Lysergic Acid Diethylamide (LSD). Cannabis is the most common illegal drug in Europe today. Unlike the opiates and cocaine where production is concentrated in the one geographical area, *Cannabis sativa* L. can be grown in many environments. The principal active ingredient in cannabis is Δ^9- tetrahydrocannabinol. It exists in three different physical forms; cannabis resin, herbal cannabis and the less common cannabis oil. In many countries, cannabis is seen as a 'soft' drug, and legal prosecutions can vary from fines to cautions. Lysergic Acid Diethylamide (LSD) is a semi synthetic hallucinogen. Produced as a sheet of absorbent paper, users tear off tiny squares for their dose. This drug is not as popular as it was in the 1960's and 1970's, and the Drug Enforcement Administration (DEA) reported seizures had declined 100% from 2000 to 2005.

Amphetamine – Type – Stimulants (ATS) is a large group of synthetic compounds of which amphetamine is the chief drug. ATS can be categorised as either stimulant or hallucinogen due to their wide and varied psychological effects, and their ultimate classification is dependent on the scientist. In total, this group comprises amphetamine, methamphetamine and all ring-substituted analogues (Figure 1.2), however, new members are frequently being synthesized. These drugs act by increasing levels of norepinephrine, serotonin, and dopamine in the brain.

Amphetamine was first synthesized in 1887 in Germany. Its chemical name is *N*,α-methylbenzeneethanamine. It is a white or off-white powder but can appear in tablet form. Purity levels range from 2% to 47% in powders and up to 40 mg in tablets (EMCDDA). It is legally used in the treatment of attention-deficit disorder (ADD) and attention-deficit hyperactivity disorder (ADHD). Brand names of the drugs that contain amphetamine include 'Adderall', 'Vyvanse', and 'Dexedrine'. Methamphetamine was first produced in Japan in 1919. Its chemical name is *N*,α-dimethylbenzeneethanamine. Like amphetamine, it is found as a white powder or in tablet form. Purity levels range from 20% to 55% in powders and up to 40 mg in tablets. It has also been used as an adulterant in ecstasy tablets and has some limited medical use.

The most common member of the ATS group is 3,4 -methylenedioxy- *N*-methylamphetamine (MDMA), or 'Ecstasy', and is categorized as a stimulant and is mildly hallucinogenic. Initially produced by Merck in 1912 it did not become popular until the 1970's. Other analogues found in tablets are MDA (3,4-methylenedioxyamphetamine), MDEA (3,4-methylenedioxy-*N*-ethylamphetamine) and MBDB (*N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine). Among the newer members of this ATS group are DOB (4-bromo-2, 5-dimethoxyamphetamine), 2C-B (4-bromo-2,5-dimethoxyphenethylamine) and DOM (4-Methyl-2,5-dimethoxyamphetamine), knowledge of whom are limited and research is ongoing.

1.2.5. Ecstasy.

Ecstasy is the generic name given to 3, 4 methylenedioxymethamphetamine (MDMA) by the World Health Organisation (WHO, 1997). MDMA is a ring substituted phenylethylamine with a methamphetamine backbone (Figure 1.2). The stereochemistry of MDMA is very important pharmacologically. The synthetic production of these compounds yield racemic mixtures. The (+) enantiomer is more stimulating and more neurotoxic than the (-) enantiomer. The difference in potency of the two isomers may vary three to ten fold (Nichols & Glennon, 1984). Pure MDMA is a white crystalline solid with a bitter taste. As a salt, the compound is chemically stable and does not decompose in heat, air or light. MDMA is usually taken orally as a tablet or capsule but may be found in powder or liquid form. It is readily absorbed

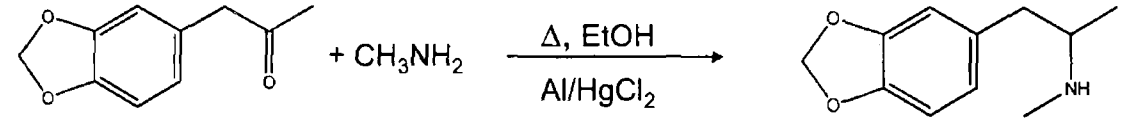
from the gastrointestinal tract into the bloodstream. More rarely, the drug is snorted, smoked or injected.

Ecstasy seizures in Ireland were at their peak in 2003 and have since declined (Figure 1.1). In Europe, 2002 was the peak, 2003-2005 seizures were stable and since then, they have declined. Ecstasy typically sells for €3 – €15 per tablet (EMCDDA National Report, 2007).

The production of synthetic drugs is not limited by geography or climate, as it is with cocaine and heroin. The Netherlands is the main country in which illicit amphetamine and ecstasy is produced. During the 1960's, the provinces in Southern Netherlands were known for large scale amphetamine production. Since the 1990's, this region continues to be used, though the focus has switched from amphetamine to ecstasy production. The major production of MDMA is reported in the Czech Republic, Estonia, France, Poland, Spain and the UK (EMCDDA Annual Report, 2003). However, production facilities have been discovered in South East Asia, China, North America, South Africa and South America (EMCDDA Annual Report, 2003). Over twenty recipes for MDMA production have been described in the literature, but only three are common, the reductive amination route, the safrole bromination route and the Leuckart reaction (Figures 1.3. to 1.6.).

1. The reductive amination route

This involves a reductive amination of a ketone at a higher temperature. Various reducing agents may be used, such as $Al/HgCl_2$, $NaBH_4$ (using low temperatures), and $NaBH_3CN$.



3, 4-(Methylenedioxy) phenyl-2-propanone

MDMA

Figure 1.3. The reductive animation route for the synthesis of MDMA.

3, 4 – (Methylenedioxy) phenyl – 2 – propanone is a controlled precursor, so chemists devised an alternative synthesis process which involved only non-controlled precursors. Safrole is known to be a common precursor in MDMA synthesis and is typically used to synthesise 3, 4 – (Methylenedioxy) phenyl – 2 – propanone.

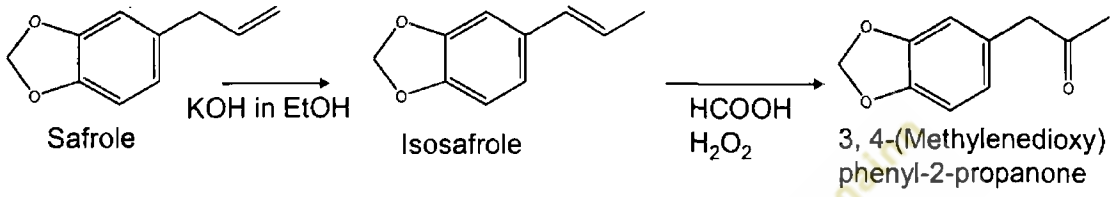


Figure 1.4. The synthesis of 3, 4 – (Methylenedioxy) phenyl – 2 – propanone using safrole as a precursor.

2. The safrole bromination route.

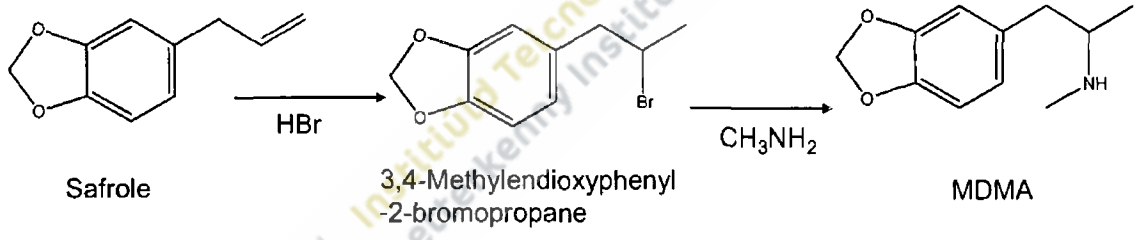


Figure 1.5. The safrole bromination route for the synthesis of MDMA.

Here, the safrole is reacted with hydrobromic acid to form 2 – bromosafrole, which in turn produces MDMA.

3. The Leuckart reaction

This is a popular synthesis method for amphetamine, but is less frequently used for the production of MDMA. $LiAlH_4$ can also be used as an alternative to H_2SO_4/HCl.

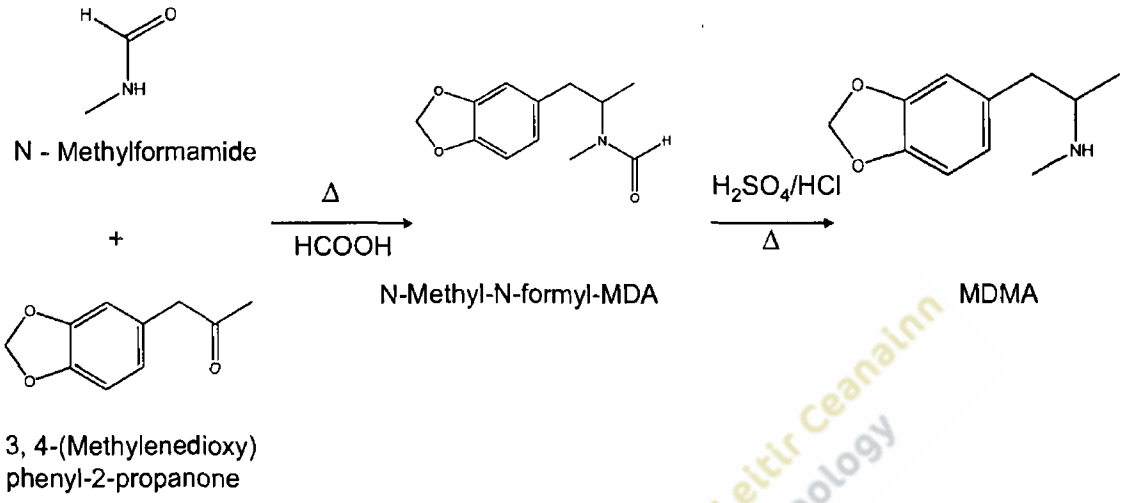


Figure 1.6. The Leuckart reaction route for the synthesis of MDMA.

MDMA is often produced as the hydrochloric salt and in tablet form. In addition to the main amphetamine ingredient tablets will also contain excipients, adulterants, adhesives or colourants.

Excipients are used by the producer to give sufficient bulk to the tablet. Excipients include binders, lubricants, disintergrants, and colourants and flavouring agents, each of which has a different function in the overall process of tablet manufacture and subsequent tablet disintegration in the body. Not every additive will necessarily be present in any given dosage and the amount may vary. In the conventional pharmaceutical industry, common excipients used are sucrose, lactose, sodium hydrogen carbonate and calcium phosphate. Clandestine manufacturers often use cheaper, more readily available excipients. Common excipients include cellulose, starch, glucose, talc and stearate salts.

Adulterants are compounds that are added to the tablet to mimic or enhance the effect of the active ingredient. These also add bulk to the tablet and can also be used to trace sources. Many tablets that are sold as ecstasy may be mixed with a variety of stimulant or hallucinogenic substances such as amphetamine sulphate or LSD. Other

amphetamine compounds such as MDA, MDEA and MBDB are commonly present. Examples of adulterants include caffeine, acetaminophen, phenacetin and acetylsalicylie acid (Figure 1.7). Adhesives also referred to as binders, are added to the powdered ingredients to increase their cohesive strength. These are usually added in concentrations between 1% and 10%. Formulations of clandestine manufacturers are likely to contain a natural gum or starch, which in paste form can function as an adhesive.

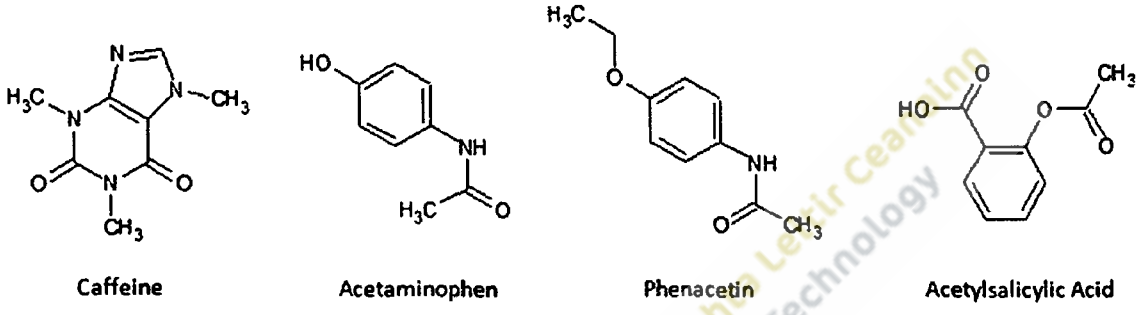


Figure 1.7. Chemical structures of the typical adulterants associated with ecstasy tablets.

Seized tablets are mainly white in colour, some are mottled or coloured. Examples of reported colours include blue, pink, green and yellow. Reasons for the addition of colorant are to increase the aesthetic appeal and distinguish product batches from others. The final colour of MDMA may change from white for the pure compound to pink or cream/yellow in the presence of by-products associated with the starting materials. Ecstasy tablets differ at this point from many other drugs of abuse. As it is in tablet form, no further excipients can be added at the different stages of trafficking. This is an essential point for profiling and linking seizures. Only those chemicals added at manufacture can be present at street level.

Producers want to differentiate their product from others by imprinting the tablets with logos or symbols, this promotes brand awareness and customer loyalty (Figure 1.8). When one 'brand' of tablets gains popularity, other laboratories will utilise the same logo punch to gain market share. It is reported that some clandestine laboratories can tailor drug combinations and customise logos for regular customers.

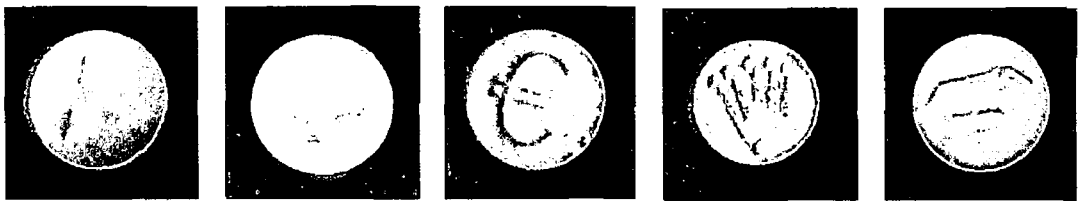


Figure 1.8. A selection of seized ecstasy tablets with different logo imprints.

1.2.6. Cocaine.

Due to its addictive nature, cocaine has always been a popular street drug, but recently it has overtaken ecstasy as the second most popular drug of abuse in Europe (EMCDDA Annual Report, 2007). Cocaine is extracted from the leaves of the coca plant *(Erythroxylon coca)*. It was originally used in South America in the mid-19th century by natives of the region to relieve fatigue. Pure cocaine (cocaine hydrochloride) was first used as a local anaesthetic for surgeries in the 1880's and was the main stimulant drug used in tonics for treatment of various illnesses in the early 1900's. The chemical name is benzoylmethyl ecgonine and it is a crystalline tropane alkaloid. It is catagorised as a stimulant; more specifically it is a dopamine reuptake inhibitor, a noradrenalin reuptake inhibitor and a serotonin reuptake inhibitor.

Cocaine is a white crystalline solid, hygroscopic in nature, odourless and has a bitter taste (Figure 1.2). It has a melting point of 197 °C. Cocaine most often appears as a white crystalline powder or an off-white chunky material. Crack cocaine typically is available in rock form. 'Street' cocaine may be contaminated accidentally during the preparation process or may be adulterated intentionally by a number of compounds in order to dilute the amount of cocaine used and increase profits. Examples of adulterants include caffeine, phenacetin, lidocaine, procaine, piracetam, and prilocaine (Barrio *et al.* 1997, Bernado, 2003, Lopez-Artiguez *et al.* 1995, Fucci & DeGiovanni, 1998)(Figure 1.9). Common excipients detected in cocaine street samples include starch, sugars, carbonates and bicarbonates (Bernado, 2003, Lopez-Artiguez *et al.* 1995).

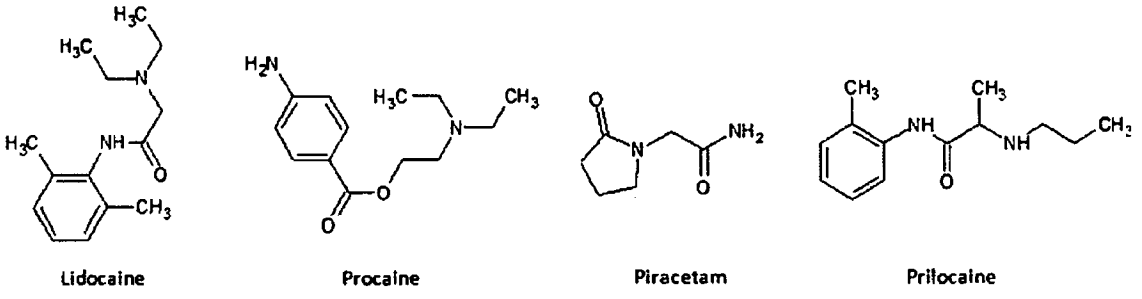


Figure 1.9. Chemical structures of the typical adulterants associated with cocaine.

Crack cocaine is derived from powder cocaine. The powder cocaine is dissolved in a solution of sodium bicarbonate and water. The solution is boiled and a solid substance separates from the boiling mixture. This solid substance, crack, is removed and allowed to dry. The crack cocaine is then broken or cut into "rocks," each typically weighing from one-tenth to one-half of a gram. One gram of pure powder cocaine will convert to approximately 0.89 grams of crack cocaine. It is estimated that crack rocks are between 75% and 90% pure cocaine.

The bulk of cocaine is produced in Colombia, Bolivia and Peru, with the traditional clandestine manufacturing process illustrated in Figure 1.10. Colombian drug organisations are responsible for producing 80% of the world's cocaine. Trafficking organisations targeted countries in Eastern and Southern Europe as stopovers for both sea and air consignments filled with large quantities of cocaine which were ultimately destined to Western Europe. The Americas made over 75% of the 2001 cocaine seizures, Western Europe around 13% and the rest of the world around 12% making Europe the second-largest cocaine market. Demand is estimated at between 120 and 150 tons per year and is on the increase. The countries of the European Union seized around 56 tons of cocaine in 2001. Spain seized 33 tons of cocaine in 2001, which is more than Mexico and almost 60% of the total quantity seized in Western Europe (EMCDDA Annual Report, 2007).

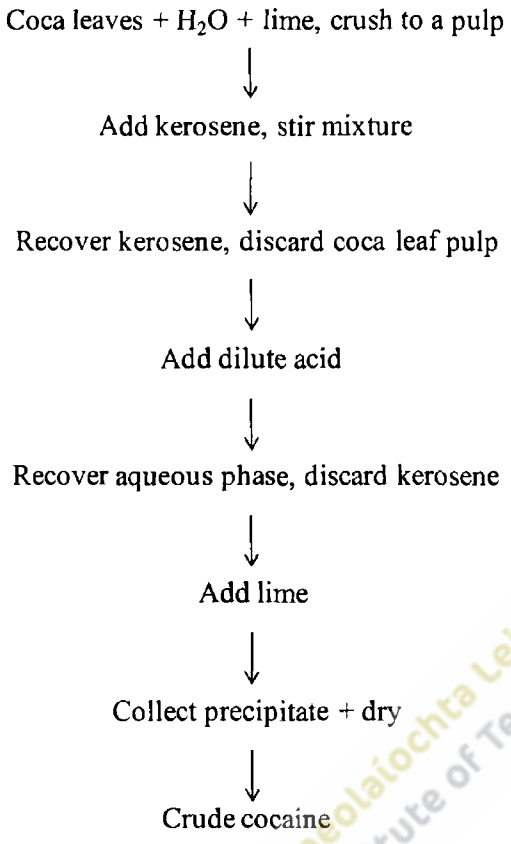


Figure 1.10. Flow diagram of each step involved in the clandestine manufacture of cocaine.

In Europe, average price for cocaine is €50 - €80 per gram (EMCDDA Annual Report, 2007). Only anecdotal information is available for the street price of drugs in Ireland. Cocaine has been sold for €30 - €40 for a half gram and crack for €50 a rock (NACD Annual Report, 2007). An overall decrease in purity was seen in cocaine in Europe from 2000-2005, with most countries reporting 30 – 60% purity (EMCDDA Annual Report, 2007). It is thought that seizures have not affected the price of cocaine but has affected the purity. Cocaine seizures have been on the rise since 2001 in Ireland, which would comply with the trend in Europe (EMCDDA Annual Report, 2010).

1.3. Current analytical techniques for forensic drug analysis.

Various spectrometric and chromatographic methods are available for both routine and forensic drug analysis. The main objective for the analytical chemist has been to introduce new, versatile techniques with high efficiency, selectivity and precision. In this section, analysis of drugs of abuse using techniques such as Infrared spectroscopy (IR), Raman spectroscopy, Nuclear Magnetic Resonance (NMR), Thin Layer Chromatography (TLC), Capillary Electrophoresis (CE), Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) are presented and their applications in the analysis of illicit drug and biological samples are reviewed.

1.3.1. Spectrometric methods.

IR spectroscopy is a long established method of analysis. The non-destructive nature of this method added with its economy of time and lack of organic solvent usage appeals to a wide variety of analysts. Sondermann and Kovar (1999) demonstrated the potential use of this technique in screening experiments for MDMA, MDEA and amphetamine in ecstasy street samples. Schneider and Kovar (2003) found IR analysis in transmission mode to be better than diffuse reflectance when analysing ecstasy tablets. IR has been used to chemically differentiate between cellulose and lactose and then applied to a number of ecstasy samples in order to differentiate between producers (Baer *et al.* 2007). A portable version of IR spectroscopy has been developed for onsite identification of ecstasy (Tsujikawa *et al.* 2008) and the contents of a clandestine laboratory for cocaine production were analysed using IR (Gostic & Klemenc, 2007). Infrared spectroscopy has been limited to identification of adulterants and excipients in cocaine samples (Lopez-Artiguez *et al.* 1995).

Raman spectroscopy is a simple, non-destructive, semi-quantitative technique that allows for rapid screening of samples and as such has become a popular technique in the forensic analysis of drugs of abuse. Bell *et al.* (2000a, 2000b and 2003) have carried out numerous studies using this technique. In 2000, initial studies demonstrated the rapid drug screening potential of Raman spectroscopy and also distinguished between geometrical isomers of MDEA and MBDB and different hydrated forms of the same drug (Bell *et al.* 2000a). Later in 2000, the group analysed 400 ecstasy tablets for their active ingredients and excipients and concluded

that the composition of the seized tablets varied significantly and even batches with similar physical appearance could not only have different active compounds but also vary in the nature of the excipients, the drug to excipient ratio and degree of MDMA hydration (Bell *et al.* 2000b). Further studies by Bell *et al.* (2003), demonstrated the potential of Raman spectroscopy as a rapid technique in the screening of a large sample number (1,500 ecstasy tablets) in characterising ecstasy tablets according to Raman composition profiling and concluded that it may be possible to recognise batch-to-batch variation in the 'signature' of tablets prepared by major manufacturers.

Other studies include the use of Raman spectroscopy in the analysis of cocaine and heroin (Ryder *et al.* 1999). The technique has also been applied to the detection of drugs of abuse from different materials and includes: MDMA in clothing (Ali *et al.* 2008), cocaine in beverages (Eliasson *et al.* 2008) and codeine, cocaine, amphetamine, barbital and nitrazepam in fingerprints (Day *et al.* 2004). Milhazes *et al.* (2007) applied the technique in the examination of synthetic precursors associated with MDMA. One of the newer members of the 'ecstasy' group, 4-bromo 2, 5 dimethoxtamphetamine (DOB) was screened using surface enhanced Raman spectroscopy (Bell *et al.* 2007). Raman has also been used as a detector for an LC method for the analysis of cocaine, heroin, amphetamine, papaverine and procaine (Sagmuller *et al.* 2003).

A more modern spectroscopic method is nuclear magnetic resonance (NMR). The ^{1}H and ^{13}C chemical shifts of NMR can provide information on the environment of the nuclei being analysed. This technique allows for differentiation between geometrical and positional isomers and also between salts and free bases of drugs. This method is also non-destructive and can be used quantitatively. However, its lack of sensitivity and the initial cost of the instrument limit this method to specific research rather than routine toxicological analysis. Molecular conformational studies using NMR were used to characterise cocaine and some of its derivatives (Airaksinen *et al.* 1999). Carter *et al.* (2002) used NMR to demonstrate its potential to study both the precursors and synthetic pathways of MDMA in order to link common batches of ecstasy tablets. A method for the analysis of excipients and impurities in ecstasy was proposed by Lee *et al.* (2000) who then further developed the method to allow for the

quantification of the percentage carbon attributed to MDMA. Billault et *al.* (2007) used NMR to study the different synthetic routes of MDMA. A new ecstasy analogue, 2-chloro-4, 5-methylenedioxymethamphetamine, was identified using the same ^{1}H NMR technique (Lewis *et al.* 2000). More recently, Zapata-Torres *et al.* (2008) used ^{1}H NMR and X-ray diffraction studies to determine different conformations of MDMA.

1.3.2. Chromatographic methods.

Low cost, speed, simplicity and its capability of simultaneous drug detection are the main advantages of thin layer chromatography (TLC). Samples are loaded onto a stationary phase, most frequently silica gel, and then the specific solvent system (mobile phase) is allowed to move through the stationary phase. The interaction of the compounds in the sample with the mobile phase allows for the separation of the sample and subsequent identification. This would be considered an inexpensive and basic technique and is typically used as a preliminary screening procedure. Within the last ten years few research articles on drugs of abuse using TLC method have been reported. Reports include the identification of adulterants in Brazilian cocaine (Bernardo, 2003) and the separation of opiate alkaloids and its derivatives (Pothier & Galand 2005). Kochana *et al.* (2003(a)) studied the markers of the Leukart production of p-methoxymethamphetamine (PMMA) by solid phase extraction (SPE) followed by TLC. In 2003, they used SPE-TLC for impurity profiling of intermediates in the synthesis of MDMA (Kochana *et al.* 2003(b)). In 2006, the same technique was applied to examine agglutinants, excipients and adulterant impurities of MDMA (Kochana *et al.* 2006).

The term 'capillary electrophoresis' describes a family of related techniques in which separations are carried out in narrow bore capillaries under the influence of an electric field with separation of analytes based on their electrophoretic mobilities. The exceptional power of separation and resolution, rapid analysis time, low mass detection limits, economy of reagents, and minimum sample requirements make CE an attractive methodology for forensic laboratories. The potential of this technique for forensic analysis was first demonstrated in 1991 by Weinberger and Lurie, who applied it to the analysis of a wide range of illicit drugs in synthetic mixtures

(Weinberger & Lurie 1991). Piette and Parmentier (2002) successfully separated seven amphetamines associated with ecstasy within eight minutes without interference from adulterants using CE and suggested that the method was an attractive alternative to GC–MS for the qualitative and quantitative determination of amphetamine and related compounds. Similarly, a chemometric approach was employed by Dahlen and Von Eckardstein (2006) for the development of a CE method to separate amphetamine and 13 amphetamine analogues. Complete separation was achieved in 23 minutes; however the authors did offer an alternative CE method which allowed for the separation of the more common amphetamines with cocaine and heroin in less than seven minutes.

The applicability of capillary electrophoresis (CE) for the purpose of drug classification was examined in the analysis of methamphetamine impurities (Iwata *et al.* 2006). Two possible precursor impurities, namely ephedrine and pseudoephedrine were detected and quantified allowing for seized drugs to be classified into three groups based on the contents of the two impurities. Chiral separations, i.e. the separation of a racemic mixture into enantiomers, using CE were accomplished by Huang *et al.* (2003). In this study the R-(-)-isomers and S-(+)-isomers of MDA and MDMA were identified in seizures of tablets and urine samples. Other applications of CE in forensic drug analysis include investigations of adulterants in street cocaine (Ishii *et al.* 2001) and impurity profiling of heroin (Lurie *et al.* 2001). A method for the separation of cocaine, opiates and amphetamines (10 compounds in total) was developed using a cyano stationary phase with capillary electrochromatography (Aturki *et al.* 2009). CE has also been extensively used in toxicological analysis, including hair (Gottardo *et al.* 2007), plasma (Boatto *et al.* 2007) and urine (Lin *et al.* 2006, Tsai *et al.* 2006).

Gas chromatography (GC) is one of the more extensively used separation techniques in the area of drug analysis. High sensitivity and excellent reproducibility has lead to the popularity of this technique; however the analyte of interest must be volatile and thermally stable. The variety of detectors associated with GC is an additional benefit for this technique. The flame ionization detector (FID) has been used regularly in the area of ecstasy profiling (Dayrit and Dumlao, 2004, Kuwayama *et al.* 2008,

Mitrevski *et al.* 2005, Puthaviriyakorin *et al.* 2002, Rashed *et al.* 2000). Dujourdy *et al.* (2008) used GC-FID to analyse cocaine samples for residual solvents that may be present and a cross border study between France and Switzerland used GC-FID to compare cocaine samples (Lociciro *et al.* 2007). GC in combination with mass spectrometer (MS) detector is considered one of the more popular tools for analysis. GC-MS has been used for the general analysis of drugs of abuse (O'Connell *et al.* 2000, Shin *et al.* 1996, Whiting *et al.* 2001) and in impurity profiling of ecstasy tablets (Cheng *et al.* 2006, Gimeno *et al.* 2002, Gimeno *et al.* 2003, Palhol *et al.* 2002, Swist *et al.* 2005). 156 street samples of cocaine in Rome were analysed for their adulterant content (Fucci *et al.* 1998). The harmonisation of MDMA profiling methods is a project undertaken by the European Commission, titled "Collaborative Harmonisation of Methods for Profiling of Amphetamine Type Stimulants" (Weyermann *et al.* 2008). Several researcher groups from different countries (Switzerland, France, The Netherlands, Finland, Germany, Czech Republic and the UK) have analysed the physical characteristics, chemical composition and organic profile of MDMA tablets. The preferred chosen method of analysis by this group for MDMA organic profiling was GC-MS.

GC lends itself to a wide variety of applications, such as testing the nails of newborns for monitoring *in utero* drug exposure (Mari *et al.* 2008), analysis of blood from drivers under the influence of drugs for cocaine and cocaine metabolites (Jones *et al.* 2008), and everyday drug profiling (Dujourdy & Besacier, 2008, Gröger *et al.* 2008, Dujourdy *et al.* 2008, Lociciro *et al.* 2008, Lock *et al.* 2007). Analysis of the newer designer drugs have been well documented (Ewald *et al.* 2007, Ewald *et al.* 2008, Theobald *et al.* 2007, Sauer *et al.* 2008). The chemical profiling of heroin utilised GC to classify different heroin samples (Esseiva *et al.* 2005). A supported liquid-liquid extraction (LLE) method for ecstasy tablets was developed by De Korompay *et al.* (2008). In this study, LLE was used to extract MDMA and was subsequently analysed by GC-MS and isotope ratio mass spectrometry (IR-MS). Furthermore, the application of GC in toxicological analysis for drug metabolites in hair (Kikura-Hanajiri *et al.* 2007, Wu *et al.* 2008, Lee *et al.* 2008), urine (Kim *et al.* 2008, Marchei *et al.* 2008, Brown *et al.* 2007, Lin *et al.* 2006), whole blood and serum (Gunnar *et al.* 2007, Kankaanpää *et al.* 2007, Ishida *et al.* 2005), meconium (Marin

et al. 2007, Salem *et al.* 2001), brain tissue (Stimpfl & Reichel 2007) and sweat (Huestis *et al.* 2008, De Martinis *et al.* 2007, Fucci *et al.* 2008).

Limitations associated with GC and TLC are overcome by the use of High Performance Liquid Chromatography (HPLC). HPLC is not limited by volatility or thermal stability as analysis is performed at room temperature. In addition, HPLC utilises a liquid mobile phase, which contributes to the separation process, making it the most widely used separation technique. Also, developments in columns, high-pressure constant delivery pumps and more sensitive detectors have contributed to its versatility. As such, HPLC is an integral analytical technique in the area of forensic drugs analysis.

The use of HPLC for the analysis of street samples has been limited. Makino *et al.* (2003) used HPLC to analyse a number of tablets on the Japanese market, more specifically, they used GC-MS to identify the different components and then LC to quantify them. Sadeghipor *et al.* (1997a, b) used LC in combination with UV and fluorescence for each of the studies. Using HPLC-UV, baseline separation was achieved for six amphetamines and four adulterants in less than eight minutes. The method was validated and application was made to eight tablets. Four amphetamines were separated using HPLC- fluorescence and the sensitivity of the fluorimetric detector was highlighted by applying the method to serum samples. HPLC-DAD was used for the study made by Cole *et al.* (2002). In this study, the chemical components of 136 ecstasy tablets were identified. Liquid chromatography with diode array detection has been used for the determination of the major alkaloids such as noscapine, papaverine, acetylcodeine, codeine, morphine, 6-monoacetylmorphine, 3-monoacetylmorphine and diacetylmorphine (Collins *et al.* 2006). In addition, this technique has been used extensively in toxicological analysis; samples include hair (Cairns *et al.* 2004, Kronstrand *et al.* 2004), urine (Namera *et al.* 2008) plasma (Concheiro *et al.* 2006), oral fluid (Concheiro *et al.* 2007, Wood *et al.* 2005) and whole blood (Chèze *et al.* 2007).

A newer development associated with both GC and LC is tandem mass spectroscopy detector. Here the fragments generated by the first MS are broken down further for a

clearer result of what the initial compound was. LC-MS/MS has been successfully applied to the separation of 14 abused drugs, including amphetamine and MDMA, in less than 2.5 minutes (Pihlainen *et al.* 2003), amphetamine, methamphetamine, MDMA and MDA in urine (Andersson *et al.* 2008) and ten amphetamine related compounds in meconium (Kelly *et al.* 2008).

1.4. Fundamentals of High Performance Liquid Chromatography.

The goal and purpose of liquid chromatography is to physically resolve the individual components of a sample from each other so that they may be quantified and/or identified. A high performance liquid chromatography instrument has a number of components, including a solvent reservoir, a pump, a sample injection system, a column and a detection system. The mobile phase (solvent) is a liquid used to carry the sample through the column (where separation occurs) and on to the detector (where data is generated). The choice of the mobile phase is dependent on the type of chromatography to be used and because of this, mobile phase can be used as a tool in the optimisation process. A high pressure pump is used to push the mobile phase and sample through the system and is capable of delivering a particular flow rate at high pressures, e.g. a flow rate of 2 mL min^{-1} at a pressure of 125 bar. When the sample is introduced into the system, it is carried by the mobile phase onto the column. It is here that separation occurs. Columns are typically steel tubes with a variety of length, diameters and packing materials. Again, the choice of stationary phase is based on the type of compounds to be separated. Detection systems are located after the column. When the separated compounds leave the column, they pass through the detector, where individual signals are recorded for each compound.

1.4.1. Chromatographic interactions.

The type of chromatography dealt with in this study is reversed phase chromatography. This essentially means a polar mobile phase is used with a non polar stationary phase and separation of the sample is based on the hydrophobic interaction of the analyte between each of the phases. The analyte is attracted to the hydrophobic surface of the particle in the stationary phase. Polar interactions, primarily hydrogen bonding, are an important secondary retention mechanism. The level of hydrophobic and polar interaction forces between solute and stationary phase will determine the

overall analyte retention time. Ion exchange interactions sometimes occur between ionisable silanols and positively charged basic groups. Some silanols ionise, gaining a negative charge. Chromatographic retention is a sum of the different interactions in which the analyte molecule participates. Differential retention results in selectivity and hence resolution. Compounds with no hydrophobic or polar groups are affected by ionic interactions only, which are much stronger interactions and more difficult to elute and therefore undesirable.

1.4.2. Mobile phase.

The type of mobile phase employed greatly influences solute retention. The mobile phase can promote or suppress ionisation of the analyte molecules, and can suppress accessible residual silanol groups or any other active adsorption centers on the adsorbent surface. The main requirement for the mobile phase is to dissolve analytes up to the concentration suitable for detection. Table 1.1 lists the most commonly used reversed phase solvent mobile phases.

Table 1.1. Chemical characteristics of typical HPLC solvent mobile phases.

Solvent	Chemical Characteristics						
	M.W.	B.P.(°C)	R.I.	UV (nm)	Viscosity (cP)	Dipole Moment	Relative Strength
Water	18	100	1.333	185	1.00	1.84	0
Acetonitrile	41	82	1.341	195	0.358	3.37	3.1
Methanol	32	65	1.326	205	0.584	1.66	2.6
Tetrahydrofuran	72	66	1.404	215	2.20	1.70	4.4

M.W. = molecular weight, B.P. = boiling point, R.I.= refractive index

The characteristics of an ideal reversed phase solvent should include low viscosity for maximum efficiency (which increases the diffusion coefficient of the analyte) with minimum backpressure, limited absorption at low wavelengths (i.e. 200 – 230 nm), available at a reasonable cost and high purity and be non-toxic and environmentally friendly for ease of disposal.

Solvent strength is a measure of solvent hydrophobicity, tetrahydrofuran is more hydrophobic than acetonitrile which is more hydrophobic than methanol, however the

three solvents do not differ significantly in their hydrophobic selectivity. Solvents differ in selectivity primarily based on their polar characteristics. Tetrahydrofuran can accept protons in hydrogen bonding, but cannot donate. Methanol can both accept and donate protons during hydrogen bonding. While with acetonitrile, solute molecules arrange themselves around the solvent molecules and is only a very weak proton acceptor.

The strength of the solvent can have a major effect on the analyte retention. With a relatively strong solvent, the equilibrium between the stationary and mobile phase favours the mobile phase resulting in short retention times and poor resolution. With a relatively weak solvent the equilibrium favours the stationary phase and results in longer retention times and maximal resolution. An intermediate solvent is required for reasonable retention. Solute retention is adjusted by mixing solvent strength – acetonitrile, methanol, or tetrahydrofuran, with water to obtain a solvent strength which results in efficient separation.

1.4.3. The column.

HPLC columns physically consist of three parts. Firstly, the matrix which refers to the chemical characteristics of the particle, secondly, the stationary phase (often added to particle) and thirdly, the hardware or steel rod housing encasing the material. The most common matrix available is silica. Others include polystyrene-divinylbenzene, alumina and zircona, which are stable across the pH range. Silica is the most common matrix used as it is very physically robust and it tolerates high pressures. It is chemically stable in virtually all solvents. Silica is versatile and is easily derivatised to form unique stationary phases through attachment of reactive silanes to surface silanols. It is available in pore sizes from 60 Å to 300 Å, choice of which depends on analyte molecular weight, and is readily available in various particle sizes.

The silica surface is not homogenous and consists of various types of silanol groups. Surface silanols vary significantly in their reactivity; 15 - 20% of surface silanols are "highly" reactive. Isolated silanols are the most acidic, geminal silanols are relatively benign and vicinal silanols form hydrogen bonds with adjacent silanols. Metals on the silica increase silanol acidity. There are approximately 8 μmoles/m^2 of silanols in

the silica surface. The type and percentage of silanols in the silica surface affect the bonding coverage and characteristics. The silica surface area affects the total amount of phase that can be bonded. The surface determines the amount and reactivity of free silanols that can produce secondary chromatographic interactions. The silica matrix is a major factor in the reproducibility of column selectivity.

During the mid – 1970's, the first silica particles specifically manufactured for HPLC were spherical in shape which resulted in much higher column efficiency and much longer column lifetimes. They naturally contain metal ions, making it acidic, which results in tailing for basic compounds. These types of columns were referred to as "Type A" columns and examples of this type of column include 'Hypersil' and 'Spherisorb'. High purity silica particles have been made since the mid 1990's. They use high purity silanes as starting materials and are essentially metal free and are referred to as "Type B" columns. They are considered to be of superior quality to previous materials. They have less tailing for basic compounds and are more reproducible. The stationary phase is formed on the surface of the silica by reacting with a silane containing a reactive group, such as chlorine, the desired phase molecule, C_8 or C_{18} and small side groups such as methyl. Many of the surface silanols remain unreacted because of the steric hindrance – the large silanes cannot physically react with every silanol. Only 3/8 is reacted due to steric hindrance. Some of the available stationary phases include; octadecyl (C_{18}), octyl (C_8), butyl (C_4), phenyl, polar (cyano, nitrile) and internal hydrophilic groups (as discussed later). All columns used in this research were type B columns.

Carbon load is the amount of carbon present in the stationary phase as a percentage of the total weight. This is usually measured by thermal gravimetric analysis and the typical carbon load is 12 - 15%. Retention is a function of carbon load-higher carbon results in longer retention. The more carbon there is the less likely to have polar interactions with silanols. The less carbon there is, the more likely to have polar interactions with silanols.

The best measurement of the hydrophobic phase density is phase coverage, measured as micromoles per meter^2 (μmoles/m^2). Maximum coverage for octadecyl groups on the surface of silica is around 3 - 4 μmoles/m^2. Many separations are sensitive to phase coverage.

Di and tri-functional silanes react not only with the silica surface but also by means of a water-catalysed reaction with adjacent silanes. This forms a cross-linked silane surface with increased pH stability and different selectivity than monomerically bonded stationary phases. Polymeric phases are more commonly available on large pore silicas because the polymers begin forming in the liquid phases and have difficulty entering small pores. Monomeric produces oil/liquid on surface whereas polymeric produces wax/solid surface. Polymeric stationary phases are more rigid than monomeric bonded phases and exhibit shape selectivity.

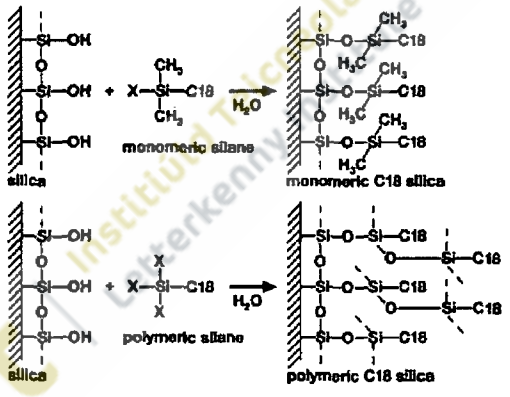


Figure 1.11. Monomeric vs. polymeric C18 bonding. (Source: Grace Vydac Application Notes)

Hydrophobic phases behave differently in a purely aqueous mobile phase. Normal C_{18} phases are solvated in the presence of about 5% or more organic modifier, giving full access of the analyte molecules to the stationary phase. When the organic solvent concentration in the mobile phase drops below 5% the C_{18} phases can no longer interact freely with analyte molecules, thus reducing performance. This is also known as phase collapse. To prevent stationary phase collapse and enhance analyte interactions with the stationary phase in highly aqueous mobile phases, polar groups

such as hydroxyls, carbamates or amides are incorporated into the hydrophobic chain near the silica surface. These are referred to as "polar embedded" columns. This also affects analyte selectivity even in mobile phases of higher organic solvent. This is typically used for hydrophilic compounds and stationary phase collapse does not occur.

While there are slightly more than 8 μmoles/m^2 of silanols on the surface of the silica, the highest practical phase density of an octadecyl ligand is approximately 4 μmoles/m^2. This means that approximately half of the silanols remain unreacted and available for interactions with solute molecules. Since these silanols can cause unwanted interactions leading to peak tailing, reversed-phase materials are often reacted a second time with a small silane molecule to "end-cap" these free silanols. Small silanes such as trimethylchlorosilane are able to access many unreacted silanols after the stationary phase has been bonded. This process, known as endcapping, renders the silanols inaccessible to solute molecules.

1.4.4. Application of monolithic columns in the area of forensic drug analysis.

Maximum resolution with minimum analysis time is the fundamental goal for any chromatographer. Previously, the chromatographer has reduced analysis time by using one or several different approaches, including the use of higher flow rates, higher column temperatures, shorter columns and reduced diameter of particles in the column. The problem of high back pressure is associated with these approaches to improve efficiency in conventional particulate columns. The development of monolithic columns provides an alternative to particulate columns and allows for faster analysis times without compromising separation efficiency.

Monolithic columns consist of a single rod-like structure containing macropores and mesopores in its structure (Figure 1.12). The large pores are typically 2 μm in size and this gives rise to a low flow resistance which allows for higher flow rates. The smaller pores are typically 12 nm and this ensures for sufficient surface area for separation efficiency. Advantages of this novel column are faster analyses without reducing resolution (Figure 1.13). These monolithic columns have greater permeability and lower plate height, whilst separation efficiency (i.e. resolution of

analytes) remains unaffected. Silica is not the only material used in monolithic columns. Other materials include various polymers such as polymethacrylates (Hebb *et al.* 2003, Lammerhofer *et al.* 2001, Peters *et al.* 1998), methacryloxypropyltrimethoxysilane (Kato *et al.* 2002), cationic stearylacrylates (Bedair *et al.* 2003), acrylamides (Hoegger *et al.* 2001) and polystyrene-divinylbenzene (Huber *et al.* 2001, Gusev *et al.* 1999). These columns have been commercially available since 2002.

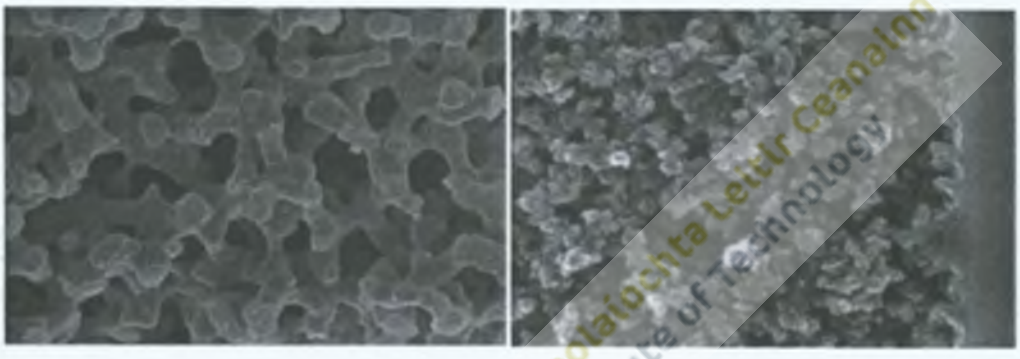


Figure 1.12. SEM – image of the porous structure of a typical monolithic silica column

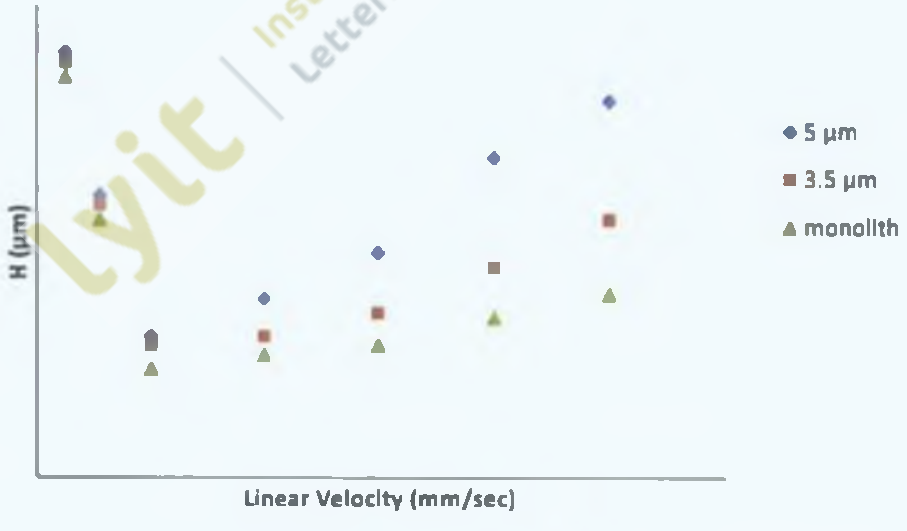


Figure 1.13. A typical Van Deemter plot for a 5 µm particle size column, a 3.5 µm particle size column and a monolithic column.

Much of the research has concentrated on the preparation and testing of the monolithic columns (Janco *et al.* 2002, Hayes *et al.* 2000, Kang *et al.* 2002, Coufal *et al.* 2002). Monolithic columns have been used most extensively in capillary electrochromatography (CEC) (Kornyšova *et al.* 2005, Zhao-Sheng *et al.* 2004, Yan *et al.* 2004, Hilder *et al.* 2004, Bandilla *et al.* 2003). Applications of the monolithic columns include DNA research (Urthaler *et al.* 2005, Forcic *et al.* 2005, Branovic *et al.* 2004, Lubbad *et al.* 2002), environmental monitoring (Cledera-Castro *et al.* 2005, Sarafraz Yazdi *et al.* 2005, Asperger *et al.* 2002) and the analysis of drugs on biological samples (Albu *et al.* 2005, Pistos *et al.* 2004, Yi Fan *et al.* 2004, Samanidou *et al.* 2004).

Monolithic columns have only been recently applied to the analysis of drugs of abuse. Most studies reported involve transfer of a method from a conventional particulate column to a monolithic column and the subsequent comparison of the two whilst using a drug of abuse as the analyte. Schneider and Kovar (2003) demonstrated the reduction in run times from 11 to 3.5 minutes for the analysis of amphetamines in ecstasy tablets. Macchia *et al.* (2004) used a Chromolith RP18 (100 x 4.6mm) to separate a mixture of nine compounds commonly found in a typical heroin sample in less than seven minutes. Aboul-Enein and Hefnawy (2005) decreased the run times by six fold in the analysis of ketamine and its two metabolites, norketamine and dehydronorketamine in human plasma. A Chromolith Performance RP-18 (100 x 4.6mm) column was used at a flow rate of 3 mL min^{-1}. Limit of quantifications (LOQs) were 25 ng mL^{-1} for ketamine and norketamine and 15 ng mL^{-1} for dehydronorketamine with run time for complete analysis occurring in less than four minutes.

Impurity profiling in ecstasy seizures can provide strategic intelligence for the determination of the synthetic origin, and tactical intelligence for the determination of whether two or more samples come from an identical source, i.e., same batch from the same laboratory. The identification of the impurities and the subsequent statistical analysis is what can link different unrelated seizures. Utilising a monolithic column Byrska and Zuba (2008) studied the different impurities that were present in ecstasy tablets. Initial studies examined the extraction process for potential ecstasy tablet impurities, tested different buffers and volumes of eluent required and optimised

HPLC conditions using a simplex method development approach. Using a Chromolith Performance RP-18e (100 x 4.6 mm) column, a gradient elution mobile phase and a flow rate of 1 mL min^{-1}, separation of the impurities were achieved in 35 minutes. The method was subsequently applied to ecstasy of known synthetic route and successfully classified samples based on the areas of 33 selected peaks. However, the use of the monolithic column under these conditions fails to take advantage of the monolithic column, i.e. low back pressure with high flow rates. Subsequently, this analysis could possibly been carried out at 4 mL min^{-1} with a run time of 10 minutes.

A noteworthy feature of monolithic columns is the high-throughput efficiency achieved particularly for toxicological samples. The simultaneous determination of 48 antidepressants and antipsychotics from human serum was achieved by Kirchherr and Kuhn-Velten (2006) using HPLC with tandem mass spectrometry. Sample preparation was limited to protein precipitation and dilution. Samples were separated using a Chromolith SpeedROD C18 (50 x 4.6 mm) column with a flow rate of 1 mL min^{-1}. The method was quantitative and had recoveries of 92 – 111% with an average of 101%. Butalbital, acetaminophen and caffeine were separated by Pistos and Stewart (2004) from human serum. Solid phase extraction was employed to extract the analytes from the serum and separation was achieved by HPLC using a Chromolith Performance RP-18e (100 x 4.6 mm) column. Although flow rate was 9 mL min^{-1}, backpressure was no greater than 177 bar and this allowed for a separation of the three analytes from serum in less than 10 minutes.

Wu *et al.* (2001) demonstrated the rapid analysis times of monolith columns in the separation of four benzodiazepines within one minute from human plasma samples. In this study tempazepam, tamoxifen, fenfluramine and alprazolam were extracted from plasma samples by solid phase extraction (SPE) and chromatographic separation was achieved using a Chromolith SpeedROD (4.6 x 50 mm) monolithic column. Similarly, Clobazam, an anticonvulsant drug, and its metabolite were separated from human plasma using a Chromolith Performance RP-18 (100 x 4.6 mm) monolithic column (Rouini *et al.* 2005). Cocaine and its metabolites (benzoylecgonine, norcocaine and cocaethylene) were separated using a Chromolith Performance RP-18 (4.6 x 100 mm) monolithic column with a flow rate of 5 mL min^{-1} with a run time of

five minutes (Caufiled & Stewart, 2002). Eight benzodiazepines (clonazepam, desalkylflurazepam, diazepam, flunitrazepam, lorazepam, midazolam, nordiazepam and oxazepam) commonly encountered in forensic toxicology were separated in less than four minutes using Chromolith Performance RP-18 (100 x 4.6 mm) (Bugey & Staub, 2004).

The development of the monolithic column has been a significant milestone in the history of separation technology. This new generation of columns has allowed for faster separations at lower backpressures without the loss in efficiency. Other advantages include the reduction of analysis times for toxicological samples and the ability to transfer methods developed for conventional particulate columns to monolithic columns. It can be expected that the monolithic column will have future applications in forensic drug analysis and be an integral tool for the war against drugs. Future applications may concentrate on the ever expanding illegal drug market and adapting analytical methods to separate and detect new chemical compounds with high abuse potential.

Since the introduction of the monolithic column, column technology has developed in several ways. Initially, manufacturers concentrated on reducing the particle size of the traditional particulate column. Particle size was reduced from 5 µm to 3.5 µm, then 2.5 µm, 1.7 µm and ultimately 1.5 µm. The efficiency of a column increases as the size of the particle decreases as the smaller particle size improves the mass transfer kinetics. However, the small particle size generates more back pressure because it gives more resistance to solvent flow. With the increase in efficiency associated with the smaller particle size, a reduction in column length was also made, from the traditional 250 mm to 150 mm and 50 mm resulting in the same efficiency as that of a 5 µm, 250 mm particulate column. The reduction in column diameter also has been used as a tool to improve efficiency. Smaller diameter columns also use less mobile phase per analysis because a slower flow rate is required to achieve the same linear velocity through the column. These changes to the physical dimensions of the particulate column required pumps of higher pressure which in turn, led to the creation of Ultra-Performance Liquid Chromatography (UPLC). Combining the advantages of the monolithic columns with the smaller particle size particulate

column, a new particle was created. Known as "fused core particle technology", "porous shell" or "core shell" the column consists of small particle, typically 1.7 µm, to which a 0.5 µm porous diffusion path is bonded. The reduction in diffusion path serves to increase the mass transfer kinetics, resulting in faster separations. Merck have since developed the next generation of monolithic column, named "Chromolith High Resolution". They state that the column performance of this new type of column is 50% greater than that of the usual Chromolith column, and is comparable to that of the sub 3 µm particle size columns.

1.5. Chemometrics and statistical profiling.

Statistics were employed throughout this study in various forms. Initially, experimental design combined with Artificial Neural Networks (ANN) were used to develop a method for the separation of cocaine components routinely detected in street samples. In addition a range of sample pre-treatment and pattern recognition methods were applied to results of the chemical analyses of ecstasy in order to profile the drugs and to establish links.

1.5.1. Experimental design.

There are two approaches that can be made in order to develop an experimental protocol, the univariate or multivariate approach. Univariate method development involves studying each factor involved in the experiment individually to assess its effect on that experiment. This process can be laborious and time consuming however all levels of each factor would be investigated. The second approach, the multivariate method, involves simultaneously investigating different factors at different levels, thus minimising the effort involved in developing the method. One of the most universal procedures used for this is a 'full factorial design'. It is based on varying the most influential chromatographic factors of the analysis at two levels. A 'fractional factorial design' uses only a subset of the experimental runs that would be involved in the full factorial design, but results in similar statistical information. Overall, this approach is more efficient and less time consuming. To illustrate the effect each chromatographic factors on analyte separation, artificial neural networks are used to generate response surfaces. From these, the optimal separation conditions can be selected.

Artificial Neural Networks is a computer based system derived from the simplified concept of the brain in which a number of nodes, called neurons, are interconnected in a netlike structure. They are capable of modeling extremely complex functions. Figure 1.14 illustrates a simple ANN. There are two input variables, one layer of six units and one output variable. The number of input variables, inner layers and output variables is dependent on the data set. For the method development, the input variables are the different levels of the different chromatographic factors for each experimental run and the output variables are the performance indicators. For the tablet classification, the input variables are the pre-treated (as defined on page 51) peak areas of the chemical components of the tablets and the output variables are the different chemical classes. A trained network should be able to model the function that relates the input variables to the output variables, and can be used to make future predictions.

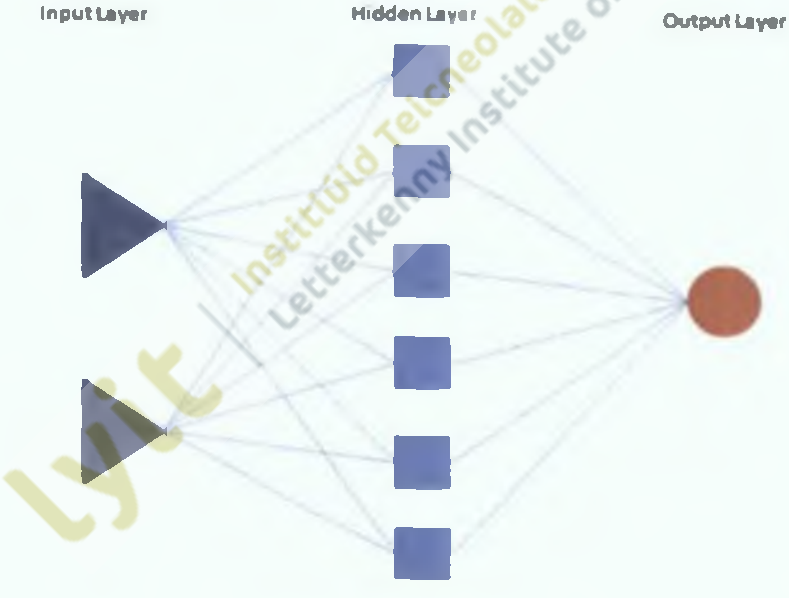


Figure 1.14. A typical neural network consisting of two input variables, one layer of six units and one output variable.

Neural networks tend to be a feed-forward structure, i.e. the signal goes through the input variables first, then the inner layers and finally the output layers. This may not always be the case as sometimes the structure may be 'recurrent', this is where the signal flows back again through from the output variable to the input variables. ANN

operates on the principal of training the data. This involves using input variables with their known output variables in order to train the network. The weights of each neuron are continuously adjusted until the errors are minimised. Once the network has been trained, predictions based on new input variables can be made.

Network architecture is an important aspect of neural networks. Two of the most common forms include MultiLayer Perceptrons (MLP) and Radial Base Functions (RBF). For each form, the *n*-dimensional pattern space is divided up using hyperplanes (MLP) and hyperspheres (RBF). MLPs are the most common type of network. RBFs tend to be slower than MLPs to use, but are very quick to train. Both hyperplanes and hyperspheres work by dividing two classes. For both models, each is trying to find the lowest point (minimum error) in the *n*-dimensional space.

1.5.2. Drug classification.

Classification is the arrangement of objects with similar properties together. In order to achieve this, the properties (i.e. chemical profiles) of the objects (i.e. drug seizures) must be determined. Once identified, the chemical profiles can be used as the data for the statistical techniques applied for the classification.

Pre-treatment methods.

Data must be pre-treated so that no one variable (in this case chemical component) dominates the others. Normalisation, standardisation, 4^{th} square root and $\log_{10}$ are the most commonly used pre-treatment methods (Andersson *et al.* 2007, Baer & Margot, 2006, Dujourdy *et al.* 2008, Lociciro *et al.* 2008).

Normalisation - For each chemical compound, their peak area was divided by the sum of all peak areas (see Equation 1.1). For metal analysis, the absorbance value for a particular metal was divided by the sum of all the absorbance values for all the metals.

$$N_i = \frac{x_i}{\sum x} \qquad \textbf{(Equation 1.1)}$$

Where x_i = area of peak i. Although this pre-treatment method will allow all data points to be on the one scale, it may happen that larger peak areas will still influence

smaller peak areas. For this reason, normalisation in combination with other pre-treatment methods is recommended.

Standardisation - For each chemical compound, their peak area was divided by the standard deviation of all peak areas (see Equation 1.2 and 1.3). This method is also referred to as 'weighting'.

$$S_i = \frac{x_i}{SD_i} \qquad \textbf{(Equation 1.2)}$$

$$\text{Where } SD_i = \sqrt{\frac{n\sum x_i^2 - (\sum x_i)^2}{n(n-1)}} \qquad \textbf{(Equation 1.3)}$$

Where x_i = area of peak *i*, n = number of samples, SD_i = standard deviation of peak *i*.

Logarithm - For each chemical compound, the $\log_{10}$ was calculated (Equation 1.4).

$$L_i = \log_{10} x_i \qquad \textbf{(Equation 1.4)}$$

Where x_i = area of peak *i*. A consideration for this method is the presence in the data set of zeros, as $\log_{10}$ of zero cannot be calculated.

4th Square root - For each chemical compound, the forth square root was calculated (Equation 1.5). Due to the inability of $\log_{10}$ to calculate zeros, this may be used as an alternative.

$$R_i = \sqrt[4]{x_i} \qquad \textbf{(Equation 1.5)}$$

Where x_i = area of peak *i*.

Pattern recognition methods.

The statistical techniques used for classification or pattern recognition can be either supervised or unsupervised. For some groups of data, prior knowledge of the samples is not known. In this case, unsupervised methods such as Hierarchal Cluster Analysis (HCA) or Principal Component Analysis (PCA) are used. Again, these specific methods may be used when general information on the data is required, e.g. natural groups or outliers within the data. Supervised methods assign class membership to the data prior to the statistics being applied. The aim is to 'train' a model so that future

samples can be classified automatically. Examples of supervised methods include Linear Discriminant Analysis (LDA) and Artificial Neural Networks (ANN).

Pearson Correlation Coefficients - This is a method used to describe the relationship or correlation between two samples (Equation 1.6). Values are given in the range -1 to 1. The closer to 1 the value is the stronger the correlation, closer to -1, the stronger the negative correlation. Values at 0 indicate no relationship whatsoever. In the context of this study, tablets from similar seizures should have a coefficient value close to 1, whilst tablets from different seizures should have a value near 0. A popular alteration to this statistic is to multiply the result by 100, as this makes for easier comparison with other statistical methods. This type of statistical method is very basic in terms of classification and relatively simple to perform and therefore is quite common in research studies.

$$r_1 = \frac{\sum_{i=1}^{n}((x_i - \bar{x})(y_i - \bar{y}))}{\sum_{i=1}^{n}(x_i - \bar{x})^2} \qquad \textbf{(Equation 1.6)}$$

Where r = correlation, $\bar{x}$ is the mean of x value and $\bar{y}$ is the value of y value.

Hierarchal Cluster Analysis (HCA) - This is a method for dividing a group of objects into classes so that similar objects are in the same class or cluster. It searches for objects which are close together in the variable space. This statistical method measures the distance between two objects and if the distance is relatively small then similar linked objects will be clustered. The similarity distance between samples is calculated by both cluster and interval methods. Typical clustering methods include Between groups, Within groups, Nearest neighbour, Furthest neighbour, Centroid, Median, and Wards. Interval methods include Euclidean, Square Euclidean, Cosine, Pearson, Chebychev, Block and Minkowski. The data variable set will determine the most appropriate interval and cluster methods. Results of HCA are typically represented as a dendogram which display the clusters associated with the data set. Figure 1.15 illustrates six cases clustered to three distinct groups at Euclidean distance 1. Increasing the Euclidean distance to 10 will generate two groups for the same six cases. The distance or similarity measure can therefore be subjective in determining the number of clusters present in the dendogram.

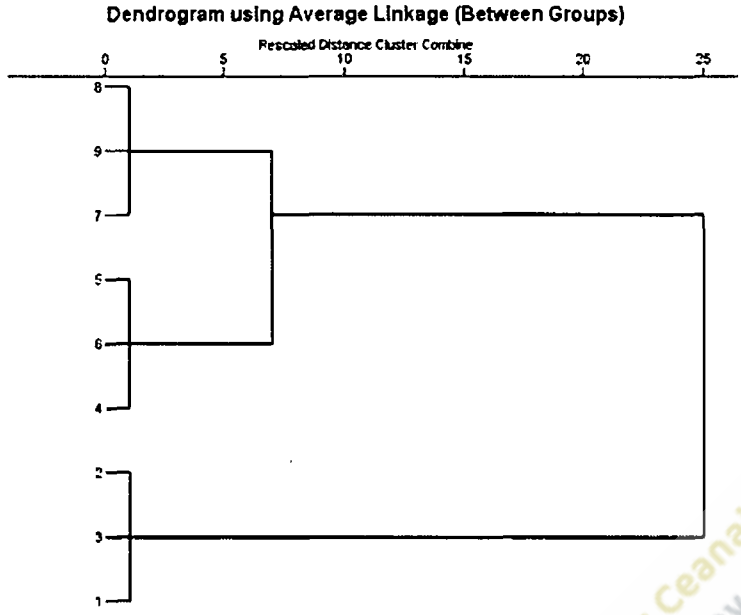


Figure 1.15. Simple HCA dendogram.

Principal Component Analysis (PCA) – This is a multivariate procedure used to reduce the dimensionality of a data set while retaining as much information as is possible. The first principal component is the combination of variables that explains the greatest amount of variation. The second principal component defines the next largest amount of variation and it is independent of the first principal. When there is significant correlation within the data, the number of principal components will be much lower than the number of variables. A scree plot is a visual display that describes how much of the variance is attributed to each of the principal components. Ideally, the first and possibly second principal components should account for the majority of the variance. After PCA is performed, scores of each principal component for each case is produced. A scores plot is a visual display of these values. This plot is important for the classification process, as this identifies the natural groupings within the data set. Ideally, related cases should be grouped together on the plot, and these linked groups should be distant from others. Figure 1.16 illustrates the score plot using the same data from the HCA dendogram above. This example shows an ideal grouping, where similar data are grouped together and groups are distant from each other.

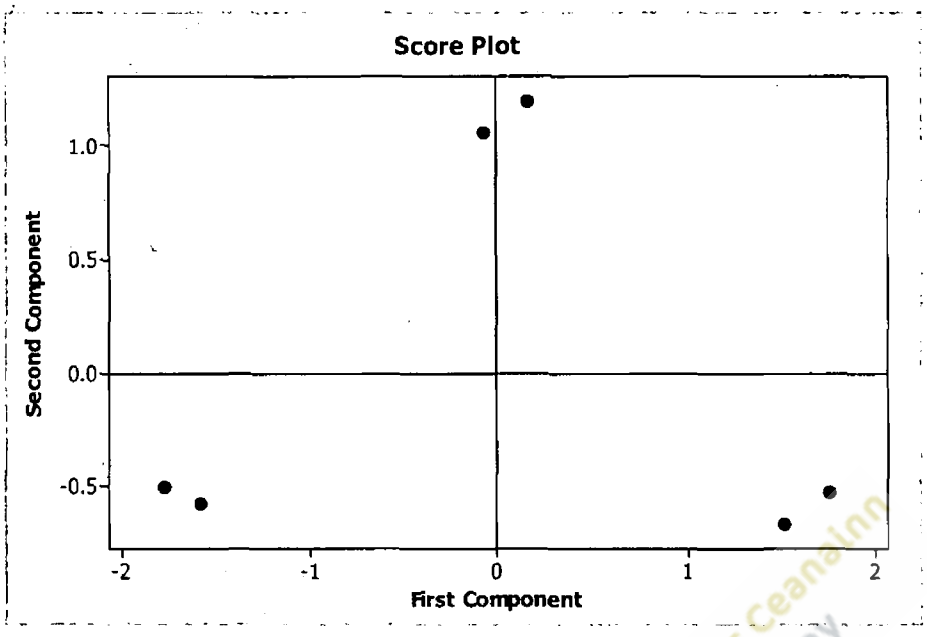


Figure 1.16. Score plot of first two principal components.

Linear Discriminant Analysis - For each seizure, the *n* data points are reduced from *n* dimensions to one dimension. Each dimension is given a coefficient which reflects the difference between seizures. This statistical approach works by finding the linear discriminant function (Y) for each class or seizure. This is a combination of the coefficients (a) of each variable (X),

$$Y = a_1X_1 + a_2X_2 + \ldots a_nX_n \qquad \textbf{(Equation 1.7)}$$

The coefficients are assigned so that Y is very different for each class. When new data is applied, the resulting value of Y will indicate which class or seizure that data set belongs to by comparison of the Y values for each class.

Artificial Neural Networks (ANN) - The major attribute of neural networks, is that once established within the laboratory as a classification tool, results of further chemical analysis can be added to the network and tablets of similar chemical profile can be highlighted. Over a period of time, this information accumulates into "drug intelligence" which can lead to dealer networks or even production laboratories being more easily identified. Artificial neural networks algorithms have the potential to provide complete solutions in the identification of patterns in data sets (Bishop, 1996). Individual networks and ensembles of networks can be created and tested using the

Intelligent Problem Solver (IPS) system function in the Trajan software. The software randomly assigns individual data sets from each sample into training, selection and test sets. Multiple networks for each data set can be tested by Multi-Layer Perceptron (MLP) and Radial Base Functions (RBF) methods. Selection of the most suitable network is based on the performance of the selection set and the difference in the error associated with the selection set and the test set. The optimal network based on performance, selection and minimum error values should be capable of handling new data and have the ability to classify new data adequately (Waddell *et al.* 2004).

1.5.3 Application of chemometrics to illicit drug profiling.

Although there are numerous reports in the literature on statistical profiling of illicit drug samples there is no clear standard protocol or approach to apply (Andersson *et al.* 2007, Baer & Margot 2007, Billault *et al.* 2007, Byrska and Zuba 2008, Cheng *et al.* 2006, Dayrit & Dumlao 2004, Dufey *et al.* 2006, Dujourdy *et al.* 2008, Dujourdy & Besacier, 2008, Esseiva *et al.* 2005, Gosav *et al.* 2006, Janhunen & Cole 1999, Klemenc 2001, Koper *et al.* 2007, Krawczyk and Parczewski 2001, Kuwayama *et al.* 2006, Lociciro *et al.* 2008, Palhol *et al.* 2002, Puthaviriyakron *et al.* 2002, Weyermann *et al.* 2008). Various pre-treatment methods, unsupervised pattern recognition methods and supervised pattern recognition methods have been applied to data sets to determine the most appropriate method. In addition many reports refer to impurities as the main target to determine patterns in illicit drug samples. Regardless, the overall aim of statistical profiling methods is to identify synthetic routes, identify geographical origin or simply highlight seizures of similar chemical profile.

Chemometrics involves the application of statistical protocols to multivariate chemical data sets. Various statistical protocols have been applied for the analysis of illicit drug samples. Baer & Margot (2007) evaluated different pre-treatment methods (i.e. normalisation, standardisation, fourth Square Root, logarithm) and different comparison methods (i.e. Pearson's correlation coefficients, Squared cosine, Similarity index, Canberra index, Euclidean distance and Manhattan distance) for the statistical profiling of ecstasy tablet samples. Fourteen data variable sets comprised of sugar and fatty acid content were examined in various combinations of data pre-treatment and comparison methods. The combination of fourth squared root with

squared cosine function was the most successful at classifying data sets with 109 ecstasy samples categorised into 67 groups. Under the European project for Collaborative Harmonization of Methods for Profiling Amphetamine Type Stimulants (CHAMP), Andersson *et al.* (2007) evaluated statistical protocols for profiling of amphetamines. Combinations of four different pre-treatment methods with six different distance methods were examined and normalisation with 4th square root pre-treatment method with Pearson's correlation coefficients comparison method was selected as the preferred protocol. Lociciro *et al.* (2008) examined sixty six statistical combinations in a cross border study of cocaine samples between France and Switzerland. Ten different data pre-treatments with six distance measurements were evaluated and the final combination of Normalisation with Standardisation for data pre-treatment with either Pearson's correlation or squared Cosine function was the most appropriate protocol for the cocaine profiling.

Impurity determination is commonly used for drug classification purposes. Puthaviriyakron *et al.* (2002) studied impurities of methamphetamine tablets from Thailand. GC peak areas of nine impurities were normalised and then analysed by HCA. At a similarity index of 0.55, five groups were identified. A similar study of methamphetamine from the Philippines, used HCA to classify the drugs based on impurities present (Dayrit & Dumlao, 2004). GC peak areas of each compound were normalised. Thirty, forty and fifty impurity compounds were tested and results indicated that by increasing the number of impurity data sets the better the resulting HCA dendogram. Kuwayama *et al.* (2006) compared different pre-treatment methods with different clustering methods using impurity data of methamphetamine samples. A combination of raw data, square root and logarithm with Euclidean, cosine and correlation coefficient distance methods was used and resulted in logarithm pre-treatment with cosine distance being the most appropriate for this analysis. Thirty-two samples were classified into nine groups based on eight impurity GC peak areas. More recently, Weyermann *et al.* (2008), was able to reduce the number of target impurities from thirty two to eight. The data was normalised with square root pre-treatment and PCA was applied. No pattern was identified, concluding that the MDMA tablets seized in Europe for this study were all produced within the same geographical area.

The identification of synthetic routes can be a key ingredient in the classification process and has been used by a number of researchers. Koper *et al.* (2007) analysed a large number of ecstasy tablets for their metal content (forty-nine metals analysed) with the intention of identifying the synthesis production method. Clustering based on Pearson's correlation together with average linkage (between groups) was found to be the best and most robust clustering technique for this type of data. The production method could be identified for eighty-nine of the ninety-seven tablets analysed. Using the element profile, thirteen links were identified within the ninety-seven MDMA tablet samples. Billault *et al.* (2007) synthesised samples of ecstasy using the five most common methods and samples were analysed by isotope ratio mass spectrometry. PCA was applied to the values from the precursors and the samples. Full discrimination was achieved for all but two samples. Byrska and Zuba (2008) divided MDMA samples into their different synthetic routes by applying HCA. The chemometric approach involved applying Wards' clustering with Pearson's distance metric was to thirty three impurities associated with samples. PCA was subsequently applied to verify the HCA findings. Palhol *et al.* (2002) investigated the most popular synthesis route for MDMA tablets in France. Within this study, thirty impurities from fifty two samples were identified. Using HCA, the reductive amination route was found to be the most popular synthesis route and 3, 4-methylenedioxyphenyl-2-propanone was the most common precursor. Similarly, Dujourdy *et al.* (2008) normalised the peak areas of forty three impurities from methamphetamine samples and the application of HCA and PCA allowed for the identification different synthetic routes.

The geographical origin of cocaine was determined using HCA and partial least squares – discriminant analysis (PLS-DA) for eighteen residues from 2863 cocaine samples (Dujourdy & Besacier, 2008). Unsupervised pattern recognition methods of HCA using Wards clustering method and Pearson's correlation coefficients were investigated. At a similarity index of 0.5 Colombian, Peruvian and Bolivian samples were distinguished. PLS-DA was used to confirm HCA results of the three groupings. A study by Klemenc (2001) used various statistical methods to observe the influence of excipients on heroin street samples. Samples were deliberately cut with the most common excipients (sucrose, glucose, mannitol, procaine, caffeine, paracetamol, citric

acid) in different combinations and concentrations and analysed by GC-MS. The peak areas were normalised and then standardised. Pattern recognition methods used were HCA, PCA and K-nearest neighbour. Results indicated that HCA and PCA were both better at classification than KNN with both having a correct classification rate of 100%. The conclusion from this study was that diluting the samples with any of the excipients had no effect on the classification. Five major constituents of heroin were successfully used to classify samples by Dufey *et al.* (2006). GC peak areas for the five targeted compounds were normalised and Pearson's correlation coefficients calculated to ascertain the capacity of the method in determining chemical links between seizures.

Many studies aim to identify general connections between seizures, without any concern regarding their synthetic route or geographical origin. In a study by Cheng *et al.* (2006), ecstasy tablets in Hong Kong were analysed by GC-MS for their impurity content. The data was normalised and HCA catagorised the eighty nine samples into four groups at 0.5 similarity index. One thousand amphetamine samples were classified into thirty seven groups in a study by Krawczyk and Parczewski, (2001). Correlation coefficients were calculated to initially classify the tablets, followed by both PCA and HCA. Linear discriminant analysis was applied to heroin samples (Janhunen & Cole, 1999). Thirty one samples were classified into eight groups with a correct classification rate of 91.9%.

The application of artificial neural networks has been limited. Gosav *et al.* (2006) built a search library for a GC-FTIR based on two neural networks for amphetamine classification. The first neural network could distinguish between amphetamines and non-amphetamines and the second neural network could distinguish between stimulant amphetamines, hallucinogenic amphetamines and non-amphetamines. The choice of neural network was based on the highest correct classification rate. A correct classification rate of 96% was achieved by Esseiva *et al.* (2005) for the classification of heroin samples. The peak areas of six compounds for 3371 samples were normalised and PCA was applied. This roughly divided the samples into different classes, from which correlation coefficients were calculated. The correlated

samples were then used to train the networks. Both MLPs and RBFs were tested. The network with the highest correct classification rate was an RBF 6:121:20.

1.6. Legislation and control efforts in Ireland.

Three treaties drawn up by the United Nations are the backbone to which drugs are controlled worldwide; The Single Convention on Narcotic Drugs 1961, The Convention on Psychotropic Substances 1971 and The Convention against Illicit Traffic in Narcotic Drugs and Psychotropic Substances 1988.

Legislation in Ireland is determined by The Misuse of Drugs Act, 1977 and 1984 and the regulations (Irish Statute Book). In 1993, the Misuse of Drugs Regulations was added to include the control of production, supply, importation, exportation and possession of precursors. In 1999, the 1977 Act was amended, Section (15A) deals with the possession of drugs of a street value over €12,700. A person in possession of drugs of this value or more is deemed to be supplying or selling and court convictions can result in a ten year minimum prison sentence.

There are five categories of drugs in the national legislation:

I. Substances with no medical use
II. Strictly controlled substances with medical use
III. Strictly controlled medicinal products
IV. Less controlled medicinal products
V. Products containing small quantities of category I-IV compounds

In Ireland, possession of cannabis or cannabis resin for personal use is punishable by a fine on first or second conviction but from a third offence onwards it incurs a fine and/or a term of imprisonment up to one year.

On the 23rd of August 2010, Ireland introduced new laws with regard to the sale of psychoactive substances that were not previously controlled under existing legislation. Retail outlets, more commonly known as 'head shops' were taking advantage of selling drugs that were new to the market and considered legal. Under the 'Criminal Justice (Psychoactive Substances) Act 2010' psychoactive substances including

synthetic cannabinoids, mephedrone, piperazine derivatives, flephedrone and methylenedioxypyrovalerone (MDPV) were made illegal (Irish Statute Book, 2010).

Various groups and organisations have been established in Ireland to address the drug problem. Reduction in supply of drugs is ultimately the responsibility of the Department of Justice, Equality and Law Reform, within which An Garda Síochána (police service), the courts, the Irish Prison Service, the Irish Customs Service and the Irish Naval Service (Department of Defence) all cooperate. Both the National Advisory Committee on Drugs (NACD) and the Alcohol and Drug Research Unit (ADRU) of the Health Research Board (HRB) are the two main bodies which conduct research on drugs and drug related issues. Drug Task Forces, both local and regional, are community based groups that assist government agencies to respond quicker and more efficiently to established and emerging drug problems within their local area. These groups are managed by the National Drugs Strategy Team (NDST), who liaises between the local and regional groups and the government. They make policy recommendations based on the conclusions of the task forces. The NDST along with the Inter-Departmental Group on the National Drugs Strategy are involved with monitoring the implementation of the National Drugs Strategy.

In Europe, many agencies have been established whose aim is to disseminate all drug related data. Each country has one or more such agencies, for example Ireland has the Drug Awareness Programme (DAP) and the Irish Needle Exchange Forum (INEF). The EMCDDA is one of the main European organisations responsible for monitoring drug developments. They work in conjunction with national agencies and Europol to provide information on the drug situation in Europe. Europol deals with all criminal intelligence in Europe and has a focus on drug trafficking. Interpol is a worldwide police organisation that supports all cross boarder activities that could reduce crime. A major focus is drugs, with an emphasis on identifying new drug trafficking developments and criminal organisations operating at the international level. The European Network of Forensic Science Institutes (ENFSI) is another organisation in which forensic laboratories across Europe collaborate on various issues. Within this large organisation are 16 'expert working groups', one of which focuses on drugs alone.

As the drug situation grows, new organisations are being established, for example the European Maritime Analysis and Operations Centre – Narcotics (MAOC-N). It is an international agency set up to coordinate anti-drug trafficking by several European countries. Based in Lisbon, its objective is to use intelligence on drug smuggling operations, by air or sea, and apply the most suitable military and/or law enforcement teams to respond to situations that arise. Ireland is a member and is represented by An Garda Síochána, the Irish Customs Service and the Irish Naval Service.

Worldwide the main policy maker is the United Nations (UN), or more specifically the United Nations Office of Drugs and Crime (UNODC). Within the UNDOC, there are two Commissions that deal directly with drugs, The Commission on Narcotic Drugs and the Commission on Crime Prevention and Criminal Justice. The former established all procedures and guidelines pertaining to illicit drugs based on observations of the world drug situation, whilst the later advises policy makers on all matters relating to crime prevention and criminal justice. The International Narcotics Control Board (INCB) implements all policies and recommendations issued by the UNODC. The World Customs Organization (WCO) provides all information of custom controls and smuggling.

1.7. Aims and objectives.

The overall aims of this research thesis are to investigate the application of High Performance Liquid Chromatography (HPLC) for the analysis of major drug constituents associated with ecstasy and cocaine illicit drug samples. Emphasis is placed on method development with univariate or multivariate experimental strategies used in the selection and optimisation of HPLC protocols. Methods developed are applied to the area of forensic drug analysis and chemical data obtained is analysed by chemometric methods to determine if statistical profiling can be beneficial in establishing links between unrelated ecstasy seizures. The research strategy was divided into following areas:

1. HPLC method development.

Univariate method development approach was applied to the design of a HPLC method for the simultaneous detection and quantification of amphetamine, methamphetamine, MDMA, MDA, MDEA, MBDB and adulterants commonly found in ecstasy tablets. Considerations with regard to the choice of chromatographic factors, solutes under investigation and the provision of quality assurance data throughout the research work are important in method development. Comparative evaluation of monolithic columns versus particulate column stationary phase materials in the separation of ecstasy analytes was performed.

Alternatively the multivariate HPLC method development approach for the analysis of cocaine samples was also evaluated. Multivariate method development simultaneously examines all chromatographic separation factors to determine optimum performance based on mathematical model response surfaces. This approach has the potential to minimise the number of chromatographic experiments required and may considerably reduce the time taken for the selection of the optimal separation conditions.

2. Analysis of seized ecstasy and cocaine samples.

Once established, the HPLC methods developed were applied to ecstasy and cocaine street samples to qualitatively and quantitatively assay for the major active ingredients and adulterants associated with street samples. The identification of sugar excipients and metal impurities is important from an investigative perspective. Knowledge of such components may provide a means of establishing the origin or the synthetic routes employed and provide evidence of a link between seized drug samples. As such, sugar composition and inorganic metal data analysis was determined to obtain a near complete chemical profile of ecstasy samples. Physical and chemical profiles were evaluated for 183 ecstasy samples seized in the Republic of Ireland during 2001-2003.

3. Chemometrics and statistical profiling of ecstasy tablets.

Recently, the applications of drug profiling methods have demonstrated the potential of these statistical techniques in establishing links between illicit drug samples. Unsupervised pattern recognition methods of PCA, HCA and Pearson's correlation coefficients and supervised pattern recognition methods of LDA and ANN were evaluated in order to determine the applicability of methods in profiling illicit drug ecstasy samples. Ecstasy chemical data sets of active ingredients, sugar components and metal composition were investigated to identifying possible groupings and thereby provide strategic intelligence and an understanding of drug trafficking in the Irish ecstasy market.

Chapter 2: Materials and methods.

2.1. General materials and instrumentation.

2.1.1. Chemicals and reagents.

All reagents used in this study were of HPLC analytical grade. Acetonitrile, ammonium dihydrogen phosphate, magnesium nitrate, nitric acid and phosphoric acid were purchased from Lennox Laboratories (Dublin, Ireland). Potassium dihydrogen phosphate and methanol were purchased from BDH Laboratory Supplies (Poole, England).

A license to purchase and possess drug standards for research purposes was obtained from the Irish Medicines Board, formally the Department of Health and Children (Drugs Section), Ireland. All amphetamine standards (amphetamine, methamphetamine, MDA, MDMA, MDEA and MBDB) were purchased from Sigma (Poole, England). All cocaine standards (cocaine, lidocaine, procaine, prilocaine and benzocaine) were purchased from Sigma (Poole, England). The Garda Forensic Science Laboratory Dublin, Ireland supplied all ecstasy and cocaine samples used in this study.

Acetaminophen, acetylsalicylic acid, caffeine, clomipramine, metoclopramide, phenacetin, propyl paraben and uracil were also purchased from Sigma (Poole, England). All sugar standards (fructose, glucose, inositol, lactose, mannitol, sorbitol and sucrose) were purchased from Sigma (Poole, England). All the metal standards (aluminium, calcium, chromium, iron, lead, magnesium, potassium, sodium and zinc) were purchased from Merck (Germany).

2.1.2. Liquid chromatography system.

The chromatography was performed on a Shimazdu HPLC system (Mason Technology, Dublin, Ireland). This was equipped with an autoinjector (SIL-10ADvp), a solvent delivery module (LC–10ATvp), a degasser (DGU-14A), a column oven (CTO-10ACvp) and a system controller (SCL-10Avp). A UV/Vis photodiode array detector (SPD-M10Avp) was used for the ecstasy and cocaine method development and a refractive index detector (RID-10A) was used for the sugar analysis. The Shimazdu ClassVp HPLC software was used for instrument control, data acquisition and data analysis.

2.1.3. Atomic Absorption Spectrometry.

A Perkin Elmer Atomic Absorption Spectrometer Model 3300 equipped with a Perkin Elmer HGA600 graphite furnace, a Perkin Elmer AS-60 Autosampler and a deuterium lamp background correction was used with hollow cathode lamps (Perkin Elmer) for the metal analysis.

2.1.4. Miscellaneous instruments.

The ultrasonic bath used was a Cole-Parmer 8890 (VWR, Dublin, Ireland). An Ohaus AS120 analytical balance was used to weigh samples and standards. The deioniser used was a Millipore 'Direct Q', ASTM Type 1, 18 MΩ (Mason Technology, Dublin, Ireland).

2.1.5. Computer software.

Principal component analysis was performed using Minitab statistical software (Minitab Inc., PA, USA). Hierarchal cluster analysis and linear discriminant analysis of ecstasy data were performed with the statistical software SPSS Version 13 (SPSS Inc., Chicago, IL, USA). A Microsoft Excel macros file was provided from the European Network of Forensic Science Institutes (ENFSI) for the determination of the Pearson's correlation coefficient. Neural networks for the classification of ecstasy data was generated by Trajan Neural Network software (Trajan Software Ltd., Durham, UK).

Minitab statistical software (Minitab Inc., PA, USA) was used to generate fractional factorial design for the multivariate cocaine method development. In addition, neural networks and response surface plots were constructed using Trajan Neural Network software (Trajan Software Ltd., Durham, UK) for the multivariate method development.

2.2. Univariate HPLC method development for the analysis of ecstasy.

2.2.1. Ecstasy standards and sample preparation.

Stock solutions of uracil and the internal standard metoclopramide HCl were prepared in methanol at a concentration of 1 μg mL^{-1}. Amphetamine standards for sample analysis and the ecstasy samples were prepared in the internal standard solution. For method development a mixed amphetamine standard solution containing; amphetamine, methamphetamine, MDA, MDMA, MDEA and MBDB was used to examine method selectivity and resolution. Uracil was used as an unretained compound. Detection of analytes was at 200 nm.

Application of the developed method was made to 183 ecstasy tablet samples. Ecstasy tablet samples were representative of the seizures throughout the Republic of Ireland (Figure 2.1.) in 2001 and 2003. Ecstasy tablets were provided as discrete individual batches (61 in total) from the Forensic Science Laboratory, Dublin. Batches 1-12 were from seizures in 2001 while the remaining batches 13-61 were seizures from 2003. Each batch contained three tablets and each tablet had a specific reference number assigned. For HPLC analysis ecstasy tablets were individually pulverised into a fine powder using a mortar and pestle. Three 10 mg aliquots of each tablet sample were dissolved in 10 ml of the internal standard solution. All solutions were sonicated for ten minutes and filtered through 0.45 μm filters (Millipore, MA., USA.) prior to HPLC injection.

Six physical parameters (weight, logo, colour, shape, thickness and diameter) were recorded to ascertain the physical characteristics of each ecstasy tablet. All weight measurements were carried out using a calibrated analytical balance. Callipers were used to measure diameter and thickness. A representative tablet from each batch was digitally photographed to record sample physical appearance.

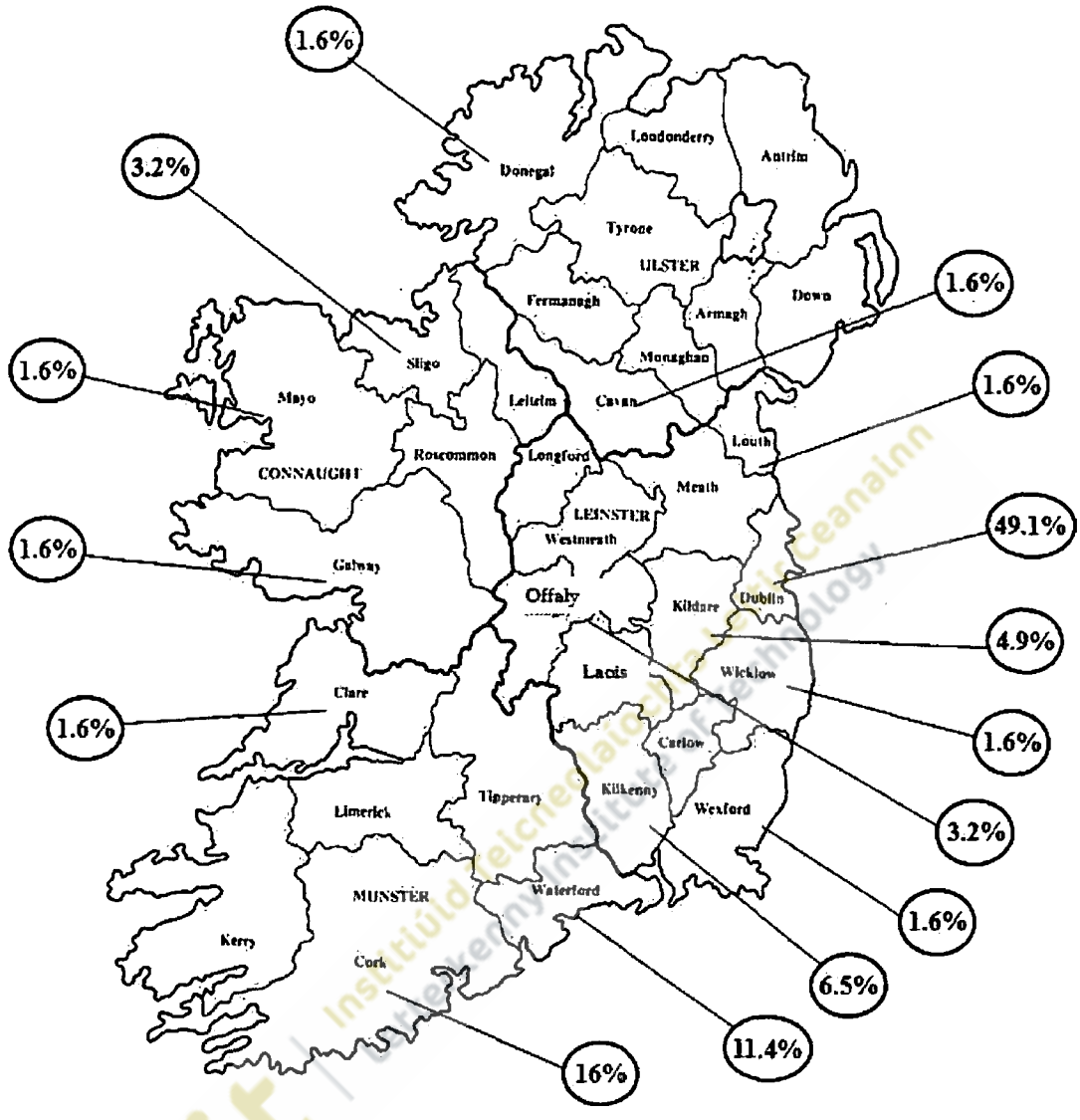


Figure 2.1. Distribution and percentage of ecstasy batch seizures used in this study.

2.2.2. Univariate method development for analysis of ecstasy.

The design of the experimental procedure concentrated on a univariate method for the development of a HPLC method for the analysis of ecstasy tablets. As such, each chromatographic factor was considered individually, and the effect of the varying factor evaluated on the performance for method development.

Assessment of column stationary phase materials was evaluated by examining seven commercially available columns. Columns of different physical and chemical characteristics were deliberately chosen (Table 2.1). Selected column types included the Luna C8 column, 5 µm, 250 x 2.0 mm, ODS2 (Phenomenex Inc.); Phenosphere, 5 µm, 250 x 4.4 mm ODS2, C18 (Phenomenex Inc.), Luna, 5 µm, 250 x 2.0 mm, ODS2, C18 (Phenomenex Inc.); a newer type 'Hybrid' column XTerra, 5 µm, 250 x 2.1 mm RP18 (Waters Corporation) and three monolithic columns, namely, Chromolith Flash RP-18, 25 x 4.6 mm, Chromolith SpeedROD, RP-18, 50 x 4.6 mm and Chromolith Performance RP-18, 100 x 4.6 mm (Merck KgaA).

Analyte separation is dependent on interactions between the mobile and stationary phases. A phosphate buffer (20 mM) was used in the preparation of the mobile phase for HPLC and was prepared by adding 2.7 g of potassium dihydrogen phosphate to 1L deionised water. Acetonitrile was used as the organic solvent within the mobile phase and its effect on the quality of the separation was determined at different solvent strengths. Solvent strengths comprising different acetonitrile concentrations ranging 0% to 30% with 20 mM phosphate buffer (pH 3.0) were evaluated. When required, gradient elution technique was applied to columns with poor chromatographic separation to enhance solute resolution. The effect of pH was also investigated by adjusting buffer pH using phosphoric acid over a pH range of 2.5, 3.0 and 3.5 (as discussed on page 85).

Table 2.1. *Physical characteristics of stationary phase columns examined for univariate HPLC method development analysis of ecstasy.*

Column	Column physical parameters								
	Pore Size (Å)	Surface Area (m^2/g)	Carbon Load (%)	Surface Coverage ($\mu moles/m^2$)	Length (mm)	Internal Diameter (mm)	Size Distribution (µm)	Particle size (µm)	Endcapping
Luna C8[1]	100	417	14.40	4.58	250	2.0	1.89	5	Yes
Phenosphere C18[1]	80	220	11.00	2.50	250	4.6	1.89	5	Yes
Luna C18[1]	100	417	18.05	3.39	250	2.0	1.89	5	Yes
XTerra RP18[2]	121	190	15.14	2.27	250	2.1	1.61	5	Yes
Performance RP-18[3]	NA	300	17.00	NA	100	4.6	NA	NA	Yes
SpeedROD RP-18[3]	NA	300	17.00	NA	50	4.6	NA	NA	Yes
Flash RP-18[3]	NA	300	17.00	NA	25	4.6	NA	NA	Yes

1. Luna and Phenosphere columns were supplied by Phenomenex Incorporation; 2. XTerra column was supplied by Waters Corporation;
3. Monolith columns were supplied by Merck Kga. NA: not applicable

Temperature effects in HPLC are not as significant as in gas chromatography. However, theory states that increasing the temperature will decrease retention factor (k') and thus decrease the retention time. The effect of column temperature was examined over a range of temperatures. The six standard solution was injected at 25°C to 45°C in 5°C increments and the most appropriate temperature was selected based on retention times and resolution.

Flow rate is an important criterion in optimising a suitable method. Optimum flow rates should give a short retention time with good analyte resolution at low pressure. In addition, optimal flow rates should consider solvent consumption and waste production, which should be kept to a minimum to reduce costs. Flow rates for the particulate columns were dependent on the internal diameter and were varied from 0.1 mL min^{-1} to 2 mL min^{-1}. The main factor which differentiates monolithic columns from the traditional particulate columns is the ability to use higher flow rates without a high backpressure. The HPLC pump capacity allowed for pump rates up to 9.999 mL min^{-1}. Monolithic columns had a maximum pressure of 200 bar. Preliminary investigations determined that 200 bar allowed for a flow rate of 9 mL min^{-1}. Therefore flow rates of 0.5 mL min^{-1} to 9 mL min^{-1} were assessed in 0.5 mL min^{-1} increments. The backpressure at each increment was recorded for each column.

2.2.3. Validation of univariate method developed.

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Although there is general agreement about what type of studies should be assessed, there is great diversity in how they are performed. The validation was carried out under the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (ICH, 1996), including selectivity, linearity, inter and intra-day precision, accuracy, limit of detection and limit of quantification.

The ideal internal standard is a compound of known purity that does not cause interference with the analysis. Clomipramine, metoclopramide and propyl paraben were all assessed for the most suitable internal standard. The selectivity of the

method was established for all amphetamine standards and the most common adulterants found in ecstasy tablets, namely, acetaminophen, acetylsalicylic acid, caffeine and phenacetin. Precision was expressed as relative standard deviation (% RSD). For intra-day, ten replicates of 50, 100 and 250 μg mL^{-1} were analysed on the same day. These standards were analysed in five replicates over five days to establish inter-day precision.

With no reference materials, the accuracy of the method was determined by analysing spiked samples at three concentration levels of 50, 100 and 250 μg mL^{-1}. Results were calculated as experimental values compared to theoretical values and were expressed as percentage recovery.

Linearity was evaluated by regression analysis and Mandel's test, which compares linear and quadratic models (Mandel, 1964). Limit of detection and limit of quantification were established by injection of 0.01 μg mL^{-1} concentrations of each of the six compounds and the signal to noise ratio was determined. The limit of detection was calculated by LOD= $3.3\sigma/S$, where σ is the standard deviation of the response of the blank and S is the slope of the calibration curve. The limit of quantification was calculated by LOQ= $10\sigma/S$ under the ICH guidelines. The limits were validated by analysing standards prepared at the concentrations of the LOQs for each standard and their precision and accuracy were assessed.

2.3. Multivariate HPLC method development for the analysis of cocaine.

2.3.1. Cocaine standards and sample preparation.

Stock solutions of uracil and the internal standard metoclopramide HCl were prepared at a concentration of 1 μg mL^{-1} in methanol. Cocaine standards were provided in salt form and were prepared in the internal standard solution. A mixed standard solution was prepared containing cocaine, lidocaine, prilocaine, procaine, benzocaine, acetylsalicylic acid, caffeine and phenacetin. Uracil was used as an unretained compound and detection of analytes was performed using a diode array detector at 200 nm.

The Forensic Science Laboratory, Dublin provided twenty eight cocaine samples seized within the Republic of Ireland during 2004. Cocaine samples were in the form of a powder, each sample weighing approximately 100 mg and 50 mg of each sample was dissolved in 10 mL of the internal standard solution. Samples were sonicated for 10 minutes to ensure total dissolution and filtered through 0.45 μm filters (Millipore, MA., USA.) prior to HPLC injections.

2.3.2. Multivariate method development for the analysis of cocaine.

The design of this experimental procedure was based on a multivariate method optimisation. As such, each chromatographic factor is assessed in combination with each other, and their combined effect on the separation is examined. The chromatographic factors that were considered important for method development were strength of organic solvent, ionic strength of phosphate buffer, temperature, pH, and flow rates. The quality of the separation was based on the performance indicators of resolution (R), retention factor (k') and efficiency (N) for each analyte. In describing the relationship between the chromatographic factors and the performance indicators, regression analysis in the form of Artificial Neural Networks (ANN) was applied using Trajan Neural Network software. The most successful neural network is then used to plot response surfaces. Response surfaces are 3D graphs that illustrate the relationship between any two of the chromatographic factors (e.g. pH and temperature) and one of the performance indicators (R, k' or N). Response surface plots indicate the levels for each chromatographic factor and determine optimal separation conditions. Although, this approach may appear complex, it produces a method without experimentally exploring each chromatographic factor at all possible levels. The combined use of neural networks and response surfaces for selection of chromatographic factor levels overall leads to a more efficient and less time consuming method development.

A 2^{5-1} fractional factorial design was created by Minitab and used as the basis of the experimental procedure (Table 2.2.). Within this, two levels (low and high) for each chromatographic factor were assigned. For each experiment, a mixed standard solution containing five cocaines (cocaine, lidocaine, prilocaine, procaine, and benzocaine), three adulterants (caffeine, phenacetin and acetylsalicylic acid) was

injected in triplicate. As part of the method development for the cocaine analysis, the mobile phase was assessed in detail and for this, potassium dihydrogen phosphate dissolved in 1 L deionised water was used as the buffer component of the mobile phase. Buffer pH was adjusted by adding appropriate amounts of phosphoric acid. The column used was a Waters XBridge C18, 50 x 4.6 mm, particle size 3.5 µm. As before with the Xterra column, this is a newer type column, using the 'Hybrid Particle Technology'. These are columns which combine the properties of both silica and polymer columns. Essentially, every third silanol is replaced with a methyl group during synthesis. This results in greater pH stability across the range and also greater peak symmetry for basic compounds.

Table 2.2. A 2^{5-1} fractional factorial experimental design for multivariate method development generated using Minitab software. Chromatographic factors examined included, A: % Acetonitrile; B: pH; C: Temperature (°C); D: Ionic strength (mM) and E: Flow rate (mL min^{-1}).

Factorial Experiment #	Chromatographic Factor				
	A	B	C	D	E
1	10	2.5	40	50	1.5
2	10	3.5	40	50	0.5
3	20	3.5	40	50	1.5
4	10	3.5	40	10	1.5
5	10	3.5	30	50	1.5
6	10	3.5	30	10	0.5
7	10	2.5	40	10	0.5
8	20	2.5	40	10	1.5
9	20	3.5	40	10	0.5
10	20	3.5	30	50	0.5
11	20	2.5	30	50	1.5
12	20	2.5	40	50	0.5
13	20	2.5	30	10	0.5
14	10	2.5	30	50	0.5
15	20	3.5	30	10	1.5
16	10	2.5	30	10	1.5

The sixteen experiments were carried out in random order to avoid systematic error. For each experiment, retention factors for each analyte, resolutions between analytes and efficiencies were calculated by the HPLC software, ClassVp. The average retention factor, resolution and efficiency (k'_{ave}, R_{ave} and N_{ave}) for each experiment were also calculated. The data from the sixteen experiments were used to test different neural networks (Figure 2.2). The five chromatographic factors at two levels were used as inputs, and the retention factors for each analyte, resolutions between analytes, efficiencies for each analyte and their averages were used as outputs. Using Trajan Neural Networks, one hundred neural networks were examined.

Every network has three layers, an input layer, a hidden layer and an output layer. The input layer is the five chromatographic factors at the two different levels for each of the sixteen experiments (Table 2.2). Every network decided whether to take all these factors or a combination within them, i.e. Acetonitrile + pH + Temperature + Ionic Strength + Flow rate or pH + Temperature + Ionic Strength, etc. The hidden layer is made up of both connections and neurons. The maximum number of neurons tested was set at twenty; the number of connections in the network is dependent on the number of neurons. The number of neurons within the hidden layer is an important factor. The greater the number of neurons the more powerful the network. This however, makes the network time-consuming to run and more complicated to train. The reverse of this is true for a small number of neurons and the balance between these two is important. The output layer is the calculated performance indicators. To test as many networks as possible within minimum time, one hundred networks were tested for each of the six outputs. Both three layer multilayer perceptron and radial base function networks were investigated. The training algorithms were a combination of back propagation and conjugate descent.

For each of the six outputs, the best neural network from the one hundred tested was selected. The selection was based on a combination of network performance and error values generated by Trajan. The best network produced high values for performance and low values for error. Using each of the six networks, the predicted values generated were plotted against the observed values. The network with the best correlation indicated that it was that network that best described the separation. This

network was then used to illustrate the separation in the form of response surfaces. Optimal conditions for the separation of the eight analytes were decided based on these response surfaces (Figure 2. 2)

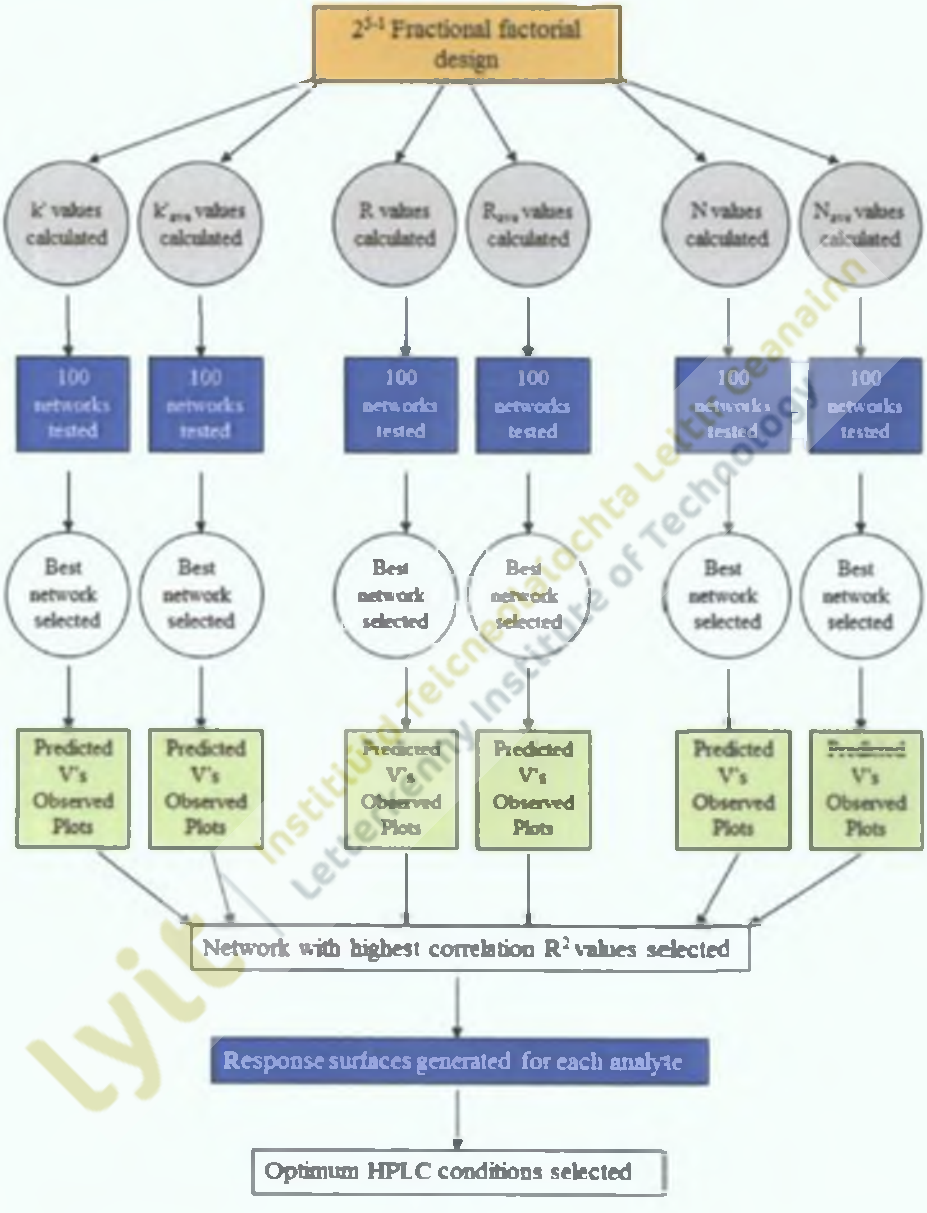


Figure 2.2. Schematic representation of the stages involved in the multivariate HPLC method development for cocaine analysis. Key for different software packages were as follows: - Minitab; - ClassVP; - Trajan; - Microsoft Excel

2.3.3. Validation of multivariate method developed.

As described previously, method validation was performed under the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (www.ich.org), including selectivity, linearity, inter and intra-day precision, accuracy, limit of detection and limit of quantification.

2.4. Sugar analysis.

Previous method development focused on the determination of active ingredients and adulterant compounds of ecstasy and cocaine. Other compounds within illicit drug samples include sugar and metal content. Ecstasy and cocaine samples were analysed for their sugar content in order to obtain a more complete chemical profile.

Ecstasy and cocaine samples were analysed by HPLC for their sugar content. Chromatography was performed on an NH_2 column from the Phenomenex Luna range, a 250 x 4.6 mm, and particle size of 5 μm. Standards for this study included fructose, glucose, inositol, lactose, mannitol, sorbitol and sucrose. Conditions for the separation were a mobile phase of acetonitrile/water (70/30), oven temperature of 30 °C and flow rate was at 0.75 mL min^{-1}. Drug samples for the sugar analysis were prepared by dissolving 50 mg of each in 20 mL rhamnose internal standard solutionin water.

2.5. Metal analysis.

Ecstasy samples were analysed for their metal content. Nine metals, aluminium (Al), calcium (Ca), chromium (Cr), iron (Fe), lead (Pb), magnesium (Mg), potassium (K), sodium (Na) and zinc (Zn) were detected and quantified by atomic absorption methods. This data was then assessed alone and in combination with the other chemical data by means of the chosen statistical procedure in order to verify its potential as another chemical parameter to aid profiling.

Glassware was soaked overnight in 2% HNO_3, and rinsed three times in deionised water before any solutions were prepared. The tablets were crushed with pestle and mortar and prepared by direct dissolution. Direct dissolution involved adding 10 ml of 5% nitric acid (HNO_3) to 25 mg of crushed tablet in a polypropylene tube. Each

mixture was vortexed for 10 seconds and made up to 40 mL with deionised water. Two forms of AA were used for metal analysis. If the concentration of the metals is in the range of parts per billion (ppb), graphite furnace is recommended. If the concentration of the metals is in parts per million (ppm), the standard flame AA can be used. Preliminary tests provided information as to which metal should be analysed by which instrument.

For the graphite furnace AA analysis, a specific method for each element was used. Each method included wavelength, charring temperature and atomisation temperature specific for the element of interest (Table 2.3). In order to reduce the volatility of the metals, matrix modifiers were used for the elements analysed by graphite furnace. 0.05 mg $Mg(NO_3)_2$ was used for Al, Cr, Fe and Zn and 0.2 mg $NH_4H_2PO_4$ was used for Pb. This was added to the sample during the autosampler run. For the flame AA, specific wavelengths, slit widths and lamp currents were stated in the manual (Table 2.4). Daily, a calibration curve was prepared and the equation of the line was used for quantification of each metal in the ecstasy tablets. Blank samples were tested after every ten samples. Each standard and sample were analysed in triplicate and the mean absorbance value of each was used. Injection volume was 20 µL.

Table 2.3. Optimum conditions for graphite furnace Atomic Absorption spectrometer.

Element	Wavelength (nm)	Charring Temp. (°C)	Atomisation Temp. (°C)
Al	309.3	1700	2500
Cr	357.9	1650	2500
Fe	248.3	1400	2400
Pb	283.3	850	1800
Zn	213.8	700	1800

Table 2.4. Optimum conditions for flame Atomic Absorption spectrometer.

Element	Wavelength(nm)	Slit Width (nm)	Lamp Current (mA)
Ca	422.7	0.7	10
K	766.5	0.4	8
Mg	285.2	0.7	10
Na	589	0.2	8

2.6. Data analysis and chemometric procedures.

Conventional profiling of illicit drugs relies on a combination of physical, chemical and statistical techniques to establish links among seizures. In this study various chemometric procedures were applied to the ecstasy chemical data sets to determine if seizures could be linked. The chemical data sets used in this study consisted of the active ingredients plus sugars (AS), metal data (MD) and all data combined (DC) for the 183 individual ecstasy tablet samples (see Appendix Table A1 & A2). Variables for AS data included MDMA, MDEA, amphetamine, caffeine lactose and sorbitol and represent only compounds detected in samples. MD data variables were Al, Zn, Fe, Mg, Ca, Cr, Pb, Na and K that were similarly detected in ecstasy samples.

2.6.1. Ecstasy data and pre-treatments.

Since all data sets were generated by different analytical techniques data pre-treatment is required due to the differences in the scales between variables. There are two standard pre-treatment methods, namely normalisation and standardisation (Miller & Miller, 2005). Both of these statistical data transformation methods can be applied in combination with logarithm and 4^{th} square root derivatisation. Data pre-treatment methods evaluated included the following:

1. Normalisation (N)
2. Normalisation & logarithm ($N + Log_{10}$)
3. Normalisation & 4^{th} square root (N + 4sqrt)
4. Standardisation (S)
5. Standardisation & logarithm ($S + Log_{10}$)
6. Standardisation & 4^{th} square root (S + 4sqrt)

To reduce the complexity and to easily identify the appropriate pre-treatment method, eight seizures were selected based on their discrete AS chemical data profile (Table 2.5, Table A1). Each seizure consisted of three tablets giving a combined sample set of twenty-four for pre-treatment analysis. Each pre-treatment method was evaluated by PCA and HCA methods to determine the most appropriate pre-treatment method(s). The appropriate pre-treatment method was then applied to the entire ecstasy data sets.

Table 2.5. Chemical composition of the eight selected ecstasy seizures used in pre-treatment studies.

Seizure Batch No.	Chemical components					
	MDMA	MDEA	Amphet	Caffeine	Lactose	Sorbitol
13	✓	✓			✓	
14	✓		✓	✓		
15	✓				✓	
17	✓			✓	✓	
22	✓	✓		✓	✓	
26	✓					✓
30			✓		✓	
55	✓				✓	✓

2.6.2. Ecstasy data pattern recognition.

Five different pattern recognition methods were evaluated to determine the suitability of methods in classifying the various ecstasy tablet data sets. The pattern recognition methods evaluated were:

1. Principal Component Analysis (PCA)
2. Hierarchal Cluster Analysis (HCA)
3. Pearson's correlation coefficient
4. Linear Discriminant Analysis (LDA)
5. Artificial Neural Networks (ANN)

Principal Component Analysis (PCA) is a relatively straightforward procedure. The data is reduced to the smallest number of components which describes the maximum information of the data. The scores of the first two principal components were plotted to produce a score plot, from which groupings, if any, are evident.

Hierarchal Cluster Analysis (HCA) is a statistical method that measure the distance between two objects, if the distance is relatively small then the objects are clustered together. The method by which the distance is measured can be calculated by different cluster and interval methods. Seven different HCA cluster methods were evaluated (Between groups, Within groups, Nearest neighbour, Furthest neighbour, Centroid, Median and Wards) using the default Squared Euclidean interval setting of

the SPSS software package. Interval methods included Euclidean, Square Euclidean, Cosine, Pearson, Chebychev, Block and Minkowski. Each of these seven interval methods were evaluated using the default 'between groups' cluster method setting of the SPSS software package. For each test, the data set of the eight seizures used for data pre-treatment studies (Table 2.5) was applied. Selection of the most appropriate cluster and interval method was determined by the numbers of clusters formed by each method. The established combination of cluster and interval methods was then applied to all ecstasy data sets.

Pearson's correlation coefficient measures the strength of the linear relationship between two samples. The stronger this relationship is, the more likely the two samples are related or linked. Pearson's correlation coefficient is currently the method of choice for the European Network of Forensic Science Institutes (ENFSI) for drug profiling. A Microsoft excel macros file to calculate Pearson's correlation was supplied by the ENFSI. In order to use this statistical method to link different seizures threshold values were assigned to each seizure. Threshold values are a number assigned to an individual seizure, above which any subsequent tablet with a coefficient equal to or higher than this value is highly correlated to that seizure and therefore is strongly linked to that seizure. Tablets that are highly correlated should have a value close to 100; those with no correlation have values close to zero. To assign threshold values for each seizure, all three replicates of all three tablets within each seizure were used. The lowest value that was returned was then used as the threshold value for that seizure. Once each seizure had a threshold value assigned, all seizures were correlated against each another. Those seizures with correlation coefficients greater than the threshold value were considered linked.

Both Linear Discriminant Analysis (LDA) and Artificial Neural Networks (ANN) require 'trained' data sets. In order to achieve this, groups within the data set had to be identified and seizure membership assigned. Linear Discriminant Analysis (LDA) was used to identify which data set and which pattern recognition method was the most suitable. The groups generated by PCA, HCA and Pearson for each of the three data sets were applied to LDA. The best data set combination and pattern recognition method should yield a classification rate close to 100%.

As before when ANN was used for method development, there are three layers to any network, an input layer, a hidden layer and an output layer. The input layer consisted of the various quantities for all variables in each tablet while the output layer was the classification groupings identified by unsupervised pattern recognition methods. As before, one hundred networks were tested, using both three layer multilayer perceptron and radial base function networks. The training algorithms were again a combination of back propagation and conjugate descent. The maximum number of neurons tested was set at fifty.

The rate at which each of the pattern recognition methods successfully classified the tablets is reported, the advantages and disadvantages of each method are evaluated and the final combination of data set, pre-treatment and pattern recognition method are discussed. The chosen data pre-treatment and pattern recognition method was applied to the ecstasy tablet data sets. Results from this highlighted possible links between previously unrelated seizures which in theory could potentially allow for law enforcement to identify dealer – user networks.

Chapter 3: HPLC method development.

3.1. Univariate HPLC method development for the analysis of ecstasy.

The design of the experimental procedure concentrated on the univariate method for the development of a HPLC method for the analysis of ecstasy tablets. As such, each chromatographic factor was considered individually, and the effect of the varying factor evaluated on the performance for method development. The separation of a large number of analytes by liquid chromatography is dependent on many variables including stationary phase, mobile phase (type and quantity of organic solvent, ionic strength of buffer, buffer pH), temperature and flow rate. For this study, all factors were assessed for the separation and quantification of amphetamine type stimulants from ecstasy. The quality of each separation was assessed by comparison of three performance indicators: resolution (R), retention factor (k') and height equivalent theoretical plate (HETP).

3.1.1. Chromatographic factors.

Columns

Analyte separations were examined on the column chemistries associated with seven different commercially available columns (Table 2.1). The analyte mixture contained all six amphetamines; amphetamine, MDA, methamphetamine, MDMA, MDEA and MBDB. The main differences between the columns are their physical dimensions and the particulate or monolith nature of the stationary phase.

Mobile Phase

Water is a weak solvent and when used alone as the mobile phase, the equilibrium between the stationary and mobile phase favors the stationary phase. This results in maximum resolution however it also results in longer retention times (i.e. high retention factors). To balance resolution with retention times, water tends to be mixed with a stronger solvent (e.g. methanol or acetonitrile), which shortens the retention times and reduces the resolution. The ratio of water to organic solvent is very specific to the separation required and is an important factor in method development. With this method development, acetonitrile was the only organic solvent chosen based on its chemical properties (Table 1.1). The UV cut off value off for acetonitrile is 195 nm, while for methanol it is 205 nm and tetrahydrofuran 215 nm. The selection of a suitable organic solvent should preferably have a lower UV absorption cut off than the

analyte UV absorption value. Since sample analysis was performed at 200 nm, acetonitrile was chosen as the preferable organic solvent due to its lower UV absorption cut off value.

With each column, 0% acetonitrile produced the greatest retention times. As the concentration of the acetonitrile increased, the retention times decreased. In addition, a decrease of retention times lead to a decrease in resolution. With each column a certain concentration of acetonitrile existed that had minimum retention times but beyond which peaks were unresolved. For example, Figure 3.1 highlights the effects of increasing acetonitrile concentration on analyte resolution. At 5% acetonitrile, all six analytes are resolved in 14.4 minutes and resolution between peak 2 and 3 was greater than 1, as calculated using the USP equation (see page 92). With increasing acetonitrile concentration the retention time decreased, however peaks 2 and 3 were not resolved when acetonitrile concentration was greater than 10%. The broad peaks that are present at the start of the separation may be due to the use of methanol as the sample solvent.

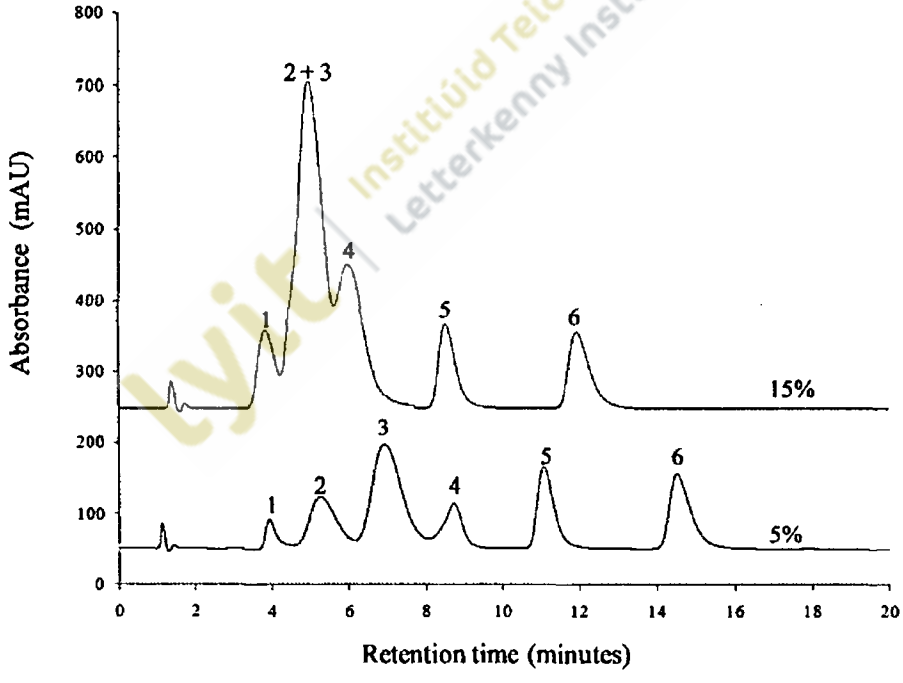


Figure 3.1. Chromatogram of the six ecstasy standard mixture resolved with the XTerra RP18 column at 5% and 15% acetonitrile solvent concentration, pH 3, 30°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4= MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

All other columns investigated followed a similar pattern to that of the XTerra RP18 column. With six of the seven columns, analytes were resolved within a minimal time of approximately 15 minutes. The final organic phase concentration was 3% for the three monolith columns, 5% for the XTerra RP18, 10% for the Luna C8 and, 13% for the Luna C18. There were slightly longer retention times with the Phenosphere C18 column with the last peak eluting at 19.445 minutes (Figure 3.2.). Due to the increased retention times for the Phenosphere C18 column, gradient elution method was assessed for this column. Gradient elution involves varying the concentration of the organic solvent in the mobile phase over the run time. The six ecstasy standard mixture was injected using varying concentrations of the organic solvent. The most appropriate gradient was 90% A (phosphate buffer): 10% B (acetonitrile) at T_0 to 60% A (phosphate buffer): 40% B (acetonitrile) at T_{10}. With this gradient elution, retention times were reduced by 5.136 minutes and all six analytes were resolved in 14.309 minutes (Figure 3.2.).

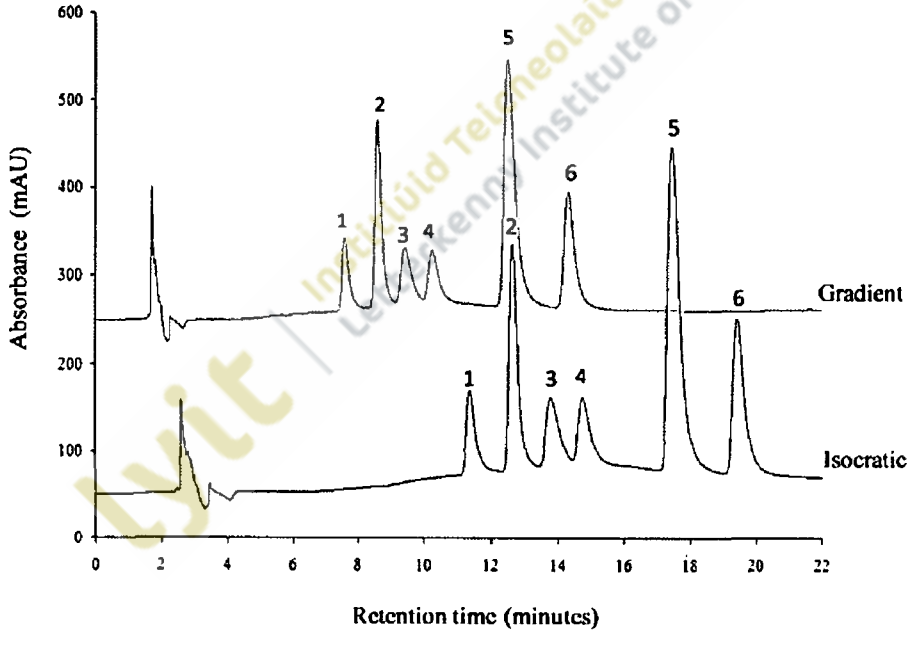


Figure 3.2. Chromatogram of the six ecstasy standard solution resolved with the Phenosphere C18 column under gradient elution and isocratic elution conditions, pH 3, 30°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4= MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

The pH of a mobile phase is an important factor as it affects the ionisability of analyte and therefore greatly influences the selectivity and retention of ionisable compounds. Phosphate buffer is a suitable buffer if analysis is at a low pH (2-3) as opposed to an acetate or citrate buffers which are suitable for slightly higher pH levels (4-5). Adequate buffer concentrations tend to be in the range 10-100 millimolar. Since silica will dissolve, at or above pH 7 and at or below pH 2, a pH range between 2 and 7 is preferred for analysis. However, pH in the middle range of 3.5 to 8.5 tend to be avoided, as large changes in retention times can occur with minor adjustments to pH resulting in difficulties with reproducibility and validation studies. In addition, analyte retention can be affected if the pH of the mobile phase is one pH unit within the pK_a of the compound being analysed. Since the pK_a's for all the amphetamines studied were approximately 10 (amphetamine = 9.8, methamphetamine = 10.1, MDMA = 9.9, MDA = 9.8, MDEA = 9.5, MBDB = 9.8), the phosphate buffer pH conditions were investigated at pH 2.5, 3 and 3.5. Slight differences in peak shape (peak symmetry was calculated using the equation As = b/a where a corresponds to the width of the front of the peak and b the width of the tail of the peak (at 10% peak height) centred around the peak apex). and a is were observed for the three pH levels examined, however pH did not affect the quality of resolution to any great extent. With the particulate columns, peak symmetry was best at pH 3 and pH 2.5 gave best peak symmetry with the monolith columns.

Temperature

By increasing the temperature, higher flow rates can be achieved and enhanced analyte mass transfer can occur, both of which ultimately would decrease retention times. Therefore the ideal temperature for each column should give the shortest retention times without jeopardising resolution. Throughout the method development, injections were made at 25°C. The effects of temperature were studied in increments of 5°C between the range of 25°C and 45°C. Slight decreases in retention times were observed for each column as temperatures increased. The optimum temperature for the Luna C8, XTerra RP18 and the three monolithic columns was 30°C, based on peaks 2 and 3. The Phenosphere C18 and Luna C18 columns were optimal at 35°C. These final temperature levels were chosen based on the resolution between the critical peak pair of peaks 2 and 3. Figure 3.3 demonstrates the minimal effect of

temperature on the separation of the six analytes at 25°C, 30°C, 35°C, 40°C and 45°C for the Luna C18 column. Total analysis time was only reduced by 0.704 minutes after the 20°C incremental increase.

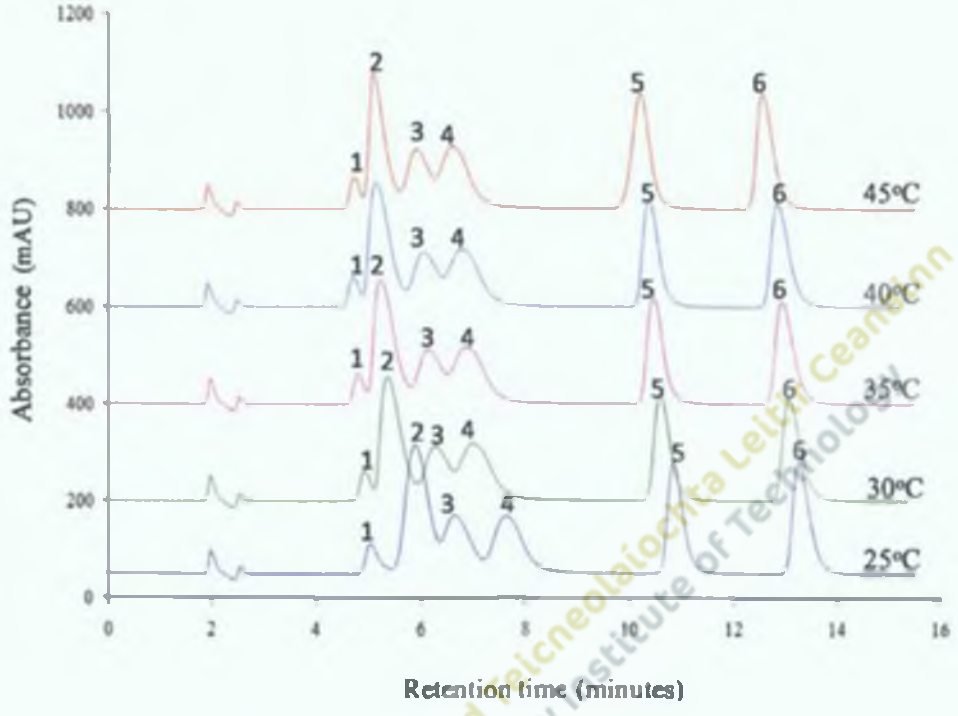


Figure 3.3. Chromatogram of the six ecstasy standard solution resolved on the Luna C18 at five temperatures: 25°C, 30°C, 35°C, 40°C and 45°C. pH at 3, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4= MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

Flow Rates

Flow rate is an important factor in method development. Solvent consumption should be kept to a minimum to limit costs. The optimum flow rate should give a short retention time, good resolution and low pressure. For Phenosphere, flow rates between 0.5 mL min^{-1} and 2 mL min^{-1} were assessed and 1.75 mL min^{-1} was chosen (Figure 3.4.). The other particulate columns had internal diameters of 2 mm and 2.1 mm and the flow rates were reduced to prevent high backpressures. The flow rates most suitable were 0.3 mL min^{-1} for the XTerra RP18 and 0.4 mL min^{-1} for the Luna

C8 and C18. These flow rates were chosen based on optimal resolution with minimal solvent consumption and column back pressure.

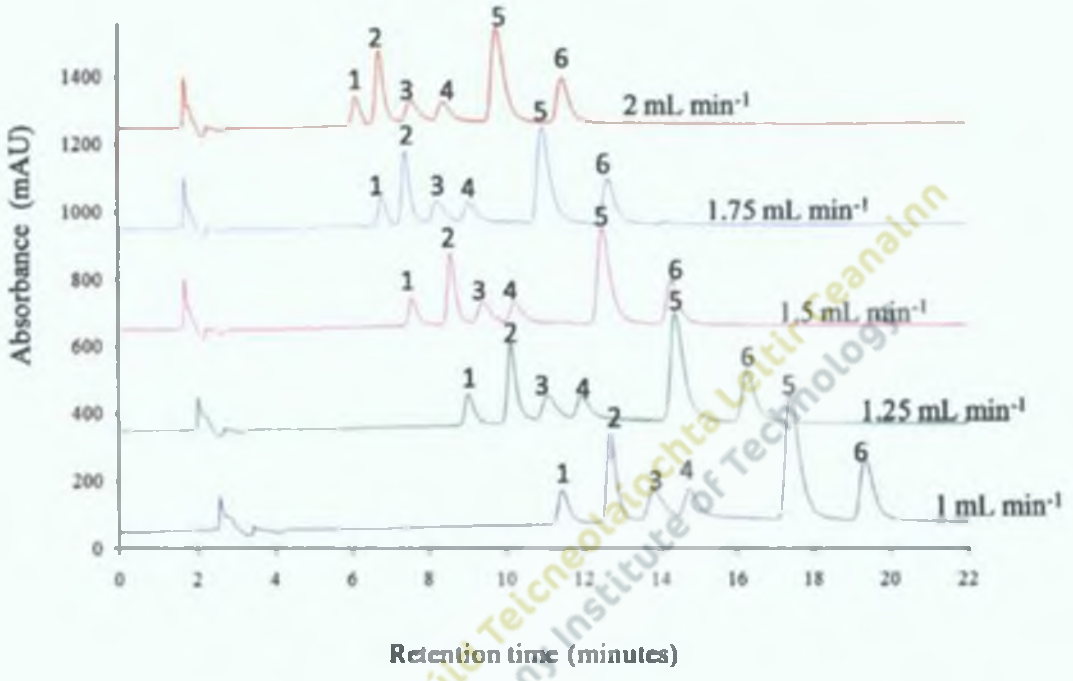


Figure 3.4. Chromatogram of the six ecstasy standard solution separated on the Phenosphere C18 column at five flow rates: 1, 1.25, 1.5, 1.75 and 2 mL min^{-1}. pH 3, 30°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4 = MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

The main factor which differentiates monolithic columns from the traditional particulate columns is the ability to use higher flow rates without the usual accompanying back pressure. The HPLC pump allowed for pump rates up to 9.999 mL min^{-1}. The flow rates for the monolithic columns could be increased significantly due to the lower backpressure. The flow rates for these columns are discussed in greater detail in Section 3.1.3. The most appropriate flow rates for Chromolith Flash, Chromolith SpeedROD and Chromolith Performance were 1.5 mL min^{-1}, 4.5 mL min^{-1} and 8.5 mL min^{-1}, respectively.

3.1.2. Selection of optimal HPLC conditions for ecstasy sample analysis.

For each column, the most suitable mobile phase (organic solvent, concentration of organic solvent, gradient elution, buffer, buffer strength and pH) temperature and flow rates were determined. Figures 3.5 to 3.11 are the chromatograms of the optimised methods for each column.

The quality of the separation of each method must be evaluated in order to select the most appropriate method. The quality of each separation was assessed by comparison of three performance indicators: resolution (R), retention factor (k') and height equivalent to theoretical plate (HETP). Chromatographic performance indicators of each column type for the separation of amphetamine standards are provided in Table 3.1.

Table 3.1. Performance indicators for each column for the separation of ecstasy compounds.

Column	R	k'	HETP (µm)
Luna C8	1.16	3.09	12.34
Phenosphere C18	1.37	5.05	20.83
Luna C18	0.74	3.23	15.00
XTerra RP18	1.14	6.71	6.44
Performance RP-18	1.28	9.40	6.58
SpeedROD RP-18	1.12	7.61	7.83
Flash RP-18	0.95	6.82	6.03

Performance indicators: R; resolution between MDA and methamphetamine, k'; retention factor for MDMA and HETP; height equivalent to theoretical plate for MDMA.

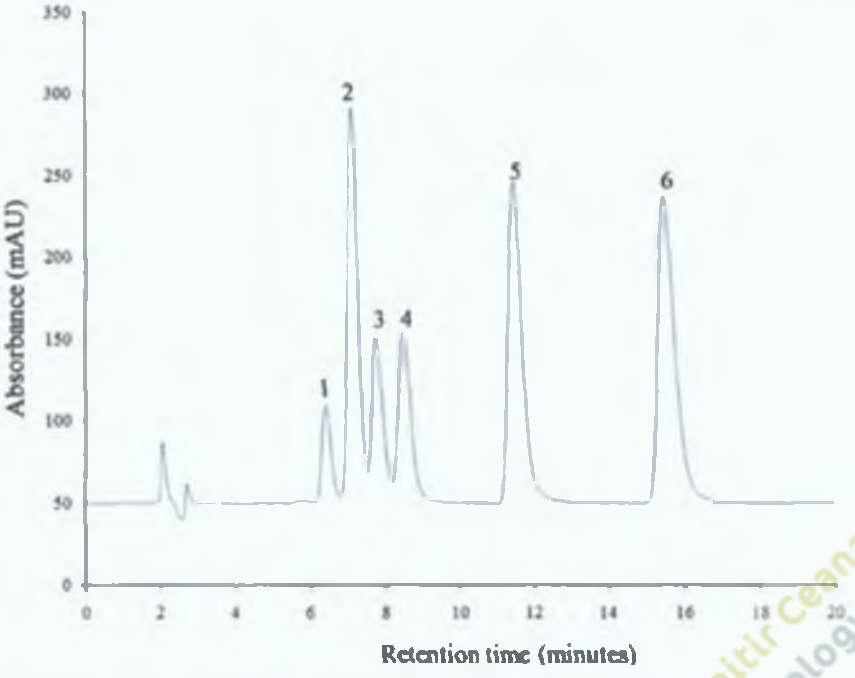


Figure 3.5. Chromatogram of the six ecstasy standard solution resolved on Luna C8. Final chromatographic conditions were 90% 20 mM buffer: 10% acetonitrile, 0.4 mL min^{-1}, pH 3, 30°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4= MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

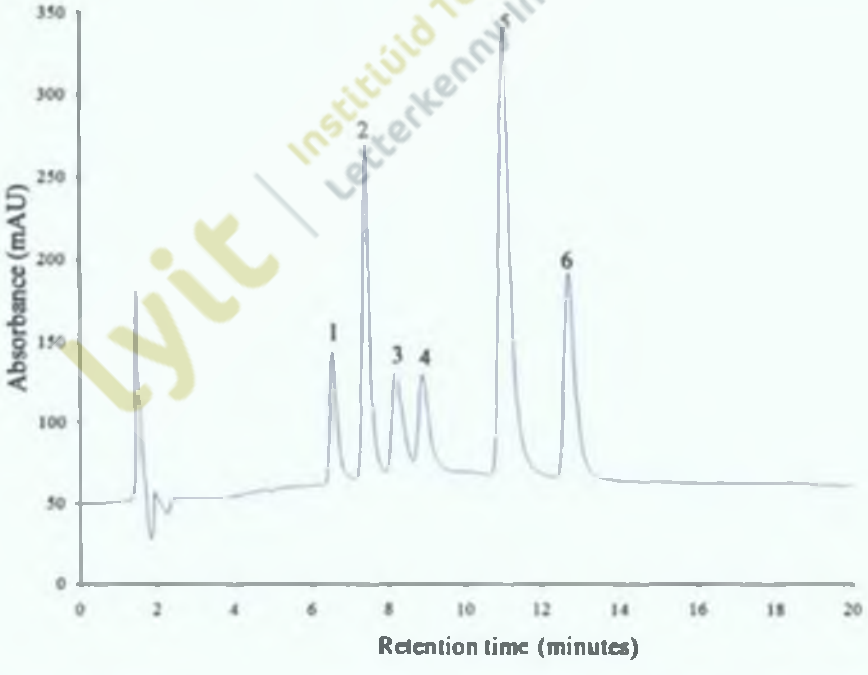


Figure 3.6. Chromatogram of the six standard solution resolved on the Phenosphere C18. Final chromatographic conditions were T_0 90% 20 mM buffer: 10% acetonitrile going to T_{10} 60% buffer: 40% acetonitrile B, 1.75 mL min^{-1}, pH 3, 35°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4= MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

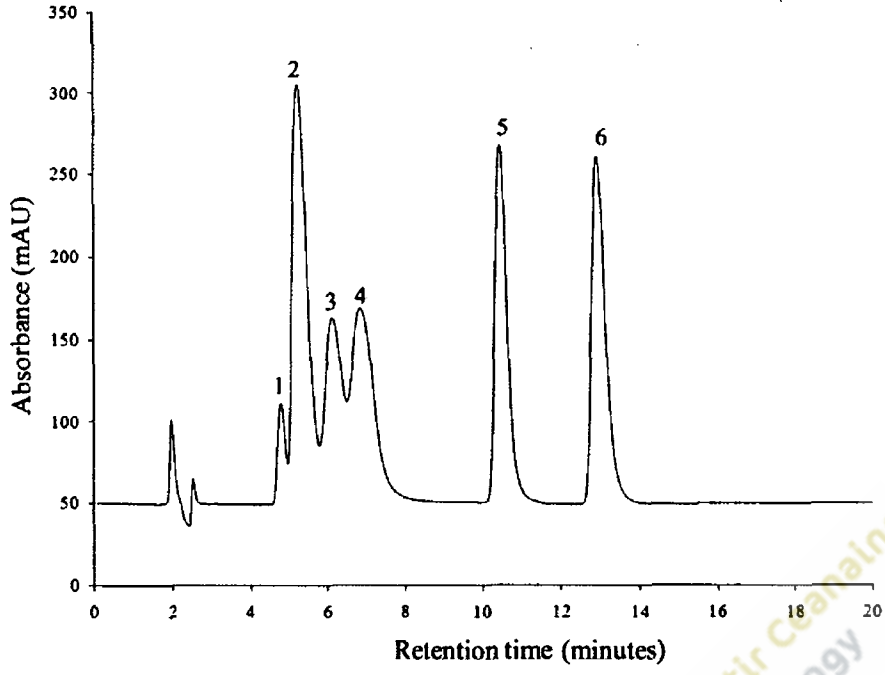


Figure 3.7. Chromatogram of the six ecstasy standard solution resolved on Luna C18. Final chromatographic conditions were 87% 20 mM buffer: 13% acetonitrile, 0.4 mL min^{-1}, pH 3, 35°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4= MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

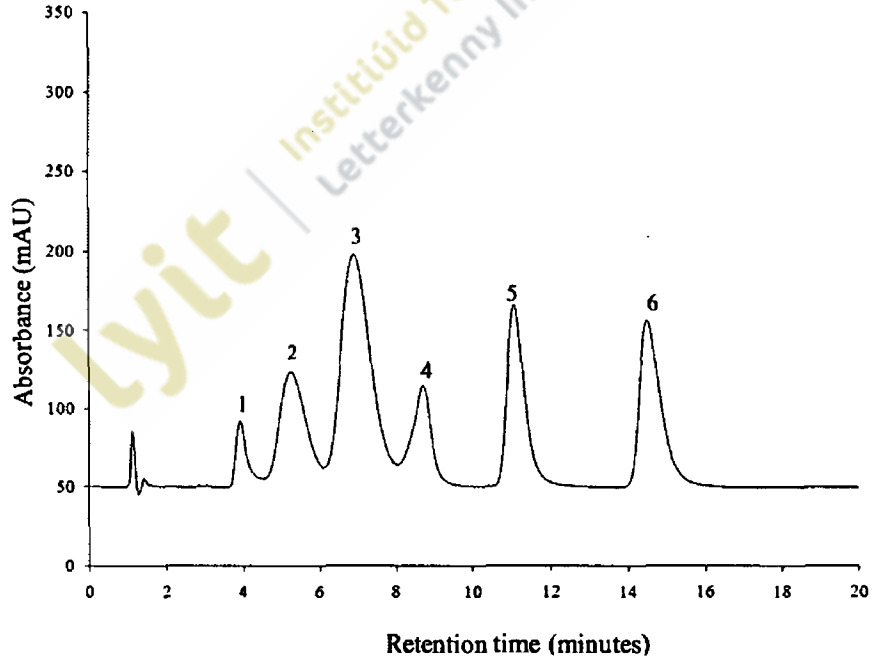


Figure 3.8. Chromatogram of the six ecstasy standard solution resolved on Xterra RP18. Final chromatographic conditions were 95% 20 mM buffer: 5% acetonitrile, 0.3 mL min^{-1}, pH 3, 30°C,20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4= MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

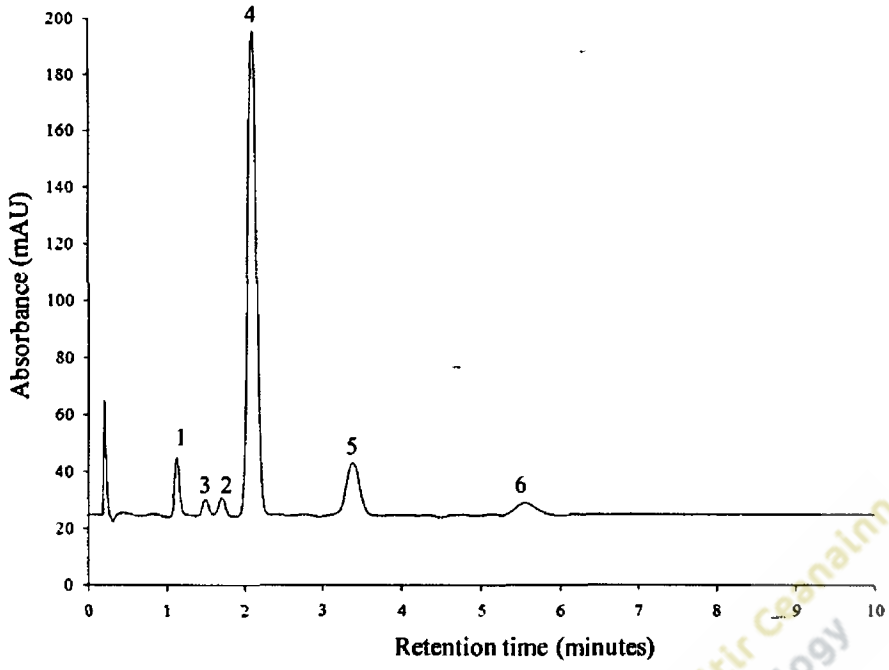


Figure 3.9. Chromatogram of the six ecstasy standard solution resolved on Chromolith Performance RP18, 100 x 4.6 mm. Final chromatographic conditions were 97% 20 mM buffer: 3% acetonitrile, 8.5 mL min^{-1}, pH 2.5, 30°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 2 = MDA, Peak 3 = methamphetamine, Peak 4= MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

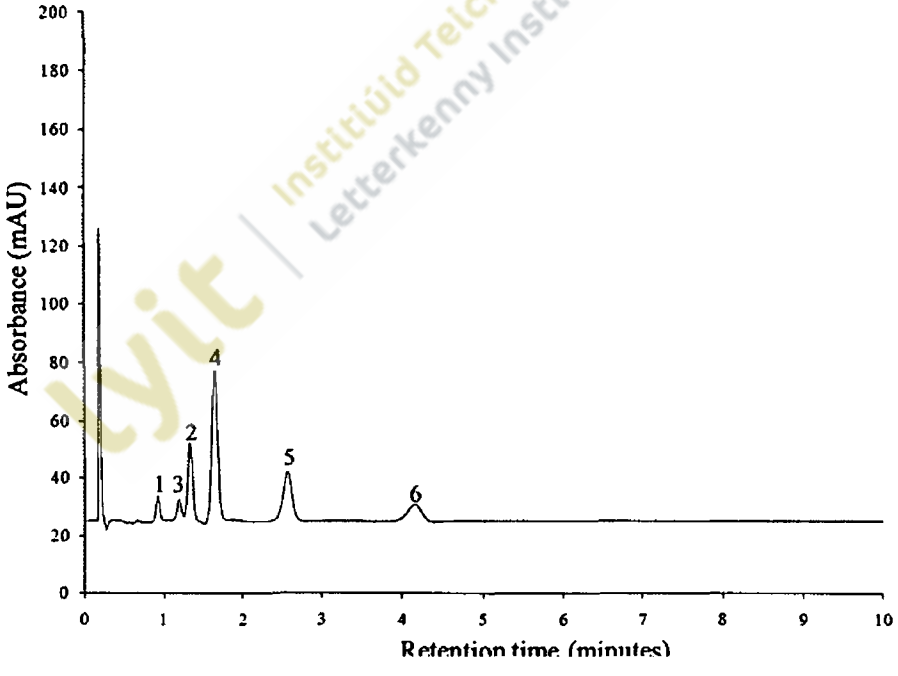


Figure 3.10. Chromatogram of the six ecstasy standard solution resolved on Chromolith SpeedROD, RP18, 50 x 4.6 mm. Final chromatographic conditions were 97% 20 mM buffer: 3% acetonitrile, 4.5 mL min^{-1}, pH 2.5, 30°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 3 = methamphetamine, Peak 2 = MDA, Peak 4 = MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

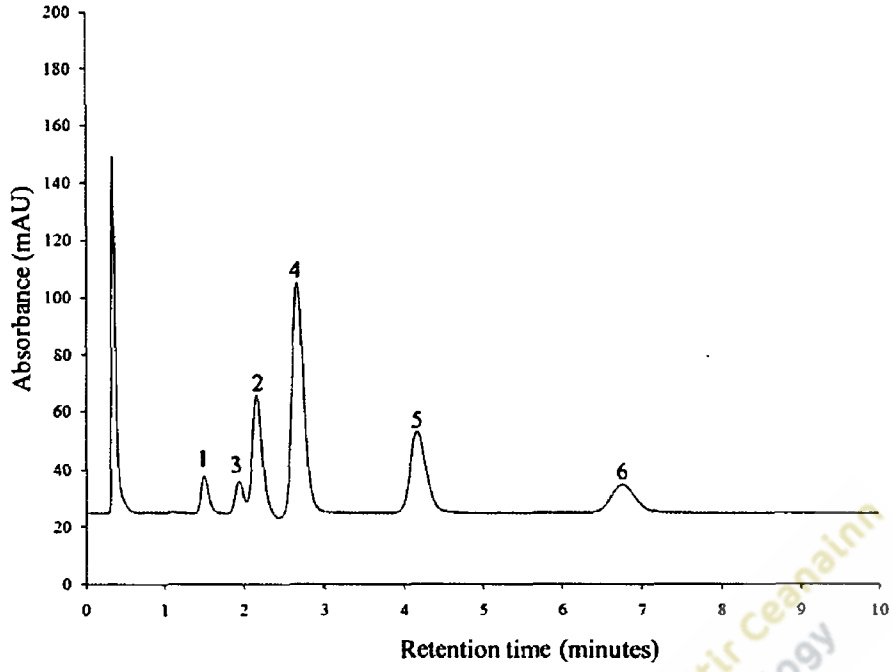


Figure 3.11. Chromatogram of the six ecstasy standard solution resolved on the Chromolith Flash RP18, 25 x 4.6 mm. Final chromatographic conditions were 97% 20 mM buffer: 3% acetonitrile, 1.5 mL min^{-1}, pH 2.5, 30°C, 20 µl sample injection, detection at 200 nm. Peak 1 = amphetamine, Peak 3 = methamphetamine, Peak 2 = MDA, Peak 4 = MDMA, Peak 5 = MDEA, Peak 6 = MBDB.

The difference in peak heights in the seven chromatograms is due to standard solutions of different concentrations being injected. A peak shift was observed with peaks 2 and 3 when moving from the particulate columns to the monoliths. This can occur and reasons may be due to silanol activity or endcapping.

For the separation of a large number of analytes, resolution is an important factor in the analysis. Chromatograms are only useful if there is a good degree of separation between each peak. Good resolution is defined as being greater than 1. Ideally, all compounds should have a resolution greater than 1.5. The resolution of each analyte was calculated by the ClassVp software, based on USP methods, using the following equation:

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1} \qquad \textbf{(Equation 3.1)}$$

Where R is the resolution between peak of interest (peak2) and the preceding peak (peak1), t_1 and t_2 are the retention times of peak 1 and peak 2, respectively and W_1 and W_2 are the widths of the base of components peak 1 and peak 2 respectively.

Methamphetamine (peak 3) and MDA (peak 2) were the two analytes that were most difficult to resolve. Therefore, in order for a column and method to be successful, it is critical that these two analytes have a resolution >1. Table 3.1 reports the resolution between MDA and methamphetamine. Two columns, the Luna C18 and the Flash RP-18, were unable to resolve these peaks satisfactorily, with resolutions <1 for both columns. All other columns had resolutions >1, however no column had an ideal resolution >1.5. From this, it can be stated that the Luna C18 and the Flash RP-18 are the least suitable columns (and method) for this analysis.

Retention factor (k') indicates the interaction between the analyte and the stationary phase, relative to the unretained compound. The optimum retention factor lies within the range of 2 to 10. The retention factor was calculated using Class Vp software package, based on USP methods, as follows:

$$k' = \frac{t_2 - t_o}{t_o} \qquad \textbf{(Equation 3.2)}$$

Where k' is the retention factor, t_2 is the retention time of the peak of interest and t_o is retention time of an unretained compound. The average retention factors for all seven columns were between 2 and 10. The values reported for the three monoliths and the XTerra RP18 were higher than those of the three particulates Luna C8, Luna C18 and Phenosphere C18 (Table 3.1).

Efficiency, defined as the number of theoretical plates in a column, is the measure of the sharpness of eluting peaks. The number of theoretical plates is affected by the size of the particles, the length of the column and the quality of the procedure used to pack the particles into the columns. The theoretical plates were calculated using ClassVp software package as follows:

$$N = 16\left(\frac{t}{W}\right)^2 \qquad \textbf{(Equation 3.3)}$$

Where N is the theoretical plates, t is the retention time of the component and W is the width of the base of the component peak using the tangent method. The higher the value of N, the more efficient the separation. As stated previously, efficiency is dependent on column length. In this study, columns of four different lengths were studied, 25 cm, 50 cm, 100 cm and 250 cm, and comparison based on their efficiency

value is not technically correct. Alternatively, Height Equivalent to Theoretical Plate (HETP)(μm) can be used to directly compare columns of different lengths. HETP is calculated as follows:

$$\text{HETP} = \frac{L}{N} \qquad \textbf{(Equation 3.4)}$$

Where L is the length of the column and N is the number of theoretical plates. Low values for HETP are desired for efficient separations. HETP values for the three monoliths were significantly lower than those of the Luna C8, Luna C18 and Phenosphere C18 (Table 3.1). Although XTerra RP18 is the same length as the other particulate columns (250 cm), its efficiency was similar to the three monolithic columns, which could be explained by the hybrid technology of this column.

When choosing any of the methods developed for the routine analysis in the laboratory, the quality of the separation is undoubtedly important. But one has to also take into account the speed of the analysis and the costs incurred. As all columns were consistent with each other in terms of separation quality, the choice of method would lean towards one of the monolithic columns, as analyte separation was achieved in shorter run times (Figure 3.9, 3.10 and 3.11). Within the monoliths, the 50 mm SpeedROD provided good quality separation with minimum solvent consumption and run time. For these reasons, the method developed using the 50 mm monolithic column was selected for the analysis of ecstasy samples (Figure 3.10).

3.1.3. Comparison of particulate and monolithic columns.

A comparative analysis of the performances of particulate and monolithic columns was performed. This involved comparing the performance of the most successful particulate column, the Waters XTerra, 5μm, 250 x 2.1 mm RP18 column with the three monolithic columns. Measurement of column backpressure and the creation of Van Deemter plots allowed for comparative assessment.

The minimum analysis times for the complete separation of the six analytes using the XTerra RP18 column was 14.512 minutes. In contrast the minimum analysis times for the complete separation of the six analytes was 6.763 minutes for the Chromolith Flash RP18, 25 x 4.6mm (1.5 mL min^{-1}), 4.160 minutes for the Chromolith

SpeedROD, RP18, 50 x 4.6 mm (4.5 mL min^{-1}) and 5.536 minutes for the Chromolith Performance RP18, 100 x 4.6 mm (8.5 mL min^{-1}). The effect of different flow rates on resolution was investigated using the Chromolith SpeedROD, RP-18, 50 x 4.6 mm column. Resolution was not affected and analysis times were decreased from 18.3 minutes to 3.8 minutes when using flow rates from 1 mL min^{-1} to 5 mL min^{-1}, respectively (Figure 3.12).

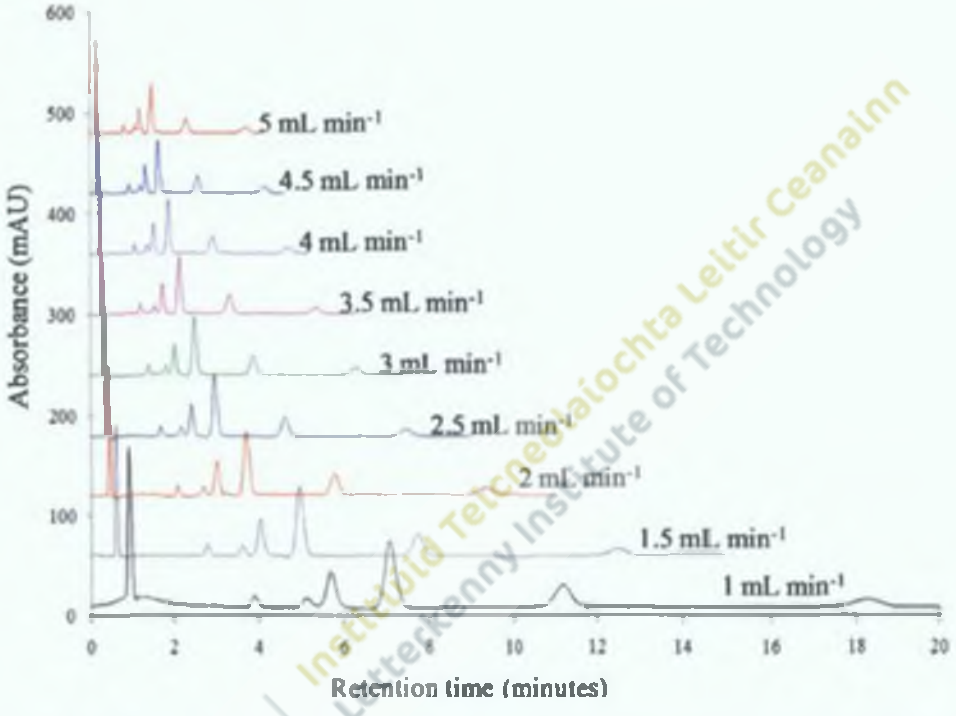


Figure 3.12. Chromatogram of the six standard solution resolved on the Chromolith SpeedROD, RP-18, 50 x 4.6 mm column at nine flow rates. The chromatographic conditions and elution order was as described in Figure 3.10.

Column backpressure was measured for each column flow rate up to recommended pressure limit. Figure 3.13 shows column backpressure as a function of flow rate for each column type tested. Maximum flow rate of 3 mL min^{-1} could only be achieved with the particulate column, while monolithic columns allowed for an increased flow rate of up to 9 mL min^{-1} with reduced back pressure. In addition the slope of the monolithic column is much lower than that for the particulate, indicating the ability of the monolithic to achieve higher flow rates without the backpressure.

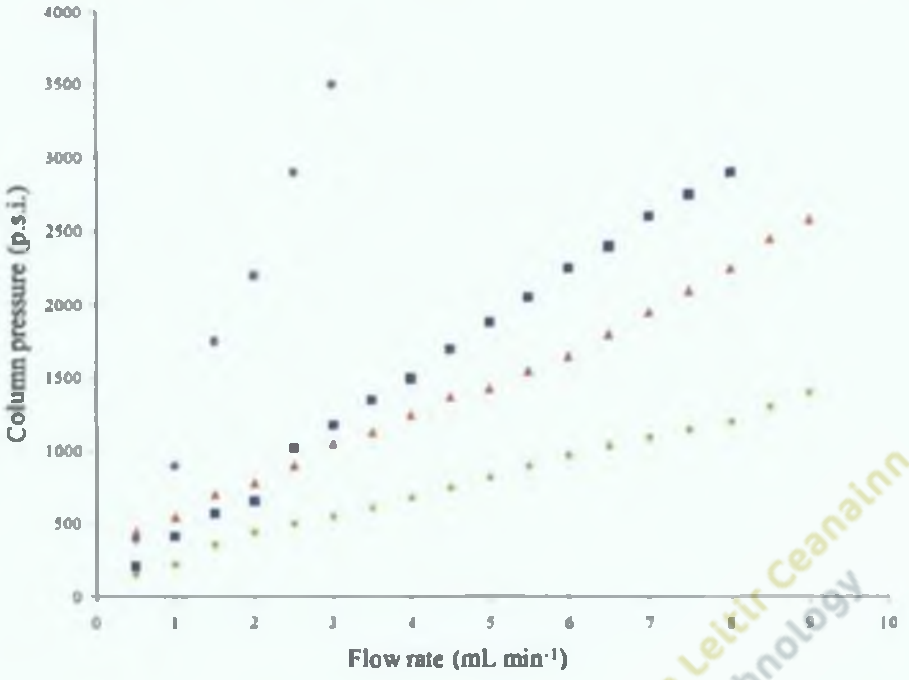


Figure 3.13. Comparison of column backpressure as a function of flow rate for the XTerra RP18 • particulate column and the three Chromolith RP18 monolithic columns. Chromolith columns were of varying lengths of 25 mm ♦ , 50 mm ▲ and 100 mm ■.

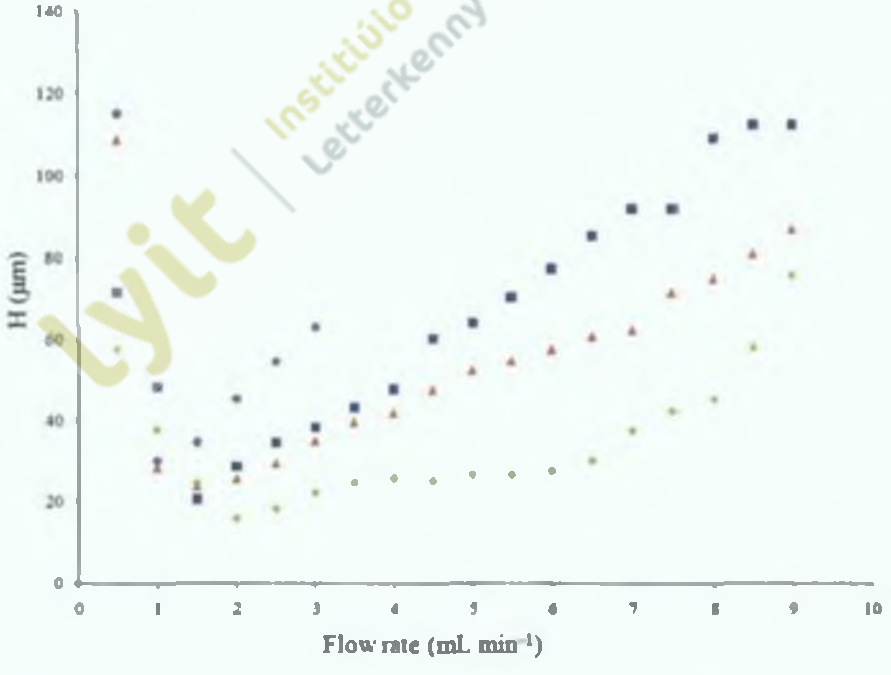


Figure 3.14. Fitted van Deemter curves of the plate height (H) vs. Flow rate for the XTerra RP18 • particulate column and three Chromolith RP-18 columns. Chromolith columns were of varying lengths of 25 mm ♦ , 50 mm ▲ and 100 mm ■.

Van Deemter curves were created for the monolithic and particulate columns. Figure 3.14 shows the resulting plots for each column type. The flatter curve for each monolithic column implies that these columns provide higher efficiency at high flow rates compared to the particulate column. For example, the plate height for the Chromolith SpeedROD, RP18, 50 x 4.6 mm at 7 mL min^{-1} is approximately the same as that for the packed column at 3 mL min^{-1}. Therefore the monolithic column can be run twice as fast as the particulate without sacrificing column efficiency.

3.1.4. Validation of univariate method developed.

Due to good separation of the six amphetamine analytes with reasonable analysis times the Chromolith SpeedROD, RP-18, 50 mm x 4.6 mm was selected for validation studies. Validation tests were carried out under the guidelines of the International Conference on Harmonisation (ICH, 1996).

The highest precision for quantitative chromatography is obtained by the use of internal standards because uncertainties produced by sample injection are avoided. Here, a compound of known concentration that does not interfere in the analysis is added to the sample mixture. Clomipramine, metoclopramide and propyl paraben are pharmaceutical compounds that are of similar chemistry to the analytes of interest, but have never been reported in any ecstasy sample. Each of the standards were prepared at concentrations of 100 μg mL^{-1} and analysed by the HPLC method developed. Metoclopramide was chosen as the most suitable internal standard. It had good resolution from other analytes and gave a good response at the wavelength chosen, 200 nm.

Selectivity is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. This was a priority due to the many possible components that may be present in ecstasy tablets. Acetaminophen, acetylsalicylic acid, caffeine and phenacetin were possible adulterants tested. Figure 3.15 shows a chromatogram of the amphetamine type compounds, adulterants and internal standard resolved on the Chromolith SpeedROD, RP-18, 50 x 4.6 mm column. Peak resolution was calculated to be greater than 1 for all analytes.

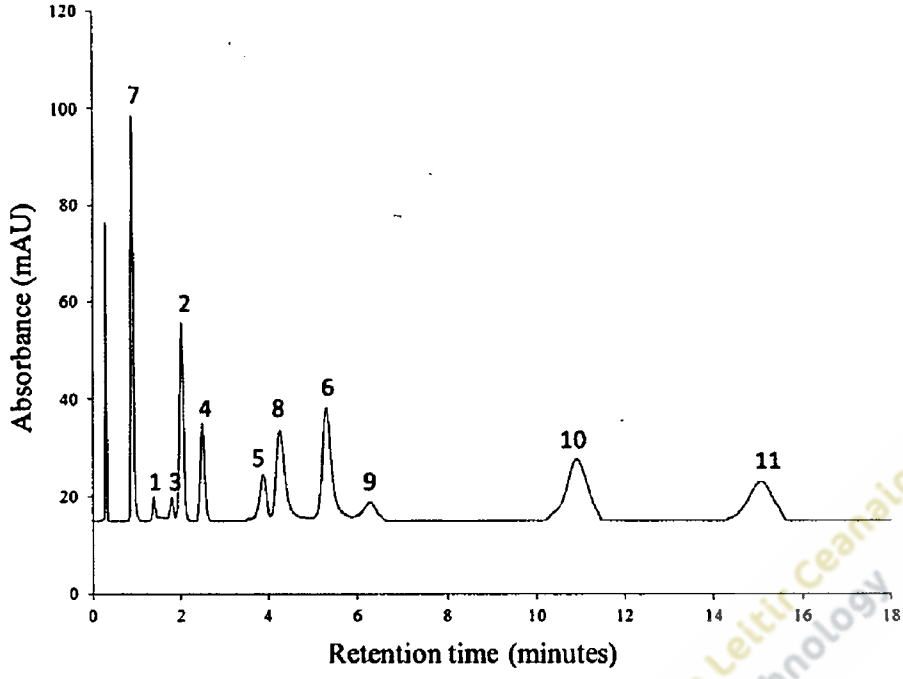


Figure 3.15. Selectivity tests showing the separation of amphetamines, adulterants and internal standard on the Chromolith SpeedROD, RP18, 50 mm x 4.6 mm. The chromatographic conditions were phosphate buffer/acetonitrile (97/3, v/v) maintained in an isocratic mode with pH at 2.5, temperature at 30 °C flow rate at 3 mL min^{-1}. The elution order was 7: acetaminophen, 1: amphetamine, 3: methamphetamine, 2: MDA, 4: MDMA, 5: MDEA, 8: caffeine, 6: MBDB, 9: acetylsalicylic acid, 10: phenacetin and 11: metoclopramide

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in samples within a given range, or proportional by means of a well defined mathematical transformation. A correlation coefficient of ≥0.999 is considered acceptable. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level. Linearity was evaluated using Mandel's test comparing linear and quadratic models (Mandel, 1964). For amphetamine, methamphetamine, MDA, MDMA and MDEA, the quadratic model was not significantly better than the linear model at the 5% level. For MBDB, deviation from linearity emerged ($F_{1, 17} = 4.91$, $P = 0.04$). Linear regression analysis was determined on sample concentrations ranging between 10 μg mL^{-1} and 250 μg mL^{-1} for six calibration points. Linear regression analysis indicated correlation coefficients of ≥0.986 for each analyte tested (Table 3.2).

Table 3.2. Linearity studies for the Chromolith SpeedROD, RP18, 50 x 4.6 mm.

Parameter	Amphetamine Standards					
	Amphetamine	Methamphetamine	MDA	MDMA	MDEA	MBDB
Correlation coefficient (R^2)	0.996	0.994	0.986	0.995	0.986	0.994
y-intercept	-1.029	2.32	4.766	2.437	5.128	2.328
Slope	0.005	0.0005	0.00016	0.006	0.00016	0.00017
Residual sum of squares	4.131	5.195	7.791	2.931	7.725	5.034

The precision of a method is defined as the closeness of a number of replicate measurements to each other and is affected mainly by the random error associated with the method. Precision was expressed as relative standard deviation (% RSD). For repeatability, ten replicates of 50, 100 and 250 μg mL^{-1} were analysed on the same day. These standards were again analysed in five replicates over five days to establish inter day precision. Repeatabilities were in the range of 0.36-1.09% and reproducibilities were in the range of 2.05-5.81% for five replicate determinations over five days (Table 3.3).

Accuracy is defined as the closeness of an analysis, or mean of replicate analysis, to the true value of the sample. With no reference materials available, the accuracy of the method was determined by analysing spiked samples at six concentration levels over the range of 25 and 500 μg mL^{-1}. Results were calculated as experimental values compared to theoretical values and were expressed as percent recovery. Accuracy of the experiment results was always within 90.8-101.2% of the theoretical values (Table 3.3).

The limit of detection (LOD) is defined as the lowest concentration that produces a response three times greater than the background noise and ten times higher for that of the limit of quantification (LOQ). LODs for all six amphetamines ranged from 0.2-3 μg mL^{-1} and LOQs ranged from 0.6-10 μg mL^{-1}. The limits were validated by analysing standards prepared at the concentrations of the LOQs for each standard and their precision and accuracy were reported (Table 3.3).

Table 3.3. Validation studies for the Chromolith SpeedROD, RP18, 50 x 4.6 mm. Six amphetamine standards were tested for intra-day precision, inter-day precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) under ICH guidelines.

Parameter	Amphetamine Standards					
	Amphetamine	Methamphetamine	MDA	MDMA	MDEA	MBDB
Intra-day Precision (% RSD)						
50 $\mu g\ mL^{-1}$	0.65	0.51	0.44	0.36	0.85	0.69
100 $\mu g\ mL^{-1}$	0.89	1.09	0.89	0.81	0.94	1.03
250 $\mu g\ mL^{-1}$	0.85	0.63	0.81	0.74	1.01	0.98
Inter-day Precision (% RSD)						
50 $\mu g\ mL^{-1}$	2.33	3.67	2.98	4.74	3.15	3.23
100 $\mu g\ mL^{-1}$	2.07	3.74	3.53	2.49	2.05	2.84
250 $\mu g\ mL^{-1}$	3.92	5.81	5.17	4.33	5.39	4.76
Accuracy (% Recovery)						
50 $\mu g\ mL^{-1}$	98.2	98.8	99.1	98.9	98.2	99.4
100 $\mu g\ mL^{-1}$	100.3	97.3	97.8	100.6	99.1	97.8
250 $\mu g\ mL^{-1}$	94.3	90.8	97.6	101.2	98.6	98.2
LOD $\mu g\ mL^{-1}$	1	2	3	3	0.2	2
LOQ $\mu g\ mL^{-1}$	3	6	9	10	0.6	6
Precision (% RSD)	1.02	0.93	0.95	0.81	0.86	0.92
Accuracy (% Recovery)	95.6	97.5	98.8	98.5	93.8	97.5

3.1.5. Conclusion.

The objective for this study was to develop a HPLC method for the separation of the major components of ecstasy tablets. A highly selective HPLC method was developed using the univariate approach. The step by step process examined chromatographic factors such as column chemistry, type and quantity of organic solvent, buffer strength, pH, temperature and flow rate. Chromatography demonstrated good separation of the amphetamines and typical adulterants. The mobile phase of a phosphate buffer/acetonitrile satisfactorily separated MDMA and its related compounds when used in conjunction with various stationary phases. The final chromatographic conditions were 20 mM phosphate buffer/acetonitrile (97/3 v/v) maintained in an isocratic mode, pH 2.5, temperature 30°C and flow rate of 3 mL min^{-1} using the Chromolith SpeedROD, 50 x 4.6 mm column.

Comparisons of column types indicate that monolithic columns are more efficient than particulate columns without loss of resolution. Column back pressure and van Deemter plots demonstrated that the monolith columns provide higher efficiency at higher flow rates when compared to the particulate column without loss of peak resolution or selectivity. Monolithic columns reduced the retention times from approximately 18.3 minutes to 3.8 minutes resulting in faster separation without sacrificing resolution (Figure 3.12). The method developed for the monolith column, the Chromolith SpeedROD 50 x 4.6 mm was validated to ICH standards. This study demonstrates the suitability of the method for the routine analysis of ecstasy tablets and the practicality of the monolith column.

3.2. Multivariate HPLC method development for the analysis of cocaine.

Multivariate method development is the application of statistical and mathematical models to the chemical separation of analytes. The separation of a large number of analytes by liquid chromatography is dependent on many variables: columns, organic solvent, pH, gradient elution and solvent strength, flow rates and temperature. The optimisation of a method could in theory be time consuming. Alternatively, multivariate method development simultaneously examines all chromatographic separation factors to determine optimum performance. Data generated by the multivariate approach can be used to calculate a mathematical model with which to obtain response surfaces (Baranda *et al.* 2005, Medenica *et al.* 2004, Soni *et al.* 2007, Wang *et al.* 2006, Zhang *et al.* 2005). A response surface is a 3D plot used to illustrate the data from the optimisation analysis and is created by the use of artificial neural networks (Boti *et al.* 2009, Jancic-Stojanovic *et al.* 2009, Tran *et al.* 2007, Webb *et al.* 2009). By using multivariate analysis, chromatographic experiments may be designed in order to give maximum information from as few experiments as possible. With the use of an optimisation strategy, the time taken for method development may be reduced considerably.

3.2.1. Multivariate method development.

The chromatographic factors that were studied were strength of organic solvent, ionic strength of buffer, temperature, pH, and flow rates. These were chosen as the most influential factors on the analyte separation based on preliminary experiments (data not shown). A 2^{5-1} fractional factorial design was created using the Minitab software and used as the basis of the experimental procedure (Table 2.2.). Resolutions between each peak and its preceding peak, retention factors and efficiencies and each of their averages were calculated for each experimental separation and referred to as 'observed' data (Table 3.4 – 3.6). Each dataset was then used to construct a neural network that would best illustrate the relationship between the chromatographic factors and the performance indicators. An alternative calculation that could be made is the 'resolution normalised' where the product of all the resolution values is divided by the average resolution value.

Both Multi Layer Perceptron (MLP) and Radial Base Function (RBF) networks were investigated. For each of the six outputs, 100 networks were tested and the best performing networks with the highest performance and lowest associated error were identified (Table 3.7). From these, the predicted values were plotted against the observed values. The correlation for each was then calculated. The combination of data set & network that best describes the separation was chosen based on the correlation values. That network with the highest correlation values was chosen as the best illustration of the separation.

Table 3.4. Experimental retention factor (k') values and k'_{ave} values obtained for eight components of cocaine in sixteen discrete experiments.

Expt. No	Cocaine analytes								Average (k'_{ave})
	Procaine	Caffeine	Lidocaine	Prilocaine	ASA*	Cocaine	Phenacetin	Benzocaine	
1	0.54	1.26	2.05	3.28	5.33	11.10	18.44	26.85	8.61
2	1.41	2.28	3.72	5.74	7.13	13.44	20.74	29.95	10.55
3	0.47	0.47	1.29	1.29	1.29	4.37	8.01	11.80	3.62
4	0.56	1.31	2.04	3.12	3.72	6.04	7.09	16.51	5.05
5	0.40	0.88	2.18	3.40	4.24	13.77	19.34	31.51	9.47
6	0.43	0.94	2.35	3.51	4.34	13.89	19.73	32.88	9.76
7	0.56	1.30	2.09	5.22	6.36	12.30	18.62	27.47	9.24
8	0.32	0.43	0.99	0.99	2.48	3.82	6.50	9.05	3.07
9	0.44	0.44	1.13	1.13	1.43	4.23	7.93	10.96	3.46
10	0.19	0.19	0.34	0.34	0.92	1.96	3.62	7.01	1.82
11	0.21	0.21	0.41	0.41	0.72	2.05	4.08	6.54	1.83
12	0.46	0.46	1.15	1.15	3.05	4.46	7.95	12.10	3.85
13	0.23	0.44	0.77	0.77	2.23	5.03	7.21	8.74	3.18
14	0.97	2.17	2.90	4.12	5.15	17.26	19.93	25.35	9.73
15	0.19	0.42	0.53	0.53	1.59	3.84	5.52	6.64	2.41
16	0.34	0.77	1.73	3.36	4.09	12.53	15.55	20.65	7.38

ASA* = Acetylsalicylic acid

Table 3.5. Experimental resolution (R) values and R_{ave} values obtained for eight components of cocaine in sixteen discrete experiments.

Expt. No	Cocaine analytes								Average (R_{ave})
	Procaine	Caffeine	Lidocaine	Prilocaine	ASA*	Cocaine	Phenacetin	Benzocaine	
1	1.87	2.18	2.11	2.68	3.03	2.58	4.43	3.67	2.82
2	4.47	2.34	3.18	3.00	1.47	2.90	3.81	3.54	3.09
3	1.13	0.00	1.13	0.00	0.00	2.96	2.15	4.48	1.48
4	2.82	2.91	2.45	3.31	1.54	1.28	1.36	7.45	2.89
5	1.52	1.42	3.01	1.69	0.63	14.92	5.38	4.59	4.15
6	2.26	1.91	4.18	1.95	0.83	3.11	2.22	5.79	2.78
7	6.01	3.02	4.36	3.94	1.66	4.58	4.36	9.99	4.74
8	1.48	0.50	5.28	0.00	8.70	2.25	4.88	2.90	3.25
9	1.75	0.00	1.59	0.00	0.55	2.76	6.00	2.78	1.93
10	1.28	0.00	0.34	0.00	3.94	0.82	3.20	4.93	1.81
11	1.45	0.00	1.68	0.00	0.84	2.49	2.85	3.04	1.54
12	1.08	0.00	1.43	0.00	3.69	1.24	4.63	3.23	1.91
13	1.45	1.27	0.82	0.00	2.66	0.93	2.95	1.06	1.39
14	3.18	3.21	1.68	1.76	0.83	6.42	1.28	2.29	2.58
15	0.98	0.92	0.22	0.00	1.28	3.96	6.91	3.47	2.22
16	1.71	1.94	3.80	2.46	0.98	6.25	2.15	2.94	2.78

ASA* = Acetylsalicylic acid

Table 3.6. Experimental efficiency (N) values and N_{ave} values obtained for eight components of cocaine in sixteen discrete experiments.

Expt. No	Cocaine analytes								Average (N_{ave})
	Procaine	Caffeine	Lidocaine	Prilocaine	ASA*	Cocaine	Phenacetin	Benzocaine	
1	413	637	955	1068	932	1452	1448	1925	1104
2	715	1156	1330	1047	957	1308	1502	1754	1221
3	104	104	289	289	289	768	162	1825	479
4	807	988	1618	2249	1850	1301	1773	1578	1521
5	451	328	809	312	157	957	1543	1620	772
6	971	496	1696	439	342	298	2117	2118	1060
7	1326	1326	2069	2921	1709	2288	2225	1961	1978
8	495	727	856	856	1651	1531	2454	1209	1222
9	412	412	212	212	346	1567	2548	1064	847
10	528	528	599	599	688	699	984	1664	786
11	355	355	533	533	195	249	285	304	351
12	149	149	310	310	903	1062	1825	912	703
13	295	315	206	206	455	1248	1705	342	597
14	481	1073	1075	496	249	1290	1541	1643	981
15	1112	257	89	89	454	1573	1856	2248	960
16	757	796	1866	636	639	1678	1970	1909	1281

ASA* = Acetylsalicylic acid

Table 3.7. The best performing ANN for each of the experimental data sets.

Data Set	ANN	Performance			Error		
		Training	Selection	Test	Training	Selection	Test
k'	RBF 5:5-2-8:8	0.854	1.309	0.849	1.329	1.416	4.628
k'_{ave}	RBF 5:5-1-1:1	0.851	0.828	0.632	0.253	0.230	0.290
R	RBF 4:4-1-8:8	0.907	0.677	0.998	0.458	0.443	0.398
R_{ave}	RBF 4:4-2-1:1	0.961	0.826	0.831	0.855	0.874	0.509
N	RBF 5:5-1-8:8	0.989	0.990	0.889	0.002	0.001	0.005
N_{ave}	RBF 5:5-1-1:1	0.997	0.963	0.987	0.003	0.004	0.006

k'. Retention factor for each analyte; k'_{ave}. Average retention factor for all analytes; R. Resolution for each analyte; R_{ave}. Average resolution for all analytes; N. Efficiency value for each analyte; N_{ave}. Average efficiency value for all analytes

Ideally, the best network should be able to predict the values exactly, thus giving a perfect correlation (i.e. $R^2 = 1$) when plotted against the experimental values. Overall, resolution as a performance indicator produced the most correlated data. The R data set resulted in correlation values ranging from 0.8026 to 0.9744 for each analyte and the R_{ave} data set had a correlation value of 0.9382. The k' data set had correlation values ranging from 0.2739 to 0.8263, with the k'_{ave} data set having a correlation value of 0.3819. Similarly, the efficiency performance indicator produced values ranging from 0.6080 to 0.9270 for the each analyte for N, and 0.8109 for the N_{ave} data set. Comparison of the correlation values for each of the predicted values versus the observed values (the experimental data) indicated that the combination of the R_{ave} data set with its network best describes the analyte separation. When plotted the R_{ave} data set produced a correlation of 0.9382 for the network RBF 4:4-2-1:1 (Figure 3.16).

The R_{ave} data set resulted in a neural network constructed as RBF 4:4-2-1:1 (Figure 3.17). This means that four of the five inputs (% acetonitrile, pH, Ionic strength, temperature) were used as inputs, two neurons were in the hidden layer and there was the one output (the R_{ave} value). The algorithm used to train the network was KM, KN, PI (K-means, center assignment, K-nearest neighbor, deviation assignment, PI, pseudo invert, linear least squares optimisation).

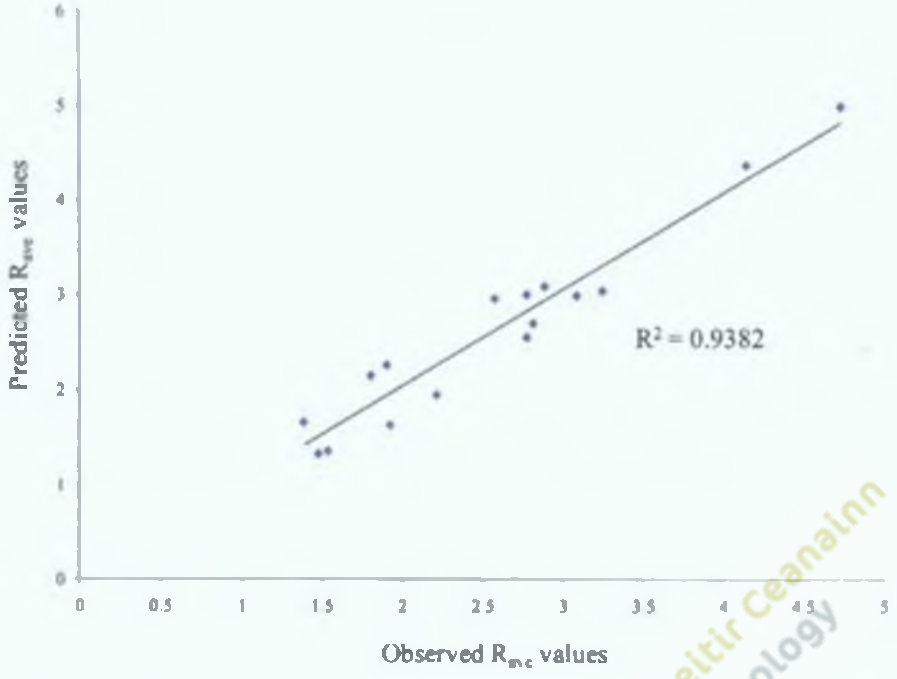


Figure 3.16. Linear regression of predicted versus observed values for R_{ave} data set using the ANN of RBF 4:4-2-1:1.

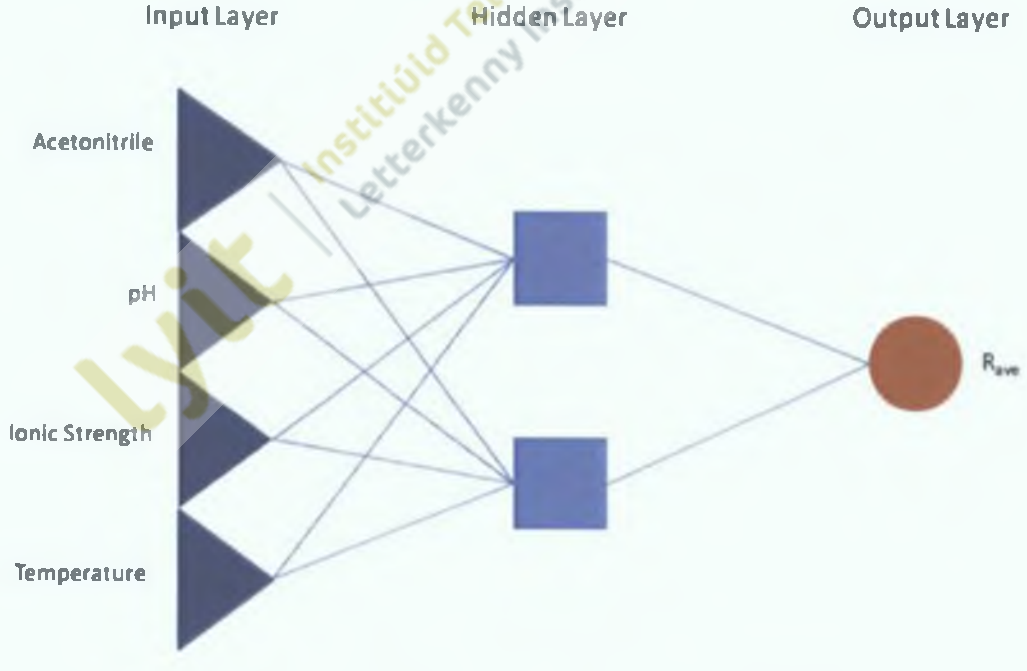


Figure 3.17. Illustration of the ANN: RBF 4:4-2-1:1.

3.2.2. Response Surfaces.

As the neural network for the R_{ave} data set was the best performing ANN, it was this network that was chosen to illustrate the separation in the form of response surfaces. Using these response surfaces the final chromatographic conditions were selected for the separation of the eight analytes (Figures 3.18 – 3.20). The red region of each response surface signifies the area in which R_{ave} value is at its highest. As it is a high R_{ave} value that is desired, it is in this area in which the optimal separation is achieved. From each of the response surfaces, there were no precise levels for each of the factors, a range of levels were produced. For optimal separation, levels of acetonitrile between 12% and 16% and pH values between 2.9 and 3.3 would be suitable (Figure 3.18), along with temperature between 32 and 36 °C (Figure 3.19) and an ionic strength between 15 and 45 mM (Figure 3.20).

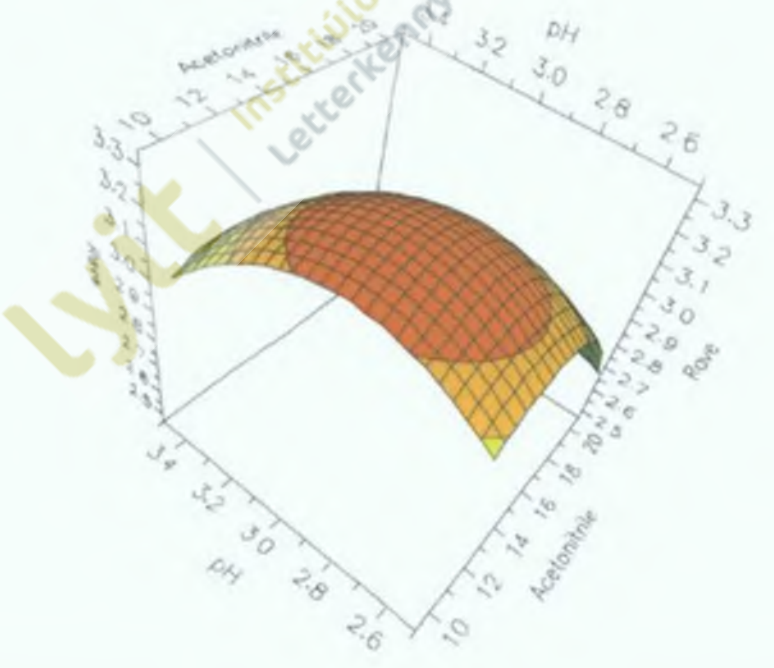


Figure 3.18. Response surface of R_{ave} values with acetonitrile and pH using the ANN of RBF 4:4-2-1:1.

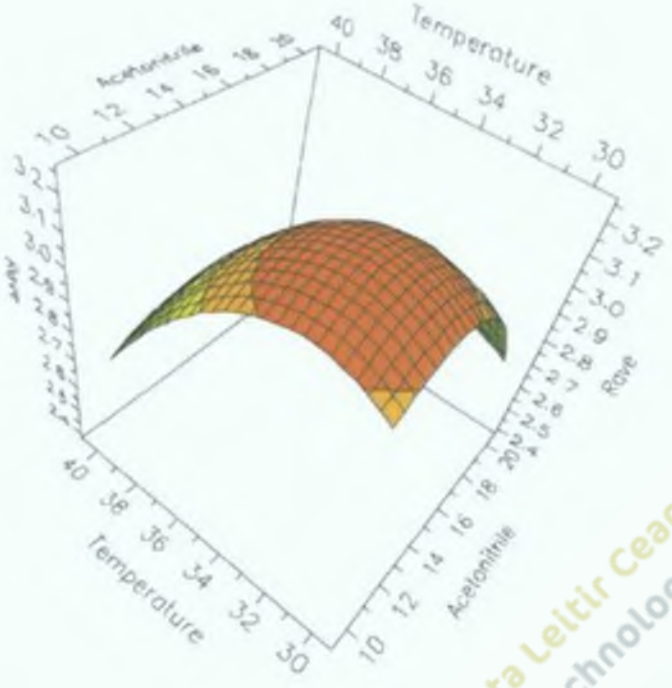


Figure 3.19. Response surface of R_{ave} values with acetonitrile and temperature using the ANN of RBF 4:4-2-1:1.

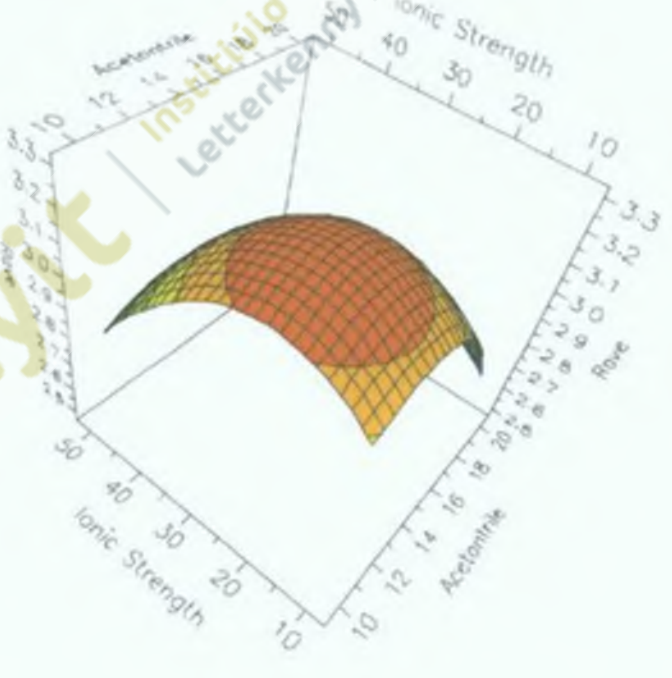


Figure 3.20. Response surface of R_{ave} values with acetonitrile and ionic strength using the ANN of RBF 4:4-2-1:1.

The final chromatographic conditions chosen were a mobile phase consisting of phosphate buffer/acetonitrile (85/15, v/v), the buffer at 20 mM ionic strength, pH 3, flow rate at 1 mL min^{-1} and a column temperature of 35°C. These levels are all within the desired range for optimal separation. Figure 3.21 is a chromatogram of the separation of the analytes under the optimised chromatographic conditions.

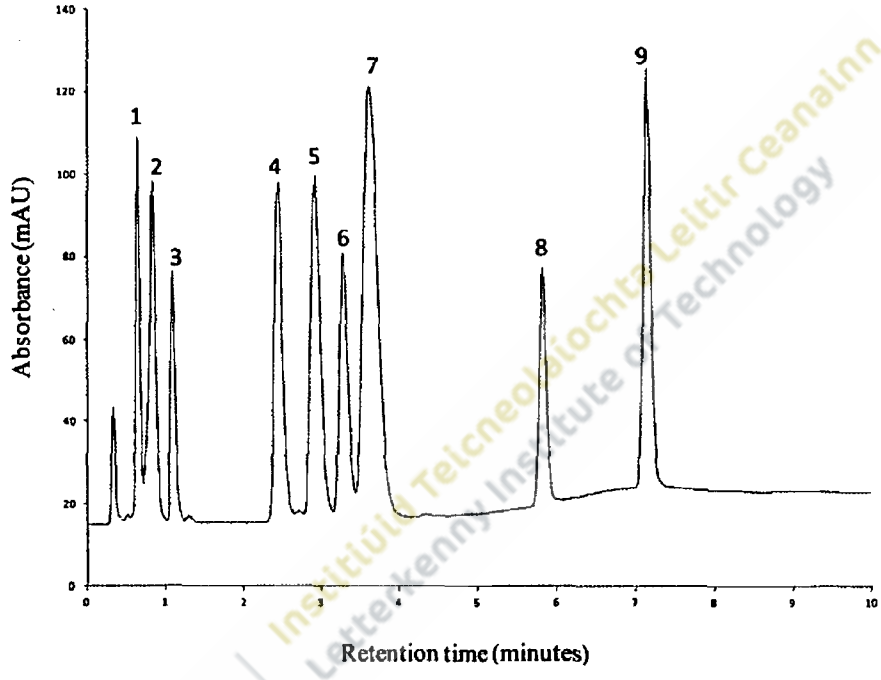


Figure 3.21. Chromatogram of the eight analytes including the internal standard, metoclopramide using the optimised chromatographic conditions. The mobile phase was phosphate buffer/acetonitrile (85/15, v/v) and flow rate was 1 mL min^{-1}. Temperature was maintained at 35°C, pH at 3, and detection was at 200 nm. The column used was a Waters XBridge C18, 50 x 4.6 mm, particle size 3.5 μm. The elution order (retention time in minutes) was: solvent peak (0.33), 1: procaine (0.64) 2: caffeine (0.83), 3: lidocaine (1.08), 4: prilocaine (2.44), 5: acetylsalicylic acid (2.91), 6: metoclopramide (3.27), 7: cocaine (3.61), 8: phenacetin (5.82), and 9: benzocaine (7.22).

3.2.3. Validation of multivariate method developed.

Validation was carried out under the guidelines of ICH. Linearity was evaluated using Mandel's test, comparing linear and quadratic models. For all the cocaine analytes tested, the quadratic model was not significantly better than the linear model at the 5% level. Linear regression analysis was performed between 50 and 500 μg mL^{-1} at five calibration points and correlation coefficients of ≥0.987 for each analyte were observed (Table 3.8).

Precision was expressed as relative standard deviation (% RSD). For intra-day, ten replicates of 100, 250 and 500 μg mL^{-1} were analysed on the same day. These standards were analysed in five replicates over five days to establish inter-day precision (Table 3.8). Intra-day precision values were in the range of 0.27-1.06% and inter-day values were in the range of 0.83-4.58% for five replicate determinations over five days (Table 3.3).

The accuracy of the method was determined by analysing spiked samples at three concentration levels (100, 250 and 500 μg mL^{-1}), with three replicates of each. Results were calculated as experimental values compared to theoretical values and were expressed as percent recovery (Table 3.8). Accuracy of the experiment results was always within 95.6-100.3% of the theoretical values (Table 3.3).

The limit of detection was calculated by LOD = $3.3\sigma/S$, where σ is the standard deviation of the response of the blank and S is the slope of the calibration curve. The limit of quantification was calculated by LOQ = $10\sigma/S$ under the ICH guidelines.
LODs for all compounds ranged from 0.2-1.9 μg mL^{-1} and LOQs ranged from 0.51-5.5 μg mL^{-1}.

Table 3.8. Validation studies for the Waters XBridge C18 50 x 4.6 mm. Cocaine and related compounds were tested for repeatability, intermediate precision, accuracy, linearity, limit of detection (*LOD*) and limit of quantification (*LOQ*).

Parameter	Cocaine Standards							
	Cocaine	Lidocaine	Prilocaine	Procaine	Benzocaine	Phenacetin	Caffeine	Acetyl. Acid
Intra-day Precision (% RSD)								
100 μg mL^{-1}	0.95	1.05	1.04	1.01	0.92	0.67	0.61	1.91
250 μg mL^{-1}	0.94	0.87	1.06	0.79	0.96	0.36	0.44	0.29
500 μg mL^{-1}	1.04	1.01	0.98	0.94	1.02	0.77	0.27	0.35
Inter-day Precision (% RSD)								
100 μg mL^{-1}	2.69	3.47	2.63	3.65	2.96	2.56	0.83	1.57
250 μg mL^{-1}	2.22	2.87	3.33	3.41	2.90	3.12	2.40	1.96
500 μg mL^{-1}	3.63	4.41	4.23	3.29	4.25	3.39	4.88	4.58
Accuracy (%Recovery)								
100 μg mL^{-1}	98.3	96.2	97.8	99.0	96.5	97.8	98.6	97.6
250 μg mL^{-1}	95.6	98.6	96.4	97.4	99.6	100.3	98.7	96.5
500 μg mL^{-1}	96.6	96.3	97.8	99.4	97.8	97.4	97.6	98.2
Linearity (R^2)	0.998	0.997	0.996	0.997	0.998	0.991	0.987	0.992
LOD μg mL^{-1}	1	1.9	0.7	1.8	2	0.25	0.2	0.5
LOQ μg mL^{-1}	3.3	5.5	1.9	5.2	6	0.77	0.51	1.52

3.2.4. Conclusions.

A HPLC method was developed for the separation of the major components of cocaine samples. This was performed using an alternative multivariate method development approach. Chromatographic factors such as quantity of organic solvent, buffer strength, pH, temperature, flow rate were assessed in combination with each other. A two level factorial design was created and used as the basis of the experimental procedure (Table 2.2.). The quality of the separation was based on the performance indicators of resolution (R), retention factor (k') and efficiency (N) for each analyte. In describing the relationship between the chromatographic factors and the performance indicators, regression analysis in the form of Artificial Neural Networks (ANN) was applied using Trajan Neural Network software. The most successful neural network was then used to plot response surfaces (Figures 3.18-3.20). Based on these response surfaces the optimal chromatographic conditions for the separation of the cocaine analytes were chosen. These conditions selected were a mobile phase consisting of 20 mM phosphate buffer/acetonitrile (85/15, v/v) maintained in an isocratic mode, pH at 3, flow rate at 1 mL min^{-1}, column temperature at 35°C with Waters XBridge C18 50 x 4.6 mm, particle size 3.5 µm column and sample detection at 200 nm. All eight analytes under investigation were separated under ten minutes with good resolution using the optimised chromatographic conditions developed (Figure 3.21). Validation studies for the method were carried out under the guidelines of the ICH (Table 3.8).

The application of neural networks and response surfaces to this method development was an attractive alternative to the laborious step-by-step process that was involved in univariate method development previously applied to the ecstasy. Although there is the initial expense of purchasing the Trajan software and the steep learning curve associated with it, once established within a laboratory, this process of method development could be applicable to any complex separation.

Chapter 4: Physical and chemical analysis of illicit drug samples.

4.1. Ecstasy analysis.

A definition of drug profiling may be summarised as "the extraction of a drug sample's chemical and/or physical profile, to be used in the application of policies against the illegal use of drugs" (Esseiva *et al.* 2007). Drug profiling involves assembling all information on all aspects of the drug of interest, most notably the physical and chemical data. Physical data pertains to the visual and measurable characteristics of the seizure. Visual characteristics include colour, format (i.e. tablet/powder/plant/capsule) or logo and physical characteristics include weight, diameter, thickness and height. Chemical data involves analytical techniques and instrumentation, where the main ingredients of the drug is identified and/or quantified. Frequently excipients, adulterants, binders and synthetic by-products are also determined. Profiling drugs enables linkage of previously unrelated seizures, thus allowing law enforcement personnel to apprehend both dealers and/or clandestine labs and in turn reduce the supply of drugs (see Chapter 5).

4.1.1. Physical profile of ecstasy.

In this study, 183 ecstasy tablets were analysed for their physical characteristics of logo, colour, weight, diameter and thickness. Recorded physical data are provided in Table A1 (Appendix). A total of 20 different logos were recorded and three tablets contained no logo (Figure 4.1). The most frequent logos were 'Mitsubishi' logo (24.6% of tablets analysed), 'Bird' logo (9.8% of tablets analysed) and 'M&M's' logo (8.2% of tablets analysed). Other logos included 'Euro', 'Durex', 'Smirnoff', 'Armani', 'Motorola', 'Chi', 'Rolex', 'Dexter', 'V2', 'Tasmanian Devil', 'Red Bull', 'Horse', 'Smiley', 'Rolls Royce', 'Superman', 'Coca Cola' and 'Heart'.

Figure 4.1. An image of all 21 logo types and corresponding seizure batch numbers for ecstasy samples used in this study.

Weight, diameter and thickness are all measurements that can be reported directly without any discrepancy. Whilst the weight of the tablet is always reported, rarely are diameter and thickness reported. In this study tablet weights ranged from 183 mg to 370.6 mg with an average of 260.9 mg (Figure 4.2). The distribution of weight of the tablets may indicate three sub groupings within the sample set. Callipers were used to determine diameter and thickness. Diameter of the tablets ranged from 8 mm to 12 mm (Figure 4.3) and thickness ranged from 2.5 mm to 6 mm (Figure 4.4).

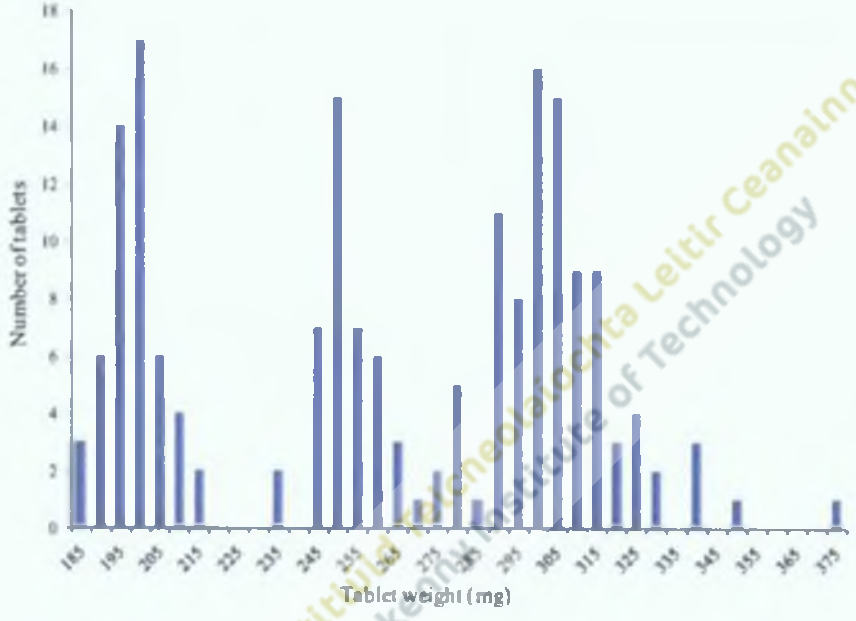


Figure 4.2. Distribution of weights within the 183 ecstasy tablets analysed.

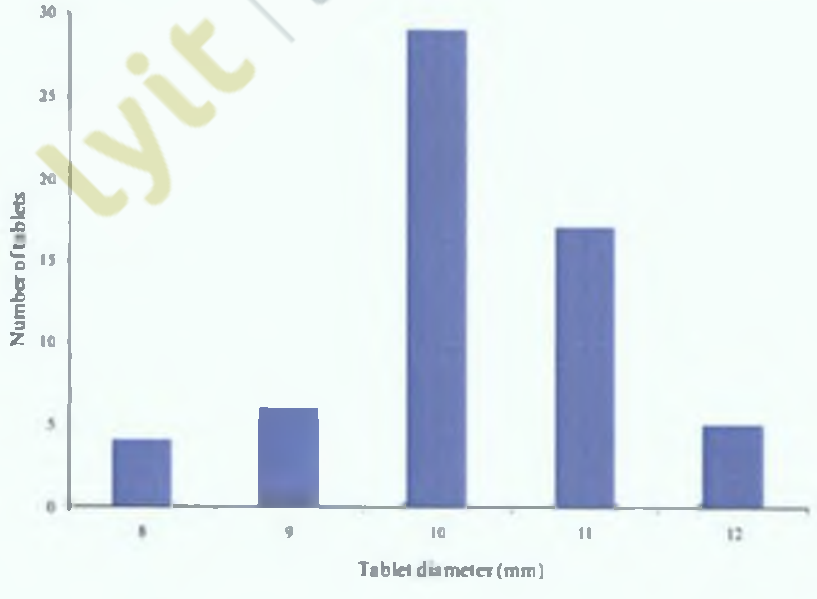


Figure 4.3. Distribution of tablet diameters within the 183 ecstasy tablets analysed.

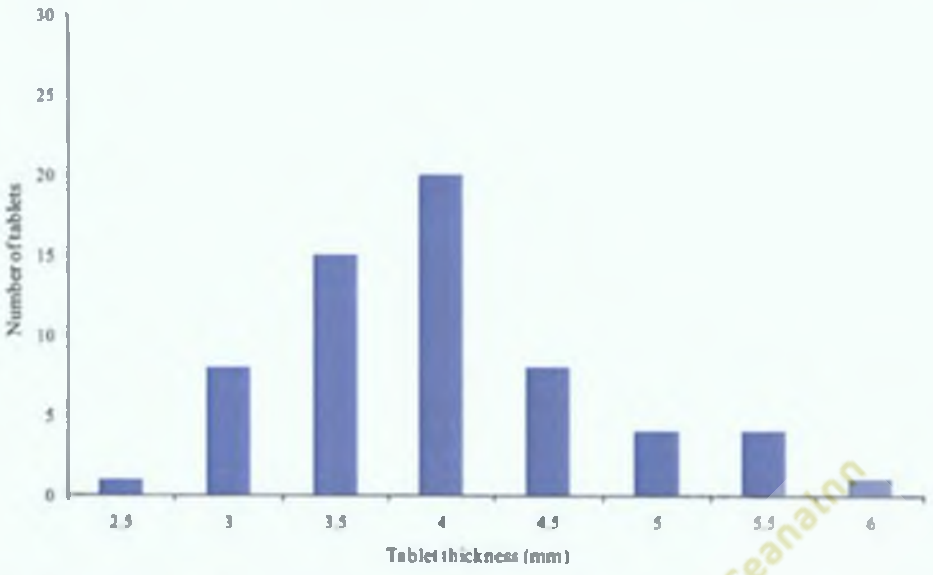


Figure 4.4. Distribution of tablet thickness within the 183 ecstasy tablets analysed.

When citing physical descriptions of tablets, logos and weights are the two most common features reported. Rarely is the colour of the tablet mentioned. The major colour reported is 'white', however differences in shade colour are possible. In this study, the majority of tablets (95%) were white, however only 55.7% of them were pure white with the remaining 39.3% being white with either brown or cream speckles. Other colours in this study included pink (3.4%) and green (1.6%) (Figure 4.5). In this study, only one seizure was triangular in shape (1.6% of total number of tablets analysed), all others were round.

Figure 4.5. Examples of ecstasy tablet colour types reported in this study.

4.1.2. Chemical analysis of ecstasy.

The determination of the active ingredients and adulterants within ecstasy tablet samples were performed using the validated HPLC method described in section 3.1. Sugar analysis was carried out by the method by described in section 2.4. Figure 4.6 is a chromatogram from a typical ecstasy sample with MDMA, amphetamine and caffeine present.

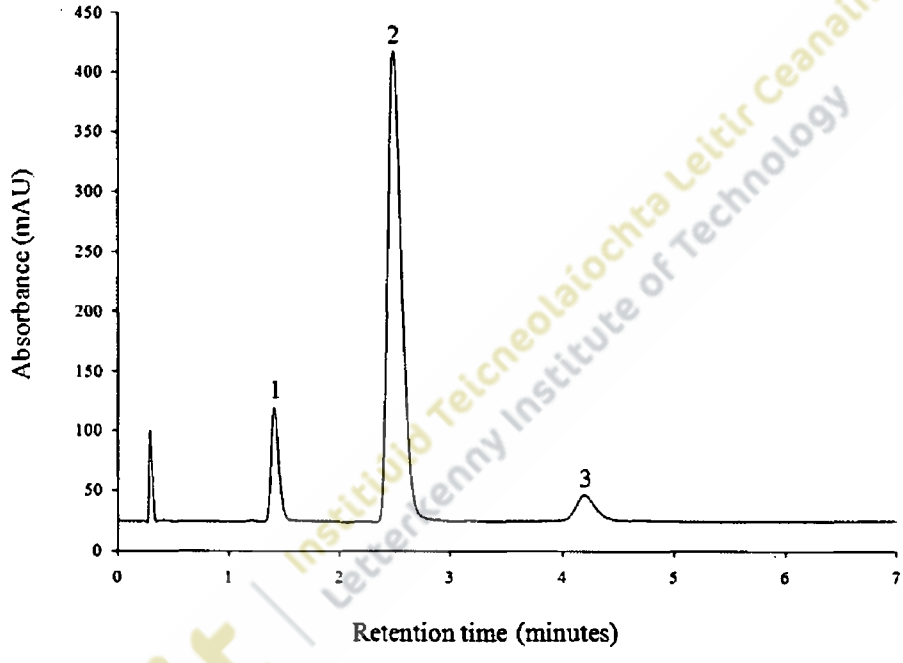


Figure 4.6. Components of a sample ecstasy tablet resolved on the Chromolith SpeedROD, RP18, 50 x 4.6 mm. The chromatographic conditions were phosphate buffer/acetonitrile (97/3 v/v) maintained in an isocratic mode, pH 2.5, temperature 30°C and flow rate of 3 mL min^{-1}. Detection was recorded at 200 nm. The elution order was 1: amphetamine, 2: MDMA and 3: caffeine.

Table 4.1. Major drug components present in 183 samples of ecstasy tablets from the Republic of Ireland seized in 2001 and 2003.

Tablet Components	% Occurrence	Concentration of component in ecstasy tablets (mg/tablet)							
		MDMA		MDEA		Caffeine		Amphetamine	
		Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
MDMA only	68.8	76.6 ± 16.7	41.4 - 125.9	-	-	-	-	-	-
MDMA & Caffeine	14.8	64.3 ± 12.3	37.6 - 89.5	-	-	1.7± 0.8	0.5 - 3.5	-	-
MDMA & MDEA & Caffeine	6.6	33.7 ± 58.3	5.6 - 69.6	5.0 ± 4.5	0.4 - 12.7	0.8 ± 0.1	0.4 - 1.1	-	-
MDMA & MDEA	6.6	78.8 ± 19.8	47.7 - 125.5	4.1 ± 3.2	0.3 - 20.0	-	-	-	-
MDMA & Amphetamine & Caffeine	1.6	27.0 ± 4.1	23.7 - 31.7	-	-	0.2 ± 0.04	0.1 - 0.2	5.4 ± 0.7	4.9 - 6.3
Amphetamine only	1.6	-	-	-	-	-	-	6.7 ± 1.6	4.9 - 8.2

Using the developed method, six possible drug components were identified and quantified in the samples, along with four commonly used adulterants. MDMA, MDEA, amphetamine and caffeine were the main components present in the 183 samples analysed, with MDMA occurring in 98.4% of samples tested (Table 4.1). Three tablets did not contain any MDMA, but amphetamine only. A wide variety of combinations of each of the four compounds were present in the samples. Quantification of the components was by the internal standard method. Depending on the batch sample analysed, MDMA concentration ranged from 5.6 - 125.9 mg/tablet. Concentrations for other compounds were much less, MDEA ranging from 0.3 - 20 mg/tablet, amphetamine ranging from 4.9 - 8.2 mg/tablet and caffeine ranging from 0.1 - 3.5 mg/tablet.

Sugars are often found in ecstasy tablets and other illegal drugs. They tend to be inexpensive and are un-controlled and as a consequence are a popular material to add bulk to the tablet. The chromatographic method used for sugar determination allowed for the detection and quantification of fructose, glucose, inositol, lactose, mannitol, sorbitol and sucrose (Section 2.4). Only two sugars, lactose and sorbitol were detected in the 183 tablets analysed. The majority of the samples (147) contained lactose only (80.3%). Eighteen samples (9.9%) contained sorbitol only. One batch, consisting of three tablets, contained both sorbitol and lactose (Figure 4.7) while the remaining fifteen tablets contained no sugars. A list of the sugars, quantities and combinations are given in Table 4.2.

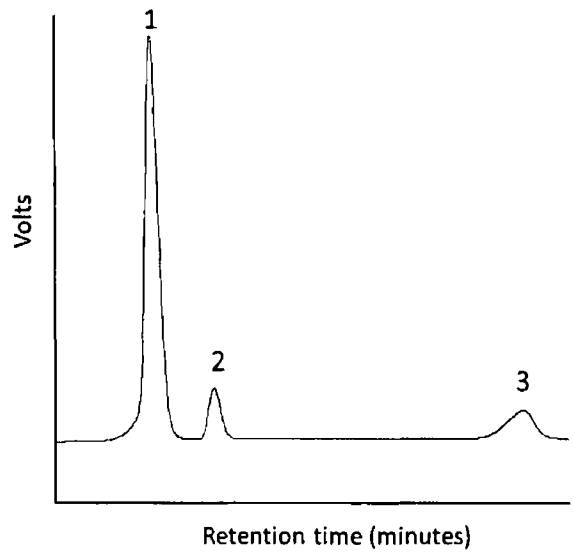


Figure 4.7. Sugar components of an ecstasy tablet resolved on the Phenomenex Luna NH_2 column, 250 x 4.6 mm. Conditions were acetonitrile/water (70/30), temperature of 30 °C and flow rate was at 0.75 mL min^{-1}. The elution order was 1: internal standard, 2: Lactose, 3: Sorbitol.

In the lactose only samples, concentrations ranged from 33.9 mg/tablet to 230 mg/tablet with an average concentration of 132 ± 53.3 mg/tablet. Expressed as percentages, 17.1% was the minimum concentration of lactose in a tablet and 76.3% was the maximum concentration in a tablet. In the sorbitol only samples, the minimum concentration quantified was 84.9 mg per tablet (28.8% sorbitol per tablet) and the maximum was 203.6 mg per tablet (70.4% sorbitol per tablet) with an average concentration of 129 ± 41.3 mg/tablet. In the case of the three tablets (one batch) that contained both lactose and sorbitol, the lactose and sorbitol were present in an approximate ratio of 55% to 45%. Active ingredients, adulterants and sugar components are provided in Table A1 (Appendix). The chemical analysis performed accounted for as little as 8.5% of the tablet ingredients to as much as 99.4 % of the tablet ingredients identified, with an average of 67%. The remaining unidentified components of the tablets could be pharmacologically inactive chemical compounds such as starch or carbonates.

Table 4.2. Sugars present in 183 samples of ecstasy seized in Ireland.

Sugars	% Occurrence	Concentration of components in ecstasy tablets (mg/tablet)			
		Lactose		Sorbitol	
		Mean ± SD	Range	Mean ± SD	Range
Lactose only	80.3	132.0 ± 53.3	33.9 – 230.0	-	-
Sorbitol only	9.9	-	-	129.2 ± 41.3	84.9 - 203.6
Lactose & Sorbitol	1.6	95.7 ± 10.4	87.3 - 107.4	76.8 ± 8.2	71.9 - 86.3
No Sugars	8.2	-	-	-	-

Inorganic element analysis provided quantitative data on nine different elements including aluminum, calcium, chromium, iron, lead, magnesium, potassium, sodium and zinc. Table 4.3 provides a summary of the inorganic elements detected in samples, the average concentration observed for all samples, the concentration range for each element and the percentage occurrence within the samples. Aluminum, iron, magnesium and potassium were present in all samples. The use of carbonates ($CaCO_3$, K_2CO_3, $MgCO_3$, and Na_2CO_3) and stearates ($Na(C_{17}H_{35}COO)$, $Ca(C_{17}H_{35}COO)_2$, $Mg(C_{17}H_{35}COO)_2$) is very common in the tablet production. The high incidence of aluminum could be an insight on how these tablet samples were produced, as aluminum is used as a reducing agent in the reductive animation route for the synthesis of MDMA. It was detected in all the tablets analysed and at concentrations up to 310.9 ppm. The high rates of zinc and iron in these tablets may possibly well be from lubricants and dyes used in the production process. A full list of all the metals present, at both their concentration levels and their combinations within each tablet, are given in Table A2 (Appendix).

Table 4.3. Concentration (ppm) of inorganic elements and occurrence in ecstasy tablet samples.

Element	% Occurrence	Average (ppm)	Range (ppm)
Al	100	43.9	1.4 - 310.9
Ca	97.8	256.7	3.5 - 2836
Cr	17.4	0.6	0.03 - 3.5
Fe	100	52.0	2.3 - 402
K	100	0.16	0.1 - 0.86
Mg	100	0.93	0.03 - 4.07
Na	93.4	0.3	0.02 - 3.1
Pb	23	0.2	0.01 - 9.5
Zn	97.8	7.9	0.3 – 92

4.1.3. General profiling of ecstasy.

In this study, the relationship between the chemical profile of ecstasy tablets and their logo was investigated to ascertain whether users could distinguish tablets based on logos only. To test the hypothesis, the four tablet logos of 'Mitsubishi', 'Bird', 'M&M's' and 'Euro' were chosen.

'Mitsubishi' Tablets Group

'Mitsubishi' tablet group comprised the largest group of seized samples. Fifteen different seizures (45 tablets) all bore this particular logo, which is consistent with the findings as being one of the most common logos seen on the European market (EMCDDA Annual Report, 2008). All tablets within this group, at first glance, seem to have the same visual, physical and chemical characteristics. All tablet weights lie within the narrow range of 277.8 mg to 324.5 mg, thickness ranges from 3.5 mm to 4.5 mm and diameters from 10.5 mm to 12 mm. All samples were the same colour and shape. All tablets contain MDMA with no other active ingredients. However, on closer inspection, the purity of the MDMA present in the tablets varies widely from 41.4 mg/tablet to 125.9 mg/tablet, or 16.6% to 39.6%. The sugar content was determined and those with lactose present had lactose concentrations ranging from

66.8 mg/tablet (23.4%) to 214.8 mg/tablet (69.7%). All metals were present at standard concentration levels.

One seizure was obviously different within the 'Mitsubishi' group of tablets. This particular seizure (seizure 35) had all physical characteristics common to the others, shape, colour weight etc, and had similar levels of MDMA present (Figure 4.8). However, when determining sugar content, this particular seizure was found to have no sugars present. Also, two of the metals, calcium and magnesium, were found in significantly higher concentrations than the others. This one seizure highlights the importance of a full chemical analysis (i.e. active ingredients and adulterants with excipients) of all samples with the intention of profiling and again emphasises the importance of quantification of the compounds.

Figure 4.8. A digital image and properties of tablets from seizure 37 and seizure 35.

Properties	Seizure No.	
	37.1	35.1
Weight	300.3 mg	293 mg
Shape	Round	Round
Colour	White	White
Thickness	4 mm	4.5 mm
Diameter	11.5 mm	10.5 mm
MDMA	107.5 mg (35.8%)	115.6 mg (39.4%)
Lactose	188.1 mg (62.6%)	0 mg
K	0.04 mg	0.02 mg
Na	0.03 mg	0.01 mg
Ca	0.01 mg	0.17 mg
Mg	0.01 mg	0.34 mg
Fe	0.02 mg	0.01 mg
Al	0.02 mg	0.01 mg

'Bird' Tablets Group

This group was comprised of six seizures, 4, 7, 8, 14, 19 and 32. Despite the lower number of seizures, the seizures within the 'Bird' group are more varied with many possible sub groups existing. Seizure 7, 8, 19 and 32 all contained MDMA and lactose. Seizure 4 contained MDMA and sorbitol, while seizure 14 contained low levels of MDMA, amphetamine and caffeine and no sugar. Seizure 14 also had

sodium, calcium and magnesium present in appreciably higher levels than other seizures. In addition, seizure 14 was pink in colour.

'M&M's' Tablets Group

A total of five seizures comprised this group, 23, 24, 25, 40 and 41. Again, they all have the same colour, shape, thickness and diameter. They all contain MDMA, caffeine and lactose. Wide ranges of purity exist with the MDMA and lactose data, 37.6 to 86 mg/tablet for the MDMA and 33.9 to 120.2 mg/tablet for the lactose. Caffeine alternatively, was present at low levels, 1.6 to 2.6 mg/tablet. Three of the seizures (23, 25 and 40) had significantly higher levels of magnesium present.

'Euro' Tablets Group

This group was comprised of three seizures and is one of the most interesting. All tablets had the same physical characteristics, shape, colour, diameter, thickness and the tablet weights ranged from 285.5 to 305 mg. All tablets contained MDMA at regular levels. None of the samples contained MDEA, amphetamine or caffeine. That is where the similarities end. Diluent analysis confirmed that the three seizures were produced separately. One had lactose as its main diluent, one had sorbitol and one had no sugar present.

Based on the results of this data, three out of four times, the logo does indicate the chemical components of the tablet. In other words, the user will be able to tell what is in the tablet just by looking at its logo, the majority of the time. All the 'Mitsubishi' tablets had MDMA only, all 'M&M's' tablets had MDMA, caffeine and lactose, and all 'Euro' tablets had MDMA. However, it must be noted that the logos do not determine the levels of those chemical component in the tablets. For those tablets with the same chemical components, levels ranged significantly.

An alternative approach is to examine the chemical profiles of tablets as opposed to their physical profiles. From the view point of the manufacturer, the logo stamp that gives the tablet the logo would be considered relatively cheap in comparison with the actual tablet ingredients. Therefore it may be very well possible that the one manufacturer has several stampers but only the one set of ingredients. This attempted to discover if one manufacturer could have one set of ingredients and recipes but

several stampers. The active ingredients and adulterants detected and quantified in the tablets were MDMA, MDEA, amphetamine and caffeine. There were six combinations of these compounds.

'MDMA only' Group

The group 'MDMA only' had the largest number of seizures. 42 seizures, or 68.8% of the total number, had MDMA as their only active ingredient. MDMA purity ranged from 41.4 to 125.9 mg/tablet (Table 4.1). Within this group however, a wide variety was observed in other aspects of the tablet composition. Tablet weights varied from 183.4 mg to 370.6 mg, thickness ranged from 4 mm to 6 mm and diameter from 8 mm to 12 mm. There were 16 different types of logo, the most popular being the 'Mitsubishi'. One seizure was pink, the others white, and one seizure was triangular in shape. Lactose was present in 33 seizures ranging from 66.6 to 230 mg/tablet. Sorbitol was present in only five of the seizures, ranging from 71.9 to 263.1 mg/tablet. There was one seizure that had both lactose and sorbitol present. These large ranges and sub groupings due to sugars suggests perhaps that more than one manufacturer produced these 'MDMA only' tablets.

'MDMA and caffeine' Group

The 'MDMA and caffeine' group was comprised of nine seizures. The range of tablet weights was much narrower, 186.7 mg to 208.7 mg. There were only three logos within the group, 'M&M's' being the most popular. All tablets were white and round, thickness varied from 3 to 4 mm and diameter from 9 to 10 mm. MDMA concentration ranged from 37.6 to 89.5 mg/tablet and caffeine ranged from 0.5 to 3.5 mg/tablet. Interestingly, lactose was the only sugar present in this group; however a large variation existed in its quantities, 33.9 to 130.2 mg/tablet. Again, allowing for within batch variability, this still would imply one manufacturer for this group. Overall, similar chemical profiles with different physical profiles does not necessarily mean the one manufacturer.

4.1.4. Discussion.

The first published report on ecstasy profiling was by Renfroe (1986) and research has continued to present day. Britain, Japan and the United States have the most published reports describing ecstasy tablets seized in their countries. Italy, the Netherlands, Israel, China and the Czech Republic have reported one-off studies. The number of ecstasy samples analysed have ranged from 13 tablets (Milroy *et al.* 1996) to 20,000 tablets (Schifano 2000), but usually sample size is in the region of 150 tablets. Results typically include the active ingredients, sometimes adulterants and very rarely sugar content. These studies also tend to include a vague description of the tablets, limiting the physical characteristics to logos and weight.

Typically amphetamine, methamphetamine, MDMA and MDA are the main compounds detected for the tablets. Other compounds that have been detected are MDEA (Makino *et al.* 2003, Cole *et al.* 2002, Teng *et al.* 2006, Sherlock *et al.* 1999), ephedrine (Makino *et al.* 2003, Teng *et al.* 2006, Sherlock *et al.* 1999), and ketamine (Cheng *et al.* 2006, Teng *et al.* 2006, Sherlock *et al.* 1999). A study based in the US (Tanner Smith, 2006), analysed for 30 compounds. The most common compounds detected were MDMA, MDEA, MDA, caffeine, ketamine and dextromethorphan (DXM). Also detected in small amounts were 2, 5-dimethoxy-4-bromoamphetamine (DOB), heroin, ketamine, phenylcyclohexylpiperidine (PCP) and paramethoxyamphetamine (PMA).

Traditionally, ecstasy was predominately MDMA, sometimes MDA, but never any other drug. Renfroe (1986) analysed 610 ecstasy samples from the years 1972 to 1985. All tablets contained either MDMA or MDA, or a mixture of both compounds. No other drug or adulterants were detected. Since its rise in popularity, other active ingredients have been detected in ecstasy tablets. In the Netherlands, a large number of tablets were analysed between 1993 and 1996 (Spruit, 1999) of which 48-60% contained MDMA alone, 12-20% contained MDEA alone and 2-5% contained MDA alone. Each year however, 6-17% contained non-amphetamine type drugs. Detected were lysergic acid diethylamide (LSD), DOB, ketamine, 4-bromo-2, 5-dimethoxyphenethylamine (2C-B), atropine, caffeine, acetaminophen and other unidentified substances. 2, 5-dimethoxy-4-methylamphetamine (DOM), DOB and 2C-B have also been identified in tablets (Kort & Cramer 1999). A report issued by

the EMCDDA (EMCDDA Report, 2008) stated that most tablets (70%) contain MDMA or MDA or MDEA as the only psychoactive substance. Spain and Portugal were exceptions with amphetamine and methamphetamine the most commonly detected compound, and often in combination with MDMA or one of its analogues. A single tablet in the Netherlands was analysed and found to contain 8 mg strychnine (The Netherlands national drug monitor, 2007).

As the chemical components of ecstasy tablets changed over the years, consequently so did the purity of each drug in the tablet. In the UK, average values were 90 to 102 mg/tablet during the years 1991 and 1995 and then fell to 73 to 88 mg/tablet during the years 1996 and 2000 (Cole *et al.* 2002). Samples analysed in the Czech Republic varied from 30 to 100 mg/tablet, also quoting concentrations of 0 mg and 152 mg (Palenicek *et al.* 2002). In Italy, between 1995 and 2000, the concentrations of MDMA ranged from 100 mg to 150 mg/tablet (Schifano 2000). In Taiwan, a study was carried out on tablets between 2002 and 2005. It was found that the average MDMA content fell over those years from 126 mg/tablet in 2002 to 73 mg/tablet in 2005 (Teng *et al.* 2006). EMCDDA reported average MDMA content across Europe to be between 5 and 72 mg/tablet for 2008. High doses (≥130 mg/tablet) have been reported in Belgium, Denmark, Germany, France, Netherlands and Norway. Purity levels for this study ranged from 41.5 to 117.5 mg MDMA/tablet for MDMA only tablets. MDMA levels were at their lowest when in combination with amphetamine and caffeine (7 to 9.6 mg MDMA/tablet) or MDEA and caffeine (5.6 to 9.6 mg MDMA/tablet).

Within batch variability also exists. That is, in the one seizure or group of similar tablets, concentrations of the drugs differ from one another. In this study, four seizures 20, 37, 43 and 57, had MDMA concentrations ranging from 57.9-98.7 mg/tablet, 61.0-117.5 mg/tablet, 50.0-103.8 mg/tablet and 41.4-99.8 mg/tablet, respectively. In 2001, Cole *et al.* (2002) analysed eighty tablets from the one batch. Concentrations ranged from 73 mg to 89 mg, with a mean of 79 mg/tablet. Limited reports have been published discussing sugar content of ecstasy tablets. In 2000, researchers in Northern Ireland identified sorbitol and glucose in a large number of tablets (Bell *et al.* 2000) and then in 2003, the same researchers found lactose to be the most common sugar, and sorbitol the second most common (Bell *et al.* 2003). In

England, lactose was identified in the tablets at an average quantity of 37% of the tablet (Cole *et al.* 2002). These reports would be consistent with this study, where lactose and sorbitol were the only sugars detected with lactose being the predominant sugar.

Europol has a catalogue of logos, (Europol Ecstasy Logo System, EELS). In this catalogue, tablets are catagorised initially by their logo (e.g. 'Mitsubishi', 'Bird') and then numbered according to their generic logo type. To date, the catalogue contains 524 logos with 402 logo type variations. The aim of this is to gather information on significant seizures and allowing this to match different seizures and further investigation by local authorities. In this study of Irish ecstasy samples 'Mitsubishi' was the most common logo, a feature which also observed by Bell *et al.* (2003) for Northern Ireland seizures for the same time period. Bell *et al.* (2003) reported that after 'Mitsubishi', the most common logos were 'Euro', 'Aladdin', 'Clover', 'Cupid', 'Unicorn', and 'Teletubby'. Similarly, 'Mitsubishi' was the most common logo found in a study in North West England (Cole *et al.* 2002). However, for Sherlock *et al.* (1999), tablets from midlands England had 'Dove' as the most common logo. Outside of Europe, Levy *et al.* (2007) studied tablets between 2001 and 2003 in Israel, and reported fifty-eight different logos, the most common being 'Bulldog', 'Feet', 'Flag', 'Superman', and 'Boss'. Interestingly, studies on the other side of the world, namely China and Japan have reported various logos but 'Mitsubishi' was a common one (Makino *et al.* 2003, Katagi *et al.* 2002, Teng *et al.* 2006). Whilst a range of logos are more common than others, 'Mitsubishi' remains very popular worldwide. Unsurprisingly, almost a quarter of the tablets analysed in this study had this logo.

The weights of the tablets analysed in this study lie within the ranges reported in other studies, 105 mg to 348 mg (Makino *et al.* 2003), 46 mg to 340 mg (Katagi *et al.* 2002), 200 mg to 390 mg (Cheng *et al.* 2006), 300 mg to 400 mg (Lueng 2002) and 148 mg to 693 mg (Sherlock *et al.* 1999).

Tablets seized in England have been reported to be white, off-white or beige (Cole *et al.* 2002). Numerous other colours have been reported, particularly in Japan, including blue, brown, yellow, grey, orange, green, purple, red, pink and rose (Katagi *et al.* 2002, Makino *et al.* 2003). The majority of research published have analysed

tablets that are round in shape. Tablets may be slightly damaged when seized, but the original shape of the tablet can usually be evident. Only one study analysed tablets that were not all round. Teng *et al.* 2006, analysed three tablets (1.6% of total number of tablets analysed) that were square with rounded edges.

Metals which may be found in ecstasy tablets include Na, Ba, Ca (sulphate, phosphate salts) from excipients, Mg, Zn, Al, Li, B from lubricants, Pd, Pt, Ni from catalyst, $LiAlH_4$, $NaBH_3CN$, $Al/HgCl_2$ from reduction of intermediate ketones, Fe/HCl, $SnCl_2$, $Pb(OAc)_2$, $PdCl_2/CuCl$ from the preparation of intermediate ketones, and I , Fe and Cu from dyes (Comment *et al.* 2001). Inorganic analysis of drugs other than ecstasy has become popular, such as amphetamine (Muratsu *et al.* 2002), methamphetamine (Marumo *et al.* 1994), heroin (Bermejo-Barrera *et al.* 1996, Bermejo-Barrera *et al.* 1997, Bora *et al.* 2002, Infante *et al.* 1999, Myors et al. 1998) and cocaine (Bermejo-Barrera *et al.* 1996, Bermejo-Barrera *et al.* 1997, Bermejo-Barrera *et al.* 1999).

The metal analysis of drugs may be valuable for linking seizures. Metals in tablets may occur from various sources, either the synthetic pathway (by – products) or the tableting process (excipients, colourants, etc). During the chemical process of MDMA synthesis, catalysts or reducing agents are used, each being very specific to each synthetic process. Knowledge of which were used would lead to information on the production of the tablets, which in turn would lead to linkage of seizures. The most common method of production of MDMA is the reductive amination route. With this method three variations of it exists. First is the high pressure method which uses platinum as a catalyst. Second is the low temperature method which uses sodium borohydride as a reducing agent. The third is known as the 'aluminum amalgam reduction' method which uses aluminum and mercury chloride as reducing agents. The safrole bromination route uses hydrobromic acid (HBr) and the less common Leukart reaction uses $LiAlH_4$. Therefore the metals that would distinguish between each synthetic route are platinum (Pt), boron (B), mercury (Hg) and lithium (Li). The determination of these metals was problematic. For mercury analysis, a hydride generator is required. This generates mercury fumes which are then analysed, which in turn creates obvious safety issues. Other specific equipment was needed for the other metals, which were not available.

4.2. Chemical analysis of cocaine.

Cocaine samples are different to ecstasy tablets and therefore profiling of cocaine samples relies on a complete chemical analysis. Ecstasy tablets generally remain intact from the production lab, through to the dealer and finally to the user on the street. This is in contrast with cocaine samples, which are in powder form and can be "cut" or diluted many times between the production lab and the user on the street. These alterations to cocaine samples from its original source can complicate profiling and makes identifying dealer networks very difficult. In addition, with ecstasy seizures, preliminary connections can automatically be based on physical characteristics, such as logos or tablet colours, linking cocaine seizures proves more difficult as most seizures show the same physical characteristics, i.e. white powders. With a view to ascertaining if cocaine samples could be profiled, a chemical analysis of the active ingredients, adulterants and sugars was performed. The determination of active ingredients and adulterants within the cocaine sample were performed using the validated HPLC method described in Section 3.2. and sugar analysis was carried out by the method by described in Section 2.4. A number of cocaine samples (28) were analysed to determine their active ingredients and adulterants. Figure 4.8 is a chromatogram from the analysis of a cocaine sample separated under the conditions described in Section 3.2.

Using the developed method, eight possible components, five active ingredients and three adulterants, were identified and quantified in the samples. The major active drug ingredients present in the samples analysed were cocaine, lidocaine and prilocaine (Table 4.4). Samples varied with the level of drug constituent present and the combinations in which they occurred in samples. Cocaine occurred in 100% of samples (n = 28) and levels of concentration ranged from 0.21 to 22.1 mg/50mg sample (0.42 to 44.2% purity) with an average value of 7.6 mg/50mg sample (15.2% purity). Lidocaine was present in 53.5% (n = 15) of samples tested and levels of concentration ranging from 1.42 to 31.5 mg/50mg sample with an average of 21.7 mg/50mg sample. Prilocaine occurred in 42.8% of samples (n = 12) and levels ranged from 0.15 to 3.3 mg/50mg sample with a mean of 1.69 mg/50mg sample. Procaine was detected in 3.5% samples (n = 1) at a concentration of 0.02mg/50 mg sample and benzocaine was only present in 3.5% samples (n = 1) and at a concentration of 0.5 mg/50 mg sample.

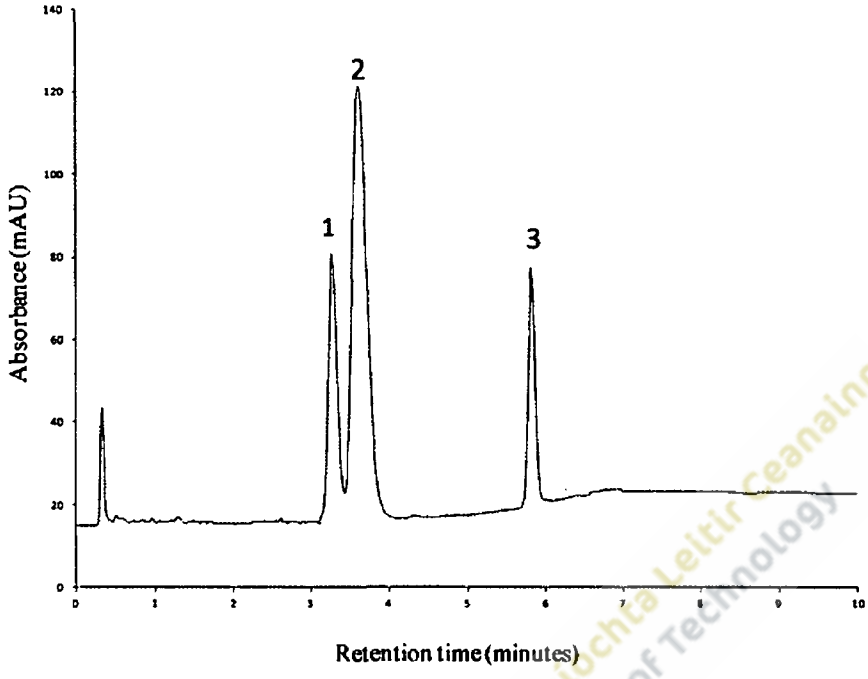


Figure 4.9. Components of a cocaine sample resolved on the Waters XBridge C18 50 x 4.6 mm, particle size 3.5 µm column. The chromatographic conditions were phosphate buffer/acetonitrile (85/15 v/v) maintained in an isocratic mode, pH 3, temperature 35 °C and flow rate of 1 mL min^{-1}. Detection was recorded at 200 nm. The elution order (retention time in min) was 1: metoclopramide (3.275), 2: cocaine (3.616) and 3: phenacetin (5.824).

Adulterants present in samples included phenacetin (53.6% of samples), caffeine (46.4% of samples) and acetylsalicylic acid (3.5% of samples). As observed with the main drug ingredients, adulterants occurred in different combinations with drug components and at different levels. Phenacetin ranged from 0.07 – 2.2 mg/50mg sample with an average value of 0.81 mg /sample. Caffeine concentrations ranged from 0.06 – 2.1 mg /50mg sample with an average value of 0.80 mg/50mg sample. Acetylsalicylic acid was only observed in one cocaine sample and at a low level, 0.63 mg/50mg sample. All concentrations and combinations of each compound are given in Table 4.3.

Table 4.4. Major drug, sugar and adulterant components (mg/50mg of sample) detected in 28 cocaine seizures used in this study

	Main drug			Sugars			Adulterants	
Sample #	Cocaine	Lidocaine	Prilocaine	Mannitol	Sucrose	Lactose	Phenacetin	Caffeine
1	15.0	-	-	6.4	-	-	-	0.1
20	8.5	-	-	2.9	-	-	1.1	-
11	6.6	-	-	4.4	-	-	0.5	-
13	7.3	-	-	5.7	-	-	0.6	-
8	12.0	13.9	-	1.4	-	-	1.0	-
17	22.1	23.5	-	3.1	-	-	1.5	-
18	0.2	29.2	-	5.2	-	-	-	-
16	15.1	14.4	-	1.5	-	-	0.1	0.1
12	0.4	30.6	-	1.9	-	-	-	1.9
7	0.4	28.7	0.4	4.3	-	-	-	0.6
9	19.3	1.4	-	5.0	-	17.7	-	0.1
28	5.4	-	1.0	2.6	-	13.2	0.7	-
14[a]	5.1	4.5	1.6	-	14.1	-	0.1	0.1
25[b]	0.2	31.5	1.4	-	7.6	-	-	-
21	3.1	26.2	2.3	-	12.7	-	0.8	-
24	10.4	18.0	2.3	-	10.8	-	-	1.1
5	6.6	-	2.5	-	16.4	-	0.7	-
26	5.1	-	1.2	-	14.5	-	0.6	-
6	18.3	-	1.2	-	1.2	12.8	2.2	0.1
27	13.9	-	3.3	-	16.1	-	-	-
3	12.8	-	2.9	-	17.0	-	-	-
4	13.5	-	0.1	-	-	20.3	-	-
22[c]	0.7	28.5	-	-	-	11.4	-	1.8
23	0.3	27.1	-	-	-	8.1	-	0.6
10[d]	0.3	-	-	-	-	-	0.2	0.4
15[e]	10.4	-	-	-	-	-	1.4	-
2	0.9	22.7	-	-	-	-	-	2.1
19	0.5	25.8	-	-	-	-	0.7	1.6
n	28	15	12	12	9	6	15	13
Min	0.2	1.4	0.2	1.4	1.2	8.1	0.8	0.1
Max	22.1	31.5	3.3	6.4	17.0	20.3	2.2	2.1
Mean	7.7	21.8	1.7	3.7	12.3	13.9	0.8	0.8
Stdev	6.7	9.4	1.0	1.7	5.1	4.4	0.6	0.8

- = not detected in samples. Other compounds present included:
a = Procaine (0.02mg/50mg sample 14); *b* = Acetylsalicylic acid (0.63 mg/50mg sample 25); *c* = Benzocaine (0.57 mg/50 mg sample 22); *d* = Sorbitol (1.57 mg/50mg sample 10) and *e* = Inositol (9.36 mg/50mg sample 15).

Sugars have been detected previously in street cocaine samples, namely glucose, sucrose, lactose and fructose (Bernardo 2003, King 1997). Mannitol, sucrose, lactose, sorbitol and inositol were the sugar components identified in cocaine samples (Table 4.4). Glucose was not detected and two cocaine samples did not contain any sugars under test conditions. Sugar components occurred as single entities for the most part in cocaine samples with only three samples having mixed sugar combinations of mannitol with lactose or sucrose with lactose. Mannitol was the main sugar component identified and it occurred as a single sugar in 35.7% of samples. Mannitol levels ranged from 1.3 – 6.3 mg/50 mg sample with an average value of 3.6 mg/50 mg sample. Sucrose was the second most common sugar occurring as a single sugar in 28.5% of samples and ranged from 1.17 – 17 mg/50 mg sample with an average value of 12.2 mg/50 mg sample. Lactose when present was at elevated levels compared to other sugar components. Lactose occurred as a single sugar in 10.7% of samples and levels ranged from 8.1 – 20.2 mg/50 mg sample with an average value of 13.9 mg/50 mg sample. Sorbitol and inositol occurred in two separate cocaine samples at levels of 1.5 mg/50 mg sample and 9.3 mg/50 mg sample, respectively.

4.2.1. Discussion.

As with the ecstasy tablets, comparisons of the results of the cocaine samples in Ireland were made with other published data. Published data includes one-off studies in Australia, UK, Spain, Italy and Brazil and comprehensive studies on cocaine purity worldwide (UNDOC), Europe (EMCDDA) and America (DEA). Data includes % purity, geographical variations in purity, temporal variations in purity, identification and/or quantification of adulterants, sugars, impurities and residual solvents. A point to note is a physical profile of these samples is limited to "white" and "powder" form. With cocaine, greater emphasis is needed for the chemical profile, i.e., the main ingredients and their quantities.

As stated previously, purity levels have been reported without any reference to the original seizure weight. However, using these reported values, the samples in this study had a lower purity level than the majority of the studies. Most European countries have reported mean values between 24% – 80% purity, with an overall average of 40% - 60% for the year 2004 (EMCDDA Report, 2006). This figure is similar for America (DEA) and Worldwide (UNDOC World Drug report 2006). In Europe, there has been a downward trend in cocaine purity from 2003 to 2008, values ranging from 13% to 62% (EMCDDA Report, 2010). This

may coincide with the increase in demand for this drug, as consumption and seizures rose over this time period.

One aspect of reporting purity values which may cause a bias is the sample weight. Seizures of a significant weight (e.g. seizures from a dealer) may be more 'pure' than seizures of a smaller weight (e.g. seizures from a drug user), which are most probably already 'cut' a number of times, thus lowering the purity value. One study (Barrio *et al.* 1997) addressed this issue by dividing the samples into two weight categories, ≥5 g and <5 g. Results did indicate a difference in the mean values for purity between the two categories. A study by Bernardo (2003) analysed samples of weights ≥5 g. Purity levels in this study ranged from 4.3 and 87.1% (mean ± S.D. = 28.1 ± 28.4). Fucci & De Giovanni (1998) analysed 156 cocaine samples, 24% of which had weights ranging from 100 to1000 g. Purity of these samples ranged from 23 to 98% and no adulterants were detected. Seizures of smaller weights may not always be low purity. Fucci & De Giovanni (1998) analysed two samples of small weights (1.7 and 5.2 g) and both had high purity levels, 90% and 99% respectively. Therefore, it would seem to be a necessity when stating purity levels, to categorise the samples into different weights or to simply state the weight of the analysed sample. Other published data did not take this factor into consideration. Darke *et al.* (2002) reported purity levels ranging from 50% to 64% from 1996 to 2000, with a peak in purity during 1997/1998. The EMCDDA Report 2010, the World Drug Report from the UNDOC, Interpol documents and DEA documents all report purities without stating or categorising the weights of the original seizures.

In this study, samples from small seizures, ≤5 g, were analysed. This may infer that the samples were seized from users as opposed to dealers; that the purity level would be low and the number and quantity of adulterants/sugars present would be high. Indeed, this was the case. Purity levels ranged from 0.4% to 44% with a mean of 15.3%. Seven different combinations of active ingredients and adulterants were detected in the samples, individually and in different combinations. Five sugars were detected, again individually and in different combinations (sucrose & lactose in one sample, mannitol and lactose in one sample and mannitol & sucrose in one sample) and quantities (Table 4.4).

Lidocaine is the most common adulterant detected in samples from other studies, closely followed by caffeine (Barrio *et al*, 1997, Bernardo, 2003, Fucci & De Giovanni, 1998, King

1997). Other adulterants detected include procaine, prilocaine, benzocaine and piracetam. In one study (Fucci & De Giovanni, 1998), fourteen different adulterants were detected. Phenacetin was also another adulterant identified along with cocaine (Fucci 2004). Pharmacologically inactive chemical compounds that may be found in samples include starch and carbonates (Lopez - Artiguez *et al.* 1995).

For classification purposes, the sample size (twenty eight) was too small to make any definite links between samples; however there are some general observations. When all the data is combined (i.e. drugs/sugars/adulterants) the twenty eight samples could only be reduced to twenty three groups. This would suggest that the samples are street cut samples due to the variations and combinations within the samples. The common samples were (11, 13 & 20); (8 & 17); (5 & 26) and (3 & 27) the remainder are all unique or have a unique combination of all data.

4.3. Conclusions.

The overall aim of this study was the application of HPLC methods developed for the chemical profiling of ecstasy and cocaine samples that were seized from the Irish market.

MDMA was the main active ingredient detected in the majority of ecstasy tablets with 62.3 % of samples containing only this active ingredient. MDEA was also identified in combination with MDMA, and 3.2% of the tablets had a greater quantity of MDEA than MDMA. Amphetamine was also found, alone and in combination with MDMA and caffeine. Caffeine was the only adulterant detected. Sugars are another major component of ecstasy tablets. Only two sugars were detected in the tablets, lactose and sorbitol which were found alone and in combination with each other. No sugars were detected in 8.2%. All detectable active ingredients, adulterants and sugars were identified and quantified for a large sample of ecstasy tablets. Upon combination of all the results, these analytes comprised of 8.5% to 99.6% of the total weight of the tablets. This study confirmed that the tablets seized in Ireland are similar to those seized elsewhere in Europe and Worldwide. This would then lead to the conclusion that these tablets are not produced in Ireland, but in other countries, most probably in the UK, Netherlands or Belgium.

The chemical components of street cocaine samples were also detected and quantified. Predictably cocaine was present in 100% of the powders analysed. The important detail to note though was the purity levels, 0.4 to 44.2%, which is considerably less than results from other research (EMCDDA report, 2006). A large number of both adulterants and sugars were detected in the samples. As each seizure was originally from a small weight of sample, one could suggest that the seizures were already "cut" a number of times, before confiscation.

In order to curtail production and sale of cocaine and indeed other drugs, efforts have been made at government level to ban the production or sale of the chemical precursors that are used in the production process these drugs, e.g. safrole for MDMA production. Whilst this is admirable, the large scale production and sale of the most common adulterants and excipients should also be prohibited. Clearly, sugars will not be made illegal, but other compounds, such as lidocaine– both of which is still legal in Ireland today - should be monitored for sales in large quantities. The onus is on the policy makers, both in Ireland and worldwide, to align themselves and ban, or restrict such compounds.

Chapter 5: Chemometrics and statistical profiling of ecstasy tablets.

5.1. Introduction.

Chemometrics can be described as the application of statistical methods to multivariate chemical data sets allowing for the statistical profiling of illicit drug seizures (Nic Daeid & Waddell 2005). Profiling of illicit drug seizures can potentially be used to monitor the extent of drug trafficking (Nic Daeid & Waddell 2005), determine the geographical origin of samples (Johnston & King 1998) and identify novel trends or developments in illicit drug samples (Jonson & Stromberg 1993). The method and types of statistical techniques commonly applied to drug profiling include specifically the use of unsupervised pattern recognition methods of PCA, HCA and Pearson's correlation and more recently supervised methods of LDA and ANN.

Unsupervised pattern recognition methods require no knowledge as to the origin of samples but can provide clusters or groupings from the data sets. PCA ascribes loadings to the classification based on the main components present in the data set. HCA gives no indication of the variables used for classification but does present all the variation in the data set in the form of a dendogram. Pearson's correlation coefficient measures the relationship between variables or samples and is currently recommended by the European Network of Forensic Science Institutes (ENFSI) as a method of choice in profiling drug samples. Discriminant analysis techniques such as LDA are parametric supervised techniques and are applied in cases where data sets are known to be normally distributed (Nic Daeid & Waddell 2005). Artificial neural networks (ANN) have the potential to provide the complete solution in identifying recognition patterns in data and new seizure data could be applied to the established neural networks to monitor developments in drug trafficking or identify new trends in illicit drug seizures. The following sections deal with the application of PCA, HCA, Pearson's correlation, LDA and ANN to determine the suitability of classification methods in identifying possible groupings within the ecstasy chemical data sets generated in this study.

5.2 Ecstasy data pre-treatment methods.

The chemical data sets used in this study consisted of the active ingredients plus sugars (AS), metal data (MD) and all data combined (DC) for the 183 individual ecstasy tablet samples (see Appendix **Table A1** & **A2**). Variables for AS data included MDMA, MDEA, amphetamine, caffeine lactose and sorbitol and represent only compounds detected in samples. MD data variables were Al, Zn, Fe, Mg, Ca, Cr, Pb, Na and K that were similarly detected in ecstasy samples. The data sets for statistical analysis were comprised of

chromatogram peak areas (AS data) or absorbance values (MD data) for each compound detected by HPLC or atomic absorption methods, respectively. Not all compounds were detected in all individual tablet samples and the presence of zeros in data sets can pose problems in particular statistical treatment of samples. In order to overcome this possible difficulty zero values were replaced with a value corresponding to half the lowest detection limit for variables of each data set (Lociciro *et al.* 2008). In addition, various pre-treatment methods were explored to determine the most appropriate method prior to applying the statistical protocol to the entire data sets.

Since all data sets were generated by different analytical techniques data pre-treatment is required due to the differences in the scales between variables. To reduce the complexity and to easily identify the appropriate pre-treatment method, eight seizures were selected based on their discrete AS chemical data profile (Table 2.5, Table A1). Each seizure consisted of three tablets with six variables giving a combined sample set of 144 data points for pre-treatment analysis. Each pre-treatment method was evaluated by PCA and HCA methods to determine the most appropriate pre-treatment method(s). Data pre-treatment methods evaluated included the following:

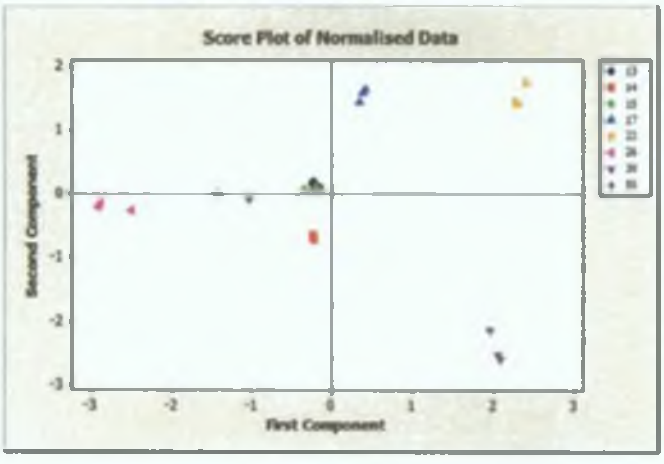
1. Normalisation (N)
2. Normalisation & logarithm ($N + Log_{10}$)
3. Normalisation & 4^{th} square root (N + 4sqrt)
4. Standardisation (S)
5. Standardisation & logarithm ($S + Log_{10}$)
6. Standardisation & 4^{th} square root (S + 4sqrt)

The calculations for each pre-treatment method are as described in Section 1.5.2. PCA allows for the visual inspection by use of score plots and the eight selected seizures should be present as discrete individual groups but distant from other seizures. Based on PCA score plots (Figure 5.1 & 5.2) the optimal pre-treatment methods of choice were normalisation & logarithm and standardisation & logarithm. Within these score plots all three data points for each seizure are cluster together while all eight seizures are distant from one another. As PCA is subjective, the same data sets using same pre-treatment methods were assessed by HCA. Pre-treatment methods should generate eight HCA clusters for the selected data set and the three tablets for each seizure should be cluster together at a Euclidean distance of 1.

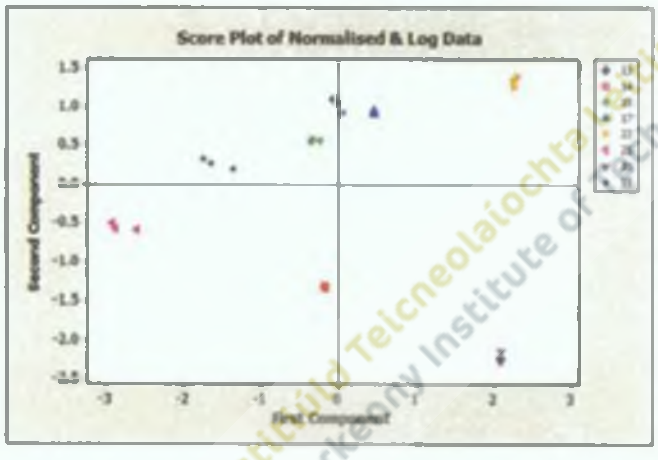
Normalisation & logarithm, standardisation & logarithm and standardisation & 4^{th} square root were suitable pre-treatment methods for identifying the eight discrete HCA clusters (Table 5.1).

Pre-treatment of data by normalisation reduces the order of magnitude for each component within a tablet to a fraction of the total concentration of all chemical components within each individual tablet. Data pre-treatment by standardisation reduces the order of magnitude for each individual chemical component in all tablets to a fraction of the total concentration for that chemical component in all tablets. Since chemical data sets were generated by different analytical techniques (i.e. HPLC peak areas and AA absorbance units) and are not in the same numerical category, data pre-treatment by normalisation is not recommended. Conversely, data pre-treatment by standardisation is suitable as it allows for the comparison of variables within each chemical data set for all tablets. As such, standardisation & logarithm was chosen as the more suitable data pre-treatment method for application to the entire 180 ecstasy tablet samples for profiling analysis. All data sets for the three ecstasy tablets from seizure 10 were excluded.

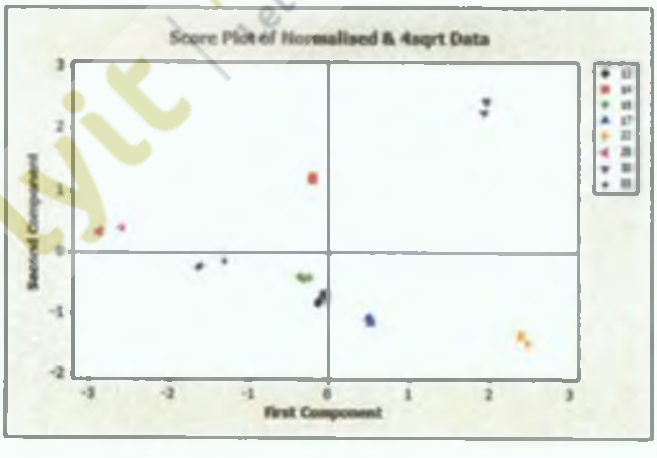
Exclusion of seizure 10 was based on the HPLC data for the active ingredients which had a reduced peak area for the internal standard suggesting a less than 20 µl sample inject volume for these samples compared to other samples. Calculation of the concentration of components in these samples is not affected as concentrations are determined by reference to the internal peak area standard. Since standardisation & logarithm data pre-treatment method reduces the order of magnitude for each individual chemical component in all tablets the inclusion of this data set could potentially be misclassified. Seizure 10 was therefore excluded from all subsequent statistical analysis.



(A)

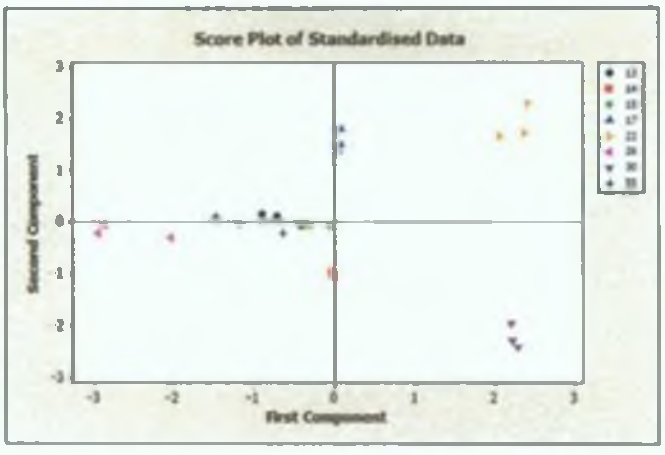


(B)

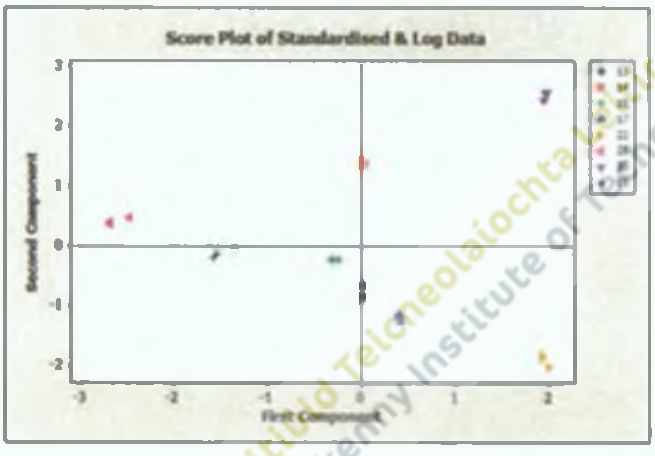


(C)

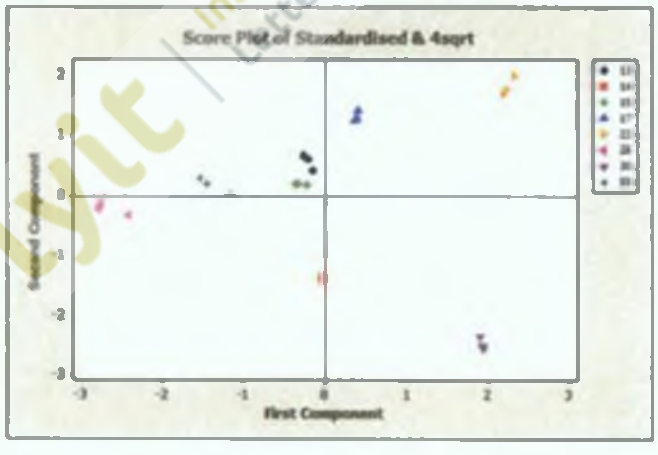
Figure 5.1. PCA score plots for normalised data pre-treatment methods. (A) normalised data, (B) normalized & logarithm data and (C) normalised & 4th square root data.



(A)



(B)



(C)

Figure 5.2. PCA score plots for standardised data pre-treatment methods. (A) standardised data, (B) standardised & logarithm data and (C) standardised & 4th square root data.

Table 5.1. HCA outcomes of data pre-treatment methods for eight selected ecstasy seizures.

Pre-treatment method	No. of clusters at a Euclidean distance of 1
Normalisation (N)	6
Normalisation & logarithm (N + Log_{10})	8
Normalisation & 4^{th} square root (N + 4sqrt)	7
Standardisation (S)	7 + 1 outlier
Standardisation & logarithm (S + Log_{10})	8
Standardisation & 4^{th} square root (S + 4sqrt)	8

5.3. Ecstasy data pattern recognition methods.

Three different unsupervised pattern recognition methods of PCA, HCA and Pearson's correlation coefficients were evaluated for all three pre-treated data sets of AS, MD and DC. Unsupervised pattern recognition methods require no prior knowledge as to the origin of samples but can provide clusters or groupings from the data sets. Groupings or clusters generated can be tested by supervised LDA to determine the percentage correct classification rate for each unsupervised pattern recognition method using each of the respective data sets.

5.3.1. Principal component analysis

PCA score plots for standardisation & logarithm pre-treated AS, MD and DC data sets are presented in Figures 5.3, 5.4 & 5.5, respectively. The score plot for the first two principal components for AS data (Figure 5.3) account for 57.1% of the total data variance and illustrated that the active ingredients & sugar variables were classified into 6 discrete groupings. Table 5.6 lists the seizure groupings for AS data identified by PCA. Individual ecstasy tablets were all clustered to discrete seizures and no outliers were detected, confirming that ecstasy tablets were linked to their original seizure number. The first two principal components for MD and DC data sets accounted for 38.7% and 29.2% of the total data variance, respectively. No distinct groups of samples were observed for the MD data (Figure 5.4). Five groups were observed for the DC data set and individual ecstasy tablets were all clustered to their original seizure numbers (Figure 5.5). In this instant, principal component analysis suggest that AS data is more important for classification purpose.

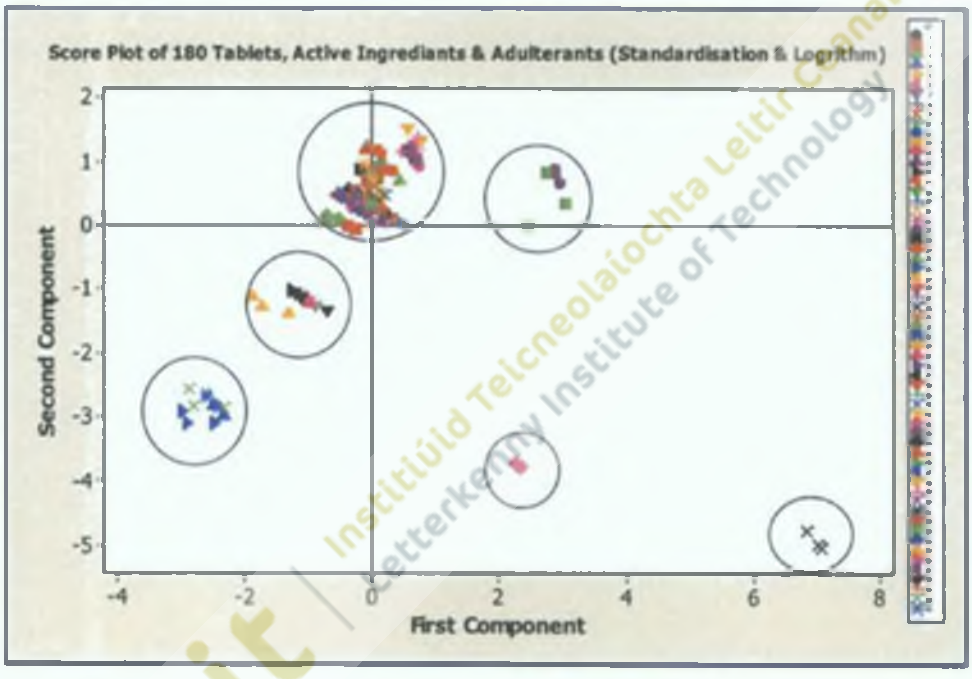


Figure 5.3. PCA score plot of the first two components using the AS data set.

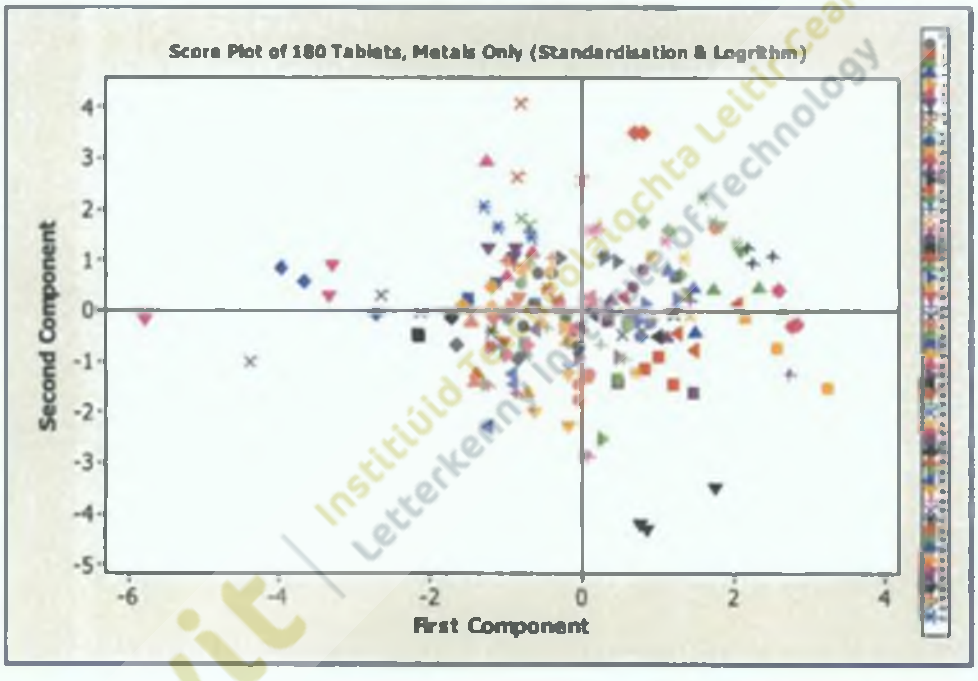


Figure 5.4. PCA score plot of the first two components using the MD data set

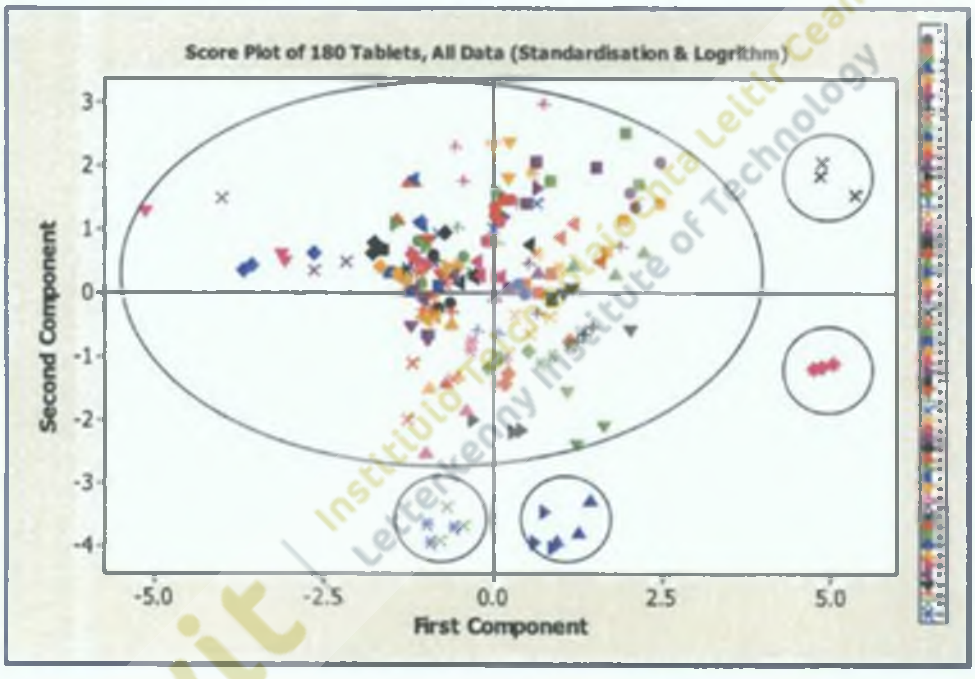


Figure 5.5. PCA score plot of the first two components using the DC data set.

5.3.2. Hierarchal cluster analysis

This statistical method measures the distance between two objects and if the distance is relatively small then similar linked objects will be clustered. The similarity distance between samples is calculated by both cluster and interval methods. In order to determine the most appropriate HCA cluster and interval method the AS data set for the eight seizures used in pre-treatment studies (Section 5.2) was evaluated prior to applying the method to the entire data sets.

Seven different HCA cluster methods were evaluated (Between groups, Within groups, Nearest neighbour, Furthest neighbour, Centroid, Median and Wards) using the default Squared Euclidean interval setting of the SPSS software package. Selection of the most appropriate cluster method was determined by the numbers of clusters formed by each method (Table 5.2). All clustering methods at a Euclidean distance of 1 generated 8 discrete clusters for the eight selected seizures and all three tablets were clustered to their respective seizure (Figure 5.6). At Euclidean distance 5 the number of clusters observed reduced from 8 to 7 or 6 regardless of clustering method employed (Table 5.2). Between groups was selected as the cluster method of choice as this is the default cluster method associated with the SPSS software for HCA. In addition, since 8 correct discrete clusters were observed at the Euclidean distance 1 this was selected as the most appropriate distance to determine group classifications for all subsequent analysis.

Table 5.2. Number of clusters for eight discrete seizures determined by different HCA clustering methods.

Cluster Method	No. of clusters at distance 1	No. of clusters at distance 5
Between groups	8	7
Within groups	8	7
Nearest neighbour	8	7
Furthest neighbour	8	6
Centroid	8	7
Median	8	7
Wards	8	7

Table 5.3. Number of clusters for eight discrete seizures determined by different HCA interval methods.

Cluster Method	No. of clusters at distance 1	No. of clusters at distance 5
Euclidean	4	8
Squared Euclidean	8	7
Cosine	8	7
Pearson	8	7
Chebychev	4	8
Block	4	8
Minkowski	4	8

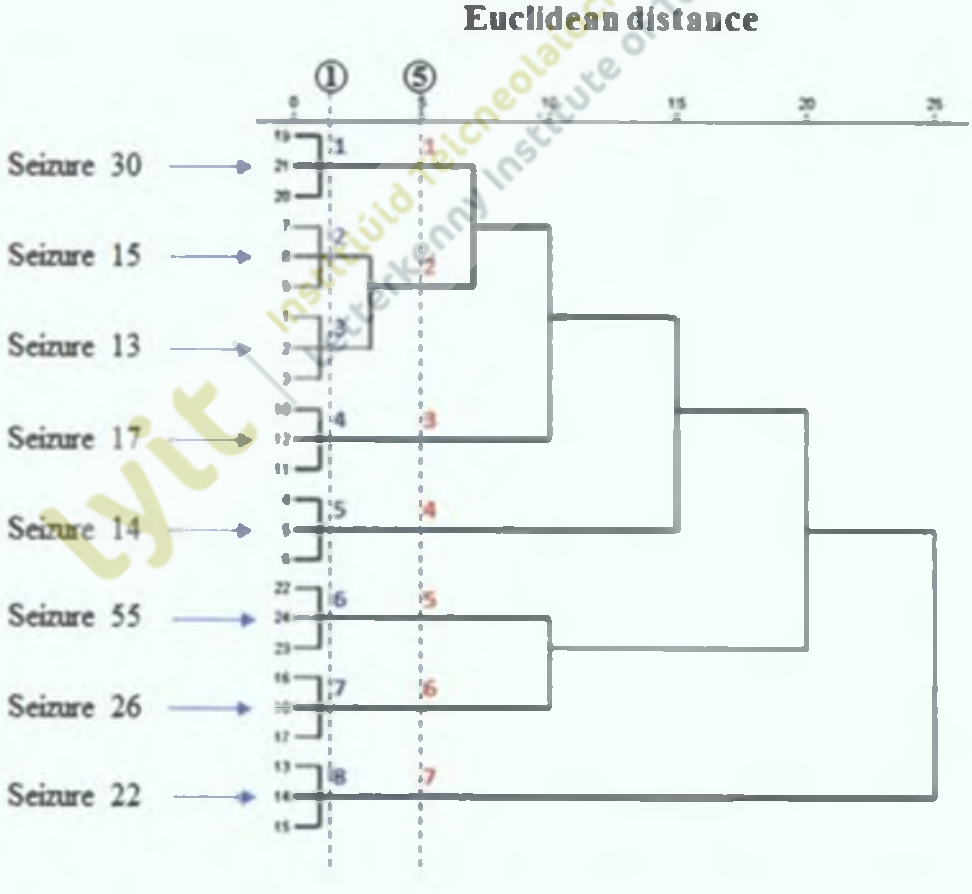


Figure 5.6. HCA dendogram using Between Groups with Squared Euclidean for eight selected seizures. The number of clusters formed was dependent on the Euclidean distance measure employed.

Using Between groups cluster method, seven interval methods were assessed (Table 5.3). Squared Euclidean, Cosine and Pearson interval methods correctly cluster all eight seizures at distance measure of 1. All other interval methods failed to correctly cluster the eight seizures. The number of clusters formed at distance 5 was reduced from 8 to 7 clusters for Squared Euclidean, Cosine and Pearson interval methods with two seizures (i.e. seizures 13 & 15) clustering as one group (Figure 5.6). As such, Between group and Squared Euclidean at distance measure of 1 were chosen as the HCA cluster and interval methods for application to the entire data sets of AS, MD and DC.

Standardisation & logarithm pre-treated AS, MD and DC data sets were analysed by HCA. The number of clusters formed, varied dependent on the data sets and Euclidean distance measured employed. Dendograms for AS, MD and DC data sets generated 11, 23 and 32 HCA cluster groups at Euclidean distance 1, respectively. MD and DC data sets did not form discrete clusters with numerous individual tablet samples distance from other corresponding members of the same seizure suggesting that these samples were incorrectly classified and represent HCA outliers. Analysis of MD and DC HCA dendograms at Euclidean distance 5 reduced the number of clusters to 7 and 19, respectively; however outliers were still present suggesting that MD and DC data sets were unreliable for HCA classification purposes.

Conversely, AS data generated 11 discrete HCA groups with all individual tablet samples linked to corresponding members from the same seizures with only two exceptions. Due to the large number of samples considered, the dendogram became complex when each sample was labelled. A simplified dendogram is illustrated in Figure 5.7 which demonstrates group linkages for AS data set, with members of each cluster identified, listed in Table 5.6. Individual ecstasy tablet samples 49.3 and 53.3 were not linked to members of the same seizure at euclidean distance 1, but could be linked with group IX and IV if measured at euclidean distance 2 and 6, respectively (Figure 5.7).

HCA identified four clustering groups of A, B, C and D with clusters further differentiated into eleven HCA groups (Figure 5.7). Clusters were differentiated based on active ingredients and sugar components present and group subdivision was based on the combination and concentration of components (Table 5.4). Cluster A was defined by two main components of MDMA and lactose. HCA groups I, VII, and XI were separated based on the presence of caffeine or sorbitol. Cluster B was comprised of groups IV, VIII, IX and X and were differentiated based on the presence of MDMA, lactose and MDEA. These groups were primarily separated based on the combination and concentration of MDMA and MDEA present (Table 5.4). Cluster C was distinguished by the absence of lactose in all samples with groups II, III, and IV separated based on the presence of caffeine, sorbitol or amphetamine. Cluster group D was comprised of the single seizure 30 and was distinguished by the lack of MDMA in either of the three tablet samples.

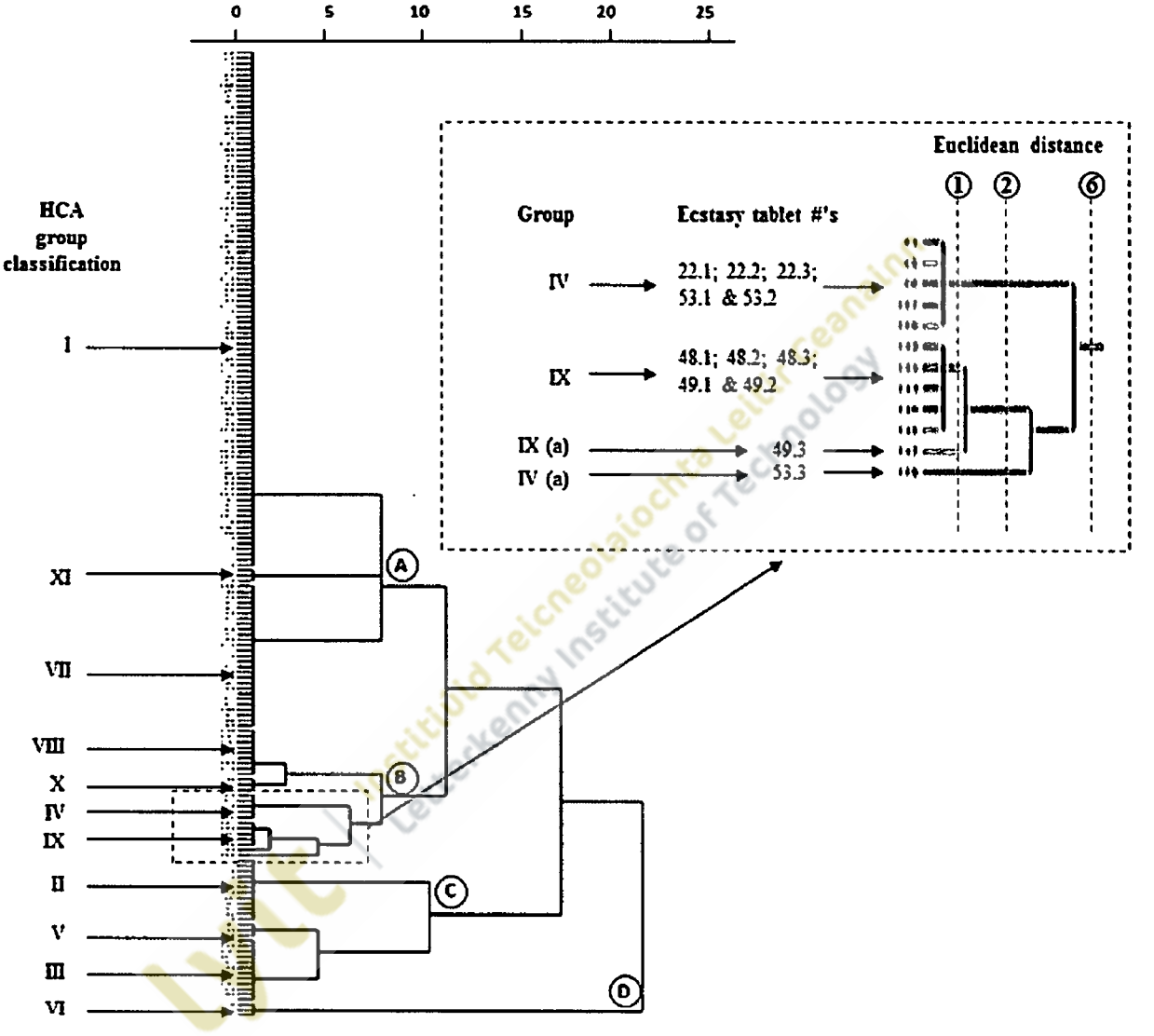


Figure 5.7. Dendogram for AS data set illustrating four clusters A, B, C, and D with eleven discrete HCA groups. Expanded section highlights the region for HCA groups IV and IX and illustrates the linkage of the two ecstasy sample outliers 49.3 and 53.3 at Euclidean distance 1, 2 or 6.

Table 5.4. Summary of the active ingredients and sugar components (mg/g) for HCA classification of ecstasy seizures used in this study. HCA cluster groups and seizure batch numbers are described in Figure 5.7 and Table 5.6, respectively.

Cluster	Group #	(N)	MDMA	Lactose	MDEA	Caffeine	Sorbitol	Amphetamine
			Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
A	I	(96)	282.1 ± 78.3	566.1 ± 103.2	-	-	-	-
	VII	(27)	331.0 ± 66.7	374.4 ± 183.3	-	9.1 ± 3.9	-	-
	XI	(3)	252.2 ± 46.2	345.6 ± 34.4	-	-	277.1 ± 24.8	-
B	VIII	(9)	288.4 ± 75.1	566.2 ± 38.7	14.1 ± 1.7	-	-	-
	X	(3)	369.5 ± 16.5	681.8 ± 7.4	1.7 ± 0.5	-	-	-
	IX	(6)	311.9 ± 44.2	422.7 ± 31.1	4.6 ± 0.3	5.1 ± 0.6	-	-
	IV	(6)	27.5 ± 5.7	481.5 ± 86.6	46.6 ± 9.0	2.8 ± 0.5	-	-
C	II	(12)	279.8 ± 42.8	-	-	-	550.4 ± 209.4	-
	V	(3)	81.5 ± 14.2	-		0.6 ± 0.1	-	16.0 ± 2.4
	III	(12)	282.9 ± 60.8	-	-	-	-	-
D	VI	(3)	-	688.6 ± 59.7	-	-	-	29.5 ± 8.6

5.3.3. Pearson's correlation coefficients.

Pearson's correlation coefficients determine the linear relationship between two samples. The more correlated the relationship the more likely two samples are linked. A Microsoft Excel macros file provided by European Network of Forensic Science Institutes (ENFSI) was used to examine Pearson's correlation matrices for the standardisation & logarithm pre-treated AS, MD and DC data sets. Application of this method for classification purposes is determined by the minimum acceptable threshold value assigned.

Threshold values between two samples are arbitrary and coefficient values equal to or greater than the minimum set value will imply a high degree of correlation between linked samples. In this study threshold values were initially determined by examining all three replicates for each of the three samples from the same seizure. Since samples are similar the minimum value obtained is considered the threshold value to link replicates of the same sample (Table 5.5). In this example, the minimum threshold values for seizure 13 and 14 were 97.97 and 98.31, respectively and both seizures are unlinked. The minimum threshold values obtained using this approach for all AS data samples ranged from 92.7 (seizure 3) to 99.9 (seizures 2 & 12) with an average value of 98.6 for all seizures. This average value of 98.6 was selected as the acceptable minimum threshold value to give a high degree of confidence between correlated AS data samples. Using the same approach the minimum average acceptable threshold values identified for MD and DC data set samples were 79.2 and 88.2, respectively. Correlation matrices values that fall below these minimum average threshold values for each of the respective data sets would imply samples were not linked while values equal to or above the value would be considered correlated.

Due to the relatively lower threshold values assigned for DC and MD data sets the number of groups identified were high (Table 5.7). The DC data set generated 35 Pearson's groups which were comprised of 10 groups and 25 unlinked seizures using the minimum acceptable threshold value of 79.2. The increased threshold value of 88.2 identified 29 groups for the MD data set, comprising 7 groups and 22 unlinked seizures. As previously observed with PCA and HCA methods the inclusion of metal data proved unreliable at differentiating unique groups from the data sets.

Table 5.5. Pearson correlation coefficients sample matrix for the three replicates of each sample seizure 13 and 14. Minimum threshold values (highlighted in red) for seizure 13 and 14 are 97.97 and 98.31, respectively. Shaded section indicates poor correlation between sample replicates of seizure 13 and 14 due to low threshold values. Pre-treated AS data set was used in this example.

	1311	1312	1313	1321	1322	1323	1331	1332	1333	1411	1412	1413	1421	1422	1423	1431	1432	1433
1311	100																	
1312	99.98	100																
1313	99.99	99.98	100															
1321	99.13	99.17	99.16	100														
1322	99.14	99.18	99.16	99.99	100													
1323	99.09	99.11	99.11	99.99	99.97	100												
1331	99.28	99.24	99.25	98.13	98.09	98.09	100											
1332	99.26	99.22	99.23	98.21	98.17	98.18	99.99	100										
1333	99.17	99.13	99.14	98.02	97.99	97.97	99.98	99.98	100									
1411	56.30	56.56	56.53	53.61	53.64	53.36	58.18	58.13	58.26	100								
1412	56.42	56.68	56.65	53.68	53.71	53.43	58.34	58.30	58.43	99.99	100							
1413	56.76	57.03	57.00	54.23	54.26	53.98	58.52	58.48	58.59	99.98	99.96	100						
1421	56.78	57.08	57.04	54.30	54.35	54.04	58.46	58.42	58.54	99.91	99.90	99.94	100					
1422	56.90	57.20	57.15	54.47	54.52	54.22	58.53	58.50	58.62	99.88	99.87	99.92	99.99	100				
1423	56.74	57.04	57.00	54.31	54.35	54.06	58.39	58.36	58.48	99.89	99.88	99.92	99.99	99.99	100			
1431	55.28	55.61	55.46	53.43	53.43	53.20	57.45	57.55	57.67	98.39	98.35	98.45	98.32	98.37	98.31	100		
1432	55.56	55.89	55.74	53.81	53.82	53.59	57.62	57.72	57.83	98.41	98.38	98.49	98.38	98.44	98.38	99.98	100	
1433	55.53	55.86	55.71	53.78	53.79	53.55	57.57	57.68	57.79	98.36	98.32	98.44	98.35	98.40	98.34	99.98	99.99	100

Pearson's correlation for the AS data set generated 13 discrete highly correlated groups (threshold value ≥ 98.6) with all individual tablet samples linked to corresponding members from the same seizures. Linked seizure numbers for each group are provided in Table 5.6. Pearson's groups for the AS data set were similar to HCA groups. The two additional groups (i.e. Group XII & XIII) are considered subsets of the HCA groups VII & I while all other groups were identical. Pearson's group XIII is unexplained as this subset did not appear significantly different from other members of Group I. Group XII can be explained based on the concentration levels of lactose in samples. Pearson's group VII had mean lactose levels of 523.19±94.3 mg/g of sample while group XII contained 188.46±12.3mg/g of sample.

Overall unsupervised pattern recognition methods of PCA, HCA and Pearson's correlation were successful at identifying groups within the 180 individual ecstasy tablet samples. From a scientific perspective, linking seizures is useful as an operational or strategic tool for law enforcement investigators. In this study, seizures could be linked based on groups formed, however the number of groups identified varied with the method applied and the sample data set utilised. The ability of PCA, Pearson's correlation and to some extent HCA to classify tablets according to seizure was clearly limited by significant variation in the metals present within each tablet and seizure (Appendix Table A2). Such variation may always be present in tablets that are produced in clandestine laboratories and will consequently limit attempts to link tablets and seizures to common batches of illicit substances.

The most successful data set for classification purposes was the active ingredients and sugar components (AS data). All three pattern recognition methods identified various groups dependent on whether PCA, HCA or Pearson's correlation was applied to the data set (Table 5.6). PCA classified the 180 ecstasy tablet samples into 6 groups based on the chemical constituents present. HCA and Pearson's correlation identified 11 and 13 groups, respectively, and were further able to classify these groups based on the concentration and combination of constituents present in samples (Table 5.4). Pearson's correlation distinguished groups based on similarity between samples while HCA provide cluster linkages of samples. Regardless, the application of these statistical methods linked seizures suggesting that the different groups may represent discrete suppliers or manufacturing processes.

There are many advantages and disadvantages with each of the three pattern recognition methods. PCA and HCA require the use of special software package such as SPSS, while Pearson's correlation is performed using a simple Microsoft excel macros file. PCA is simple and produces visual score plots of potential groups formed but are subject to the analyst's interpretation. HCA and Pearson's correlation are more informative and provide similarity linkage between samples but require a more in-depth knowledge of the statistical protocol to apply.

Table 5.6. Seizure numbers for groups identified using unsupervised pattern recognition methods of PCA, HCA or Pearson's correlation for active drug and sugar (AS) data sets.

Group #	Pattern recognition method																							
	PCA								HCA								Pearson's							
Group I	1	2	3	5	6	7	8	9	1	2	3	5	6	7	8	9	1	5	6	7	8	9	11	12
	11	12	13	15	17	19	20	21	11	12	15	19	20	21	27	28	15	19	20	21	27	28	29	31
	23	24	25	27	28	29	31	32	29	31	32	33	34	37	38	44	32	33	34	37	38	44	46	47
	33	34	36	37	38	39	40	41	46	47	50	51	54	56	57	59	50	51	54	56	57	59		
	42	43	44	45	46	47	48	49																
	50	51	52	54	56	57	59																	
Group II	4	26	60	61					4	26	60	61					4	26	60	61				
Group III	16	18	35	55	58				16	18	35	58					16	18	35	58				
Group IV	22	53							22	53							22	53						
Group V	14								14								14							
Group VI	30								30								30							
Group VII									17	23	24	25					17	25	39	45	52			
									39	40	41	45	52											
Group VIII									36	42	43						36	42	43					
Group IX									48	49							48	49						
Group X									13								13							
Group XI									55								55							
Group XII																	23	24	40	41				
Group XIII																	2	3						

Table 5.7. Linear discriminant analysis percentage correct classification rates for the number of groups identified by Principal component, Hierarchal cluster and Pearson's correlation pattern recognition methods.

Data Sets	Number of Variables	Pattern recognition method					
		Principal Component Analysis		Hierarchal Cluster Analysis		Pearson's Correlation Coefficient	
		No. of groups	% Correct	No. of groups	% Correct	No. of groups	% Correct
DC	15	5	98.4%	19	96.7%	35[a]	80.6%
MD	9	1	ND	7	93.9%	29[b]	76.8%
AS	6	6	93.4%	11	99.5%	13[c]	99.5%

DC = all data combined; MD = metal data only; AS = active ingredients & sugar data. Minimum threshold values differed for each data set; a = 88.2, b = 79.2 and c = 98.6. ND = not determined

5.4 Supervised pattern recognition methods.

Both LDA and ANN were applied to the chemical data sets of the 180 individual ecstasy tablet samples to determine both the correct classification rate and possible linked networks in samples. LDA was applied to all groups identified by unsupervised pattern recognition methods to confirm percentage correct classification rates (Table 5.7). LDA percentage correct classifications ranged from 76.8% to 99.5% depending on the pattern recognition method and data group set tested suggesting that the data groupings were reasonably assigned. As observed previously LDA classification rates were poorest for MD data set regardless of pattern recognition method employed. When MD and AS were combined classification rates improved suggesting that AS data variables were more important in determining group classifications. Both HCA and Pearson's correlation groups for the AS data set provided the best classification rates of 99.5%. The 0.5% error was associated with the single ecstasy sample 49.3 which was classified with samples from Group IV. Since HCA and Pearson's correlation AS data groups had similar LDA classification profiles both groupings were further tested by ANN. Neural networks were applied to samples in order to determine the correct classification rate of pattern recognition method and also to establish a neural network that could be beneficial in classifying new drug seizures.

Artificial neural networks algorithms have the potential to provide complete solutions in the identification of patterns in data sets (Bishop, 1996). In this study, individual networks and ensembles of networks were created and tested using the Intelligent Problem Solver (IPS) system function in the Trajan software. AS data was inputted into the software as individual sets of data coded according to group seizure numbers identified by HCA or Pearson's correlation methods. The software randomly assigned individual data sets from each sample into training, selection and test sets. One hundred networks for each of the six chemical variables were tested for the three layers (input, hidden and output layers) by Multi-Layer Perceptron (MLP) and Radial Base Functions (RBF) methods. The training algorithms were a combination of back propagation and conjugate descent. Selection of the most suitable network was based on the performance of the selection set and the difference in the error associated with the selection set and the test set. The errors associated with the selection and test sets should be relatively small to ensure the network is capable of generalising new data.

The performance of the training and selection tests should be similar and close to 1 to indicate that the model does not suffer over-learning. The performance of the test should also be close to 1 to indicate that the network can classify new data adequately (Waddell *et al.* 2004). The final neural networks selected for the HCA and Pearson's group classification of AS data set are provided in Table 5.8.

Table 5.8. Optimal artificial neural networks selected for HCA and Pearson's group classification of AS data set.

ANN Criteria	HCA	Pearson's
Network Profile	MLP 6:6-10-11:1	RBF 6:6-46-13:1
Training Performance	1	0.9139
Selection Performance	1	0.8880
Test Performance	1	0.9555
Training Error	0.0002	0.0067
Selection Error	0.0003	0.0681
Test Error	0.0188	0.0665
Correct Classification Rate	100%	85.7%
Training	BP100, CG20, CG41b	KM, KN, PI

The optimal network selected was based on the best performing network of the 100 networks tested for both groups. In the case of the Pearson's AS data groupings the optimal network was an RBF 6:6-46-13:1. This is a radial base function network architecture with six input chemical variables, forty six neurons in the hidden layer and thirteen output groups. Classification rate for this network was 85.7% with a selection performance of 0.888 suggesting mis-classification within the Pearson's group structure. The optimal neural network for the HCA AS data groupings was an MLP 6:6-10-11:1 and yielded a perfect 100% correct classification. This is a multilayer perceptron network architecture with six input chemical variables, ten neurons in the hidden layer and eleven output groups. Error values were low and training, selection and test performance values were the ideal value of 1. This neural network structured according to the HCA AS data groupings is more than capable of handling, generalising and classifying new data. Future chemical data based on the combination and concentrations of MDMA, MDEA, amphetamine, caffeine lactose or sorbitol applied to this trained neural network could potentially serve as a model to monitor trends and developments for ecstasy drug trafficking in Ireland.

The particular advantage of chemometrics is that it allows for the statistical analysis of multivariate chemical data into discrete reliable groups for profiling illicit drug samples from clandestine manufacturing processes. In this study, ecstasy seizures were linked based on HCA clustering groups and confirmed by the MLP 6:6-10-11:1 artificial neural network. Group classification was based on the combination and concentration of active ingredients and sugar components present in samples. Physical data and seizure location data can be combined with these linked groups to investigate whether any patterns exist in ecstasy drug trafficking in Ireland during the period 2001-2003.

Group I contained the greatest number of seizures, 32 of the 60 seizures analysed. This group was distinguished based on the MDMA and lactose content. It is more than likely that ecstasy tablets within all 32 seizures from different regions were prepared by the same manufacture. This group contained numerous different logo types and included; 'Mitsubishi' (14 seizures), 'Bird' (4 seizures), 'Horse' (2 seizures), 'Smirnoff' (2 seizures) and the remaining ten seizures all had unique logos of 'Armani', 'Chi', 'Dextran', 'Euro', 'Heart', 'Motorola', 'Rolls Royce', 'Smiley', 'V2' and no logo (Figure 4.1) suggesting that the manufacturer was applying logo's randomly. The wide diversity of logo types imply that this group could not be classified based on logos but required chemical profiling. The majority of Group I was seized in the Dublin metropolitan area (21 seizures) however the same samples were seized in other regions (Cork, Kildare, Kilkenny, Sligo, Waterford and Wexford) strongly suggesting that Dublin is the main port of entry with distribution regionally via the main North Western, South Western and Southern transport routes (Figure 5.8).

Group II was similar to Group I in chemical composition but contained sorbitol instead of lactose as the main adulterant. This group was seized in Dublin (3 seizures) and Donegal (1 seizure) and could not be distinguished based on physical characteristics or brand logos. Logos recorded for this group include 'Heart' (2 seizures), 'Bird' (1 seizure) and 'Euro' (1 seizure). This group represents a possible network between Dublin and Donegal. Bell *et al* (2003) reported MDMA/sorbitol ecstasy tablets seized within Northern Ireland during the same period suggesting cross-border activity between the two jurisdictions.

Group III was comprised of 4 seizures, three seized in the Dublin area and the fourth seized in Galway. All four seizures had four different logos of 'Mitsubishi', 'Euro', 'Smiley' and 'Tasmanian Devil'. This group was unique since it only contained MDMA with no adulterants present. Group IV contained MDMA/MDEA/caffeine/lactose and all contained a 'Crown' logo. In addition all samples were white in colour and had the same physical dimensions supporting the argument that these seizures were produced from the same manufacturing process. This group was seized in Louth and Wicklow and may possible be a distribution network along the East coast corridor from Northern Ireland as these samples were not present in other seizures from the Republic of Ireland.

Group V was a single seizure from Dublin, containing MDMA/caffeine/amphetamine with a 'Bird' logo and was pink in colour. This group may represent a transient seizure since it was not present in other regions of Ireland (Figure 5.8). Group VI was a single seizure from Cavan and was distinctly unique based on the Superman brand logo, green colour and contained amphetamine and lactose. The uniqueness of this seizure may suggest the introduction of a new manufacturing process as amphetamine is rarely seen as the only active ingredient in ecstasy tablets.

Group VII was the second largest group of linked seizures and samples were seized in Cork (3 seizures), Kildare (2 seizures), Offaly (2 seizures) and one seizure each from Dublin and Mayo (Figure 5.8). This group was similar to Group I but contained caffeine in addition to MDMA/lactose and had various logo types. Brand logo's included 'M&M's' (5 seizures), 'Red Bull' (3 seizures) and 'Coca-Cola' (1 seizure). Group VIII was comprised of three seizures and all seizures contained a Durex brand logo with the main chemical components of MDMA/MDEA/lactose. Samples were seized from Cork (2 seizures) and Kilkenny (1 seizure). Group IX was similar to Group VIII but contained caffeine in addition to MDMA/MDEA/lactose and the two seizures were seized in Cork and Waterford. Both seizures contained the 'Coca-Cola' brand logo. Since the majority of Groups VII, VIII and IX were seized in Cork the possibility exists that Cork is the importation port with distribution regionally via the northern, north eastern and eastern transportation routes.

Group X and XI were single seizures seized in Waterford and Clare. Group X contained MDMA/MDEA/lactose with a 'Smirnoff' logo while Group XI contained MDMA/lactose/sorbitol with a 'Heart' logo and was pink in colour. It is possible that both these seizure are not part of a major network but represent samples for personal consumption obtained abroad.

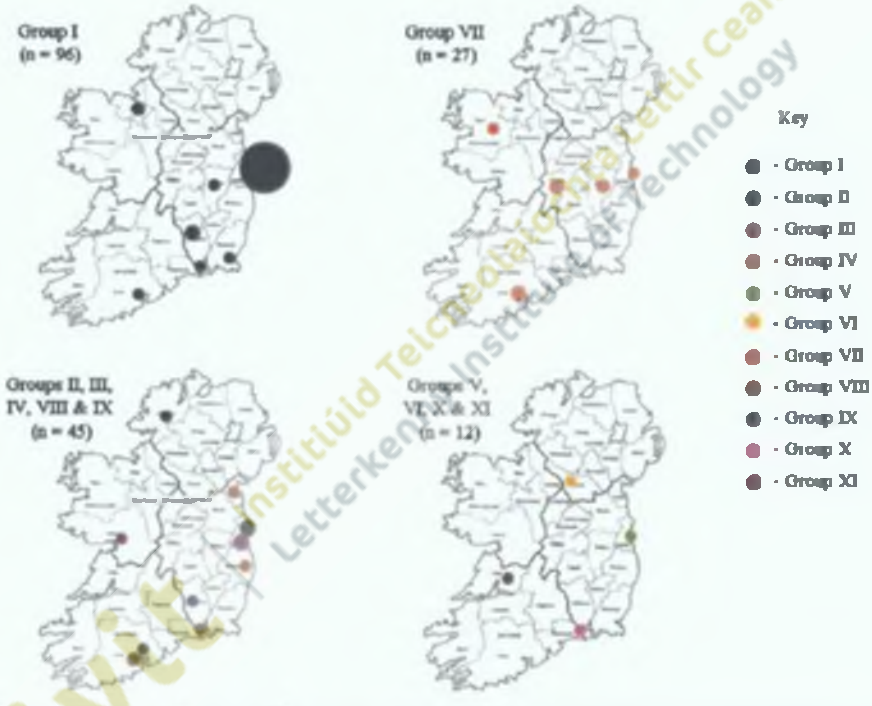


Figure 5.8 Relative distribution of ecstasy seizures examined in this study by HCA and MLP 6:6-10-11:1 neural network group classification.

5.5. Conclusions.

Recently, the application of drug profiling methods have demonstrated the potential of these techniques in establishing links between illicit drug samples and thereby are advantageous in drug intelligence (Locicero *et al.* 2008, Weyermann *et al.* 2008, Andersson *et al.* 2007, NicDaeid & Waddell 2005). Physical and chemical data can provide useful information however no clear structure can be identified regarding the manufacturing process involved. Chemometrics allows for the statistical comparison of samples to determine if patterns exist within the population sample. Difficulties can arises in directly comparing ecstasy seizures produced by different synthetic routes (Section 1.2.5) from unregulated clandestine laboratories. To overcome these barriers data sets generated must be pre-treated to allow for statistical comparisons (Miller & Miller, 2005) and an appropriate pattern recognition method must be selected (Nic Daeid & Waddell 2005).

In this study, the recommended chemometric protocol involved the generation of validated HPLC ecstasy data for the main active ingredients and sugar components (Sections 2.2 & 2. 4) followed by standardisation & logarithm pre-treatment of HPLC peak areas. Data sets for the six chemical variables for all 180 individual ecstasy samples were grouped by HCA unsupervised pattern recognition method using SPSS software. Artificial neural networks using Trajan software were applied to determine classification rates and establish a neural network pattern linking unrelated ecstasy seizures. The neural network MLP 6:6-10-11:1 was more than capable of handling new data and has the potential to monitor future trends in the Irish ecstasy market. Metal data set, alone or in combination with AS data, was on unreliable at classifying tablets according to seizure and was clearly limited by significant variation in the metals present within each tablet and seizure (Appendix Table A2).

Although the number of seizures examined was limited to those supplied by the Garda Forensic Laboratory, the application of chemometrics demonstrated the potential of this approach in establishing links between unrelated illicit ecstasy drug seizures in Ireland. The neural network MLP 6:6-10-11:1 identified eleven discrete groups for the 60 seizures analysed suggesting eleven different manufacturing processes. Alternatively, HCA provided four cluster linkages of samples (Figure 5.7) with

groups identified within clusters (Table 5.4) being possible random batch variations in a standard manufacturing method. With more extensive data it may be possible to recognize the signature of tablets prepared by major manufacturers.

Seizure locations illustrated a random geographical network distribution primarily based on the major Irish traffic routes and linked seizures were not specifically confined to a particular region. Since ecstasy clandestine laboratories have not been reported in Ireland the drug trafficking source is presumably Europe with U.K., Belgium or Holland the more than likely sources. Since Dublin and Cork contained the greatest number of seizures it is assumed that these sites are the main ports of entry to the island of Ireland. A possible alternative entry route, suggested by linked seizures in this study, is via the neighbouring Northern Ireland jurisdiction and as such future profiling studies should examine the whole of Ireland. The advantage of the established neural network MLP 6:6-10-11:1 would allow for the addition on new data to build on the database to monitor developments and trends in the Irish ecstasy market.

Chapter 6: Conclusions and future work.

Forensic analysts are faced with the challenge of continually developing sophisticated methods of analysis to combat the increasing variability that occurs in illicit drug samples. The main objective for the analytical chemist has been to introduce new, versatile techniques with high efficiency, selectivity and precision. Research work for this thesis has focused on the development of the analytical method HPLC (High Performance Liquid Chromatography) for the analysis of major drug constituents associated with ecstasy and cocaine illicit drug samples.

6.1. Method development.

Emphasis in this thesis has been placed on method development with univariate or multivariate experimental strategies used in the selection and optimisation of procedures. Considerations with regard to the choice of chromatographic factors, solutes under investigation and the provision of quality assurance data throughout the research work have been the main criteria in methods developed. Methods developed have undergone validation studies including, intra- and inter- reproducibility, accuracy, linearity of calibration, limit of detection (LOD) and limit of quantification (LOQ) and the use of internal standards. Two HPLC methods have been developed and these methods have been successfully applied to the analysis of ecstasy and cocaine samples seized from Ireland during the period 2001 to 2004.

The univariate experimental approach was applied to the development and validation of a HPLC method that could be used to qualitatively and quantitatively assay for MDMA and its analogues in ecstasy tablets. Chromatography performance was based on resolution, retention factor and efficiency. A highly selective HPLC protocol with DAD using the Chromolith SpeedROD, RP-18, 50 x 4.6 mm was developed to quantify MDMA and its analogue derivatives found in ecstasy tablets. Chromatography, using monolithic columns, demonstrated good separation of the amphetamines and the typical adulterants (Figure 3.15). Comparisons of column types indicate that monolithic columns are more efficient than particulate columns without loss of resolution. Monolithic columns reduced the retention time from approximately 18.3 to 3.8 minutes resulting in faster separation without sacrificing column efficiency (Figure 3.12). A single system that could successfully separate MDMA and its related compounds, MDA, MDEA and MBDB, is highly desirable as

these related compounds are increasingly found in combination with the illicit drug. The application of the method to a large number of ecstasy tablets demonstrated the suitability of the method for the routine analysis of ecstasy tablets and the practicality of monolithic columns (McFadden *et al.* 2006).

Cocaine analysis was performed using the alternative multivariate method development approach. The group of analytes under investigation comprised cocaine and it's more commonly occurring components present in street samples. This group included cocaine, lidocaine, prilocaine, procaine, benzocaine caffeine, phenacetin and acetylsalicylic acid. A two level fractional factorial design was created and used as the basis of the experimental procedure. Performance indicators such as resolutions, retention factors and efficiencies were calculated for each experimental run. Artificial neural networks were applied to create response surfaces illustrating the relationship between the chromatographic factors and the performance indicators.

The optimal chromatographic conditions selected for cocaine analysis using this experimental approach were a mobile phase consisting of 20 mM phosphate buffer/acetonitrile (85/15, v/v) maintained in an isocratic mode, pH at 3, flow rate at 1.0 mL min^{-1}, column temperature at 35°C with Waters XBridge C18 50 x 4.6 mm, particle size 3.5 µm column and sample detection at 200 nm. All eight analytes under investigation were separated under ten minutes with good resolution using the optimised chromatographic conditions developed (Figure 3.21). The method developed was applied to seized cocaine samples and major drug constituents and adulterants were identified (McFadden *et. al.* In preparation).

6.2. Analysis of illicit drug samples.

Ecstasy differs from many other drugs of abuse in that it is in tablet form and no further excipients can be added at the different stages of trafficking. This is an essential point for profiling and linking seizures as only those chemicals added at manufacture can be present at street level. Linking seizures of ecstasy tablets is primarily based on the similarity between seizures of visual, chemical and physical data. Physical data includes colour, logo, shape, weight, diameter and thickness of the tablet. Chemical profiling investigates the major amphetamine components, possible

adulterants and excipients used in the manufacture of tablets. The levels of chemical components present in samples will vary in composition and quantities present.

As part of this study, physical and chemical data sets for 183 ecstasy tablets seized from Ireland during the period 2002 to 2003 have been recorded as discrete data sets. Physical characteristics recorded include tablet shape, colour, presence or absence of brand logos, tablet weight, diameter and thickness. Chemical data sets include both the quantification and occurrence in individual tablets of the major amphetamine components (i.e. MDMA, MDA, MDMA, MDEA, MBDB and amphetamine), adulterant components (i.e. caffeine, paracetamol, phenacetin and aspirin), diluent components (i.e. sucrose, glucose, lactose, fructose, mannitol, sorbitol and inositol) and inorganic components (i.e. Al, Ca, Cr, Fe, K, Mg, Na, Pb and Zn).

With a view to investigative purposes, a detailed chemical analysis of cocaine was conducted. Whereas with ecstasy seizures, preliminary connections can automatically be based on physical characteristics, such as logos or tablet colours, linking cocaine seizures proves more difficult. All samples were analysed for active ingredients, adulterants and sugars. The occurrence of drug ingredients, adulterants and sugar components present in street samples were as follows; cocaine (100%), prilocaine (42.8%), lidocaine (53.5%), benzocaine (3.5%), procaine (3.5%) , caffeine (57.1%), phenacetin (64.2%), acetylsalicylic acid (3.5%), mannitol (42.9%), sucrose (35.7%), lactose (17.9%), sorbitol (3.6%) and inositol (3.6%). Samples varied with the level of constituents present and in the combinations in which they occurred in each sample. Due to the complexity of the chemical profiles associated with cocaine samples and the small sample size, statistical analysis was not performed.

6.3. Chemometrics and statistical profiling of ecstasy samples.

Chemometrics, including unsupervised methods of PCA, HCA and Pearson's correlation, as well as supervised methods of LDA and artificial neural networks was applied to the chemical data sets from ecstasy samples to demonstrate the ability of the statistical approach to linking seizures with samples (Chapter 5). HCA and ANN were the statistical methods that most efficiently distinguished between linked and unlinked seizures. Eleven groups were identified from the chemical data sets with

group classification dependant on the main amphetamine, adulterant and diluent present. Further analysis with increased sample size is required to assure the statistical reliability of this approach. Nevertheless profiling results by chemometrics has the potential to classify drug seizures and to provide links between seized samples or between materials found at the crime scene. The benefits from this study will provide strategic intelligence and an understanding of the operational level in the Irish ecstasy market and help evaluate the changing profile or dynamics associated with this illegal market supply.

6.4. Establishing a profiling protocol in a forensic laboratory.

This study used ecstasy as the sample for chemically and statistically profiling seizures. However, the protocols developed could lend themselves easily to other types of drug seizures. The equipment required for profiling studies is minimal, as most forensic laboratories would have a basic LC system. A HPLC with a diode array detector proved to be an adequate instrument for the chemical analysis of ecstasy and cocaine (qualitative and quantitative data). The data generated was sufficient for classification purposes. More sophisticated instruments are available, such as an LC-MS/MS. This would provide the same data and have the added advantage of being able to detect impurities at very low concentration levels that might be present in the sample. Impurity analysis is popular with researchers whose aim is to classify the drugs. However, as proven in this study, determination of impurities is not essential for classification purposes. Therefore, a simple LC system with a UV detector is sufficient for forensic laboratories establishing a drug profiling protocol. The actual sample preparation and chromatographic analysis could be adopted into the routine drug analysis already established in the laboratory which would minimise any additional time required. Creating the statistical protocol may involve training; however, as discussed previously in Chapter 5, once established in the laboratory, the neural network software will be of major advantage for classification purposes.

Future methods of analysis should focus on developing a single LC system for the simultaneous determination of all the major drug components commonly detected in street samples. By using the multivariate approach for the method development, this would ensure minimal experiments with maximum results. Also, the use of a

monolithic column would allow for a large number of analytes to be separated in minimal time.

Metals are popular components of drug samples used for classification purposes (Waddell *et al.* 2004, Koper *et al*, 2007). In this study, metal data was unsuitable for classification. The key to metal analysis is the choice of metals, those which identify the synthetic pathways in which the ecstasy tablets were produced. These important metals are platinum, boron, mercury and lithium. Any future profiling studies should include these metals.

6.5. Future studies on the current major drugs in Ireland.

A temporal analysis of the major drugs from year to year would provide valuable information on purity levels, types and levels of adulterants and also provide information on physical characteristics of the tablets such as trends in logos. An in-depth geographical comparative analysis, either by county or by province, of each of the major drugs could offer additional evidence to support investigations by providing insights into dealer networks.

Considering Ireland is an island, one of the most important law enforcement agencies is the Irish Customs service. Most of the synthetic and semi-synthetic drugs seized in Ireland are produced outside of Ireland, so therefore the points of entry to this country are essential to control. An interesting study would be to monitor the drug seizures at each customs point for both physical and chemical profiling of all drug types. Comparisons between each port and airport would be beneficial. Such a study should also include points of entry in Northern Ireland. Cross border cooperation to identify points of entry and dealer networks is imperative for both the Garda Siochana and the Police Service of Northern Ireland (PSNI). Data gathered could be used to generate a drug intelligence data bank which would be beneficial to national and international law enforcement agencies.

The general trend in drug seizures in Ireland has changed in recent years. In 2003 ecstasy was the most seized drug in Ireland after cannabis, hence the reason for choosing ecstasy as the drug to study in this thesis. After 2004, cocaine overtook

ecstasy as the most seized drug, after cannabis. Currently ecstasy is no longer one of the most common illegal drugs seized. As reported in the Annual Garda Report 2010, seizures of ecstasy have plummeted to 398 tablets, a decline of 95% since its height in 2000 (Figure 1.1). Cocaine quantities seized still remains very high in comparison with other drugs, 94,804 grams of cocaine versus 26,462 grams of amphetamine and 30,158 grams of heroin (Annual Garda Report 2010). Recently there has been a significant change in the types of drugs seized due to new legislation in March 2009, when benzylpiperazine (BZP) was made illegal. In 2010, 351,536 tablets, 3,271 grams and 371 capsules of this drug were seized. Also, there has been an increase in seizures of abused prescription drugs. For example, Alprazolam or 'Xanax' as it is more commonly known, is a benzodiazepine and is listed as a Schedule IV medicine under the Misuse of Drugs Act 1984. Over seventy thousand tablets of this drug were seized in 2010 alone. Equally, nearly 146000 tablets of diazepam, or Valium as it is known as, were seized. Other abused prescription drugs that have been seized include Clonazepam, Flunitrazepam (Rohypnol), Lorazepam, Prazepam, Temazepam, Triazolam. Future studies on drugs in Ireland should reflect this shift in drug use.

At European level, the current major concern is the new psychoactive substances or 'legal highs' as they are known as. The EMCDDA received reports on forty one new drugs in 2010, a huge increase from twenty four substances in 2009 and thirteen in 2008. The three main categories of emerging drugs include pipazerines, synthetic cathinones and synthetic cannabinoids. They are all slightly modified versions of existing illegal drugs. The piperazines include BZP, CPP (chlorophenylpiperazine), TFMPP (1-(3-trifluoromethylphenyl) piperazine) and DBZP (1, 4-dibenzylpiperaine). Synthetic cathinones derived from Khat (*Catha edulis* Forsk), are closely related to the phenethylamine family and the most common types are mephedrone and methylone. Synthetic cannabinoids are compounds that have the same effect as cannabis but are not structurally similar to Δ^9-THC, the active ingredient in cannabis. This group of compounds include 'Spice' and twenty six other similar compounds. The major concern regarding these products is the ease at which they can be purchased and the escalating rate of their popularity. As they are new to the market, in-depth and long term studies have not yet been performed on their possible health effects.

Given the rise in seizures of BZP pills and prescription drugs, illegal drug purchases may not be conducted by traditional street deals but are more likely internet purchases. This poses problems for the law enforcement agencies, as identifying dealer networks and apprehending the main dealers is much more complex. Ghost IP addresses can be created easily by a computer enthusiast thus hiding the location of the dealer. The dealer network may be more intricate and more global. Efforts must be made not only by the analytical chemist but also the computer forensic scientist to assist the law enforcement agencies in the crackdown in internet sales of illegal drugs.

In summary this research project, has been undertaken with the support and co-operation of the Garda Forensic Science Laboratory and provided an excellent opportunity for the development and application of a liquid chromatographic method for the chemical profiling of ecstasy and cocaine drugs of abuse. From an investigative point of view linking drug seizures with chemical profiles provide a means of determining the synthetic or natural origins of the sample and provide evidence of links between seized samples or between materials found at illegal laboratories.

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Appendix: Table A1. Physical and chemical data (i.e. active & sugar components) for the 183 ecstasy tablet samples used in this study.

Sample #	Physical characteristics					Chemical data (mg/g)					
	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
1.1	Mitsubishi	White	302.3	4.5	11.5	285.1	0.0	0.0	0.0	291.8	0.0
1.2	Mitsubishi	White	285.2	4.5	11.5	337.3	0.0	0.0	0.0	234.2	0.0
1.3	Mitsubishi	White	309.7	4.5	11.5	222.1	0.0	0.0	0.0	407.7	0.0
2.1	Armani	White	313.8	4.5	10.5	259.4	0.0	0.0	0.0	430.1	0.0
2.2	Armani	White	323.2	4.5	10.5	232.9	0.0	0.0	0.0	534.6	0.0
2.3	Armani	White	327.4	4.5	10.5	182.3	0.0	0.0	0.0	444.5	0.0
3.1	Motorola	White	183.5	3.5	10.0	433.4	0.0	0.0	0.0	528.7	0.0
3.2	Motorola	White	206.9	3.5	10.0	394.5	0.0	0.0	0.0	567.0	0.0
3.3	Motorola	White	210.2	3.5	10.0	410.0	0.0	0.0	0.0	574.7	0.0
4.1	Bird	White	246.2	4.0	12.0	259.9	0.0	0.0	0.0	0.0	668.1
4.2	Bird	White	256.3	4.0	12.0	281.5	0.0	0.0	0.0	0.0	586.9
4.3	Bird	White	255.2	4.0	12.0	337.7	0.0	0.0	0.0	0.0	654.0
5.1	No logo	White	243.3	4.0	10.5	421.1	0.0	0.0	0.0	357.9	0.0
5.2	No logo	White	246.1	4.0	10.5	423.0	0.0	0.0	0.0	404.8	0.0
5.3	No logo	White	244.0	4.0	10.5	351.6	0.0	0.0	0.0	408.3	0.0
6.1	Chi	White	314.9	3.0	10.0	285.2	0.0	0.0	0.0	576.4	0.0
6.2	Chi	White	324.9	3.0	10.0	301.1	0.0	0.0	0.0	588.3	0.0
6.3	Chi	White	319.9	3.0	10.0	260.5	0.0	0.0	0.0	560.8	0.0
7.1	Bird	White	256.4	4.0	11.5	334.8	0.0	0.0	0.0	641.2	0.0
7.2	Bird	White	250.6	4.0	11.5	272.1	0.0	0.0	0.0	629.9	0.0
7.3	Bird	White	253.7	4.0	11.5	271.7	0.0	0.0	0.0	668.2	0.0

Table A1 (contd.)

Sample #	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
8.1	Bird	White	260.6	3.0	10.0	286.6	0.0	0.0	0.0	732.8	0.0
8.2	Bird	White	264.5	3.0	10.0	266.7	0.0	0.0	0.0	696.3	0.0
8.3	Bird	White	253.4	3.0	10.0	291.6	0.0	0.0	0.0	722.8	0.0
9.1	Mitsubishi	White (BS)	302.4	3.5	9.5	380.2	0.0	0.0	0.0	575.4	0.0
9.2	Mitsubishi	White (BS)	317.3	3.5	9.5	396.7	0.0	0.0	0.0	583.6	0.0
9.3	Mitsubishi	White (BS)	311.2	3.5	9.5	289.8	0.0	0.0	0.0	601.4	0.0
10.1	Crown	White	322.6	5.5	10.0	312.7	0.0	0.0	0.0	610.2	0.0
10.2	Crown	White	298.9	5.5	10.0	326.4	0.0	0.0	0.0	625.3	0.0
10.3	Crown	White	313.3	5.5	10.0	328.4	0.0	0.0	0.0	622.9	0.0
11.1	Dexter	White	370.6	4.0	10.0	297.1	0.0	0.0	0.0	589.6	0.0
11.2	Dexter	White	349.6	4.0	10.0	316.5	0.0	0.0	0.0	574.8	0.0
11.3	Dexter	White	338.7	4.0	10.0	271.6	0.0	0.0	0.0	596.3	0.0
12.1	V2	White	288.4	4.5	10.5	324.3	0.0	0.0	0.0	610.1	0.0
12.2	V2	White	296.9	4.5	10.5	355.4	0.0	0.0	0.0	624.3	0.0
12.3	V2	White	285.9	4.5	10.5	295.2	0.0	0.0	0.0	622.7	0.0
13.1	Smirnoff	White (BS)	194.4	4.0	9.0	365.3	2.1	0.0	0.0	686.7	0.0
13.2	Smirnoff	White (BS)	195.2	4.0	9.0	355.4	1.9	0.0	0.0	685.4	0.0
13.3	Smirnoff	White (BS)	183.0	4.0	9.0	387.7	1.1	0.0	0.0	673.2	0.0
14.1	Bird	Pink	336.2	5.0	9.0	70.8	0.0	14.2	0.5	0.0	0.0
14.2	Bird	Pink	328.0	5.0	9.0	97.7	0.0	18.7	0.7	0.0	0.0
14.3	Bird	Pink	336.0	5.0	9.0	76.1	0.0	15.1	0.5	0.0	0.0

Table A1 (contd.)

Sample #	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
15.1	Smirnoff	White (BS)	193.2	3.5	10.0	302.3	0.0	0.0	0.0	594.7	0.0
15.2	Smirnoff	White (BS)	193.8	3.5	10.0	339.4	0.0	0.0	0.0	461.3	0.0
15.3	Smirnoff	White (BS)	183.4	3.5	10.0	357.3	0.0	0.0	0.0	483.1	0.0
16.1	Taz Devil	White (BS)	243.5	3.5	11.5	257.0	0.0	0.0	0.0	0.0	0.0
16.2	Taz Devil	White (BS)	247.6	3.5	11.5	321.1	0.0	0.0	0.0	0.0	0.0
16.3	Taz Devil	White (BS)	246.5	3.5	11.5	306.7	0.0	0.0	0.0	0.0	0.0
17.1	Red Bull	White	194.8	4.0	10.0	331.7	0.0	0.0	6.2	369.1	0.0
17.2	Red Bull	White	200.5	4.0	10.0	419.1	0.0	0.0	7.6	366.5	0.0
17.3	Red Bull	White	196.5	4.0	10.0	359.8	0.0	0.0	7.5	355.2	0.0
18.1	Smiley	White (BS)	275.8	4.0	11.0	278.5	0.0	0.0	0.0	0.0	0.0
18.2	Smiley	White (BS)	288.3	4.0	11.0	278.3	0.0	0.0	0.0	0.0	0.0
18.3	Smiley	White (BS)	279.1	4.0	11.0	336.4	0.0	0.0	0.0	0.0	0.0
19.1	Bird	White	305.4	4.0	12.0	253.0	0.0	0.0	0.0	715.4	0.0
19.2	Bird	White	304.5	4.0	12.0	217.3	0.0	0.0	0.0	655.1	0.0
19.3	Bird	White	305.8	4.0	12.0	283.3	0.0	0.0	0.0	637.6	0.0
20.1	Euro	White	301.5	3.5	11.0	192.2	0.0	0.0	0.0	762.8	0.0
20.2	Euro	White	305.0	3.5	11.0	240.2	0.0	0.0	0.0	696.3	0.0
20.3	Euro	White	298.7	3.5	11.0	330.6	0.0	0.0	0.0	722.8	0.0
21.1	Mitsubishi	White	309.3	4.0	11.0	244.2	0.0	0.0	0.0	576.4	0.0
21.2	Mitsubishi	White	297.8	4.0	11.0	216.0	0.0	0.0	0.0	588.3	0.0
21.3	Mitsubishi	White	297.4	4.0	11.0	166.2	0.0	0.0	0.0	560.8	0.0

Table A1 (contd.)

Sample #	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
22.1	Crown	White	242.9	4.5	10.0	37.4	58.7	0.0	3.0	652.1	0.0
22.2	Crown	White	245.2	4.5	10.0	26.6	52.1	0.0	2.8	457.5	0.0
22.3	Crown	White	260.6	4.5	10.0	21.8	52.2	0.0	2.1	486.1	0.0
23.1	m&m's	White (BS)	186.7	3.5	10.0	297.2	0.0	0.0	10.7	189.0	0.0
23.2	m&m's	White (BS)	195.6	3.5	10.0	315.9	0.0	0.0	10.2	206.0	0.0
23.3	m&m's	White (BS)	188.7	3.5	10.0	259.5	0.0	0.0	11.4	205.0	0.0
24.1	m&m's	White (BS)	193.8	4.0	10.0	316.0	0.0	0.0	10.5	200.2	0.0
24.2	m&m's	White (BS)	197.1	4.0	10.0	342.4	0.0	0.0	11.6	184.1	0.0
24.3	m&m's	White (BS)	196.7	4.0	10.0	285.1	0.0	0.0	10.9	185.0	0.0
25.1	m&m's	White (BS)	192.6	3.5	10.0	291.8	0.0	0.0	9.5	624.0	0.0
25.2	m&m's	White (BS)	195.9	3.5	10.0	234.2	0.0	0.0	8.8	550.2	0.0
25.3	m&m's	White (BS)	191.2	3.5	10.0	407.7	0.0	0.0	8.5	573.2	0.0
26.1	Euro	White	285.5	4.0	12.0	280.5	0.0	0.0	0.0	0.0	737.6
26.2	Euro	White	287.2	4.0	12.0	239.3	0.0	0.0	0.0	0.0	916.0
26.3	Euro	White	289.3	4.0	12.0	208.3	0.0	0.0	0.0	0.0	807.4
27.1	Mitsubishi	White	311.9	3.5	10.5	221.2	0.0	0.0	0.0	648.2	0.0
27.2	Mitsubishi	White	309.6	3.5	10.5	173.8	0.0	0.0	0.0	666.0	0.0
27.3	Mitsubishi	White	311.0	3.5	10.5	180.1	0.0	0.0	0.0	615.7	0.0
28.1	Mitsubishi	White	301.1	3.5	11.0	229.1	0.0	0.0	0.0	613.0	0.0
28.2	Mitsubishi	White	292.0	3.5	11.0	240.2	0.0	0.0	0.0	588.3	0.0
28.3	Mitsubishi	White	304.2	3.5	11.0	187.1	0.0	0.0	0.0	567.0	0.0

Table A1 (contd.)

Sample #	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
29.1	Rolls Royce	White (BS)	206.6	5.0	8.0	357.9	0.0	0.0	0.0	395.4	0.0
29.2	Rolls Royce	White (BS)	197.4	5.0	8.0	404.8	0.0	0.0	0.0	426.5	0.0
29.3	Rolls Royce	White (BS)	203.7	5.0	8.0	408.3	0.0	0.0	0.0	462.9	0.0
30.1	Superman	Green	214.4	2.5	11.0	0.0	0.0	38.5	0.0	634.3	0.0
30.2	Superman	Green	234.4	2.5	11.0	0.0	0.0	21.3	0.0	752.5	0.0
30.3	Superman	Green	233.7	2.5	11.0	0.0	0.0	28.6	0.0	679.0	0.0
31.1	Mitsubishi	White	303.7	4.0	12.0	308.4	0.0	0.0	0.0	669.4	0.0
31.2	Mitsubishi	White	302.4	4.0	12.0	208.7	0.0	0.0	0.0	697.7	0.0
31.3	Mitsubishi	White	311.5	4.0	12.0	167.2	0.0	0.0	0.0	647.1	0.0
32.1	Bird	White	306.4	4.0	11.0	258.2	0.0	0.0	0.0	660.2	0.0
32.2	Bird	White	312.6	4.0	11.0	241.4	0.0	0.0	0.0	682.0	0.0
32.3	Bird	White	307.7	4.0	11.0	177.3	0.0	0.0	0.0	662.0	0.0
33.1	Mitsubishi	White	300.2	3.5	11.0	234.0	0.0	0.0	0.0	690.5	0.0
33.2	Mitsubishi	White	309.9	3.5	11.0	227.4	0.0	0.0	0.0	622.7	0.0
33.3	Mitsubishi	White	310.9	3.5	11.0	226.7	0.0	0.0	0.0	639.1	0.0
34.1	Mitsubishi	White	321.5	4.5	11.5	208.1	0.0	0.0	0.0	668.1	0.0
34.2	Mitsubishi	White	315.2	4.5	11.5	183.0	0.0	0.0	0.0	586.9	0.0
34.3	Mitsubishi	White	298.3	4.5	11.5	166.5	0.0	0.0	0.0	654.0	0.0
35.1	Mitsubishi	White	293.0	4.5	10.5	394.5	0.0	0.0	0.0	0.0	0.0
35.2	Mitsubishi	White	298.4	4.5	10.5	283.0	0.0	0.0	0.0	0.0	0.0
35.3	Mitsubishi	White	296.7	4.5	10.5	319.0	0.0	0.0	0.0	0.0	0.0

Table A1 (contd.)

Sample #	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
36.1	Durex	White (BS)	245.9	4.0	10.5	249.4	11.6	0.0	0.0	633.5	0.0
36.2	Durex	White (BS)	248.6	4.0	10.5	323.8	13.3	0.0	0.0	617.8	0.0
36.3	Durex	White (BS)	248.9	4.0	10.5	204.3	12.9	0.0	0.0	584.9	0.0
37.1	Mitsubishi	White	300.3	4.0	11.5	391.2	0.0	0.0	0.0	659.6	0.0
37.2	Mitsubishi	White	297.3	4.0	11.5	205.4	0.0	0.0	0.0	643.4	0.0
37.3	Mitsubishi	White	300.3	4.0	11.5	229.1	0.0	0.0	0.0	634.7	0.0
38.1	Mitsubishi	White (BS)	295.1	4.0	11.0	253.6	0.0	0.0	0.0	616.0	0.0
38.2	Mitsubishi	White (BS)	290.7	4.0	11.0	221.9	0.0	0.0	0.0	642.9	0.0
38.3	Mitsubishi	White (BS)	299.0	4.0	11.0	203.5	0.0	0.0	0.0	640.1	0.0
39.1	Red Bull	White (BS)	199.0	3.0	9.0	325.1	0.0	0.0	3.9	654.2	0.0
39.2	Red Bull	White (BS)	199.5	3.0	9.0	448.7	0.0	0.0	3.6	610.0	0.0
39.3	Red Bull	White (BS)	189.3	3.0	9.0	297.3	0.0	0.0	4.2	567.3	0.0
40.1	m&m's	White (BS)	200.3	3.5	10.0	317.9	0.0	0.0	11.2	171.2	0.0
40.2	m&m's	White (BS)	205.2	3.5	10.0	282.5	0.0	0.0	8.3	194.9	0.0
40.3	m&m's	White (BS)	190.9	3.5	10.0	197.0	0.0	0.0	8.8	183.3	0.0
41.1	m&m's	White (BS)	199.3	3.5	10.0	431.7	0.0	0.0	13.5	171.6	0.0
41.2	m&m's	White (BS)	197.3	3.5	10.0	327.7	0.0	0.0	11.9	196.6	0.0
41.3	m&m's	White (BS)	194.1	3.5	10.0	443.3	0.0	0.0	13.1	174.6	0.0
42.1	Durex	White	248.1	4.0	11.5	336.3	13.4	0.0	0.0	538.0	0.0
42.2	Durex	White	247.2	4.0	11.5	306.1	13.2	0.0	0.0	559.8	0.0
42.3	Durex	White	246.8	4.0	11.5	193.1	14.8	0.0	0.0	555.9	0.0

Table A1 (contd.)

Sample #	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
43.1	Durex	White (BS)	246.0	3.5	10.5	422.3	16.9	0.0	0.0	519.5	0.0
43.2	Durex	White (BS)	241.7	3.5	10.5	332.7	14.5	0.0	0.0	531.2	0.0
43.3	Durex	White (BS)	241.7	3.5	10.5	227.7	16.2	0.0	0.0	555.2	0.0
44.1	Mitsubishi	White	304.8	5.0	11.0	247.4	0.0	0.0	0.0	578.7	0.0
44.2	Mitsubishi	White	301.5	5.0	11.0	252.7	0.0	0.0	0.0	591.7	0.0
44.3	Mitsubishi	White	291.6	5.0	11.0	184.9	0.0	0.0	0.0	551.7	0.0
45.1	Red Bull	White (BS)	195.9	3.0	10.0	263.1	0.0	0.0	3.6	573.2	0.0
45.2	Red Bull	White (BS)	187.3	3.0	10.0	326.6	0.0	0.0	3.3	547.7	0.0
45.3	Red Bull	White (BS)	190.8	3.0	10.0	269.5	0.0	0.0	2.8	571.8	0.0
46.1	Smirnoff	White (BS)	195.7	3.0	10.0	408.4	0.0	0.0	0.0	482.8	0.0
46.2	Smirnoff	White (BS)	192.9	3.0	10.0	264.9	0.0	0.0	0.0	469.6	0.0
46.3	Smirnoff	White (BS)	191.8	3.0	10.0	327.1	0.0	0.0	0.0	507.8	0.0
47.1	Mitsubishi	White	303.4	4.0	11.5	198.5	0.0	0.0	0.0	652.6	0.0
47.2	Mitsubishi	White	287.3	4.0	11.5	294.3	0.0	0.0	0.0	635.0	0.0
47.3	Mitsubishi	White	295.5	4.0	11.5	176.2	0.0	0.0	0.0	633.5	0.0
48.1	Coca-Cola	White (BS)	202.9	3.0	10.0	294.1	4.8	0.0	5.0	413.5	0.0
48.2	Coca-Cola	White (BS)	193.4	3.0	10.0	359.9	4.9	0.0	5.8	453.9	0.0
48.3	Coca-Cola	White (BS)	208.7	3.0	10.0	238.1	4.4	0.0	5.9	448.0	0.0
49.1	Coca-Cola	White (BS)	186.8	3.5	9.5	352.5	4.9	0.0	4.6	367.2	0.0
49.2	Coca-Cola	White (BS)	191.5	3.5	9.5	318.2	4.2	0.0	4.6	430.2	0.0
49.3	Coca-Cola	White (BS)	185.8	3.5	9.5	308.5	4.3	0.0	4.6	423.5	0.0

Table A1 (contd.)

Sample #	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
50.1	Mitsubishi	White	295.8	4.0	11.5	268.7	0.0	0.0	0.0	528.7	0.0
50.2	Mitsubishi	White	296.8	4.0	11.5	265.3	0.0	0.0	0.0	567.0	0.0
50.3	Mitsubishi	White	287.8	4.0	11.5	235.8	0.0	0.0	0.0	574.7	0.0
51.1	Smiley	White	198.4	4.5	8.5	455.9	0.0	0.0	0.0	418.3	0.0
51.2	Smiley	White	196.8	4.5	8.5	410.2	0.0	0.0	0.0	436.4	0.0
51.3	Smiley	White	200.7	4.5	8.5	529.9	0.0	0.0	0.0	415.5	0.0
52.1	Coca-Cola	White (BS)	199.0	3.0	10.0	385.4	0.0	0.0	11.4	503.5	0.0
52.2	Coca-Cola	White (BS)	196.6	3.0	10.0	439.5	0.0	0.0	18.9	490.8	0.0
52.3	Coca-Cola	White (BS)	203.0	3.0	10.0	321.6	0.0	0.0	13.2	491.1	0.0
53.1	Crown	White	253.6	5.0	10.0	29.1	36.2	0.0	3.5	421.1	0.0
53.2	Crown	White	256.7	5.0	10.0	27.9	42.1	0.0	2.5	434.7	0.0
53.3	Crown	White	253.8	5.0	10.0	22.3	38.1	0.0	3.0	437.3	0.0
54.1	Heart	White (BS)	279.2	6.0	10.0	306.3	0.0	0.0	0.0	469.2	0.0
54.2	Heart	White (BS)	273.5	6.0	10.0	289.2	0.0	0.0	0.0	410.0	0.0
54.3	Heart	White (BS)	289.9	6.0	10.0	362.2	0.0	0.0	0.0	468.7	0.0
55.1	Heart	Pink	269.2	5.5	10.0	209.9	0.0	0.0	0.0	324.2	268.2
55.2	Heart	Pink	278.7	5.5	10.0	301.5	0.0	0.0	0.0	385.3	257.9
55.3	Heart	Pink	282.8	5.5	10.0	245.3	0.0	0.0	0.0	327.4	305.1
56.1	Horse	White (BS)	251.0	4.0	10.0	261.4	0.0	0.0	0.0	454.5	0.0
56.2	Horse	White (BS)	249.8	4.0	10.0	278.4	0.0	0.0	0.0	430.3	0.0
56.3	Horse	White (BS)	253.1	4.0	10.0	235.8	0.0	0.0	0.0	459.1	0.0

Table A1 (contd.)

Sample #	Logo	Colour	Weight (mg)	Thickness (mm)	Diameter (mm)	MDMA	MDEA	Amp	Caffeine	Lactose	Sorbitol
57.1	Horse	White	248.3	4.5	10.5	383.7	0.0	0.0	0.0	430.1	0.0
57.2	Horse	White	242.6	4.5	10.5	170.9	0.0	0.0	0.0	534.6	0.0
57.3	Horse	White	245.2	4.5	10.5	407.2	0.0	0.0	0.0	444.5	0.0
58.1	Euro	White (BS)	290.9	3.5	12.0	216.6	0.0	0.0	0.0	0.0	0.0
58.2	Euro	White (BS)	294.1	3.5	12.0	158.6	0.0	0.0	0.0	0.0	0.0
58.3	Euro	White (BS)	286.1	3.5	12.0	244.7	0.0	0.0	0.0	0.0	0.0
59.1	Mitsubishi	White	296.0	3.0	9.0	304.1	0.0	0.0	0.0	568.5	0.0
59.2	Mitsubishi	White	304.0	3.0	9.0	246.1	0.0	0.0	0.0	577.6	0.0
59.3	Mitsubishi	White	277.9	3.0	9.0	205.9	0.0	0.0	0.0	517.0	0.0
60.1	Heart	White (BS)	294.1	5.5	8.0	255.4	0.0	0.0	0.0	0.0	351.1
60.2	Heart	White (BS)	257.8	5.5	8.0	265.9	0.0	0.0	0.0	0.0	372.1
60.3	Heart	White (BS)	257.1	5.5	8.0	252.1	0.0	0.0	0.0	0.0	515.7
61.1	Heart	White (BS)	294.9	5.5	8.0	344.4	0.0	0.0	0.0	0.0	287.9
61.2	Heart	White (BS)	273.9	5.5	8.0	291.0	0.0	0.0	0.0	0.0	316.2
61.3	Heart	White (BS)	295.7	5.5	8.0	341.5	0.0	0.0	0.0	0.0	391.6
Mean	-	-	260.39	3.98	10.4	280.42	18.28	22.73	7.01	525.49	495.72
Min	-	-	183	2.5	8.0	21.8	1.1	14.2	0.5	171.2	257.9
Max	-	-	370.6	6	12.0	529.9	58.7	38.5	18.9	762.8	916.0
SD	-	-	47.40	0.74	0.98	89.54	17.87	9.29	4.29	141.44	217.59
% Occur.	-	-	-	-	-	98.36	13.11	3.28	22.95	85.24	8.19

White (BS) = white with brown speckles

Appendix: Table A2. Metal data for the 183 ecstasy samples used in this study.

Sample #	Metal concentration (µg/g)								
	Al	Zn	Fe	Mg	Ca	Cr	Pb	Na	K
1.1	17.739	5.757	23.313	0.209	43.350	0.000	0.000	0.080	0.055
1.2	22.120	8.875	24.094	0.803	78.339	0.000	0.000	0.019	0.032
1.3	24.175	6.611	18.474	0.329	54.640	0.000	0.000	0.054	0.044
2.1	44.141	40.672	79.253	2.957	189.602	0.000	0.000	0.131	0.113
2.2	47.524	45.939	89.699	1.563	198.492	0.000	0.000	0.118	0.156
2.3	41.713	26.653	81.044	4.080	211.196	0.000	0.000	0.125	0.125
3.1	70.508	3.060	36.377	1.497	353.774	0.446	0.000	0.000	0.091
3.2	58.984	2.229	27.119	1.170	397.924	0.330	0.000	0.000	0.086
3.3	74.896	1.694	37.159	1.203	409.574	0.803	0.000	0.000	0.056
4.1	185.045	12.304	25.000	1.092	287.169	0.000	0.000	0.113	0.160
4.2	204.036	18.213	29.848	1.199	354.354	0.000	0.000	0.182	0.128
4.3	174.969	21.559	40.295	1.281	293.900	0.000	0.000	0.127	0.194
5.1	14.963	18.019	67.943	0.533	214.765	0.000	0.000	0.131	0.135
5.2	12.421	24.649	60.663	0.492	189.785	0.000	0.000	0.118	0.109
5.3	13.129	32.474	64.203	0.858	143.235	0.000	0.000	0.125	0.128
6.1	25.708	2.455	55.328	0.669	252.174	0.000	1.241	0.127	0.206
6.2	43.350	2.803	62.763	0.463	287.169	0.000	1.083	0.092	0.176
6.3	25.140	2.739	61.163	0.395	330.827	0.000	0.430	0.046	0.190
7.1	47.814	1.186	6.901	1.322	79.253	0.000	0.000	0.099	0.197
7.2	43.587	1.110	10.763	1.241	89.699	0.000	0.000	0.129	0.183
7.3	28.659	1.170	4.576	1.204	63.409	0.000	0.000	0.118	0.229
8.1	78.339	16.960	30.208	1.694	174.969	0.621	0.000	0.140	0.220
8.2	54.260	18.694	28.022	1.556	245.739	0.571	0.000	0.155	0.206
8.3	54.640	15.159	48.593	1.766	185.045	1.886	0.000	0.242	0.176
9.1	29.236	9.736	4.435	0.755	89.699	0.000	0.000	0.040	0.148
9.2	30.349	8.289	7.196	0.803	70.508	0.000	0.000	0.078	0.162
9.3	20.833	8.333	6.894	0.501	56.398	0.000	0.000	0.098	0.142
10.1	2.572	4.580	5.068	0.162	121.615	0.000	0.000	0.073	0.127
10.2	4.065	4.435	4.576	0.250	109.307	0.000	0.000	0.096	0.142
10.3	2.079	3.740	2.337	0.305	150.187	0.000	0.000	0.114	0.150
11.1	39.441	16.527	22.241	0.190	98.908	0.572	0.000	1.700	0.164
11.2	62.948	23.318	28.571	0.140	105.981	0.195	0.000	1.556	0.107
11.3	40.295	25.140	41.763	0.155	140.533	0.670	0.000	1.856	0.187

Table A2 (contd.)

Sample #	Al	Zn	Fe	Mg	Ca	Cr	Pb	Na	K
12.1	13.875	40.672	61.399	0.319	170.257	0.000	0.000	0.236	0.114
12.2	16.041	45.939	58.273	0.233	220.414	0.000	0.000	0.180	0.160
12.3	17.601	26.653	71.674	0.252	234.198	0.000	0.000	0.223	0.194
13.1	44.141	9.974	121.615	1.224	122.715	0.276	9.571	0.169	0.156
13.2	79.253	6.057	402.062	2.139	153.191	0.756	6.955	0.232	0.156
13.3	47.524	8.939	49.764	1.262	141.161	0.211	3.641	0.189	0.142
14.1	71.009	78.756	18.662	1.948	24.739	0.000	0.433	3.052	0.141
14.2	74.074	92.063	30.952	1.958	21.264	0.000	0.177	3.188	0.146
14.3	89.699	80.324	28.935	1.933	24.063	0.000	0.615	3.067	0.116
15.1	41.713	7.196	30.387	1.478	205.374	0.000	0.271	0.207	0.180
15.2	30.208	6.273	18.750	0.891	142.066	0.000	0.242	0.220	0.162
15.3	67.720	7.239	83.791	1.566	216.319	0.000	0.356	0.234	0.165
16.1	28.022	7.706	19.918	1.277	165.775	0.000	0.000	0.137	0.110
16.2	81.044	7.940	24.863	1.250	157.439	0.000	0.000	0.206	0.124
16.3	55.288	8.113	19.231	1.262	155.556	0.000	0.000	0.240	0.096
17.1	48.593	6.894	109.307	0.974	141.287	0.064	0.864	0.173	0.162
17.2	36.099	6.207	45.905	1.034	166.667	0.067	0.648	0.280	0.162
17.3	74.500	12.150	52.833	1.083	89.163	0.130	0.755	0.383	0.250
18.1	70.508	12.168	26.367	0.898	73.836	1.242	0.000	0.176	0.215
18.2	32.309	5.932	71.186	1.081	182.143	1.471	0.000	0.148	0.127
18.3	56.398	9.348	33.175	1.090	116.603	3.506	0.000	0.190	0.142
19.1	58.984	10.176	30.078	0.430	42.226	0.000	0.000	0.098	0.215
19.2	22.120	6.901	4.804	0.438	63.439	0.000	0.000	0.035	0.150
19.3	74.896	6.100	16.701	0.456	74.830	0.000	0.000	0.093	0.124
20.1	245.739	15.341	57.528	1.932	125.926	0.000	0.000	0.057	0.142
20.2	237.854	5.051	62.652	1.447	286.573	0.000	0.000	0.051	0.132
20.3	310.976	9.736	64.837	2.622	269.311	0.000	0.000	0.142	0.305
21.1	72.932	8.289	30.827	0.432	52.154	0.000	0.000	0.094	0.150
21.2	47.842	12.788	40.647	0.432	33.755	0.000	0.000	0.108	0.126
21.3	185.045	15.893	62.277	0.491	30.899	0.000	0.000	0.156	0.201
22.1	36.094	12.031	43.906	1.844	153.247	0.000	0.306	1.688	0.141
22.2	63.667	8.333	49.167	1.017	122.000	0.000	0.000	2.467	0.133
22.3	51.796	9.296	36.377	2.201	236.715	0.000	0.000	2.335	0.135

Table A2 (contd.)

Sample #	Al	Zn	Fe	Mg	Ca	Cr	Pb	Na	K
23.1	51.093	6.639	79.098	1.844	277.778	0.683	0.000	0.082	0.109
23.2	52.821	0.000	69.615	1.731	0.000	0.000	0.000	0.103	0.128
23.3	42.284	0.000	92.284	1.790	0.000	0.000	0.000	0.170	0.123
24.1	77.181	6.628	16.527	0.034	5.063	0.356	0.000	0.067	0.168
24.2	94.175	5.649	12.500	0.035	6.263	0.245	0.000	0.035	0.106
24.3	123.517	10.763	27.119	1.186	10.236	0.621	0.000	0.127	0.233
25.1	104.036	7.354	23.318	1.110	150.915	0.460	0.446	0.157	0.179
25.2	83.283	4.292	23.069	1.170	272.500	0.369	0.426	0.064	0.161
25.3	94.796	11.803	18.401	1.180	100.000	0.571	1.140	0.074	0.232
26.1	205.556	4.944	76.806	1.597	323.034	0.000	0.000	0.028	0.181
26.2	248.188	5.797	104.348	1.993	343.750	0.000	0.000	0.018	0.145
26.3	266.129	6.624	59.101	1.671	252.174	0.036	0.000	0.035	0.115
27.1	47.739	1.411	18.568	0.467	330.882	0.000	0.000	0.052	0.124
27.2	63.409	1.466	37.159	0.455	310.078	0.000	0.000	0.068	0.114
27.3	68.921	0.558	22.120	0.458	821.596	0.000	0.000	0.092	0.170
28.1	2.712	5.094	25.708	0.542	106.481	0.000	0.000	0.000	0.189
28.2	7.685	0.825	43.350	0.468	567.164	0.000	0.000	0.000	0.111
28.3	1.439	0.000	25.140	0.517	0.000	0.000	0.000	0.000	0.140
29.1	40.672	4.580	44.403	1.315	287.169	1.886	1.172	0.345	0.326
29.2	45.939	4.226	54.569	1.497	354.354	0.863	0.000	0.406	0.190
29.3	26.653	3.130	49.690	1.405	448.845	0.114	0.622	0.331	0.134
30.1	55.328	5.068	47.814	0.533	105.121	0.119	0.183	0.178	0.314
30.2	31.630	4.435	43.587	0.435	98.039	0.108	0.330	0.174	0.391
30.3	36.125	1.186	28.659	0.434	365.714	0.156	0.209	0.081	0.190
31.1	15.289	3.740	12.328	0.475	127.072	0.000	0.025	0.083	0.193
31.2	29.073	5.056	45.787	0.478	94.444	0.000	0.000	0.197	0.281
31.3	21.904	4.576	35.780	0.528	115.288	0.000	0.000	0.183	0.149
32.1	16.960	6.222	49.670	0.551	88.496	0.000	0.000	0.055	0.121
32.2	18.694	2.337	25.000	0.490	209.524	0.000	0.803	0.045	0.096
32.3	15.159	2.595	28.889	0.492	189.602	0.000	0.127	0.032	0.190
33.1	23.039	3.060	33.403	0.448	146.453	0.000	0.020	0.056	0.091
33.2	15.378	2.229	28.536	0.428	191.882	0.000	0.000	0.058	0.090
33.3	10.768	1.694	18.019	0.433	255.591	0.000	0.000	0.060	0.092

Table A2 (contd.)

Sample #	Al	Zn	Fe	Mg	Ca	Cr	Pb	Na	K
34.1	14.912	3.000	24.649	0.439	146.199	0.000	0.000	0.044	0.132
34.2	14.820	2.249	32.474	0.432	191.977	0.000	0.000	0.039	0.097
34.3	10.575	1.920	22.241	0.483	251.497	0.000	0.000	0.040	0.103
35.1	12.234	3.537	32.979	1.170	330.827	0.000	0.000	0.594	0.080
35.2	16.823	4.164	28.571	1.203	288.939	0.000	0.000	0.536	0.019
35.3	17.176	3.975	24.910	1.196	300.905	0.000	0.000	0.755	0.054
36.1	29.789	5.757	48.659	1.140	198.003	0.000	0.000	0.546	0.000
36.2	22.448	8.875	41.763	0.969	109.150	0.000	0.329	0.122	0.116
36.3	30.095	6.611	67.417	1.173	177.419	0.000	0.000	0.166	0.320
37.1	62.763	4.287	66.517	0.511	119.089	0.000	0.041	0.113	0.143
37.2	53.416	2.605	45.419	0.517	198.492	0.000	0.000	0.111	0.131
37.3	43.115	15.082	151.639	0.533	35.326	0.000	0.501	0.156	0.131
38.1	12.304	2.572	38.613	0.543	211.196	0.000	0.000	0.098	0.118
38.2	18.213	4.065	29.848	0.533	131.175	0.000	0.000	0.125	0.125
38.3	21.559	2.079	30.829	0.527	253.378	0.000	0.000	0.091	0.316
39.1	32.463	3.750	150.187	0.858	228.856	0.000	0.000	0.121	0.140
39.2	46.455	3.945	165.455	0.900	228.111	0.000	0.573	0.136	0.155
39.3	59.662	5.145	99.034	1.486	288.732	0.000	0.000	0.229	0.242
40.1	25.278	5.889	65.972	2.139	363.208	0.000	0.000	0.125	0.319
40.2	15.855	1.556	80.517	0.885	568.690	0.000	0.000	0.055	0.065
40.3	30.037	4.304	140.110	1.511	351.064	0.000	0.819	0.046	0.128
41.1	29.656	6.457	63.462	0.030	4.702	0.000	4.816	0.192	0.121
41.2	25.268	3.795	64.375	0.036	9.412	0.000	2.069	0.161	0.732
41.3	29.911	16.815	81.845	0.060	3.540	0.000	2.999	0.327	0.134
42.1	24.007	6.570	78.339	1.155	175.824	0.000	0.000	0.099	0.063
42.2	23.071	7.974	54.260	1.166	146.169	0.000	0.000	0.129	0.105
42.3	20.914	5.042	54.640	0.963	190.934	0.000	0.000	0.118	0.111
43.1	19.788	6.384	16.694	1.148	179.847	0.000	1.202	0.114	0.122
43.2	31.579	9.487	102.895	1.908	201.110	0.000	3.087	0.197	0.184
43.3	18.536	4.914	45.249	1.114	226.624	0.000	1.332	0.086	0.164
44.1	8.761	1.766	49.217	0.506	286.290	0.000	0.000	0.028	0.107
44.2	6.460	1.510	55.769	0.534	353.774	0.000	0.224	0.036	0.064
44.3	7.078	1.813	29.063	0.555	306.034	0.000	0.000	0.031	0.188

Table A2 (contd.)

Sample #	Al	Zn	Fe	Mg	Ca	Cr	Pb	Na	K
45.1	13.846	1.853	98.718	0.737	397.924	0.000	0.000	0.032	0.077
45.2	10.505	2.093	131.107	1.050	501.946	0.000	0.000	0.024	0.187
45.3	9.595	2.482	98.908	1.092	439.716	0.000	0.000	0.044	0.220
46.1	14.963	2.835	105.981	1.199	422.951	0.000	0.807	0.056	0.260
46.2	12.421	1.714	140.533	1.281	747.706	0.000	0.000	0.063	0.181
46.3	13.129	3.255	58.273	1.322	406.077	0.000	0.000	0.081	0.189
47.1	8.798	3.176	71.674	0.494	155.405	0.000	0.000	0.236	0.376
47.2	7.765	1.259	49.811	0.464	368.421	0.000	0.000	0.180	0.862
47.3	6.781	3.724	65.411	0.916	245.977	0.000	0.000	0.223	0.214
48.1	18.426	3.307	36.853	1.285	388.554	0.000	0.000	0.966	0.319
48.2	10.310	2.298	48.450	0.964	419.355	0.000	0.000	0.276	0.263
48.3	12.135	3.659	93.978	1.241	339.152	0.000	0.000	0.447	0.319
49.1	8.488	1.728	49.614	1.204	696.429	0.000	0.772	0.448	0.370
49.2	5.870	1.883	68.927	1.083	575.269	0.000	0.000	0.294	0.233
49.3	27.606	0.344	56.270	0.977	2836.879	0.000	0.000	0.326	0.252
50.1	63.012	0.730	85.041	0.430	589.888	0.000	0.000	0.000	0.143
50.2	29.288	0.836	53.779	0.480	573.913	0.000	0.000	0.000	0.073
50.3	12.410	0.000	209.532	0.432	0.000	0.000	0.000	0.000	0.099
51.1	19.206	2.017	68.348	0.826	409.574	0.000	0.000	0.086	0.204
51.2	13.011	1.465	39.441	0.572	390.698	0.000	0.000	0.061	0.102
51.3	25.326	6.270	62.948	0.806	128.571	0.000	0.000	0.090	0.138
52.1	40.823	2.215	40.295	1.350	609.524	0.000	0.000	0.232	0.570
52.2	30.825	2.597	98.908	1.468	565.421	0.000	0.000	0.303	0.279
52.3	41.627	2.225	105.981	1.220	548.387	0.000	0.000	0.311	0.251
53.1	37.796	2.959	140.533	1.324	447.500	0.000	0.000	1.324	0.111
53.2	45.741	5.009	56.759	1.472	293.900	0.000	0.000	1.370	0.093
53.3	4.437	4.340	22.565	1.028	236.908	0.000	0.000	1.315	0.070
54.1	5.125	0.569	13.875	0.400	702.525	0.000	0.000	0.000	0.056
54.2	4.794	1.241	16.041	0.333	268.293	0.000	0.000	0.000	0.073
54.3	13.407	2.263	51.814	0.670	296.167	0.000	0.000	0.000	0.103
55.1	29.639	1.045	94.459	0.799	764.488	0.000	0.000	0.876	0.155
55.2	12.885	0.959	191.176	0.973	1014.599	0.000	0.000	1.085	0.112
55.3	16.507	3.050	67.943	0.766	250.980	0.000	0.000	2.022	0.203

Table A2 (contd.)

Sample #	Al	Zn	Fe	Mg	Ca	Cr	Pb	Na	K
56.1	12.896	1.960	60.663	0.706	360.294	0.000	0.000	0.029	0.115
56.2	13.841	4.609	64.203	0.725	157.233	0.000	0.000	0.043	0.109
56.3	15.644	1.700	38.531	0.669	393.491	0.000	0.000	0.040	0.096
57.1	23.313	3.036	19.578	0.536	176.587	0.000	0.000	0.078	0.114
57.2	15.564	1.556	21.201	0.551	354.331	0.000	0.000	0.098	0.092
57.3	24.110	1.856	39.110	0.598	322.314	0.000	0.000	0.107	0.123
58.1	14.236	2.813	29.236	1.229	437.037	0.000	0.000	0.160	0.479
58.2	61.163	4.419	30.349	1.698	384.211	0.000	0.000	0.198	0.558
58.3	12.639	3.028	20.833	1.257	415.138	0.000	0.000	0.125	0.486
59.1	24.094	1.775	9.894	0.491	276.596	0.000	0.000	0.128	0.113
59.2	39.806	4.005	18.689	0.534	133.333	0.000	0.000	0.194	0.182
59.3	21.866	2.455	8.507	0.463	188.450	0.000	0.000	0.142	0.127
60.1	18.618	2.803	18.618	0.355	126.761	0.000	0.000	1.158	0.092
60.2	18.474	2.739	28.952	0.588	214.765	0.000	0.000	1.994	0.046
60.3	31.818	1.188	30.861	0.395	332.326	0.000	0.000	2.285	0.084
61.1	23.295	0.795	21.429	0.292	367.722	0.000	0.000	1.047	0.057
61.2	33.556	0.691	35.333	0.311	450.161	0.000	0.000	2.167	0.067
61.3	14.603	0.901	9.675	0.325	360.000	0.000	0.000	0.980	0.030
Mean	43.945	8.204	52.013	0.932	262.473	0.636	1.225	0.360	0.168
Min	1.439	0.344	2.337	0.030	3.540	0.036	0.020	0.018	0.019
Max	310.976	92.063	402.062	4.080	2836.879	3.506	9.571	3.188	0.862
SD	50.531	12.609	44.253	0.591	258.889	0.714	1.892	0.617	0.110
% Occur.	100	97.8	100	100	98.4	17.5	23	93.4	99.5