Statement of Authenticity

I certify that except where due acknowledgement has been made, the work is that of the author alone; neither the thesis nor the original work contained therein has previously been submitted to any Institution for a higher degree; the content of this thesis is the result of work which has been carried out since the official commencement date of the approved research program; and any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Blagoj S. Mitrevski
I dedicate this thesis to my daughter Elena, my little princess who was 5 days old only when I started this project.
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### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>1D GC</td>
<td>(conventional) one-dimensional gas chromatography</td>
</tr>
<tr>
<td>2D GC</td>
<td>two-dimensional gas chromatography</td>
</tr>
<tr>
<td>3,4-MDP-2-P</td>
<td>3,4-methylenedioxyphenyl-2-propanone</td>
</tr>
<tr>
<td>6-MAM</td>
<td>6-monoacetyl morphine</td>
</tr>
<tr>
<td>AA</td>
<td>anabolic agents</td>
</tr>
<tr>
<td>AAS</td>
<td>atomic absorption spectroscopy</td>
</tr>
<tr>
<td>AS</td>
<td>anabolic (androgenic) steroids</td>
</tr>
<tr>
<td>ANN</td>
<td>artificial neural network</td>
</tr>
<tr>
<td>BSTFA</td>
<td>$N,O$-bis(trimethylsilyl)trifluoroacetamide</td>
</tr>
<tr>
<td>CCC</td>
<td>$cis$-cinnamoylcocaine</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CI</td>
<td>chemical ionization</td>
</tr>
<tr>
<td>CID</td>
<td>collision induced dissociation</td>
</tr>
<tr>
<td>DEA</td>
<td>Drug Enforcement Administration</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DVB/PDMS</td>
<td>divinylbenzene/polydimethylsiloxane</td>
</tr>
<tr>
<td>ECD</td>
<td>electron capture detector</td>
</tr>
<tr>
<td>EMD</td>
<td>epimethendiol</td>
</tr>
<tr>
<td>EME</td>
<td>ecgonine methyl ester</td>
</tr>
<tr>
<td>FID</td>
<td>flame ionization detector</td>
</tr>
<tr>
<td>FPD</td>
<td>flame photometric detector</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infra red</td>
</tr>
<tr>
<td>GC×GC</td>
<td>comprehensive two-dimensional gas chromatography</td>
</tr>
<tr>
<td>GCcIRMS</td>
<td>gas chromatography combustion isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>GC-EAIRMS</td>
<td>gas chromatography-elemental analysis isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>GC-qMS</td>
<td>gas chromatography-quadrupole mass spectrometry</td>
</tr>
<tr>
<td>HCA</td>
<td>hierarchical cluster analysis</td>
</tr>
<tr>
<td>hGH</td>
<td>human Growth Hormone</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
<tr>
<td>HS</td>
<td>head space</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>inductively coupled plasma-atomic emission spectroscopy</td>
</tr>
<tr>
<td>IRMS</td>
<td>Isotope-ratio mass spectrometry</td>
</tr>
<tr>
<td>IS</td>
<td>internal standard</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LLE</td>
<td>liquid-liquid extraction</td>
</tr>
<tr>
<td>LMCS</td>
<td>longitudinal modulation cryogenic system</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>LOD</td>
<td>limit of detection</td>
</tr>
<tr>
<td>M1</td>
<td>17α-methyl-5α-androstane-3α,17β-diol (metabolite 1)</td>
</tr>
<tr>
<td>M2</td>
<td>17α-methyl-5β-androstane-3α,17β-diol (metabolite 2)</td>
</tr>
<tr>
<td>MAM</td>
<td>minimum acceptable match</td>
</tr>
<tr>
<td>MDA</td>
<td>3,4-methylenedioxyamphetamine</td>
</tr>
<tr>
<td>MDMA</td>
<td>3,4-methylenedioxyamphetamine</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>MSTFA</td>
<td>N-methyl-N-(trimethylsilyl)-trifluoroacetamide</td>
</tr>
<tr>
<td>MTBE</td>
<td>methyl tert-butyl ether</td>
</tr>
<tr>
<td>MTWRD</td>
<td>maximum tolerance window of relative difference</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPD</td>
<td>nitrogen-phosphorus detector</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PDMS/DVB</td>
<td>polydimethylsiloxane/divinylbenzene</td>
</tr>
<tr>
<td>PMK</td>
<td>piperonyl methyl ketone</td>
</tr>
<tr>
<td>qMS</td>
<td>quadrupole mass spectrometry</td>
</tr>
<tr>
<td>RMM</td>
<td>relative molecular mass</td>
</tr>
<tr>
<td>S/N</td>
<td>signal-to-noise ratio</td>
</tr>
<tr>
<td>SA</td>
<td>South America</td>
</tr>
<tr>
<td>SEA</td>
<td>Southeast Asia</td>
</tr>
<tr>
<td>SIM</td>
<td>selected ion monitoring</td>
</tr>
<tr>
<td>SNIF-NMR</td>
<td>site specific natural isotope-ratio fractionation - deuterium nuclear magnetic resonance</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>SPME</td>
<td>solid phase microextraction</td>
</tr>
<tr>
<td>STRL</td>
<td>Special Testing and Research Laboratory</td>
</tr>
<tr>
<td>SWA</td>
<td>Southwest Asia</td>
</tr>
<tr>
<td>T/E</td>
<td>testosterone/epitestosterone ratio</td>
</tr>
<tr>
<td>TCC</td>
<td>trans-cinnamoylcocaine</td>
</tr>
<tr>
<td>TFAA</td>
<td>trifluoroacetic anhydride</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion current</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>TOFMS</td>
<td>time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>TPC</td>
<td>tropacocaine</td>
</tr>
<tr>
<td>UNODC</td>
<td>United Nations Office on Drug and Crimes</td>
</tr>
<tr>
<td>UPC</td>
<td>urine positive control</td>
</tr>
<tr>
<td>UPLC-MS/MS</td>
<td>ultra performance liquid chromatography – tandem mass spectrometry</td>
</tr>
<tr>
<td>WADA</td>
<td>World Anti-Doping Agency</td>
</tr>
<tr>
<td>WDR</td>
<td>world drug report</td>
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Synopsis

It is almost 10 years since the famous athlete Marion Jones won 5 medals at the Sydney Olympics 2000, a success now known to have been highly contributed to by performance-enhancing drugs. She was not the first athlete to win so many medals at one Olympic game, but probably she was the first top athlete to plead guilty whilst never having been caught by anti doping authorities. In December 2007 the International Olympic Committee stripped her of the medals won in Sydney, and she was banned from the Beijing Olympics in 2008 in any capacity. Interestingly, 10 years after this case the screening for anabolic agents and stimulants in sports still relies on classical gas chromatographic – mass spectrometry (GC-MS) analysis, the “transparency” of which was the major cause of this failure to catch Jones.

Gas chromatographic analysis has been continuously improving since its introduction in 1950s. The two milestones in this technological progress were the introduction of capillary columns for the separation part and mass spectrometry for the detection part of the analysis. Capillary GC-MS was soon established as a golden standard in many areas of analysis, dominating the analytical methodologies up to today. Improvement has not stopped, but continues apace. Probably the next milestone, along with the improvement in MS technology, is the introduction of multidimensional separations, i.e. heart-cut GC and more recently its comprehensive two-dimensional GC counterpart. This thesis represents an original work in which the applicability of comprehensive two-dimensional gas chromatography (GC×GC) in performance enhancing and illicit drug analysis has been investigated.

The thesis covers a diverse range of analytes (endogenous sterols, anabolic agents, ecstasy, heroin, cocaine), matrices (urine, illicit preparations), sample preparation techniques (solid phase micro extraction, solid phase extraction, derivatization), separation and detection approaches (one dimensional GC, two-dimensional GC, quadrupole MS, fast time-of-flight
MS, accurate mass TOFMS, flame photometric detection). The scope of the thesis justifies the extensive comprehensive introductory part given in Chapter 1, where a major focus is devoted chronologically on developments in profiling and analysis methods usually applied in forensic and anti-doping laboratories.

Chapter 2 provides details on the reagents and samples used in the study as well as the sample preparation steps applied prior to analysis. While some general instrumental approaches are given in this chapter, the details on the specific experimental set up for each study are included in the subsequent chapters.

The emerging need for better separation has arisen from the limitation of conventional one dimensional (one column) GC separation, which was unable to solve the perpetual problem where coelution causes imprecise or erroneous analytical results. This becomes especially true in cases where detection and unambiguous identification of ever smaller amounts of analytes in the presence of a complex matrix becomes mandatory due to lower required detection limits. As the ratio between the analyte and the matrix changes in favour of the latter, the use and relevance of highly selective detectors has diminished. An alternative and logical approach to solve the problem is to improve separation instead of resorting to altering detector selectivity. This subject has been discussed in Chapter 3, where special focus has been given to a literature review on application of multidimensional separations in performance enhancing and illicit drug analysis, supported by several examples arising from the work in this thesis. A clear in-depth classification of multidimensional GC techniques, based on multidimensionality arising from separation and multidimensionality arising from detection, has been given for the first time.

The endogenous sterols profile has become an important part of every top athlete’s “biological passport” and increased importance has been given to this baseline measurement in the last several years. The applicability of GC×GC-TOFMS in this area has been discussed in Chapter 4. The synergic effect of the highly selective TOFMS detector
and improved separation of GC×GC has been shown as particularly advantageous over classical GC in terms of net information content and component identification. A new metric named the minimum acceptable match (MAM) has been proposed for the first time as an additional criterion when defining the limit of detection (LOD). The new LOD definition incorporates not only a reproducible signal magnitude (a simple single number) but also a “named” signal whose identity is based on full mass spectral similarity to a specific reference component.

Key World Anti-Doping Agency (WADA) anabolic agents, the most difficult to analyse by using GC, have been chosen as model components of exogenous doping substances. The application of GC×GC in this area has been discussed in Chapter 5. One of the most important parameters in separation of anabolic agents in urine samples has been shown to be the column set. Better separation followed by improved identification of anabolic agents has been obtained by using a thicker film on the 2D column. The poor similarity of GC×GC-TOFMS experimental spectra against commercial MS databases has been ascribed to the bias of TOFMS detector towards higher masses. Therefore it was necessary to create an in-house TOFMS based library of anabolic agents and sterols which has greatly improved spectra match similarity, and further improved the limit of detection when the MAM criterion was applied. Other benefits of the proposed methods are detection and identification of non-target components and retaining the full mass spectra of the matrix. This could be very beneficial to doping control authorities since it enables retrospective search for new “designer” drugs through data files only, without need for sample re-analysis.

The World Anti-Doping Agency has set up rigorous criteria for minimum required performance limit (MRPL) of the method applied in doping control and for identification of anabolic agents in urine. Chapter 6 focuses on unique evaluation of the GC×GC format, for results obtained in Chapter 5 against the strict WADA criteria in doping control. Retention time tolerance, relative abundance tolerance window, presence of abundant isotopes (Cl, Br), presence of diagnostic ions, linearity and signal-to-noise ratio of data in
GC×GC format are shown here to largely comply with WADA criteria. The TOFMS bias toward higher masses has been found to have both advantages and disadvantages compared to the traditional GC-qMS analysis. The lack of at least three diagnostic ions with abundance higher than 5% in TOFMS spectra has been compensated by the high spectral similarity against the in-house library. Many more advantages of GC×GC over GC have been discussed in details.

Illicit drug profiling is another area where the applicability of GC×GC should have obvious advantages. The pitfalls of classical GC separations in this area, reported in several occasions in the literature, have been overcome by improvement in the separation phase of the process rather than by making the detection system more “blind” (i.e. more highly specific to just one or a few single compounds). The applicability of GC×GC-TOFMS in ecstasy profiling has been discussed in Chapter 7, and heroin and cocaine profiling in Chapter 8.

SPME sampling of volatiles in ecstasy tablets and subsequent separation on a non-polar – polar column set has shown the most versatile impurity profile patterns for sample differentiation. Sixteen components have been selected for profiling purposes amongst the great number of components which have been detected and identified. All 24 ecstasy samples were correctly classified according to their post-tableting characteristics and intelligence information. The advantages of GC×GC over GC have been discussed.

In contrast to the endogenous and exogenous sterols work where low MS similarity was obtained in the absence of a dedicated in-house library, in this work high TOFMS spectral similarity has been obtained against commercial (e.g. NIST05) MS databases for the volatiles in the illicit drugs. This has been attributed to the lower molecular mass of the components analysed, since the sample preparation procedure was based on SPME (headspace) extraction. It has been shown that GC×GC has many advantages over classical GC. Many more components have been detected, which in turn means that there are opportunities for new relationships between their presence and their origin in the sample
to be established, and indeed are required before their potential use as markers for the synthesis route or origin differentiation is proposed.

An alternative approach to close the gap between comprehensive, information-rich and tedious methods, and fast, simple and less powerful profiling methods, has been proposed in heroin and cocaine profiling, discussed in Chapter 8. A fast, simple and information-rich method based on SPME extraction of volatiles and semi-volatiles in drug samples, followed by GC×GC separation, has been shown as a good base for development of new profiling approaches. 21 heroin samples have been classified in 9 groups, and 18 cocaine samples in 8 groups according to their volatile profiles. Links between the presence and the origin of the new components revealed in the GC×GC profiles need to be established, which in turn will define their importance in the profiling process. The advantages and disadvantages compared to traditional drug profiling methods have been discussed in detail.

The thesis concludes with Chapter 9, where the most important findings, outcomes and specific conclusions of each study are summarised. The perspectives of the research undertaken are discussed in this chapter and recommendations for further study are proposed.
Chapter 1

1 INTRODUCTION
1.1 GLOBAL DRUG PROBLEM

Currently illicit drug use has been estimated to be less than 5% of the world adult population aged 15-64 [1]. In other words, one in every twenty people used illicit drug at least once in the past 12 months. The number of problem drug users or people severely drug dependant is estimated to be one tenth of that number or 26 millions in total, comprising about 0.6% of the world’s adult population.

According to the United Nations World Drug Report (WDR) for 2008 [1], this is an impressive achievement when considered in the historical perspective of a century of drug control. The result is even fascinating when compared to the consumption of tobacco or alcohol, the two most addictive legal psychoactive drugs that are used by at least one quarter of the world’s adult population, and cause millions of deaths every year. In the absence of the drug control system, illicit drug use may well have reached such levels, with devastating consequences for public health.

On the other hand, WDR provides evidence of a surge in illicit drug supply in 2007. The world’s opium production almost doubled between 2005 and 2007, with Afghanistan alone accounting for 82% of the total area harvested. Coca cultivation has also increased in the Andean countries. Amphetamine-Type Stimulants (ATS) is the only drug type for which production and consumption remained steady in the last few years. However, the prevalence rate of ATS abusers (0.6%) is higher than the cocaine (0.4%) and double the heroin (0.3%) prevalence rate. 24.7 million people aged 15-64 consumed ATS at least once in 2006, of which 9 million were ecstasy abusers. Interestingly, Australia has the highest prevalence rate of ecstasy abusers in the world (4.4%).

Drug abuse is one of the major problems of the modern world. Illicit drugs are harmful to individuals due to: (a) their side effects and physical harm; (b) potential for addiction or
the tendency of the drug to induce dependence; and (c) the effect of drug use on family and society [2]. Based on these three main factors the drugs are classified as more or less harmful, where heroin and cocaine are graded as the most harmful, and ecstasy and anabolic agents as less harmful. Nevertheless of their harm, the total burden of drug misuse only in Britain, in terms of health, social, and crime-related costs, has been estimated to be between £10 billion and £16 billion per year [3].

Illegal drug production and trafficking is a “lucrative” underground business. The United Nations has estimated that organized crime earns $1.83 trillion a year from heroin related activities alone [4]. Drug empires are international, corrupting, and operating with impunity, often outside the reach of law enforcement agencies [5]. An excellent compilation of data on drugs, especially on heroin and amphetamines in Australia, is the PhD thesis of Bruce Myor [5].

1.2 GLOBAL FIGHT AGAINST DRUG PRODUCTION AND TRAFFIKING

The global drug problem requires a global response. Efforts to establish linkage between samples seized nationally or internationally, or to find out the geographic origin of drugs is never-ending. As the drug problem spreads around the world and filled every pore of the people’s life, more detailed analysis are performed in order to increase the net background information content of drugs. The needs for intelligence and data exchange between countries have never been greater, resulting in several harmonised methods [6-10]. The Commission on Narcotic Drugs (CND) at the UN passed a resolution that was particularly relevant to drug profiling [6]. Resolution 47/5, ‘Illicit drug profiling in international law enforcement: maximizing outcome and improving cooperation’, expressed the following statements (selected by the author):
... recognizing also the value of illicit drug characterization and profiling in supporting law enforcement intelligence gathering and operational work and the international fight against illicit drugs ...

Noting the need for the effective exchange of drug profiling information between States to optimize the intelligence capacity of drug profiling programmes and facilitate the identification of illicit drug sources, trafficking patterns and distribution networks ...

(The CND) Affirms the need to develop and, subsequently, to harmonize the illicit drug profiling activities of the international drug law enforcement community;

The CND requested action by the United Nations Office on Drugs and Crime (UNODC) to encourage member states to:

- seek to enhance their capacity to undertake profile analysis of seized illicit drugs, including heroin and other narcotics in the opiate group, cocaine and amphetamine-type stimulants;

- participate in the international exchange of profile analysis information and samples of seized illicit drugs for profile analysis purposes.

The RMIT Australian Centre for Research on Separation Science (ACROSS) group has commenced a program to contribute to the profiling of both illicit and sports drugs by using comprehensive two-dimensional gas chromatography (GC×GC).
1.3 DRUG PROFILING

Drug profiling, like any other form of profiling, is basically an intelligence-gathering process. We profile people, religious attitude, politicians, food, wine etc. As we gather more information about the subject being “profiled”, the more we learn about the subject and we can use the information for our benefit [7].

1.3.1 Drug characterization and impurity profiling

Illicit drugs are rarely pure substances. Among the main (active) component, they contain many other substances, thus representing a complex mixture. According to the UNODC [8] illicit drugs may contain one or more of the three different types of key components: natural components, by-products and cutting agents.

Natural components naturally occur in the plants (coca leaf, opium) from which plant-based drugs (cocaine, heroin) are produced. As a result of their chemical similarities to the main component (solubility, polarity), they are co-extracted with the main compound through the production process and remain in the final product. Even though it is hard to establish a strict classification, generally components which are present in more than 1% relative abundance to the main component are considered as major and those present in less that 1% as minor.

By-products are formed during drug manufacture (synthesis) and they are method dependant. Because of the crude production and purification processes they are also co-extracted into the final product and remain as key markers for the particular route of synthesis. They usually belong to the minor components group.
**Cutting agents** are intentionally added substances to the drugs in order to increase the weight, and therefore the profit, or to enhance some effect of drugs, i.e. volatility of drugs for smoking, to relieve the pain when injected etc. Psychologically active substances or adulterants are used for latter purposes (caffeine, procaine, paracetamol, aspirin) and psychologically inactive substances or diluents are used for the former purpose (sugars, talc, starch).

The variety of components found in the drugs and their variability in the amount allow more comprehensive analysis to be done, resulting in chemical profiles, also known as “signatures” or “impurity profiles” of the drug. Profiling typically involves several analyses that are designed to produce a detailed picture (profile) of a drug sample, usually in the form of chromatographic data. These data are further processed by means of statistical and chemometric evaluation in order to classify material from different seizures into groups of related samples and/or to identify the origin of samples, if appropriate samples with known origin are available for comparison. Impurity profiling studies together with the drug characterization, where physical features of drugs as well as chemical composition are determined, can be valuable scientific tools to support law enforcement operations.

While “characterization” in organic chemistry is often utilized to describe the process of exact structure determination, in drug characterization it means describing the main features of the drug samples, both physical and chemical. Physical characteristics of samples, like colour, texture, logo and other toolmarks on tablet exhibits, packages (wraps) of drug blocks, are equally important as chemical characteristics, i.e. major and minor components composition, trace metals and occluded solvents, organic impurities, stable isotope abundance etc.

Impurity profiling is used to describe the process of gathering a large number of data (both in number of samples and in unique data per sample) from variety of instrumental analyses (usually in form of chromatograms), visual or statistical evaluation of the results,
and finally an interpretation of the results in terms of sample relations and/or origin/production route determination. The results of impurity profiling are called drug “signatures” or impurity profiles. The importance of “signatures” originated from the region-specific routes of drug production and the concept of batch variation [9].

1.3.2 Batch concept

Discrete and separate “batches” of the final product are usually processed at any time in clandestine drug production. Because production conditions can not be reproduced exactly each time variations in batch to batch composition will occur, i.e. different batches from the same clandestine laboratory or operator will have different and unique signature (inter-batch variation). In addition, the illicit drugs are usually non-homogenous and small variations in composition within the same batch are to be expected (intra-batch variation). Normally, it is expected inter-batch variations to be significantly greater than intra-batch variations. If sufficient data are gathered by applying different analytical methods and proper discrimination criteria are established based on these variations, then successful common batch origin classification of samples is possible. Samples with a similarity greater than the established statistical intra-batch variation are considered as linked and in contrast, samples with a lower similarity are considered as non-linked. In practice, it is much more difficult to establish a link between samples than to exclude the linkage. The data “density” acquired and its uniqueness, as well as the frequency of the particular pattern of profile defines the strength of the linkage. Sample similarity based on larger and more unique data sets is stronger, as well as similarity between samples with unusual profile, i.e. of low frequency compared to the previously encountered patterns. However, several factors may complicate the establishment of specific links between drug samples. Post-production sample decomposition due to the sun/heat expose, hydrolysis, dilution with some aggressive cutting agents (ascorbic acid), may cause significant differences in the profiles of linked samples. Highly purified or highly diluted samples might limit the gathering of large amounts of data to support strong sample similarity links.
1.3.3 Geographic origin concept

It is well known that some plant-derived drugs have a unique compositional pattern which depends on the geographical region where the plant is grown. In addition, clandestine production methods in any one region are believed to be similar or the same. As a consequence, samples from that region may contain similar impurities in similar proportions, and despite the batch-to-batch variation in the composition, can be classified together. A representative signature or impurity profile can be assigned to the region by running authentic samples or samples originating from known geographic regions.

1.3.4 Drug profiling and law enforcement

Generally, impurity profiling applications fall into one of two overlapping categories: tactical and strategic. The establishment of a drug distribution network and/or trafficking links based on sample similarity is an example of tactical application. The results are usually presented at the court as evidence, complementary to the intelligent investigation. Geographic origin determination for naturally based drugs, and production route determination for synthetic drugs, based on the sample characterization are examples of strategic application and the results are part of the intelligent information used in the fight against drug production on regional and global scale. Well experienced chemists who are very familiar with all the target components and close cooperation / data exchange with law enforcement personal are the two most important prerequisites for successful application of sample characterisation and impurity profiling.
1.3.5 Data handling and interpretation of results

Large amounts of data in the form of numbers and chromatograms are usually generated in such a comprehensive approach. If visual inspection and comparison of chromatograms were the only way of data evaluation in the early years of impurity profiling applications, commercial and dedicated in-house computer programmes are used for data handling and statistical processing in modern objective methods. Sample composition expressed in absolute values offers long-term reproducibility, possibility of quality control and data exchange between laboratories [10]. However, the magnitude of the analytical variance, especially for less abundant components, and the lack of reference materials of such a great number of diverse impurities, make this approach less practical. Component ratio method is preferred in impurity profiling since it overcomes the limitations of the former method. Basically it takes into account the ratio of impurities to the main component or to an appropriate internal standard.

The results of impurity profiling, i.e. the sample similarity or geographic origin determination, have to be interpreted carefully, taking all relevant considerations into account. Good understanding of the purpose of impurity profiling and drug production/distribution pattern, and close cooperation with law enforcement personal are equally important as knowledge and skill in relevant chemistry methodologies. A well experienced chemist can recognize the uniqueness of some impurities and can give them more weight in the discrimination process, thus making the evidence (linkage) stronger.

1.4 ILLEGAL PRODUCTION OF HEROIN

Heroin is a semi synthetic drug obtained from morphine by acetylation with acetic anhydride. Morphine is the main alkaloid of the papaveraceae family, and Papaver
*Somniferum* (more commonly referred to as opium) being the most exploited in this manner.

### 1.4.1 History of opium

Cultivation of opium poppies for food, anesthesia, and ritual purposes dates back to at least the Neolithic Age. The Sumerian, Assyrian, Egyptian, Greek, Roman, Persian and Arab Empires each made widespread use of opium, which was the most potent form of pain relief then available. By the sixteenth century, residents of Persia and India began eating and drinking opium mixtures as a purely recreational euphoric. Portuguese and Dutch merchants traded ever-increasing cargoes of Indian opium to China, which forced China’s Emperor to ban opium, addressing the rising opium addiction problem. The law failed to slow the opium smuggling by British and Americans merchants and the Emperor was forced to rescind the opium ban after British fought two successful Opium Wars (1839 and 1856) against China. At the turn of the 18th century, 13.5 million addicts were estimated in China’s population of 400 million, consuming 39000 tons of smoking opium [11].

Several conventions, treaties and acts (Shanghai Opium Commission, 1909; Harrison Narcotic Act, 1914; Geneva Convention, 1925; League’s Limitation Convention, 1931) were enforced in the beginning of the 20th century in order to reduce the legal production of opium and heroin for recreational use. However, the reduction in legal opium cultivation and heroin production had a negligible effect on global heroin production and consumption. Instead, opium cultivation and heroin production were driven underground and made even more profitable.
1.4.2 Opium poppy cultivation and opium production

About three months after planting, each stem produces a brightly coloured flower hiding a seedpod, which synthesises a milky white sap (opium) soon after the petals have fallen away [5]. The maximum content of morphine in the poppy is reached when the capsules (seedpod) are still in the green stage. At this stage a series of shallow incisions through the capsules are made which allow latex to “bleed” onto the surface. Most commonly, the latex is collected the next day, when it is partially dry on the capsule surface, by scraping with a special hand tool. The fresh dried latex is like a gum with light to dark brown colour, turning dark to black material by ageing, known as opium. The yield varies from 10 kg/ha in Myanmar (South East Asia, SEA) to as much as 50 kg/ha in Afghanistan (South West Asia, SWA) [1]. The differences in the yield are mainly due to the growing conditions and the differences in the opium poppy varieties. More than 30 alkaloids are found in this product, together with resins, triterpenoid alcohols, sterols, fatty acids, polysaccharides etc.

According to the WDR for 2008 [1] opium poppy cultivation continues to increase in 2007, with Afghanistan accounting for 82% (193,000 ha) of the world’s area under cultivation. Other regions contributing to the global opium cultivation are South East Asia (Lao PDR, Myanmar, Thailand, Viet Nam), South West Asia (Pakistan), Latin America (Colombia) and Mexico. The estimated annual opium production for 2007 is 8870 metric tons, with Afghanistan alone accounting for 92% of the world’s production [1].

The alkaloid content of opium latex will vary from region to region, from field to field, or even from plant to plant. It is quite clear that the composition is affected the most by the climatic and agronomic conditions. However, nearly always the bulk of the alkaloidal fraction is comprised of 5 main alkaloids in different proportions: morphine (1), codeine (2), thebaine (3), noscapine (4) and papaverine (5). The first three are classified as phenanthrene alkaloids and the other two as isoquinoline alkaloids. Their structures are given in Figure 1.1.
Figure 1.1 Structures of main opium alkaloids: 1. morphine, 2. codeine, 3. thebaine, 4. papaverine, 5. narcotine (noscapine).

According to a study in which 1414 raw opium samples were quantified [12], morphine is the most abundant, followed by noscapine. Even though opium has a long history, the first isolation of the main alkaloid was in 1803 by the German pharmacist Friedrich Sertürner, who named it *morphium* after Morpheus, the Greek god of dreams. The details of the alkaloidal composition of opium are given in Table 1.1.

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Min (%)</th>
<th>Max (%)</th>
<th>Aver. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>3.1</td>
<td>19.2</td>
<td>11.4</td>
</tr>
<tr>
<td>Codeine</td>
<td>0.7</td>
<td>6.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Thebaine</td>
<td>0.2</td>
<td>10.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Papaverine</td>
<td>&lt;0.1</td>
<td>9.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Noscapine</td>
<td>1.4</td>
<td>15.8</td>
<td>8.1</td>
</tr>
</tbody>
</table>
1.4.3 Extraction of morphine

There are numerous methods for extraction of morphine from raw opium. However, the most exploited in illegal production is “the lime method” [12]. Opium pieces are dissolved in hot water, stirred and filtered. The filtrate is reduced to half by boiling and poured into a solution of boiling calcium hydroxide. The precipitate formed in this step is filtered, re-extracted and residues are discarded. The combined filtrates are reduced to half by boiling and filtered again till clear solution is obtained. After that, the solution is boiled and ammonium chloride is added. When the solution cools morphine base will precipitate, and the raw base is collected by cloth filtration. If there is a demand for a pure heroin, or morphine is the final step in production, the base is further purified by dissolving in a minimum volume of warm hydrochloric acid. As the solution cools, morphine hydrochloride precipitates, whereupon it is collected by filtration. Further purification involves boiling with a charcoal and filtration.

The lime method is not the only one used for the extraction of morphine from opium in clandestine laboratories. However, its simplicity, and most importantly in illegal production, the use of only a few simple and widely available materials (lime, ammonium chloride, drums, cloths) makes it widely spread.

1.4.4 Synthesis of heroin

The first reported synthesis of heroin was in 1874 and the first commercial production in 1898 by the “Bayer” company, who named this new drug as heroin [12]. Shortly after its appearance on the market the drug popularity grows as a cough suppressant reducing morphine and codeine use, and was widely used for tuberculosis and other respiratory disease treatments. A few years after the 1925 International Convention on Narcotics, international control begun to limit the supply of heroin, and the illegal production of heroin began.
Almost with no exceptions, clandestine heroin synthesis is a one step acetylation process involving boiling of morphine with acetic anhydride. The process is completed in several hours (usually overnight) and the product is removed by treating the cooled reaction mixture with sodium carbonate and collecting the precipitate (heroin base) by filtration. There are at least two regions in the world, New Zealand [13] and Poland and some regions in Russia [14], where the clandestine process differs from the traditional. However, both processes are thought to be exclusively used to produce small scale quantities for individual consumption.

The heroin base can be a final product distributed on the black market, as in the case of southwest Asia (SWA), or can be further processed into heroin hydrochloride. This process involves dissolution of heroin base in a minimum volume of organic solvent, i.e. diethyl ether, and precipitation by adding concentrated hydrochloric acid. The precipitate is removed by filtration and further processed by decolourisation and pre-crystallization if highly purified heroin hydrochloride is required. A block of SWA heroin base with stamped logo, seized during an international smuggling operation through the Macedonian/Serbian border, is shown in Figure 1.2.

Figure 1.2 Heroin block (~0.5 kg) with a stamp on its surface, seized in 2006 in Macedonia
1.4.5 Impurities in heroin

As previously stated, there are three main ways which contribute to the total impurities in heroin samples:

1. co-extracted **natural products** originating from the opium, which do not change their chemical nature during the production process;
2. **by-products** or components generated during the acetylation process, which include the components capable of undergoing acetylation or components decomposed by the rigorous acetylation environment;
3. **cutting agents** and their impurities added after the completion of the production process, usually during the distribution chain.

Opium minor components and their acetylation and degradation by-products encountered in heroin samples have been extensively studied in a series of works done at the Special Testing and Research Laboratory (STRL) at Drug Enforcement Agency (DEA) [19-25]. Many components were synthesized and characterized by using gas chromatography (GC), high performance liquid chromatography (HPLC), preparative and semi-preparative HPLC, high resolution mass spectrometry (HRMS), nuclear magnetic resonance (NMR), infra red spectroscopy (IR), and the data generated by the analyses were extensively used in many further works in this field.

Natural products can be divided in three main groups according to their chemical structures. These include morphine-related (morphine, codeine and thebaine), benzyloquinoline (papaverine) and phthalideisoquinoline alkaloids (narcotine, narceine), as well as their acetylation products and by-products.
1.4.5.1 Morphine-related compounds

Morphine (1, Figure 1.1) is a major constituent in opium (see Table 1.1). Acetylation of morphine by using warm acetic anhydride generates diacetylmorphine or heroin (6) via two stable intermediates. Dybowski and Gough [15] have shown that acetylation of hydroxyls in morphine predominantly involves the hydroxyl at position 3, giving 3-monoacetylmorphine (7), followed by the acetylation of hydroxyl at position 6 (Figure 1.3). Hydrolysis of acetyl group at position 3 in heroin (phenolic hydroxyl) gives 6-monoacetylmorphine (8), one of the most important morphine-related by-products. Further hydrolysis, obviously, will give morphine. These compounds are present in heroin samples at varying amount, 8 being the most abundant after the main drug.

![Chemical structures](image)

Figure 1.3 The major acetylated products in heroin samples: 6. diacetylmorphine (DAM, heroin), 7. 3-monoacetylmorphine (3-MAM), 8. 6-monoacetylmorphine (6-MAM), 9. acetylcodeine.

Codeine (2) is structurally very similar to morphine, thus it is very often co-extracted with morphine. Similarly to morphine, codeine undergoes acetylation giving 6-acetyl codeine.
and its relative concentration is often used as a marker for geographic origin determination. N-oxide forms of morphine (10) and codeine (11), as well as their acetylated products (12, 13), can be found as impurities in raw processed heroin samples (Figure 1.4).

Figure 1.4 N-oxide forms of morphine and codeine, and their respective acetylation products: 10. normorphine, 11. norcodeine, 12. N,3,6-triacetylnormorphine, 13. N,O-diacetylnorcodeine

Thebaine (3) is a highly toxic alkaloid found in opium, but rarely found in processed heroin samples due to its degradation in the acetylation step. Allen et al. [16] have described at least nine degradation products of thebaine when boiled in acetic anhydride; acetyl thebaol being the main degradation product. Some of the products encountered in heroin and used in impurity profiling procedures (14-18) are given in Figure 1.5.
Figure 1.5 Thebaine degradation products: 14. thebanol, 15. acetylthebanol, 16. 3,6-dimethoxy-4,5-epoxyphenanthrene, 17. thebaine degradation product 1, 18. thebaine degradation product 2.

Neopine (19), oripavine (20) and their acetylated products (21, 22) are minor morphine-related alkaloids which can be found in heroin samples at varying amounts, depending upon the climate and soil conditions. Apomorphine (23) and morphothebaine (24), as respective degradation products of morphine and thebaine, are also found. Their structures are given in Figure 1.6.
1.4.5.2 Benzoquinoline-derived impurities

Papaverine (4) is one of the principal opium alkaloid, derived from the benzylisoquinoline structure. Laudanosine (25), reticuline (26), codamine (27) and laudanine (28) are minor alkaloids from this group (Figure 1.7), which undergo degradation in boiling acetic anhydride giving at least 18 degradation products [17]. They are found as precursors for papaverine and others alkaloids in *papaver somniferum*. Other impurities from this group are norlaudanosine (29) and its acetylated product *N*-acetylnorlaudanosine (30).
1.4.5.3 Phthalideisoquinoline-derived impurities

Narcotine (5) is the second most abundant alkaloid in opium, after morphine. This wrongly named non-narcotic alkaloid (lately renamed as noscapine) does not undergo acetylation but forms many degradation products when boiled with acetic anhydride (Figure 1.8). *N*-acetylnornarcotine (31), *N*-acetylanhydronornarceine (32), 1-acetoxy-*N*-acetyl-1,9-dihydroanhydronarceine (33) and (E)-3-[2-(2-(*N*-methylacetamido)ethyl)-4,5-methylendioxy-6-methoxyphenyl]acrylic acid (34) have been detected in heroin samples [18].
1.4.5.4 Other alkaloid impurities

Many other minor alkaloids, often at very low concentrations, can be found in heroin samples (Figure 1.9): protopine (35), cryptopine (36), aporeine (37), rheadine (38), hydrocotarnine (39) etc. [5].
1.4.5.5 Cutting agents

A large number of other substances have been seen in heroin samples, especially at “street” level. There are two main reasons for this: a) to increase the mass of the drug and to make an extra profit and b) to pronounce some effects or properties of the drug. Diluents are used for the former, mostly sugars, talc, starch, and adulterants for the latter, most exploited being paracetamol, caffeine, procaine, phenobarbitone etc. For example, procaine is used in heroin as a local anaesthetic to relieve the pain at the injection point and caffeine is used to facilitate the volatility of heroin base when smoked. Cutting agents are usually not origin-related or process-related compounds but some low frequently agents can be seen as valuable information in sample comparison, distribution chain establishment or even geographic origin determination.

The most exploited impurities in heroin profiling are major components, minor organic impurities, stable isotope-ratio abundances, metal contaminants and solvents occluded in the drug crystal. Gas chromatography coupled to flame-ionisation (GC-FID) and mass
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spectrometry (GC-MS) detection, high performance liquid chromatography (HPLC), isotope ratio mass spectrometry (IRMS) and atomic absorption spectroscopy (AAS) are widely used for gathering analytical data. However, the literature review on heroin profiling in the thesis is mainly focused on GC methods, because of its pre-eminence in this area. The high separation power and versatility of the detectors used are the two main reasons for this.

1.4.6 Gas chromatographic methods for heroin impurity profiling

It has been a quarter of a century since Neuman and Gloger [19] developed a capillary gas chromatography method for heroin impurity profiling. The work was one of the first on use of high resolution GC on trace organic components in heroin samples. The method is based on an extraction of neutral and acidic impurities with toluene from an acidic heroin solution. About 70 peaks were detected in a highly complex chromatogram obtained in the case of a near/middle East Asia heroin sample, showing distinct advantage over methods previously used for forensic comparison of illicit heroin samples based on packed columns. Furthermore, it was found that the profile of a heroin sample from Turkey was totally different from that from Malaysia, and small but significant differences appear when compared to that from Lebanon. In conclusion, the method clearly demonstrated its practical value in forensic case work.

The same method [19], slightly modified, was used also for opium and crude morphine profiling with even more components (150) detected in chromatograms [20]. The author concluded that greater similarities are obtained between opium and morphine profiles than between heroin and opium/morphine profiles. It is expected if we have in mind that the processing of morphine from opium involves only extraction and precipitation steps, but processing heroin from opium/morphine involves acetylation, which changes the impurity pattern drastically.
Barnfield et al. [21] have developed a method for routine profiling of heroin samples based on the analysis of the main components. The similarity between samples is obtained by comparing the relative ratio of each of the analysed components in samples to that of diacetylmorphine. The data obtained have been used as the basis for providing the courts with information about the possible links between heroin samples. The method has been shown to fulfil the criteria given by the authors concerning the simplicity, robustness and separation efficiency of the procedure as well as the homogeneity of the sample. Neumann [22] has commented on the huge solvent peak in the chromatograms arising from this work [21], which have a negative effect on the determination of the first several eluting components.

In contrast to earlier heroin profiling works which were based on visual comparison of chromatograms [23], multidimensional statistical methods like principal component analysis (PCA) and hierarchical cluster analysis (HCA) have been applied later. A HCA approach has been used to classify selected heroin samples seized in Germany in 1991 into four impurity profiles [24]. Furthermore, impurity profile analysis results were used as a valuable second method besides the results from the main components analysis for sample comparison.

A theoretical consideration of comparative analysis and its importance in forensic drug profiling has been discussed by Perillo et al. [25] and Huizer [10]. While trace level chemical analysis of a heroin sample, which produces a signature characteristic of its origin, is a very important step, the ballistic comparison must not be negligible. All manufacturing techniques (compressing powder into tablets or blocks, imprinting logo designs) can leave unique and reproducible toolmarks on the final product, amenable to ballistic (toolmark) comparison with other exhibits or authentic samples from a reference collection [25]. Ballistics thereby represents a complementary but completely independent source of strategic and/or tactical intelligence.
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Analysis of the major components has been used to predict the batch membership of heroin samples [26, 27], or to predict the country of origin [28, 29]. A brief review on comparative analysis of heroin and cocaine samples was published by Chiarotti [30], summarising the analytical methods used in the field. However, no comments on data analysis were given, beside the variety of techniques covered (TLC, HPLC, GC, CE, GC-IRMS, AAS). A desire for a single robust method able to resolve any possible significant marker compounds, and a standardized general procedure suitable for use on an international level has been expressed.

Soon after this review was published, a harmonized study for retrospective heroin impurity profile comparison was published [31], as a collaboration study initiated by three well equipped forensic laboratories. The majority of the GC conditions and the sample extraction procedure, based on the previously published methods [18, 19], were optimized, 16 variables (compounds) were selected and the criteria of similarity were defined. The quotient method (Q-method) [32], a software program developed at the National Laboratory of Forensic Science (SKL), Linkoping, Sweden, was used for statistical treatment of data. Briefly, it calculates for each selected peak in the chromatogram the ratio between that peak in sample A and that peak in sample B, and the similarity between the two samples is expressed as the number of peaks giving similar ratios (within a predefined window). It has been shown that, despite the intensive research and quality control checks between the three well equipped and experienced forensic laboratories, the variation of the inter-laboratory results are much larger than the variation of the intra-laboratory results. The main source of the irreproducibility is shown to be the GC or precisely the limitations of GC (which have been reached) for this specific application using the selected 16 variables.

As instrumental development progresses the choice of method for heroin samples comparison and profiling becomes wider. Besacier et al. [33] have extended the two step procedure (major/minor components analysis and impurity profiling) with a third step – stable isotope ratio of major components. They have described a case where impurity
profiling was not as efficient as the isotope ratio method. However, the three step procedure proved to be suitable for complete comparative chemical analysis of drug samples. Myors et al. [34] have used a GC-MS procedure for heroin samples profiling, based on 25 discriminating factors (selected organic impurities). HCA was used to investigate the similarities between samples, explaining 85-100% of the variations.

Trends in heroin profiling throughout 1990s, a decade proclaimed by the United Nations as the ‘Decade for Eradicating Drug Abuse’, were reviewed by Dams et al. [35]. The authors stated that due to its speed and high resolution, capillary GC was the most often applied technique during the covered period. Capillary electrophoresis (CE) and hyphenated LC-MS have been viewed as promising techniques in drug profiling in the next period, offering solution of some problems encountered with the previously used techniques. In contrast to the previous review [30], a great part of Dams’ work was devoted to data treatment and interpretation, since the authors concluded that the most significant trend during the 1990s was the way that large amount of quantitative data are handled. While in the early 1990s the comparison between samples was performed by visual inspection of physical properties and chromatograms, statistical programmes for extensive data handling, i.e. chemometrics, were introduced later. Both unsupervised (HCA, PCA) and supervised (linear discriminant analysis, k-nearest neighbours) methods were used in heroin profiling, allowing the formation of accurate and more objective profiles and fingerprints. However, more sophisticated statistical methods are proposed to be explored in the future.

Various statistical approaches in heroin profiling have been implemented in a series of research conducted at the Institute de Police Scientifique, University of Lausanne (Switzerland). Cosine function [43, 44] and artificial neural network (ANN) [36] have been used on results from the major components analysis in heroin, while the graph theory [37] was used in profiling of cutting agents in heroin samples. The authors stated that the major components method offers advantages over the minor components method in terms of simplicity, sample consumption and speed, and 98% of the results from the former one
were confirmed by the latter one. Because of this, the Laboratoire de Police Scientifique de Lyon (France) switched the routine profiling procedure from minor components to major components method [38]. However, the fact that the number of variables when using major components method is limited can not be ignored.

The application of machine learning algorithms (spectral clustering and kernel-PCA) on heroin and cocaine gas chromatography data have been recently published [39]. The results have shown that the classical methods miss the inherent structure of data, which is highlighted by methods such as spectral clustering and its variants. Once again, the data was based on major components analysis, taking into consideration only 7 variables (meconin, acetycodeine, acetylthebaol, monoacetylmorphine, diacetylmorphine, papaverine and narcotine).

The most comprehensive heroin profiling procedure applied on a large heroin seizure was reported by Collins et al. [40]. The authors have used many analytical techniques (HPLC, CE, GC-MS, GCcIRMS, GC-EAIRMS) for major and volatiles (solvents) component analysis, organic impurity profiles and stable isotope-ratio abundance. While alkaloid ratios (major components) and volatile components profiles were consistent with heroin from Southeast Asia (SEA), the acid/neutral impurity profile was significantly different. Such a detailed analysis revealed a new heroin origin and/or production route which was classified as unknown at the time of the case report.

1.4.6.1 Prerequisites in heroin comparison and profiling

Several problems associated with heroin profiling are cited in the literature, and researchers should be aware of them.

Transacetylation, or the transfer of an acetyl group of one compound to another, can occur in the heroin molecule when injected in mixture with some hydroxyl-containing components capable of acetylation. Mixtures of heroin and codeine can give trace levels of 6-MAM and acetycodeine as a result of transacetylation [15]. Similarly, mixture of heroin
and acetaminophen (paracetamol) will give 6-MAM and acetylaminophen [41].

Transacetylation of heroin has not been observed by using HPLC and so was attributed to occur in the injection port of the GC. The problem can be avoided by lowering the injector temperature and by using non-alcoholic solvents.

Klemenc has reported a case [42] where major components analysis were performed on 22 blocks of seized heroin weighing 11 kg in total. While 20 samples contained unusual high concentration of noscapine, heroin was absent from the remaining two, even though they resembled the common SWA heroin samples. Detailed analysis revealed 88% of noscapine and 6% of papaverine, with no measurable amount of heroin, acetyl codeine, 6-monoacetylmorphine and morphine. The research based on 22 case samples has shown that noscapine can be used as an adulterant in heroin samples, increasing the noscapine/whole morphine (WM) ratio up to 3.5. In this way the major components criteria for classification of samples could be wrong, or even the organic impurity profiles can be inconsistent due to the unusual variability of the noscapine and its related by-products. Similar case samples were encountered in the author’s previous laboratory - Forensic Science Laboratory at the Ministry of Internal Affairs, Skopje, Macedonia, during 1999-2001 (B. Mitrevski, unpublished results). The report suggested that the significance of the noscapine should be evaluated with care in cases of comparative sample analysis or in predicting the sample origin.

Similarly, a case where three illicit drug seizure thought to be “brown heroin” completely lacked diacetylmorphine, but contained 6-acetylmorphine, acetyl codeine, morphine, codeine, paracetamol and mannitol hexaacetate (MHA), has been reported [43]. After series of experiments it has been shown the MHA was a genuine heroin ingredient, resulting from the addition of mannitol before the acetylation step of heroin production. The lack of heroin in the samples was explained by its incomplete acetylation due to the presence of the mannitol as a big consumer (contains 6 hydroxyls) of the acetylating agent (acetic anhydride). The case has shown the alkaloid proportions sometimes can lead to a wrong conclusion and inconsistency to the previously established criteria. However, the
presence of an unusual adulterant can be used as a very useful indicator in heroin profiling enabling the connection of batches that underwent the same production sequence to the same production source.

All current methodologies for heroin profiling are based on acetic anhydride acetylation of morphine and opium-related alkaloids, although there are at least two other reported acetylation reagent (acetyl chloride and ethylidene diacetate). Odell et al. [44] have developed a method for detection and discrimination of heroin samples produced by trifluoroacetic anhydride (TFAA) acetylation, suggesting 6-monoacetylmorphine/whole morphine (WM) ratio window as a new criteria. TFAA acetylation is an extremely clean, efficient and fast route which yields high quality heroin without heat and large reagent excess. From a drug enforcement perspective the use of this method in clandestine heroin production is significant, not only because of the high yield, but also because neither TFAA nor acetic acid is restricted precursors.

All current heroin signature programs are based on determination of the origin of heroin between the major world producers (SEA, SWA, South America, Mexico), with limited success for determination of samples from between sub-regions. Odell et al. [45] have developed a profiling signature of heroin produced from Tasmanian opium which accounts for 25% of the world’s legal supply of opium straw. The signature is based on the presence of the unique marker compound in opium – oripavine and its several acetylated products and by-products. While most of them were detected in selected heroin samples seized in Australia and suggesting they originate from Tasmanian opium, none of these compounds has been detected in heroin samples from SWA, SEA and South America (SA). The authors’ opinion is that the unique marker impurities signature has advantage over traditional heroin signature programmes (HSP) which rely on the differences in production processes that result in quantitative variation in the ratio of commonly encountered major and minor compounds.
1.4.6.2 Profiling of volatile compounds (solvents occluded) in heroin samples

The production procedure of almost all drugs involves some dissolution and precipitation steps. Trace amount of solvents used as a medium can be trapped into the crystal structure of the drugs during the crystallization step, giving valuable information about the drug production history. Since the clandestine production does not comply with any quality control / quality assurance protocol (QA/QC), residual solvent are often left behind in crudely dried drugs. Some solvents found in illicit drugs are due to degradation processes. Acetic acid, which is the product of hydrolysis of heroin, gives its characteristic “acidic” odour. The variety and the number of solvents encountered in the drugs can reveal the production route, the number and the type of purification steps applied, or the storing conditions. Volatile components profiles of the samples can be used as a valuable additional tool in sample comparison or in drug origin determination.

Chiarotti and Fucci [46] completed one of the first works on volatile components analysis in heroin samples. Static head space (HS) technique was used for sampling, three different type of packed column were used for separation, and FID for detection of components. One to five volatiles were found in all analysed samples with acetic acid, acetone and diethyl ether being the most frequent. The authors stated that the sample ageing does not impair the detection of volatiles by this non-destructive sampling methodology.

GC-MS with HS [47], solid phase adsorption [48] and solid phase microextraction (SPME) [49] sampling techniques was used for identification and confirmation of volatiles in heroin and cocaine samples. Much more volatile components are detected in heroin samples when compared to the previous work [46], probably thanks to the capillary column used. However, the variety and the number of occluded solvents were in favour of cocaine samples, probably because generally it has larger crystal size than heroin which may account for its ability to retain larger amount of occluded solvents [47]. The volatile components heroin profile must be seen as a complement to other profiles (major components, organic impurities, trace metal contaminants) in sample comparison since the limited number of variables (solvents) in the profile can lead to wrong conclusions [48].
The heroin and cocaine sample odour, represented by its volatile components composition, have been used in detector dog training programs [49]. SPME sampling technique was used to develop a “pseudo heroin” and “pseudo cocaine” formulation for initial scent association training. It enables screening of stock materials in order to minimize the risk of dogs being trained on a wrong scent and fail to alert when they encounter real drug samples.

### 1.4.7 Profiling of trace metal contaminations in heroin samples

The variety of trace metals and their different ratio in heroin samples are used as a complementary method in heroin profiling. Some of trace metals are accumulated in plants from soil [50], some are added during the trafficking chain (Ca), and some can originate from the metal utensils used in production processes (Fe, Zn, Cu). The importance of this approach has arisen from the similarity of the impurity profiles obtained from heroin samples originated from SEA, which are usually very pure and contain organic contaminants in almost constant proportions [51]. Atomic absorption (AAS) [52], inductively coupled plasma atomic emission (ICP-AES) [50, 53] and ICP-MS [51, 54] have been applied for atomisation and detection.

Myors et al. [51] have successfully used a number of statistical methods (HCA, PCA, k-means cluster) to differentiate SEA from non-SEA heroin samples using the results from analysis of 73 elements. However, they have found it difficult to discriminate the subgroups within the SEA samples. Broad similarity has also been obtained between 44 SWA originated heroin samples, based on data from 10 elements [55]. Tactical intelligence (sample-to-sample comparison) is more likely to profit from the results of this profile than strategic, since more reliable data from analysis of authentic samples is required in order to correlate trace metal composition to the geographic origin. Another obstacle is
difficulties in the rationale for presence/absence of trace elements in samples from different geographic regions.

1.4.8 Isotope-ratio mass spectrometry in heroin profiling

The stable isotope content of a plant, or its constituents, is characterized by its photosynthetic cycle and the metabolic pathways of the fixation of carbon, nitrogen, oxygen, hydrogen, which in turn depends on the environmental conditions (humidity, soil, temperature, isotope ratio of the ambient CO$_2$) in which the plant has grown [56]. Therefore, stable isotope abundance of carbon, nitrogen and oxygen in heroin samples will differ according to the geographic origin of its natural precursor – opium. The isotope-ratio mass spectrometry (IRMS) technique is capable of measuring these small differences in the isotope ratio, which in turn can be attributed to the geographical origin of heroin samples, or its natural precursor. Since partial sample degradation or multiple sample dilutions do not alter the isotope-ratio, the results can be used as a valuable contribution to the overall drug origin determination procedure, combining the results of the major components analysis, organic impurity profiling, volatile components and trace metal composition.

Isotope ratios are usually expressed as δ-values (in permille, ‰) versus a standard:

\[
\delta^{15}N = \frac{\left(\frac{^{15}N}{^{14}N}\right)_{sample} - \left(\frac{^{15}N}{^{14}N}\right)_{std}}{\left(\frac{^{15}N}{^{14}N}\right)_{std}} \times 1000
\]

δ$^3$C and δH ratios are calculated correspondingly. Pure gaseous air (for δ$^5$N), hydrogen (for δH) or carbon dioxide (for δ$^3$C), previously calibrated against standard reference materials (SRM), are pulsed (20-30 s) in the MS before and after the analysed component.

Desage et al. [57] have applied IRMS for the first time in heroin profiling, determining the δ$^3$C isotope ratio of samples from different origins. While samples from Turkey were
significantly distinguished from all other analysed samples, the samples from Pakistan, Niger and Thailand were quite close to each other and could not be distinguished based solely on the $\delta^{13}$C ratio of the whole heroin molecule. Further discrimination is proposed by measuring the individual $\delta^{13}$C ratio: from the morphine moiety and the acetyl substituent after heroin hydrolysis. This has been done by Besacier et al. [56]. They have shown that the $\delta^{13}$C ratio in the heroin molecule can be greatly contributed by the two acetyl groups. The obtained differences between $\delta^{13}$C enrichment of heroin molecules and morphine molecules after hydrolysis were in the range from +0.17 ‰ to -4.99 ‰ and are solely contributed by the acetyl moiety from the reagent used to derivatise the molecule. After this correction, no significant differences in $\delta^{13}$C are obtained between samples from the same geographic origin. However, some samples from various origins showed very close $\delta^{13}$C and it was difficult to distinguish them. The authors stated that either the samples’ origin is inaccurate or the $\delta^{13}$C is not informative enough. For example, at least 0.6 ‰ difference in $\delta^{13}$C ratio is necessary to differentiate two values in the enrichment range of 3 ‰.

Ehleringer et al. [58] have extended the research, including also the $\delta^{15}$N ratio along with the $\delta^{13}$C ratio. 76 heroin samples from the four major growing regions (SEA, SWA, SA and Mexico), supplied by Drug Enforcement Administration (DEA) of USA with a high certainty of their authenticity, were used in the study. The results have shown that heroin samples from all four geographic regions are statistically distinguished, except the limited overlap of $\delta^{13}$C and $\delta^{15}$N values of samples from Mexico and SA. Similarly to the previous work [56], $\delta^{13}$C and $\delta^{15}$N ratio values of corresponding morphine molecules (obtained after hydrolysis of heroin) have shown clearer separation of samples from different origin, with no overlapping. In this way, the $\delta^{13}$C ratio variation of the four carbons from the two acetyl, which in term can show great difference in $\delta^{13}$C, is eliminated, leaving the variation solely on the pure plant-origin compound – morphine. It has been concluded that samples from different geographic origin can be correctly distinguished on the bases of stable isotope-ratio data of the heroin molecule, and even more completely through the
morphine molecule. IRMS has been applied in heroin profiling on a limited number of samples by Idoine et al. [59] and Galimov et al. [60].

Casale et al. [61] have reported a case in which heroin samples from a big seizure were not classified in any of the four major geographic regions based on organic impurity profiles [40], even though the major components and occluded solvents profiles resembled the SEA origin. IRMS was applied to confirm the authenticity of the samples from the possible newly encountered heroin production region or production process, according to their distinct and unique $\delta^{13}$C and $\delta^{15}$N ratios.

Site specific natural isotope-ratio fractionation measured by deuterium nuclear magnetic resonance (SNIF-NMR) was used in geographic determination of heroin samples [62], offering separate $\delta^2$H enrichment for every labelled site in the molecule. Even though the method is capable to measure the “synthetic” deuterium sites independently (i.e. the two acetyl groups in heroin), the high sample consumption of about 1 g of purified heroin (due to the relatively inactive and low abundant deuterium nuclei) and the peak overlapping in the low resolved deuterium NMR spectra, make this technique difficult to apply in the case of heroin. The use of higher field magnets on smaller naturally occurring molecules, where availability is not limited, was expected to increase the sensitivity and improve the peak resolution.

1.4.9 Other methods used in heroin profiling

Capillary gas chromatography is not the only technique used in heroin analysis/profiling of main constituents, minor impurity composition or cutting agents. However, its high separation power and sensitivity has not left much room for other techniques. Thin layer chromatography (TLC) has been mainly used in adulterant identification and comparison purposes based on main alkaloidal composition [35]. Other spectroscopic techniques such as FTIR [72, 73] or Raman spectroscopy [74, 75] have been used as non-destructive
methods but exclusively for the main active compound (diacetylmorphine). Two techniques which are worth mentioning are HPLC and CE.

1.4.9.1 HPLC methods in heroin profiling

Liquid chromatography coupled to various detectors has also been used in heroin analysis/profiling. It overcomes some problems associated with GC separation as absorption, decomposition, transesterification [41], inability to elute higher boiling point components etc. However, the lower separation power due to the lower peak capacity limits the applicability of liquid chromatography techniques in separation of highly complex heroin impurity matrixes. On the other hand, preparative and semi-preparative LC are widely used in isolation and characterisation of many impurities present in heroin samples.

Lurie and Allen [63] have developed a HPLC method for separation, isolation and detection of acidic and neutral acetylated products arising from the rearrangement of opium alkaloids when reacted with acetic anhydride. Ultraviolet (UV), flame ionisation (FID) and electron capture (ECD) detectors in series have produced highly sensitive and selective detection of studied compounds. Similarly, a HPLC-UV method for simultaneous analysis of heroin, basic impurities and the most common adulterants in heroin samples has been developed [64]. Improved peak capacity over classical HPLC was obtained by applying a 1.7 µm particle size column at elevated pressure [65]. It has been shown that the peak capacity and sensitivity can be even greater than that of capillary electrophoresis (CE) by 2 and 15 fold, respectively.

Further improvement in application of LC in heroin profiling was contributed by coupling MS to the separation system. Single MS [66] was applied for fast separation (5 min run time) and quantification of major alkaloid components. The lack of the method sensitivity was compensated by its high throughput, and since the major components are usually found at higher concentration the method has been shown to suit heroin profiling.
Tandem mass spectrometry coupled to ultra performance liquid chromatography (UPLC-MS/MS) [67] has also been applied in heroin profiling. Many unreported solutes, amongst the basic impurities, were detected in heroin samples from SWA. UPLC-MS/MS has shown significant increase in selectivity and sensitivity versus photo diode array (PDA) detection. However, several problems encountered in the separation steps, such as hydrolysis of heroin at basic pH, double peaks obtained when using acidic injection solvent and decreased sensitivity when aprotic solvent (acetonitrile) was used, were still reported.

1.4.9.2 CE in heroin profiling

Capillary electrophoresis (CE) like HPLC has been primarily used for separation and quantification of major components and adulterants in heroin samples [81-83], rather than used for impurity profiling. It shows greater peak capacity than HPLC, generally shorter run time and economy of use [68].

Lurie et al. [69] have developed a sensitive determination of acidic and neutral impurities in heroin samples based on PDA and laser-induced fluorescence detection. The sensitivity for phenanthrene-related impurities, which show high native fluorescence under 248 nm wavelength excitation, was up to 500 times (acetylthebaol) higher with laser induced fluorescence (LIF) detection when compared to UV detection. However, its application in organic impurities profiling is limited due to the lower peak capacity compared to capillary GC methods.

1.5 ILLICIT COCAINE PRODUCTION

Cocaine is a plant-derived illicit drug and one of the most frequently abused. Coca leaf chewing has a long history (over one thousand years) among South American indigenous peoples. It has been used in the past as an ingredient in wines, medicines, soft drinks
(Coca Cola) and cigarettes [70]. Today it represents one of the biggest global problems, with an estimated 16 million people aged 15-64 who had consumed it in 2006/07 [1].

1.5.1 Coca leaves

Whole cocaine which is available in the clandestine market derives from the leaves of the genus *Erythroxylum*, a plant indigenous to the Andean region of South America. The plant resembles a blackthorn bush, and grows to a height of 2–3 m. The branches are straight, and the leaves, which have a green tint, are thin, opaque, oval, and taper at the extremities. A marked characteristic of the leaf are the two longitudinal curved lines, one line on each side of the midrib.

The leaf of the plant has a long history as a stimulant, local anesthetic, appetite and fatigue suppressant, cure for many illnesses, and it was chewed for centuries by some indigenous communities. Ancient Peruvian mummies have been found with the remains of coca leaves and in 2005 cocaine and benzoylecgonine were detected in nails and hair of mummies dating almost 1000 BC [71]. The plant is grown historically as a source of homeopathic medicine but the cultivation for production of illicit cocaine has become a major global problem in the last century.

There are four recognized varieties of two species that contain significant level of cocaine [72]: *Erythroxylum coca* var. *coca* (ECVC), *Erythroxylum coca* var. *ipadu* (ECVI), *Erythroxylum novogranatense* var. *novogranatense* (ENVN) and *Erythroxylum novogranatense* var. *truxillense* (ENVT). ECVC (Bolivian coca) has been considered historically as the source of most of the world’s supply of cocaine. It is found on the eastern slopes of the Andes in areas of humid montane forests. ECVI (Amazonian coca) was considered to be cultivated only sparingly in the western part of the Amazon Basin. ENVN (Colombian coca) existed as a plantation crop in Colombia and as an ornamental plant throughout the tropics. ENVT, mostly confined to the desert areas of northern Peru, was cultivated for ‘coca-chewing’ and in the
19th and early 20th centuries, for flavouring of an internationally popular, cola-based soft drink.

According to the WDR for 2008 [1] the total area under coca cultivation in 2007 was 181600 ha, the highest level since 2001. Fifty-five per cent of coca bush was cultivated in Colombia, followed by Peru (30%) and Bolivia (15%).

1.5.2 Cocaine extraction

Although the stimulant and hunger-suppressant properties of coca had been known for many centuries, the isolation of the coca alkaloid was not achieved until 1855, when cocaine (40, Figure 1.10) was first isolated by the German chemist Friedrich Gaedcke [70].

The sun-dried coca leaves (100-150 kg) are mixed with an alkaline substance, usually lime, and the resulting pulp is crushed. Kerosene is added and the mixture is stirred, allowing alkaloids extraction into the organic phase. After separation and filtering, kerosene is mixed with acidified water (usually H$_2$SO$_4$) extracting alkaloids into aqueous layer. The two immiscible solvents are separated, aqueous phase being made alkaline to force alkaloid precipitation while the kerosene is usually recycled for the next batch. The precipitate is filtered and dried as a final product (coca paste, ~1 kg) of the “pasta lab” [73]. Coca paste can be processed to cocaine base by further purification which is achieved by dissolution in diluted H$_2$SO$_4$ and adding KMnO$_4$ solution. The olefinic moiety of cinnamoylcocaines, as the main impurities in coca paste, is being attacked by KMnO$_4$ giving a water soluble products which are removed after precipitation of cocaine base by adding lime or ammonia. The purity of this product is usually 70-90% [74]. If further purification is required, coca base is converted to cocaine hydrochloride (cocaine HCl) by dissolution in diethyl ether in which concentrated HCl in acetone is added. The white precipitate (cocaine HCl) is filtered and dried. The purity of this product can be as high as 99% [75]. In 2007, global potential production of cocaine reached 994 metric tons [1].
1.5.3 Impurities in cocaine

Many components are co-extracted with cocaine during the production process to the final product. They originate from the two main sources: naturally occurring components in coca leaves and processing by-products.

Naturally occurring minor alkaloids are often found in the final product, even in the highly refined cocaine. Their similarity in structures and properties to the major components result in co-extraction and “survival” during the production process. Since their variety and relative concentration depend on climate conditions, geographic region, taxonomy, processing steps applied etc. they represent a valuable source of data for sample comparison and drug profiling. Tropacocaine (41), cis-cinnamoylcocaine (42), trans-cinnamoylcocaine (43) and truxillines (44, 45, Figure 1.10) are the most common found in illicit cocaine samples. Hydroxycocaines (46) and trimethoxycocaines (47-50) isomers are present at lower levels in coca leaves and cocaine samples. While cuscohygrine (51) and hygrine (52) are present at higher levels in coca leaves their solubility in water and volatility make them less encountered in the refined cocaine samples. A summary of coca leaves composition is given in Table 1.2 and their structures are given in Figure 1.10.

In addition to the naturally occurring minor alkaloids, processing by-products are another group of components found in the final product. They can be formed in a variety of chemical or physical processes. One of the most common is the hydrolysis of the major alkaloid – cocaine.
Figure 1.10 Major and minor alkaloidal components originating from coca leaves: 40. Cocaine, 41. tropacocaine, 42. cis-cinnamoylcocaine, 43. trans-cinnamoylcocaine, 44. α-truxilline, 45. β-truxilline, 46. 6-hydroxytocaine, 47. trimethoxycocaine, 48. trimethoxytropacocaine, 49. trimethoxy-cis-cinnamoylcocaine, 50. trimethoxy-trans-cinnamoylcocaine, 51. cysohygrine, 52. hygrine. (Continued on page 41)
The hydrolysis of cocaine (and tropane alkaloids in general) usually occurs at ester linkages, thus producing benzoylecgonine (53), ecgonine methyl ester (54), ecgonine (55) and benzoic acid (56) [75]. Minor alkaloids can also undergo hydrolysis at ester sites, giving rise to the complexity of the impurity profiles of cocaine samples: cinnamic acid (57) is formed from cinnamoylcocaines, and truxinic/truxillic acids (58, 59) are formed from truxillines (Figure 1.11).
Figure 1.11 Cocaine and truxillines hydrolysis products: 53. benzoylecgonine, 54. ecgonine methyl ester, 55. ecgonine, 56. benzoic acid, 57. cinnamic acid, 58. truxinic acid, 59. truxillic acid.

Another type of impurities, so called “N-nor” series (Figure 1.12), are components oxidized by the peroxides which are usually present in diethyl ether. N-norcocaine (60), N-formylnorcocaine (61) and N-benzoynorecgonine methyl ester (62) are formed during the demethylation process of the respective components.

Figure 1.12 Nor-series of impurities found in cocaine samples: 60. N-norcocaine, 61. N-formylnorcocaine, 62. N-benzoynorecgonine methyl ester.
The use of strong bases can cause epimerization and rearrangement which results in formation of traces of some pseudo-epimers [76] and N-nor esters [74]. The excess use of KMnO₄ in the purification step can oxidize cocaine to yield trace levels of N-formylnorcocaine (61) [73, 74]. The quantitative level of these by-products can vary by several orders of magnitude in cocaine samples. Except for cocaine hydrolysis products, which are usually detected at levels over 1%, the rest are present at levels well below 1%. Major alkaloids in coca leaves, and cocaine impurities, are given in Table 1.2.

Recently, two new groups of cocaine impurities have been identified in cocaine samples: six tropane ethyl esters (63-66, Figure 1.13) have been detected in samples purified by using ethanol instead of KMnO₄ [77], and four incompletely oxidized cinnamoylcocaines (67-70, Figure 1.14) in samples oxidized by KMnO₄ from neutral instead of acidic medium [78]. Cocaethylene (63) has been detected in high amount (up to 20%) in smuggled cocaine samples dissolved in ethanol (wine or liquor) [79]. A cocaine sample smuggled in a bottle of wine and seized at the airport in Skopje (Macedonia) is shown in Figure 1.15.

Figure 1.13 Ethyl esters of cocaine alkaloids encountered in cocaine samples: 63. cocaethylene, 64. 3',4',5'-trimethoxybenzoylecgonine ethyl ester, 65. cis-cinnamoylcocaine ethyl ester, 66. trans-cinnamoylcocaine ethyl ester.
Figure 1.14 Recently encountered incompletely oxidised cinnamoylcocaines by-products: 67. (2S,3R)-dihydroxy-3-phenylpropionylecgonine methyl ester, 68. (2R,3S)-dihydroxy-3-phenylpropionylecgonine methyl ester, 69. (2R,3R)-dihydroxy-3-phenylpropionylecgonine methyl ester, 70. (2S,3S)-dihydroxy-3-phenylpropionylecgonine methyl ester

Figure 1.15 Cocaine smuggled in a wine bottle and seized at Macedonian borders.
Solvents used in dissolution/precipitation steps can be trapped in the cocaine crystals, representing another valuable dataset for sample comparison, geographic origin and process determination. The most common encountered are acetone and methyl ethyl ketone, followed by diethyl ether, benzene, toluene etc. Their usual concentration is from zero to several hundreds ppm [80].

Table 1.2 Alkaloidal composition of coca leaves and illicit cocaine samples, expressed as % values relative to cocaine content. Data compiled from ref. [75].

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Coca leaf (%)a</th>
<th>Illicit cocaine (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>0.23-0.96</td>
<td>80-97</td>
</tr>
<tr>
<td>Cis-cinnamoylcocaine</td>
<td>0-53</td>
<td>1.9</td>
</tr>
<tr>
<td>Trans-cinnamoylcocaine</td>
<td>0-170</td>
<td>1.2</td>
</tr>
<tr>
<td>Tropacocaine</td>
<td>0.2-5</td>
<td>0.02</td>
</tr>
<tr>
<td>Truxillines</td>
<td>2-60</td>
<td>2-13</td>
</tr>
<tr>
<td>Ecgonine methyl ester</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hydroxycocaines</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Trimethoxycocaines</td>
<td>0.1-1</td>
<td>0-0.1</td>
</tr>
<tr>
<td>Cuscohygrine</td>
<td>10-80</td>
<td>-</td>
</tr>
<tr>
<td>Hygrine</td>
<td>1-25</td>
<td>-</td>
</tr>
</tbody>
</table>

a All % values are relative to cocaine content, except for the cocaine (absolute content).
b Present in cocaine samples due to cocaine hydrolysis.

Among the naturally occurring alkaloids and by-products, a variety of other substances can be seen in illicit cocaine samples. They are used as cutting agents to increase the mass of the drug, thus the profit. Procaine, benzocaine, lidocaine, caffeine, phenacetine and sugars (inositol, mannitol, lactose, dextrose) are the most common.

1.5.4 Gas chromatographic methods for cocaine impurity profiling

While heroin profiling studies were first initiated during the 1960s and by the mid-1970s such studies were ongoing in several national laboratories [9], one of the first work on
cocaine profiling was published by Casale and Waggoner in 1991 [81]. The knowledge gathered during the past 20 years in the area of heroin profiling, the greater number and frequently the higher relative concentration of impurities encountered in cocaine samples compared to heroin samples, plus the fact cocaine alkaloidal impurities are not modified by a synthetic step, facilitated the development of this procedure and several cocaine impurity and sample comparison methods were published in the next few years [88, 89, 97-104]. Several other works, even though not related directly to impurity profiling, have given a great contribution in this field by characterizing a large number of impurities present in coca leaves and cocaine samples. Among other components, 11 truxillines [82], N-formyl cocaine [73], several N-nor series [74] and hydroxycocaine impurities [83], four trimethoxy-substituted analogs of cocaine [84], hygrine and cuscohygrine [85], and recently detected four cinnamoyl-oxidation products [78] and 6 tropane ethyl ester homologues [77], have been characterized. Furthermore, about 100 uncharacterized impurities have been detected in South American coca leaves [86], many of them being removed during the production/purification process.

LeBelle et al. [87] have reported a sample comparison method based on the relative ratio of the three main alkaloids: cocaine, cis- and trans-cinnamoylcocaine, supported by visual inspection of chromatograms and occluded solvents present in samples. The methodology was similar to the methodology used in heroin profiling [19]. The high frequency occurrence of cinnamoylcocaines in cocaine samples and their stability have shown to be a good marker for distinguishing large production batches or manufactures. Janzen et al. [88] have added two more alkaloids (tropacocaine and norcocaine) in their sample comparison method based on NPD detection and statistical evaluation of similarity. However, the final confirmation was achieved after visual inspection of the chromatograms of possibly related cocaine samples. The method has been successfully applied in solving a real forensic case.

A review of potentially good impurities for profiling of cocaine samples has been given by Ensing et al. [89]. After detailed consideration of the known cocaine alkaloids, selection
has been made of 6 components, excluding the main drug. The fingerprint profile of each sample was constructed in this manner: a) each peak’s height is measured; b) the highest peak is given a reference value 10; c) the value for all other peaks is calculated as the ratio to the highest peak multiplied by 10. The calculated component values drawn against their retention times, so called pictograms, represent an easy way of sample comparison, similar to the mass spectra similarity comparison. The method results have been seen as useful contributing evidence to the total investigation rather than a definite proof of linked samples.

A simple one-step derivatization procedure for obtaining chromatographic impurity signature profile analysis (CISPA) of cocaine samples has been developed by Casale and Waggoner [81]. 14 components from the chromatogram have been selected as variables for statistical evaluation of sample similarities by using PCA. Good overall absolute error of the assay (less than 1%) has been reported, most probably because of the derivatization step applied and the very good chromatographic profiles obtained during separation of the components on DB-1701 capillary column. It has been shown that different and unique impurity profiles were obtained for hundreds of samples from different batches, and very similar profiles from linked samples (from same batch). Based on this method, Casale and Watterson [90] have added two new impurities and have applied a neural network pattern recognition software to ascertain whether the impurity signature comes from the same batch. Only 7 out of 50 laboratory reports provided for cocaine conspiracy investigation were inconclusive, the rest confirming the linked or non-linked samples. However, the authors have suggested that in no instance the conclusion should be based solely on computerized search. Visual comparison of the reference chromatogram to narrow field of possible matches was necessary.

An excellent review on in-depth chromatographic analysis of cocaine and coca leaves has been published in 1994 by Moore and Casale [75]. The rationale for presence of impurities and artefacts in cocaine samples has been discussed, and a comprehensive compilation of mass spectra of derivatized and underivatized major components and impurities is given.
A brief review on analytical methods used in comparison of heroin and cocaine samples has been given later by Chiarotti and Fucci [30].

Recently, an effort has been made for harmonization of cocaine profiling method for data exchange between two laboratories from two different countries [9, 106], in a similar manner as previously done on amphetamine-like substances (ALS) [91] and heroin [31]. Eight main alkaloids have been selected and the most influential parameters have been optimized for the best performance. It has been demonstrated that the varied storage conditions (temperature and humidity) do not influence significantly the impurity profiles during the studied period of at least three months and the selected eight alkaloids were convenient for cocaine profiling. The chromatographic conditions were based on previously published methods [81, 88]. Six pre-treatment steps and eleven data treatment methods were tested for best discrimination between linked and non-linked samples. Normalization plus standardization followed by cosine function or Pearson correlation coefficient (N + S / cosine and N + S / Pearson) have been shown to be the best combinations. The correlation coefficient, based on similarity of linked samples and dissimilarity of non-linked samples, was lower for between-laboratories comparison than within-laboratory, but still discrimination of linked from non-linked samples based of the data from two laboratories was possible. This has demonstrated that sharing of databases and chemical profile results between two laboratories using instruments from different producers is possible and apparently there is no need for analyses to be centralized (done in a unique laboratory) in order to compare cocaine sample. However, the authors have stressed the precautions when more than two laboratories are to be included in the harmonisation process, as an expected flattening of the conclusive discriminations, lowering the threshold level for similarity of linked samples and overall reduction in method efficiency would occur.
1.5.4.1 Profiling of solvents occluded in cocaine

Most of the clandestine amine drugs, including cocaine, are produced in hydrochloride salt form. This is obtained either by adding concentrated HCl dispersed in water miscible organic solvent or by bubbling HCl gas into the organic phase of the respective free base [47]. The rapid precipitation of the salt is followed by trapping significant quantities of solvent into the crystal matrices, so called occluded solvents. Residual solvents due to incomplete drying can also be detected, especially in freshly prepared samples. The variety of these solvents and their relative ratio can be used to support both strategic (origin determination) and tactical (sample-to-sample comparative analysis) intelligence.

Detection and characterisation of solvents encountered in illicit cocaine samples [48] have proceeded on the occluded solvents profiling methods. One of the first applications of solvent profiles in sample comparison was published in 1991 [87]. It has been demonstrated that this profile can further discriminate an accidental coincidence of linked cocaine samples and erroneous conclusions based on major alkaloid ratio similarity. However, the authors did not comment on the possibility of different post-production treatment of the linked samples, which in fact can produce a change in the solvent profiles.

A great contribution in solvent profiling has been done by the work of Morello et al. [47] in which the residual solvents composition in 75 cocaine and 826 heroin samples were determined. From 2 to 14 solvents were detected in cocaine HCl samples, compared to 0 to 8 solvents detected in heroin HCl exhibits, which is mainly due to the much larger cocaine HCl crystals compared to heroin. Static head space was used for concentration and sampling of volatiles and the few co-elutions reported did not interfere with determination due to the selectivity of MS detection. The method can also be applied on diluted samples since adulteration had no significant influence on quantitative data.

Activated charcoal has also been used for trapping and concentration of solvent vapour in cocaine samples [48] with subsequent elution with carbon disulfide (CS₂). A larger number of solvents (16) have been detected in cocaine samples than in heroin samples (12), which
is in agreement with the previous finding [47]. However, combination of CS$_2$ from different producers is recommended to use in this method since some impurities found in commercial CS$_2$ interfered with the determination.

A method for occluded solvents profiling based on SPME concentration and sampling has been developed by Chiarotti et al. [92]. The method overcame the abovementioned problem with impurities present in CS$_2$ since no solvent was needed for extraction of volatiles. 32 different volatiles have been detected in 47 analysed cocaine samples, with acetone, diethyl ether, ethyl- and propyl acetate, 2-butanone, methyl chloride and hydrocarbons being the most encountered. Statistical evaluation of 11 selected solvents by using PCA and HCA has successfully classified the samples in four distinct groups. The method has been proposed as an additional tool for comparative analysis of cocaine samples, adding more details about the chemical fingerprinting of compared samples.

A comprehensive method for solvent residue profiling of cocaine samples has been recently published [93]. The method is based on HS sampling and GC separation on a non-polar HP-1 column with FID detection. 18 residual solvents were selected for statistical evaluation of samples similarity by using computerized and fully automated data analysis. Cosine function, already applied in cocaine [94] and heroin [38] impurity profiling, showed a good discrimination between linked (correlation value 100) and non-linked (correlation value 0) samples, based solely on the residual solvent profiles. The threshold value which differentiates these two groups was fixed at 99.4 providing minimum false positives (0.01%) and false negatives (0%). However, several coelutions of solvents have been reported.

1.5.5 Stable isotope-ratio profiling for cocaine samples

The isotopic composition of plant materials, i.e. cocaine, is fixed during biochemical synthesis and is highly dependant on the variations in the metabolic pathway or
environmental conditions during growth [60]. For example, plants grown under high humidity or water-rich soil may have $\delta^{13}$C values up to 4-6 ‰ more negative than plants produced under low humidity or water-stressed conditions. Similarly, the type of soil and its microbial N$_2$ conditions can influence the $\delta^{15}$N composition. This feature is used to trace the geographic origin of natural materials, i.e. cocaine, or the “natural” part of the semi-synthetic products, i.e. heroin. Unlike other methods, isotope-ratio profiling is production-process independent and is solely related to the growing region and conditions.

Ehleringer et al. [58] have shown for the first time that successful growth-region classification of cocaine samples can be done according to their $\delta^{13}$C and $\delta^{15}$N stable-isotope abundances. Whilst based on a limited number of cocaine samples (28) all samples were correctly classified according to their geographic origin (Bolivia, Peru, Ecuador and Colombia). In an extended work conducted soon after the above [95], 90% of 200 cocaine samples have been clearly classified according to their geographic origin, based solely on $\delta^{13}$C and $\delta^{15}$N abundances. When these results were combined with the impurity profiling results of the same samples, the ratio of successfully classified samples was 96%. Similar results were obtained later by Galimov et al. [60] by using both GC-combustion (GCcIR) and elemental analysis (EAIR) isotope ratio. However, while the former is recommended for diluted samples, the latter one is more suitable for undiluted samples.

The conclusions drawn for application of SNIF-NMR in heroin profiling [62] are applicable also for cocaine samples. The only difference is the completely natural moiety of cocaine molecule and the lower molecular mass, which partially improve the S/N ratio of NMR spectrum.

### 1.5.6 Trace metal contaminants profiling

The general conclusions regarding trace metal profiling of cocaine samples are basically the same as the ones given for heroin. Difficulties in data interpretation and rationale for
elements present make this profile less useful in geographic origin determination, while sample-to-sample comparison can be greatly enhanced by using this data. Limited number of works has been published in the literature.

Violante et al. [53] have applied ICP-AES for cocaine sample comparison based on data from analysis of 11 elements but no conclusive relationships have been attained due to the largely uncertain sources of the samples analysed. On the other hand, cocaine samples entering Spain at Galicia have been successfully grouped into three groups, two of them related to the international airline flights coming from Colombia and Venezuela [96]. 15 elements have been analysed in 46 samples and pattern recognition analysis (PCA, HCA) have been used for classification. Authentic cocaine samples and additional data on volatiles and impurity profiling are necessary in order to elucidate the geographical origin.

### 1.5.7 Other methods used in cocaine profiling

Several other techniques have been used in selected cocaine trace components and by-products analysis rather than in comprehensive impurity profiling. HPLC has been used for separation of the main acidic components (benzoic acid, cinnamic acid, isomers of truxillic/truxinic acids) and some minor components present in cocaine samples [97]. CE has been found superior in heroin and cocaine impurity analysis, separating twice as many peaks as in HPLC, but at an increasingly lower sensitivity [98]. However, CE coupled to laser induced fluorescence (LIF) detector has been found 100 times more sensitive than PDA for cocaine components exhibiting native fluorescence [99]. Although these detectors can be useful in drug analysis, their highly selective response to analytes makes them unsuitable for comprehensive impurity profiling [100]. The recent advance in coupling with MS will probably widen the applicability of these liquid phase separation techniques in drugs impurity profiling.
1.6 ECSTASY PROFILING

Ecstasy is a street name of the tablets (pills) containing the purely synthetic drug 3,4-methylenedioxyamphetamine. Synthesized at the beginning of the last century and seemingly forgotten until a few decades ago, the drug became a widespread especially among youth. It is estimated that 9 million people worldwide aged 15-64 had consumed ecstasy in 2006 [1].

1.6.1 MDMA history

3,4-methylenedioxyamphetamine (MDMA, ecstasy, 71) is a ring-substituted phenethylamine, the core structure (the blue in Figure 1.16) of central nervous stimulants (CNS) – amphetamines. Although synthesized in 1913 by Merck and patented in 1914 as an appetite suppressant, the drug was never marketed and was rapidly forgotten.

![Figure 1.16 Chemical structure of MDMA (71)]

The drug re-appeared in the late 1970s when it was first used to aid psychotherapy (the results of which are poorly documented) and later for recreational use. In 1990’s its popularity rocketed and illegal production has risen significantly. Users of the drug say it produces a positive sense of well being and empathy to others but prolonged abuse seems to cause brain damage and impairments in visual and verbal memory. After several reported MDMA fatalities, it has been prohibited since 1986 as a drug with no legitimate medical use, and is probably best known as the controlled substance in the club drug
“ecstasy”. Today, ecstasy tablets refer to clandestine produced tablets containing purely MDMA (in Europe and North America) or MDMA and/or other controlled drugs such as 3,4-methylenedioxyamphetamine (MDA), ketamine, amphetamine, methamphetamine etc. (in Asia). They have become a “necessary part” of rave scenes organized all around the world.

1.6.2 MDMA synthesis

Many precursors and routes of clandestine MDMA (71) production have been reported in the literature and the choice is generally driven by the precursors’ availability, the simplicity of the synthesis, the “underground chemist’s” skill etc. However, most of the synthesis routes start with materials with pre-formed methylenedioxyphenyl ring such as safrole (72), isosafrole (73), piperonylmethylketone (PMK, 74) and piperonal (79). PMK is mainly obtained by oxidation of isosafrole, which is obtained by isomerisation of safrole extracted from sassafras oil (Figure 1.17).

![Figure 1.17 Scheme of PMK production from safrole: 72. safrole, 73. isosafrole, 74. PMK](image)

It seems reductive amination of 3,4-methylenedioxyphenyl-2-propanone (3,4-MDP-2-P, PMK, 74) with methylamine via a Schiff base (75) formation is the most used synthesis route in clandestine ecstasy production (Figure 1.18a). At least 6 different routes have been reported in the literature [101]. Borohydride (NaBH₄) reduction at low temperature, dissolving metal reduction (Al/Hg) and cyanoborohydride (NaBH₃CN) reduction are the most often encountered. The general scheme of this reaction is given in Figure 1.18a.
a) 

\[
\begin{align*}
\text{O} & \text{O} \\
\text{O} & \text{N} \\
\text{NaCNBH}_3 & \\
\text{NaBH}_4 & \\
\text{Al(Hg)} & \\
\text{O} & \text{O} \\
\text{H}_3\text{NCHO} & \\
\text{HCOOH} & \\
\text{HCONHCH}_3 & \\
\text{hydrolysis} & \\
\end{align*}
\]

Figure 1.18 Schemes of the main ecstasy production routes: a) reductive amination, b) Leuckart reaction, c) safrole bromination.

Leuckart reaction (Figure 1.18b), which involves reaction of PMK with formamide in presence of formic acid and consequent hydrolysis of the obtained intermediate (76), is less encountered, though it is predominantly used in clandestine amphetamine synthesis. Safrole bromination is also used where safrole is brominated by HBr to 77 followed by methylamine substitution (Figure 1.18c).

In 2007 a new pre-precursor (methyl 3-[3’,4’-(methylenedioxy)phenyl]-2-methyl glycidate, 78 in Figure 1.19) was encountered in Australia, as an alternative route for 3,4-MDP-2-P production, the main MDMA precursor [102]. It seems the list of the precursors and production methods of designer drugs is never ending.

According to the batch concept (see section 1.3.2), each sample from the same batch has the same, or almost the same, compositional pattern which depends on the precursors
used, the synthetic route and the purification processes applied in the production. Minor amount of precursors and their impurities, and by-products formed during the synthesis are usually co-extracted in the final product, and together with the components added as binders, lubricants and excipients in the tablettng process, comprise the chemical profile of samples. The variety of components encountered in clandestine produced ecstasy and their relative ratio enables comparative analysis of exhibits. Moreover, the synthetic method can be assigned based on the specific markers (precursors, by-products and intermediates) characteristic to that route. Selected impurities and by-products found in ecstasy samples are given in Figure 1.19.

Swist et al. [103] have compared impurities found in MDMA samples which were synthesized by five different routes of production and they found several unique markers or groups of components characteristic for only a particular route. Reductive amination has been found to be the predominant route of synthesis of ecstasy tablets seized in France based on some key markers [118, 119]. However, the possibility of different origin for the same impurity, i.e. the overlap between impurities generated by different routes [104], which may be interpreted as “inefficient chromatographic separation of some impurities or their low levels” [105], hinder the determination.

Once the synthesis is completed, the final product is purified by liquid/liquid extraction (LLE), vacuum distillation and crystallization. The powder from a single production batch, or more likely from several production batches, is then mixed with substances necessary to produce solid form of drugs (excipients), artificial colours, lubricants (palmitic and stearic acids) and adulterants (caffeine, ketamine), homogenized and pressed into tablets. Tabletting usually occurs at remote locations. Different logos are usually stamped on the top as a sign of “quality”, and the list of logos is ever increasing. Some of the tablets seized in Macedonia in the period from 2000 to 2003 are given in Figure 1.20.
Figure 1.19 Selected precursors and by-products frequently encountered in ecstasy tablets (adapted from ref. [106]).
The ecstasy production process does not include purely natural components as in the case of heroin (opium) and cocaine (coca leaves), and is not region-related. Clandestine synthesis can be done virtually anywhere, from rural sheds and farms, through storage facilities, to kitchens in urban areas. The production scale is from personal use level (several grams) to as large as a ton. The estimated annual production for 2006 was 4.5 metric tonnes and the largest producers are The Netherlands and Belgium [1]. According to the WDR for 2008 [1], regional shift in ecstasy production has been observed in the last decade. Despite the dominance of West and Central Europe in the ecstasy trade, the general trend has been towards an increase in ecstasy production, trafficking and abuse in North America. This is clearly reflected in seizure statistics where the share of West and Central Europe in global ecstasy seizures fell from 79% in 1995 to 43% in 2006. In contrast, in 1995 North America accounted for 20% of ecstasy seizures, rising to 34% by 2006. Similarly, Oceania's proportion increased from 1% to 12%. According to Australian authorities the main origin countries in 2005/06 for shipments of ecstasy to Australia were Canada, Belgium, the UK and France.
1.6.3 Gas chromatographic methods for ecstasy impurity profiling

While volatile components and metal contaminants have been reported in ecstasy profiling, minor organic impurities and stable isotope-ratio are the most exploited. Extraction of impurities with organic solvent is a common practice since some markers are present at a very low level. However, the impurity profile is strongly influenced by the pH. Most of the basic impurities are efficiently extracted from alkaline medium but the huge MDMA peak can interfere with some co-eluting key markers. On the other hand, MDMA is almost absent in the acidic extract, but far fewer impurities, with those being at low levels, are obtained in this way.

Palhol et al. [101] have developed a method for profiling of ecstasy tablets based on capillary gas chromatography separation of the impurities extracted with dichloromethane from alkaline aqueous solution. Almost 30 compounds (in 52 samples) were identified as precursors, intermediates and by-products. The presence/absence of the key marker components and their relative ratio were used as criteria for grouping the samples in three main classes, while further discrimination is obtained by applying HCA to the 27 impurities as variables. Two case samples have been reported as an example of applicability of the method in forensic sample comparison.

Soon after this work, a similar method was developed [104], based on impurities extracted by diethyl ether from an alkaline (pH = 12.8) aqueous solution. Several new impurities such as p-methoxymethamphetamine (PMMA, 85) and 3,4-methylenedioxy-N,N-dimethylbenzylamine (91) were detected and characterized. Chemical ionisation (CI) and tandem MS were used for confirmation along with electron ionisation mode. The overlap of the impurity origin, or the possibility for some markers to originate from different routes, was stressed. The cosine function was used for comparison of chromatographic profiles and for evaluating similarity/dissimilarity among exhibits.
Gimeno et al. [107] developed an optimized and reproducible method for ecstasy impurity profiling, since they had found great variation in the extraction procedures and conditions used and reported in the literature. Slow evaporation of the diethyl ether extract obtained at pH of 11.5 with solvent volumes precisely controlled, were among the main conclusions. For best results the extracts should be analysed the same day they are prepared. 8.5% within-day and 10.5% between-days RSD for repeated extraction under optimized condition were achieved.

Profiling of ecstasy tablets based on major components and physical characteristics (ballistics) has been reported on exhibits seized in Japan [108], and profiling based on minor organic impurities in Hong Kong [109] and The Netherlands [106]. In the latter case a statistical approach (Pearson correlation) was used for sample similarity assessment.

An excellent review covering organic impurity, composition and trace metal profiling, as well as future directions in ecstasy profiling, has been published [110]. SPME, ICP-MS and ANN have been recognized as the areas where significant development is to be expected in the future.

The most comprehensive ecstasy profiling work so far has been conducted under the auspices of the European project “Collaborative Harmonisation of Methods for Profiling of Amphetamine Type Stimulants” (CHAMP), in cooperation between 6 European countries [124, 125]. Since MDMA is mostly distributed in form of tablets, and the tableting process is separated from the production process, pre-tableting (pre-TB) and post-tableting (post-TB) batch terms have been introduced. In this way irrespective of the post-TB process (i.e. tablets with different colour, logo and dimensions), samples from the same pre-TB batch can be linked based on their impurity profiles. Moreover, irrespective of their pre-TB process (i.e. tablets from different production batches) samples from the same post-TB can be linked based on the physical characteristic of the tablets. While diameter, thickness, weight and score are reliable and reproducible parameters colour, shape side, edge profile
and logo are excluded as highly operator-dependent (low reproducibility for a given manufacturing process). However, the link between samples based on physical characteristic has been found far less reliable than the link based on impurities present in samples. The discrimination power of the final target eight selected components was found to be nearly the same as the discrimination power of the 32 previously selected impurities. 46 organic impurities in total have been detected in chromatograms of the samples analysed, based on the method developed by van Deursen et al. [106]. The applicability of the method on an international basis was confirmed through a case in which two tablets seized in different countries (The Netherlands and Switzerland) were linked based on their impurity profiles.

1.6.4 Stable isotope-ratio methods for ecstasy profiling

The synthesis of the majority of the designer drugs starts from natural products (essential oils) and their characteristic native isotope abundance is transferred to the final product, or at least on the “natural part” of the final product. As already stated, safrole, isosafrole and piperonal are widely used natural products in precursors and/or ecstasy production, and catechol, apiol, sesamol and myristicine aldehyde use are reported in the literature [126, 127]. Broader explanation of the stable isotope fixation in natural products is given in section 1.4.8. Grouping the samples according to their isotope-ratio similarities gives additional criteria in sample differentiation and common-batch origin relations.

One of the first works on stable isotope-ratio in ecstasy profiling was by Mas et al. [111]. 16 ecstasy tablets have been classified in four groups according to the δ¹³C values of MDMA molecules. Further discrimination was obtained by applying δ¹⁵N ratios, which resulted in improved selectivity of the method. Some samples which were previously belonged to the same δ¹³C group were discriminated according to their δ¹⁵N values. Palhol et al. [112] have extended the research, by analysing 106 ecstasy tablets. The large range of δ¹⁵N (from -17 ‰ to +19 ‰) allowed discrimination between different samples and the formation of five
groups with very similar profiles, which probably have similar synthetic pathway. Since the \( \delta^{15}N \) range of commercially available precursors commonly used in clandestine production has been found narrow (from -5.8 \( \% \) to +5.5 \( \% \)), the \( \delta^{15}N \) variation in MDMA was attributed to isotopic fractionation during the production process, which vary depending the routes of production, reagents and conditions applied [126, 127]. However, classification of the samples based solely on \( \delta^{15}N \) was in good agreement with the observed impurity profiles (synthetic route dependent).

Further discrimination between ecstasy samples has been obtained by adding \( \delta^2H \) ratio to the \( \delta^{13}C \) and \( \delta^{15}N \) results [129, 130]. Recently published work [113] has demonstrated that \( \delta^2H \) ratio can discriminate ecstasy samples produced by the same route (reductive amination) through three different reduction systems (Al/Hg amalgam, NaBH\(_4\) and Pt/H\(_2\)). Accurate discrimination of the synthetic route was achieved only when \( \delta^2H \) data was included on its own or in conjunction with \( \delta^{13}C \) and/or \( \delta^{15}N \). The discrimination power of the stable isotope-ratio enrichment follows this order: \( \delta^2H > \delta^{15}N > \delta^{13}C \). This is expected since the variation of \( \delta^2H \) was found the widest (-93.9 \( \% \) to +6.3 \( \% \)) and the \( \delta^{13}C \) was the narrowest (-26.5 \( \% \) to -28.1 \( \% \)). The \( \delta^{15}N \) variation was in the range from -3.6 \( \% \) to 23.8 \( \% \) and it was attributed to the fractionation during the production rather than due to the precursor. Similarly to the applications in heroin and cocaine profiling, SNIF-NMR has also been applied with limited success in ecstasy samples discrimination [114].

### 1.6.5 Trace metal profiling of ecstasy tablets

Possible relation between ecstasy samples with common origin has been demonstrated on the basis of 10 elements analysed [115]. Nine clusters have been defined from 91 unrelated samples. An extensive statistical data treatment has been applied on the results based on analysis of 14 metals in 99 ecstasy tablets obtained by ICP-MS [116]. Artificial neural network algorithms were superior compared to PCA and HCA in trace metal classification of ecstasy according to the seizure, showing 96-99\% successful prediction of origin. The
limited ability of PCA and HCA was attributed to the significant variation in the intensity of metals present within each seizure.

The catalyst or reducing agents most used in reductive amination were used as a base for synthetic route determination of ecstasy tablets [117]. The great variation in Pt, B, Hg, Li, Pd and Ni abundances has been shown to be a good discrimination parameter. The correct synthetic route (including the catalyst/reducing agent) was properly assigned in 48 out of 57 tablets based on Pt, B and Hg content. Al and Na, even though present in some reducing agents, were excluded from the calculation and classification because they are found to be present in many excipients and adulterants used in ecstasy tabletting. The authors concluded that the elemental composition analysis is the only way to discriminate different variations of the same reductive amination route of ecstasy production. However, the great variation in metal intensities between batches from the same production site limited the method applicability in production-specific origin determination. On the other hand it has been shown to be a valuable tool next to profiling based on the organic impurities in sample-to-sample comparison.

1.6.6 Volatile organic components profiling of ecstasy tablets

As for the other drugs, volatile components encountered in ecstasy tablets can play an important role in impurity profiling. The relatively volatile precursors and by-products are expected to be amenable for complex volatile profiles giving an additional tool in sample comparison. However, relatively few works so far have been conducted on volatiles in ecstasy.

The first work in ecstasy profiling by using SPME for extraction and pre-concentration has been published by Kongshaug et al. [118]. A mixed phase SPME fibre polydimethylsiloxane-divinylbenzene (PDMS/DVB) was found superior compared to the non-polar polydimethylsiloxane (PDMS), extracting both polar and non-polar impurities. In order to
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suppress the recovery of the bulky MDMA, and owing to its basic nature, slightly acidic medium (pH 5.0) was chosen for extraction. Under this condition the majority of MDMA is ionized and principally retained in the aqueous layer. Discrimination power of the volatile impurity profiles has been demonstrated on four non-linked ecstasy samples seized in Norway.

Recently, a comparison between LLE and SPME extraction of organic impurities in ecstasy has been conducted [135, 136]. LLE extraction has shown higher efficiency extracting 46 components compared to 31 extracted by SPME. This discrepancy could be explained by the fact that in the case of SPME the extraction of components is based on its volatility, while for LLE the extraction relies on its solubility. However, the most discriminative components related to the production route have been detected by both techniques. The applicability has been demonstrated in a case where three different specimens were linked based on both SPME and LLE extraction impurity profiles. The simpler sample preparation and the absence of solvents in the SPME procedure represent an unquestionable gain compared to LLE, considering health and environmental issues.

1.6.7 Other methods applied in ecstasy profiling

Other methods for ecstasy profiling have also been reported in the literature. A simple and fast procedure for sample screening before profiling has been proposed [119] by using SPE extraction and TLC separation of impurities. Different composition patterns were observed under UV light (366 nm and 254 nm) between samples produced by different synthetic routes. The method has been proposed as an inexpensive screening before more advanced and expensive methods (GC-MS, HPLC) are applied.

Raman spectroscopy has been used as a fast (50 tablets per hour) and simple method for discrimination of ecstasy tablets based on the relative ratio of the major components
(MDMA and excipients) [120, 121]. The inhomogeneity of sample composition, even within the same tablet, has been identified as the major problem in this method.

HPLC at elevated pressure with 1.7 µm particles has been found superior over conventional HPLC, offering more separated components and higher peak capacity for ecstasy impurity profiling [65]. An impurity profiling method based on SPE extraction and subsequent HPLC separation with 2 µm particles has been proposed [122]. Proper classification based on 30 selected impurities was achieved for all analysed samples according to their synthesis route.

1.7 INTRODUCTION TO DOPING AND DOPING CONTROL

It is in a human nature to compete against others in every field of endeavour. The sports arena is probably the most competitive. Athletes will attempt to gain advantage over their opponent in order to achieve superiority and to win the competition. However, there are many different pathways to the win.

Special diet and its effect on athletes’ performance had been recorded in ancient times, as early as 668 BC during the Ancient Olympic Games [123, 124]. The sporting industry has capitalized on the desire for superiority among athletes, spending millions of dollars in development and continuously improvement of exercise equipment and apparel. Professional coaching and special training methods [124], as part of the “winning principles” provided by many professional sports trainers, are other legal ways to “beat” competitors.

But, what happen when all legal methods are exhausted and the desire for superiority and advantage over other competitors is still sought? Ergogenic aids in the form of natural products, bland chemicals and animal extracts have been known since Greco-Roman
times. In recent times, the remarkable advances in science and biotechnology have favoured the introduction of synthetic molecules, recombinant hormones and genetic manipulation of athletes in the attempt to push human performances to the limit.

### 1.7.1 Brief history of doping in sport

The word doping originates from “dop”, a term used by tribes in South Africa that conventionally refers to a stimulant drink used in tribal ceremonies during the eighteenth century [125]. Its first appearance in English dictionary was in 1879, where it describes “the use of a drug in an attempt to enhance sporting performance” [123].

Doping has a long history, contrary to the opinion that it is a recent “fashion”. Ancient Greeks have used dried figs, honey, a portion of brandy and wine, bread with spices from juice of the opium poppy as a part of their special diet [124]. Various performance-enhancing plants extracts and drinks were also used during the ancient Olympic Games. Roman gladiators have used caffeine and strychnine to overcome fatigue and injuries. Most of these stimulants were derived from plants. The 19th century stimulant list was extended to include cocaine, nitro-glycerine, sugar cubes dipped in ether, mixture of champagne, brandy, hot drops of morphine, belladonna and strychnine.

In the 19th century doping was mainly associated with cycling and it is not surprising that the first reported fatality occurred during such an event. The English cyclist Arthur Linton died in 1886 during the 600 km race between Bordeaux and Paris. He is alleged to have overdosed on “tri-methyl”, thought to be a compound containing either caffeine or ether [123].

The beginning of the 20th century was characterized by the discovery of the potential of testosterone in sports. The use of testosterone skyrocketed when an 18-year-old horse won many races after testosterone treatment. Many sport trainers have realized the potential of
this and many more anabolic steroids, and began to advocate their use [124]. The unrealistic body shape and extremely large muscles of many body-builders at that time have been mainly attributed to testosterone and other anabolic steroids. Not surprisingly, anabolic steroids (AS) were referred sometimes as “breakfast for champions” in 1970s.

Stimulants were also prevalent in cycling. The 1960s and 1970s were known also as the “amphetamine decade” for anyone competing in cycling. Another English cyclist died during the 1967 Tour de France due to methamphetamine overdose [124]. However, not only anabolic steroids and stimulants were used in sport competition. Performance enhancing potential of many other molecules and methods has been discovered, used and abused during the last few decades of the “bio-technology revolution”. As the doping revolution “progresses” the awareness of the side effects and health risks in competition push authorities to tighten doping control in sport.

1.7.2 Brief history of anti-doping control

Although several athletes died during the late 19th and early 20th century, sports authorities remained passive. The first reported fatality at the modern Olympic Games which triggered an official action happened in 1960 when a Danish cyclist died during a road race in Rome. It was obvious that doping had been practiced for many years and the giant gap between use and control needed to be closed. The Union Cycliste Internationale (UCI) began to develop a set of rules and in 1967 the International Olympic Committee (IOC) created a “Medical Commission” (IOC-MC) to combat the misuse of drugs in Olympic Sports. This resulted in the first IOC banned substances list, produced in 1968 [123].

The first large scale doping control at modern Olympic Games occurred in Mexico (1968) when the first known positive case was recorded. At the 1972 Munich Olympic Games urine samples were screened for stimulants and narcotics by gas chromatography and for the first time mass spectrometry was introduced in doping control [126]. Seven positive
cases were reported. By the time of the 1976 Montreal Olympic Games, IOC had banned anabolic steroids (AS) due to their widespread misuse in sport. As a result, eleven athletes tested positive for AS. The number of positive samples at several consecutive Olympic Games is presented graphically in Figure 1.21.

![Figure 1.21 Number of adverse analytical findings (positive cases) at several consecutive Olympic Games that resulted in sanction [126]](image)

In the decades to follow, the use of performance enhancing drugs changed - rather than decreased - and became more secretive. As doping control tests improved in their identification of synthetic steroids, users moved on to testosterone (T) and other endogenous sterols. Unfortunately, neither GC nor LC can distinguished T produced by human body from exogenous T from the pharmacy or the black market. Extensive studies conducted between Moscow and Los Angeles Games resulted in introduction of a testosterone/epitestosterone ratio (T/E). T/E was introduced in 1983 as a criterion for exogenous testosterone doping and re-analysis of anonymous urine samples collected during Moscow Olympics in 1980 showed administration of testosterone in a considerable percentage of the samples [126]. In spite of this clear contravention of the spirit of the Games, no positive case was reported at the Moscow Games.

One of the most featured positive cases in the modern Olympics was the stripping of Ben Johnson’s gold medal at the 1988 Seoul Olympics, after setting a new world record of 9.79 s in the 100 m race. Two stanozolol metabolites were detected in the urine free fraction
after LLE, derivatization and GC-MS analysis. Interestingly, the subsequent investigation has shown that “at least half of the athletes who competed in Seoul used anabolic steroids to enhance their performance” [127].

However, the use of AS in sports posed a new problem for sport authorities. Unlike stimulants, which act immediately, the effects of AS have a gradual onset. Among the fact they allow more intense and efficient training, the athletes learned to “bulk-up” during training and discontinue the drug intake when the test is to be conducted, in order to avoid a positive test result. This resulted in out-of-competition testing, first introduced in 1991 by the International Association of Athletic Federations (IAAF) [128].

In the next decade other ways of enhancing performance were revealed. As a result, hormones and related substances (erythropoietin (EPO), human growth hormone (hGH), insulin etc.), beta-2 agonists, diuretics and other masking agents, beta-blockers, and others were added to the list of banned substances. The list was further extended with prohibited methods: plasma expanders, blood transfusion, gene doping and others. The 1996 Atlanta Olympic Games were termed the “Growth Hormone Games” by some athletes [124].

The need for harmonisation of the procedures and rules between different International and National Sports Federation has pushed IOC to formation of a new anti-doping agency acting as a central body. After considerable discussion between IOC and participating governments, the World Anti-Doping Agency (WADA) was created in 1999. After WADA was created, the term doping was extended not only to intake of prohibited substances but also prohibited methods by an athlete. The Code [129], which spells out the anti-doping rules, now states that doping is also an attempt to use a prohibited substance or method, refusing or failing to submit for sample collection, violation of applicable requirements regarding athlete availability for out-of-competition testing, trafficking in any prohibited substances or methods etc.
An extraordinary example of how doping and anti-doping efforts compete is the Marion Jones case at Sydney Olympic Games in 2000. She won five medals, and although she later admitted to be using steroids, she had never been caught. The reason was the “transparency” of the screening procedure for anabolic agents at that time. The screening approach even today is still based on monitoring a few characteristic fragmentation ions of each of the substances on the list by using for example gas chromatography with mass spectrometric detection (GC-MS) in selected ion monitoring (SIM). If the substance is not on the list and it doesn’t give a mass fragment which is monitored, the screening method is blind to that substance. Probably that is the main reason why the newly designed molecule used by Marion Jones – tetrahydrogestrinone (smartly derived from gestrinone to avoid detection) has been known as “The Clear”. It is often the case in the history of sport, the athletes found a way to circumvent the testing method before scientists could develop a suitable method [124]. It wasn’t until 2007 when Jones pleaded guilty, that the IOC stripped her of the Sydney Olympic medals. Eleven adverse analytical findings had been reported but the large number of articles discussing performance enhancing drugs at the games caused the games to be nicknamed as “the dirty games” [124]. In contrast, the 2004 Athens Games are known as “the cleanest games” even though the number of doping violations was doubled (23). Two Greek top athletes were removed right before the Games for skipping a mandatory test, and seven medals in total were taken from athletes who tested positive or refused tests [123].

1.7.3 Endogenous sterols

Steroids are compounds possessing the skeleton of cyclopenta[a]phenanthrene, or so called sterane structure, and other functional groups. The sterane carbon core consists of four fused rings, labelled as A, B, C and D shown in Figure 1.22. Methyl groups are normally present at C-10 and C-13 and an alkyl side chain may also be present at C-17.
Figure 1.22 Sterane carbon skeleton, the core structure of steroids. Note that the rings are denoted with A, B, C and D, and the carbons are numbered from 1 to 17.

Estranes are steroids with a methyl group present at C-13 but not at C-10, and with no side chain at C-17. If methyl groups are present at both C-10 and C-13, with no side chain at C-17, steroids are called androstanes. Cholestanes and pregnanes are steroids with side chain at C-17. Steroids with 21 carbons containing ethyl functional group at C-17 are called pregnanes and those 27 carbon steroids containing -(CH₃)(CH₂)₃CH(CH₃)₂ at C-17 are called cholestanes.

There is no strict definition of sterols. However, they refer to a special group of steroids carrying a hydroxyl group at position 3 and most of the skeleton of cholestane. Additional carbon atoms may be present in the side chain. Hundreds of different sterols are found in animals (zoosterols) and plants (phytosterols). They are widely distributed and are important in cell membrane structures. Cholesterol is the most well known zoosterol and is the precursor of almost all other sterols. It is mainly produced in the cell membrane and its role is to establish proper membrane permeability and fluidity. A small portion is produced by the brain.

In the 1930s it was already known that testes contained a powerful androgen, and several years later the hormone was isolated and identified. It was named the hormone testosterone, from the stems of testicle and sterol, and the suffix of ketone. The two most credited scientists (Ruzicka and Butenandt) were offered the 1939 Nobel Prize for Chemistry for their work on testosterone synthesis. Clinical trials on humans began soon after. There are often reported rumours that German soldiers were administered anabolic steroids during the Second World War, in order to increase their aggression and stamina, but these are unproven.
Among the exogenous steroids used for doping in sport, endogenous (i.e. naturally occurring) anabolic steroids have also been used for performance enhancement. The best known natural anabolic androgenic steroids are testosterone and dihydrotestosterone (DHT).

The urinary steroid profile, recently coined as part of the “biological passport” of athletes, represents concentrations and ratios of a range of endogenously produced steroids, their precursors and metabolites. The majority of their parameters are not affected by exercise, severe physical endurance performance nor menstrual cycle, and their concentration and relative ratio is a valuable tool in doping control. However, the administration of endogenous steroids in the body produces variations of steroidal profile following partial suppression or enhancement of the endogenous steroids. These changes in the steroid profile can be used as a marker for endogenous doping [130].

In 1983 Donike et al. [131] described a method for the detection of the administration of testosterone in urine samples based on increased concentration of $T$ in comparison to its epimer – epitestosterone ($E$). $E$ is normally present in the urine but it does not increase after testosterone administration. However, the $T/E$ ratio can be artificially kept in the “desired” range by administration of $E$ together with $T$. That is the reason why WADA had listed epitestosterone as a masking agent and laboratories have to report its urinary concentration exceeding 200 ng mL$^{-1}$. The endogenous doping with DHT, similarly to $T$ doping, is based on monitoring the Androsterone/Eticholanone ratio ($A/Etio$). Androsterone is a metabolite of DHT and elevated ratio $A/Etio$ is a good marker for DHT doping.

Many steroids are sold over-the-counter as food supplements and they can be used by athletes for performance enhancement. Dehydroepiandrosterone (DHEA) is an orally ingested weak endogenous androgen. It is used by athletes in order to increase the levels of more potent androgens such as testosterone and DHT, which are part of its metabolic
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pathway [132]. The $T/E$ value and the urinary concentrations of testosterone, epitestosterone, androsterone, etiocholanolone and other endogenous steroids are quantitated precisely during the screening procedure in doping control.

### 1.7.4 Anabolic agents

Anabolic androgenic steroids or simply anabolic steroids (AS) are drugs derived from natural male sex hormone testosterone. They stimulate protein synthesis resulting in an acceleration of the food conversion rate, muscle growth, body mass build up and enhanced performance. Because of this they are used as therapeutics to stimulate the recovery of protein deficiency and protein-wasting disorders (e.g. osteoporosis, cancer, AIDS, bone marrow failure anaemia). However, athletes have recognised the “positive” effects on their performance and they became the most abused doping substances today. Through a number of mechanisms anabolic steroids stimulate the formation of muscle cells and hence cause an increase in the size of skeletal muscles leading to increased strength.

Historically IOC banned AS in 1976, but later this group of substances was renamed to anabolic agents (AA) because some anabolic substances abused in sport competition are not steroids, i.e. clenbuterol. The name comes from anabolic, meaning induction of protein synthesis and muscle build up (growth), and androgenic meaning pronouncing male phenotype such as development of the vocal cords, secondary sex organs, facial and body hair, etc.

Anabolic androgenic steroids are derived from testosterone structure (Figure 1.23) mainly by altering C-3 and C-17 positions. Many other powerful anabolic agents have been developed with slightly different androgenic or physiological properties. Alkylation at C-17 position with methyl or ethyl group created orally active compounds because it slows the degradation of the drug by the liver, and esterification at the C-17 position allows the substance to be administered parenterally. In this way the duration of effectiveness is
increased because agents soluble in oily liquids may be present in the body for several
months. Finally alterations of the ring structure were applied for both oral and parenteral
agents to obtain different anabolic to androgenic effect ratios. Higher anabolic /
androgenic ratio steroids are preferred in sport doping.

Figure 1.23 Chemical structure of testosterone

There are two known techniques of steroids use by athletes: “cycling” and “stacking” [130]. Cycling refers to the timing of steroid use. The perceived benefit is that after prolonged steroid use muscles’ receptor sites fail to recognize the steroid and if this occurs the steroid may lose its effectiveness and even higher doses may not provide significant increase in muscle. Accordingly, “on” and “off” cycles are designed for maximum benefit of the steroid, by allowing the cells to become receptive to steroid once again. The use of two or more different agents at the same time is referred to as stacking. It is based on the belief that this will saturate the muscles’ steroid receptors and will provide a synergistic effect. Three different agents with a cycling time of 5-10 weeks is the preferred technique according to a recent internet based study [130].

Even though the cycling and stacking techniques reduce the risk of adverse side effects of illegal use of steroids, the usual doses are 5-100 times greater than the normal body production of testosterone, and side effects can occur. The most pronounced is the suppression of the normal production of gonads (testis and ovary), which can reduce the sperm count to minimal levels and even cause infertility. Conversion of testosterone to dihydrotestosterone (DHT) can accelerate the rate of premature baldness for males who are genetically predisposed, but testosterone itself can produce baldness in females. Virilisation is another side effect, more dramatic in women. Acne and inappropriate facial
and body hair, combined with deepening of the voice are the most predominant. High doses of oral anabolic steroid compounds can cause liver damage, as the steroids are metabolized (C-17-alkylated) in the digestive system to increase their bioavailability and stability. The list of side effects is much larger and exceeds the scope of this thesis.

The rapid metabolization of steroids leading to low concentration levels of the parent compound, combined with the habit of athletes to stop using illicit substances before competition, has pushed anti-doping authorities to lower the minimum required performance limit (MRPL) of the techniques used for detection. Most of the detection methods are based on monitoring steroid metabolites rather than the parent compound, since they can often be detected much longer. Although all anabolic steroids are considered as doping agents, most of the positive cases detected by the IOC anti-doping laboratories refer to the illicit use of four specific compounds (methandienone, methyltestosterone, nandrolone and stanozolol). In addition to androgenic steroids, also beta-2-agonists have been banned by the IOC for their putative anabolic actions; among them, clenbuterol is supposed to be illicitly used by some athletes due to its “repartitioning effects”. The IOC Medical Commission has therefore stated that each WADA accredited laboratory should detect the presence of clenbuterol and/or the metabolites of the four anabolic androgens at a certain minimum required performance limit for the technique used for detection. The WADA technical document TD2004MRPL [133] stated the MRPL for anabolic agents to be 10 ng mL$^{-1}$. However, the MRPL for clenbuterol, 19-norandrosterone (nandrolone metabolite), epimethendiol (metandienone metabolite), methyltestosterone metabolite 2 (M2 metabolite) and 3’hydroxystanozolol (stanozolol metabolite) has been set at 2 ng mL$^{-1}$, making them the most difficult to detect of the anabolic agents. That is the reason why they are called the “key” WADA anabolic agents, required to be analysed by WADA accredited laboratories.

Nandrolone (Figure 1.24a) is an exogenous anabolic androgen steroid which is used in the treatment of osteoporosis in postmenopausal women (though now not recommended). It is also used for some aplastic anaemias, osteoporosis and some forms of neoplasia
including breast cancer. Apart from its legal use in medicine, nandrolone (19-nortestosterone) is one of the most popular anabolic androgens used by athletes to increase muscle mass, red blood cell production and bone density. The list of publicized nandrolone positive cases is relatively large [134].

Nandrolone is administered via intra muscular injection and its two major metabolites 19-norandrosterone (Figure 1.24b) and 19-noretiocholanolone (Figure 1.24c) can be detected for a long time past-administration. The screening procedure in doping control is common for all anabolic agents and consists of SPE or LLE, β-glucuronidase hydrolysis of the conjugates, extraction, derivatization and GC-MS analysis. The same methods for confirmation are used as in the case of clenbuterol. The limit of detection for this potent AS is the lowest ever set (1 ng mL⁻¹). Some false positive and incorrect urine analysis have been reported due to its low concentration levels and the presence of metabolites from other anabolic agents [134].

![Figure 1.24](image)

**Figure 1.24 Structure of nandrolone (a) and its major metabolites 19-norandrosterone (b) and 19-noretiocholanolone (c)**

Metandienone (methandrostenolone, Figure 1.25a) is another exogenous anabolic androgenic steroid; its popularity among bodybuilders persists. Despite the lack of any known therapeutic applications, the drug remained legal from its discovery in early 1960s until the early 1990s. It is typically being combined (stacked) with injectable compounds, such as testosterone, nandrolone or trenbolone. Several successful athletes and professional bodybuilders have come forward and admitted long-term metandienone use before the drug was banned, including Arnold Schwarzenegger [135].
Introduction

Metandienone anabolic effects are similar to the effects of other AS and it is screened and confirmed by using the same abovementioned methods. Epimethendiol (EMD, Figure 1.25b) is one of the metabolites of metandienone which can be detected for a longer period of time after administration and the screening is based on monitoring some characteristic mass fragments of this metabolite.

![Figure 1.25 Structure of metandienone (a) and its major metabolite epimethendiol (b)](image)

Stanozolol is an anabolic steroid derived from testosterone but in a different way. Unlike most of the AS, it contains pyrazole nucleus attached to the A ring (Figure 1.26a), which makes its extraction and GC analysis eminently difficult [136]. It is one of the most difficult AS to analyse by GC.

Its anabolic effects are similar to others AS with a slightly better anabolic/androgenic ratio. That is the reason why it is preferred by women [137]. Despite the ban, this AS is still illegally used as a growth promoter in cattle and widely used as a doping agent in sport competition. The list of positive cases is large, but probably the most featured case is Ben Johnson’s test result in 1988 Seoul Olympic Games.

The methods for screening and confirmation are the same as for the other AS, where the most characteristic fragment masses of its major metabolite 3’hydroxystanozolol (3’OH-stanozolol, Figure 1.26b) are monitored. This is one of the longest lasting metabolite in the urine, enabling doping violation to be detected long after stanozolol administration.
Figure 1.26 Structure of stanozolol (a) and its major metabolite 3’OH-stanozolol (b)

Methyltestosterone (17-α-methyltestosterone, MT, Figure 1.27a) is a 17-methyl derivative of testosterone with similar properties and effects to the other AS. It hasn’t been detected in urine unchanged and this, along with the structure similarity to testosterone-derived AS, is the main reason why it is the most frequently used internal standard in doping control. Its detection and confirmation is based on monitoring its two main metabolites, namely 17α-methyl-5α-androstane-3α,17β-diol (M1 metabolite, Figure 1.27b) and 17α-methyl-5β-androstane-3α,17β-diol (M2 metabolite, Figure 1.27c).

Figure 1.27 Structures of 17α-methyltestosterone (a), and its two major metabolites (b) M1 and (c) M2

Clenbuterol (Figure 1.28) is a beta-2 receptor agonist administered as a bronchodilator for the treatment of respiratory diseases, i.e. bronchial asthma [138, 139]. It shows “repartitioning effect” defined as the increase of lean muscle mass and the concomitant decrease of fat deposition. The effect is achieved through both an increased muscle protein deposition and an augmented lipolysis. Its ability to increase the muscle-to-fat body ratio makes its illegal use in livestock popular to obtain leaner meats. However, several massive poisonings have happened around the world (China, USA, Italy) by eating pork
contaminated by clenbuterol that had been fed to the animals to keep their meat lean. Athletes have recognized the benefits of this effect and clenbuterol has been extensively used in the past several decades.

Clenbuterol is mainly excreted in urine as unchanged and almost all methods for its detection are based on the parent drug. Even though the average urine level following oral administration of therapeutic doses is above 10 ng mL$^{-1}$, WADA has set the threshold at 2 ng mL$^{-1}$. This is mainly because of the “bulk up” of athletes, especially in the case of in-competition testing, where its effect can be “enjoyed” a long time after drug intake stops, which occurs long time before urine sample collection.

Detection methods are based on gas or liquid chromatography separation and UV or MS detection. GC-qMS is a golden standard for anabolic agents in doping control, where screening is based on monitoring the most characteristic fragment ions of beta-2 agonist fragmentation. In case of positive screening result, confirmation is achieved by applying HRMS or tandem MS. Amendola et al. [138] have proposed a GC-MS$^3$ method for clenbuterol confirmation, offering significant background reduction and reliable identification.
1.8 SCOPE OF THE THESIS

The thesis will commence with an introductory section – Chapter 1 – that describes the role of general profiling for drugs analysis, how it is conducted, and the use of gas chromatography for this purpose. Chapter 2 gives details for the samples and analytical methods. A thorough consideration of multidimensionality in analysis follows – Chapter 3 – with particular attention on the two key platforms of importance to gas chromatography technology – the separation dimension and the detection dimension. This theme will then be developed through the applications in the drugs area, which will be the bulk of the thesis original research.

Steroids in doping control occupy three chapters – Chapters 4-6 – and constitutes firstly the development of understanding of the technology of GC×GC and GC×GC-MS, then the use of GC×GC for doping analysis, and finally an evaluation of the requirements of the World Anti-Doping Authorities for steroids analysis in national drug testing laboratories.

Based on the success of the above research, attention will then focus on illicit drugs, the relevance of GC×GC for such studies, and the new capabilities which GC×GC can bring to ecstasy – Chapter 7 – and heroin and cocaine – Chapter 8 – analysis.

The conclusion – Chapter 9 – summarises the major outcomes and recommendations that arise from the research reported.
Chapter 2

2 MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 Endogenous sterols

The following endogenous sterols (min. 99% purity) were obtained from Steraloids Inc. (Newport, RI, USA) except as noted: 5α-androstan-3β-ol (1), 5β-androstan-3β,17β-diol (2), 5β-estrane-3α-ol-17-one (3), 5α-androstan-3α-ol-17-one (4), 1,3,5(10)-estratrien-3,17β-diol (5), 5-androsten-3β-ol-17-one (6), 5α-androstan-3β-ol-17-one (7), 5α-androstan-3α,11β-diol-17-one (8), 5α-androstan-17β-ol-3-one (9), 5-androsten-3β,16α-diol-17-one (10), 5β-androstan-3α-ol-11,17-dione (11), 4-androsten-17α-ol-3-one (12), 4-androsten-17β-ol-3-one (13), 5α-cholestane (14), 1,3,5(10)-estratrien-3-ol-17-one (15), 5-cholesten-3β-ol (16), 4-androsten-3,17-dione (17), 5,22-cholestadien-24β-ethyl-3β-ol (18), 5-cholesten-3β-ol n-butyrate (19), 5β-pregnane-3α,20α-diol (20) (from Acros Organics <www.acros.com>), 5β-pregnan-3α,17,20α-triol (21), 5β-pregnnan-3α,11β,21-triol-11,20-dione (22), 5β-pregnnan-3α,17α,21-triol-11,20-dione (23), 5β-pregnnan-3α,17,20α,21-tetrol-11-one (24), 5β-pregnnan-3α,17,21-triol-11,20-dione (25), 5α-pregnnan-3α,11β,17,21-tetrol-20-one (26), 5β-pregnnan-3α,11β,17,21-tetrol-20-one (27). Methyl testosterone (IS, 99.3% purity) used as an internal standard was purchased from Sigma-Aldrich. Their structures are given in Figure 2.1.

![Figure 2.1 Structure of sterols used in the study (Continued on page 83)](image-url)
Continued…

![Chemical structures](image-url)

2.1.2 WADA key sterols

Clenbuterol (Clen.), 19-norandrosterone (19-nor.), epimethendiol (EMD), 17α-methyl-5α-androstane-3α,17β-diol (M1), 17α-methyl-5β-androstane-3α,17β-diol (M2), 3′-hydroxy-
stanozolol (3’OH-stan.), methyltestosterone (IS, 99.3% purity) were purchased from National Measurement Institute (NMI, Pymble, Australia). Their structures are given in Chapter 1, section 1.7.4.

2.1.3 Illicit drugs

24 ecstasy tablets in total were used in the experiments. Nine were seized in Macedonia during the period end-2007 – early-2008 and were provided by Macedonian Police, and 15 were seized in Australia at the end of 2009 and were provided by Australian Federal Police (AFP). Macedonian samples were from 9 different seizures (one tablet from each seizure), but were classified in three groups each of three tablets according to their post-tabletting (post-TB) physical characteristics (logo, colour, diameter and thickness). Three tablets were off-white in colour with “dove” logo (XTC3 in Figure 2.2), three were brown with “bird” logo (XTC4), and three were pink with “Toyota” logo (XTC5). The three XTC3 tablets are labelled as XTC3-1, XTC3-2 and XTC3-3. The three XTC4 and three XTC5 tablets were labelled accordingly. Intelligence information provided by Macedonian Police indicated that the tablets with the same post-TB are likely related. The Australian samples were from one recent seizure and were classified in five groups, each of three tablets according to their physical characteristics. Their labels and logos are given in Figure 2.2.

Figure 2.2 Photos of the ecstasy tablets used in the study, showing all their logos
Heroin profiling study was performed by analysing samples from three different seizures made in Macedonia in 2006-2008 (Ha, Hb and Hc). Each sample was divided in three separate aliquots (subsamples) simulating different but linked seizures and each were labelled as Ha1, Ha2, Ha3, Hb1, Hb2 etc. They were light-brown to brown in colour and their physical appearance and intelligent information, combined with the major components profile, suggested they resemble SWA heroin. Three blend samples (B1, B2, B3) composing of equal amount of Ha, Hb and Hc was used to test the statistical methods for sample classification. Additionally, the profiles of nine heroin samples seized in Australia and provided by AFP were compared to the SWA heroin sample profiles. Australian heroin samples (H1 to H9) were white to pale brown in colour and their major components profiles likely resemble SEA heroin.

Three cocaine samples seized in Macedonia (Ca, Cb, Cc) in 2005-2007 were used in the cocaine profiling study. They were white to off-white powders in colour, each weighted 0.6 g. Each sample was divided in three subsamples, labelled as Ca1, Ca2, Ca3, Cb1, Cb2 etc. simulating different but linked samples. Sample Cc was taken from the biggest ever single seizure of cocaine in Macedonia: 438 kg. Additionally, nine cocaine samples (C1 to C9) seized in Australia (provided by AFP) were included in the study. No information related to their origin was supplied. All cocaine samples were white in colour.

2.2 REAGENTS AND CHEMICALS

Methanol (HPLC grade), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS), N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), heneicosane, toluene and methyl tert-butyl ether (MTBE) were purchased from Sigma–Aldrich, sulfuric acid, anhydrous sodium sulfate, ammonium iodide (NH4I) and potassium carbonate (K2CO3) from BDH Chemicals (Kilsyth, Australia), phosphorouspentoxide (P2O5) and ethanethiol from Merck (Darmstadt, Germany), and
pyridine from Alltech. β-glucuronidase (from *E. Coli*, K12) was supplied from Roche (Mannheim, Germany). All chemicals and reagents were of analytical grade or higher. Water used in the experiments was of Milli-Q® (Millipore Corp., MA, USA) grade.

2.3 SAMPLE PREPARATION

2.3.1 Endogenous sterols sample preparation

Two stock solutions of sterols were prepared – one a 27 component mixture, the other a 19 component mixture when pregnanes were excluded – by dissolving a known amount of each sterol in HPLC grade methanol to a concentration of each between 0.3–1.2 mg mL$^{-1}$. Working solutions were prepared by progressive dilution of the stock solution. A stock solution of internal standard was prepared by dissolving 10 mg of MT in 10 mL methanol and the working solution by further dilution of 100 μL of stock solution in 10 mL methanol. Solutions were stored at 4 °C when not in use.

Free sterols were extracted from spiked and blank urine samples by a procedure described elsewhere [139]. Hydrolysis of steroid glucuronide conjugates, as is customary in many steroid preparation protocols, was not performed for the present studies. Briefly, 5 mL urine was spiked with 19 sterols at a concentration from 0.1 ng mL$^{-1}$ to 10 ng mL$^{-1}$ and to each sample 50 μL of internal standard (10 μg mL$^{-1}$) was added. The samples were made alkaline with 0.5 mL of 5% K$_2$CO$_3$ to pH of 9–10 and shaken well for 1 min. Sterols were extracted with two aliquots of 2.5 mL TBME, after shaking for 5 min and centrifuging for 20 min at 3500 rpm. The two extracts were combined, dried and kept in a desiccator over P$_2$O$_5$ for 20 min. Extracts were then derivatized by adding 50 μL pyridine, 50 μL BSTFA and heated for 30 min at 80 °C. After transferring the derivatized mixture to micro inserts, 3 μL were injected using splitless injection mode. Blank urine samples were processed in the same manner without addition of sterols.
Standard mixtures of sterols were prepared by drying aliquots of diluted solutions, and derivatized as described. Each of the 27 sterols were sampled and derivatized individually by drying an aliquot of each then derivatizing as described above to a final concentration of 100 μg mL⁻¹.

2.3.2 WADA key sterols sample preparation

Stock solutions of anabolic agents were prepared by dissolving a known amount of each in HPLC grade methanol to a concentration of 0.2 mg mL⁻¹. Working solutions and standard mixtures were prepared by progressive dilution of the stock solutions. A stock solution of internal standard (methyltestosterone) was prepared at a concentration of 1 mg mL⁻¹ in methanol, and the working solution at a concentration of 5 μg mL⁻¹. The derivatization mixture (MSTFA-NH₄I-ethanethiol) was prepared in a ratio 1000:2:6 (v/w/v). Solutions were stored at 4 °C when not in use.

Urine samples spiked with anabolic agents were prepared in the concentration range from 0.5 ng mL⁻¹ to 20 ng mL⁻¹ by adding an appropriate volume of the standard mixture and 25 μL of the internal standard solution to the blank urine extracts and they were prepared according to the widely accepted sample preparation procedure [139, 140] for anabolic agents. Briefly, 2.5 mL urine was applied to a 500 mg C-18 SPE column (Bond Elut, Varian), previously conditioned with 3 mL methanol and 3 mL water. Anabolic agents were eluted with 3 mL methanol and the solvent was evaporated under a steam of nitrogen. 1 mL of phosphate buffer (pH 7.0) and 50 μL β-glucuronidase were added to the residue and the mixture was incubated in a water bath at 55 °C for 1 h. After cooling, 0.75 mL potassium carbonate solution (5%) was added, shaken for 5 min and anabolic agents were extracted with two portions of 2.5 mL TBME. The two extracts were combined, dried under a steam of nitrogen, and spiked with anabolic agents and IS at appropriate concentration, dried again under nitrogen and kept in a desiccator over P₂O₅ for at least 20
min. Prior to analysis, the residue was derivatized by dissolving in 50 μL of derivatization mixture (MSTFA-NH₄I-ethanethiol) with heating at 80 °C for 30 min. Standard mixtures of derivatized anabolic agents were prepared by drying aliquots of diluted solutions, and derivatized as described. Bis-TMS derivatives (2TMS) of clenbuterol, 19-norandrosterone, EMD, M1, M2, and methyltestosterone (IS) were monitored and tris-TMS (3TMS) derivative of 3’OH-stanozolol.

A urine positive control sample (UPC) spiked with 5 anabolic agents at a concentration of 5 ng mL⁻¹ was prepared at the National Doping Control Centre (NDCC), Mahidol University in Bangkok, Thailand (WADA accredited laboratory). M1 metabolite was not spiked in the UPC sample, since this epimer is not monitored at the MRPL of 2 ng mL⁻¹. The sample was used for checking the performance of the present method.

2.3.3 Illicit drugs sample preparation

2.3.3.1 Ecstasy samples preparation
Ecstasy (MDMA) tablets were quickly crushed and homogenized by using mortar and pestle. 20 mg of the fine powder was placed in a 4 mL septum-sealed vial, equilibrated for 30 min at 80 °C and then a PDMS/DVB SPME fibre (Supelco, Bellefonte, PA, USA) was inserted and exposed to the headspace for another 30 min. The samples were desorbed in the GC injection port for 5 min at 250 °C. No additional chemicals or reagents were used in the ecstasy profiling study.

2.3.3.2 Heroin samples preparation
Organic impurities present in heroin samples were extracted by the procedure described by Stromberg et al. [31]. Briefly, a 30 mg sample is dissolved in 5 mL of 0.5 N sulfuric acid and mixed vigorously for 5 min. 5 mL MTBE (or toluene, for comparison purpose) is added, mixed vigorously for 10 min and centrifuged for 10 min at 3500 rpm. 3.2 mL of the
upper organic layer is removed, dried under nitrogen and reconstituted with 200 μL of the extracting solvent. Two blanks (one of each extracting solvent) are prepared in the same manner for compensation of the background components.

Volatile components present in heroin samples were extracted by means of SPME extraction. Because there was no method described in the literature, except the one applied on a macro scale (10 g sample) in a dog training program [49], we applied SPME procedure similar to the one described for ecstasy profiling, with slightly prolonged equilibration and absorption times and increased sample amount. Briefly, heroin samples were homogenized and 40 mg of each was transferred to a 4 mL septum-sealed vial and equilibrated for 40 min at 80 °C. After the equilibration period, PDMS/DVB or divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibres were inserted and exposed to the headspace for another 40 min. Volatile components were desorbed in the GC injector (for 5 min at 250 °C).

2.3.3.3 Cocaine samples preparation

Cocaine samples for liquid injections were prepared by the method described in [141]. Briefly, 30 mg finely powdered sample was dissolved in 0.5 mL chloroform/pyridine (5:1) containing 1 mg mL⁻¹ heneicosane (C21) as internal standard. 100 μL of MSTFA were added to the mixture, shaken vigorously and derivatized at 80 °C for 60 min.

The comprehensive cocaine impurity profiling method, as described by Moore and Casale [142] and currently used by the Special Testing and Research Laboratory (STRL) in USA, has not been applied due to three main reasons:

1. the high cocaine amount required (500 mg);
2. the complex and lengthy procedure where alumina column chromatography is used for impurity fractionation, collection and consequent analysis;
3. relatively simple chromatographic profiles of each of the fractions.
The average amount of cocaine samples available was 600 mg per sample, so it was decided to explore the new possibility of profiling rather than to use the whole amount for one sample preparation and analysis only which would prevent replicate sampling.

Volatile components present in cocaine samples were extracted by using the same SPME sample preparation method previously described for heroin.

### 2.4 INSTRUMENTATION

An Agilent 6890 gas chromatograph (Palo Alto, CA, USA) equipped with split/splitless injector and Agilent 7683 series auto samplers was used in all one dimensional (1D) and two-dimensional (2D) GC experiments. The modulator used in 2D GC experiments was a Longitudinal Modulation Cryogenic System (LMCS, Chromatographic Concepts Pty Ltd., Doncaster, Australia) retrofitted to the GC. Liquid CO₂ was used for cryotrapping of components and nitrogen at 15 psi as a flush gas for prevention of sticking the column to the cooled (frozen) trap.

The GC was equipped with a flame ionisation detector (FID) for the GC-FID and GC×GC-FID experiments or coupled to an Agilent 5973 quadrupole mass selective detector (qMS) upgraded with fast electronics for GC-MS experiments. Unless otherwise stated, the MS transfer line and ion source temperatures were 280 °C and 230 °C, respectively, and the MS detector voltage and electron impact (EI) ionization energy were 1.8 kV and 70 eV, respectively.

A LECO time-of-flight mass spectrometer (TOFMS) model Pegasus III (LECO Corp., St. Joseph, MI, USA) was coupled to the GC in all GC-TOFMS and GC×GC-TOFMS experiments. The applied MS transfer line temperature, ion source temperature and
electron ionization energy were the same as in GC-MS experiments. The detector voltage was 1.6 kV, unless otherwise stated.

Experiments in high resolution mass spectrometry were conducted using a GCT Premier (Waters, Manchester, UK) accurate TOFMS, connected to an Agilent 7890 gas chromatograph. The TOFMS detector was operating at 7000 FWHM (full width at half maximum) resolution with specified mass accuracy of less than 5 ppm.

### 2.5 METHODS

The GC columns and column sets used in 1D and 2D GC experiments are listed in the following Chapters (under Experimental subheadings). All BP columns were products of SGE International (Ringwood, VIC, Australia) and DB-1 and DB-5 were products of Agilent J&W Scientific. Unless otherwise stated, experiments in 1D GC were carried out under the same chromatographic conditions as in 2D (including the column configuration), but without activating the cryogenic modulation process. The modulation period and the modulator temperature were different for different type of analytes and details are given in the following Chapters.

Helium was used as a carrier gas in qMS and TOFMS experiments and hydrogen in FID experiments. One μL sample volume was injected in all methods, except in P/NP column configuration in GC×GC-TOFMS methods for endogenous sterols, where 3 μL were injected. The acquired mass range was from 45 m/z to 700 m/z in TOFMS and from 45 m/z to 650 m/z in qMS experiments, unless otherwise stated.

A separate GC×GC-TOFMS based in-house library of endogenous and WADA key sterols was generated using standard solutions at a concentration of 1 μg mL⁻¹ and 0.5 μg mL⁻¹, respectively. Spectra similarity was performed against NIST05 commercial MS database.
and the in-house created TOFMS library in sterol work by using NIST algorithm and against NIST05 and Wiley7 commercial databases in illicit drug study by using both NIST and PBM spectral search algorithms.

2.5.1 Data acquisition and processing

FID data were acquired at 100 Hz and processed by Agilent ChemStation software. The data were then exported to csv (comma separated values) file format and converted in two-dimensional matrix by using an in-house (RMIT University, Melbourne, AU) developed software. Transform (Fortner Research, VA, USA) was used for 2D and contour plots presentation. Agilent MSD ChemStation software was used for data acquisition and processing in GC-qMS study.

TOFMS data were acquired and processed by ChromaTOF (LECO Corp., St. Joseph, MI, USA) software ver. 2.32. Data acquisition rate was 100 Hz in WADA sterols experiments and 50 Hz in endogenous sterols and illicit drugs studies, both conducted in 2D GC mode. The sampling frequency in 1D GC mode in all experiments was adjusted to 20 Hz to closely resemble the usual acquisition rate in classical gas chromatography. 2D image and contour plots of TOFMS data were created by ChromaTOF software, and all other chromatograms and 2D plots were constructed by using Origin ver. 7.5 (OriginLab Corp., Northamptoni, MA, USA), after conversion of the raw data into “csv” files.
Chapter 3

3 MULTIDIMENSIONAL GAS CHROMATOGRAPHY
WITH SPECIAL EMPHASIS ON ITS USE IN DOPING
CONTROL AND DRUG ANALYSIS
3.1 INTRODUCTION TO MULTIDIMENSIONALITY

That there is a need for improved methods of analysis for many sample types is a truism. Rarely will advances in analysis techniques be rejected as inappropriate or undesirable, except for the simplest analysis task. The most pressing questions that exercise the strategies of analysts are:

- What constitute truly advanced methodologies?
- How do they compare in delivery of improved analytical capability?
- Are they needed for the task at hand?

Usually these questions revolve around the ability of the method to measure compounds at lower abundances, perhaps over a greater order of magnitude response, and with better selectivity in the presence of other compounds. These in turn lead to a greater capacity to record, measure and quantify more of the total analytical response of a sample.

Here, the focus is on gas chromatography (GC) with particular reference to how multidimensional methods offer new capabilities for analysis. The first part presents an introduction to considerations of multidimensionality and hyphenation, with discussion of the role of the separation step in GC, then the detection step, and finally multidimensionality of the total method. Here, drugs analysis and anti-doping control will be used for demonstration of the different concepts in multidimensionality.
3.2 HYPHENATION vs. MULTIDIMENSIONALITY IN CHROMATOGRAPHY

The term “hyphenated” technique has a variety of meanings in the scientific community. While the meaning for some authors is almost limited to the coupling of a separation technique to the mass spectrometer (MS), for others it includes coupling of a sample preparation technique to one or more separation techniques, coupled to one or more detection systems [143]. For some authors [144] the hyphenation just means close relationships between two devices or techniques, and does not even mean on-line coupling, and for some [145] it is even equivalent to the term multidimensionality. Ali et al. [147] have stated that hyphenation is nothing but coupling of a sample preparation unit with the core analytical instrument. Having in mind the abovementioned, sometime there is contradiction in the terminology, e.g. if hyphenation is equal to multidimensionality, how can SPE coupled to HPLC be defined as a multidimensional technique? Or can offline L/LE and subsequent injection in GC be considered as a hyphenated technique? However, most analysts agree in the following: appropriate hyphenation brings faster analysis time, lower detection limits, involves less manpower and reduces chances of human error, limits sample contamination and analyte degradation, with enhanced analytical precision etc. [146].

In the author’s best knowledge, the review of Bogusz [147] best describes the term hyphenation so far. He compares the hyphenated technique to the hyphenated man: a naturalized citizen believed to be ambivalent in his loyalty; so called of his tendency to style himself according to his former and present nationalities, using hyphen (as defined in Webster Encyclopaedic Unabridged Dictionary). In this meaning the freshly combined techniques are seldom treated as a complete and integer system, in the same manner as a freshly naturalized citizen is still ambivalent. Historically, the operation of the hyphenated techniques derives from developments by specialists from two or more different fields,
who usually were confident in only one analytical aspect (e.g. separation or detection problems). As the hyphenated techniques “mature” and the bench top instruments become commercially available, the “second generation” of users (like in the case of GC-MS, HPLC-DAD) do not regard these techniques as hyphenated but take them for granted and as a fully integrated “single” system. As a conclusion, Bogusz [147] stated that the term “hyphenated” seems to apply only to those techniques which still have not reached their maturity.

Even if it is hard to establish a strict definition of a hyphenated chromatography technique, most would agree that in the widest meaning it is a carefully adjusted (usually involving compromises) and on-line coupling of two or more techniques, or techniques and devices, in one, while retaining the appropriate fluid flow through the system as a whole. Sample preparation/pre-concentration techniques (SPE, SPME) and detectors (FID, NPD, ECD, UV, RID etc.), which are not techniques by themselves belong to this group when coupled to the analytical techniques. Off-line coupling of two analytical techniques and/or sample preparation techniques is excluded in this definition, since discrete flow operation does not mean hyphenation. Also it does not mean the coupling must consist solely of separation and mass spectrometry techniques. As an example, GC coupled with MS or FTIR is considered as hyphenated, as well as when interfaced with an FID, NPD or ECD detector. In the same manner, GC-AED (atomic emission detector), SFC-FTIR (supercritical fluid chromatography-Fourier transform infra red), CE-MS (capillary electrophoresis-mass spectrometry), LC-GC, SPE-LC are all hyphenated techniques. GC coupled to GC (GC-GC, GC×GC), or LC coupled to LC (LC-LC, LC×LC) are classified in this group even though they do not comply with the Bogusz definition [147]. In order to be fully functional, the “hyphenation” has to be done through an adjustment of the conditions, implementing an interface between the two GC columns, adjustment of the detector to be able to handle the data etc. However, the term “hyphenated” is usually omitted for the mature hyphenated techniques.
3.3 MULTIDIMENSIONAL GAS CHROMATOGRAPHY TECHNIQUES

Today, many published articles deal with terms such as ‘two-dimensional’, ‘three-dimensional’ and even ‘four-dimensional’ techniques, with one common goal: they are all intended to accelerate the analysis by time, by number of components resolved, or by number of components detected. Here, an interpretation can be given to the relative merits and implementation strategies of multidimensional analyses, with various applications from the literature on doping and drugs analysis.

Generally, multidimensionality comprises the coupling of two or more dimensions, each of which is technique in their own right. Mondello et al.’s text [148] is one of the most recent book covering this general topic, although much new research has appeared in the years since this book was first published. For example, both GC coupled to mass spectrometry (MS) as a detection system giving GC-MS, and high performance liquid chromatography (HPLC) coupled to ultraviolet-visible spectroscopy (UV) giving HPLC/UV-vis are multidimensional techniques. In contrast, a technique which comprises a separation dimension coupled to a simple detection system which is not itself a technique, may be considered as hyphenated, but not multidimensional. Thus GC-flame ionisation detection (FID), GC-electron capture detection (ECD), HPLC-refractive index detection (RID) are some examples of this group. Another category arises where dimensions of the same type (or category) are coupled, such as GC with GC, GC with LC, or LC with LC. Are these couplings considered as hyphenated or multidimensional techniques? This question will be explored below. It is widely accepted that all multidimensional techniques are hyphenated, but the reverse does not necessarily apply. This is not to imply that the information content of hyphenated systems are equivalent, or for multidimensional systems is equivalent. This will depend on the capabilities for molecular specificity and/or capacity of each dimension to characterise an individual component. Kidwell [149] expressed the informing power of different multidimensional
systems (based on separation with mass spectrometry), but this should now be updated with a view to included recent technical developments in instrumentation. Giddings has discussed the general concept of multidimensionality of analysis and of separations in a number of key works [150-152].

Even though there is a lack of unique nomenclature defining multidimensionality, liberty can be taken to classify GC techniques into two distinguishable classes: GC techniques where multidimensionality arises from the separation process (nSEP), and GC techniques where multidimensionality arises from the selectivity/specificity of detection (nSEL). Since these techniques are widely accepted in many areas of volatiles analysis, focus will be only on the application of GC techniques in the field of drugs, their metabolites, and sterols in anti-doping control. As a starting point, and given that MS methods will feature herein, Figure 3.1 [153] proposes how the GC techniques above are distinguished.

![Figure 3.1](image)

**Figure 3.1** (A) Gas chromatography normally uses single column, but (B) dual column operation (multidimensional GC or GC×GC) is now increasingly common. In combination with single dimension MS (quadrupole, high resolution, time-of-flight) or multidimensional MS (MS/MS), various types and capabilities of analysis are possible [153]. Reproduced with permission of Future Science Ltd.
3.3.1 Multidimensionality arising from selectivity (n-SEL)

Here, the focus is on detection selectivity, but since a separation step is still needed, only a single dimension of separation is considered.

3.3.1.1 One dimension: One dimension of separation, no detection selectivity (1SEP-0SEL)

There are still many laboratories where the detection system coupled to the separation techniques simply registers a signal, rather than constitutes a separate technique. Their continued use is because of their robustness, wide linear response range and versatility, as in the case of a GC-FID for blood alcohol analysis [154, 155], or their sensitivity for instance in the case of the GC-ECD in toxicological analysis of chlorinated pesticides, or drugs after conversion to an electron-affinity derivative [156]; or for selectivity toward particular groups of compounds in the application of the GC-nitrogen-phosphorus detector (NPD) for stimulants and opiate screening in forensic, toxicological and doping analysis [157]. However, the availability of a variety of analytical techniques able to be coupled to the separation technique, considering their sensitivity, specificity and selectivity capabilities toward the analytes and the matrix, reduces the applicability of ‘1SEP-0SEL’ techniques. Because of this, a focus on multidimensional analytical techniques is appropriate, representing a separation technique coupled on-line with a spectroscopic detection technique.

3.3.1.2 Two dimensions: One dimension of separation, one dimension of detection selectivity (1SEP-1SEL)

Techniques where sample determination is based on two distinguishable and independent dimensions, generating unique data sets, are defined as two-dimensional analytical techniques. Conceptually, two different separation mechanisms or two different detection-based dimensions may constitute the two-dimensional method. However, since it is hard –
if not impossible – to couple two detection-based techniques without using a separation dimension (0SEP-2SEL), apart from some novel tandem mass spectrometry (MS/MS) methods, the 2D approach almost exclusively comprises of a separation technique coupled to a selective detection technique – MS, Fourier-transform infra-red (FTIR) or nuclear magnetic resonance (NMR). Whilst FTIR is a viable and useful detection method for GC [158], and NMR has recently been described for GC [159] and with multidimensional GC (MDGC) [160], today it is largely MS methods which comprise the majority of the viable multidimensional methods, and have largely replaced 1D GC methods.

Many applications have been developed for routine bioanalysis in a wide diversity of matrices – blood, urine, saliva, hair, tissue, etc. In addition to its robustness, simplicity, sensitivity and selectivity, the most important attribute is its identification power. Scan mode, with full mass spectra of separated compounds acquired, permits widely accepted use of MS spectral libraries for identification, but this is best accomplished with well separated components, or with spectral deconvolution. Selected ion monitoring mode (SIM), where pre-selected ions are monitored, dramatically increases sensitivity since the signal is increased by allowing more time for measurement of the few ions monitored; ion noise is suppressed, but spectral identification is compromised as few ions are monitored. GC-MS is irreplaceable for routine analysis in toxicology, forensic and doping control, and it is widely accepted and recommended by many international organisations, e.g. World Anti-Doping Agency (WADA), as a “gold standard”. Preliminary screening in almost every WADA accredited laboratory for anabolic agents, stimulants and opiates is first by GC-MS in SIM mode. The core instrumental tool applied in systematic toxicological analysis (STA) is GC-MS in scan mode, with identification through matching mass spectra with MS libraries. In forensic analysis the role of GC-MS is critical when dealing with unknown components or new designer drugs in seized or biological matrices.

Notwithstanding the widespread acceptance of GC-MS (full scan and SIM modes) many endogenous components similar in structure and/or mass to the target analytes are co-extracted and the probability of co-elution on the chromatographic column is high.
Similarity of structure means matrix interferences can bear common mass fragments as the analytes, making quantification difficult or almost impossible. This can especially happen at low levels of concentration required by the minimum required performance limit (MRPL), where minor mass fragments of endogenous components (usually present at much higher amount) may strongly interfere with trace amounts of analytes. Thus refinements to the basic MS instrumental technique may be imperative. The two main improvements in single dimension mass spectroscopy for bioanalysis are the introduction of time-of-flight mass spectrometry (GC-TOFMS) and high resolution mass spectrometry (GC-HRMS).

Further selectivity of the detection system, accompanied by an increase in signal-to-noise (S/N), is obtained through exact mass measurement of ions in the mass spectra through HRMS. Differentiation of coeluting components is possible, according to their exact mass-to-charge ratio (m/z), depending on the magnitude of their fractional mass difference. The selectivity of the method depends on the mass resolution, expressed as m/Δm. Routine analysis using HRMS is not as common as might be suspected. Magnetic sector instruments, and more so Fourier transform MS systems, are expensive and often are unsuited to very fast scanning. Recently, high resolution TOFMS (HRTOFMS) with moderate resolution of about 7,000-10,000 has become available [161, 162], but is still beyond the price of a low resolution spectrometer. However their sensitivity and power for empirical and structure elucidation remains attractive.

GC-HRMS has been explored for doping control over the last decade. This technique along with tandem mass spectrometry is recommended by the International Olympic Committee (IOC) [163] and WADA [164] for confirmation of trace levels of anabolic androgen steroids (AS) in urine of athletes. Kokkonen et al. [163] have found GC-HRMS to be 2-10 times more sensitive than GC-MS (low resolution). The number of positive samples reported by the Cologne anti doping laboratory in 1995 increased dramatically following the introduction of this technique; 75 positive samples out of 116 were identified on the basis of GC-HRMS [165] demonstrating increased sensitivity and selectivity when compared to
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GC-MS, even though the two metabolites of stanozolol were not separated under the tested conditions. The same group concluded that in order to enhance sensitivity, selective isolation procedures could be implemented since the metabolite signal is hampered by high biological background. The coelution of one of the 3’OH-stanozolol TMS derivatives with an endogenous component which interfered with its mass spectral analysis, was resolved by preparation of the tributylsilyl (TBS) derivative of the metabolite.

Few literature articles have used GC-HRMS in drug analysis with most referring to improved detection limit and selectivity. Grosse et al. [166] used GC-HRMS to study the pharmacokinetics of remifentanil (a fentanyl derivative with potent analgesic activity) in blood, with 10 times lower limit of quantification compared to low resolution GC-MS. Another study showed even greater difference in sensitivity (20 fold) between these two techniques [167].

The coupling of TOFMS to GC is a second identified improvement of the MS detection. Recently available with GC, TOFMS provides a mass fragmentation pattern (MS spectra) that is effectively instantaneous, so provides an unbiased spectrum for each spectral scan over the chromatographic peak [162]. This detector provides a very fast acquisition rate of e.g. up to 500 spectra/s, and is of advantage especially when narrow and sharp peaks are obtained in fast gas chromatography, or in comprehensive two-dimensional gas chromatography [168], where the peak width at the base can be as narrow as 50 ms. The high acquisition rate with full mass spectral scanning offers mathematical deconvolution for coeluting components so as to generate the unique spectrum of each overlapping component, provided they each temporally differ by a few scans.

It has been shown in a recently published study [169] that GC-TOFMS, combined with LC-TOFMS, can be used as a screening method for a wider number of prohibited drug substances, offering great sensitivity and selectivity. However, it seems that the method lacks sensitivity, since the authors analysed several AS at 10 ng mL⁻¹, which exceeds the required LOD of 2 ng mL⁻¹ (1 ng mL⁻¹ for 19-norandrosterone) [133]. Furthermore, several
prohibited substances, including methyltestosterone metabolites M1 and M2 failed the test. Despite these limitations, this dual blind screening approach covered a wide range of molecular features, from substances difficult to derivatise but amenable to LC-TOFMS (ESI), to substances difficult to ionise, but amenable to GC conditions and derivatization.

3.3.1.3 Three dimensions: One-dimension of separation, two dimensions of detection (1SEP-2SEL)

Further improvement in sensitivity and selectivity amongst GC-MS methods can be obtained by adding an additional dimension in mass spectrometry selectivity – that of tandem mass spectrometry (MS/MS). The coupled mass spectrometry dimensions allows the first mass spectrometry dimension (MS1) to serve as a mass filter for pre-defined ion(s) (precursors) and the second (MS2) to scan either a single or multiple product ions of the precursor ion(s), with the latter formed during the process of collision with a reagent gas. This process is termed collision induced dissociation (CID). In this way the pre-defined precursor ion(s), which might be a mixture of mass fragments from coeluting components which share common fragment ions, is ‘extracted’, filtered (at MS1) and passed to the collision chamber. Here further dissociation to well defined product ions (generally free from isotope ions since monoisotopic ions will be selected) occurs, and finally scanned (at MS2) as a full mass spectrum, or through monitoring as specific product ions. Figure 3.2 proposes how such an analysis is carried out, with Figure 3.2(A) the triple quadrupole example for compounds separated by the GC first dimension. Figure 3.2(B) shows that the ions generated by the ionisation process can be scanned or selected by SIM at Q1, dissociated at Q2, and then the products interpreted at Q3. Here the wavy line represents an ion at Q1 selected by SIM, and then two product ions of the dissociation are monitored at Q3. The sensitivity and selectivity is dramatically increased by monitoring only the expected product ions from the analyte, while rejecting the product ions of co-filtered ions through MS1 (i.e. having the same mass as originally selected analyte ion). This arises (ideally) because the primary isobaric ions from interfering compounds will have different structure and different CID product(s) to the target ion; specificity is obeyed provided
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different ion structure holds. GC-MS/MS represents an extremely sensitive and selective method, and is often the only solution for detection and confirmation of low level of analytes in complex matrices such as urine, hair, meat and meat products, environmental samples, food etc. Kidwell [149] has reported the informing power (IP) of the GC-MS/MS experiment to be the greatest (~6 × 10⁹), amongst the methods chosen for comparison; EI-MS was the more informing technology compared with CI-MS, due to the spectral selectivity of the full spectral EI-MS scan. The comparative selectivity of GC (IP ~ 550 separation ‘bits’) when combined with MS spectral matching gave IP ~ 6 × 10⁶ for GC-MS. The main limitation of GC-MS/MS is the requirement for prior knowledge about the analyte, since it functions by choice of pre-defined precursor and product ions. Thus the technique is ideal for confirmation, and therefore widely accepted in doping control, food analysis etc.

Enhanced S/N and selectivity compared to GC-MS justifies the use of GC-MS/MS in doping control. Whilst the sensitivity gain for GC-HRMS over GC-MS is 10-20 fold, the reported gain in sensitivity for GC-MS/MS over GC-MS was somewhat variable. Bowers and Borts [170] applied GC-MS/MS for separation and confirmation of anabolic sterols in urine using large volume injection (25 μL). After carefully selecting both GC and ion trap MS/MS conditions, a factor of 100 fold better detection for 19-norandrosterone (5 pg mL⁻¹) was achieved when compared to GC-MS analysis. On the other hand, in other reports [171, 172] using GC-MS/MS for analysis of anabolic agents and β-blockers, the reported limit of detection was from 0.5 - 20 ng mL⁻¹. The variation most likely resulted from use of compromise conditions required for best overall sensitivity (choice of precursor ions, CID conditions, selection of product ions etc.). However, it has been demonstrated that tandem mass spectrometry offers increased S/N, with concomitant lower detection limit for sterols in a complex urine matrix. Reproducible mass spectra at 1 ng mL⁻¹ for anabolic steroids in urine were possible by using this technique [170].
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Drugs analysis using GC-MS/MS has proved beneficial, in urine, saliva and hair matrices. Detection limits were in the range of 0.1-5 ng mL\(^{-1}\) for cocaine and related compounds in saliva, 0.4-1 ng mL\(^{-1}\) for amphetamines in urine and 6-52 pg/mg for drugs of abuse in hair. Gambelunghe et al. [173] reported successful application of GC-MS/MS in four forensic cases related to recent or past abuse of drugs, with analysis of different lengths of hair from the root providing unambiguous evidence of the drug abuse history.

A coupled chromatographic separation with a 2D detection system comprising GC-HRMS/TOFMS was applied successfully for screening and confirmation of the key anabolic agents in urine at a concentration from 1-3 ng mL\(^{-1}\) [174]. An excellent S/N in full scan MS2 mode was supported by the high acquisition rate of TOFMS. The authors reported this system to be useful for confirmation, when the signal was close to the
detection limit of GC-HRMS, and justified the addition of the extra dimension to the detection system.

However, coelution of components which share the same fragment masses (possibly produced by the same fragmentation process) may still occur on the chromatographic column, especially where the complex matrix comprises components similar in structure (sterols/metabolites in urine and blood; PAHs in urban air; PCBs in environmental samples, etc.). Measuring a minor amount of analyte in highly complex matrixes is still a challenging task even for the most selective detection systems and further chromatographic separation is highly desirable. Bowers and Borts [170] stated that none of the five tested columns with different selectivity was able to separate all of the tested sterols (epimers, isomers, isobaric compounds), and so required the more highly selective detection system of tandem mass spectrometry.

3.3.1.4 Four Dimensions: One dimension of separation; three dimensions of detection (1SEP-3SEL)

The group of Bowers and Borts [170] have used GC tandem MS to further fragment the unusually stable m/z 545 ion from 3’OH-stanozolol, derived from the fragmentation process and selected in MS2. After isolation and storage of this ion, a further CID voltage waveform was applied, producing fragmentation of that ion to yield a second generation mass spectrum (MS3). In this way an additional dimension is added to the tandem MS to obtain (MS/MS/MS) giving a four-dimensional technique. Notwithstanding the potential power of such a tool, there appears to be little routine analysis in this area so perhaps robustness or broad applicability remains to be proven.
3.3.2 Multidimensionality arising from separation (n-SEP)

3.3.2.1 One dimension: One dimension of separation, no detection selectivity (1SEP-0SEL)

Classical one dimensional gas chromatography (1D-GC) still occupies the majority of past and present separation methods, although there is both a long history of multidimensional separations, with more recently an increased activity in this area. Separation based on a single column coupled to a single, or multiple, detector(s) via splitting the effluent at the end of the column, may be supplemented for increased separation power by using two separate – or parallel – columns of different phase, or longer and/or narrower I.D. columns. There is a singular aim in this work – to achieve better resolution of components. This usually increases the separation time, but there is a diminishing advantage when increasingly complex mixtures have to be analysed, at lower levels, with major component interferences. To overcome the many coelutions in the chromatogram, which is unavoidable due to the peak capacity limitations of a single column, off-line purification and concentration steps can be developed, leading to simpler chromatograms, increased resolution, and greater S/N. These extra steps are time-consuming, operator intensive, may involve use of toxic solvents or expensive equipment, and can introduce extra contaminants or involve critical component losses especially in trace analysis. Ultimately, these steps may have little success. A new paradigm may be required. Hyphenated with a non-spectroscopic detector, the information content is likely to be incompatible with component identification needs, and so for all but the simplest analysis, alternative methods must be sought.

3.3.2.2 Two dimensions: Two dimensions of separation, no detection selectivity (2SEP-0SEL)

The obvious question is whether a coupling of two techniques of the same type, like GC to GC or LC to LC, can be considered as a multidimensional technique or just a hyphenated
technique? Or maybe we have to ask ourselves if the coupling of two techniques of the same type is hyphenation? We would argue that the multidimensional realm can include the area of coupled column methods, such as comprehensive two-dimensional GC (GC×GC), multidimensional GC (MDGC), multidimensional LC (MDLC) etc. If “dimensionality” comes from the number of uncorrelated structure-specific signals, than the separation of the components on two orthogonal columns will fall well within this group. One of the most important criteria for multidimensionality is the availability of the reading (signal) in each dimension, and keep it as much as possible uncorrelated to the signal from the previous dimensions. Orthogonality of the column sets applied in MDGC is responsible for this criterion.

According to this classification, if the separation is based on two distinct mechanisms and the method can be applied in a manner that permits a measure of orthogonality or independence of these mechanisms, then a 2D separation technique is defined. This approach achieves selectivity from separation alone, thus uses non-spectroscopic detection, however multidimensionality of detection will be an additional capability to the technique (see below).

Several techniques comprise this group: heart-cut GC and GC×GC (explained in detail below), coupled to e.g. FID, NPD or ECD detectors. They usually exhibit high separation, but low detection confirmation power. Their application is mainly in the field of profiling in the petrochemical industry, essential oil analysis, or in method development. If there is one advantage, it is probably the price, since these detectors are usually inexpensive.

Neumann et al. [175] have observed that some problems, such as inefficient separation of certain parts of the impurity profile of raw processed heroin samples and complete masking of important components by interfering matrix compound, remain unsolved beside the use of a highly efficient and perfectly deactivated capillary column. He has realized that the use of two coupled capillary columns with different polarity, as previously used in tobacco smoke separation [176], can solve the problem. As it was
expected, the three heart-cuts from the first column (SE-54) giving 6, 5 and 6 components, were transferred to the second column coated with a more polar OV-17 stationary phase, giving an increase in the number of resolved peaks (17, 13 and 12, respectively). Similarly, the important impurity – acetylthebaol, which was completely masked by the huge peak of rosin matrix on the first column, was nearly quantitatively separated on the second column. However, the extreme complexity of the technology involved in the separation has prevented the application of column-switching on a routine base.

3.3.2.3 Three dimensions: Two separation dimensions, one dimension of detection (2SEP-1SEL)

‘Heart-cut’ gas chromatography

Increased separation of a region of interest in 1D GC can be obtained if the target zone (termed a heart-cut; H/C) is redirected to a second column with different separation mechanism. Usually, the transferred segment is just a minor part of the first column separation, so is limited in both number of H/C and duration of each sampled zone. Sample transfer may be achieved by a number of specialty devices – sampling valves, storage trapping zones, mechanical switching, and pressure-based microfluidic devices; the Deans switch is the most popular of these. This should achieve quantitative and reproducible transfer of small, unresolved pre-selected fractions of effluent from the first column, to the second column. The extent of separation improvement will be related to the magnitude (peak capacity; total number of peaks; mechanism of separation) of the sampled zone, to the available peak capacity and capability of the second column to separate the transferred components. The process will clearly demand a significant increase in peak capacity for the transferred zone, and although there is much application of MDGC in the literature of petrochemical, essential oil and environmental analysis, in the drugs and doping control area the number of applications do not reflect its potential for improved analysis. Generally there is no special requirement placed on detection systems in MDGC since the second column tends to be rather ‘classical’, so FID, NPD,
ECD, MS and IR can be connected to the MDGC. It should however be appreciated that as separation improves, detection methods should work better.

As a demonstration of this principle, the use of heart-cut GC with olfactometry, where human ‘sniffers’ are used to detect ‘character-impact odorants’ allows a better correlation of resolved peaks with the perception of odour [177]. Human olfaction can be very sensitive and selective toward odour-active components, and so this detector constitutes an additional dimension for GC analysis. Complex mixtures of essential oils and component coelution lead to odour-active component assignment being difficult or impossible. Transfer of a narrow H/C fraction of a first dimension (\(1^D\)) column effluent to the second dimension (\(2^D\)) column allows ‘quantitative’ separation of odour-active components. Eyers et al. [177] employed heart-cut GC to determine odours responsible for coriander leaf and hop essential oil aromas. Whilst not classified as an instrumental technique, the human nose possesses all the hallmarks – with its sensitivity and selectivity attributes – of a spectroscopic detector in GC. This example demonstrates the principles of accelerated analysis by adding additional dimensions to the analysis. Although the separation efficiency of heart-cut MDGC is very high for target separation regions, frequent heart-cut events during a single analysis cannot be easily accommodated due to the time demand of each \(2^D\) analysis. This limits the gain in total peak capacity.

**Comprehensive two-dimensional gas chromatography**

In 1991 Phillips and Liu [178] introduced a new approach to expand the peak capacity of 1DGC and H/C MDGC. The key to this new approach was in the use of a short, fast-eluting \(2^D\) column, combined with a sampling strategy which was faster than the peak width of compounds eluting from the \(1^D\) column. The technique of comprehensive 2D GC (GC×GC) relies on continuous transfer of small time-fractions of effluent from \(1^D\) to \(2^D\) in a pre-defined time (the modulation period; \(P_M\)), where \(P_M\) normally ranges from 3 s to 8 s. Maximum retention on \(2^D\) (\(= 2t_{R_{(max)}}\)) should be less than \(P_M\) so that all the components elute prior to launching the next portion of effluent to \(2^D\). Effluent from \(1^D\) may be either (i) ‘sub-sampled’ with only a small portion of each peak taken via use of a diaphragm...
Multidimensional gas chromatography

valve, (ii) sampled in a differential flow modulator to collect most of the $^1$D peak before flushing it to the $^2$D column using a high flow rate, or (iii) refocused at the beginning of the $^2$D column using a (cryogenic) interface at the column confluence which traps the effluent and introduces it to $^2$D as a narrow band. The first cryogenically driven modulator was the longitudinally modulated cryogenic system (LMCS, see Figure 3.3), upon which most cryogenic modulators available today are based, with cooling by air, or liquid N$_2$ or CO$_2$.

The efficient trapping and fast releasing of the components is usually achieved by cryo-focusing (CO$_2$), producing ultra-narrow peaks at the end of the $^2$D column. The moving part of the modulator (mp on Figure 3.3) is continuously cooled down to the optimal trapping temperature, condensing the components in the column (going through the moving part) at the cooled position 1. At predefined time intervals the modulator moves down to position 2, leaving the trapped components at location 1 exposed to the oven temperature, which is usually 100-150 °C higher than the $T_M$. This enables fast releasing of the trapped components onto the $^2$D column, followed by increased S/N due to the compression effect. Co-eluting components on $^1$D, where the separation is based on the first mechanism, are separated on the orthogonal $^2$D column, where the separation is based on the second mechanism.

Figure 3.3 Schematic presentation of GC×GC system with LMCS based modulator
The GC×GC format for data collection and transformation is presented schematically in Figure 3.4. Each 5 s modulation slice of 1D effluent, represented by the series of vertical lines in B, is trapped and released on 2D column and this is represented as a narrow band spot in B. Four modulation slices are observed for the first eluting peak in 1D (B) and only 2 slices for the second peak, which coelutes with the former in 1D (B), but is completely separated in GC×GC format (A). The most interesting case is the last eluting component in 1D, where GC×GC reveals a minor peak completely overlapped by the major peak. This is due to the separation of these two components on the 2D column, which is preferably orthogonal to the 1D. The signal height is usually described by different colours, each representing different response magnitude. Note that the y-scale in A is 5 s only, the same as the modulation period.

![Fig. 3.4 1D GC chromatogram peak modulation and GC×GC data format transformation and presentation to a so called 2D plot.](image)

Narrow peaks obtained at the end of the separation process (width at half-height ~ 50-150 ms) require fast detection for adequate quantification. Normally, acquisition rates of 20+ Hz is required, and requirements for GC×GC detection have been reviewed [179]. The overall separation power will depend on orthogonality exhibited by the two columns, thus
their separation mechanism. One common variant is to employ a non-polar (NP) $^1$D column phase, then a polar (P) $^2$D phase. Thus two co-eluting components on $^1$D will have similar volatility, and provided they sufficiently differ in their ‘polarity’, they will be separated by the $^2$D column. The total analysis time should be similar to that of conventional GC, since the $^1$D column approximates a conventional column; Junge et al. [180] demonstrated fast analysis (5 min) for allergen compounds by using GC×GC. It is proposed that some off-line sample purification steps applied in 1DGC may no longer be required, since peak overlap in 1DGC can be compensated by the greater component separation in GC×GC, thus increasing overall sample throughput [181]. The maximum overall peak capacity is often reported as $^1n \times ^2n$, with components separated over the time plane defined by $^1$D and $^2$D retention times. This should facilitate sample-to-sample comparison or fingerprinting. In addition, since various classes of substances related by structural features and/or property (homologues, congeners) are positioned on the plane according to their structure, this generates ‘structured chromatograms’ in ways that are not possible in 1DGC. Signal-to-noise ratio enhancement arising from cryogenic compression of the sample band at the modulator is another benefit, which, when considered with resolving power of GC×GC makes the identification of individual components more secure.

Quadrupole MS (GC×GC-qMS) and TOFMS (GC×GC-TOFMS) have both been reported for detection in GC×GC. More recently, Tobias et al. [182] have applied isotope-ratio MS (IRMS) methods to the GC×GC experiment. However, the relatively slow scanning or sampling capabilities of qMS when applied in full mass scan mode limit its suitability for quantitative data acquisition in GC×GC. Selected ion monitoring (SIM) decreases the MS duty cycle, but only for a small number of selected ions. Narrow mass range scanning with qMS [183] was used to increase sampling rate for an essential oil, and Song et al. [184] employed the same strategy with GC×GC-qMS for separation and detection of 77 drugs with reduced mass scan range to obtain greater scan speed. Omitting the molecular ion was shown to hinder similarity matching against the MS library. Use of GC×GC-qMS in
doping control for horse racing analysis [185] was shown to increase peak height and reduce interference ions in the spectrum, when compared to classical GC-qMS.

High data acquisition rates of TOFMS (up to 500 spectra/s) improves compatibility with GC×GC [162]. This high ‘data density’ supports peak deconvolution capabilities, where coeluting peaks differing in retention maxima by only a few scans can be reliably detected. This additional feature is still available in GC×GC analysis, with residual unresolved peaks deconvoluted to provide identification. Recent work to assess applicability of GC×GC-TOFMS for endogenous sterol analysis and metabolites compared results with GC-TOFMS [186]. An increase of 30 fold in signal height arising from signal modulation was gained in the GC×GC-TOFMS experiment, when using the same acquisition rate. This produced ~ 1 order of magnitude lower limit of detection. Two additional criteria for component identification were proposed when compared to 1D GC at the low detection limits required: 1) retention time matching in the 2D space, and 2) full mass spectral similarity. For example, estrone was detected in the free fraction in urine at a concentration of 0.1 ng mL\(^{-1}\) with a similarity of 940 (on a scale from 0-999). At this level, it is normally necessary to employ SIM methods, and so conventional library matching is not possible. Since GC×GC-TOFMS spectra of sterols differ sufficiently from the commercial quadrupole based MS spectra, this justified creation of an in-house TOFMS database. It was also concluded that GC×GC offers much higher similarity against in-house TOFMS spectra than 1D GC, under the same conditions, due to increased ion abundance. However, the most critical benefit of using GC×GC-TOFMS remains the separation of components on the 2D plane. The separation of analytes from each other, from the matrix, and from column bleed at higher elution temperatures all benefit from the additional separation dimension.

In the area of doping control analysis by using GC×GC-TOFMS, Silva et al. [187] showed improved signal response and separation compared to GC-MS. Similar conclusions were achieved in the work [188], extending the research on separation optimization, mass spectral similarity, quantification and compliance to World Anti-Doping Agency (WADA)
identification and detection limit criteria. Thus coelution of components with similar structure and fragmentation pattern can occur when using 1D GC, while they are successfully separated using GC×GC (Figure 3.5). Better separation was obtained by using a thicker second dimension column film thickness (0.2 μm). Examples of coelutions on the 1D column include an endogenous component with clenbuterol for extracted ion m/z 86 (but not at m/z 335), and the coelution of an endogenous component with methyltestosterone metabolites for extracted ion m/z 255. In each case they are baseline separated on the 2D column in GC×GC.

Differences noted between TOFMS and quadrupole MS spectra compromises the choice of ions for 1D (qMS) and GC×GC (TOFMS) analysis. While the most abundant ions in the qMS of the majority of sterols are at the higher masses, the relative abundance of the higher mass fragments in TOFMS spectra are often dramatically reduced. Since quantification ions are preferably higher mass ions, this reduces the apparent sensitivity of TOFMS. However, this observation is compensated by component refocusing on the 2D column in GC×GC, giving narrow and tall peaks. Since the quality of mass spectra depends on the ratio of ions originating from the component over other ions originating from the matrix or column bleed, better differentiation of components is highly desirable.

Figure 3.5 Extracted ion three dimensional plot for 19-norandrosterone, showing its coelution on 1D with an endogenous component with similar mass fragments. (A) 405 m/z and (B) 420 m/z. Note that these two mass fragment are the most characteristic for 19-norandrosterone [153]. Reproduced with permission of Future Science Ltd.

Song et al. [189] applied GC×GC-TOFMS to drug analysis, where improved separation, sensitivity and identification were obtained, compared to GC-qMS. It was shown that the
method exhibits a good linearity ($R^2$ in the range 0.978-0.998) and precision. The latter was confirmed by quantification of diazepam in a real forensic blood sample, where the same indicative concentration was obtained as that with a well established GC-NPD method.

Recently, a comprehensive two-dimensional gas chromatography was applied for separation of impurities in heroin samples and pixel-based PCA was used for chemometric processing [190]. Twenty-one heroin samples were classified in several groups, depending on the cutting level of the dendrogram obtained from the pixel-based analysis. The grouping has been found to match the results from the well-established comparative studies and/or the forensic background of the samples. In addition, the most discriminating components can be selected according the Fisher criteria and subsequently can be used as markers for analysis of future illicit drug seizures. Even though the study was based on the data of minor component analysis (impurity profiling) only 9 components were taken for calculations. The authors stated that the rest of the 16 components used by some criminal police authorities in Europe were not found in the samples or there were no spectra accessible. According to the author’s experience in using time-of-flight mass spectrometry (TOFMS) as a detector in GC×GC, the missed components can be partially contributed to the observed differences in the TOFMS spectra when compared to the quadrupole based entries in the commercial MS libraries (NIST05, Wiley7) [186]. However, the study represents a new way of improvement of the quality of GC data, based on separation of the components on two orthogonal columns. At the author’s best knowledge, this is probably the most remarkable step in heroin profiling and generally, in drug profiling. The research should be extended to more heroin samples in order for the results to be generally applicable.

3.3.2.4 Four dimensions: Three separation dimensions, one dimension of detection (3SEP-1SEL)

Watson et al. [191] have developed a three-dimensional gas chromatographic separation system by coupling three columns in series, interfaced by two six-port diaphragm valves.
Another example is the work of Edam et al. [192], where LC-GC×GC was applied for characterisation of complex petrochemical samples. In both cases an FID detector provided identification of separated components, although TOFMS can be applied to form a real four dimensional technique. Whether such a system provides improved separation power in a procedure that can usefully implemented in practice is still to be demonstrated, considering also the novel presentation formats needed of such a system.

3.3.2.5 **Four dimensions: Two separation dimensions, two dimensions of detection (2SEP-2SEL)**

Improvement in multidimensional methods will continue in future, for instance, the combination of multidimensional separation capabilities with multidimensionality in the detection system. The use of MDGC with MS/MS would seem logical, but GC×GC coupled with MS/MS would present a more technically challenging task. Whilst there is no reported case of such a combination, coupling GC×GC or H/C GC to the tandem MS experiment seems a likely development. Such a future technique will probably combine the relatively high acquisition capabilities of a filter type MS1 (preferable HRMS, but more likely qMS) coupled in tandem with full scan MS2 (probably TOFMS) as a detection system. In combination with enhanced separation power of GC×GC, this real four-dimensional technique (GC×GC-HRMS/TOFMS) will represent best detector selectivity with best separation power.

3.4 **CONCLUSIONS**

Gas chromatography has a rich and extensive history in the study of drugs, natural products, and compounds derived from biological processes. The multidimensional GC tools (GC-MS, GC-TOFMS, GC-MS/MS, GC-HRMS, MDGC-MS, GC×GC-TOFMS) that are currently available to the analyst for drugs and metabolic profiling are mature and flexible enough to allow for their widespread use in the study of biochemical processes.
Whilst the emerging GC×GC technique has thus far shown encouraging results, albeit for limited applications studies, it should play a major role in future drugs and metabolic profiling. Current applications in GC×GC still address basic tasks such as optimization of various hardware configurations, development of software tools which provide the capacity to handle large data sets produced by high-resolution instrumentation, and MS detection protocols.

The availability of a more complete drugs and metabolite complement of isotopically labelled standards would assist in quantification. Whilst dedicated MS libraries are available, construction of more comprehensive databases continues. This progressive development suggests that more work is still required for precise peak identification. GC×GC may assist through 3-dimensional correlation of $t_R^1$, $t_R^2$ and MS data, along the lines of present retention index / MS spectral matching in 1D GC-MS.

Multidimensionality, arising from both detection dimensionality and separation dimensionality, leads to more selective determination. Detection selectivity focuses on detection precision, usually by making it increasingly ‘blind’ to the matrix, and sensitivity by improving signal-to-noise, whilst method separation focuses on the separation efficiency with an aim to resolve more components. This generally increases the method accuracy and precision, and the gross number of components separated, detected and quantified. The ideal technique should be, as a logical consequence of the above, the most powerful separation technique coupled to the most selective and sensitive detection system. The search for such a system is still in progress.
Chapter 4

4 APPLICATION OF COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY TO ENDOGENOUS STEROLS ANALYSIS
4.1 INTRODUCTION

The analysis of sterols is challenged by their usually low concentration in samples, and complex matrices in which they are found. It requires extraction/pre-concentration, followed by a separation technique, normally chromatographic, coupled to a sensitive and selective detector, such as mass spectrometry (MS). Complexity of samples, as well as low level analytes, and the need for precise results demands best possible identification and quantification.

Sterols are important for a number of reasons. The occurrence of estrogenic sterols in surface water is environmentally important. Endocrine-disrupting chemicals (EDC) can interfere with the function of the endocrine systems in humans and animals, and estrogen sterols are one of the most obvious and important of these. Concentrations may be low (ng L⁻¹), but can be sufficient to interfere with the normal reproduction and development of wildlife [193]. Naturally produced estrogens such as estrone and 17β-estradiol, are mainly released in water by inefficient removal from waste-water treatment plants (WWTPs), where they are found in humans and livestock excreta [194].

Many sterols indicated as estrogens, gestagens and androgens (EGAs) are forbidden in products of animal origin due to the possible toxicological effect on consumer's health [195]. The minimum required performance limit (MRPL) of the methods for analysis of these banned hormonal growth promoters is as little as 2 μg L⁻¹, as reported in the Italian Monitoring Plan [196]. Plasma or urine levels of some sterols can be used as a marker of biosynthesis deficiency [197].

The desire for maximum athletic performance in sporting arenas, in addition to social recognition and financial rewards, leads some competitors to use performance enhancing
drugs. Among the most important are anabolic steroids similar in structure and activity to the male hormone testosterone. They are used by competitors to improve muscle mass and to accelerate recovery times after strenuous exercise [198]. Because of this, they are banned in sport competition, and the MRPL for the method of analysis defined by the World Anti-Doping Agency (WADA) is set at 2 ng mL\(^{-1}\) [133].

The separation power of gas chromatography (GC) combined with the selectivity offered by mass spectrometry (MS) detection, makes GC-MS the method of choice for sterols analysis. Methods such as solid phase extraction (SPE) and liquid/liquid extraction (LLE) are applied prior to analysis to effect a concentration step since sterol concentration is usually very low. Most of the sterols and their metabolites are polar compounds containing one or more functional groups (-OH, >C=O, -COOH). In order to decrease polarity and to increase volatility and thermal stability of sterols, derivatization can be applied. Of the derivatization reagents available, BSTFA [193, 194, 199, 200] and MSTFA [139, 194, 195, 200-202] are the most common; trimethylsilyl (TMS) derivatives of sterols are stable, giving informative fragmentation ions under positive electron ionization (EI). This allows identification and quantification of sterols in complex matrices at a low concentration, if sufficient separation from chemical noise is achieved.

Comprehensive two-dimensional gas chromatography (GC×GC) has proved to be advantageous over conventional gas chromatography (1D GC) in terms of greatly increased peak capacity (separation power), signal-to-noise ratio enhancement, and it also offers unique structured chromatograms when structurally related classes of substances (analogues, congeners, isomers) are analysed. Principle of operation of this technique is given in Chapter 3 (section 3.3.2.3). This technique has been successfully applied in many areas, including petrochemicals [179, 201], essential oils [168, 180], food analysis [204, 205], environmental [202-204], forensic [189, 205] amongst others. Our pilot study on application of GC×GC-FID in endogenous sterol analysis [186] showed an increased separation power over 1D GC and signal enhancement of up to 35-fold. A previous study of derivatized environmental steroids, including steroids derived from the gut such as
coprostanol [206], revealed nine detectable faecal sterols of interest in GC×GC instead of five incompletely resolved in 1D GC.

The present study investigates the applicability of GC×GC coupled to both flame ionization (GC×GC-FID) and time-of-flight mass spectrometry (GC×GC-TOFMS) detectors for analysis of 27 endogenous sterols in human urine. LLE of the free sterols fraction and derivatization with $N,O$-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were performed prior to analysis. Comparison between 1D GC and GC×GC in terms of separation efficiency, sensitivity and identification power of TOFMS detector are reported.

### 4.2 EXPERIMENTAL

#### 4.2.1 GC×GC-FID

The gas chromatograph, equipped with a flame ionisation detector (GC×GC-FID), used in the study was an Agilent 6890 system (Palo Alto, CA, USA) with a Longitudinal Modulation Cryogenic System (LMCS, Chromatographic Concepts Pty Ltd., Doncaster, Australia). Two column configurations were used with this system, differing only in the length of the 1D column: (1) 30 m length of BPX5 phase, 0.25 mm I.D., 0.25 μm film thickness ($d_i$) as a first dimension column (1D) coupled to a 1 m length of BPX50 phase, 0.1 mm I.D., 0.1 μm $d_i$ as a second dimension column (2D); and (2) the same configuration as (1) except the column length of 1D was 18 m. These column sets will be abbreviated as NP/P and NP(s)/P respectively according to the non-polar, non-polar (short), and polar phase nature of the column sets. All columns were from SGE International, Ringwood, Australia. Oven program for the first system was from 240 °C to 270 °C at 2 °C min$^{-1}$, then to 320 °C at 4 °C min$^{-1}$ (hold for 10 min), and for the second system was from 240 °C to 260 °C at 2 °C min$^{-1}$, then to 320 °C at 10 °C min$^{-1}$ (hold for 5 min). The injector and detector temperatures were 320 °C and 330 °C, respectively, and the sampling frequency was 100 Hz, both for GC and GC×GC. Hydrogen was used as a carrier gas at a flow rate of
1.2 mL min$^{-1}$ for (1) and 1.0 mL min$^{-1}$ for (2). The modulation period was varied from 3 s to 6 s, and temperature of the modulator system ($T_M$) from 50 °C to 170 °C, during the optimization study. CO$_2$ was used as a coolant in the LMCS and nitrogen as a flush gas at a pressure of 15 psi. The modulator in GC×GC operation was commenced at a time from 2 min to 5 min prior to the elution of first sterol, and turned off after the elution of the last sterol. Agilent ChemStation was used for data acquisition and processing.

### 4.2.2 GC-qMS

The gas chromatography–quadrupole mass spectrometry system (GC-qMS) consisted of an Agilent model 6890 GC, coupled to a model 5973 MS detector with fast electronics upgrade. A single 30 m long BPX5 column (0.25 mm I.D.; 0.25 μm $d_f$) was used for separation of sterols. Oven program was from 200 °C to 250 °C at 2 °C min$^{-1}$, then to 320 °C at 4 °C min$^{-1}$ (hold for 15 min). The injector was set at 320 °C, transfer line at 280 °C and ion source at 230 °C. The scanning mass range was from 45 m/z to 650 m/z at applied electron ionisation voltage of 70 eV. Data acquisition and processing was done by Agilent MSD ChemStation software.

### 4.2.3 GC×GC-TOFMS

A LECO time-of-flight (TOF) mass spectrometer model Pegasus III (LECO Corp., St. Joseph, MI, USA) connected to an Agilent 6890 GC was used in GC×GC-TOFMS experiments. The modulator was a LMCS system, and subsequent to optimization, the cryotrap was operated isothermally at 120 °C and the modulation period was 5 s. Two column configurations were used in this experiment: the first was the same as (1) listed for GC×GC-FID analysis above, and the second was 30 m BPX50 (0.25 mm I.D.; 0.25 mm $d_f$) as ¹D and 1 m BPX5 (0.1 mm I.D.; 0.1 μm $d_f$) as ²D column (the latter referred to as a P/NP set). Oven program for the first column configuration was from 240 °C for 1 min, increased
to 260 °C at 2 °C min\(^{-1}\) then immediately increased to 320 °C at a rate of 10 °C min\(^{-1}\) (hold for 10 min). Data collection rate was 100 Hz over the mass range from 45 m/z to 570 m/z.

The oven program for the second column configuration was adjusted for splitless injection, so the starting oven temperature was 80 °C, hold for 1 min, increased to 240 °C at 40 °C min\(^{-1}\), increased to 260 °C at 2 °C min\(^{-1}\), and finally increased to 330 °C at a rate of 6 °C min\(^{-1}\) (hold for 5 min). Helium was used as a carrier gas in both methods at a flow rate of 1.5 mL min\(^{-1}\). The temperature of injector, transfer line and ion source were 300 °C, 280 °C and 230 °C, respectively. The detector was operated at 1550 V, and applied electron ionisation voltage was 70 eV. Data collection rate was 50 Hz over the mass range from 45 m/z to 570 m/z. A separate GC×GC-TOFMS based library was generated for identification purposes. Data acquisition and processing were performed by ChromaTOF software (LECO Corp., St. Joseph, MI, USA).

### 4.3 RESULTS AND DISCUSSION

#### 4.3.1 Derivatization of sterols

The efficacy of the derivatization procedure was checked by running the derivatized individual sterols on the GC-qMS system. For their names and annotations see Chapter 2 (section 2.1.1). All of the estranes (2, 5, 15), cholestanes (14, 16, 18, 19) and androstanes (except 8) gave single products with no measurable amount of partially-derivatized or underivatized products. 8 gave two products (mono- and di-TMS), with the mono-TMS favoured in an approximate 2.5:1 ratio at 80 °C. When temperature and/or time of derivatization are increased the ratio is decreased, but mono-TMS was detected in measurable amount under all experimental conditions applied. In respect of derivatized pregnanes, a single product was obtained only for one sterol (20), with multiple products for four (21, 22, 23, 25), and three pregnanes gave low or no signal at all (24, 26, 27). Because of this pregnanes were excluded from the experiments dealing with urine.
4.3.2 GC×GC-FID separation and identification

The GC×GC separation of the standard mixture of sterols was initially accomplished by GC×GC-FID using column configuration NP/P, where modulation period and modulator temperature \((T_M)\) were optimized. An acceptable separation was achieved in 32 min (Figure 4.1a.), at 5 s modulation period, but the best modulation temperature was found to be dependent on retention time of the sterols and presumably their boiling points.

![GC×GC-FID separation of (a) 27 BSTFA derivatized sterols on a 30 m BPX5 / 1 m BPX50 column set. The small dots (minor components) are partially-derivatized pregnanes; (b) 19 derivatized sterols on 18 m BPX5 / 1 m BPX50 column set. Good separation is achieved in almost 16 min.](image)

Generally, it was found that increasing retention time of sterols accompanies their optimum \(T_M\) being shifted towards higher temperature. Using column configuration NP(s)/P, the \(T_M\) study involved analysing a standard mixture of sterols at isothermal modulation temperatures of 50 °C, 80 °C, 110 °C, 130 °C, 150 °C and 170 °C. The curves in Figure 4.2 show the influence of \(T_M\) on peak widths, and their direct effect on the
selectivity and sensitivity. The narrowest peak width for the first eluted sterol (1) with a retention time of 4 min is obtained at 50 °C, but for the last eluted sterol (19) with a retention time of 16 min, the best $T_M$ is found to be 170 °C. For the rest of the sterols the $T_M$ producing narrowest peak widths tends to increment proportionally with retention time of sterols.

![Figure 4.2 The influence of modulator temperature ($T_M$) on peak width for 1 (androstanol), 6 (DHEA), 21 (pregnatriol) and 19 (cholesteryl butyrate).](image)

By applying a $T_M$ program from 80 °C to 160 °C (in four steps incremented by 20 °C), good separation for all sterols was achieved in only 16 min (Figure 4.1b), with negligible reduction in the separation achieved on the 1D column compared to the separation on the 30 m BPX5 column (column configuration NP/P). Fine “tuning” of the modulation temperature was then tested by running the same sample using a modulation temperature program which tracks the oven temperature program, with a differential of −120 °C, −140 °C, −160 °C and −180 °C. The best peak shapes were achieved by tracking the oven temperature program by about −160 °C. Presently, the only way to set the $T_M$ is by tedious manual adjustment. From these results, an automated method of tracking the oven
temperature at a given differential setting appears desirable, and a method for this is presently being evaluated. However, by setting the $T_M$ isothermally at 120–130 °C a compromise can be achieved for best sterol peak shapes using this column configuration (see Figure 4.2).

![Figure 4.3 2D plot of 12 androstanes separated on a 30 m BPX5 / 1 m BPX50 column set. The peaks are presented at only 2 pA from the baseplane, showing almost no peak tailing or fronting. Peak 8* is that for the respective mono-TMS sterol.](image)

The GC×GC separation that can be achieved may be compared to conventional 1D GC separation, where no modulation is applied. Two completely overlapping pairs of sterols predicted to arise in the 1D chromatogram of the 12 androstane standard mixture on a 30 m BPX5 phase (Figure 4.3) occur at 15.0 min (9 and 11) and 16.8 min (13 and 17). They can be successfully separated in GC×GC, under the same chromatographic conditions, accompanied by dramatic enhancement of the signal. Here, on average, a 30-fold increase of signal height is observed in GC×GC compared to 1D GC, under the same chromatographic conditions (see Figure 4.4). Note that the split ratio in GC×GC is 50:1, compared to 20:1 in 1D GC. A peak width at half height of 60 ms to 100 ms and nearly ideal peak symmetry is obtained when the optimized $T_M$ program is applied. The androstanes peaks in the 2D plot in Figure 4.3 are presented at only 2 pA response above the baseplane, exhibiting almost no peak tailing, nor fronting.
Figure 4.4 Chromatograms of 12 derivatized androstanes on (a) 1D GC-FID and (b) GC×GC-FID. For GC×GC analysis, the split ratio was increased by 2.5 times. The insets show the two pairs of co-eluting broad sterol peaks in 1D (# and *, and $ and &, respectively), which are completely resolved in GC×GC analysis. Note that about three modulated peaks are obtained for each sterol under GC×GC operation.

Whilst peak tailing may be observed when the temperature of the modulator is too low and peak fronting occurs for non-overloaded peaks when the temperature of the modulator is too high (Figure 4.5), it was found that, provided $T_M$ is optimized, the sensitivity of GC×GC-FID approaches the sensitivity of GC-qMS in SIM mode when multiple ions are monitored. Where sterols are well resolved from the chemical noise in GC×GC, the sensitivity is further improved and does not suffer the sensitivity loss of GC-qMS (SIM mode) when many ions are selected to monitor many compounds simultaneously. However, the lack of identification power of the FID detector limits its applicability in sterols analysis in complex mixtures, since the analyte signals may be impossible to assign amid the much higher signals from the matrix. The retention time window, now locked by $^1$D and $^2$D retention times, can give information of the possible presence of a particular sterol, but confirmation is still required, or at least, desirable.
4.3.3 GC×GC–TOFMS separation and identification of sterols

Knowledge about sterol separation, the 1D and 2D retention times, and optimized conditions from the above studies, were used in subsequent GC×GC-TOFMS experiments. Conditions were focused on maximising sensitivity, so 3 μL splitless mode injections were made in order to establish lower limit of detection. A 50 Hz data collection rate was adequate for proper peak shape, identification and for maximum sensitivity, since higher sampling rate will decrease S/N magnitude (acquisition rate $\propto N^{0.5}$).

Separation of the standard mixture of BSTFA derivatized sterols at a concentration of 10 μg mL$^{-1}$ on the NP/P column configuration (BPX5/BPX50) is shown in Figure 4.6a. This column configuration showed better separation, with no co-elution, when compared to the
separation of sterols on the P/NP column configuration (BPX50/BPX5), shown in Figure 4.6b, where three co-elutions are observed (4/3, 7/6, 12/11). Whilst better spread of sterols is obtained on the former column set, unfortunately it is accompanied by poorer peak shape. The average peak width (at half height) on the NP/P configuration is almost 300 ms, but for the P/NP column configuration the average is only 120 ms. Because of this, the second column configuration was chosen for further experiments, which then focused on maximizing sensitivity. Since mass spectral selectivity is determined by choice of the proper extracted ion(s) from the full mass spectra offered by TOFMS detection as established by spectral information, this parameter is consistent from 1D to 2D operation.

Figure 4.6 GC×GC-TOFMS separation of (a) 27 derivatized sterols on BPX5/BPX50 column set and (b) 19 derivatized sterols on BPX50/BPX5 column set.

For the P/NP column configuration, a peak table (Table 4.1) with the 1D and 2D retention times was created, and a GC×GC-TOFMS laboratory-based mass spectral library of all BSTFA-derivatized sterols was generated using TOFMS spectra. This in-house library was tested for searching and identification purposes in subsequent experiments. Library spectra were recorded from an extract of spiked water samples with sterols at a concentration of 20 ng mL⁻¹, except for IS (100 ng mL⁻¹) and cholesterol (10 μg mL⁻¹).
An approximate inversion of position of the sterols within the 2D plot is obtained for the two column configurations. For instance 17 has the greatest retention time when BPX50 is the 2D column, but has the least retention time when BPX5 is the 2D column. More revealing is the relative positions of 10, 13 and 15 when the column configuration is inverted. Taking the example of 15, this component is assumed to be more polar since it elutes later on the polar BPX50 2D phase. Consequently, in the inverted configuration column, it will be more strongly retained when the BPX50 phase is used as the 1D phase, being retained to a higher elution temperature. This results in 15 being poorly retained when the BPX5 low polarity phase column is used in 2D. To a first approximation, the relative position of steroids in the second dimension may be expected to depend upon the number of derivatized hydroxyl groups, and the presence of keto functional groups on the macrocycle. Since the BPX5/BPX50 column set comprises a more polar second column, the more polar steroid should elute later in this dimension. It appears that the number of derivatized hydroxyl groups does not affect too greatly the relative 2D position, but as more keto functions are added, retentions in 2D increase. In the inverted polarity set, and using data from Table 4.1, the average 2D retention for sterols with one hydroxyl and no keto group is 1.90 s. For sterols with one hydroxyl and one keto group it is 1.77 s, but sterols with two hydroxyl and one keto groups it is 2.05 s, and those with two keto groups it is 1.56 s. Results in Figure 4.6 demonstrate that position inversion with functional group composition is apparent when the two different column sets are used.

After identification of all derivatized sterols on the 2D plot, creating a peak table and recording a TOFMS based library, next experiments involved the separation and identification of sterols in urine. The data processed chromatogram of the 100 μL blank urine extract from 5 mL urine showed about 2000 peaks (data not shown) at a peak width threshold of 50 ms and a minimum signal-to-noise ratio (S/N) threshold of 10. The peaks prior to the retention time of the first eluting sterol, and just after the last eluting sterol, are excluded in this number of matrix peaks.
Table 4.1 Peak table of sterols by using GC\times GC-TOFMS on BPX50/BPX5 column configuration, with S/N for selected m/z ion value, LOD, match quality and peak width at half height indicated.

<table>
<thead>
<tr>
<th>Sterol</th>
<th>$^{1}t_{R}$ (s)</th>
<th>$^{2}t_{R}$ (s)</th>
<th>conc. (ng mL$^{-1}$)</th>
<th>m/z</th>
<th>S/N</th>
<th>match (TOFMS)</th>
<th>$W_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>690</td>
<td>1.72</td>
<td>0.25</td>
<td>333</td>
<td>20</td>
<td>946</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>750</td>
<td>2.20</td>
<td>0.25</td>
<td></td>
<td>S</td>
<td>951</td>
<td>120</td>
</tr>
<tr>
<td>3</td>
<td>945</td>
<td>1.82</td>
<td>0.25</td>
<td>216</td>
<td>14</td>
<td>953</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>950</td>
<td>1.88</td>
<td>PB</td>
<td>75</td>
<td>156</td>
<td>958</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>1040</td>
<td>2.00</td>
<td>0.25</td>
<td>285</td>
<td>24</td>
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<td>88</td>
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<tr>
<td>6</td>
<td>1045</td>
<td>1.82</td>
<td>PB</td>
<td>129 (360)</td>
<td>272 (11)</td>
<td>942</td>
<td>92</td>
</tr>
<tr>
<td>7</td>
<td>1050</td>
<td>1.80</td>
<td>0.5</td>
<td>347</td>
<td>26</td>
<td>878</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>1050</td>
<td>2.02</td>
<td>1.0</td>
<td>156</td>
<td>67</td>
<td>978</td>
<td>100</td>
</tr>
<tr>
<td>IS</td>
<td>1055</td>
<td>2.20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>1070</td>
<td>1.82</td>
<td>0.5</td>
<td>257</td>
<td>14</td>
<td>978</td>
<td>95</td>
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<tr>
<td>10</td>
<td>1105</td>
<td>1.98</td>
<td>0.5</td>
<td>214</td>
<td>95</td>
<td>923</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>1120</td>
<td>1.74</td>
<td>PB</td>
<td>232</td>
<td>252</td>
<td>951</td>
<td>79</td>
</tr>
<tr>
<td>12</td>
<td>1125</td>
<td>1.70</td>
<td>1.0</td>
<td>73</td>
<td>254</td>
<td>918</td>
<td>90</td>
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<tr>
<td>13</td>
<td>1160</td>
<td>1.78</td>
<td>0.5</td>
<td>73</td>
<td>120</td>
<td>974</td>
<td>117</td>
</tr>
<tr>
<td>14</td>
<td>1180</td>
<td>2.06</td>
<td>PB</td>
<td>217</td>
<td>89</td>
<td>927</td>
<td>97</td>
</tr>
<tr>
<td>15</td>
<td>1215</td>
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<td>940</td>
<td>77</td>
</tr>
<tr>
<td>16</td>
<td>1360</td>
<td>2.06</td>
<td>0.5</td>
<td>91</td>
<td>77</td>
<td>964</td>
<td>97</td>
</tr>
<tr>
<td>17</td>
<td>1355</td>
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<td>368</td>
<td>242</td>
<td>933</td>
<td>120</td>
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<td>18</td>
<td>1455</td>
<td>2.00</td>
<td>PB</td>
<td>129</td>
<td>243</td>
<td>965</td>
<td>104</td>
</tr>
<tr>
<td>19</td>
<td>1675</td>
<td>2.00</td>
<td>1.0</td>
<td>71 (368)</td>
<td>38 (14)</td>
<td>968</td>
<td>106</td>
</tr>
</tbody>
</table>

PB: present in the blank; S: sum of 225 masses, chosen by the software.
* The LOD concentration reported is the lowest concentration of the spiked standard in urine for which the match statistic exceeds 800. This ensures that the library match is still adequate to provide reasonable certainty of peak identity, at the concentration given.

4.3.3.1 Location of sterol peaks in 2D total ion and extracted ion plots

The complexity of the endogenous background in the 2D total ion current (TIC) plot of the spiked urine extract (2 ng mL$^{-1}$) obscures most of the sterol peaks, apart from a few that are well separated from the matrix (Figure 4.7). The internal standard (IS) alone is clearly visible since its concentration (100 ng mL$^{-1}$) is about 50 times higher than the sterol concentration.
Figure 4.7 GC×GC-TOFMS separation of spiked urine extract with sterols at concentration of 2 ng mL\(^{-1}\) (a) and (b) inset showing the location of 5, 6, 7, 10, 11 and 14 in the 2D TIC plot. Note that the endogenous compounds are much higher levels than the spiked sterols.

Much more specific information can be obtained from extracted ion queries of the full scan TOFMS data, facilitated by the strong signal response obtained with prior separation by GC×GC. The full mass spectra capability provided by TOFMS allows choice of any ion in post-analysis data processing, for example according to the best selectivity and/or the best sensitivity. In the extracted ion chromatogram for 5 (416 m/z; M\(^{+}\)), only one peak in the spiked urine extract of sterols at a concentration of 2 ng mL\(^{-1}\) appeared, whereas no peaks are observed in the blank urine sample (data not shown). However, in the extracted ion chromatogram for 360 m/z, characteristic for testosterone (13), epitestosterone (12) and dehydroepiandrosterone (DHEA) (6), more peaks appeared than those from just these three sterols (data not shown). Furthermore, if a more common and abundant ion for most of the sterols is chosen, e.g. 129 m/z, even more peaks are observed in the chromatogram.
Finally, if 73 m/z ion is chosen, (a diagnostic ion for TMS derivatives) all TMS-derivatized sterols and at least all TMS-derivatized endogenous compounds will appear in the extracted chromatogram. Usually, the 73 m/z ion is the most abundant ion in the mass spectra of BSTFA-derivatized sterols, but at the same time the 73 m/z ion is not such a unique ion and so the extracted ion chromatogram of 73 m/z ion is more complex. Figure 4.8 displays selected extracted ions from the TIC data, to illustrate a particular sterol as indicated in the caption.

![Figure 4.8 Extracted ion chromatogram (a) and extracted ion 2D plot (b) for 347 m/z (5α-androstan-3β-ol-17-one) of spiked urine sample with sterols at 2 ng mL$^{-1}$, demonstrating the specificity of position of 7 combined with extracted mass in GC×GC. Note that the same time window as in Figure 4.7 is used.](image)

### 4.3.3.2 Identification against NIST05 versus laboratory-created TOFMS library

In order to compare the identification power of TOFMS when searching against a commercial quadrupole-based MS database (NIST05) and a GC×GC-TOFMS-based in-house laboratory library, the extract of the sterol spiked urine sample at 2 ng mL$^{-1}$ was processed under the two different data processing methods, differing only in the MS library against which searching is performed. When a match threshold is set to 800, only
four of the sterols are identified as a first match against the NIST05 library, but all 19 sterols are positively identified against the TOFMS based laboratory library, with an average match quality of 950 (Table 4.2). Whilst the mass spectra from qMS and TOFMS are visually similar, they differ sufficiently to justify the need for creating a purpose constructed TOFMS based library.

Comparison in terms of searching the different MS libraries was made also between chromatograms in 1D GC and GC×GC. An extract of urine spiked with sterols at a concentration of 10 ng mL$^{-1}$ was run in 1D GC and GC×GC modes, under the same chromatographic conditions, except in the first case the modulator is off. Only three of the sterols in the 1D GC chromatogram are identified against NIST05 at a match threshold of approximately 800 (on a 0 - 999 scale), but 15 out of 19 are identified against the laboratory made TOFMS library at the same match threshold (Table 4.2). The average match quality for the identified sterols ($n = 15$) in 1D GC using the TOFMS library was 867, and 826 for the identified sterols ($n = 3$) in 1D GC versus the NIST05 library. As a comparison, in the GC×GC chromatogram all the sterols are identified against the laboratory-generated library with an average match of 959. At higher concentration the standard mixture of sterols at concentration of 10 μg mL$^{-1}$, revealed an average match quality of 972. The key to this high match is probably in the algorithm of finding the unique masses and apexes of each compound, thus making deconvolution easier. It is much easier to differentiate sharper peaks from each other and from the matrix, than broader peaks that suffer interference, and GC×GC is superior in this respect compared to 1D GC.
Table 4.2 Library match of TOFMS spectra obtained from spiked sterols in urine at 2 ng mL\(^{-1}\) (GC×GC mode) and 10 ng mL\(^{-1}\) (1D GC and GC×GC mode) against TOFMS laboratory-based and NIST05 libraries. Comparative data for sterols at 10 μg mL\(^{-1}\) in standard solution also shown (BPX5/BPX50 column configuration).

<table>
<thead>
<tr>
<th>Sterol</th>
<th>GC×GC</th>
<th>2 ng mL(^{-1})</th>
<th>10 ng mL(^{-1})</th>
<th>10 μg mL(^{-1})</th>
<th>10 ng mL(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIST05</td>
<td>TOFMS</td>
<td>TOFMS</td>
<td>TOFMS rep. 1</td>
<td>TOFMS rep. 2</td>
</tr>
<tr>
<td>1</td>
<td>764*</td>
<td>889</td>
<td>966</td>
<td>997</td>
<td>991</td>
</tr>
<tr>
<td>2</td>
<td>846</td>
<td>951</td>
<td>951</td>
<td>971</td>
<td>984</td>
</tr>
<tr>
<td>3</td>
<td>675 (6(^{th}) match)</td>
<td>968</td>
<td>971</td>
<td>963</td>
<td>956</td>
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<tr>
<td>4</td>
<td>750*</td>
<td>958</td>
<td>977</td>
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<td>758*</td>
<td>978</td>
<td>978</td>
<td>996</td>
<td>981</td>
</tr>
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<td>804*</td>
<td>978</td>
<td>978</td>
<td>945</td>
<td>941</td>
</tr>
<tr>
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<td>947</td>
<td>976</td>
<td>949</td>
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<td>11</td>
<td>783*</td>
<td>951</td>
<td>960</td>
<td>959</td>
<td>948</td>
</tr>
<tr>
<td>12</td>
<td>723 (2(^{nd}) match)</td>
<td>918</td>
<td>949</td>
<td>984</td>
<td>989</td>
</tr>
<tr>
<td>13</td>
<td>800</td>
<td>974</td>
<td>974</td>
<td>997</td>
<td>993</td>
</tr>
<tr>
<td>14</td>
<td>784 (2(^{nd}) match)</td>
<td>958</td>
<td>938</td>
<td>997</td>
<td>994</td>
</tr>
<tr>
<td>15</td>
<td>869</td>
<td>940</td>
<td>976</td>
<td>980</td>
<td>969</td>
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<td>918</td>
<td>998</td>
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<td>17</td>
<td>757 (3(^{rd}) match)</td>
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<td>996</td>
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<td>19</td>
<td>885*</td>
<td>968</td>
<td>968</td>
<td>957</td>
<td>957</td>
</tr>
</tbody>
</table>

* Match quality shown, but for an incorrect compound match.
† Data for duplicate analyses shown.

4.3.3.3 LOD and the minimum acceptable match (MAM)

The time window of an individual sterol, locked by 1D time and 2D time, as well as the full mass spectra of the deconvoluted compounds in complex matrices such as urine, offers unparalleled identification power for compounds. Using GC×GC-TOFMS, we propose a minimum acceptable match (MAM) which can be applied as an additional criterion for sterols identification at the limit of detection, and at low concentrations usually found in sport doping control. Here, the MAM metric is proposed to offer an additional level of
specificity to the identification of steroids, especially at low levels. In GC×GC there are two unique positions of each sterol in the $^1$D and $^2$D. Compared with 1D GC (which usually requires SIM mode with one or more ions – one for quantification and one or more for diagnostic or confirmatory ions), the proposed MAM uses all the ions and so offers the opportunity for routine library matching. Thus in this proposal a given quality match comparison between the experimental spectrum and the library spectrum is offered as part of the metric, as distinct from the use of a few confirmatory ions and the ratio of these ions (within an expressed uncertainty) as the indication of the identity of a steroid. The question here is whether a library match metric, as opposed to confirmatory ion(s) and their ratio, is a suitable and adequate measure of the identity of the steroid.

Clearly the library match quality will depend on the (proprietary) method employed for matching the spectra, and for a routine procedure between laboratories an agreed method should be used for this matching algorithm. However in the case of the proposed MAM, it should be possible to state with a higher probability that a given steroid is indeed present, compared with a SIM experiment. Here, a minimum match of 800 is applied for the library match, and to establish a limit of detection that incorporates this metric. Thus if a response to a spiked standard in urine is recorded at low concentration (for example S/N of 10), but the MAM is <800, then this is not accepted as a suitable match, nor as the limit of detection. This will generally lead to an increased LOD, but provides improved certainty of positive identification.

GC×GC-TOFMS can offer screening, even for unknown compounds present in a sample that only become recognised at some later date through “retro-searching” of full MS data, and provides identification and quantification of sterols below the detection limit of present analytical methods in GC-qMS. For doping control, GC×GC-TOFMS offers either the possibility for manual searching of unexpected compounds in a sample, with the chance that a “new” designer drug may be identified (admittedly a tedious task), or if such a compound is identified in the future, it should be a relatively straightforward task to search data files for this compound, since full mass spectral data are available.
In order to obtain the LOD, a series of spiked urine samples were processed as described and analysed using the P/NP column set and TOFMS based library for identification. The lowest limit of detection, when MAM is set to 800, S/N to 10 and peak width to 50 ms, is obtained for estrone (15) at 0.1 ng mL\(^{-1}\), and the highest for 8, 12 and 19 at 1 ng mL\(^{-1}\) (Table 4.1). Even though S/N for some sterols may be much higher than 10, unless the criterion of MAM > 800 is satisfied, the sterol concentration is not taken to represent the LOD. Here, the requirement of achieving a minimum in the match statistic (>800) is taken as an integral part for defining the LOD. For sterols detected in the blank urine samples (4, 6, 11, 14, 16, 18) LOD cannot be calculated. The LOD for all analysed sterols, including the quantification ions, S/N, match quality and peak width, is given in Table 4.1. The average match quality at LOD is 940 and the average peak width at half height is 100 ms. The S/N is calculated for the corresponding quantification ion chosen by the software, and this automated decision is not always apparent. Thus the S/N value of 29 calculated for 2 is obtained from the sum of 225 quantification ions chosen by the software. Any other combination of ions gave lower S/N ratio.

### 4.3.3.4 Linearity

The linearity was checked by running a series of extracts of spiked urine samples with sterols at various concentrations, from 0.1 ng mL\(^{-1}\) to 10 ng mL\(^{-1}\). Linearity equations and correlation coefficients for 5, 6 and 9 are given in Table 4.3. The presence of some of the sterols in the blank urine is visible from the linearity curve (Figure 4.9), e.g. for DHEA, where the intercept is not zero; for sterols absent in the blank urine the intercept is close to zero (5 and 9). Using the calibration curve, the calculated concentration of DHEA in the blank urine is 0.95 ng mL\(^{-1}\).
Table 4.3 Linearity equations and correlation coefficients for selected sterols obtained for the given quantification ions.

<table>
<thead>
<tr>
<th>sterol</th>
<th>Quant. ion</th>
<th>equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>androstanol (5)</td>
<td>285 m/z</td>
<td>$Y = 0.12802x + 0.0079$</td>
<td>0.999</td>
</tr>
<tr>
<td>DHEA (6)</td>
<td>360 m/z</td>
<td>$Y = 0.02102x + 0.0209$</td>
<td>0.997</td>
</tr>
<tr>
<td>stanolon (9)</td>
<td>257 m/z</td>
<td>$Y = 0.12329x - 0.0056$</td>
<td>0.999</td>
</tr>
</tbody>
</table>

$Y = A/A_{IS}; x$ – concentration of sterols in ng mL$^{-1}$

Figure 4.9 Linearity curves for androstanol (5), DHEA (6) and stanolon (9) spiked in urine in the concentration range from 0.1 ng mL$^{-1}$ to 10 ng mL$^{-1}$

4.4 CONCLUSIONS

This study shows that comprehensive two-dimensional gas chromatography can be successfully applied to sterols analysis in complex samples, offering better separation and signal enhancement when compared to conventional GC. Although GC×GC coupled to FID lacks suitable identification power, GC×GC coupled to the TOFMS detector is promising for screening, quantification and confirmation of sterols in complex matrices such as urine, even at a concentration below 1 ng mL$^{-1}$. The results show that TOFMS
offers greater information content (peak positions and full mass scanning, at lower levels) when coupled to GC×GC, compared to 1D GC. Furthermore, it was found that the match quality is dramatically improved if searching is performed against a TOFMS laboratory-created library compared to the commercial qMS-based library.

A new criterion for determination of the limit of detection is introduced here, which allows identification of the measured component at a predefined minimum acceptable match quality, in conjunction with the signal-to-noise ratio for a particular mass ion. In this way, instead of measuring a signal of a predefined mass ion in a predefined 1D time window, as in a traditional GC-MS-SIM mode analysis, here the signal of a component is identified according its full mass spectrum, with a match quality higher than the defined match quality. Once the steroid is confirmed, then a suitable m/z ion of choice, locked by predefined 1D and 2D retention times, can be used for quantification. In this case, it is relatively easy to confirm that the ion chosen for quantification does not suffer interference. The present method contrasts with the conventional GC-MS (SIM) analysis method because the latter will require choice of a small number (e.g. two) of confirmation ions for identification of the steroids, and then choice of a quantification ion. In this case, spectral identification relies solely upon the validity of the confirmation ions to correctly represent the target compound, which is not as secure as a full mass spectral match.

The GC×GC-TOFMS method, acquired with full mass scan data, can permit identification of unknown compounds in a sample either (i) if the mass spectrum of the compound can be interpreted based on spectral interpretation, or (ii) if a new compound (e.g. designer steroid) is found in the future and its retention and mass spectrum are then established, the acquired GC×GC data can be re-interrogated to determine if a doping offence had been committed. Since the limit of detection for the analysed endogenous sterols is 1 ng mL⁻¹ or less, this method is in accordance with the MRPL for the anabolic sterols defined by the World Anti-Doping Agency (WADA).
Chapter 5

5 APPLICATION OF COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY TO WADA KEY ANABOLIC AGENTS ANALYSIS
5.1 INTRODUCTION

The very low concentrations of anabolic agents (AA), and the complex matrices in which they are found (urine, sera, and other biological materials), require a powerful technique for separation and unambiguous identification. At present gas chromatography coupled to a quadrupole mass analyser (GC-qMS) is a technique of choice which exhibits high specificity and sensitivity, especially when selected ion monitoring (SIM) mode is applied. The separation is based on using a traditional narrow bore capillary column, and detection necessarily relies on monitoring pre-defined diagnostic ions in pre-defined time windows.

The extraction process is generally standardized, comprising of solid-phase extraction (SPE), hydrolysis of conjugates (metabolites) with β-glucuronidase and liquid-liquid extraction (LLE) by using diethyl ether or methyl tert-butyl ether (MTBE). Prior to GC-MS analysis, the AA are derivatized to their corresponding trimethylsilyl (TMS) derivatives since they usually contain one or more hydroxyls and/or keto groups and generally show poor chromatographic behaviour if not derivatized [207]. The most characteristic and preferably the most abundant ion is used for quantification, and 2–3 other ions (qualifiers) are used for confirmation purpose.

Most of the current methods in doping analysis, if not all of them, are still “transparent” to the potentially new “designer” AA at the lowest level of detection, since they rely on pre-defined ions to be monitored. Full scan mass spectral techniques coupled to classical one-dimensional GC (1D GC) with classical injection are not sensitive enough to detect low level concentrations down to 1 ng mL$^{-1}$, as defined by the World Anti-Doping Agency (WADA) [133]. In the last few decades many attempts have been made to improve the selectivity and the sensitivity of the GC-MS approach. Improvements in the oven temperature program [208, 209] in order to obtain better signal-to-noise ratio (S/N), the use
GC×GC of anabolic agents

of ion-trap tandem mass spectrometry [170, 172], high-resolution mass spectrometry [163, 165, 208], hybrid mass spectrometry (high-resolution–time-of-flight) [174], and combustion/isotope ratio mass spectrometry [139], are amongst the most common.

An attempt was made by Mazzarino et al. [209] to shorten the runtime while retaining the resolution and limit of detection by applying fast GC with a 5 m narrow bore column (0.1 mm I.D.; 0.1 μm film thickness). However, most methods are based on improvements of the selectivity, although usually by making the detector increasingly “blind” to the matrix. Such an example is the shift from full scan mode to SIM mode in low resolution MS, then to high resolution MS (in SIM mode), and finally to tandem MS (MS/MS or MSn). Several new approaches are proposed for increasing the sensitivity and selectivity of the methods in doping control by using an extra step in purification of the samples. In this manner immunoaffinity chromatography (IAC) [210] and high performance liquid chromatography (HPLC) fractionation [211] are employed. None of these approaches is based on chromatographic separation improvements since the limit of the separation efficiency of the 1D GC step has largely been reached.

Recently a new approach for separation improvement has been proposed, by using comprehensive two-dimensional gas chromatography (GC×GC) [186, 187] in doping analysis. The use of GC×GC is perhaps one of the most significant innovations in terms of separation efficiency improvement since the introduction of narrow bore capillary columns. GC×GC also reportedly exhibits improved sensitivity, alongside separation power, compared to traditional 1D GC [212, 213]. The separation is performed on two sequential columns: the first one (1D) approximates a conventional 1D GC separation, while the second one (2D) must act as a fast-eluting high-efficiency column, usually 0.5-2 m in length, with 0.1 mm I.D. and 0.1 μm film thickness (df). The separation mechanisms of the stationary phases should be as different as possible in order to maximize their orthogonality, giving rise the significant increase in separation power. The interface between the two columns (modulator) has to trap/accumulate in the predefined period (3–8 s) all the components which elute from the 1D column and then release them rapidly as
narrow adjacent fractions into the 2D column. The efficient trapping and fast releasing of the components is usually achieved by cryo-focusing, producing ultra-narrow peaks at the end of the 2D column. Co-eluting components on 1D, where the separation is based on the first mechanism, are separated on the orthogonal 2D column, where the separation is based on the second mechanism. GC×GC has been successfully applied in petrochemical [201, 214], food [201, 217], environmental [204], forensic [189, 205], essential oils analysis of complex matrices [168, 180], showing for the first time some new features such as structured chromatograms. Apart from improved peak capacity and structured chromatograms, GC×GC exhibits an increase in the signal height when compared to classical GC [186, 215].

Silva et al. [187] reported analysis of key WADA AA in urine using GC×GC-TOFMS at the lowest purported concentration, demonstrating that this technique is highly sensitive and specific in screening of these AA. Alternatively, it can be employed as a full spectral confirmatory method when coupled to TOFMS as a detector. The separation on a second dimension column was based on a 1 m OV-1701 column (0.1 mm×0.1 μm), however, the AA were spread in a rather narrow band of approximately 1 s out of the 6 s modulation period, along with matrix compounds also within this region. This tends to reduce the extent of matrix separation. No data were provided on linearity and TOFMS spectra similarity to a commercial or in-house MS library.

The present report describes further improvements in doping analysis, specifically the increased peak capacity (separation power) through better spread of AA and matrix components over the 2D space, and sensitivity and identification power of the TOFMS as a detector coupled to GC×GC. Linearity ranges and limit of detections for the tested substances are established, and advantages of GC×GC over 1D GC in term of sensitivity, separation and identification power are demonstrated through several examples. The bias of TOFMS detector against the higher masses, observed in the results of Silva et al. [187] but not reported, has been stressed in respect of the 19-norandrosterone case.
The performance of the present proposed method was confirmed using spiked urine extracts obtained after SPE, hydrolysis with β-glucuronidase and LLE with MTBE. The proposed method is “non-transparent”, since it permits full mass spectral information to be retained, both for targets or non-targets. Finally the method was quantified for a urine positive control sample (UPC) spiked with the key WADA anabolic agents at a concentration of $5 \text{ ng mL}^{-1}$, which was prepared at NDCC (a WADA accredited anti-doping laboratory in Bangkok, Thailand).

### 5.2 EXPERIMENTAL

#### 5.2.1 GC×GC-FID

The GC equipped with a flame ionization detector (GC×GC-FID) used in the study was an Agilent 6890 system (Palo Alto, CA, USA) with a longitudinal modulation cryogenic system (LMCS; Chromatography Concepts Pty Ltd., VIC, Australia). The column configuration used with this system was 30 m BPX5 (0.25 mm I.D.; 0.25 μm film thickness ($d_f$)) as a first dimension ($1^\text{D}$) column coupled to a 1 m BPX50 (0.1 mm I.D.; 0.1 μm $d_i$) as a second dimension ($2^\text{D}$) column, both columns from SGE Scientific (Ringwood, Australia). The oven temperature program was from 140 °C (hold for 1min) to 200 °C at 40 °C min$^{-1}$, then to 240 °C at 4 °C min$^{-1}$, then to 330 °C at 15 °C min$^{-1}$ (hold for 5min). The injector and detector temperatures were 280 °C and 320 °C, respectively, and the sampling frequency was 100 Hz. Hydrogen was used as a carrier gas at a flow rate of 1.1 mL min$^{-1}$ and 1 μL of the sample was injected in split mode at a split ratio of 10:1. The modulation period was 5 s, and temperature of the modulator system ($T_M$) was varied from 0 °C to 200 °C, during the optimization study. CO$_2$ was used as a coolant in the LMCS and nitrogen as a flush gas at a pressure of 15 psi. Agilent ChemStation software was used for data acquisition and processing.
5.2.2 GC×GC-TOFMS

A LECO time-of-flight mass spectrometer (TOFMS) model Pegasus III (LECO Corp., St. Joseph, MI, USA) connected to an Agilent 6890 GC was used in GC×GC-TOFMS experiments. The TOFMS detector was operated at 1600 V and applied electron ionization voltage was 70 eV. Data collection rate was 100 Hz over the mass range from 45 to 700 m/z. The temperatures of the transfer line and ion source were 280 °C and 230 °C, respectively, and data acquisition and processing were performed by ChromaTOF software (LECO Corp., St. Joseph, MI, USA). A separate GC×GC-TOFMS-based in-house library for improved identification was generated using standard solutions at a concentration of 1 μg mL⁻¹ for all AA, in the same manner as in our previous work [186]. The National Institute of Standards and Technology algorithm (NIST MS Search 2.0 Program) was used for mass spectra searching. Two complementary column configurations were used for AA separation and identification in this experiment: polar/non-polar (P/NP) and non-polar/polar (NP/P).

5.2.2.1 Polar/non-polar column configuration (P/NP)
P/NP consisted of 30 m BPX50 (0.25 mm I.D.; 0.25 μm df) as ¹D and 1 m BPX5 (0.1 mm I.D.; 0.1 μm df) as ²D column. Oven temperature program was from 80 °C (1 min) to 180 °C at 40 °C min⁻¹, then to 240 °C at 4 °C min⁻¹ and finally to 330 °C at 15 °C min⁻¹ (hold for 5 min). Temperature of the injector was 280 °C and 1 μL of sample was injected in splitless mode (1 min purge time) at a carrier gas (helium) flow rate of 1.5 mL min⁻¹.

5.2.2.2 Non-polar/polar column configuration (NP/P)
Two different ²D columns were applied in NP/P, differing only in df. The first one (NP/P1) was the same as listed for GC×GC-FID analysis above and the second one (NP/P2) was of the same length and I.D., but with a 0.2 μm df BPX50 phase. Two chromatographic methods for each NP/P set were applied, differing only in the oven temperature program.
and carrier gas flow rate. The oven program for the first (NP/P1s; S: short runtime) was from 120 °C (hold for 1 min) to 200 °C at 20 °C min\(^{-1}\), then to 230 °C at 3 °C min\(^{-1}\), and finally to 320 °C (hold for 5 min) at 8 °C min\(^{-1}\), at a flow rate of 1.5 mL min\(^{-1}\), while the oven program for the second one (NP/P1l; L: long runtime) was from 120 °C (hold for 1 min) to 320 °C (hold for 5 min) at 4 °C min\(^{-1}\), at a flow rate of 1.3 mL min\(^{-1}\). Accordingly, a 0.2 μm \(d_f\) 2D column film thickness was used in NP/P2s and NP/P2l chromatographic methods. 1 μL sample was injected in splitless mode (2.5 min purge open time).

### 5.2.3 Experiments in 1D GC mode

All experiments using 1D GC were carried out under the same conditions as in GC×GC, except the modulator was off and the acquisition rate of TOFMS detector was 20 Hz. Thus in this case the 1D system will comprise a long column directly coupled to a short column and whilst this can be termed a multichromatography system according to Hinshaw and Ettre [216] and discussed elsewhere [217], the second very short column is anticipated to lead to negligible variation in peak properties such as width.

### 5.3 RESULTS AND DISCUSSION

#### 5.3.1 GC×GC-FID separation of anabolic agents

It was found in the previous work [186] that the peak shape of steroids on the 2D depends on the modulator temperature \((T_M)\). The GC×GC separation of the standard mixture of anabolic agents was initially accomplished by GC×GC-FID, where \(T_M\) was optimized for the best separation and narrowest peak width. An acceptable separation was achieved in 21 min, except for 4 and 5 which co-elute (Figure 5.1), at 5 s modulation period but the best modulation temperature was found to be dependent on the retention time of the anabolic...
agents, and presumably their boiling point. The peak width at half height ($W_{1/2}$) of each anabolic agent was constant over a $T_M$ range from 0 °C until a characteristic (steroid-dependant) temperature, at which stage the peak width of the anabolic agent starts to increase. For instance, the peak of clenbuterol (first eluted compound) started to broaden at 60 °C $T_M$ (Figure 5.2), but the peak of 3'OH-stanozolol maintained its $W_{1/2}$ of ~45 ms up to 160 °C. Onset of peak broadening for the rest of the anabolic agents was observed between these two temperatures.

Figure 5.1 GC×GC-FID contour plot of the TMS derivatized WADA key AA on BPX5/BPX50 column configuration: clenbuterol (1), 19-norandrosteron (2), epimethendiol (3), M1 metabolite (4), M2 metabolite (5), and 3’OH-stanozolol (6)

In contrast to the previous work [186], here an increase of the peak width towards lower temperatures (down to 0 °C except for 6 where the minimum achieved $T_M$ at its elution temperature was 60 °C) was not observed. This is probably due to the different nature of some of the anabolic agents, and the use of a different derivatization reagent capable of derivatising keto-enol groups also, and not just the hydroxyls. In order to maximize the separation efficiency (narrowest peaks) and to minimize the CO$_2$ consumption, we decided to perform all further experiments by holding the $T_M$ at 80 °C. The 2D plot in Figure 5.1 shows the separation of the 6 anabolic agents in a standard mixture at a concentration of 10 μg mL$^{-1}$ (split ratio 1:10), at 80 °C $T_M$.

The anabolic agents were detected at a concentration down to 0.2 μg mL$^{-1}$ in a standard mixture (splitless mode), which theoretically corresponds to 4 ng mL$^{-1}$ spiked in urine (at a
concentration factor of 50), but the lack of identification power of the FID detector limits its applicability in complex mixtures, since the analyte signals may be poorly recognized in the presence of much higher signals from the matrix.

![Figure 5.2 The influence of modulator temperature \(T_M\) on peak width \(W_{1/2}\) for the key WADA anabolic agents](image)

### 5.3.2 GC×GC-TOFMS separation and identification of anabolic agents

Since BPX50/BPX5 (P/NP) has been successfully applied in endogenous sterols separation [186] this column configuration has also been used for AA separation. Despite the good peak shapes (data not shown) of 1, 2, 3, 4 and 5, with an average \(W_{1/2}\) of 90 ms, it was not possible to elute 3’OH-stanozolol at a concentration lower than 0.5 μg mL\(^{-1}\). This concentration corresponds to a concentration of 10 ng mL\(^{-1}\) of AA spiked in urine at a concentration factor of 50. The injector temperature was varied from 260 °C to 320 °C, initial oven temperature from 80 °C to 180 °C, and the flow rate from 0.8 mL min\(^{-1}\) to 1.5 mL min\(^{-1}\), and 6 could not be eluted at concentrations below 0.5 μg mL\(^{-1}\). This is probably because of the length (30 m) and the polarity of the 1D column (BPX50) and its activity towards polar components. 3’OH-stanozolol is known as the most problematic steroid in anti doping control when GC is applied [136, 218]. Because of this, experiments were continued on the non-polar/polar (NP/P) column configuration BPX5/BPX50.
5.3.2.1 Short runtime vs. long runtime

Since the focus of this work was the separation and identification of the key WADA anabolic agents in a urine matrix, and not on the extraction efficiency, they were directly spiked to the blank urine extracts. A 2D plot of a blank urine extract spiked with the anabolic agents at a concentration of 2 ng mL\(^{-1}\) and IS at a concentration of 50 ng mL\(^{-1}\), by using the method NP/P1\(_s\), is shown in Figure 5.3A. As the result of the poor spread of the components on the 2D column, unsatisfactory separation from the matrix and identification of the key anabolic agents are obtained. For instance, 2, 3, 4 and 5 were detected with a similarity below 600, and 1 and 6 were not detected at all. In order to improve the separation efficiency, especially the spread of the components on the 2D, a slower oven temperature program was applied (NP/P1\(_l\)). The obtained separation and identification of anabolic agents were better, but at an overall runtime of 50 min (Figure 5.3B).

5.3.2.2 0.2 µm 2D column vs. 0.1 µm 2D column

In order to further improve the spreading of components on the 2D plot, a 2D column with 0.2 µm \(d_l\) instead of the original 0.1 µm was applied, keeping other conditions the same as in NP/P1\(_l\). The 2D plot of the blank urine extract spiked with AA at 2 ng mL\(^{-1}\) is shown in Figure 5.3C, where the improvement of the peak separation is apparent. A peak table with \(1^D\) retention times (\(t_{R1}\)), \(2^D\) retention times (\(t_{R2}\)), \(W_{1/2}\), S/N for selected quantification ions and the similarity to the in-house created TOFMS library for the WADA key anabolic agents at 0.1 µg mL\(^{-1}\) in standard solution was created (Table 5.1) under these conditions (NP/P2\(_l\)). The concentration of 0.1 µg mL\(^{-1}\) in the standard solution theoretically corresponds to a concentration of 2 ng mL\(^{-1}\) of anabolic agents when spiked in an original urine sample, at the concentration factor of 50 (2.5 mL urine to 50 µL final volume of the urine extract). The improvement of the separation on the 0.2 µm \(d_l\) 2D column over 0.1 µm \(d_l\) is clearly seen from the example of 19-norandrosterone, given in Figure 5.4. The increased retention of 2 and the coeluting endogenous component provided by the thicker film produces a better separation, followed by a better deconvolution of their MS spectra.
Figure 5.3 2D plots of spiked urine extracts with the WADA key anabolic agents at a concentration of 2 ng mL⁻¹, analysed under NP/P1s (A), NP/P1L (B) and NP/P2L (C) chromatographic conditions.
Figure 5.4 Extracted ion chromatogram at 405 m/z for 19-norandrosterone (2), showing the achieved separation from the co-eluting endogenous component on 0.1 µm (A) and 0.2 µm (B) d$_2$D column.

5.3.3 Linearity and Limit of Detection (LOD)

The method linearity was recorded by obtaining data over the concentration range from 0.5 ng mL$^{-1}$ to 20 ng mL$^{-1}$ and the limit of detection (LOD) was established under these conditions (NP/P2r). The correlation coefficients were in the range from 0.995 (for 3’OH-stanozolol) to 0.999 (for epimethendiol). LOD was defined as the lowest concentration which gave S/N above 10 for the quantification ion (see Table 5.1) and the minimum acceptable match (MAM) criterion [186] to the in-house library of 700 or higher. This is a rigorous criterion when defining LOD for steroids in anti doping control, and only possible by using GC×GC-TOFMS at the lowest limit required for reporting the WADA check solution. The obtained LOD was from 1 ng mL$^{-1}$ (for 1, 2, 4 and 5) to 2 ng mL$^{-1}$ (for 3 and 6).
Table 5.1 Peak data table of the WADA key anabolic agents, including retention time on each dimension and peak width at a half height. TOFMS similarity against the in-house TOFMS-based library, selected quantification ions and corresponding signal-to-noise ratios were reported for a concentration of 0.1 µg mL\(^{-1}\) in standard solution. Linearity data are for a concentration range of 2-20 ng mL\(^{-1}\).

<table>
<thead>
<tr>
<th>Anabolic agent</th>
<th>(t_R^1) (s)</th>
<th>(t_R^2) (s)</th>
<th>(W_{1/2}) (ms)</th>
<th>TOFMS similarity</th>
<th>Quant. ion</th>
<th>S/N</th>
<th>(R^2)</th>
<th>Equation(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clen-2TMS(^a)</td>
<td>1670</td>
<td>2.48</td>
<td>108</td>
<td>963</td>
<td>335(86)</td>
<td>51</td>
<td>(542)</td>
<td>0.996</td>
</tr>
<tr>
<td>19-Nor-2TMS</td>
<td>2125</td>
<td>2.58</td>
<td>103</td>
<td>986</td>
<td>405</td>
<td>51</td>
<td></td>
<td>0.996</td>
</tr>
<tr>
<td>EMD-2TMS(^a)</td>
<td>2165</td>
<td>2.64</td>
<td>110</td>
<td>970</td>
<td>358(143)</td>
<td>18</td>
<td>(398)</td>
<td>0.999</td>
</tr>
<tr>
<td>M1-2TMS (4)</td>
<td>2340</td>
<td>2.44</td>
<td>117</td>
<td>984</td>
<td>255</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2-2TMS (5)</td>
<td>2345</td>
<td>2.45</td>
<td>119</td>
<td>982</td>
<td>255</td>
<td>30</td>
<td></td>
<td>0.995</td>
</tr>
<tr>
<td>IS</td>
<td>2500</td>
<td>2.91</td>
<td>115</td>
<td>981</td>
<td>301</td>
<td>1808</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3’OH-Stan-3TMS(^c)</td>
<td>2920</td>
<td>3.08</td>
<td>128</td>
<td>840</td>
<td>143</td>
<td>34</td>
<td></td>
<td>0.996</td>
</tr>
</tbody>
</table>

\(^a\) For clenbuterol and EMD two ions of each are shown with their respective S/N. The former ions are more unique, but are of lower abundance.

\(^b\) \(Y = \text{Area}_{AA} / \text{Area}_{IS}; x = \text{AA concentration in ng mL}^{-1}\)

\(^c\) Equation obtained for 254 m/z

### 5.3.4 GC×GC-TOFMS vs. GC-TOFMS

The high acquisition rate of TOFMS as a detector in GC allows full scan mass spectral information to be acquired not only for the target components, but also for non-targets and the matrix. The similarity matching of each compound’s MS spectrum against the MS libraries, even at the LOD, is another benefit of TOFMS. In addition, it allows choosing the best ion for quantification from the full mass spectrum, whether it be the most abundant and/or the ion that has least interferences from the matrix. The sensitivity, separation and identification efficiency of the present method (NP/P2L) were checked on a UPC sample (Figure 5.5) and a spiked urine extract with WADA key anabolic agents at a concentration of 20 ng mL\(^{-1}\) (data not shown), in GC×GC and 1D GC mode. In the latter case the modulator was turned off and the acquisition rate was reduced to 20 Hz (in order to closely match the data acquisition conditions in classical 1D GC-MS), while the rest of the conditions were kept the same.
The results of a comparison (see Table 5.2) showed that not one anabolic agent in the UPC sample was detected in 1D GC, except 2 with a very low similarity (match 520 on a scale from 0 - 999). Furthermore, at the highest concentration level tested in urine (20 ng mL\(^{-1}\)), only 2 and 5 were properly detected in 1D GC (matches 854 and 817). 3 was poorly identified (match 591) and 4 was recognized with a higher similarity (match 761) but at a low S/N (14). Clenbuterol and 3’OH-stanozolol were not detected at all under the defined criteria. In comparison, all the anabolic agents in both samples were identified in GC×GC mode with an average match quality of 890 (UPC sample) and 910 (20 ng mL\(^{-1}\)). The results of the comparison are given in Table 5.2.

<table>
<thead>
<tr>
<th></th>
<th>5 ng mL(^{-1}) (UPC sample)</th>
<th>20 ng mL(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1D GC</td>
<td>GC×GC</td>
</tr>
<tr>
<td>clen-2TMS</td>
<td>-</td>
<td>875</td>
</tr>
<tr>
<td>19-nor-2TMS</td>
<td>520</td>
<td>928</td>
</tr>
<tr>
<td>EMD-2TMS</td>
<td>-</td>
<td>822</td>
</tr>
<tr>
<td>M1-2TMS(^*)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M2-2TMS</td>
<td>-</td>
<td>932</td>
</tr>
<tr>
<td>3’OH-stan-3TMS</td>
<td>-</td>
<td>870</td>
</tr>
</tbody>
</table>

* M1 was not spiked in the UPC sample, since this epimer is not monitored at the MRPL of 2 ng mL\(^{-1}\) [133].

As can be seen from the 2D plot of the UPC sample in Figure 5.5, the extract prepared at NDCC is significantly cleaner than the extracts prepared at the ACROSS laboratory, presumably because of the experience of NDCC and optimized conditions applied in the sample preparation. The extraction conditions applied at the ACROSS laboratory were not optimized since the focus of the research was the separation efficiency. The separation and improved dispersion of the components on the 2D plot by using 0.2 μm \(d_l\) column is even more apparent on this sample.
Figure 5.5 2D plot of the UPC sample, analysed under NP/P2\textsubscript{2} conditions

Here a number of advantages of GC\texttimes GC over the classical 1D GC method were observed:

In the extracted ion chromatogram (data not shown) and 3D plot at 86 m/z for clenbuterol (Figure 5.6), an endogenous component co-elutes with \textbf{1} on 1\textsubscript{D}, making quantification difficult in 1D GC if this ion is used. For the 335 m/z extracted ion chromatogram (Figure 5.6B) there is no interference (co-elution), but this ion is much less abundant compared to 86 m/z under these conditions, thus this drastically reduces the sensitivity. As a comparison, S/N of 239 is obtained at the concentration of 0.5 ng mL\textsuperscript{-1} in urine if 86 m/z is selected, with no background interference for GC\texttimes GC. However, the match quality was not satisfactory to confirm the presence of clenbuterol at this level of concentration, using the applied criteria.

In the extracted ion chromatogram and 3D plot at 405 m/z, 420 m/z and 315 m/z for \textbf{2} (Figure 5.6C-E), an endogenous component co-elutes with \textbf{2}, with some common mass fragments (405 m/z, 420 m/z) as in \textbf{2}. The extracted ion area ratio is in favour of the endogenous component at lower concentrations (see Figure 5.4, at 2 ng mL\textsuperscript{-1}), and can not be ignored in 1D GC. It is obvious from Figure 5.6E that 315 m/z will give the least interference in 1D GC if chosen for quantification, with some loss of sensitivity compared
to 405 m/z. However, GC×GC offers in this case choice of any ion for quantification, since full mass spectra are obtained even at the lowest detectable concentrations. The same sensitivity as for 405 m/z can be obtained if 225 m/z were chosen, with no interfering component (data not shown).

In the extracted ion chromatogram (data not shown) and 3D plot at 255 m/z for M2 metabolite (Figure 5.6F) an endogenous component co-elutes on 1D, making the quantification in 1D GC difficult if this ion is selected. GC×GC overcomes this problem since M2 and the interfering component are separated on the 2D column. However, in the 435 m/z, and especially in the 345 m/z extracted ion chromatograms (data not shown), there is no interference, but these two ions are less abundant under these conditions when compared to 255 m/z, thus reducing the sensitivity by approximately 2 fold.

On the other hand, the extracted ion chromatogram and 3D plot at 143 m/z (Figure 5.6G) lacks interference and can be used as a quantification ion for sensitive determination since 143 m/z is the base ion in the mass spectra of M2. From Table 5.2, the similarity of the mass spectrum of M2 at this level of concentration to the in-house TOFMS library is 932. A similar sensitivity as for 255 m/z was obtained by selecting 270 m/z ion (Figure 5.6H), with no coelution on the 1D column. All these ions (255 m/z, 143 m/z, 345 m/z, 435 m/z and 270 m/z) are common for both M1 and M2 metabolites, but at different relative abundances, which helps to differentiate them even though they are separated by only one modulation period (5 s) on 1D column. Deconvolution of their mass spectra is possible if their retentions differ on the 2D column. Figure 5.7 shows the two metabolites present in a standard solution. The ion abundance for 255 m/z is higher for M1, and that of 270 m/z is higher for M2. Since the ratio of ions 255 m/z and 270 m/z are not constant in the two peak pulses in Figure 5.7, this shows that M1 and M2 are partially resolved on the 1D column. The retention of each compound on the first column can be predicted by a recently proposed metric based on modulated peak distribution [219]. The peaks almost completely overlap on the 2D column.
Figure 5.6 Extracted 3D plots for clenbuterol at 86 m/z (A) and 335 m/z (B); 19-norandrosterone at 405 m/z (C), 420 m/z (D) and 315 m/z (E); M2 metabolite at 255 m/z (F), 143 m/z (G) and 270 m/z (H), obtained from the UPC sample.

Cryo-focusing and releasing of components on the 2D column permits an elegant integration of the peak area of 6, even though it showed a high degree of tailing on 1D. The expanded extracted ion chromatogram at 254 m/z in Figure 5.8 shows 9 modulation slices.
of 6 (due to the tailing on ¹D) at a concentration of 0.5 μg mL⁻¹ in standard solution, automatically integrated and summed up by ChromaTOF software. At a concentration of 5 ng mL⁻¹ in urine (UPC sample) 4 slices are detected, integrated and summed up. The identification and integration of tailing peaks is a great problem in 1D GC.

Figure 5.7 Mass spectral deconvoluted modulated peaks for ions 255 m/z and 270 m/z, corresponding to M1 and M2 metabolites in a standard solution, showing that the peaks strongly overlap on both the first and second columns.

Figure 5.8 Extracted ion chromatogram at 254 m/z of 3’OH-stanozolol at 10 ng mL⁻¹ in standard solution showing the ¹t_R range in which 9 modulations were detected and integrated automatically by ChromaTOF software. The extracted 3D plot in the inset shows the integrated surface.
Among the full spectral information of the target components, GC×GC-TOFMS also offers the complete spectral information of the matrix (e.g. endogenous sterols) and non-target components, allowing post-run search and even quantification if reference material is available, without the need for re-analysis of the samples. The UPC sample was post-run processed against the in-house created library of 19 endogenous sterols, which were investigated in our previous work [186]. 10 of them were detected and identified with an average similarity of 922. The corrected T/E ratio (testosterone/epitestosterone) for the UPC sample was 0.85, which is within the expected window for normal athlete’s urine [207, 220, 221].

Forty to seventy other endogenous compounds, generally low molecular mass, were usually identified with a match quality much higher than 900. Higher molecular mass compounds exhibiting higher molecular mass fragments in the mass spectra showed lower similarity to the commercial (quadrupole based) MS libraries, since bigger difference is observed in the ion intensities to the TOFMS obtained spectra. However, if a more comprehensive TOFMS-based library of identified endogenous compound was created, the average similarity would be expected to be much higher.

The main drawback of using TOFMS as a detector for GC×GC in sterol analysis is some loss of sensitivity when higher mass fragments are chosen for quantification. For instance, 405 m/z is the base ion in the MS spectrum of 2 in NIST05 (quadrupole based) library, and 73 m/z is 73% abundant compared to the base ion, but in the TOFMS spectrum of 2 (recorded at 1 µg mL⁻¹ in standard solution), the base ion is 73 m/z and the ion 405 m/z is 2.8% abundant only (Figure 5.9). This is the main reason for the low similarity of TOFMS spectra against the quadrupole based MS library, and why best library matching is with a laboratory generated TOFMS library, as observed in the previous work [186]. It is worth to point out that the TOFMS was properly tuned according the producer’s recommendation.
The applicability of GC×GC coupled to TOFMS has been demonstrated in this study. The main advantages of GC×GC over 1D GC, as the increased peak capacity, enhanced sensitivity and improved identification power, were proved through the analysis of the key WADA anabolic agents spiked in urine extracts.

In contrast to the traditional GC-MS method in SIM mode, the proposed method offers some unique features. The 2D retention times, the full mass spectra of target and non-target components, as well as the retained full mass spectral information of the matrix, are the benefits of the proposed method. Additionally, the MAM criterion, introduced in the previous work [186], was successfully applied along the established WADA rigorous criteria for identification and quantification of anabolic agents in urine. Furthermore, the method provides a retro-search (post-run) in a case new “designer” drug is present in the sample, but not detected at the time of analysis due to the lack of its spectral information. In this case the search is performed only on the acquired data, with no need of re-analysis.
Moreover, GC×GC revealed its real power through a few examples of component coelution on 1D column in extracted ion mode. The extreme case, where the co-eluted components share the same m/z masses originating from the same structure fragments, can not be solved even by using high resolution or tandem mass spectrometry. Separation of co-eluted components in this case is the only solution. We have shown in our experiment a coelution of 19-norandrosterone with an endogenous component, sharing some of the most characteristic m/z fragments in their spectra.

A new dimension in doping control analysis has been implemented in this study, this time on the separation side. Instead of increasing the method performance by making the detector more “blind” (like in SIM mode, HRMS, tandem MS) this study proposes an improvement in method capabilities by adding a new “separation” dimension, while retaining the full mass spectral information. Further coupling of the best in separation (GC×GC) to the best in detection (MS/MS) will be probably just a matter of time.
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Chapter 6

6  EVALUATION OF WORLD ANTI-DOPING AGENCY CRITERIA FOR ANABOLIC AGENT ANALYSIS BY USING COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY–MASS SPECTROMETRY
6.1 INTRODUCTION

The following work represents a logical continuation of the first two studies on sterols and anabolic agents. While general applicability of comprehensive two-dimensional gas chromatography has been demonstrated on endogenous sterols, with the limit of detection established at reasonably high concentrations of up to 100 ng mL⁻¹, the real applicability has been confirmed on the WADA key anabolic agents. Their low required limits of detection and strict identification criteria are established by WADA and described in its two technical documents: TD2003IDCR [164] and TD2004MRPL [133]. In this chapter a point-by-point assessment of data in GC×GC-TOFMS format against each criterion is presented.

Anabolic steroids (AS) and β2-agonists with anabolic effect are the pharmacological substances most frequently abused in doping control. The International Olympic Committee (IOC) and other organizations responsible for anti-doping control (i.e. WADA) have banned these substances because of their effect on athlete’s performance and consequently on the results of competitions. More details about the importance of these substances in doping control, the matrix of choice and the current methodology for analysis are given in the introduction part of Chapter 4 and Chapter 5. Here the focus is given to the compliance of the GC×GC results to the present WADA criteria [164].

6.2 EXPERIMENTAL

The same sample preparation method used for WADA key anabolic agents analysis by GC×GC-TOFMS (Chapter 5) has been applied also in this study. Since the focus of the study was the separation and identification of AA, and not the extraction efficiency, the
AA were spiked in urine extracts and not directly to the urine samples. In this way the eventually encountered problems and/or limitations can be easily associated to the GC×GC technology and not to the extraction step. All the results used for method performance assessment were generated by using GC×GC-TOFMS method described in Chapter 5.

6.3 RESULTS AND DISCUSSION

6.3.1 Retention time tolerance criteria

**Present WADA criterion:**
- The retention time of the analyte shall not differ by more than 1% or ±0.2 minutes (whichever is smaller) from that of the same substance in a spiked urine sample or reference material analysed contemporaneously [164].

The relative standard deviation (RSD) of the first dimension ($t_R^1$) and second dimension ($t_R^2$) retention times of the proposed method has been calculated from a series of spiked urine samples with anabolic agents at different levels of concentration and the results are given in Table 6.1. A representative 2D plot of urine extract spiked with sterols at a concentration of 10 ng mL$^{-1}$ is given in Figure 6.1. The reproducibility (RSD) of $t_R^1$ has been found to range from 0% for clenbuterol-2TMS and 19-norandrosterone-2TMS to 0.18% for EMD-2TMS, which agrees with the previous findings for GC×GC reproducibility [222].

The relative error of the retention times of the reference compounds are well within the tolerance window of 1% [164]. The lowest deviation (Table 6.1) has been obtained for clenbuterol-2TMS and 19-norandrosterone-2TMS (0%), and the highest for EMD-2TMS (0.23%). The 0% RSD values for clenbuterol-2TMS and 19-norandrosterone-2TMS are due to the “quantised” values of the $t_R^1$ derived from the modulation process and data processing software.
Specifically, ChromaTOF assigns the same $^1t_R$ for all components in the same modulation period, regardless of their $^2t_R$ values. In addition, since each component generates more than one modulation peak, the $^1t_R$ of the component is assigned the retention time of the highest modulated peak. Obviously, the highest modulated peak for clenbuterol-2TMS has been found in the same modulation event at 1670 s in each run, and likewise for the 19-norandrosterone-2TMS peak at 2125 s to give an ‘arbitrary’ $^1t_R$ variation evaluated to be 0%. Thus it is not necessarily true that each replicate of each of these compounds has exactly the same $^1t_R$, but rather is an artefact of the data presentation. This group has proposed an algorithm for exact $^1t_R$ determination in GC×GC [219].

Figure 6.1 A representative 2D plot of urine extract spiked with anabolic agents at a concentration of 10 ng mL$^{-1}$. Note that the $^2t_R$ is shifted vertically by -1 s to provide a better presentation format.
### Table 6.1 \( t_R \) and \( 2t_R \) retention time reproducibility and the relative error to the reference compounds

<table>
<thead>
<tr>
<th></th>
<th>Clen-2TMS</th>
<th>19-nor-2TMS</th>
<th>EMD-2TMS</th>
<th>M2-2TMS</th>
<th>3'OH-stan-3TMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA average</td>
<td>( t_R ) (s)</td>
<td>( t_R ) (s)</td>
<td>( t_R ) (s)</td>
<td>( t_R ) (s)</td>
<td>( t_R ) (s)</td>
</tr>
<tr>
<td>(n=13)</td>
<td>1670</td>
<td>2.498</td>
<td>2125</td>
<td>2.577</td>
<td>2170</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.00</td>
<td>0.65</td>
<td>0.00</td>
<td>0.75</td>
<td>0.18</td>
</tr>
<tr>
<td>ref. average</td>
<td>( t_R ) (s)</td>
<td>( t_R ) (s)</td>
<td>( t_R ) (s)</td>
<td>( t_R ) (s)</td>
<td>( t_R ) (s)</td>
</tr>
<tr>
<td>(n=2)</td>
<td>1670</td>
<td>2.480</td>
<td>2125</td>
<td>2.565</td>
<td>2165</td>
</tr>
<tr>
<td>rel. error (%)</td>
<td>0.00</td>
<td>0.72</td>
<td>0.00</td>
<td>0.47</td>
<td>0.23</td>
</tr>
</tbody>
</table>

The \( 2t_R \) relative error of the reference compounds, even though not defined as part of the established WADA criteria, fall within the tolerance window of 1%. The reproducibility is in good agreement with the previous results [222], and the relative error is from 0.47% (for 19-norandrosterone-2TMS) to 0.85% (for M2-2TMS metabolite). Reproducibility in \( 2t_R \) values is found to depend on maintaining constant CO\(_2\) cryogen supply, since the temperature of the modulator can influence the \( 2t_R \); clearly the values found here, being less than 1% RSD, are acceptable for purposes of this criterion. The high reproducibility of \( t_R \) produces very reproducible elution temperature for all anabolic agents, which in turn means constant and stable \( 2t_R \). If the modulation process is properly conducted the \( 2t_R \) is very robust so the same criteria (1% relative error) can be applied also for this additional parameter. The retention time reproducibility was carried out on spiked urine samples at different concentration levels, over the range from the limit of detection (2 ng mL\(^{-1}\)) to the highest tested concentration level (20 ng mL\(^{-1}\)), which demonstrates a rugged reproducibility assessment of \( t_R \). In the event that WADA adopts GC×GC technology, then a new criterion specific for \( 2t_R \) will need to be considered.
6.3.2 Relative abundance tolerance window criteria

**Present WADA criteria [164]:**
- All diagnostic ions with relative abundance greater than 10% in the reference spectrum must be present in the spectrum of the unknown peak, and

- The relative abundance of three diagnostic ions shall not differ by more than the amount shown in Table 6.2 from the relative intensities of the same ions from that of a spiked urine or reference material.

<table>
<thead>
<tr>
<th>rel. abundance (% of base peak)</th>
<th>(EI)GC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 50</td>
<td>± 10% (absolute)</td>
</tr>
<tr>
<td>25 to 50</td>
<td>± 20% (relative)</td>
</tr>
<tr>
<td>&lt; 25</td>
<td>± 5% (absolute)</td>
</tr>
</tbody>
</table>

The mass spectrometric detection and identification in selected ion monitoring (SIM) mode is usually based on monitoring of one quantification ion (for estimation of concentration) and two or more qualification ions (qualifiers, for identification criteria). The relative abundance of these diagnostic ions in unknown samples compared to the relative abundances of the same ions from the reference compounds is used as criteria for positive identification. The graphical view of the maximum tolerance window of relative difference (MTWRD) against the relative abundance of the ions, based on WADA criteria [164], is given in Figure 6.2.
Figure 6.2 Graphical view of the maximum tolerance window of the relative difference (MTWRD) against the relative abundance, according to the WADA criteria [164]

Several diagnostic ions and ion ratios for each anabolic agent have been tested against their compliance with the WADA relative abundance criteria. The results are summarized in Table 6.3.

The results in Table 6.3 demonstrate that all ions and ion combinations for clenbuterol-2TMS, except for 86 m/z alone, comply with the WADA criteria. The low reproducibility of the relative abundance of 86 m/z is due to two main factors:

1. The low uniqueness of the base ion 73 m/z in TOFMS spectra. This ion is present in all trimethylsilyl (TMS) derivatized compounds - endogenous or exogenous - since it is a fragment pertaining to derivatizing agent. In contrast, the base ion in quadrupole based MS spectra (NIST05) is 86 m/z, which is more characteristic than 73 m/z.

2. The narrower permitted tolerance window of the relative difference for ions with higher relative abundance (see Figure 6.2).

However 335 m/z, 337 m/z and their combinations, including the combinations with 86 m/z, have complied with the criteria. The relative abundance ratio has shown higher reproducibility than the relative abundances itself. All ion combinations fell well within the tolerance window.
Table 6.3 Relative abundances of diagnostic ions and their combinations for AA spiked in urine extract over the concentration range from 2 ng mL\(^{-1}\) to 20 ng mL\(^{-1}\)

<table>
<thead>
<tr>
<th></th>
<th>ion (m/z)</th>
<th>86</th>
<th>335</th>
<th>337</th>
<th>335/86</th>
<th>337/86</th>
<th>337/335</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clen-2TMS</strong></td>
<td>average (%)</td>
<td>65.7</td>
<td>10.2</td>
<td>8.2</td>
<td>12.2</td>
<td>9.0</td>
<td>74.6</td>
</tr>
<tr>
<td></td>
<td>STD</td>
<td>27.1</td>
<td>3.1</td>
<td>2.3</td>
<td>0.6</td>
<td>0.6</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>out of range(^a)</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>19-nor-2TMS</strong></td>
<td>average (%)</td>
<td>35.4</td>
<td>14.9</td>
<td>13.4</td>
<td>41.9</td>
<td>36.6</td>
<td>81.0</td>
</tr>
<tr>
<td></td>
<td>STD</td>
<td>20.4</td>
<td>9.7</td>
<td>8.7</td>
<td>3.5</td>
<td>5.1</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>out of range(^a)</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>EMD-2TMS</strong></td>
<td>average (%)</td>
<td>1.9</td>
<td>67.1</td>
<td>3.4</td>
<td>2.9</td>
<td>5.0</td>
<td>57.6</td>
</tr>
<tr>
<td></td>
<td>STD</td>
<td>0.3</td>
<td>9.0</td>
<td>0.7</td>
<td>0.4</td>
<td>0.7</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>out of range(^a)</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td><strong>M2-2TMS</strong></td>
<td>average (%)</td>
<td>2.7</td>
<td>86.9</td>
<td>2.8</td>
<td>3.3</td>
<td>3.5</td>
<td>94.7</td>
</tr>
<tr>
<td></td>
<td>STD</td>
<td>0.4</td>
<td>19.0</td>
<td>1.3</td>
<td>0.4</td>
<td>1.3</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>out of range(^a)</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><strong>3’OH-stan-3TMS</strong></td>
<td>average (%)</td>
<td>15.1</td>
<td>51.6</td>
<td>6.1</td>
<td>29.5</td>
<td>12.7</td>
<td>44.6</td>
</tr>
<tr>
<td></td>
<td>STD</td>
<td>6.5</td>
<td>23.3</td>
<td>2.8</td>
<td>3.1</td>
<td>3.3</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>out of range(^a)</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) Number of spiked urine samples (out of the total number of analysed samples) which do not comply with the WADA relative abundance criteria

The relative abundance of the diagnostic ions and their combinations for EMD-2TMS fell within the tolerance window except the 143 m/z ion. Again, the main reasons for this are the same as for clenbuterol-2TMS: the low uniqueness of 73 m/z as a base ion in TOFMS spectra of EMD-2TMS, and the narrower tolerance window for ions with higher abundances. Any tested combination, except 143 m/z alone, can be used as a qualifier.
Methyltestosterone metabolite M2-2TMS gives similar results to EMD-2TMS. 143 m/z alone cannot be used as a diagnostic ion since it shows low reproducibility and does not comply with the established identification criteria. Another combination which cannot be used is the relative ratio of the 255 m/z and 435 m/z relative abundance; because they are both of low abundance (less than 3% of the base ion), their ratio can therefore vary greatly. All other ions and ion combinations (255 m/z, 435 m/z, 255/143 m/z and 435/143 m/z) can be used as qualifiers.

The 143 m/z ion alone, as well as 254 m/z alone, did not pass the test as qualifiers for 3’OH-stan-3TMS. However, 545 m/z, 545/143 m/z and 254/143 m/z gave results within the tolerance window. 560 m/z cannot be used as a diagnostic ion in samples with a concentration of 3’OH-stanozolol-3TMS lower than 4 ng mL\(^{-1}\), because of the observed bias of TOFMS towards higher masses [186]. The comparison of TOFMS and quadrupole MS spectra of 3’OH-stanozolol-3TMS is given in Figure 6.3. The relative abundance of 560 m/z in TOFMS spectrum of 3’OH-stan-3TMS acquired at 0.5 µg mL\(^{-1}\) concentration in standard solution (Figure 6.3a) shows approximately 20 times lower relative abundance when compared to the quadrupole based MS spectra (Figure 6.3b) [188]. However, the TOF mass spectra similarity of 3’OH-stan-3TMS to the in-house created TOFMS library ensures positive identification of this sterol even at the lowest level of concentration spiked in urine extracts.

Each of the diagnostic ions alone for 19-nor-2TMS (405 m/z, 420 m/z and 315 m/z) have failed the criteria despite the good similarity of TOFMS spectra to the entries in the in-house library. One of the reasons could be the coelution of 19-nor-2TMS with the endogenous component which shares some of the diagnostic ions [188], but most probably the reasons are the same as for the other anabolic agents. However, their combinations (with few exceptions at the lower levels of concentration) gave results within the tolerance window (Table 6.3).
Figure 6.3 Comparison of (a) TOFMS spectra of 3’OH-stan-3TMS acquired at 0.5 µg mL⁻¹ in standard solutions and (b) its entry from the NIST05 MS database. Note that the abundance of several consecutive mass intervals in TOFMS spectrum is enhanced by factors given above the intervals.

Furthermore, the spectral similarity even at the lowest level of concentration is high enough to ensure unambiguous identification. The similarity of 19-nor-2TMS spiked in urine extract at different concentrations is given in Table 6.4. The general trend of increased similarity for higher concentrations has been observed, even though the variation of similarity has been found to be quite large (within ±10%).
Table 6.4 TOFMS similarity of 19-norandrosterone-2TMS spiked in urine extracts at different levels of concentration against the in-house TOFMS library

<table>
<thead>
<tr>
<th>conc. level (ng mL⁻¹)</th>
<th>similarity (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 (std)</td>
<td>945</td>
</tr>
<tr>
<td>20</td>
<td>926</td>
</tr>
<tr>
<td>10</td>
<td>867</td>
</tr>
<tr>
<td>8</td>
<td>891</td>
</tr>
<tr>
<td>6</td>
<td>888</td>
</tr>
<tr>
<td>4</td>
<td>799</td>
</tr>
<tr>
<td>2</td>
<td>784</td>
</tr>
</tbody>
</table>

The lower relative abundance of higher masses observed in TOFMS spectra of anabolic agents can be seen as an advantage over GC-qMS identification since the maximum tolerance window of the relative difference for lower abundance diagnostic ions is wider (see Figure 6.2), and the reproducibility of TOFMS spectra even at the lowest levels of concentration is good. On the other hand, the diagnostic ion ratio, which is not implemented in the WADA criteria, gave reproducible and consistent results allowing this to be used as a criterion in anabolic agent identification.

6.3.3 Other criteria

6.3.3.1 Presence of diagnostic ions

*Present WADA criteria:*
- Diagnostic ion with relative abundance of less than 5% in the reference must be present in the unknown.

All the diagnostic ions with abundance above 10% which were present in the reference spectra of the anabolic agents have been detected also in the spiked urine samples. When ions with abundance lower than 5% were chosen as the qualifier, these ions were also
detected in spiked samples, even at the lowest concentration tested. As stated previously, the commonly used 560 m/z ion for 3’OH-stan-3TMS was not chosen as a qualifier because of the decreased sensitivity of TOFMS at higher masses. Figure 6.3 shows the comparison of TOFMS spectra of 3’OH-stan-3TMS and its entry in NIST05 MS database (qMS based spectrum). For the same reason the ion 448 m/z is not considered in EMD-2TMS identification criteria.

This creates the biggest challenge in strictly applying the WADA criteria in GC×GC-TOFMS: the lack of three diagnostic ions with abundance above 5%. As can be seen from Table 6.3, the abundances of 358 m/z and 216 m/z for EMD-2TMS, and 255 m/z and 435 m/z for M2-2TMS metabolite, are below 5%. As a comparison, the abundance of these ions in NIST05 library entries of the same sterols is between 10% (216 m/z) and 30% (435 m/z). However, the high similarity of TOFMS spectra from spiked urine samples to the TOFMS spectra of the reference compounds permits unambiguous identification based on all ions in the spectrum. This has a range of ramifications for establishing a new criterion based on adequacy of library matches to sterol spectra as opposed to simply comparing ion ratios in SIM spectra [188].

### 6.3.3.2 Isotope ratio criteria

**Present WADA criteria:**
- A second ion belonging to the same isotopic cluster may also be used as diagnostic only when the peculiarity of the atomic composition of the fragment so justifies (e.g. presence of Cl, Br, or other elements with abundant isotopic ions).

The clenbuterol molecule contains two chlorine atoms and its isotope pattern can be another useful criterion in identification of this anabolic agent. The ion abundance ratios of 337/335 m/z and 339/335 m/z have been compared against the theoretical values calculated by the isotope calculator from the NIST MS Search software package (ver. 2.0). The average ion abundance ratios were calculated from 12 spiked urine samples with anabolic agents over the concentration range from 2 ng mL\(^{-1}\) to 20 ng mL\(^{-1}\). The results are given in Table 6.5.
Table 6.5 Experimental and calculated isotope ratio patterns for clenbuterol-2TMS analysed in GC×GC-TOFMS format

<table>
<thead>
<tr>
<th>ion abundance ratio</th>
<th>area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>337/335 m/z</td>
</tr>
<tr>
<td>average (%; n=12)</td>
<td>74.6</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>3.4</td>
</tr>
<tr>
<td>calculated (%)</td>
<td>73.7</td>
</tr>
</tbody>
</table>

Good agreement has been obtained between the experimental and the calculated values for both ion ratios: 337/335 m/z and 339/335 m/z (Table 6.5). The 337/335 m/z and 339/335 m/z ratios, when the peak area was used instead of ion abundances, were slightly higher. However, the reproducibility of the 337/335 m/z ion abundance and area ratio was better than the 339/335 m/z ratios.

The good agreement between the experimental results obtained from clenbuterol-2TMS spiked at different concentrations in urine extract to the theoretical values is a result of the deconvolution capabilities of the ChromaTOF software. The high acquisition rate (100 Hz) contributes to the correct deconvolution. A series of deconvoluted MS spectra of clenbuterol-2TMS spiked in urine extract at different concentrations, showing the chlorine isotope pattern, are given in Figure 6.4.

Furthermore, the appearance of 334 m/z in the experimental results (but not in the calculated isotope pattern) and the differences arising in the isotope pattern at 336 m/z and 338 m/z between experimental and calculated results (Figure 6.5) may be explained by presuming the formation of a species with A-1 m/z (that is, the 334 m/z ion). The isotope pattern of the dominant fragment ion at m/z 335 is shown in Figure 6.5. The experimental spectrum in this region extends from 334 – 341 m/z. Note that ion 334 m/z will not arise from fragment mass loss from the parent ion, but arises from an A-1 ion species.
Taking the predicted isotope pattern for the 335 m/z ion, including the Cl isotopes, gives the pattern labelled as ‘calculated without A-1 ion’. This is a very poor match with the experimental pattern. In order to calculate the isotope pattern correctly, the presence of the A-1 ion has been presumed, and since it contains 2 × Cl atoms (in the ratio 9 : 6 : 1), it will have a strong isotope contribution at 336 m/z. This leads to the large discrepancy between the experimental pattern and the calculated pattern in the absence of the A-1 species. Once the A-1 species is included as an overlapping ion pattern, very good agreement between experimental and calculated patterns arises.
6.3.3.3 Linearity

The linearity of the proposed method has been calculated from spiked urine extracts with anabolic agents at different concentrations from 0.5 ng mL\(^{-1}\) to 20 ng mL\(^{-1}\). The correlation coefficients and the linearity curve equations are given in Table 6.6. The highest correlation (R\(^2\)) was obtained for EMD-2TMS and 19-nor-2TMS (0.996) and the lowest for M2-2TMS metabolite (0.992). Please note than even though the quantification ions of the anabolic agents are detected down to the lowest tested concentration (except for EMD-2TMS at 0.5 ng mL\(^{-1}\)), unambiguous identification is obtained only at 1 ng mL\(^{-1}\) and above for clenbuterol-2TMS, 19-norandrosterone-2TMS and M2-2TMS, and at 2 ng mL\(^{-1}\) and above for EMD-2TMS and 3’OH-stanozolol-3TMS [188]. The good correlation coefficients are the result of the temporal deconvolution of the anabolic agents’ mass spectra from the background components, and this is a direct result of the separation of the components in the 2D space. The high acquisition rate of the TOFMS detector (100 Hz) further facilitates the deconvolution in the mass spectral domain. As previously shown [188], the poorer separation of the components on the 0.1 µm \(d^2\)D column gave lower similarity which is
based on the poorer deconvolution. Furthermore, the low acquisition rate of the TOFMS detector (20 Hz) in 1D GC experiments, and the separation based on classical 1D GC, gave even lower mass spectra similarity against TOFMS entries in the in-house library.

Table 6.6 Linearity parameters for the anabolic agents spiked in urine extracts at different concentrations from 0.5 ng mL\(^{-1}\) to 20 ng mL\(^{-1}\)

<table>
<thead>
<tr>
<th>anabolic agent</th>
<th>quant. ion</th>
<th>linearity equation(^{*})</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMD-2TMS</td>
<td>358 m/z</td>
<td>(Y = 0.0081x + 0.0074)</td>
<td>0.996</td>
</tr>
<tr>
<td>19-nor-2TMS</td>
<td>405 m/z</td>
<td>(Y = 0.0061x + 0.0094)</td>
<td>0.996</td>
</tr>
<tr>
<td>M2-2TMS</td>
<td>255 m/z</td>
<td>(Y = 0.0144x + 0.0070)</td>
<td>0.992</td>
</tr>
<tr>
<td>clen-2TMS</td>
<td>335 m/z</td>
<td>(Y = 0.0143x + 0.0050)</td>
<td>0.994</td>
</tr>
<tr>
<td>3’OH-stan-3TMS</td>
<td>254 m/z</td>
<td>(Y = 0.0043x + 0.0003)</td>
<td>0.994</td>
</tr>
</tbody>
</table>

* \(Y = \text{Area(AA)} / \text{Area(IS)}\); \(x = \text{concentration of AA (ng mL}\(^{-1}\)\)

6.3.3.4 Testosterone/epitestosterone (T/E) ratio

Testosterone/epitestosterone ratio (\(T/E\)) values can be calculated from the same run with unambiguous identification of both sterols. The average similarity of testosterone and epitestosterone TOFMS spectra against the in-house library, across the concentration range of AA from 0.5 ng mL\(^{-1}\) to 20 ng mL\(^{-1}\) in urine samples, was 915 and 820, respectively.

6.3.3.5 Signal-to-noise (S/N) ratio

Present WADA criteria:
- The signal-to-noise ratio of the least intense diagnostic ion must be greater than 3:1.

The final criterion which must be complied with is the signal-to-noise (S/N) ratio for the least intense diagnostic ions. The lowest S/N permitted in WADA criteria is 3:1. S/N ratio for quantification ions obtained at 0.1 µg mL\(^{-1}\) in standard solution (which corresponds to 2 ng mL\(^{-1}\) in spiked urine samples at a concentration factor of 50) has been found to be above 18 [188]. The lowest S/N (4) has been obtained for EMD-2TMS (358 m/z) spiked in urine extracts at 2 ng mL\(^{-1}\). This is again due to the bias of TOFMS towards the higher
masses, combined with the low relative abundance of 358 m/z. The lack of potential diagnostic ions in the TOFMS spectra of EMD-2TMS limits the choice.

19-nor-2TMS and 3’OH-stan-3TMS are the only anabolic agents which completely comply with defined WADA criteria, since all others do not contain at least 3 diagnostic ions with abundance higher than 5% relative to the base ion. However, WADA permits a laboratory to establish its own identification criteria which can be based on minimum MS similarity against the reference compounds. A minimum acceptable match (MAM) has been defined as a criteria for identification of minor components in a complex matrices such as urine, where a library match is required to be above a certain match quality in order to provide satisfactory identification of the component. The MAM in doping control depends on the reproducibility of the MS similarity and probably is best to be set at the average MS similarity of anabolic agents at the positive urine control level, corrected for the standard deviation of the reproducibility. It has been found that for unambiguous identification the MAM can be set at 800 when analysing free sterols in non-hydrolysed urine samples [186], and to 700 in hydrolysed urine samples [188]. The main reason is the more complex urine extract from hydrolysed samples. Obviously, MS similarity is just a confirmation of the other identification parameters, such as relative abundance ratio and retention time tolerance windows. In this case the MAM serves the purpose of providing a minimum value for the quality of full mass spectral similarity matching against, here, the in-house library for sterols. By all measures this should represent a sound basis for confirmation (subject to the metric by which library searches are performed). The compounds can still be quantified based on a selected single quantification ion if so desired.

### 6.3.4 Other benefits of the GC×GC-TOFMS method

Amongst T and E, another 8 sterols which were investigated in a previous work [186] were detected in the urine extract with an average similarity above 900. Once full MS data are available, the presence of sterols can be confirmed in post-run processing, allowing retro-
searching for newly designed sterols, should the analyst become aware of such sterols or any other illicit compounds in the future.

Another advantage of having access to the full mass spectral information is characterisation of the interfering components and the matrix. Just as the deconvolution works for the target anabolic sterols, so it works also for matrix components, and their spectra are likewise deconvoluted. This is a simple and inexpensive way to characterize trace components in urine matrix by their full deconvoluted mass spectra, and not just a limited suite of sterols defined by selected SIM ions and retention windows.

Finally, as previously stated, several tens of other non-target components were identified with high similarity. Most of them are low molecular mass components, because lower mass components have higher similarity against the NIST05 database than high mass counterparts. At present only sterols are included in the custom in-house library. A dedicated comprehensive TOFMS library is needed for proper identification of higher molecular mass components because of the previously stated bias of TOFMS against the higher masses, and consequent larger differences between qMS (used for most library entries) and TOFMS spectra.

### 6.4 CONCLUSIONS

The results presented here confirm that GC×GC-TOFMS of anabolic agents largely complies with the established WADA identification criteria. This technique has been shown to be a powerful tool for detection and unambiguous identification of trace amounts of anabolic agents in complex matrix as urine, so proved to be a promising choice for doping control in sport competition.
Retention time reproducibility on both columns has been found to be below 0.83%, allowing relative error to the reference components to be within the WADA tolerance window of 1%. The second dimension retention time relative error also complies with these criteria, although it is not explicitly required in the WADA criteria.

The relative abundance tolerance criteria have been complied with by most of the diagnostic ions selected, except for the high abundance ions or the ratio between two low abundance ions. Spectral deconvolution has been demonstrated in the case of clenbuterol-2TMS, where very good agreement has been obtained between experimental and theoretical calculations for the isotope cluster from 334-339 m/z.

Other general criteria complied with include the linearity and minimum spectral match quality for positive identification. The deconvoluted full mass spectral information of the non-target components and the matrix are other benefits from this method. The least-complied criterion of the proposed method is the presence of at least three diagnostic ions in the TOFMS spectra with abundance above 5% of the base peak. This appears to be due to the predominance of the 73 m/z in sterol spectra, and if scans were to exclude the 73 m/z ion by presenting data at > 73 m/z, then the WADA criteria may be complied with. Because of the observed bias towards the higher masses, only 19-norandrosterone-2TMS and 3’OH-stanozolol-3TMS comply with the criteria. However, the high similarity based on the full mass spectra offers an unambiguous identification even at the lowest tested concentrations. This latter benefit of using full scan TOFMS data is not available for most classical methods used in doping control that rely on SIM analysis.

The present work suggests that the GC×GC-TOFMS method can play an important role in doping control and drug testing in the future. Separation of components on 2D space relaxes the eventually coelutions with the matrix components and deconvoluted full mass TOFMS spectra offers unambiguous identification. This makes the method a good alternative for anti-doping screening and if sensitivity is improved and confirmed when
recovery is taken into consideration, the method can be a promising option for AA confirmation.
Chapter 7

7 PROFILING OF ECSTASY TABLETS BY USING COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY
7.1 INTRODUCTION

Amphetamines are the second most commonly used illicit drug type in Australia, after cannabis [223]. Ecstasy, a term referring to tablets containing 3,4-methylenedioxy-methamphetamine as an active component, are very popular among young party-goers for their short-term effects, which include a heightened sense of well-being, reduced inhibitions, increased energy levels and self-confidence. According to the World Drug Report 2008 [1], at 4.4% Australia has the highest rate of ecstasy users in the world for those aged 15-64. The composition of these illicit preparations can vary significantly under the influence of synthetic route, purification methods, source materials and the presence of cutting agents and tabletting excipients. As clandestine laboratories rarely adopt good manufacturing practices or standard preparation procedures, the presence and relative concentrations of trace impurities and additives may serve as chemical ‘fingerprints’ that are unique to a particular manufacturer, ‘cook’ or batch of drugs.

In the last 2 decades considerable international effort has been made in the fight against illicit production and distribution of amphetamine-type stimulants (ATS), including ecstasy. In addition to cataloguing the physical characteristics (colour, shape, logo, dimension and mass) of seized samples [224, 225], other chemical features including the presence of major components, trace organics [106, 118, 230, 231], metals [116, 117] and the isotopic ratio of $^{15}\text{N}/^{14}\text{N}$ [111-114, 226] have been profiled. Several laboratories have harmonised their methods of analysis in the interests of efficient data sharing across jurisdictions [7, 106].

Impurity profiling in this sense is understood to be the chemical analysis, collection of data and its use for evidential or intelligence purposes [7, 10, 106, 233]. As in any type of profiling, the more information that is gathered during the analytical phase, the greater
the opportunity to derive some useful information from the relationship between the seized specimen and the database [7]. Trace organic component analysis is most often used for profiling or developing a sample ‘signature’ because it has the capacity to provide higher information content than other forms of analysis.

Many reported methods for organic impurity analysis involve liquid-liquid extraction (LLE) [104, 106, 230, 231] and subsequent analysis by gas chromatography coupled to a flame-ionisation detector (GC-FID) [118, 234] or mass spectrometer (GC-MS) [104, 106, 229]. More recently, solid phase micro extraction (SPME) of volatile components [227, 228] in ecstasy samples was found to give comparable results to LLE for the detection of the most discriminative components. The attraction to SPME was reported to be a simpler and shorter sample preparation and a reduction in the sources of error.

The analysis of chemical ‘signature’ has been reported in a variety of ways. Principal component analysis (PCA) [112, 231] and hierarchical cluster analysis (HCA) [112, 113, 116, 117] have been used for classifying samples according their similarities (intelligence) while Pearson correlation coefficients [106, 227, 229-231] and cosine functions [104, 231] have been employed for differential comparisons or evidential purposes. Most of the reported drug profiling methods have used single-column (or one-dimensional, 1D) chromatographic techniques. Due to the very large number of trace components found in the majority of illicit drug samples, most of these 1D chromatographic methods have had insufficient separating power to map important regions in the chromatogram. Co-elution can mask the presence of trace impurities that are definitive to the chemical signature and therefore reduce the informing power of the analysis. While mass spectrometry provides a tool that can be used for deconvolution of coeluting peaks, this method is only useful where there is either sufficient mass spectral power or where the coeluants are well resolved in the mass domain. In most cases, coeluant resolution to evidential standard relies on either a reduction in the informing power of the mass spectrum or the application of a tandem technique.
Conventional approaches to increasing informing power when employing 1D-techniques include multiple analyses on different columns or the use of complementary methods that are able to increase the information content of the total analysis. Typically for a single technique, increasing the informing power for one aspect of the analysis results in either reduced throughput and increased reporting times (larger data set) or a decrease in the sample cross-section and therefore a loss of information peripheral to the identity of the key components (increased specificity or selectivity). While both approaches are valid, neither is ideal for the efficient chemical fingerprinting of samples. These strategies not only limit the throughput in the laboratory but also risk the loss of important components that could have contributed to the unique signature of a sample.

Recent advances in multidimensional chromatographic technologies provide a facile means of achieving high separation efficiency with a single injection and acceptable analysis times. One reported example from Neumann and Meyer [175] used “heart-cut” multidimensional GC (GC-GC) for the analysis of illicit heroin. In their experimental setup, two GC columns of highly different selectivity were connected by a pneumatic Deans switch at the confluence of the two columns. The role of the switching device was to direct specific (co-eluting) bands from the first column (1D) to the second column (2D) for further analysis. This approach is particularly useful when specific parts of a 1D chromatogram contain severely overlapping analytes. The different selectivity of the 2D GC column affords enhanced resolution power to reveal trace impurities, which might otherwise be obscured. Depending on the routing of the two outlet columns to detectors, the technique can provide an additive increases in the information content.

In comprehensive two-dimensional gas chromatography (GC×GC), instead of sampling only subsections of the 1D column chromatogram, the entire eluted volume from the first column is subjected to further analysis on the 2D column. With only one detector, the increase in separation power is multiplied within the same analysis time as that of a single column GC experiment.
The opportunity to maximise the separating power of the two column set is reliant on selecting phases that have orthogonality in their selectivity towards the analytes of interest and so it is to be expected that column sets will be tuned to particular tasks. The 2D column in GC×GC must act as a fast-eluting high-efficiency column and is typically 0.5–2 m in length and 0.1 mm I.D. with a 0.1 μm film thickness (df). The interface between the two columns (modulator) has to trap/accumulate all the components which elute from the 1D column for a predefined period (typically 3–8 s) and then release the ‘focussed’ band in narrow fractions to the 2D column. The efficient trapping and fast release of components is usually achieved by cryo-focusing, which yields ultra-narrow peak widths at the end of the 2D column.

GC×GC has been successfully applied in the analysis of petrochemical [201, 214], food [201, 229], environmental [204], forensic [189, 190, 205, 230, 231], doping [186, 187], and essential oil [168, 180] samples in complex matrices. In addition to improved peak capacity, GC×GC yielded highly structured chromatograms and improved sensitivity from increased signal height when compared to classical GC [185, 221].

Although GC is an important technique for ATS profiling, to the author’s knowledge, no reports have been made describing the application of GC×GC in this area. GC×GC has characteristics that make it ideally suited for drug profiling purposes [21]. In this chapter, chemical profiling of 24 ecstasy tablets by GC×GC has been described and its potential as a new chemical profiling tool has been explored.
7.2 EXPERIMENTAL

An Agilent 7890 GC was used in both GC-accurate time-of-flight mass spectrometry (GC-accTOFMS) and GC-flame photometric detection (GC-FPD) experiments and an Agilent 6890 GC fitted with a longitudinal modulation cryogenic system (LMCS) modulator (Chromatography Concepts, Doncaster, Victoria, Australia) was used in all other 1D GC and GC×GC experiments. A LECO Pegasus III MS instrument was used in fast acquisition TOFMS (fastTOFMS) experiments, an Agilent 5973 MSD in quadrupole MS (qMS) and Waters GCT Premier in accTOFMS experiments. 1D GC experiments were carried out either on a single column (qMS and accTOFMS) or under the same conditions as in GC×GC, except the modulator was off and the acquisition rate of fastTOFMS detection was reduced to 20 Hz. Thus in this case the 1D system will comprise a long column directly coupled to a short column. Whilst this can be termed a multi-chromatography system according to Hinshaw and Ettre [216] and discussed elsewhere [217], the second very short column is anticipated to lead to negligible variation in peak properties such as width.

The GC conditions were kept the same for each column configuration, except the column head pressure which depends on the column dimensions and outlets. Column sets used for separation are listed in Table 7.1. Columns denoted BP were from SGE Analytical Science (Ringwood, Victoria, Australia), and the HP column was from Agilent (Folsom, CA, USA). Conditions are given below, unless otherwise specified. Injector and detector temperatures were both 250 °C and oven temperature program was from 40 °C (hold for 2 min) to 250 °C at 10 °C min⁻¹ (hold for 7 min). Hydrogen was used as a carrier gas at a flow rate of 1 mL min⁻¹ in all FID/FPD and helium at 1.3 mL min⁻¹ in all MS experiments. Splitless mode was applied for injection (SPME desorption) with a purge off time of 2 min. The modulation period (P_M) was varied from 3 s to 6 s and modulation temperature (T_M)
was set at -30 °C, 0 °C, 40 °C and 80 °C during the optimization stage. Temperature tracking was applied in all GC×GC-TOFMS experiments by keeping a constant temperature difference between the oven and modulator temperature of 130 °C, starting at 0 °C for the LMCS cryotrap.

**Table 7.1 Column sets used in all 1D GC and GC×GC experiments**

<table>
<thead>
<tr>
<th>system</th>
<th>1D column</th>
<th>2D column</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC×GC-FID</td>
<td>BP20</td>
<td>BPX5</td>
</tr>
<tr>
<td></td>
<td>BPX50</td>
<td>BPX50</td>
</tr>
<tr>
<td></td>
<td>BPX5</td>
<td>BP20**</td>
</tr>
<tr>
<td>GC×GC-FPD</td>
<td>BPX5</td>
<td>BP20</td>
</tr>
<tr>
<td>GC×GC-fastTOFMS</td>
<td>BPX5</td>
<td>BP20</td>
</tr>
<tr>
<td>GC-accTOFMS</td>
<td>HP5</td>
<td>BP20</td>
</tr>
<tr>
<td>GC-qMS</td>
<td>BPX5</td>
<td>BP20</td>
</tr>
</tbody>
</table>

* 30 m x 0.25 mm; 0.25 μm
** 1 m x 0.1 mm; 0.1 μm, except otherwise stated
*** 1.4 m x 0.1 mm; 0.1 μm

MS detectors were operated at ionisation energy of 70 eV over the mass range from 45 m/z to 500 m/z. One scan per second acquisition rate was used with all single column experiments (qMS and accTOFMS), 20 Hz in all 1D GC and 50 Hz in all GC×GC experiments with two-column sets (FID, FPD and fastTOFMS). Components were tentatively identified against NIST05 (National Institute of Standards and Technology, Gaithersburg, USA) and Wiley 7 (John Wiley & Sons, New York, USA) commercial MS databases by using PBM (qMS only) or NIST algorithm. When in doubt, accTOFMS was used for component confirmation. Automated spectra deconvolution and peak finding features of ChromaTOF (LECO Corp.) acquisition and data processing software have been applied in all fastTOFMS experiments. Signal-to-noise ratio (S/N) threshold of 100 was set for peak detection (except otherwise stated) and minimum acceptable match threshold [186] of 800 (on 0 – 999 scale) for component identification. All 2D plots are presented with 50 contour levels from 10±2 pA to 50 pA (FID) or 180k – 680k counts (TOFMS).
7.2.1 Statistical analysis

Data were processed using the Statistica Software release 8.1 package (Statsoft, Maison-Alfort, France). The 24 ecstasy tablets were gathered in eight groups by their post-tabletting (post-TB) physical characteristics (logo, colour, diameter and thickness) and intelligence information. The raw dataset (53 observations x 16 variables) consisted of the abundance of the targeted compounds determined by GC×GC-TOFMS for each replication of each tablet sample plus 5 replicates of one sample (XTC5). The data were normalized according to the procedure described by Bonadio et al. [227, 228]. The procedure involves calculation of the 4th square root of the ratio between the abundance of the targeted compound and the sum of the abundance of all compounds selected for the profiling. To determine which compounds distinguished between the eight groups, a one-way analysis of variance (ANOVA) was performed on raw and normalized datasets according to the model: abundance of the compound : group of tablets, p<0.05. Principal component analysis was performed on raw and normalized data sets to visualize the structure of the data. In order to confirm the ability of some compounds to differentiate the samples according to their country of seizure, a one-way ANOVA was performed on the normalized data set according to the model : country, p<0.05.

In order to determine a set of “orthogonal” compounds enabling discrimination of the eight groups of ecstasy tablets, a linear discriminant analysis (LDA) was carried out on the normalized dataset. For processing the LDA, the dataset was divided into two independent subsets: (i) a “learning” dataset (n=35) for the generation of candidate discriminative models and (ii) a “test” dataset (n=18) for the assessment of the robustness of the model. For the DA, only the three most relevant variables were selected according to the “best subset” algorithm. The best subset of three variables was selected from the learning dataset according to the best subset algorithm where the misclassification rate was chosen as the criterion of ranking between all the models constructed on three variables.
7.3 RESULTS AND DISCUSSION

7.3.1 Separation of volatile components in ecstasy on different column sets

The concept of orthogonality in GC×GC aims to maximise the utility of the 2D separation space by having minimum correlation between the retention mechanisms of 1D and 2D [232]. In cases where this extreme is desirable and can actually be achieved, the GC×GC approach is capable of generating a large number of points of reference and so is effective for profiling complex mixtures. In the case of chemico-legal profiling of illicit substances, differential analyses of this type permits ideally results in the derivation of relationships such as batch-to-batch association, supply chain tracking and mapping of synthetic and geographic origins.

The selection of column sets that have demonstrable orthogonality is sample dependent and must be derived from either a detailed understanding of the sample composition or by empirical methods. As the former is rarely achieved without first completing the orthogonal analysis, phase polarity is usually deduced using the ad hoc application of solvation parameter models such as that derived by Abraham et al. [233]. In practice, the phase polarity is more commonly interpreted in terms of a simplified polarity scale, such as that developed by Wynne et al. [234], in which spatial restriction to interaction are normalised and bonding considered according to the π- or n-type character in the analyte-phase interactions.

In the case of ecstasy and its likely precursors, by-products and contaminants, predictive analyte chemistry suggests that orthogonality to a simple van der Waals type retention is likely to be found by using an aromatic chemistry (on the basis of the 3,4-methylenedioxybenzyl moiety) or a hydrogen bonding or n-electron chemistry (on the basis of the amine moiety) [234]. Accordingly, these polar chemistries (P) have been
coupled with less polar phases (NP) in two dimensional sets to test for separation of components in the ecstasy samples.

### 7.3.1.1 BP20 / DB-1

A BP20 / DB-1 column combination was used as a P – NP column set to test the separation of volatiles in ecstasy samples. Despite the reasonably good spread of the components on the 2D plot and their very good peak shape (Figure 7.1), the lowest number of components has been detected with this column set (proved when all column sets were tested). This fact together with the strongly tailed MDMA peak on the 1D column, which is most likely due to the strong H-bonding and weak n-π* interaction with the stationary phase, has limited the applicability of this column set in ecstasy volatiles profiling.

![Figure 7.1 2D plot of ecstasy sample XTC4 analysed by SPME GC×GC-FID on BP20 / DB-1 column set. The highly tailed MDMA peak on 1D column is annotated.](image)

### 7.3.1.2 BPX50 / BPX5

The second P – NP column set applied for separation of volatiles in ecstasy tablets was BPX50 / BPX5 (Figure 7.2). Similarly to the separation on the opposite phase column set (BPX5 / BPX50, see Figure 4 later), increased peak signal was obtained due to the very narrow peaks on the 2D column, with the components spread in a narrow band on the 2D plot along the 1D time. Three different ecstasy samples (which were later proved to be different) showed similar GC×GC profiles here, but this is due to the poor separation on the 2D column which compresses the volatile components into the narrow band noted
above. The separation mechanism in BPX5 based on the weak H-bonding and stronger π-π* interaction is not enough to separate the ecstasy constituents on 2D. This makes the column set unsuitable for GC×GC profile comparison since it does not offer much advantage over classical 1D GC separation. The major feature of this column set was the clear separation of highly NP components, i.e. alkanes and siloxanes, from the rest of the ecstasy constituents, which have the longest retention on BPX5. However, peaks as narrow as 50 ms peak width at half height ($W_{1/2}$) on 2D column have been obtained.

Figure 7.2 Representative 2D plots of ecstasy samples XTC3 (A), XTC4 (B), XTC5 (C) and a blank (D) analysed on BPX50 / BPX5 column set. Note the similarity of the profiles, even though the samples proved to be different.
7.3.1.3 BPX5 / BPX50

The separation of volatiles in ecstasy tablets on a NP – P column set was first conducted using BPX5 / BPX50. While increased peak signal was obtained when compared to the other column set (BP20 / DB-1), the components are located in a narrow band in the 2D axis on the 2D plot along the 1D time, showing poor spread in 2D space (Figure 7.3). This makes the column set unsuitable for analysis of volatiles since it does not offer much advantage over classical 1D separation. However, good peak shape (narrow and symmetrical) has been obtained. The separation on this and the previous column set incorporating the BPX50 polar column suggests that the selectivity of the BPX50 phase is relatively poor towards chemical constituents of the sample. However, this phase is known to be more selective (retaining) towards aromatic compounds. It seems that all sample constituents show similar “aromaticity” and the selectivity towards BPX50 is in some way “normalized” to give only a narrow separation band. The separation appears to be superficially the same as a boiling point elution, thus reducing the orthogonality of this column set.

Figure 7.3 2D plot of sample XTC4 analysed by SPME on BPX5 / BPX50 column set

7.3.1.4 BPX5 / BP20

The second NP – P column set tested was BPX5 / BP20. This column set showed a good spread of components over the 2D space, as well as having good peak shape (Figure 7.4).
Despite the unresolved cluster of components at the lower right side region of the 2D plots, which is partially contributed by siloxanes from the SPME fibre and column bleed, most of the components are well separated and spread within 2D space. It seems the spread of the components on the 2D column (BP20) is mainly due to the column selectivity towards the aliphatic hydrocarbon chain in sample constituents rather than the selectivity towards the aromatic moiety.

This column set reveals a high number of volatiles in ecstasy samples, offering complex sample profiles for easier sample comparison. Three different volatile ecstasy profiles are shown in Figure 7.4, along with a representative blank. Closer examination of the profiles reveals some easily noticeable differences in component ratios between samples, and some differences in presence/absence of other components. Because of this, all further experiments were conducted on this column set. However, unambiguous identification of the components is necessary in order to avoid comparison of samples based on non drug-origin related markers. Whilst a large number of reference components is required for identification of components based on GC×GC-FID, availability of MS detection provides valuable molecular information of sample composition.

Flame photometric detection (FPD) is a highly sensitive and selective detection system toward P- and S-containing components. Its potential in GC×GC format for ecstasy profiling has been investigated by using this column set. However, only a few components (data not shown) have been detected on both the P- and S-channel. Although some infrequent and unusual components can be of great value in sample-to-sample comparison, the few detected components with similar ratio in the analysed ecstasy samples has been shown to be of little value in ecstasy volatile profiling by using FPD.

On the basis of the good separation found for the present NP – P column set, it was decided that a subset of 16 compounds would be selected as representative of different ecstasy samples, to demonstrate sample similarity and heterogeneity.
Figure 7.4 Representative 2D plots of three different ecstasy samples analysed by SPME on BPX5 / BP20 column set, at -30 °C modulation temperature and 6 s modulation period: sample XTC3 (A); sample XTC4 (B); sample XTC5 (C) and a blank (D). Note the differences between profiles, especially as suggested by the circled regions. The annotated components in sample XTC3 are selected for $^2$R and area reproducibility.
7.3.2 Optimization of some GC×GC parameters

Modulation period, $P_M$. It has been shown in subsequent experiments that 5-6 s modulation period is necessary in order to elute some polar components from the highly polar $^2$D column, under the proposed conditions, within one $P_M$ value. The most retained components from the selected 16, along with various fatty acids, were the most polar 1,3-benzodioxole-5-MeOH and benzyl alcohol. Although they showed a high level of tailing on the $^1$D column, they were well modulated on the $^2$D column which makes identification and integration much easier [188].

Modulation temperature, $T_M$. Not surprisingly, it has been found that lower modulation temperature offers better peak shape. However, consistent with conclusions in previous work on sterols [186], experience reveals that higher modulation temperature favours better elution of some of the higher boiling point components (data not shown). Whilst a modulation temperature of -30 °C offers the best overall peak shapes, modulation at 0 °C gave acceptable results since none of the early eluting components were selected for profiling. All subsequent experiments, unless otherwise stated, were carried out at this $T_M$.

7.3.3 Reproducibility of peak area and $^2$D retention time in GC×GC

The reproducibility of the GC×GC method was confirmed by running five fresh aliquots of the same sample on the above column set. Very reproducible profiles were obtained, even in terms of the minor components usually not seen in 1D GC chromatograms. The 2D plots of five replicates of ecstasy sample XTC3 are given in Figure 7.5.
Figure 7.5 2D plots of five replicates of ecstasy sample XTC3 obtained by GC×GC-FID on BPX5 / BP20 column set. The three components selected for $t_R$ and peak area reproducibility are circled in the top plot.

The $t_R$ and the peak area reproducibility of three selected components (shown in Figure 7.4A, and in the top plot of Figure 7.5) are given in Table 7.2. Relative standard deviation
(RSD) of ~1% was obtained for $t_R$ reproducibility and 6-7% RSD for peak area reproducibility of the selected components. The results for $t_R$ reproducibility here are in good agreement with the previous findings in GC×GC format [188, 222].

**Table 7.2 Reproducibility of $^2$D retention time and peak area of three selected components obtained from five replicates of ecstasy sample XTC3, analysed by SPME GC×GC-FID. Selected components are annotated in Figure 7.4A.**

<table>
<thead>
<tr>
<th></th>
<th>component 1</th>
<th>component 2</th>
<th>component 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_R$</td>
<td>Average (s)</td>
<td>2.736</td>
<td>3.228</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.20</td>
<td>0.83</td>
<td>1.15</td>
</tr>
<tr>
<td>Area</td>
<td>Average (pA s)</td>
<td>13.19</td>
<td>14.56</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>6.2</td>
<td>7.0</td>
<td>6.1</td>
</tr>
</tbody>
</table>

### 7.3.4 Ecstasy volatiles profiling on GC×GC-TOFMS

The two column sets incorporating BPX50 and BPX5 were eliminated from further experiments due to the low components spread over the 2D separation space. This is greatly contributed by the poorer orthogonality of these column sets towards sample constituents compared to the orthogonality of BPX5 / BP20 and BP20 / DB-1. The phase selectivity of BP20 based on H-bonding interactions apparently plays a key role in the orthogonality of these column sets for this kind on components.

Both, BP20 / DB-1 and BPX5 / BP20, gave good spread of the ecstasy volatiles in the 2D plots. However, because of the strong tailing of the large MDMA peak on the $^1$D BP20 column, all further experiments were proceed on the BPX5 / BP20 column set. One method difference involved replacing the 1.4 m $^2$D column with a 1.0 m long $^2$D column of the same type, to avoid any wraparound (ensuring $t_R < P_M$) whilst still keeping good spread of the components on the 2D plots.
### 7.3.4.1 Identification of components on GC×GC-TOFMS

Automated deconvolution and peak finding data processing of GC×GC-TOFMS chromatograms on this column set revealed the identity of most of the higher abundance components with high match quality. The high match quality of TOFMS spectra against commercial MS databases (NIST05 and Wiley7) agreed with our previous conclusions [186, 188] that match quality decreases as molecular mass and higher mass ion fragmentations in the mass spectra increase. The highest molecular mass components which were detected and identified in HS-SPME sampling were $N$-formyl- (RMM 221) and $N$-acetyl-MDMA (RMM 235), compared to as high as RMM 560 for derivatized components in the previous study of sterols (see Chapter 5 and Chapter 6).

Through use of ChromaTOF software automated detection and identification, an extensive number of components in ecstasy volatile profiles have been recorded. In some ecstasy samples up to 450 components out of a total of 1200 have been tentatively identified at a S/N threshold of 100 and minimum acceptable match threshold of 800, of which several tens were replicates and background components (mainly siloxanes, see Figure 7.4D). In comparison, only 50 out of 200 detected peaks in total were identified against MS databases by using PBM algorithm in GC-qMS experiments under similar chromatographic and integration conditions (data not shown). This demonstrates that ecstasy volatile profiles in GC×GC format are complex, that many more peaks can be positively identified, and that GC×GC approaches should be appropriate for sample-to-sample comparison and grouping. Representative GC×GC-TOFMS 2D plot of an ecstasy sample analysed on BPX5 / BP20 column set is given in Figure 7.6.
7.3.4.2 Selection of components for profiling and their location on the 2D plot

Selection of components for profiling was firstly based on previous work [104, 106, 227, 235], according to components that have been previously employed for profiling in 1D GC methods. Among several precursors (safrole, isosafrole, piperonal), intermediates (PMK) and by-products (piperonyl alcohol, PMK-OH, N-formyl- and N-acetyl-MDMA) with known origin, several unknown or known components with unexplained origin have also been selected. The structures of the 16 selected components which showed good reproducibility and were present in all tested samples in reasonable but variable concentrations are given in Figure 7.7. Their location on a representative 2D plot of an ecstasy sample is given in Figure 7.6, and the peak table with their names, \( t_{R1} \), \( t_{R2} \), and quantification ions for peak area calculation is given in Table 7.3.
Peak area reproducibility of the selected components for profiling has been confirmed on the BPX5 / BP20 column set also in GC×GC-TOFMS format, similarly to the procedure where GC×GC-FID had been applied. An average of 9.1% RSD has been obtained with the lowest being for 1,3-benzodioxole-5-MeOH (6.0%) and N-acetyl-MDMA (5.8%) and the highest for benzyl alcohol (15.7%) and benzothiazole (15.5%). The average RSD has reduced on 7.9% when the three least discriminative components (3,4-MD-benzyl-Cl, benzyl alcohol and benzothiazole) were excluded from the calculation. The last two components do not contain the 3,4-methylenedioxyphenyl bridge. The higher RSD in GC×GC-TOFMS format over GC×GC-FID format is likely related to the lower mass spectrometry reproducibility over FID reproducibility. Peak area reproducibility is a key issue when the aim is to discriminate samples according to their similar profiles. A poor reproducibility is a source of unexplained variability and contributes to lower the discrimination power of the distinctive compounds.
Even though the number of selected components for profiling is quite large (16), only one component (5) in only one sample (XTC3) was not detected at a defined S/N threshold of 100, but was detected at S/N ratio of 20. This is partially contributed by the strong tailing of 5 on the 1D column, where up to 6 modulation slices were automatically detected, integrated and summed up. Adequate integration was facilitated by the excellent trapping and releasing of 5 on the 2D column, giving narrow peaks with an average $W_{1/2}$ of ~200 ms. It has been shown latter that this tailing and low abundant component could be one of the most discriminative in the proposed statistical approach.

On the other hand, the TOFMS detector was saturated with some of the components listed in the literature at their characteristic ions, i.e. $N,N$-dimethyl-3,4-methylenedioxyamphetamine at 72 m/z. Because of this, the component was rejected from selection. An inconvenience due to the overloading has been encountered also with other higher abundance components in the profiles such as PMK, piperonal and PMK-OH. Sometimes different modulation slices of the same component have been identified and integrated as different species, so manual inspection and proper data processing method (match similarity threshold for combining modulation slices, peak width on 2D, S/N threshold) are required. This problem was not observed with lower abundance components in the profiles.

Many production-specific components, generally those containing a 3,4-methylenedioxyphenyl bridge, co-elute on the 1D column with some matrix components or cutting agents, but they then are subsequently separated on the 2D column. Figure 7.8 shows co-elution of benzodioxole-5-MeOH (5) with two other matrix components, with which they share the two most characteristic mass fragments of 5: 135 m/z with component x and 152 m/z with component y. Note that the Macedonian ecstasy sample presented in Figure 7.8 (sample XTC3) contains minor amount of 5, in contrast to the Australian ecstasy sample presented in Figure 7.6.
Table 7.3 Peak table of the 16 selected components chosen for profiling with their retention times and quantification ions used for area calculation

<table>
<thead>
<tr>
<th>Compound</th>
<th>¹D time (s)</th>
<th>²D time (s)</th>
<th>Quant. ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>safrole</td>
<td>960</td>
<td>1.58</td>
<td>162</td>
</tr>
<tr>
<td>isosafrole-2</td>
<td>995</td>
<td>1.78</td>
<td>162</td>
</tr>
<tr>
<td>3,4-methylenedioxyphenylpropane (3,4-MD propane)</td>
<td>920</td>
<td>1.48</td>
<td>164</td>
</tr>
<tr>
<td>piperonal</td>
<td>970</td>
<td>2.86</td>
<td>149</td>
</tr>
<tr>
<td>1,3-benzodioxole-5-MeOH (benzodioxole-5-MeOH, piperonyl alcohol)</td>
<td>1005</td>
<td>4.54</td>
<td>152</td>
</tr>
<tr>
<td>3,4-methylenedioxyphenyl-2-propanone (Piperonylmethylketone, PMK)</td>
<td>1080</td>
<td>2.58</td>
<td>178</td>
</tr>
<tr>
<td>3,4-methylenedioxyphenyl-2-propanol (PMK-OH)</td>
<td>1085</td>
<td>2.88</td>
<td>180</td>
</tr>
<tr>
<td>unknown 147</td>
<td>1150</td>
<td>2.420</td>
<td>147</td>
</tr>
<tr>
<td>N-formyl-MDMA</td>
<td>1400</td>
<td>3.080</td>
<td>162</td>
</tr>
<tr>
<td>N-acetyl-MDMA</td>
<td>1415</td>
<td>2.78</td>
<td>162</td>
</tr>
<tr>
<td>3,4-methylenedioxyacetophenone (3,4-MDAcPh)</td>
<td>1050</td>
<td>2.62</td>
<td>164</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>670</td>
<td>4.24</td>
<td>108</td>
</tr>
<tr>
<td>3,4-methylenedioxytoluene (3,4-MD toluene)</td>
<td>740</td>
<td>1.68</td>
<td>135</td>
</tr>
<tr>
<td>benzylmethylketone (BMK)</td>
<td>760</td>
<td>1.98</td>
<td>134</td>
</tr>
<tr>
<td>benzothiazole</td>
<td>870</td>
<td>2.28</td>
<td>135</td>
</tr>
<tr>
<td>3,4-methylenedioxybenzylchloride (3,4-MD-benz-Cl)</td>
<td>990</td>
<td>2.30</td>
<td>170</td>
</tr>
</tbody>
</table>

The highly abundant and strongly tailing peaks of polar organic acids on the ¹D column can strongly interfere with some of the co-eluting components in the profiles. For example, Figure 7.6 shows that 13 co-elutes with heptanoic acid, 1 with decanoic acid and 8 with several matrix components on the ¹D column. However, they are all well resolved on the ²D column and this, facilitated by the deconvolution and automated peak finding features.
of ChromaTOF software, resulted in high similarity to the commercial MS database entries.

Figure 7.8 Extracted ion 2D plot of Macedonian ecstasy sample XTC3 showing the co-elution of 5 on the 1D column with two other components which share some common mass fragments: component x and component y have abundant 135 m/z and 152 m/z daughter ions, respectively. Note that these two ions are the two most characteristic for 5. 1D GC will have difficulty in identifying these peaks.

Two out of four of the most discriminative components listed in the work of Weyermann et al. [235] elute after N-formyl- and N-acetyl-MDMA, and they were not detected in the samples due to their poor sorption on SPME fibre at the applied temperature (80 °C). Several other higher boiling point components listed in the literature were not detected in the samples due to the SPME sampling limitation. However, many lower molecular weight components are detected in the profiles which could represent minor by-products, intermediates or impurities present in precursors used in ecstasy production and the list of the components selected for profiling can be further extended.

The average match quality of the 15 selected components listed in Table 7.3 (there is no entry for Unknown 147 in the MS databases) against NIST05 and Wiley7 was 920, the lowest being for N-formyl-MDMA (813) and N-acetyl-MDMA (901) and the highest for isosafrole-2 (957) and 3,4-MDAcPh (965). The mass spectrum of Unknown 147, in a latter stage confirmed as 3-(3,4-methylenedioxyphenyl)-3-buten-2-one by accTOFMS at 3 ppm
error, showed high similarity to the literature spectra [106] of Unknown 44. The high match quality for low RMM compounds, facilitated also by the deconvolution capabilities offered by ChromaTOF software, precludes the need for generation of a TOFMS dedicated in-house library, which was necessary in the previous study on higher molar mass sterols [186] and anabolic agents [188].

GC×GC has demonstrated its improved separation and enhanced sensitivity over 1D GC through more separated and detected components in the profiles. The fastTOFMS detector performance (high speed in full mass spectra mode, non-skewed spectra across the peak, spectral deconvolution and peak finding features) fits perfectly with the GC×GC performance and simply testifies to its superior identification power over 1D GC when coupled to this detector. However, the larger number of separated and detected components requires an extensive study on relationship between the newly detected components and their significance in ecstasy profiling.

### 7.3.5 Authentication of ecstasy tablets based on GC×GC-TOFMS profiling

The 24 tablets were initially classified in eight groups by their post-TB characteristics, even though samples from Macedonia (XTC1, XTC2 and XTC3) were all from different seizures and samples from Australia (ECS1 to ECS5) were from a single seizure. In order to determine whether the sixteen variables chosen for profiling enable to discriminate these groups (supposing they all have different profiles), one-way ANOVA was performed on the raw dataset and confirmed the relevance of each of the sixteen selected volatile compounds (p<0.05). The first map of the PCA performed on the dataset (53 analysed samples x 16 compounds) shows that it was possible to make a clear distinction between 6 of these groups based on the raw data (peak area) provided by the GC×GC-TOFMS analysis (Figure 7.9A). In contrast, ECS3 and ECS5 or ECS2 and ECS4 can not be clearly distinguished. Figure 7.9B shows the first map of the PCA performed on the same dataset after its normalization by the procedure proposed by Bonadio et al. [229, 230]. Whereas the
percentage of variation explained by the first map was roughly the same. Figure 7.9B shows a clear separation of the 8 groups after normalisation and confirms both the relevance of this data treatment and the suitability of the sixteen selected compounds for authentication purpose.

![Graph](image)

**Figure 7.9** First map PCA differentiation of 24 ecstasy tablets carried out from the raw (A) and normalized (B) abundances of the 16 selected components. The eight groups are annotated by their names beside each group and the country of seizure is annotated by empty circles (Macedonia) or empty squares (Australia).
In addition to the clustering of tablets from different seizures with the same post-TB (XTC samples) and differentiation between the eight groups, Figure 7.9B also evidenced a clustering of Australian and Macedonian samples. The examination of the variable projection on the PCA first map (data not shown) shows that Macedonian samples were found to be rich in N-formyl-MDMA and N-acetyl-MDMA and poor in 3,4-MD-propane and 3,4-MD-acetophenone as compared to Australian samples. One-way ANOVA confirmed this significant country effect (p<0.05) for the four variables with Fisher-F values ranging from 57 to 386.

In order to determine the best subsets of variables enabling the discrimination of the eight groups, a linear discriminant analysis (LDA) was applied on the normalized data matrix. Several combinations of three compounds chosen out of the sixteen were shown to be suitable to classify 100% of the samples in both learning and test subsets of the dataset. Figure 7.10 shows the discrimination obtained in the 3D orthogonal space generated by 3,4-methylenedioxyacetophenone, 1,3-benzodioxole-5-MeOH and Unknown 147.

Figure 7.10 3D score plot of normalized abundances of three components selected by LDA, showing clear discrimination of the eight groups of ecstasy tablets.
Even though synthesis route elucidation was not the focus of the present thesis, several components specific for country of seizure have been detected. 2-(dimethylamino)-2-methyl-3-(3,4-methylenedioxyphenyl)-propanenitrile, shown to be a marker for reductive amination by using cyanoborohydride (NaBH₃CN) [103], has been detected in different proportions only in samples from Macedonia. Its GC×GC-TOFMS spectrum is given in Figure 7.11. The difference in the reduction pathway could be one of the reasons for obtaining different profiles between samples according the country of seizure. Interestingly, the presence of this component in samples from Macedonia could not be confirmed with accTOFMS even though the relative location on 1D GC chromatogram and accurate mass were available. The reason is probably the presence of this component at low levels, where the compression effect of the modulator and the relatively low m/z of the base mass fragment (97 m/z) favour its detection and identification in GC×GC format. As a reminder, the sensitivity of fastTOFMS is biased against higher masses [186, 188, 236].

Two other unknown components, whose mass spectra are given in Figure 7.12, have been detected only in samples from Australia. This could be another reason for the difference in the profiles between samples from the two countries. Even though the initially annotated Unknown 166 has not been identified against MS databases, the mass spectrum (Figure 7.12A) resembles the mass spectrum of piperonyl methyl ether, a component previously
reported as an impurity in ecstasy samples [106]. The identity of initially annotated Unknown 208 is still unknown but the presence of 135 m/z ion fragment in its mass spectrum (Figure 7.12B) suggests it could be a 3,4-methylenedioxymethylphenyl derivative. Many more components are detected in extracted 135 m/z GC×GC chromatograms (data not shown) which suggest that the number of potential markers could be much higher than the selected 16 components.

![Figure 7.12 Mass spectra of Unknown 166 (A) and Unknown 208 (B), two components detected only in samples from Australia](image)

### 7.4 CONCLUSIONS

The purpose of this work is to show the enhanced separation efficiency of GC×GC over classical 1D GC, and to prove its applicability in ecstasy impurity profiling. Different column set combinations gave different profiles with components spread over 2D space in a different pattern. The BPX5 / BP20 column set has shown the best separation with maximum use of the 2D separation space among the tested column sets.

General advantages of GC×GC over 1D GC applies also in ecstasy volatiles profiling. The modulation process, combined with SPME concentration and sampling, leads to almost all selected components being detected at S/N threshold of 100 and tentatively identified with
an average MS similarity of 920. Some components previously reported in the literature as being relevant to ecstasy profiling are rejected due to the TOFMS saturation at its characteristic mass fragment. The incorporation of highly tailed components on the 1D column in the presented metric, which is always problematic in classical 1D GC and limits the selection of these components for profiling purposes, is facilitated due to the modulation process involved in GC×GC which captures a larger area proportion of such peaks. Combined with the increased separation efficiency, enhanced sensitivity and retaining the full mass spectra information content of targets, non-targets and the matrix, extends the possibility of detecting more markers and route-specific components, especially low abundance polar components (i.e. component 5). However, the work should include a larger sample set in order to construct robust and generic models for sample classification and discrimination.

PCA has clearly classified the 24 analysed ecstasy tablets into 8 groups, which corresponds to their post-tabletting features. PCA score plot has also shown distinction between Macedonian and Australian samples based on the relative abundance of the selected 16 components for profiling purpose. In a latter stage, several components characteristic either for Macedonian or Australian samples only have been detected, which makes differentiation between countries of seizure trivial.

GC×GC-TOFMS can be successfully applied to ecstasy impurity profiling. The main drawback of the method is its high investment cost. Peak integration, even facilitated by the compression effect of the modulator, can be an issue since more “slices” have to be integrated, properly identified, and combined in one total peak area. This is especially true for highly abundant components such as PMK, piperonal and PMK-OH. This makes the method more operator-dependent than the current GC-MS profiling methods. On the other hand, automation in data processing (detection, identification and integration) with low abundance components appears to work better than in GC-qMS.
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Chapter 8

8 APPLICATION OF COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY IN HEROIN AND COCAINE PROFILING
8.1 INTRODUCTION

Cocaine is a purely natural component derived from coca leaves (Erythroxylum coca). Its illegal production involves several consecutive extraction and purification steps, leaving the main component unchanged from its source to the final product. However, many naturally occurring components in coca leaves are co-extracted and so are present in the final product, giving rise to its total profile. Tropacocaine (TPC), ecgonine methyl ester (EME), cis- and trans-cinnamoylcocaine (CCC and TCC, respectively), cuscohygrine are the most frequently encountered in cocaine samples. Others i.e. hygrine, trimethoxycocaines, trimethoxy-tropacocaines, are present in minor amounts in coca leaves or are largely removed during the extraction process; they are less frequently found in cocaine samples [84, 85]. Special extraction methods such an ion-pairing and column fractionation [84] have been developed in order to effectively quantify the presence of natural impurities. Some of them i.e. truxillines show poor chromatographic behaviour so reduction to simpler molecules and their derivatization is required for their detection [75, 86].

The process of purification usually involves an oxidation step by potassium permanganate, where many by-products are generated. The bulk of them are intentionally removed, i.e. cis- and trans-cinnamoylcocaine, but many more are unintentionally formed during the poorly controlled oxidation step. N-norcocaine, N-benzoynorecgonine methyl ester and N-formylcocaine have been formed in over-oxidized cocaine samples [74]. Four diastereomeric of 2,3-dihydroxy-3-phenylpropionylecgonine methyl esters from incomplete oxidation of cis- and trans-cinnamoylcocaine have been detected in cocaine samples [78]. Due to their highly polar character, derivatization prior to GC analysis is required. Another group of by-products has been detected in Peruvian cocaine base samples purified by using ethanol instead of permanganate oxidation. Several ethyl homologues of natural tropane alkaloids have been detected in these samples due to the
Cocaine and heroin profiling by GC×GC

transesterification [77], after ion-pair chromatography fractionation and concentration. Finally, many hydrolysis products are formed during the production steps or during prolonged storage under inappropriate conditions. Hydrolysis usually occurs at the ester linkage so cocaine yields benzoylecgonine, ecgonine methyl ester, ecgonine and benzoic acid. Trace amounts of other hydrolysis products are formed from minor tropane alkaloids.

Heroin on the other hand is a semi-synthetic drug obtained by acetylation of morphine, a purely plant-derived alkaloid isolated from the latex of opium poppy (*Papaver somniferum*). Many naturally occurring alkaloids in opium poppy (codeine, thebaine, noscapine, papaverine etc.) are co-extracted with morphine and undergo acetylation giving many by-products. This is especially true for the raw processed morphine, where the variety in the impurities, their acetylation products and degradation by-products, and variety in their concentrations give rise to the total heroin profile. At least nine degradation products of thebaine [16] and eighteen of papaverine [17] have been detected when these components had been boiled in acetic anhydride. On the other hand, acetylation of highly purified morphine samples results in heroin which lacks these trace components, leading to simpler profiles.

The level of different manufacturing by-products and naturally occurring alkaloids in cocaine and heroin samples vary significantly, about 2-3 orders of magnitude [75]. The concentration range of the impurities as well as the variety in their chemistry require various methods for extraction, concentration, derivatization and detection. This is especially true for cocaine where many different cocaine profiling methodologies have been applied.

The generic approach to major component profiling of cocaine and heroin [27, 29, 38, 94, 140, 243] is based on sample dissolution, optional prior derivatization and injection into the GC port. Sample comparison is based on the normalized area of each of the selected analytes against the peak area of cocaine, the peak area of whole morphine (sum of
morphine, 6-monoacetyl morphine and diacetylmorphine), or the peak area ratio against an internal standard. The chromatograms do not appear complex because of the very few target components at relatively high concentrations in the profiles. Cocaine, CCC, TCC, tropacocaine, ecgonine, benzoylecgonine, EME are mainly used in cocaine profiling; meconine, acetyl codeine, 6-monoacetyl morphine (6-MAM), papaverine and noscapine are often used in heroin profiling.

A separate cocaine and heroin profiling method based on solvents occluded in their crystals have been applied as a complementary technique by using head space (HS) [47, 48, 93] or solid-phase microextraction (SPME) [92] sampling. The information content has been limited to several solvents detected in the samples, originating from the purification and base-to-salt conversion process applied. The most frequently encountered solvents are toluene, acetone, diethyl ether, methyl ethyl ketone and methylene chloride in cocaine samples [93] and acetic acid, acetone and diethyl ether in heroin samples [46, 47]. Additional methods for sample-to-sample comparison and geographic origin determination are based on isotope ratio [56, 58, 60, 62, 95] or trace metal contaminants [51-53, 55, 96].

Advanced heroin profiling methodology has been generally based on acidic and neutral organic impurity extraction [19], concentration and subsequent analysis on GC coupled to FID [19, 33] or MS detector [17, 34]. Sixteen impurities have been selected in the most comprehensive study aimed at harmonizing the method for retrospective comparison and data exchange between three well-equipped and experienced forensic laboratories [31]. Various limitations of the GC technique have been identified as the main reason for the poor inter-laboratory reproducibility. Recently, comprehensive two-dimensional gas chromatography has been applied in heroin and cannabis impurity profiling with pixel-based chemometric processing [190]. Even though only 9 out of 16 components reported in the literature have been detected in a relatively simple 2D profile, the group classification has matched the results found by well-established 1D GC impurity profiling methods and/or the forensic background of the samples. However, the applicability of GC×GC is poorly justified for
samples where no coelutions on 1D column have been observed and the problem could be solved by using well established 1D GC.

The most comprehensive cocaine profiling approach so far has been developed and used by the Special Testing and Research Laboratory (STRL) at the Drug Enforcement Administration (DEA) in USA. The approach is based on six independent cocaine profiling methods for sample comparison analyses, geographic origin studies and solvent determinations [142]. It combines different and separate preparation steps, from simple derivatization, through head space sampling of volatiles, to component reduction and column chromatography fractionation. Furthermore, different detection systems have been used for each class of the components (FID, ECD, MSD).

However, none of the profiling methods available offers high information content and simplicity at the same time. Either the methods are complex and time consuming but offering high amount of data, or they are simple but offering limited information content.

In this chapter an application of comprehensive two-dimensional gas chromatography as an alternative simple and reasonably high information content method for cocaine and heroin sample discrimination is presented. The method is based on SPME sampling of volatiles and semi-volatiles in cocaine and heroin samples, subsequent separation on a non-polar / polar column set and TOFMS detection. Even though many components currently used in profiling methods, especially those which require reduction and derivatization (truxillines), and higher molecular mass components (i.e. CCC, TCC, papaverine, noscapine, thebaine acetylation by-products) are not detected, the method offers a complex profile composed of many other sample constituents. Several potential markers have also been detected in highly purified heroin samples from SEA. To author’s best knowledge, this is the first ever application of GC×GC in cocaine profiling and the second one in heroin profiling, but this time focused on more complex profiles of heroin volatile and semi-volatile components.
8.2 EXPERIMENTAL

Gas chromatography and mass spectrometry conditions for profiling of volatiles in heroin and cocaine samples were generally the same (unless otherwise stated) as the conditions applied in ecstasy profiling in Chapter 7.

Analysis of impurities in heroin samples extracted by LLE was performed in 1D and GC×GC mode by using BPX5 / BPX50 and BPX50 / BPX5 column sets. For column dimension details see Experimental section for ecstasy profiling (Chapter 7). 1D GC experiments were carried out either on 1) a single column (qMS and accTOFMS) or 2) on coupled columns under the same conditions as in GC×GC, except the modulator was off and the acquisition rate of fastTOFMS and FID detectors was reduced to 20 Hz. Thus in this case the 1D GC system will comprise a long column directly coupled to a short column and whilst this can be termed a multi-chromatography system according to Hinshaw and Ettre [216] and discussed elsewhere [217], the second very short column is anticipated to lead to negligible variation in peak properties such as width. The applied temperature program [31] was from 160 °C to 320 °C at 6 °C min⁻¹ (hold 6 min) at a flow rate of 1.3 mL min⁻¹ (He). Splitless injection with a purge time of 1 min was applied.

Comparison between major component analysis of cocaine samples in 1D GC and GC×GC mode has been done on the same column set, under different oven temperature program [141]. The following conditions in 1D GC analysis were applied: isothermal temperature at 180 °C was kept for 1 min, then increased to 275 °C at 4 °C min⁻¹ rate, and finally hold at this temperature for 2.25 min. One µL of the MSTFA derivatized sample was injected in split mode at 20:1 split ratio.
8.2.1 Statistical analysis

Several pre-treatment methods on the raw data followed by PCA analysis have been tested in order to find the best statistical approach for separation of linked (subsamples) and non-linked (different seizures) samples. Applied pre-treatment methods are given in Table 8.1.

<table>
<thead>
<tr>
<th>Description</th>
<th>abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Normalization</td>
<td>N</td>
</tr>
<tr>
<td>2 Normalization + square root</td>
<td>N + SQR</td>
</tr>
<tr>
<td>3 Normalization + 4th square root</td>
<td>N + 4SQR</td>
</tr>
<tr>
<td>4 Logarithm</td>
<td>Log</td>
</tr>
<tr>
<td>5 Normalization + logarithm</td>
<td>N + Log</td>
</tr>
</tbody>
</table>

20 volatiles in 21 heroin and 23 volatiles in 18 cocaine samples have been selected for profiling purposes. The raw dataset of heroin/cocaine samples consisted of the abundance of the targeted components determined by GC×GC-TOFMS. Each sample was run in duplicate, unless otherwise stated. However, some components were absent in some samples (i.e. heroin samples seized in Australia) either because the actual components were absent from the samples, thus representing characteristic of the sample profile, or they were below the limit of detection of the instrument [94]. In such a case an arbitrary area has been assigned to the component, which corresponds to the concentration of that component at the limit of detection under the given conditions. Data were processed by using the Minitab 15 software package (Minitab Inc., State College, PA, USA).
8.3 RESULTS AND DISCUSSION

8.3.1 1D GC vs. GC×GC in major component analysis of cocaine and heroin

Based on author’s previous results and experience in illicit drug analysis, GC×GC was not expected to offer much advantage over 1D GC in term of major components analysis. The only advantage expected was seen in some increase of sensitivity for low abundant components. A comparison of GC×GC-FID and 1D GC-FID chromatograms of the same cocaine sample is presented in Figure 8.1.

Figure 8.1 Comparison of (A) GC×GC-FID and (B) GC-FID results for cocaine major component analysis. BPX5 has been used in 1D GC and BPX5 / BP20 column set in GC×GC format. Note the difference in the peak width of cocaine on 1D column.

Despite the two well separated pairs of coeluting components in 1D GC at 11 min and 19 min, there is not much improvement in the GC×GC 2D plot. In contrast, several components eluting immediately after the cocaine in 1D GC (Figure 8.1B) are overlapped by its huge and tailing spot on 1D column in GC×GC format (Figure 8.1A). Peak tailing on 1D of overloaded components in GC×GC is mainly contributed by the inefficiency of the
modulator to effectively remobilize the trapped (overloaded) portion onto the 2D column. The spot width on 1D can be minimized either by increasing the temperature of the modulator (to ease the remobilization), or by decreasing the modulation period (less component cryotrapped per cycle). However, both can significantly alter the overall 2D plot, either by inefficient trapping of other components (at higher $T_M$) or by increasing the chances of wraparund (shorter $P_M$). In general, the example confirmed the proposition that GC×GC has no advantages over 1D GC when dealing with simple component mixtures. Similar results for comparison between 1D GC and GC×GC analysis of main components have been obtained for heroin samples (data not shown).

### 8.3.2 1D GC vs. GC×GC in impurity profiling of heroin

Heroin organic impurities have been analysed on both, BPX5 / BPX50 and BPX50 / BPX5 column sets, the second one being similar to the column configuration used in [190]. The first column configuration gave good spread of components on 1D BPX5 column with relatively strong retention and almost no coelution revealed on 2D BPX50 column (Figure 8.2A). The retention on BPX50 is probably contributed by the high aromaticity content of the impurities. Despite the expected enhancement in sensitivity, there was not much improvement in separation compared to the well established 1D GC heroin profiling methods (Figure 8.2B).

Since the column choice for separation of high boiling point components in GC×GC is limited to the few high temperature limit columns, the logical step was to check the separation on BPX50 / BPX5 column configuration. As expected (Figure 8.3), most of the aromatic impurities have shown strong retention on the 1D BPX50 column, with some better spread of components on 2D BPX5 column, and few coelutions of minor components revealed on 1D BPX5 column. However, the strong tailing of the major polar components on 1D column, especially the latter eluting, has rejected this column set as an appropriate for heroin impurity profiling. The major benefit of using this column set, among the good
spread and peak shape of early eluting components, was the clear separation of straight chain hydrocarbons and siloxanes from other components (Figure 8.3). Less tailing on 1D and more on 2D column has been obtained in the results of Groger et al. [190].

Figure 8.2 Comparison of a heroin impurity profile in (A) GC×GC-FID with the profile in (B) GC-FID

Figure 8.3 A representative heroin impurity profile on BPX50 / BPX5 column set. Note the significant tailing of components on 2D column, and especially on 1D column for late eluting components. Siloxanes and n-alkanes homologues are denoted.

8.3.3 Cocaine and heroin volatile profiles in 1D GC

A sampling procedure similar to the one explained in the previous chapter on ecstasy profiling (see Chapter 7) has been applied to cocaine and heroin samples in order to check if their volatile and semi-volatile profiles are complex enough for profiling in GC×GC format. Generally, heavier molecules and consequently less volatile components were
expected in cocaine and heroin samples, so prolonged equilibration and absorption time was applied in the SPME sampling procedure. Chromatograms of three different cocaine samples analysed on GC-qMS are given in Figure 8.4. Reasonably complex profiles were obtained, showing many coelutions. However, only a small number of components were identified with a similarity above 800 (out of 999). The number of identified components was improved by applying the AMDIS32 (NIST), an automated deconvolution and identification software, but still the number was far below the number of components identified by GC×GC-TOFMS in a later stage. Similar results were obtained also for three different heroin samples. In author’s opinion, the profiles looked complex enough and promising for the next step - comprehensive two-dimensional gas chromatography.

Figure 8.4 Comparison of GC-qMS volatile profiles of three different cocaine samples: sample Ca (A), sample Cb (B) and sample Cc (C)
8.3.4 Separation of volatiles in cocaine and heroin samples on different column sets in GC×GC format

Comprehensive two-dimensional gas chromatography offers more choices in term of column selection over 1D GC because two-column combinations are in play in each system. As stated previously, the choice for higher molecular weight components is limited due to the temperature limitation of highly polar columns. On the other hand, the choice is much wider for volatiles and semi-volatile components eluting up to 260 °C. The separation of volatiles in cocaine and heroin samples has been tested on four different column sets: two non-polar (NP) – polar (P) and two P – NP. Different profiles for each column set have been obtained, each offering advantages and disadvantages over the other column sets.

8.3.4.1 BPX50 / BPX5

The first column set applied in cocaine volatiles separation was BPX50 / BPX5. Three different cocaine samples (proved to be different in a later stage) have shown different profiles, but the components were spread on a rather narrow band along the 1D column (Figure 8.5). It seems that the separation mechanism in BPX5 based on the weak H-bonding and moderate π-π* interaction is not enough to separate the cocaine constituents on 2D column. This makes the column set unsuitable for GC×GC profiles comparison since it does not offer much advantage over classical 1D GC separation.
Figure 8.5 Representative 2D plots of volatiles in cocaine samples Ca (A), Cb (B), Cc (C) and a blank (D) analysed by SPME on BPX50 / BPX5 column set.

The major feature of this column set was the clear separation of highly non-polar n-alkanes from the rest of the matrix, having the longest retention time on 2D column (BPX5). However, narrow peaks on 2D column have been obtained. Interestingly, very similar dispersion on 2D column has been obtained also for volatiles in heroin samples, which were spread rather in a narrow band along the 1D column. Similar profiles have been obtained for samples Ha and Hb and slightly different for sample Hc (Figure 8.6), even though all three samples proved to be different.
8.3.4.2 BP20 / DB-1

A BP20 / DB-1 column combination was used as a second P – NP column set to test the separation of volatiles in cocaine and heroin samples. Very few components in sample Cc (Figure 8.7) have been detected in contrast to the 2D plot of the same sample obtained on BPX50 / BPX5 column set (Figure 8.5C). Please note that the same contour level has been used in all FID 2D plots. However, good spread on 2D space and good peak shape have been obtained, excluding some components showing tailing on 1D column, (most probably the polar ones). Similar results have been obtained on 15 m FFAP as a 1D column, showing similar profiles; however at a shorter total runtime (due to the reduced 1D column length).
Figure 8.7 2D plot of volatiles in cocaine sample Cc analysed by SPME on BP20 / DB-1 column set

Chromatographic behaviour of volatiles in heroin on this column set (Figure 8.8) has been found similar to the cocaine volatiles, not because of their same identity, but simply because the spread over 2D space. Compared to separation on BPX50 / BPX5, smaller number of components has been detected; however they showed good peak shape and they were better spread on the 2D plot. It seems the separation mechanism of DB-1, based on weaker aromatic and hydrogen bonding but strong van der Waals interaction, shows higher discrimination of cocaine and heroin constituents on 2D column, thus better spread over the 2D space.

Figure 8.8 2D plot of volatiles in sample Ha analysed on BP20 / DB-1 column set

8.3.4.3 BPX5 / BPX50

The separation of volatiles in cocaine samples on NP – P column sets was initially conducted on the BPX5 / BPX50 column set (Figure 8.9A). While slightly increased peak signal for components was obtained when compared to BPX50 / BPX5 column set, and
significantly more components detected than in the case of BP20/DB-1, the components are rather located in a narrow band on the 2D plot along the 1D retention time, showing poor spread on 2D space. The “orthogonality” of this column set based on the different aromaticity content of the two phases (5% vs. 50%) does not work well for cocaine constituents. The poor spread on 2D plot is undesirable in GC×GC, which makes the column set unsuitable for analysis of volatiles since it does not offer much advantage over classical 1D GC separation. Similarly to the separation on BPX50/BPX5, good peak shape has been obtained. Again, similar results for spread of components on 2D plots have been obtained also for volatiles in heroin samples (Figure 8.9B).

Figure 8.9 2D plots of cocaine sample Cc (A) and heroin sample Ha (B) analysed by SPME on BPX5/BPX50 column set. Note that the same contour levels are used in all FID 2D plots.

8.3.4.4 BPX5/BP20

BPX5/BP20 was the second NP–P column set tested for volatiles in cocaine and heroin samples. It has shown better spread of components on the 2D space, as well as good peak shape. This column set revealed the highest number of separated volatiles in cocaine (Figure 8.10) and heroin (Figure 8.11) samples compared to the other column sets, offering complex sample profiles for easier (or reliable) sample discrimination. The “orthogonality” of this column set, based on strong van der Waals and weak aromatic interactions of BPX5
phase vs. strong H-bonding and weak π- and van der Waals bondings of the BP20 column, is more discriminative towards heroin and cocaine volatile constituents. A representative GC×GC-FID profile of volatiles and semi-volatiles in cocaine is shown in Figure 8.10 along with a 2D plot of a blank, where only a few siloxanes are detected as background components.

![Figure 8.10 Representative 2D plots of (A) cocaine sample Cc and (B) a blank, analysed by SPME on BPX5 / BP20 column set at 0 °C modulation temperature and 5 s modulation period.](image)

In contrast to heroin volatile profiles of samples Ha, Hb and Hc obtained on BPX50 / BPX5 column set (given in Figure 8.6), profiles on BPX5 / BP20 showed more individual components and could serve reliable sample comparison based on much more individually separated components. However, unambiguous identification of the components is necessary in order to avoid comparison of samples based on non-origin related markers. Unfortunately, a high number of reference components is required with GC×GC-FID to identify all components. A closer look at the heroin profiles in Figure 8.11 can distinguish sample Ha from sample Hb and the differences are easily noticeable at the circled regions. Because of this, all further experiments were conducted on this column set, including the application employing a flame photometric detector (FPD). Differentiation of these two samples on the other column set was almost impossible. The largest spot in
sample Hb at 16t of 20-23 min is due to the high abundance peak of caffeine, which in fact is not a good component for sample comparison.

![Representative 2D plots of heroin samples](image)

Figure 8.11 Representative 2D plots of heroin samples Ha (A), Hb (B) and Hc (C) analysed by SPME on BPX5 / BP20 column set at 0 °C modulation temperature and 5 s modulation period, along with a 2D plot of a blank (D), analysed under the same conditions. Note the difference in the Ha and Hb profiles at circled regions.

8.3.4.5 Heroin and cocaine profiling on BPX5 / BP20 with FPD detection

The findings for presence of S- and P-containing components in heroin and cocaine volatiles are similar to the findings for ecstasy profiling. Very few components at similar ratio have been detected on both S- and P-channels, which makes this highly selective and sensitive detector not suitable for profiling purposes.
8.3.5  Cocaine and heroin volatiles profiling by GC×GC-TOFMS

GC×GC-TOFMS separation and identification were conducted on the BPX5 / BP20 column set under the same conditions as in GC×GC-FID experiments, but with two differences: 1.4 m ²D column was replaced with 1 m long ²D column of the same type and helium was used as a carrier gas instead of hydrogen.

8.3.5.1  Identification and location of components in GC×GC-TOFMS

As expected, most of the higher molecular weight components usually used in cocaine and heroin profiling have not been detected in profiles by SPME sampling. The main reason, beside the low concentration of some of them, is the sampling technique. Some are not amenable for direct detection without prior reduction and/or derivatization (i.e. truxillines). Head space SPME is generally not efficient in sampling higher molecular weight components, components which show low vapour pressure and polar components on PDMS/DVB. However, GC×GC-TOFMS chromatograms of volatiles and semi-volatiles in four different cocaine samples (Figure 8.12) have revealed a large number of components, with some of them as potentially good markers for profiling purpose.

Good spread of the volatiles on this column set has also been obtained for heroin samples. Figure 8.13 shows different profiles of different heroin samples seized in Australia. It is well known that highly purified heroin samples from SEA (the majority of heroin samples seized in Australia come from this region) have simple organic impurity profiles making them less suited for sample-to-sample comparison by using current profiling 1D GC methods. However, volatile profiles of these samples are still of reasonable complexity and represent a good base for selecting many markers for sample discrimination. The huge peak at $t_{R}$ of 1350 s in Figure 8.13A and Figure 8.13C is due to the heavy sample adulteration with caffeine.
Cocaine and heroin profiling by GC×GC

Figure 8.12 Representative GC×GC-TOFMS 2D plots of cocaine samples Ca (A), Cb (B), Cc (C) and a cocaine sample seized in Australia (D), analysed by SPME on BPX5 / BP20 column set. Visually different profiles have been obtained for different samples.

In contrast to the previous methods for cocaine volatile profiling [93, 244], where components eluting up to acetophenone (RMM 120) have been detected and selected for profiling, in the present method components with a molecular weight up to 289 (norcocaine) have been detected. The list of components preliminarily selected for cocaine profiling purposes consisted of 23 components (Table 8.2) and the list of selected components for heroin profiling consisted of 20 components (Table 8.3).
Figure 8.13 Representative GC×GC-TOFMS 2D plots of heroin samples H6 (A), H7 (B), H8 (C) and H9 (D) seized in Australia, analysed by SPME on BPX5 / BP20 column set. Visually different profiles have been obtained for different samples.

Some of them are well known as constituents of cocaine (tropanone, ecygonidine ME, EME, tropacocaine and norcocaine) and heroin samples (meconine, hydroxycotarnine, acetyl codeine), some of them are structurally similar to cocaine-related (methyl benzoate, trimethoxybenzaldehyde, methyl- and ethyl cinnamate) and heroin- or opium-related components (cresol acetate isomers, 4-methoxy quinoline) and are related or possibly related to them, but many of them apparently have not been previously reported as cocaine (methyl nicotinate, 1-methyl pyrrole, 2,5-pyrrole-dione) or heroin sample constituents (1,2,4-trimethoxy benzene, 3,4- and 3,5-dimethoxy benzaldehyde, xanthene isomers) and therefore their origin is unknown. An extensive study on a larger number of
samples is required in order to establish a strong relation between these components and their origin. However, most of them are prevalent in all analysed cocaine samples, with few exceptions where some selected components are unique for Macedonian or Australian samples. The latter makes classification according to the country of seizure trivial.

Highly purified heroin samples from SEA (i.e. samples seized in Australia) lack many of the components selected for profiling making differentiation between SEA and SWA samples trivial. However, the lack of markers in these samples makes differentiation within a region difficult and probably an increase of extraction temperature and sample amount are a possible solution to reveal more components for reliable sample discrimination.

The list of components for cocaine profiling can be further extended with components frequently encountered in cocaine samples but originating from solvents (or impurities in solvents) used in cocaine extraction and purification. Toluene, xylene isomers, ethyl benzene, trimethylbenzenes, straight-chain hydrocarbons, benzyl alcohol, benzoic acid etc. are detected in many cocaine samples, but they are not taken into consideration. In the same manner, acetic acid, toluene, n-alkanes, xylene isomers, phenyl acetate, benzaldehyde etc. are detected in heroin samples but not taken for profiling purpose.

The lowest boiling point solvents such as diethyl ether, acetone, methanol, ethanol, methylene chloride etc. are not determined because of their poor trapping at the operating temperature of the modulator (-30 °C to 0 °C). Formation of some degradation products due to the long equilibration and absorption time (80 min in total), even though unlikely, is not excluded. However, such a confirmation requires an extensive research undertaking.

A number of pyridine and quinoline derivatives have been detected in some cocaine samples from Macedonia only, which probably represent a unique profile of samples originated from that batch or particular supply chain. Two pesticides have been detected at different levels in some drug samples. γ-lindane has been detected in some cocaine
samples from Macedonia and in one sample from Australia, and trifluraline has been detected in all heroin samples from Macedonia and in one sample from Australia.

Table 8.2 Selected 23 components for cocaine profiling with their retention times, ions used for area calculation and similarity against NIST05 and Wiley7 MS libraries

<table>
<thead>
<tr>
<th>Component</th>
<th>( t_R^1 ) (s)</th>
<th>( t_R^2 ) (s)</th>
<th>similarity ( ^a )</th>
<th>quant. ion (m/z)</th>
<th>RMM ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1-methyl pyrrole</td>
<td>340</td>
<td>1.64</td>
<td>946</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>2 phenyl methyl ketone</td>
<td>700</td>
<td>2.04</td>
<td>961</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>3 methyl benzoate</td>
<td>725</td>
<td>1.76</td>
<td>938</td>
<td>136</td>
<td>136</td>
</tr>
<tr>
<td>4 2,5-pyrrole-dione</td>
<td>750</td>
<td>3.22</td>
<td>947</td>
<td>113</td>
<td>113</td>
</tr>
<tr>
<td>5 methyl nicotinate</td>
<td>775</td>
<td>2.12</td>
<td>943</td>
<td>137</td>
<td>137</td>
</tr>
<tr>
<td>6 1-phenyl-1,2-propanedione</td>
<td>800</td>
<td>2.06</td>
<td>952</td>
<td>105</td>
<td>148</td>
</tr>
<tr>
<td>7 tropanone</td>
<td>835</td>
<td>1.92</td>
<td>905</td>
<td>139</td>
<td>139</td>
</tr>
<tr>
<td>8 6-caprolactam</td>
<td>905</td>
<td>3.34</td>
<td>940</td>
<td>113</td>
<td>113</td>
</tr>
<tr>
<td>9 ( \alpha )-oxo-benzeneacetic acid methyl ester</td>
<td>915</td>
<td>2.40</td>
<td>966</td>
<td>105</td>
<td>164</td>
</tr>
<tr>
<td>10 2-chloroacetophenone</td>
<td>915</td>
<td>2.66</td>
<td>956</td>
<td>105</td>
<td>154</td>
</tr>
<tr>
<td>11 ecgonidine methyl ester</td>
<td>1010</td>
<td>1.56</td>
<td>809</td>
<td>181</td>
<td>181</td>
</tr>
<tr>
<td>12 ecgonine methyl ester</td>
<td>1085</td>
<td>1.80</td>
<td>899</td>
<td>199</td>
<td>199</td>
</tr>
<tr>
<td>13 4-ethoxybenzoic acid ethyl ester</td>
<td>1100</td>
<td>1.68</td>
<td>930</td>
<td>194</td>
<td>194</td>
</tr>
<tr>
<td>14 Unknown-1</td>
<td>1110</td>
<td>2.18</td>
<td>-</td>
<td>199</td>
<td>-</td>
</tr>
<tr>
<td>15 3,4,5-trimethoxybenzaldehyde</td>
<td>1165</td>
<td>2.38</td>
<td>925</td>
<td>196</td>
<td>196</td>
</tr>
<tr>
<td>16 phenyl benzoate</td>
<td>1210</td>
<td>1.64</td>
<td>972</td>
<td>105</td>
<td>198</td>
</tr>
<tr>
<td>17 2-ethylhexyl benzoate</td>
<td>1260</td>
<td>1.02</td>
<td>888</td>
<td>105</td>
<td>234</td>
</tr>
<tr>
<td>18 (4-chlorophenyl)phenyl methanone</td>
<td>1355</td>
<td>1.56</td>
<td>907</td>
<td>216</td>
<td>216</td>
</tr>
<tr>
<td>19 tropacocaine</td>
<td>1430</td>
<td>1.76</td>
<td>909</td>
<td>245</td>
<td>245</td>
</tr>
<tr>
<td>20 Unknown-2</td>
<td>1445</td>
<td>1.30</td>
<td>-</td>
<td>182</td>
<td>-</td>
</tr>
<tr>
<td>21 1,3-diphenyl-1,3-propanedione</td>
<td>1505</td>
<td>3.42</td>
<td>904</td>
<td>223</td>
<td>224</td>
</tr>
<tr>
<td>22 norcocaine</td>
<td>1600</td>
<td>3.52</td>
<td>928</td>
<td>168</td>
<td>289</td>
</tr>
<tr>
<td>23 Unknown-3</td>
<td>1755</td>
<td>2.96</td>
<td>-</td>
<td>105</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Calculated from duplicate analysis obtained from samples where the component has been detected at S/N of 100 or above.

\(^b\)RMM: relative molecular mass.
The trace amount of pesticides could be good batch markers if they originate from the coca and opium crops treatment. Their presence in the samples (only when present in higher amount) has been confirmed by GC-accTOFMS with an average mass accuracy of less than 5 ppm. It is worth to mention that GC-accTOFMS was not able to detect and confirm the presence of pesticides in samples with lower abundance even though they were detected and tentatively identified by using GC×GC-TOFMS. The sample carry-over was excluded as an option for this discrepancy, since none of the pesticides were detected in the blanks.

Table 8.3 Selected 17 components for heroin profiling with their retention times, ions used for area calculation and similarity against NIST05 and Wiley7 MS libraries

<table>
<thead>
<tr>
<th>Name</th>
<th>$t_R^1$ (s)</th>
<th>$t_R^2$ (s)</th>
<th>similarity</th>
<th>quant. ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 4-cresol acetate</td>
<td>770</td>
<td>1.76</td>
<td>940</td>
<td>150</td>
</tr>
<tr>
<td>2 3-cresol acetate</td>
<td>805</td>
<td>1.76</td>
<td>905</td>
<td>150</td>
</tr>
<tr>
<td>3 naphthalene</td>
<td>825</td>
<td>1.74</td>
<td>940</td>
<td>128</td>
</tr>
<tr>
<td>4 Unknown 129 isomer1</td>
<td>905</td>
<td>2.12</td>
<td>-</td>
<td>129</td>
</tr>
<tr>
<td>5 Unknown 129 isomer2</td>
<td>930</td>
<td>2.74</td>
<td>-</td>
<td>129</td>
</tr>
<tr>
<td>6 1,2,4-trimethoxybenzene</td>
<td>975</td>
<td>2.02</td>
<td>945</td>
<td>168</td>
</tr>
<tr>
<td>7 3,5-dimethoxybenzaldehyde</td>
<td>1060</td>
<td>2.18</td>
<td>920</td>
<td>166</td>
</tr>
<tr>
<td>8 3,4-dimethoxybenzaldehyde</td>
<td>1090</td>
<td>2.44</td>
<td>925</td>
<td>166</td>
</tr>
<tr>
<td>9 4-methoxyquinoline</td>
<td>1135</td>
<td>2.34</td>
<td>850</td>
<td>159</td>
</tr>
<tr>
<td>10 3,4-dimethoxybenzyl acetate</td>
<td>1165</td>
<td>2.14</td>
<td>835</td>
<td>210</td>
</tr>
<tr>
<td>11 xanthene isomer 1</td>
<td>1195</td>
<td>1.64</td>
<td>-</td>
<td>181</td>
</tr>
<tr>
<td>12 xanthene isomer 2</td>
<td>1205</td>
<td>1.74</td>
<td>-</td>
<td>181</td>
</tr>
<tr>
<td>13 xanthene isomer 3</td>
<td>1215</td>
<td>1.76</td>
<td>-</td>
<td>181</td>
</tr>
<tr>
<td>14 Unknown 197 isomer 1</td>
<td>1210</td>
<td>1.20</td>
<td>-</td>
<td>197</td>
</tr>
<tr>
<td>15 Unknown 197 isomer 2</td>
<td>1215</td>
<td>1.26</td>
<td>-</td>
<td>197</td>
</tr>
<tr>
<td>16 Unknown 197 isomer 3</td>
<td>1250</td>
<td>1.32</td>
<td>-</td>
<td>197</td>
</tr>
<tr>
<td>17 meconine</td>
<td>1275</td>
<td>3.78</td>
<td>885</td>
<td>194</td>
</tr>
<tr>
<td>18 hydrocotarnine</td>
<td>1320</td>
<td>1.92</td>
<td>840</td>
<td>220</td>
</tr>
<tr>
<td>19 acetyl codeine</td>
<td>1395</td>
<td>3.44</td>
<td>830</td>
<td>341</td>
</tr>
<tr>
<td>20 trifluraline</td>
<td>1200</td>
<td>1.26</td>
<td>810</td>
<td>306</td>
</tr>
</tbody>
</table>
Once the full mass spectra of the components of the matrix are retained, cutting agents and other non-target components can be easily included in the list if such is needed. On average 400 components out of 1400-1700 detected in total have been tentatively identified in the samples with MS similarity above 800 and S/N threshold of 10. Although several tens are background components (mainly siloxanes from the SPME fibre and column phase bleeding) the majority are still cocaine and heroin related components.

The large number of components detected in heroin and cocaine volatile profiles in this experiment is mainly contributed by three factors:

i. The extraction and concentration capabilities of SPME sampling method, where the whole sorbed extract to the SPME fibre is introduced directly into the GC system. In contrast, in liquid injection methods only small part of the extract is injected (usually 1 µL out of 100-500 µL).

ii. The compression effect of the modulator, where the whole effluent in the time interval of 5-6 s has been condensed and released to generate sharp bands (~200 ms) on the 2D column.

iii. The separation of the complex profiles based on two different mechanisms results in increased number of components resolved and detected as unique compounds.

While the first factor is purely due to the sample preparation step, the last two are exclusively contributed by the comprehensive two-dimensional gas chromatography. Full mass spectral data recorded at high acquisition rate, combined with the automated deconvolution capabilities of ChromaTOF software, have also contributed to the total number of detected components.

One of the drawbacks in GC×GC-TOFMS is the “artificial” increase in the number of detected components due to the modulation process. Namely, each “slice” of the same
component is first deconvoluted, detected, integrated and identified, and then all the slices with MS similarity above the pre-defined value are combined as a unique component with common retention times on 1D and 2D columns, area and MS identity for all combined slices. In cases where the MS similarity between different slices is below the pre-defined value, or the difference in their retention times on the 2D column is above the tolerance window, each different slice is identified as a different component, which “artificially” increases the number of detected unique components, which then requires manual adjustment. Some of the reasons for this include inability to accommodate proper S/N and peak width thresholds in samples with large variability of component abundance, multiple slicing of high abundance components, MS similarity threshold too high, high fluctuation in the modulation temperature etc. However, this can be minimized with proper tuning of the data processing method or can be completely eliminated by manual inspection and correction of the results. Approximately several tens of components are “artificially” added to the real number of unique components in both heroin and cocaine volatile profiles.

While the number of components detected in Macedonian samples in GC×GC format was around 2000 (several tens were replicates and background components), the number of components in the corresponding samples in 1D GC mode was 3-4 times less. Some of the lower abundance selected components for profiling of heroin and cocaine samples have not been detected in 1D GC mode analysis of the same samples. Six out of the 20 selected heroin volatiles (1, 4, 7, 11, 13 and 19 in Table 8.2) and eight out of the 23 selected cocaine volatiles (1, 5, 7, 15, 19-22 in Table 8.3) have not been detected or identified with a minimum MS similarity of 800 in corresponding samples analysed on GC-fastTOFMS. The two main reasons are the lack of the compression effect of the modulator and the lack of the second separation mechanism in 1D GC, which reduces the sensitivity and leaves the eventual coelutions on 1D column unresolved. Reduced acquisition rate of TOFMS in 1D GC mode (20 Hz) is another reason for worse deconvolution and identification of lower abundance components in complex matrixes.
GC×GC-TOFMS offers unprecedented information content for volatiles and semi-volatiles in cocaine and heroin samples. The high match quality of TOFMS spectra against commercial MS databases (NIST05 and Wiley7) agreed with previous conclusions [186, 188] that match quality decreases as molecular weight and thus mass fragments in the mass spectra increases. The general trend of similarity decreasing as molecular weight increases ($R^2 = 0.60$), as calculated for the components given in Table 8.2, is shown in Figure 8.14. The average similarity of selected cocaine profiling components (excluding two outliers) was 925 and the average similarity for the identified heroin components was 880. The lower average similarity for heroin components is probably contributed by their higher average molecular mass (data not given) when compared to cocaine components. The high similarity of TOFMS spectra to the commercial library entries excludes the need of a TOFMS dedicated in-house library, one which was necessary in the studies on sterols and anabolic agents [186, 188, 236].

![Figure 8.14](image-url)  
Figure 8.14 The average similarity of TOFMS spectra of selected volatiles in cocaine samples against NIST05 and Wiley7, versus their molecular weight.
8.3.6 PCA discrimination and classification of samples

The best PCA discrimination of different heroin samples (non-linked) and the best grouping of the subsamples to the corresponding samples (linked) was obtained by applying the N + SQR pre-treatment (Table 8.1). Four different groups were obtained from Macedonian heroin samples (including the blend sample), and five groups from Australian heroin samples. Although all 9 Australian samples were (presumably) originated from SEA region, 5 groups have been observed. The five Australian samples in the same group (H1-H5, Figure 8.15) were samples from the same seizure, and the rest of the samples were discriminated between each. The PCA result for heroin samples is given in Figure 8.15. Similar differentiation into five groups has been obtained also by using Australian samples only, and by selecting components which were present in at least one of the samples.

Figure 8.15. Principal component analysis of 21 heroin samples based on the selected volatiles (samples Hb and Hc in triplicate). N + SQR pre-treatment was applied on raw data. The first two components account for 66% of the variability.
When blend samples (B1-B3) were plotted against the original samples only (Ha, Hb and Hc), the PCA score plot showed they are located at the gravity center of the three clusters (Figure 8.16). It was expected since the variability set of the blend samples is composed of the variables from the three original samples used to make the blends. This observation confirms the reliability of the analysis and strengthen the confidence in the selected set of variables (the components selected for profiling).

![Figure 8.16 The location of blend samples B1-B3 on the PCA score plot when plotted against the original samples only](image)

Similarly to the statistical analysis applied on heroin data, the same approach has been applied also for discrimination of cocaine samples. The best sample discrimination has been obtained by applying the Log pre-treatment on the raw data. PCA has clearly distinguished the three Macedonian samples (Ca, Cb and Cc) while all subsamples (Ca1, Ca2, Ca3, Cb1 etc.) have been well grouped into their corresponding sample origin (Ca, Cb etc.). Six Australian cocaine samples originated from the same seizure (C1-C6) have been classified in one group according to the PCA analysis of their volatile profiles. The rest of the Australian samples represent separate groups. The score plot of all selected volatiles in
18 cocaine samples is shown in Figure 8.17. The first two principal components account for 71% of the variation, slightly higher than in the case of heroin.

Figure 8.17. Principal component analysis of 18 cocaine samples based on the selected volatiles (each sample in replicate). Log pre-treatment was applied on raw data. The first two components account for 71% of the variation.

8.4 CONCLUSIONS

The method presented here represents a compromise between the complex and time consuming profiling methods offering high information content and much simpler methods based on major components profiling which offer limited information content. Even though the method lacks some components usually used in profiling of cocaine and heroin, mainly higher molecular mass components, the method offers clear and trivial discrimination between samples from different countries, and more important – discrimination between samples from the same country (region). This is especially valuable for highly purified heroin samples usually originating from SEA region, where the low abundance of impurities in the profiles prevents their discrimination using 1D GC methods.
Volatile and semi-volatile component profiles are complex, containing a great number of compounds for reliable comparison. However, the number of resolved components is strongly related to the column configuration applied for separation, and BPX5 / BP20 has been shown to offer maximum spread on the 2D plots for both, heroin and cocaine volatiles. It seems the orthogonal separation mechanisms of BPX5 phase based on moderate $\pi$-$\pi^*$ interaction and BP20 phase based on strong hydrogen bonding are best suited for volatiles in cocaine and heroin samples. This gives different profile patterns of samples with maximum number of individually separated components, which in turn offers most reliable sample comparison and differentiation.

The lists of cocaine and heroin selected components for profiling can be further extended with new components from profiles once a good relationship is established between them and their origin in the samples. Solvents used in the preparation of drugs, as well as their impurities, are good markers for sample-to-sample comparison and can be easily added to the list. Exceptions are lowest boiling point solvents such as diethyl ether, ethanol, methanol, acetone etc. which are not amenable for proper modulation in GC×GC format at the operating temperature of LMCS.

Although heroin samples originated from SEA region usually exhibit poor impurity profiles, around 1000 components have been detected in GC×GC-TOFMS at S/N threshold of 10 in samples from Australia which presumably come from the same region. On the other hand approximately double the peaks in SEA samples have been detected in samples from Macedonia, which presumably come from SWA region. In any case the number of components detected in GC×GC format is much higher than in any current profiling method based on 1D GC separation. The success of this study can be attributed to the improved separation based on two “orthogonal” separation mechanisms, to the enhanced sensitivity due to the compression effect of the modulator, and to the powerful deconvolution capabilities of the LECO ChromaTOF software facilitated by the high acquisition rate.
PCA followed by Log pre-treatment of raw data for cocaine and N + SQR pre-treatment of raw data for heroin samples has shown best discrimination between different samples (non-linked) and good grouping of all subsamples to the corresponding samples (linked). While discrimination between heroin samples from different countries and presumably from different production regions is trivial even with the current 1D GC profiling methods, the differentiation between samples within regions is greatly facilitated by the large number of peaks detected and selected for profiling purpose when GC×GC is employed.

The full mass spectral data of non-targets and matrix components, retained due to the TOFMS detector capabilities, permit many more components to be tested and used as markers. Some of them could be very unique for particular group of samples or geographic origin determination if strong relationships are established. Pesticides used in crop treatment could be potentially good geographic origin markers once strong relationships are established between their presence in samples and known regions of their specific use.
CHAPTER 9

9 CONCLUSIONS AND FUTURE WORK
The infamous Marion Jones had never been caught by anti-doping authorities, not because she was “negative”, nor had she avoided the frequent testing that all elite athletes must submit themselves to, but simply because of the “transparency” of the screening methods applied at that time – the inadequacy of the analytical method! This thesis proposes a new approach for screening in doping control, which overcomes the “transparency” of the current screening methods; by having significantly more separation performance, then full scan MS can be applied to better characterise separated components. The work in this area, presented in this thesis, is far from complete, but it offers a fertile ground for further improvement and tightening the screw on sports-drug cheating. There is a long way to go through performance testing on larger numbers of real samples, and further validation against current and hopefully new established criteria in GC×GC format in the future. Hopefully, the scope of the work presented here, will direct sports drug analysts along the path that has been illuminated by this study.

Mass selective detectors are well established in analysis of complex matrixes, and very often is the only way to identify minor amount of analytes in presence of high abundance background components. However, as lower detection is mandated, mass interference becomes prominent due to matrix coelutions. Selected ion monitoring (SIM) can significantly increase the sensitivity but at the price of lower net information content. Further sample separation without losing the full mass spectral capability is highly desirable in analysis of such a complex matrix. Comprehensive two-dimensional GC coupled to fast time-of-flight MS has been proved advantageous over classical GC in doping control. As a result, the minimum acceptable match (MAM) has been proposed as an additional criterion when defining limit of detection. The new criterion proposes that the LOD be taken as the lowest concentration of analyte which gives S/N of a pre-defined value or greater (the same is in current methods), plus a further metric of MS similarity of the measured signal equal to or above a pre-defined value. The metric slightly compromises the LOD but it greatly improves the reliability of the detection and identification, at the lowest concentrations.
The criterion has been successfully applied in endogenous sterol analysis, and in the proposed GC×GC-TOFMS method for analysis of anabolic agents. The ultimate result reported in this thesis is the detection of the free fraction of estrone at a concentration of 0.1 ng mL⁻¹ spiked in blank urine, with a MS similarity of 94%.

Furthermore, full mass spectral information of non-targets and the matrix are retained and can be used for future retro-searching – re-examining the data alone in the light of newly identified compounds, to identify if a doping offence has been committed. The biggest issue identified in this study was the poor similarity of TOFMS spectra to the entries in the commercial MS libraries. The bias of the TOFMS detector towards higher masses has been identified as the cause of the problem. Further work needs to be done in order to find the law (if there is) which relates TOFMS and quadrupole MS sensitivity across the mass range. In that case the reliability of searching of “altered” TOFMS spectra against well established and huge commercial MS libraries (mainly based on quadrupole MS spectra), will be enhanced. Note that this appears to be a limitation of the LECO Pegasus system, since TOFMS should ideally be able to generate an unbiased spectrum.

GC×GC-TOFMS has been proven advantageous over one dimensional GC when coupled to the same detector in analysis of anabolic agents in urine. More anabolic agents, with higher similarity, have been detected in GC×GC format. The bias of TOFMS towards higher masses has been addressed again. Another field of further research in this area can be coupling of GC×GC with another type of TOFMS, namely accurate mass TOFMS. Preliminary results have shown this detector lacks the bias towards higher masses reported above, which makes it a perfect detector for minor amount of anabolic agents in such a complex matrix as urine. Furthermore, accurate mass will give another dimension in mass selectivity which will further increase reliability of the method. However, the acquisition rate of this detector will need to be improved from the currently 20 Hz to at least 50 Hz, in order to be applied in GC×GC format. Knowing the development of technology, it seems it is just a matter of time.
The proposed GC×GC-TOFMS method has been evaluated against strict WADA criteria for identification and minimum required performance limits. It has been shown that proposed methods largely comply with the criteria. The lack of at least three diagnostic ions with abundance above 5% has been compensated by the high MS similarity. Among the MAM criterion, an additional criterion has been proposed, namely second dimension retention time tolerance window. It adds weight to the total reliability of the method, and it seems the present criterion for retention time tolerance window defined by WADA can be freely applied also for the second dimension retention time in GC×GC format. A full mass spectrum of “everything” which elutes from the columns is retained and can be used for retro-search for e.g. new designer steroids and new drugs in the future. It is also now of interest to apply the method more generally to all drugs of relevance to sports drug testing.

The orthogonal separation of impurities in ecstasy samples combined with enhanced signal due to the modulation process, plus full mass spectra of the matrix has been shown advantageous over current ecstasy profiling methods. A large number of components have been revealed in profiles when components have been separated on the best (preferred) column set (BPX5 / BP20), many of them can be used as potential markers for synthesis route identification or sample differentiation. Piperonyl methanol, a low abundance, a polar and coeluting component with other background components (which share some diagnostic ions), has been shown to be one of the most discriminative components in ecstasy profiling. Proper identification and integration of low abundance components which show tailing in classical GC can be very difficult. 24 ecstasy tablets have been correctly classified in 8 groups according to their post-tabletting characteristics. This approach can now be validated for a much wider range of ecstasy samples, covering as wide a sample set as possible, of different synthetic route, and other relevant factors, to appreciate the power of the GC×GC method for profiling.
The full mass spectral data of targets, non-targets and matrix components detected in illicit drugs permit many more compounds to be tested and used as markers. Although heroin samples originated from SEA region usually exhibit poor impurity profiles, around 1000 volatile components have been detected in heroin samples from Australia (which presumably come from the same region) by using GC×GC-TOFMS. Some of them could be very unique for a particular group of samples or geographic origin if strong relationships are established.

PCA followed by Log pre-treatment for cocaine and N + SQR pre-treatment of the raw data for heroin samples has shown best discrimination between non-linked samples and good grouping of linked samples. While discrimination between heroin samples from different countries and presumably from different production regions is trivial even with the current 1D GC profiling methods, the differentiation between samples within regions is greatly facilitated by the large number of peaks detected and selected for profiling purpose when GC×GC is employed.

By virtue of the improved separation and enhanced sensitivity of GC×GC a high number of components have been detected in the profiles, which in turn requires further research on establishing links between their presence and their origin in drugs to be undertaken. Once a strong link is established, they can be used as new markers for particular geographic origin of plant materials used as precursor for drug production, for specific route of production of synthetic drugs, or for establishing routes of trafficking. Another advantage is that the same method conditions can be used for profiling of both heroin and cocaine volatiles. In currently employed methods, the conditions for heroin and cocaine profiling are different.

This study is not the first to investigate the application of GC×GC to drugs or to sterols in general, but it is differentiated by the novel methods, proposals, and investigations that have been instituted. The study using WADA key anabolic agents sought to apply established WADA criteria to the GC×GC results, and in doing so bring some measure of
knowledge to the ‘harmonisation’ of this area where GC×GC could be a critical new testing tool. This is done through demonstration of equivalency of the data so obtained.

Profiling should be the most logical use of the GC×GC method, and its inherent 2D data presentation format. That this resembles a two-dimensional picture of the sample, where the brush now paints a picture of the unique chemical properties of compounds within the axes, offers considerable molecular knowledge to a sample’s composition. This distinctive pattern can be likened to a fingerprint, to compare samples across a related sample set. Whilst strategies have been proposed for sample-to-sample comparison, it is not clear that the ‘simplicity’ or robustness of fingerprint comparison has been established yet. The activity in GC×GC will continue to advance the technique. Different – and new – MS methods, different column geometries, alternative modulation methods... The field will continue to provide the GC community with further advanced methods into the foreseeable future, and forensic and law enforcement community will benefit as well. It is hoped that this thesis will be recognised as playing its part in the total literature and science of comprehensive two-dimensional gas chromatography.
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25 References


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