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Innovative solutions for enhanced illicit drugs profiling using comprehensive two-dimensional gas chromatography and mass spectrometry technologies

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Acronyms

1D	one dimensional
1tR	first dimension retention
2D	two dimensional
2tR	second dimension retention
AAs	anabolic agents
AAS	anabolic androgenic steroids
fastTOFMS	fast acquisition TOFMS
FID	flame ionisation detector
FPD	flame photometric detector
GC	gas chromatography
GC-accTOFMS	GC-accurate time-of-flight mass spectrometry
GC-MS	gas chromatography-mass spectrometry
GCxGC	comprehensive two-dimensional gas chromatography
HCA	hierarchical cluster analysis
LDA	linear discriminant analysis
L/LE	liquid/liquid extraction
LMCS	longitudinal modulation cryogenic system
MAM	minimum acceptable matching
MDGC	multidimensional gas chromatography
MDMA	3,4-methylenedioxy-methamphetamine
MS	mass spectrometry
NIST	National Institute of Standards and Technology
PCA	principal component analysis
post-TB	post-tabletting
qMS	quadrupole MS
RSD	relative standard deviation
SIM	selected ion monitoring
S/N	signal-to-noise
SPME	solid phase microextraction
TMS	trimethylsilyl
TOFMS	time-of-flight mass spectrometry
WADA	World Anti-Doping Agency

Summary of Final Report

Aim

The aim of this project is to determine whether comprehensive two-dimensional (2D) gas chromatography (GC×GC—the new technology developed at RMIT University) is suitable for routine use in a forensic laboratory for profiling illicit substances.

Format

This report is divided into three main parts described in detail in Chapters 1, 2 and 3, as well as a Background and Conclusion.

Chapter 1

Evaluation of world anti-doping agency criteria for anabolic agent analysis by using comprehensive two-dimensional gas chromatography/mass spectrometry

Steroid analysis by GCxGC was used as a model study to determine whether the method would comply with the strict and demanding analytical protocol for substances tested in athletes as determined by the World Anti-Doping Agency (WADA). The study at RMIT was one that was therefore subject to levels of scrutiny to ensure that it met the demands of steroid testing in a number of national laboratories around the world. The final result was four published papers and a new grant for continued study of different classes of WADA-controlled substances.

Chapter 2

Organic impurity profiling of ecstasy tablets using GCxGC

GCxGC analysis of illicit substances that are of concern to police jurisdictions such as ecstasy, heroin and cocaine, was carried out. This Chapter focuses on ecstasy.

Chapter 3

Application of GCxGC in heroin and cocaine profiling

GCxGC analysis of heroin and cocaine as an alternative to current profiling methods is presented in this Chapter. GCxGC analysis offers a relatively simple, yet highly informative method for cocaine and heroin sample discrimination. This results in a complex profile that includes many other sample constituents that give additional information. Furthermore, several potential makers have also been detected in highly purified samples. This report describes the first ever application of GCxGC in cocaine profiling and the second one in heroin profiling to date.

Background

GCxGC

GCxGC analysis can separate and detect very low levels of complex, volatile substances such as drugs of abuse. The new methods are based on comprehensive GC×GC, whereby a 2D presentation of volatile components in a sample is obtained that provides much greater separation power for the composition of the sample. This is achieved by using a two-column gas chromatography method, with a device called a modulator, located between the columns such that each component is subjected to continuous separation on the two columns, and separate and independent retention properties on each column are attained. The result is a 2D presentation of chemical constituents, much like a contour plot of each compound in 2D space.

SPME

The sample itself may consist of a complex biological matrix from which the target compound must be extracted from samples using a reproducible, highly accurate and specific method such as solid phase microextraction (SPME).

GCxGC–SPME analysis

Therefore, not only is greater separation achieved using high powered GCxGC, but also improved detection of compounds is realised. This significantly aids the process of visualising peaks in two dimensions, enables compounds to be 'targeted' and allows many more minor peaks to be located in a sample. These minor peaks now detectable using GCxGC may include 'marker' compounds/metabolites useful for identification and which provide further invaluable information such as sample origin/history, process of manufacture, common features that can link samples etc.

Results and detailed analysis

This is described in Chapters 1–3.

Overall conclusions and recommendations

- GC×GC does provide the necessary improved separation power to isolate many target steroid compounds for a complex urine matrix. The same can be expected for illicit drugs and studies to date suggest this to be true.
- SPME is a suitable technology for headspace analysis of organic components contained in illicit drugs.
- The reproducibility of the GC×GC method is adequate for supervised analysis of component identity (and in this respect, is similar to that of GC analysis); however, for unsupervised analysis as required for some chemometric methods, advanced data treatment methods such as alignment of data may be required (as is also the case for GC). Alignment in GC is easier than in GC×GC methods.
- Software is an issue for routine application of GC×GC methods and work is currently being carried out on this at RMIT University.
- If mass spectrometry detection is required for GC×GC separations, the LECO Pegasus system is the one that is used most often and has been used in the present research project at RMIT University. We have experience in use of quadrupole MS (qMS) with GC×GC (note that qMS is often believed to be not capable of sufficiently fast data acquisition for the very fast peaks produced by GC×GC).

- The quality of library matches with the LECO ToFMS has necessitated the creation of our own in-house libraries for steroid compounds. This is suitable where the known steroids are available, but for general analysis of many compounds, this may present a challenge. Note that the LECO ToFMS appears to bias against higher mass spectrometry ions, but if the illicit drug profiling is limited to lower molar mass compounds, the National Institute of Standards and Technology (NIST) library may be adequate. Some literature reports suggest this to be true.
- A recent paper (see *Appendix*, ref 7) has tested the criteria for detection and identification of steroids set by WADA and has found that for the most part, these mandatory steroids to be detected can be suitably analysed by using GC×GC-ToFMS. An advantage is that full mass spectral data acquisition is available and so a more complete interpretation of all matrix compounds can be achieved, compared with the usual methods in WADA laboratories that rely on mass spectrometry operated in selected ion monitoring mode.
- Considerable differences have been found in the analysis of ecstasy, heroin and cocaine by using our SPME-GC×GC technology and while one dimensional (1D) GC will also have recognised differences, we will now be applying data interpretation methods to gauge and evaluate the relative advantages of having much greater separation for the matrix.
- The turnaround time for analysis of drug-capture samples depends upon the availability of suitable sampling equipment for automated SPME analysis (at present, we have used manual SPME). This will usually require a CombiPAL autosampler system. The GC×GC system requires about 40 minutes per sample; however, data analysis is a more time-consuming process.
- Note that for profiling, as opposed to simply measuring the presence of the active drug in a sample, this requires more substantial efforts on a chemometric method to compare and contrast samples against a library or database of samples analysed in the same manner. It is hoped that automated methods for such processing may become available in future arising from other work at RMIT, but this will probably need to be deferred to a future project.

Most recent work

In addition to the studies reported in mid-2009, the most recent work we have completed or proposed includes:

- the need to establish the long-term reproducibility of the GC×GC method for the different phase columns and geometries used in the experiment. An alternative will be to develop a metric for peak position correction in the 2D experiment based on a proposed standard or test mixture which is sensitive to changes in column properties with time. This will be important for concepts such as establishing a database of peak positions in GC×GC and for the process of retro-searching; and
- the study of WADA anabolic agents (AAs; *Appendix* reference 8) that are required for laboratories that seek WADA accreditation and re-accreditation purposes. This study established criteria for analysis of the AAs under conditions developed for GC×GC analysis. Specific observations are reported below:

Outcomes and further work

The work described in the papers (Refer to *Appendix* papers 1, 3, 6, 7) demonstrates that:

- A new mass spectrometry library must be generated in order to adequately match spectra from the time-of-flight spectrometer. Conventional spectra in the NIST and related libraries are often based on quadrupole MS data, and in order to have a suitable match, in-house spectra need to be generated for authentic standard material. This is especially so for higher mass molecules, such as derivatised compounds.
- In order to optimise the process of cryotrapping, which is a thermal process, it was necessary to temperature program the cryotrap device as the oven temperature increases. This reduces consumption of CO2 cryogen. In essence, this produces efficient cooling for compound trapping, but does not excessively cool the column such that it retards the re-mobilisation process of the compound.

- We developed a new metric for establishing the detection limits of a compound in GC analysis. This is termed Minimum Acceptable Matching (MAM), such that only a library match above the MAM is accepted as sufficient confirmation of the compound presence. This necessarily gives a detection limit that is higher than the conventional concept of 3 x S/N ratio. However, we feel that it is important to have correct confirmation of compound identity that recognises the use of a library matched statistic.
- We introduced the concept of a retro-searching capability for analysed samples, such that once a sample is analysed by the GC×GC technique, under conditions of full mass spectrometry data acquisition, it should be possible to subsequently identify steroids (or indeed any compound that is able to be analysed by GC) that are present in the sample but whose presence was neither anticipated nor known at the time. This will mean that a sample need not be re-analysed if, in future, a new designer drug was suspected or needed to be tested for in the sample. Once the gas chromatography (GC) and mass spectrometry (MS) properties of the new compound are established, previously collected data can be screened for the presence of these new target compounds. This will be a potentially invaluable deterrent for future use of illegal compounds in for example the sports competition arena.

Implications for law enforcement agencies with respect to GC×GC technologies

- Access to technologies, systems availability and cost

  GC×GC and multidimensional GC (MDGC) are advanced operational methods for GC analysis. They are more expensive than single dimensional GC and GC/MS. If mass spectrometry detection is required for GC×GC, there are few options other than LECO Corporation who have an office in Sydney. This is, understandably, more expensive again. GC×GC-FID (flame ionisation detector) systems are also suitable for routine analysis, however, if identification of components is needed, mass spectrometry is mandatory. It is possible to have an analysis of a representative sample on a GC×GC/TOFMS system and translate this to FID analysis, and we have experience in this, but this requires access to a TOFMS system that is available for use. Our experience is that we always seek identification of peaks by re-analysis on GC×GC/TOFMS. GC×GC/TOFMS may cost in the order of $200–250K. The many studies that are currently being investigated overseas (and in our RMIT labs) probably means that this is a technology of the future that eventually will become more of a mainstream technology and so forensic laboratories will have to adopt this eventually.

- Training of personnel

  There is a major need for training of personnel for GC×GC analysis. We have much experience in training and have had in excess of 35 visiting overseas researchers in our laboratory in the past five years. They all come to study GC×GC and become somewhat expert after this time. We are prepared to undertake this training if required.

- Necessity of chemometrics

  All GC analyses should require chemometrics in order to profile samples and seek underlying information. We show our preliminary chemometric studies herein and will be expanding our research in this in the future. The important ability to provide the links between samples and compare and contrast the key features of samples especially in the area of profiling is critical.

- Future collaboration

  The opportunity to collaborate with leading researchers in Australia who have much experience in GC×GC and MS analysis will maximise the skills transfer and technical capabilities of GC×GC methodologies. This would be welcomed by this research group.

- Further research needs

  There is a continuing need for research, since this project only touched on the opportunity for illicit drug profiling. This will require greater access to samples and close collaboration with law enforcement

agencies for background intelligence of samples. The focus here was on GC×GC, but MDGC needs to be also tested for advanced information on volatile chemical analysis. We have much experience in GC×GC and MDGC, and would suggest that the success of the present project would serve as a useful springboard for continuing research, again with closer collaboration.

Ongoing work

GCxGC analyses produce vast amounts of raw data that require chemometric interpretation to extract meaningful results. Software programs have been developed at RMIT to do this. They are currently being refined and optimised as part of other concurrent studies.

Conclusions and recommendations

The results presented in this final report indicate that there is considerable scope to be optimistic about the future for profiling of illicit drug samples by using GC×GC technology.

Chapter One:

Evaluation of World Anti-Doping Agency Criteria for Anabolic Agent Analysis by Using Comprehensive Two-Dimensional Gas Chromatography Mass Spectrometry

Abstract

This work presents the validation study of comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (GC×GC-TOFMS) method performance in analysis of the key WADA anabolic agents in doping control. The relative abundance ratio, retention time, identification and other method performance criteria have been tested in the GC×GC format to confirm if they comply with those set by WADA. Furthermore, tens of other components were identified with an average similarity of >920 (on the 0–999 scale), including 10 other endogenous sterols and full mass spectra of 5,000+ compounds were retained. The testosterone/epitestosterone ratio was obtained from the same run.

A new dimension in doping analysis has been implemented by addressing separation improvement. Instead of increasing the method sensitivity that is accompanied by making the detector increasingly 'blind' to the matrix (as represented by selected ion monitoring mode, high-resolution mass spectrometry, tandem MS), the method capabilities have been improved by adding a new 'separation' dimension, while retaining full mass spectral scan information. Apart from the requirement for the mass spectral domain that a minimum of three diagnostic ions with relative abundance of five percent or higher in the MS spectra, all other WADA criteria are satisfied by GC×GC operation. The minimum of three diagnostic ions arises from the need to add some degree of specificity to the acquired mass spectrometry data; however, under the proposed full MS scan method, the high MS similarity to the reference compounds offers more than the required three diagnostic ions for an unambiguous identification. This should be viewed as an extension of the present criteria to a full scan MS method.

Introduction

Anabolic androgenic steroids (AAS) and β2-agonists with anabolic effect are the pharmacological substances most frequently abused in doping control. The International Olympic Committee and other organisations responsible for anti-doping control (ie WADA) have banned these substances because of their effect on athlete's performance and consequently on the results of competitions.

The biological fluid of choice in current doping control methods is urine. Urine is a complex matrix containing many components in different concentrations and with similar structures, many of which are unknown. Anabolic agents are extracted from this matrix by solid phase extraction (SPE) (Buiarelli et al. 2001; Kokkonen et al. 1999; Schanzer & Donike 1993) or liquid/liquid extraction (L/LE) (Ayotte, Goudreault & Charlebois 1996; Geyer et al. 1998), conjugates are hydrolysed and the free agents are extracted again and derivatised into corresponding trimethylsilyl (TMS) derivatives. Beside this, many endogenous components are co-extracted to some extent and introduced in the chromatographic system. GC-MS is the preferred technique for analysis, because it offers high sensitivity and selectivity. However, the coelution of anabolic agents with endogenous components and the high background noise give rise to false signals in mass spectra and therefore it is possible to make erroneous interpretation (Munoz-Guerra et al. 1997). High resolution mass spectrometry (Horning & Schanzer 1997; Kokkonen et al. 1999; Schanzer et al. 1996; Thieme, Grosse & Mueller 1997) and

tandem mass spectrometry (Bowers & Borts 1996; Munoz-Guerra et al. 1997) have been proposed to solve this problem and to achieve ever lower limits of detection. Several approaches are proposed for increasing the sensitivity and selectivity of the methods by using an extra step in purification of the samples. In this manner, immunoaffinity chromatography (Machnik et al. 1997) and high performance liquid chromatography fractioning (Gotzmann, Geyer & Schanzer 1997) are employed. Recently, a new approach for separation improvement has been proposed by using GC×GC in sterol (Mitrevski et al. 2008, 2007; Silva et al. 2009, 2007) and drug analysis (Kueh et al. 2003). GC×GC is a relatively new technique that exhibits improved separation power compared with traditional 1D GC. The principles of GC×GC have been reported elsewhere (Adahchour et al. 2006a, 2006b).

The applicability of GC×GC to doping analysis has been demonstrated previously (Mitrevski et al. 2008; Silva et al. 2009) through its improved separation and identification power when combined with time-of-flight mass spectrometry (TOFMS) as a detector. However, no evaluation of the method performance, as pertaining to assessment against specific WADA criteria has been presented so far. Therefore, herein a systematic and coherent evaluation of analysis of AAS against WADA criteria (World Anti-Doping Agency 2003) and a point-by-point assessment of GC×GC/TOFSM related to each criterion is presented. The five key anabolic agents (clenbuterol, 19-norandrosterone, epimethendiol, methyltestosterone M2-metabolite and 3'OH-stanozolol) that WADA had listed at the low ng mL-1 level (World Anti-Doping Agency 2004) were used as model components.

Experimental

Reagents and chemicals

Clenbuterol, 19-norandrosterone, epimethendiol (EMD), 17a-methyl-5b-androstane-3a,17b-diol (methyltestosterone M2-metabolite), 3'OH-stanozolol and methyltestosterone (IS) were purchased from National Measurement Institute (Pymble, Australia). N-methyl-N-(trimethylsilyl)-trifluoroacetamide was purchased from Sigma Aldrich and β-glucuronidase (from E. Coli, K12) was supplied from Roche (Mannheim, Germany). All other reagents and chemicals were of analytical grade or higher. Water used in the experiments was of Milli-Q (Millipore) grade.

Sample preparation

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 solutions and 25 µL of the internal standard solution (5 µg mL-1) to the blank urine extracts. The extracts were prepared according to the previously published sample preparation procedure (Geyer et al. 1998; Saudan et al. 2006). Prior to analysis, the residue was derivatised by dissolving in 50 µL of derivatisation mixture (MSTFA-NH4I-ethanethiol, 1000:2:6) with heating at 80°C for 30 minutes. Standard solutions of anabolic agents were prepared at a concentration of 0.5 µg mL-1 for the purpose of acquiring reference TOFMS spectra. Solutions were stored at 4°C when not in use.

Instrumentation

A Pegasus III time-of-flight mass spectrometer (LECO Corporation, St. Joseph, Michigan, United States) connected to an Agilent 6890 gas chromatograph fitted with a Longitudinal Modulation Cryogenic System (LMCS) (Chromatographic Concepts Pty Ltd, Victoria, Australia) was used in GC×GC-TOFMS experiments. The detector was operated at 1600V and applied electron ionization voltage was 70eV. Data acquisition rate was 100Hz over the mass range from 45amu to 600amu. Data acquisition and processing were performed by ChromaTOF software (LECO Corporation, St. Joseph, Michigan, United States). A separate GC×GC-TOFMS

based in-house library for improved identification was generated using standard solutions of anabolic agents at a concentration of 0.5 µg mL-1. The NIST algorithm was used for mass spectra searching.

The column configuration applied was non-polar/polar consisting of 30 m BPX5 (0.25 mm I.D.; 0.25 µm df) as a ID column and 1 m BPX50 (0.1 mm I.D.; 0.2 µm df) as a 2D column. The oven temperature program was from 120°C (hold for 1 minute) to 320°C (hold for 5 minutes) at 4°C min-1, at a flow rate (He) of 1.3 mL min-1. 1 µL samples were injected in splitless mode (2.5 minute purge time).

Results and discussion

Retention time tolerance criteria

Present WADA criterion:

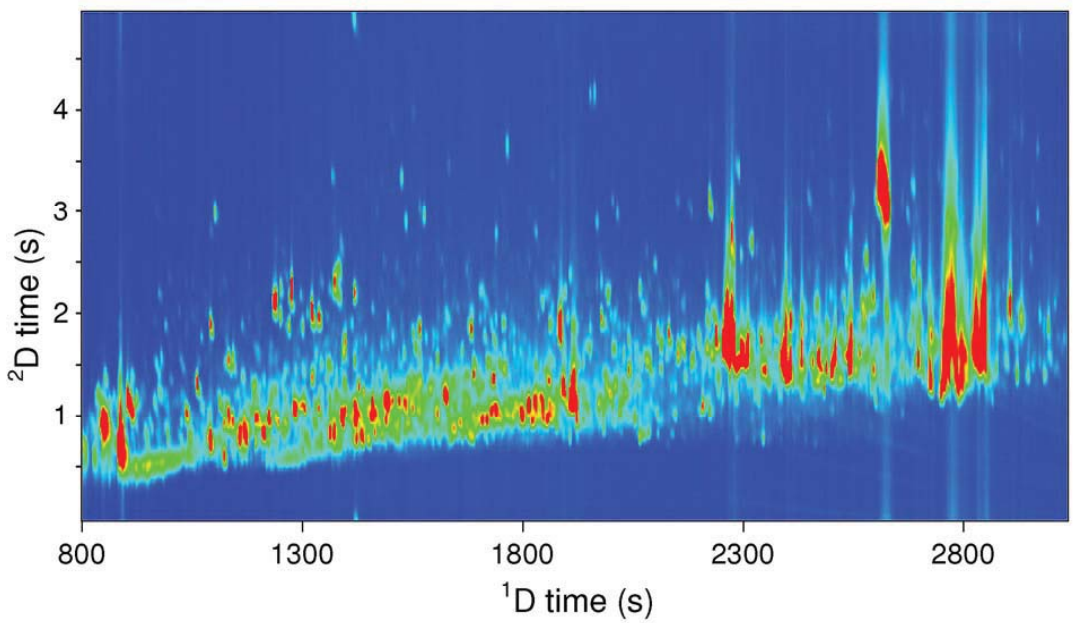
> The retention time of the analyte shall not differ by more than 1% or ±0.2 minutes (whichever is smaller) from that of the same substance in a spiked urine sample or reference material analysed contemporaneously (World Anti-Doping Agency 2003, page 1).

The relative standard deviation (RSD) of the first dimension (1tR) and second dimension (2tR) 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 1.1. A representative 2D plot of urine extract spiked with sterols at a concentration of 10 ng mL-1 is given in Figure 1.1. The reproducibility (%RSD) of 1tR has been found to range from zero percent for clenbuterol-2TMS and 19-norandrosterone-2TMS to 0.18 percent for EMD-2TMS, which agrees with the previous findings for GC×GC reproducibility (Shellie, Xie & Marriott 2002). The relative error of the retention times of the reference compounds are well within the tolerance window of one percent (World Anti-Doping Agency 2003). The lowest deviation (see Table 1.1) has been obtained for clenbuterol-2TMS and 19-norandrosterone-2TMS (0%) and the highest for EMD-2TMS (0.23%). The zero percent RSD values for clenbuterol-2TMS and 19-norandrosterone-2TMS are due to the 'quantised' values of the 1tR values derived from the modulation process and data processing software. Specifically, ChromaTOF assigns the same 1tR for all components in the same modulation period, regardless of their 2tR values. In addition, since each component generates more than one modulation peak, the 1tR 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' 1tR variation evaluated to be zero percent. Therefore, it is not necessarily true that each replicate of each of these compounds has exactly the same 1tR, but rather is an artefact of the data presentation. This group has proposed an algorithm for exact 1tR determination in GC×GC (Adcock et al. 2009).

Table 1.1 1tR and 2tR retention times reproducibility and the relative error to the reference compounds

	clenbuterol-2TMS		19-norandrosterone-2TMS		EMD-2TMS		M2-2TMS metabolite		3'OH-stanozolol-3TMS	
	1tR (s)	2tR (s)	1tR (s)	2tR (s)	1tR (s)	2tR (s)	1tR (s)	2tR (s)	1tR (s)	2tR (s)
AAS average (n=13)	1670	2.498	2125	2.577	2170	2.627	2343	3.071	2918	3.075
RSD (%)	0.00	0.65	0.00	0.75	0.18	0.83	0.10	0.64	0.09	0.57
ref. average (n=2)	1670	2.480	2125	2.565	2165	2.605	2340	3.045	2920	3.055
rel. error (%)	0.00	0.72	0.00	0.47	0.23	0.84	0.13	0.85	0.07	0.65

Figure 1.1 2D plot of urine extract spiked with anabolic agents at a concentration of 10 ng mL-1



Note: The 2tR is shifted vertically by -1 (s) to provide a better presentation format

The 2tR relative error of the reference compounds, even though not defined as part of the established WADA criteria, fall within the tolerance window of one percent. The reproducibility is in agreement with the previous results (Shellie, Xie & Marriott 2002) and the relative error is from 0.47 percent (for 19-norandrosterone-2TMS) to 0.85 percent (for M2-2TMS metabolite). Reproducibility in 2tR values is found to depend on maintaining constant CO2 cryogen supply, since the temperature of the modulator can influence the 2tR; clearly the values found here, being less than one percent RSD, are acceptable for purposes of this criterion. The high reproducibility of 1tR produces very reproducible elution temperature for all anabolic agents, which in turn means constant and stable 2tR. If the modulation process is properly conducted, the 2tR is very robust so the same criteria (1% relative error) can be applied also for this additional parameter. We stress that 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 1tR. In the event that WADA adopts GC×GC technology, then a new criterion specific for 2tR will need to be considered.

Relative abundance tolerance window criteria

Present WADA criteria:

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 1.2 from the relative intensities of the same ions from that of a spiked urine or reference material (World Anti-Doping Agency 2003, page 2)

Table 1.2 Maximum tolerance windows for relative ion intensities to ensure appropriate uncertainty in identification

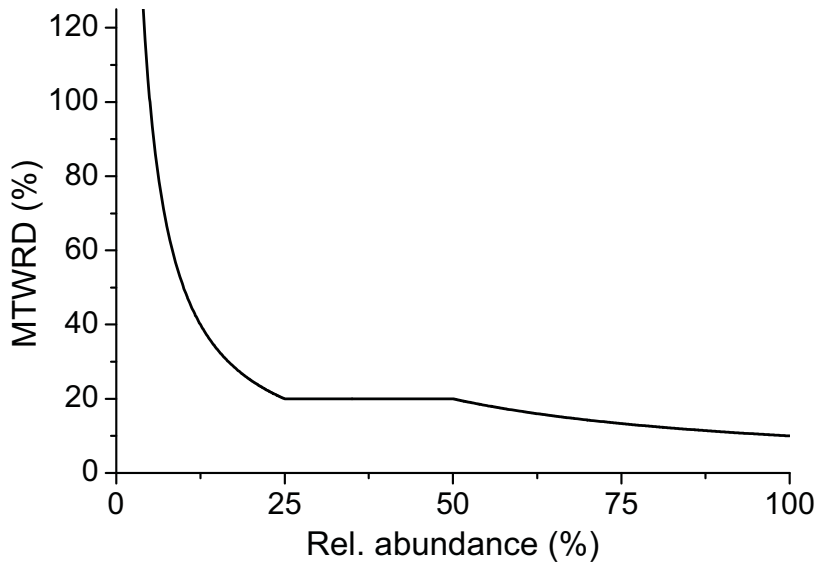
Rel. abundance (% of base peak)	EI-GC/MS
>50%	± 10% (absolute)
25% to 50%	± 20% (relative)
<25	± 5% (absolute)

Source: World Anti-Doping Agency 2003

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 with 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 against the relative abundance of the ions, based on WADA criteria (World Anti-Doping Agency 2003), is given in Figure 1.2.

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 summarised in Table 1.3.

Figure 1.2 Graphical view of the maximum tolerance window of the relative difference against the m/z abundance, according to the WADA criteria



Source: World Anti-Doping Agency 2003

Table 1.3 Relative abundance of diagnostic ions and their combinations

Clenbuterol-2TMS (n=12)	ion (m/z)	86	335	337	335/86	337/86	337/335
	average (%)	65.7	10.2	8.2	12.2	9.0	74.6
	STD	27.1	3.1	2.3	0.6	0.6	2.6
	out of range[a]	8	-	-	-	-	-
19-norandrosterone-2TMS (n=11)	ion (m/z)	405	420	315	420/405	315/405	315/420
	average (%)	35.4	14.9	13.4	41.9	36.6	81.0
	STD	20.4	9.7	8.7	3.5	5.1	13.5
	out of range[a]	8	6	4	-	1	2
EMD-2TMS (n=12)	ion (m/z)	358	143	216	358/143	216/143	358/216
	average (%)	1.9	67.1	3.4	2.9	5.0	57.6
	STD	0.3	9.0	0.7	0.4	0.7	9.0
	out of range[a]	-	4	-	-	-	1
M2-2TMS metabolite (n=10)	ion (m/z)	255	143	435	255/143	435/143	255/435
	average (%)	2.7	86.9	2.8	3.3	3.5	94.7
	STD	0.4	19.0	1.3	0.4	1.3	29
	out of range[a]	-	4	-	-	-	3
3'OH-stanozolol-3TMS (n=9)	ion (m/z)	254	143	545	254/143	545/143	545/254
	average (%)	15.1	51.6	6.1	29.5	12.7	44.6
	STD	6.5	23.3	2.8	3.1	3.3	12.9
	out of range[a]	1	6	1	1	-	1

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

The results in Table 1.3 demonstrate that all ions and ion combinations for clenbuterol-2TMS, except for 86m/z alone, comply with the WADA criteria. The low reproducibility of the relative abundance of 86m/z is due to two main factors:

- The low uniqueness of the base ion 73m/z in TOFMS spectra. This ion is present in all TMS derivatised compounds—endogenous or exogenous—since it is a fragment pertaining to derivatising agent. In contrast, the base ion in quadrupole-based MS spectra (NIST05) is 86m/z, which is more characteristic than 73m/z.
- The narrower permitted tolerance window of the relative difference for ions with higher relative abundance (see Figure 1.2).

However, 335m/z, 337m/z and their combinations, including the combinations with 86m/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.

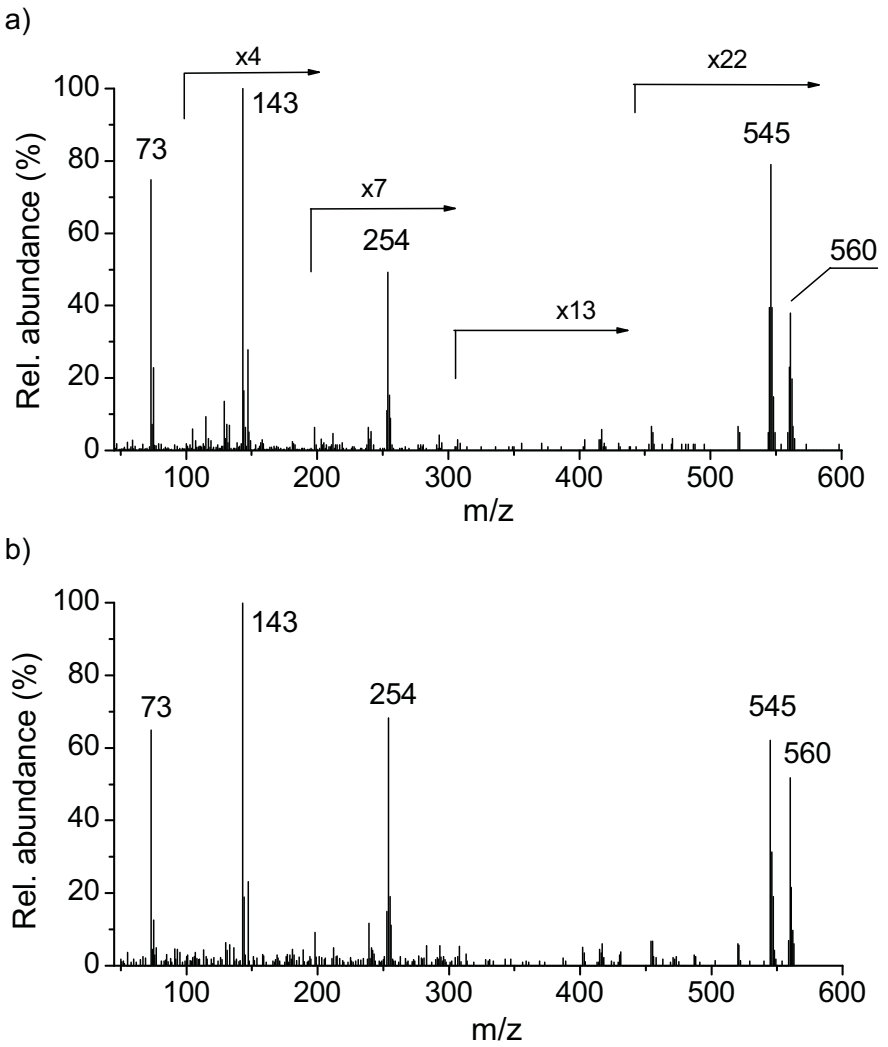
The relative abundance of the diagnostic ions and their combinations for EMD-2TMS fell within the tolerance window, except the 143m/z ion. Again, the main reasons for this are the same as for clenbuterol-2TMS—the low uniqueness of 73m/z as a base ion in TOFMS spectra of EMD-2TMS and the narrower tolerance window for ions with higher abundances. Any tested combination, except 143m/z alone, can be used as a qualifier.

Methyltestosterone metabolite M2-2TMS gives similar results to EMD-2TMS. 143m/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 255m/z and 435m/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 (255m/z, 435m/z, 255/143m/z and 435/143m/z) can be used as qualifiers.

The 143m/z ion alone, as well as 254m/z alone, did not pass the test as qualifiers for 3'OH-stanozolol-3TMS. However, 545m/z, 545/143m/z and 254/143m/z gave results within the tolerance window. 560m/z cannot be used as a diagnostic ion in samples with a concentration of 3'OH-stanozolol-3TMS lower than 4ng mL-1, because of the observed bias of TOFMS towards higher masses (Mitrevski, Wilairat & Marriott 2010a). The comparison of TOFMS and quadrupole MS spectra of 3'OH-stanozolol-3TMS is given in Figure 1.3. The relative abundance of 560m/z in TOFMS spectrum of 3'OH-stanozolol-3TMS acquired at 0.5µg mL-1 concentration in standard solution (see Figure 1.3a) shows approximately 20 times lower relative abundance when compared to the quadrupole based MS spectra (see Figure 1.3b) (Huenerbein et al. 2003; Schanzer et al. 1996). However, the TOF mass spectra similarity of 3'OH-stanozolol-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.

Figure 1.3 Comparison of (a) TOFMS spectra of 3'OH-stanozolol-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



Each of the diagnostic ions alone for 19-norandrosterone-2TMS (405m/z, 420m/z and 315m/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-norandrosterone-2TMS with the endogenous component which shares some of the diagnostic ions (Mitrevski, Wilairat & Marriott 2010a), 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 (see Table 1.2). Furthermore, the spectral similarity even at the lowest level of concentration is high enough to ensure unambiguous identification. The similarity of 19-norandrosterone-2TMS spiked in urine extract at different concentrations is given in Table 1.4. 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%).

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 1.2) and the reproducibility of TOFMS spectra even at the lowest levels of concentration is good. Conversely, 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.

Table 1.4 TOFMS similarity of 19-norandrosterone-2TMS spiked in urine extracts at different levels of concentration against the in-house TOFMS library

conc. level (ng mL-1)	similarity (n=2)
500 (std sol.)	945
20	926
10	867
8	891
6	888
4	799
2	784

Other criteria

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 (World Anti-Doping Agency 2003, page 1)

All the diagnostic ions with abundance above 10 percent that 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 five percent 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 560m/z ion for 3'OH-stanozolol-3TMS was not chosen as a qualifier because of the decreased sensitivity of TOFMS at higher masses. Figure 1.3 shows the comparison of TOFMS spectra of 3'OH-stanozolol-3TMS and its entry in NIST05 MS database (qMS based spectrum). For the same reason the ion 448m/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 five percent. As can be seen from Table 1.2, the abundances of 358m/z and 216m/z for EMD-2TMS, and 255m/z and 435m/z for M2-2TMS metabolite, are below five

percent. As a comparison, the abundance of these ions in NIST05 library entries of the same sterols is between 10 percent (216m/z) and 30 percent (435m/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 (Mitrevski et al. 2008).

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) (World Anti-Doping Agency 2003, page 4)

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 337/335m/z and 339/335m/z have been compared against the theoretical values calculated by the isotope calculator from the NIST MS Search software package (version 2.0). The average ion abundance ratios were calculated from 12 spiked urine samples with anabolic agents over the concentration range from 2ng mL-1 to 20ng mL-1. The results are given in Table 1.5.

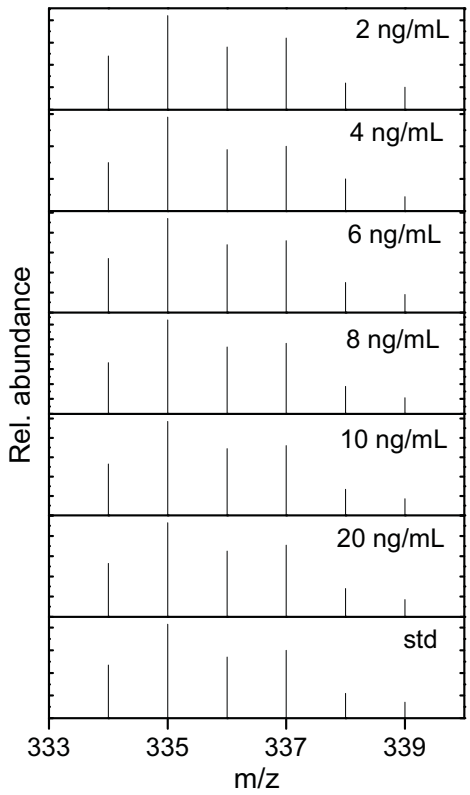
Table 1.5 Experimental and calculated isotope ratio patterns for clenbuterol-2TMS

	ion abundance ratio		area ratio	
	337/335m/z	339/335m/z	337/335m/z	339/335m/z
average (%) (n=12)	74.6	17.8	76.5	21.1
RSD (%)	3.4	11.7	2.6	11.8
calculated (%)	73.7	16.7	-	-

Good agreement has been obtained between the experimental and the calculated values for both ion ratios—337/335m/z and 339/335m/z (see Table 1.5). The 337/335m/z and 339/335m/z ratios, when the peak area was used instead of ion abundances, were slightly higher. However, the reproducibility of the 337/335m/z ion abundance and area ratio was better than the 339/335m/z ratios.

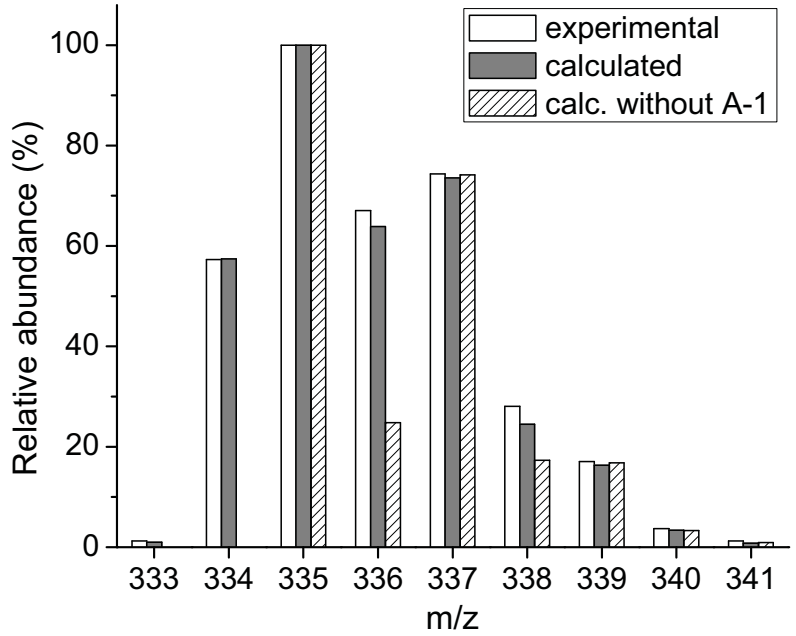
The good agreement between the experimental results obtained from clenbuterol-2TMS spiked at different concentration in urine extract to the theoretical values is a result of the deconvolution capabilities of the ChromaTOF software. The high acquisition rate (100Hz) contributes to the correct deconvolution. 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 1.4.

Figure 1.4 Deconvoluted TOFMS spectra of clenbuterol-2TMS spiked in urine extracts at different concentrations, expanded over the 334–339m/z cluster and normalized to 335m/z as the base ion



Furthermore, the appearance of 334m/z in the experimental results (but not in the calculated isotope pattern) and the differences arising in the isotope pattern at 336m/z and 338m/z between experimental and calculated results (see Figure 1.5) may be explained by presuming the formation of a species with A-1m/z (ie the 334m/z ion). The isotope pattern of the dominant fragment ion at m/z 335 is shown in Figure 1.5. The experimental spectrum in this region extends from 334–341m/z. Note that ion 334m/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 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, we invoke the presence of the A-1 ion, and since it contains 2 x Cl atoms, it will have a strong isotope contribution at 336m/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.

Figure 1.5 Cluster isotope pattern of clenbuterol-2TMS in standard solution at 0.5µg mL-1 for experimental data, calculated data (by assuming the A-1 fragment has been formed in a ratio 1:2 against A), and calculated data without taking the A-1 fragment formation into account



Linearity

The linearity of the proposed method has been calculated from spiked urine extracts with anabolic agents at different concentrations from 0.5ng mL-1 to 20ng mL-1. The correlation coefficients and the linearity curve equations are given in Table 1.6. The highest correlation was obtained for EMD-2TMS and 19-norandrosterone-2TMS (0.996) and the lowest for M2-2TMS metabolite (0.992). Please note than even the AA's quantification ions are detected down to the lowest tested concentration (except for EMD-2TMS at 0.5ng/mL), unambiguous identification is obtained only at 1ng/mL and above for clenbuterol-2TMS, 19-norandrosterone and M2-2TMS, and at 2ng/mL and above for EMD-2TMS and 3'OH-stanozolol-3TMS (Mitrevski, Wilairat & Marriott 2010a). 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 two-dimensional space. The high acquisition rate of the TOFMS detector (100Hz) further facilitates the deconvolution in the mass spectral domain. As we have previously shown (Mitrevski, Wilairat & Marriott 2010b), the poorer separation of the components on the 0.1µm df 2D column gave lower similarity which is based on the poorer deconvolution. Furthermore, the low acquisition rate of the TOFMS detector (20Hz) 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 1.6 Linearity parameters for the anabolic agents spiked in urine extracts at different concentrations from 0.5ng mL-1 to 20ng mL-1

anabolic agent	quant. ion	linearity equation[a]	correlation coefficient
epimethendiol-2TMS	358m/z	Y=0.0081x + 0.0074	0.996
19-norandrosterone-2TMS	405m/z	Y=0.0061x + 0.0094	0.996
M2-2TMS metabolite	255m/z	Y=0.0144x + 0.0070	0.992
clenbuterol-2TMS	335m/z	Y=0.0143x + 0.0050	0.994
3'OH-stanozolol-3TMS	254m/z	Y=0.0043x + 0.0003	0.994

a: Y=Area(AAS) / Area(IS); x=concentration of AAS (ng mL-1)

Testosterone/epitestosterone ratio

Testosterone/epitestosterone ratio 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 AAS from 0.5ng mL-1 to 20ng mL-1 in urine samples, was 915 and 820, respectively.

Signal-to-noise ratio

Present WADA criteria:

> The signal-to-noise ratio of the least intense diagnostic ion must be greater than 3:1 (World Anti-Doping Agency 2003, page 3)

The final criterion that 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 0.1µg mL-1 in standard solution (corresponding to 2ng mL-1 in spiked urine samples at a concentration factor of 50 has been found to be above 18 (Mitrevski, Wilairat & Marriott 2010a). The lowest S/N (4) has been obtained for EMD-2TMS (358m/z) spiked in urine extracts at 2ng mL-1. This is again due to the bias of TOFMS towards the higher masses, combined with the low relative abundance of 358m/z. The lack of potential diagnostic ions in the TOFMS spectra of EMD-2TMS limits the choice.

19-norandrosterone-2TMS and 3'OH-stanozolol-3TMS are the only anabolic agents which completely comply with defined WADA criteria, since all others do not contain at least three diagnostic ions with abundance higher than five percent 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 has been defined (Mitrevski et al. 2008) 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 minimum acceptable match 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. We have found that for unambiguous identification, the minimum acceptable match can be set at 800 when analysing free sterols in non-hydrolyzed urine samples (Mitrevski et al. 2008) and to 700 in hydrolysed urine samples (Mitrevski, Wilairat & Marriott 2010b). 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 minimum acceptable match 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.

Other benefits of the GC×GC-TOFMS method

Among testosterone and epitestosterone, another eight sterols which were investigated in our previous work (Mitrevski et al. 2008) 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 characterise 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 their high mass counterparts. At present, we have only chosen sterols to be in our 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.

Conclusion

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 percent, allowing relative error to the reference components to be within the WADA tolerance window of one percent. 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 334–339m/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 five percent of the base peak. This appears to be due to the predominance of the 73m/z in sterol spectra and if scans were to exclude the 73m/z ion by presenting data at >73m/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 co-elutions 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 AAS confirmation.

Chapter Two:

Organic Impurity Profiling of Ecstasy Tablets by Using Comprehensive Two-Dimensional Gas Chromatography

Introduction

Amphetamines are the second most commonly used illicit drug type in Australia, after cannabis (McKetin & McLaren 2004). Ecstasy, a term referring to tablets containing 3,4-methylenedioxy-methamphetamine (MDMA) as an active component, are very popular among young party goers for their short-term effects, which include a heightened sense of wellbeing, reduced inhibitions, increased energy levels and self-confidence. According to the World Drug Report 2008 (UNODC 2008), at 4.4 percent Australia has the highest rate of ecstasy users in the world for those aged 15–64 years. 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 two decades, considerable international effort has been made in the fight against illicit production and distribution of amphetamine-type stimulants, including ecstasy. In addition to cataloguing the physical characteristics (colour, shape, logo, dimension and mass) of seized samples (Marquis et al. 2008; Milliet, Weyermann & Esseiva 2009), other chemical features including the presence of major components, trace organics (Kongshaug et al. 1999; Milliet, Weyermann & Esseiva 2009; Weyermann et al. 2008; van Deursen, Lock & Poortman-Van Der Meer 2006), metals (Koper et al. 2007; Waddell, NicDaeid & Littlejohn 2004) and the isotopic ratio of 15N/14N (Buchanan et al. 2008; Carter et al. 2002; Collins et al. 2007; Mas et al. 1995; Palhol et al. 2004; Palhol, Lamoureux & Naulet 2003) have been profiled. Several laboratories have harmonised their methods of analysis in the interests of efficient data sharing across jurisdictions (Collins et al. 2007; van Deursen, Lock & Poortman-Van Der Meer 2006).

Impurity profiling is understood to be the chemical analysis, collection of data and its use for evidential or intelligence purposes (Collins et al. 2007; Esseiva et al. 2007; Huizer 1994; van Deursen, Lock & Poortman-Van Der Meer 2006); 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 (Collins et al. 2007). 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 L/LE (Gimeno et al. 2002; Milliet, Weyermann & Esseiva 2009; Weyermann et al. 2008; van Deursen, Lock & Poortman-Van Der Meer 2006) and subsequent analysis by gas chromatography coupled to a GC-FID (Kongshaug et al. 1999; Mitrevski & Zdravkovski 2005) or GC-MS (Bonadio et al. 2008; Gimeno et al. 2002; van Deursen, Lock & Poortman-Van Der Meer 2006). Solid phase micro extraction (SPME) of volatile components (Bonadio et al. 2009, 2008) in ecstasy samples was found to give comparable results to L/LE for the detection of the most discriminative components. SPME was reported to be a simpler, faster and to reduce the sources of error.

Chemical 'signatures' have been interpreted and reported in a variety of ways. Principal component analysis (PCA) (Palhol et al. 2004; Weyermann et al. 2008) and hierarchical cluster analysis (HCA) (Buchanan et al. 2008; Koper et al. 2007; Palhol et al. 2004; Waddell, NicDaeid & Littlejohn 2004) have been used for

classifying samples according their similarities (intelligence), while Pearson correlation coefficients (Bonadio 2009, 2008; Milliet, Weyermann & Esseiva 2009; Weyermann et al. 2008; van Deursen, Lock & Poortman-Van Der Meer 2006) and cosine functions (Gimeno et al. 2002; Weyermann et al. 2008) have been employed for differential comparisons or evidential purposes. Most of the reported drug profiling methods have used single-column (or 1D) chromatographic techniques. The large number of trace components found in the many illicit drug samples results in 1D chromatographic methods have insufficient separating power to map important regions in the chromatogram. Co-elution masks the presence of trace impurities that are definitive to the chemical signature and reduces the informing power of the analysis. While MS can be used for deconvolution of co-eluting peaks, this method is only useful where there is either sufficient mass spectral power or where the components are well resolved in the mass domain. In most cases, resolution to evidential standard relies on either a reduction in the informing power of the mass spectrum or the application of a tandem technique.

Multidimensional chromatographic technologies provide a facile means of achieving high separation efficiency with a single injection in acceptable analysis times. Neumann and Meyer (1987) used 'heart-cut' multidimensional GC for the analysis of illicit heroin. Thus, two GC columns of highly different selectivity were connected by a pneumatic Deans switch at the confluence of the two columns. The switching device directs specific (co-eluting) bands from the first column (1D) to the second column (2D) for further analysis. This approach is particularly useful when specific regions 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.

In comprehensive GC×GC, rather than sampling subsections of the 1D column chromatogram, the entire eluted volume from the first column is subjected to further analysis on the 2D column. Maximising the separating power of the two-column set is reliant on selecting phases that have orthogonality 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 0.5–2m in length and 0.1mm I.D. with a 0.1µm 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 'focused' 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 (Adahchour et al. 2004; Beens, Blomberg & Schoenmakers 2000), food (Adahchour et al. 2004; Khummueng et al. 2006), environmental (Focant, Sjodin & Patterson 2004), forensic (Frysinger & Gaines 2002; Groger et al. 2008; Kueh et al. 2003; Song et al. 2004; Song 2006), doping (Mitrevski et al. 2008; Silva et al. 2009) and essential oil (Junge et al. 2007; Shellie, Marriott & Morrison 2001) 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 (Mitrevski et al. 2008, 2007).

Although GC is an important technique for amphetamine-type stimulant profiling, to the authors' 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 (Barnfield et al. 1988). In the present paper, we describe the chemical profiling of 24 ecstasy tablets by GC×GC from multiple jurisdictions and describe its potential as a new chemical profiling tool.

Materials and methods

Samples

Twenty-four ecstasy tablets in total were used in the experiments, of which nine were seized in Macedonia (2006–07) and provided by Macedonian Police, and 15 were seized in Australia (2009) and provided by Australian Federal Police. Macedonian samples were from nine different seizures, but were classified into three groups of three tablets according to their post-tabletting (post-TB) physical characteristics (logo, colour, diameter and thickness) and intelligence information. The Australian samples were from one recent seizure and were classified into five groups of three tablets according to their physical characteristics (post-TB).

Sample preparation

MDMA tablets were quickly crushed and homogenised by using mortar and pestle. A 20mg portion of the fine powder was placed in a 4mL septum-sealed vial. After 30 minutes equilibration of the sample at 80°C, a 65µm polydimethylsiloxane/divinylbenzene SPME fibre (Supelco, Belafonte, United States) was inserted and exposed to the headspace for another 30 minutes. The samples were desorbed in the GC injection port for five minutes at 250°C.

Instrumentation

An Agilent 7890 GC was used in GC-accurate time-of-flight mass spectrometry (GC-accTOFMS) and flame photometric detection experiments and an Agilent 6890 GC fitted with a 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 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 detector was reduced to 20Hz. Therefore, in this case, the 1D system comprised a long column directly coupled to a short column. While this can be termed a multi-chromatography system according to Hinshaw and Ettre (1986) and discussed elsewhere (Marriott & Kinghorn 2000), 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 column head pressure which depends on the column dimensions. Column sets used are listed in Table 2.1. Columns denoted BP were from SGE Analytical Science (Ringwood, Victoria, Australia) and the HP column was from Agilent (Folsom, California, United States). Conditions are given below, unless otherwise specified. Injector and detector temperatures were both 250°C. The oven temperature program was from 40°C (hold 2 minutes) to 250°C at

10°C min-1 (hold 7 minutes). Hydrogen was used as a carrier gas at a flow rate of 1 mL min-1 for FID/FPD (flame photometric detector) and helium at 1.3mL min-1 for MS experiments. Splitless injection mode (SPME desorption) employed a purge off time of two minutes. The modulation period was varied from three second to six seconds and modulation temperature was set at -30°C, 0°C, 40°C and 80°C during the optimisation. Temperature tracking was applied in all GC×GC-TOFMS experiments, keeping a constant ΔT of 130°C between the oven and modulator, starting at 0°C for the LMCS cryotrap.

Table 2.1 Column sets used in all 1D and 2D GC experiments

System	1D column[a]	2D column[b]
GC×GC-FID	BP20	DB-1
	BPX50	BPX5
	BPX5	BPX50
	BPX5	BP20[c]
GC×GC-FPD	BPX5	BP20
GC×GC-fastTOFMS	BPX5	BP20
GC-accTOFMS	HP5	
GC-qMS	BPX5	

a: Dimension: 30m x 0.25mm; 0.25µm

b: Dimension: 1m x 0.1mm; 0.1µm, except otherwise stated

c: 1.4m x 0.1mm; 0.1µm

All MS detectors were operated at -70 eV ionisation energy in the mass range from 45m/z to 500m/z. One scan s-1 acquisition rate was used with all single column experiments (qMS and accTOFMS), 20Hz in all 1D and 50Hz 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, United States) and Wiley 7 (John Wiley & Sons, New York, United States) commercial MS databases by using probability-based matching algorithm (qMS only) or NIST algorithm. When in doubt, accTOFMS was used for component confirmation. Automated spectra deconvolution and peak finding features of ChromaToF (Leco Corporation) acquisition and data processing software have been applied in all fastTOFMS experiments. S/N threshold of 100 was set for peak detection (except otherwise stated) and minimum acceptable match threshold (Mitrevski et al. 2008) 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).

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-TB physical characteristics (logo, colour, diameter and thickness) and intelligence information. The raw dataset (53 observations x 16 variables) consisted in the abundance of the targeted compounds determined by GC×GC-TOFMS for each replication of each tablet sample plus five replicates of one sample (XTC5). The data were normalised according to the procedure described by Bonadio et al. (2009, 2008). The procedure consists of the 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 normalised datasets according to the model—abundance of the compound—group of tablets, $p<0.05$. Principal component analysis was performed on raw and normalised data sets to visualise 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 normalised data set according to the model—country, $p<0.05$.

In order to determine a set of 'orthogonal' compounds enabling to discriminate at best the eight groups of ecstasy tablets, a linear discriminant analysis (LDA) was carried out on the normalised dataset. For processing the LDA, the dataset was divided into two independent subsets

- a 'learning' dataset (n=35) for the generation of candidate discriminative models; and
- a 'test' dataset (n=18) for the assessment of the robustness of the model.

For the discriminant analysis, 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.

Results and discussion

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 (Ryan, Morrison & Marriott 2005). 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.

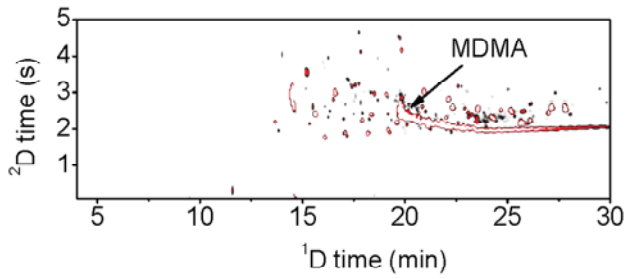
The selection of column sets that have demonstrable orthogonality is sample dependent and must be derived from either a detailed understanding of the sample composition or by empirical methods. As the former is rarely achieved without first completing the orthogonal analysis, phase polarity is usually deduced using the ad hoc application of solvation parameter models such as that derived by Abraham, Ballantine and Callihan (2000). In practice, the phase polarity is more commonly interpreted in terms of a simplified polarity scale, such as that developed by Wynne, DiFeo and Dawes (nd) 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). Accordingly, we have coupled these polar chemistries (P) with less polar phases (NP) in two dimensional sets to test for separation of components in the ecstasy samples.

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 (see Figure 2.1), 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.

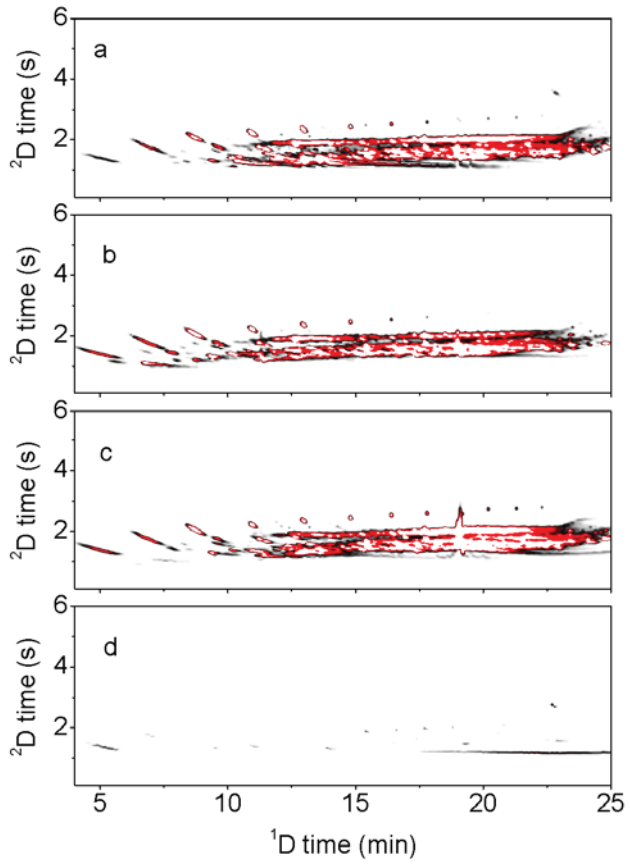
Figure 2.1 2D plot of ecstasy sample XTC4 analysed by SPME on BP20/DB-1 column set. The highly tailed MDMA peak on 1D column is annotated



BPX50/BPX5

The second P–NP column set applied for separation of volatiles in ecstasy tablets was BPX50/BPX5 (see Figure 2.2). Similarly to the separation on the opposite phase column set (BPX5/BPX50, see Figure 2.4 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; that is, alkanes and siloxanes, from the rest of the ecstasy constituents, which have the longest retention on BPX5. However, peaks as narrow as 50ms peak width at half height (w1/2) on 2D column have been obtained.

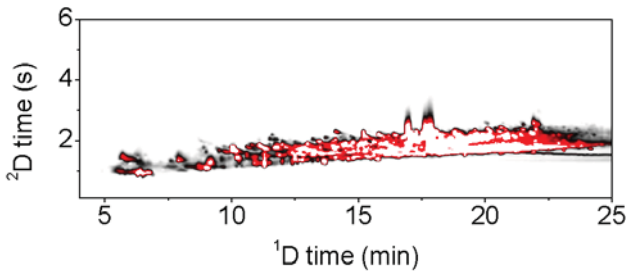
Figure 2.2 Representative 2D plots of ecstasy samples XTC3 (a) XTC4 (b) XTC5 (c) and a blank (d) analysed on BPX50/BPX5 column set. Note the similarity of the profiles, even though the samples proved to be different



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 (see Figure 2.3). 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 'normalised' 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 2.3 2D plot of ecstasy sample XTC4 analysed by SPME on BPX5/BPX50 column set



BPX5/BP20

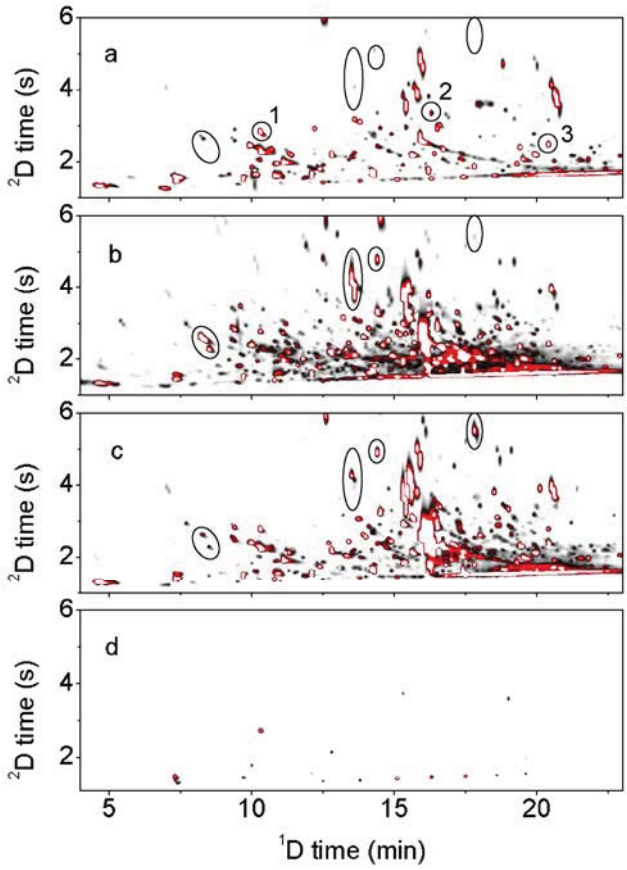
The second NP–P column set tested was BPX5/BP20. This column set showed a good spread of components over the 2D space, as well as having good peak shape (see Figure 2.4). Despite the unresolved cluster of components at the lower right side region of the 2D plots, which is partially contributed to by 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 2.4, 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. While a large number of reference components are required for identification of components based on GC×GC-FID, availability of MS detection provides valuable molecular information of sample composition.

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. This is further discussed below.

Figure 2.4 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: (a) sample XTC3; (b) sample XTC4; (c) sample XTC5 and (d) a blank. Note the differences between profiles, especially as suggested by the circled regions. The annotated components in sample XTC3 are selected for 2tR and area reproducibility



Optimization of some GC×GC parameters

Modulation period

It has been shown in subsequent experiments that a five to six second modulation period is necessary in order to elute some polar components from the highly polar 2D column, under the proposed conditions, within one modulation period 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 1D column, they were well modulated on the 2D column which makes identification and integration much easier (Mitrevski, Wilairat & Marriott 2010a).

Modulation temperature

Not surprisingly, we have found that lower modulation temperature offers better peak shape. However, consistent with conclusions in previous work on sterols (Mitrevski et al. 2008), 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 modulation temperature.

Reproducibility of peak area and 2D retention time in GC×GC-FID

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 (replicate traces not shown), even in terms of the minor components usually not seen in 1D GC chromatograms. The 2tR and the peak area reproducibility of three selected components (shown in Figure 2.4a) are given in Table 2.2. Around one percent RSD for 2tR and six to seven percent RSD for summed modulated peak area were obtained for all three selected components. The results for 2tR reproducibility here are in good agreement with previous findings (Mitrevski, Wilairat & Marriott 2010a; Shellie, Xie & Marriott 2002).

Table 2.2 Reproducibility of the 2D retention time and the peak area of three selected components in GC×GC-FID analysis of ecstasy sample XTC3. Selected components are annotated in Figure 2.4a. n=5

		component 1	component 2	component 3
2tR	Average (s)	2.736	3.228	2.328
	RSD (%)	1.20	0.83	1.15
Area	Average (pA s)	13.19	14.56	48.05
	RSD (%)	6.2	7.0	6.1

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 to by the poorer orthogonality of these column sets towards sample constituents compared with 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 1D BP20 column, we decided to proceed with all further experiments on the BPX5/BP20 column set. One method difference involved replacing the 1.4 m 2D column with a 1.0 m long 2D column of the same type, to avoid any wraparound (ensuring 2tR<PM) while still keeping good spread of the components on the 2D plots.

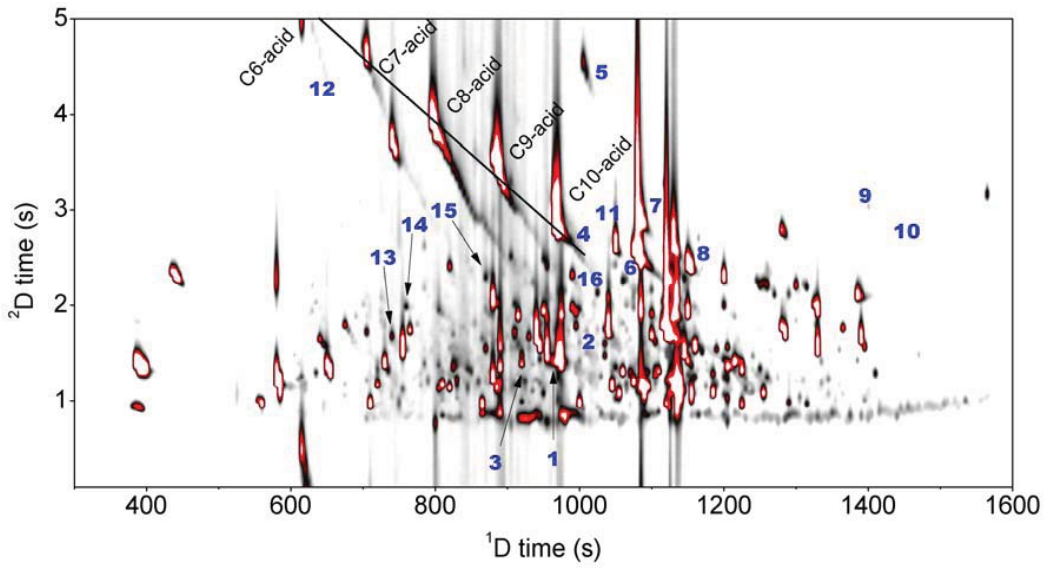
Identification of components on GC×GC-TOFMS

Automated deconvolution and peak finding data processing of GC×GC-TOFMS chromatograms on this column set revealed the identity of the most of the higher abundant components with high match quality. The high match quality of TOFMS spectra against commercial MS databases (NIST05 and Wiley7) agreed with our previous conclusion (Mitrevski et al. 2008; Mitrevski, Wilairat & Marriott 2010a) 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 with as high as RMM 560 components in our previous study.

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 1,200 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 2.4d). By 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 2.5.

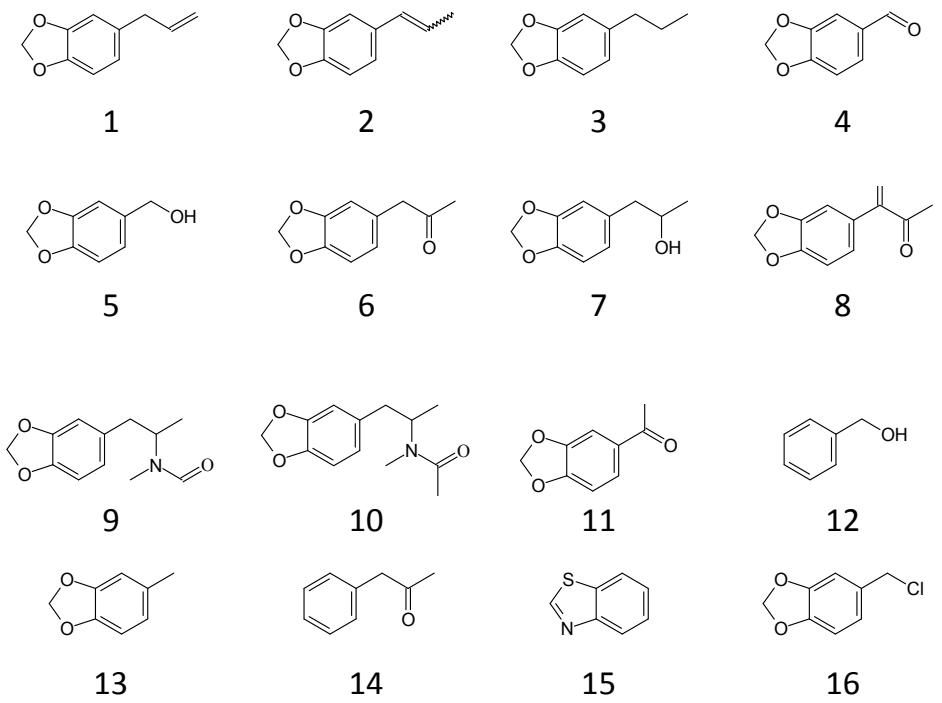
Figure 2.5 Representative GC×GC-TOFMS 2D plot of ecstasy sample analysed on BPX5/BP20 column set. The location of selected 16 components for profiling, along with the identified fatty acids, is shown on the plot



Selection of components for profiling and their location on the 2D plot

Selection of components for profiling was firstly based on previous work (Bonadio et al. 2008; Gimeno et al. 2002; Weyermann et al. 2008; van Deursen, Lock & Poortman-Van Der Meer) 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 that showed good reproducibility and were present in all tested samples in reasonable but variable concentrations are given in Figure 2.6. Their location on a representative 2D plot of an ecstasy sample is given in Figure 2.5 and the peak table with their 1tR, 2tR and quantification ions for peak area calculation is given in Table 2.3.

Figure 2.6 Structures of the 16 components selected for profiling. Note that most compounds incorporate similar aromatic functionality; this choice of compounds was not a deliberate strategy, but rather was completely fortuitous



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 percent 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 dropped on 7.9 percent 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 five on the 1D column, where up to six modulation slices were automatically detected, integrated and summed up. Adequate integration was facilitated by the excellent trapping and releasing of five on the 2D column, giving narrow peaks with an average w1/2 of ~200ms. It has been shown (later) that this tailing and low abundant component could be one of the most discriminative in the proposed statistical approach.

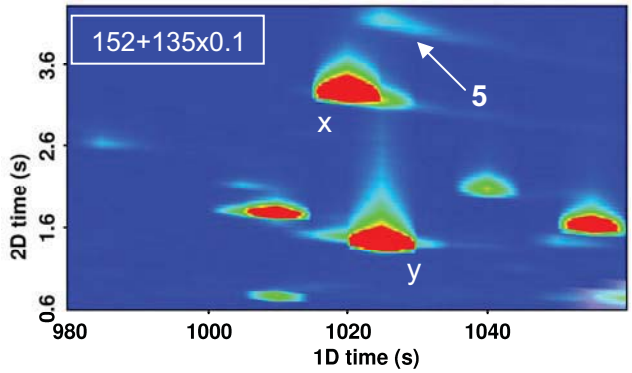
Table 2.3 Peak table of the 16 selected components chosen for profiling with their retention times and quantification ions used for area calculation

	Compound	1D time (s)	2D time (s)	Quant. ion (m/z)
1	safrole	960	1.58	162
2	isosafrole-2	995	1.78	162
3	3,4-methylenedioxyphenylpropane (3,4-MD propane)	920	1.48	164
4	piperonal	970	2.86	149
5	1,3-benzodioxole-5-MeOH (benzodioxiole-5-MeOH, piperonyl alcohol)	1,005	4.54	152
6	3,4-methylenedioxyphenyl-2-propanone (Piperonylmethylketone, PMK)	1,080	2.58	178
7	3,4-methylenedioxyphenyl-2-propanol (PMK-OH)	1,085	2.88	180
8	unknown 147	1,150	2.420	147
9	N-formyl-MDMA	1,400	3.080	162
10	N-acetyl-MDMA	1,415	2.78	162
11	3,4-methylenedioxyacetophenone (3,4-MDAcPh)	1,050	2.62	164
12	Benzyl alcohol	670	4.24	108
13	3,4-methylenedioxytoluene (3,4-MD toluene)	740	1.68	135
14	benzylmethylketone (BMK)	760	1.98	134
15	benzothiazole	870	2.28	135
16	3,4-methylenedioxybenzylchloride (3,4-MD-benz-Cl)	990	2.30	170

On the other hand, the TOFMS detector was saturated with some of the components listed in the literature at their characteristic ions, for example N,N-dimethyl-3,4-methylenedioxy-amphetamine at 72m/z. Because of this, the component was rejected from selection. An inconvenience due to the overloading has also been encountered with other higher abundant 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 abundant components in the profiles.

Many production-specific components, generally those containing a 3,4-methylene-dioxyphenyl 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 2.7 shows co-elution of benzodioxole-5-MeOH (5) with two other matrix components, with which they share the two most characteristic mass fragments of 5:135m/z with component x and 152m/z with component y. Note that the ecstasy sample presented in Figure 2.7 (sample XTC3) contains minor amount of five, in contrast to the ecstasy sample presented in Figure 2.5. The highly abundant and strongly tailing 1D peaks of polar organic acids can strongly interfere with some of the co-eluting components in the profiles. For example, Figure 2.5 shows that 13 co-elutes with heptanoic acid, one with decanoic acid and eight 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 have resulted in high similarity to the commercial MS database entries.

Figure 2.7 Extracted ion 2D plot showing the co-elution of 5 on the 1D column with two other components that share some common mass fragments—component x and component y have abundant 135m/z and 152m/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. (2008) 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 2.3 (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 later stage confirmed as 3-(3,4-methylenedioxyphenyl)-3-buten-2-one by accTOFMS at 3 ppm error, showed high similarity to the literature spectra7 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 our previous study on higher molar mass sterols (Mitrevski et al. 2008) and anabolic agents (Mitrevski, Wilairat & Marriott 2010a).

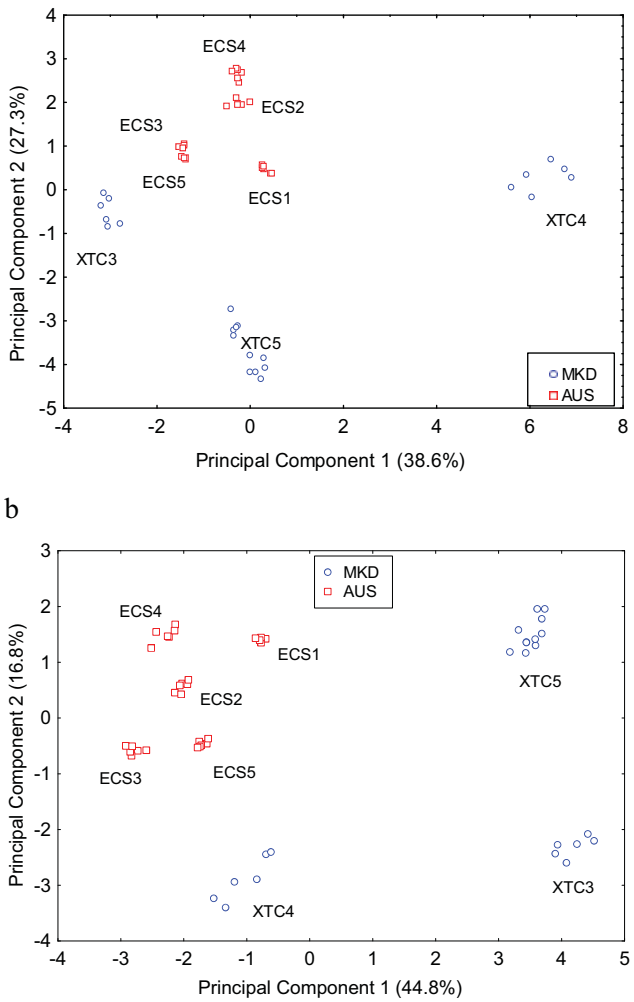
GC×GC has demonstrated its improved separation and enhanced sensitivity over 1D GC through more separated and detected components in the profiles. The fastTOFMS detector performance (high speed in full mass spectra mode, non-skewed spectra across the peak, spectral deconvolution and peak finding features) fits perfectly into the GC×GC part 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.

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 16 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 six of these groups based on the raw data (peak area) provided by the GC×GC-TOFMS analysis (see Figure 2.8a). By contrast, ECS3 and ECS5 or ECS2 and ECS4 cannot be clearly distinguished. Figure 2.8b shows the first map of the PCA performed on the same dataset after its normalisation by the procedure proposed by Bonadio et al. (2008, 2009). Whereas the percentage of variation explained by the first map was roughly the same, Figure 2.8b shows a clear separation of the eight groups after normalisation and confirms both the relevance of this data treatment and the suitability of the 16 selected compounds for authentication purpose.

Figure 2.8 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)

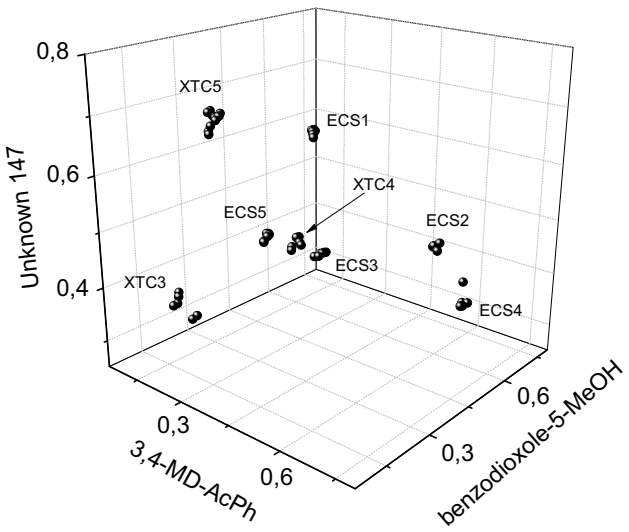


In addition to the clustering of tablets from different seizures with the same post-TB (XTC samples) and differentiation between the eight groups, Figure 2.8b also evidenced a clustering of Australian and Macedonian samples. The examination of the variable projection on the PCA first map (data not shown) 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 compared with 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, an LDA was applied on the normalized data matrix. Several combinations of three compounds chosen out of the 16

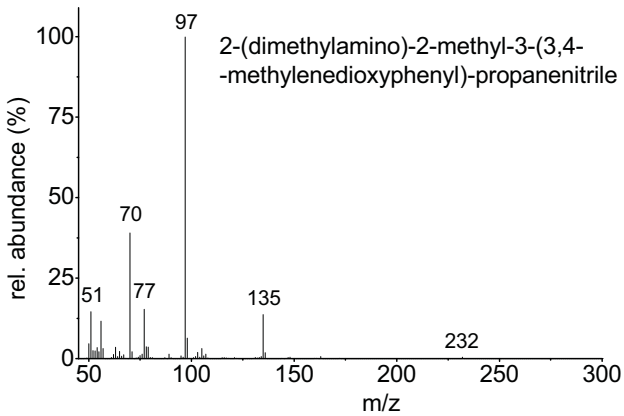
were shown to be suitable to classify 100 percent of the samples in both learning and test subsets of the dataset. Figure 2.9 shows the discrimination obtained in the three dimensional orthogonal space generated by 3,4-MD-Acetophenone, 1,3-benzodioxole-5-MeOH and Unknown 147.

Figure 2.9 Three dimensional score plot of normalised abundances of three components selected by LDA, showing clear discrimination of the eight groups of ecstasy tablets



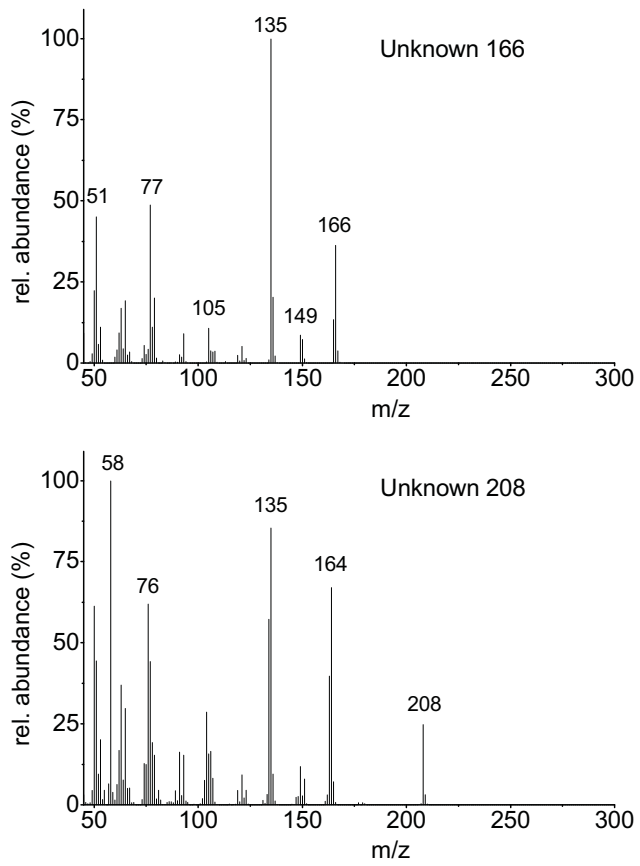
Even though synthesis route elucidation was not focus of the present paper, several components specific for country of seizure have been detected. 2-(dimethylamino)-2-methyl-3-(3,4-methylenedioxyphenyl)-propanenitrile, shown to be a marker for reductive amination by using cyanoborohydride (NaBH3CN)45, has been detected in different proportions only in samples from Macedonia. Its GC×GC-TOFMS spectrum is given in Figure 2.10. 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 (97m/z) favour its detection and identification in GC×GC format. As a reminder, the sensitivity of fastTOFMS is biased against higher masses (Mitrevski et al. 2008; Mitrevski, Wilairat & Marriott 2010a, 2010b).

Figure 2.10 Mass spectra of 2-(dimethylamino)-2-methyl-3-(3,4-methylenedioxyphenyl)-propanenitrile, a marker for cyanoborohydride reduction of PMK, found only in samples from Macedonia



Two other unknown components, whose mass spectra are given in Figure 2.11, 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 (see Figure 2.11a) resembles the mass spectrum of piperonyl methyl ether, a component previously reported as an impurity in ecstasy samples (van Deursen, Lock & Poortman-Van Der Meer 2006). The identity of initially annotated Unknown 208 is still unknown but the presence of 135 m/z ion fragment in its mass spectrum (see Figure 2.11b) suggests it could be a 3,4-methylenedioxyphenyl derivative. Many more components are detected in extracted 135m/z GC×GC chromatograms (data not shown), which suggests that the number of potential markers could be much higher than the selected 16 components.

Figure 2.11 Mass spectra of Unknown 166 (a) and Unknown 208 (b) two components detected only in samples from Australia



Conclusion

The purpose of this Chapter 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. 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 abundant polar components (ie component 5). However, the work should include larger sample set in order to construct robust and generic model for sample classification and discrimination.

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 abundant components appears to work better than in GC-qMS.

Chapter Three:

Application of Comprehensive Two-Dimensional Gas Chromatography in Heroin and Cocaine Profiling

Introduction

Cocaine is a purely natural component derived from coca leaves (Erythroxilym 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) and cuscohygrine are the most frequently encountered in cocaine samples. Others such as hygrine, trimethoxycocaines and 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 (Casale & Moore 1994; Moore et al. 1995). Special extraction methods, such an ion-pairing and column fractionation (Casale & Moore 1994) have been developed in order to effectively quantify the presence of natural impurities. Some of them, such as truxillines, show poor chromatographic behaviour so reduction to simpler molecules and their derivatisation is required for their detection (Moore & Casale 1994; Moore et al. 1994).

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, such as cis- and trans-cinnamoylcocaine, but many more are unintentionally formed during the poorly controlled oxidation step. N-norcocaine, N-benzoylnorecgonine methyl ester and N-formylcocaine have been formed in over-oxidised cocaine samples (Ensing & Hummelen 1991). Four diasteromeric of 2,3-dihydroxy-3-phenylpropionylecgonine methyl esters from incomplete oxidation of cis- and trans-cinnamoylcocaine have been detected in cocaine samples (Casale et al. 2007). Due to their highly polar character, derivatisation 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 transesterification (Casale, Boudreau & Jones 2008), 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 numerous 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 (Allen et al. 1984) and 18 of papaverine (Toske et al. 2006) have been detected when these components have been boiled in acetic anhydride. Acetylation of highly purified morphine samples result in heroin that 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 two to three orders of magnitude (Moore & Casale 1994). The concentration range of the impurities, as well as the variety in their chemistry, require various methods for extraction, concentration, derivatisation 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 (Dufey et al. 2007; Geyer et al. 1998; Johnston & King 1998; Klemenc 2001; Lociciro et al. 2008) is based on sample dissolution, optional prior derivatisation and injection into the GC port. Sample comparison is based on the normalised area of each of the selected analytes against the peak area of cocaine, the peak area of whole morphine (sum of morphine, monoacetylmorphine and diacetylmorphine), or the peak area ratio against internal standard. The chromatograms do not appear complex because of the very few target components at relatively high concentrations in the profiles. Cocaine, cis- and trans-cinnamoylcocaine, tropacocaine, ecgonine, benzoyl ecgonine and 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 (Cartier, Gueniat & Cole 1997; Dujourdy & Besacier 2008; Morello & Meyers 1995) or SPME sampling (Chiarotti, Marsili & Moreda-Pineiro 2002). 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 (Dujourdy & Besacier 2008) and acetic acid, acetone and diethyl ether in heroin samples (Morello & Meyers 1995; Chiarotti & Fucci 1988). Additional methods for sample-to-sample comparison and geographic origin determination are based on isotope ratio (Besacier et al. 1997; Ehleringer et al. 1999; Galimov et al. 2005; Hays et al. 2000; Ehleringer et al. 2000) or trace metal contaminants (Myors et al. 1998; Infante et al. 1999; Violante et al. 1992; Bora, Merdivan & Hamamci 2002; Bermejo-Barrera et al. 1999).

Advanced heroin profiling methodology has been generally based on acidic and neutral organic impurity extraction (Neumann & Gloger 1982), concentration and subsequent analysis on GC coupled to FID (Neumann & Gloger 1982; Besacier et al. 1997) or MS detector (Toske et al. 2006; Myors et al. 2001). Sixteen impurities have been selected in the most comprehensive study aimed at harmonising the method for retrospective comparison and data exchange between three well-equipped and experienced forensic laboratories (Stromberg et al. 2000). Various limitations of the GC technique have been identified as the main reason for the poor inter-lab reproducibility. Recently, comprehensive 2D GC has been applied in heroin and cannabis impurity profiling with pixel-based chemometric processing (Groger et al. 2008). Even though only nine 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 GCxGC is poorly justified for samples where no coelution on the 2D 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 at the Drug Enforcement Administration. The approach is based on six independent cocaine profiling methods for sample comparison analyses, geographic origin studies and solvent determinations (Moore & Casale 1998). It combines different and separate preparation steps, from simple derivatisation, through head space sampling of volatiles, to components 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 offer high amount of data, or they are simple but offer limited information content. In this paper, we present the application of comprehensive two-dimensional gas chromatography as an alternative, with simple and reasonably high information content method for cocaine and heroin sample discrimination. The method is based on SPME sampling of volatiles and semi-volatiles in cocaine and heroin samples, subsequent separation on non-polar/polar column set and TOFMS detection. Even though many components currently used in profiling methods, especially those that require reduction and derivatization (truxillines), and higher molecular mass components (ie cis-, trans-cinnamoylcocaines and their analogous, papaverine and 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 the best of our 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.

Experimental

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

Analysis of impurities in heroin samples extracted by L/LE was performed in 1D and GC×GC mode by using BPX5/BPX50 and BPX50/BPX5 column sets. For column dimension details see *Experimental* section in ecstasy profiling in Chapter 2. 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 detector was reduced to 20 Hz. Therefore, in this case, the 1D GC system comprised a long column directly coupled with a short column and while this can be termed a multi-chromatography system according to Hinshaw and Ettre (1986) and discussed elsewhere (Marriott & Kinghorn 2000), the second very short column is anticipated to lead to negligible variation in peak properties such as width. The applied temperature program (Stromberg et al. 2000) was from 160°C to 320°C at 6°C min-1 (hold 6 minutes) at a flow rate of 1.3mL min-1 (He). Splitless mode with a purge time of one minute was applied in both modes because of the low sensitivity of the method in 1D GC mode.

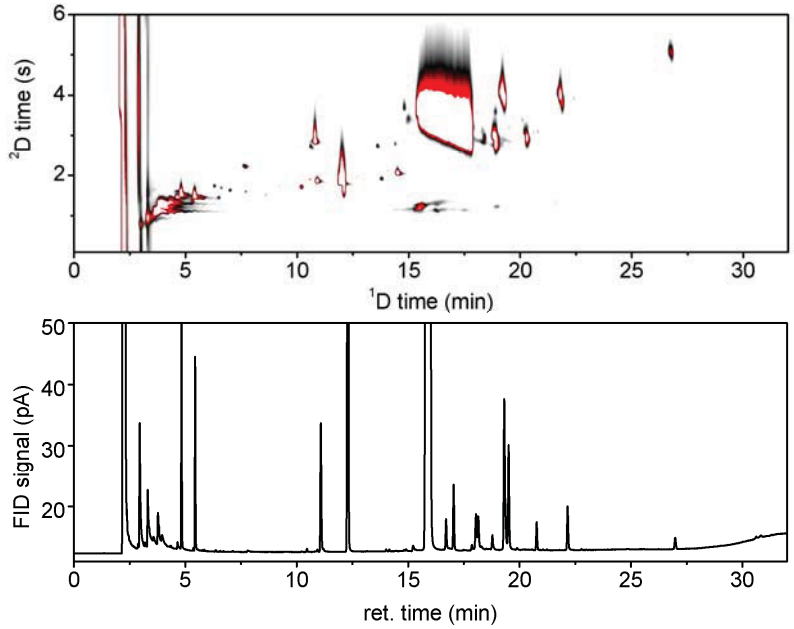
Comparison between major component analysis of cocaine samples in 1D GC and GC×GC mode has been done on the same column set, under different oven temperature program (Lociciro et al. 2007). The following conditions were applied—isothermal for one minute at 180°C then increased to 275°C at 4°C min-1 rate and hold at this temperature for 2.25 minutes. One µL of the sample was injected in split mode at 20:1 split ratio.

Results and discussion

GC×GC versus 1D GC in major component analysis of cocaine and heroin

Based on previous results and our 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 3.1.

Figure 3.1 Comparison of (A) 1D GC and (B) GC×GC cocaine major components profiles. 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 pair of coeluting components in 1D GC at 11.0 minutes and 19.5 minutes, there is no much improvement in GC×GC 2D plot. By contrast, several components eluting immediately after the cocaine in 1D GC are overlapped by its huge and tailing spot on 1D column in GC×GC format. Peak tailing on 1D of overloaded components in GC×GC is mainly contributed to by the inefficiency of the modulator to effectively remobilise the trapped (overloaded) portion onto the 2D column. The spot width on 1D can be minimised either by increasing the temperature of the modulator (to ease the remobilisation), 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 modulation temperature) or by increasing the chances of wraparound (shorter program temperature). In general, the example confirmed our proposition that GC×GC has no advantages over 1D GC when dealing with simple component mixtures. Similar results for comparison between 1D and 2D GC of main components have been obtained for heroin samples (data not shown).

GC×GC versus 1D 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 Groger et al. (2008). 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 (see Figure 3.2A). 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 (see Figure 3.2B).

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 (see Figure 3.3), most of the components 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 later eluting, has rejected this column set as an appropriate one 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 (see Figure 3.3). Less tailing on 1D and more on 2D column has been obtained in the results of Groger et al. (2008).

Figure 3.2 Comparison of a heroin impurity profile in (A) GC×GC with the profile in (B) 1D GC

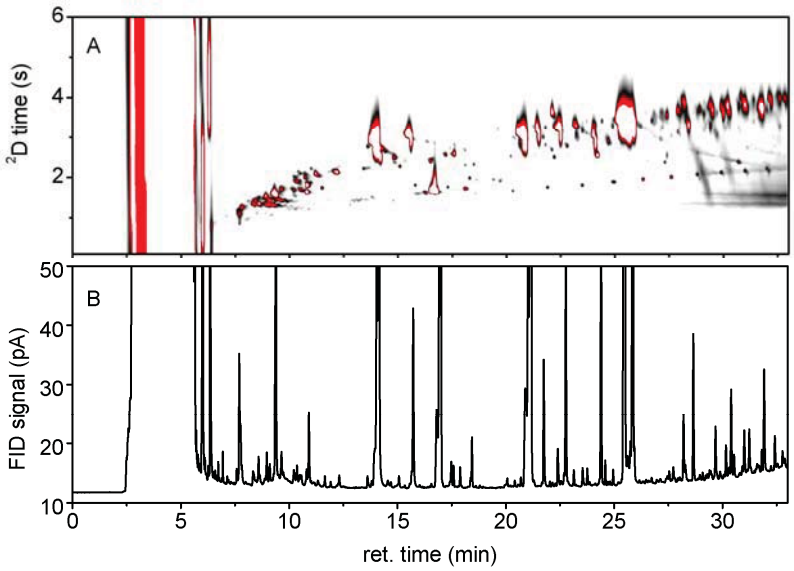
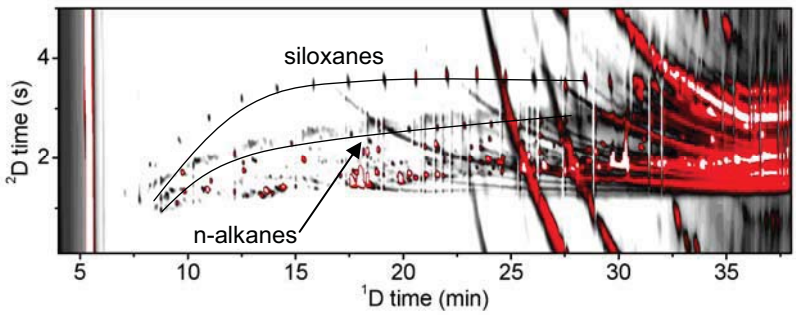


Figure 3.3 Heroin impurity profile on BPX50/BPX5 column set. Note the significant tailing of components on 2D, and especially on 1D column for late eluting components. Siloxanes and n-alkanes homologues are denoted

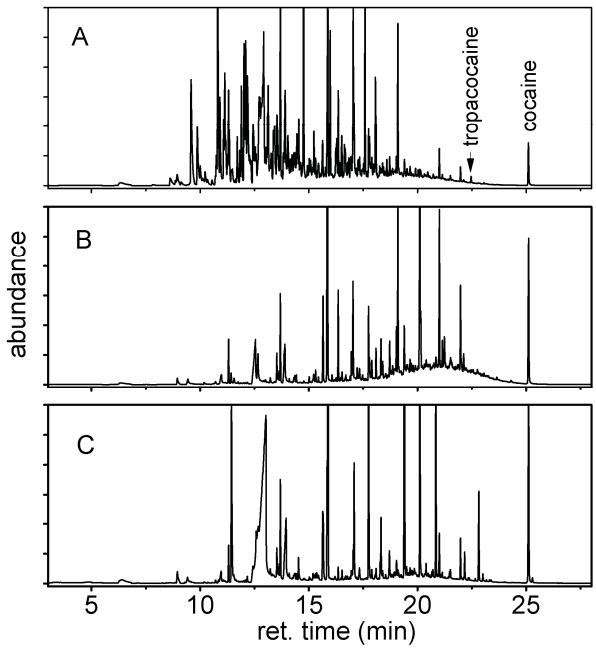


Cocaine and heroin volatiles in 1D GC

A sampling procedure similar to the one explained in our previous work on ecstasy profiling (see Chapter 2) has been applied in order to check if volatiles and semi-volatiles cocaine and heroin profiles are complex enough for profiling in GC×GC format. Since known components used in cocaine and heroin profiling methods are expected to be generally less volatile than the ones used in ecstasy samples, prolonged equilibration and absorption time was applied. Chromatograms of three different cocaine samples analysed on GC-qMS are given in Figure 3.4. Reasonable complex and different profiles are 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 of components was far below the number of components later identified by GC×GC-TOFMS. Similar results have also been obtained for three different heroin samples. At least the profile looked promising for the next step, comprehensive two-dimensional gas chromatography.

Figure 3.4 GC-qMS comparison of volatile and semi-volatile component profiles of three different cocaine samples. (A) sample Ca, (B) sample Cb, (C) sample Cc



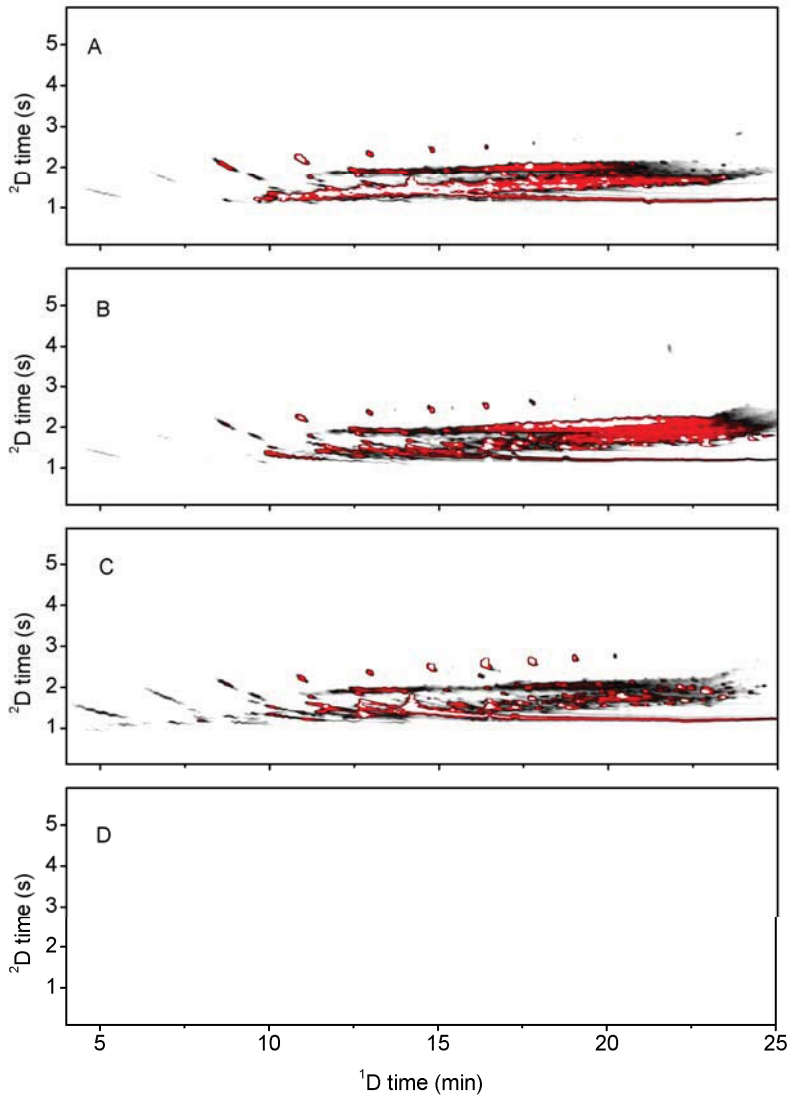
Separation of volatile and semi-volatile components in cocaine and heroin samples on different column sets in GC×GC format

2D GC 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. Conversely, 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.

BPX50/BPX5

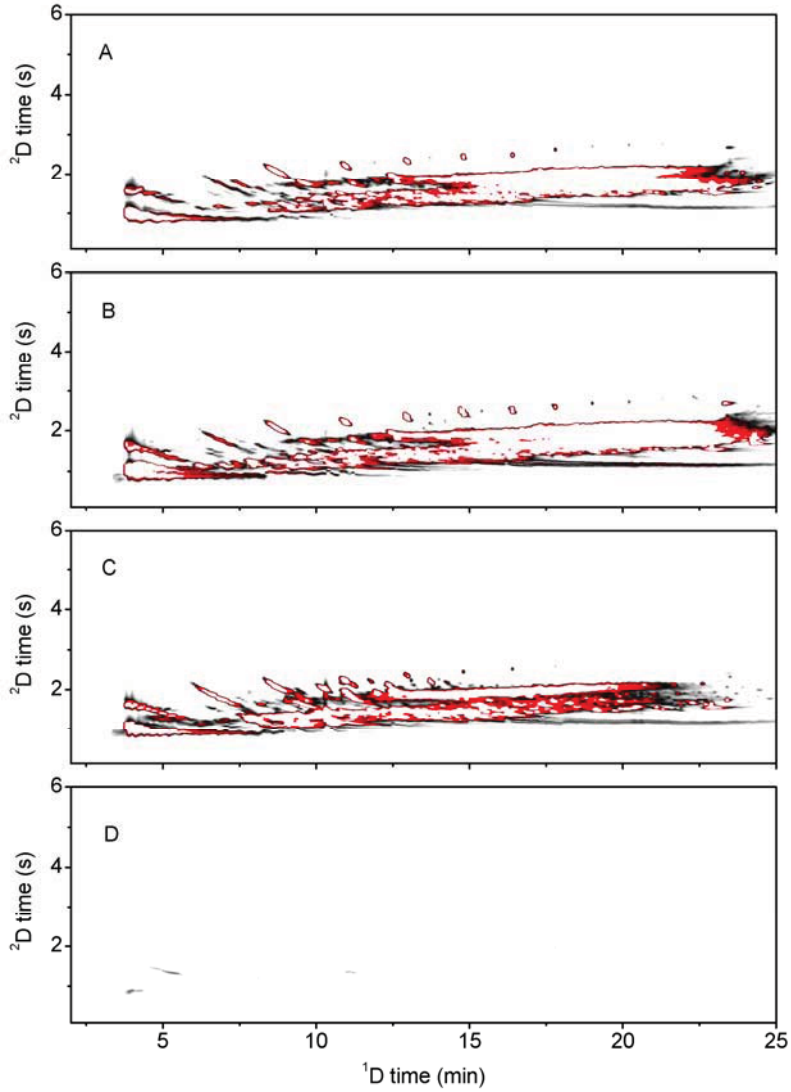
The first column set applied in cocaine volatiles separation was BPX50/BPX5. Three different cocaine samples (proved to be different in a later stage) have shown different profiles, but the components were spread on a rather narrow band along the 1D column (see Figure 3.5). It seems that the separation mechanism in BPX5 based on the weak H-bonding and stronger 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 3.5 Representative 2D plots of volatiles in cocaine samples Ca (A), Cb (B), Cc (C) and a blank (D) analyzed by SPME on BPX50/BPX5 column set. Note the similarity of the profiles, even the samples have proven to be different



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 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 (see Figure 3.6), even though all three samples proved to be different.

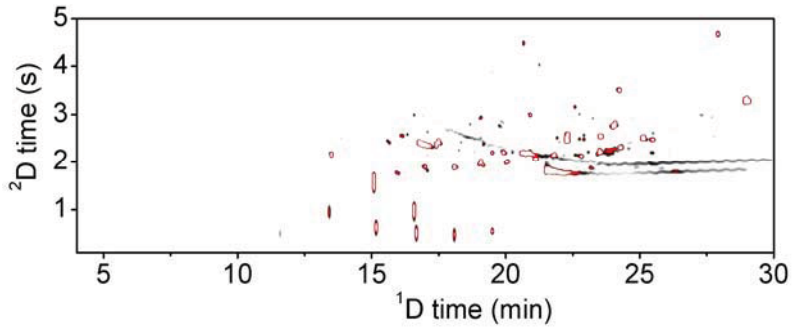
Figure 3.6 Representative 2D plots of volatiles in heroin samples Ha (A), Hb (B), Hc (C) and a blank (D) analysed by SPME on BPX50/BPX5 column set. Note the similarity of the profiles in (A) and (B), even the samples have proved to be different



BP20/DB-1

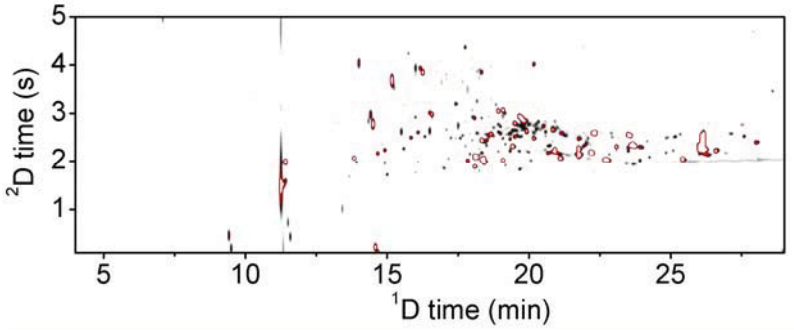
A BP20/DB-1 column combination was used as a second P–NP column set to test the separation of volatiles in cocaine and heroin samples. Very few components in sample Cc (see Figure 3.7) have been detected by contrast with the 2D plot of the same sample obtained on BPX50/BPX5 column set (see Figure 3.5C). 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 length).

Figure 3.7 2D plot of volatiles in cocaine sample Cc analysed by SPME on BP20/DB-1 column set



Chromatographic behaviour of volatiles in heroin (see Figure 3.8) on this column set has been found similar to the cocaine volatiles, not because of their same identity, but simply because the spread is greater over 2D space. Compared with separation on BPX50/BPX5, a smaller number of components has been detected; however, they show 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 the cocaine and heroin constituents, thus better spread on the 2D column.

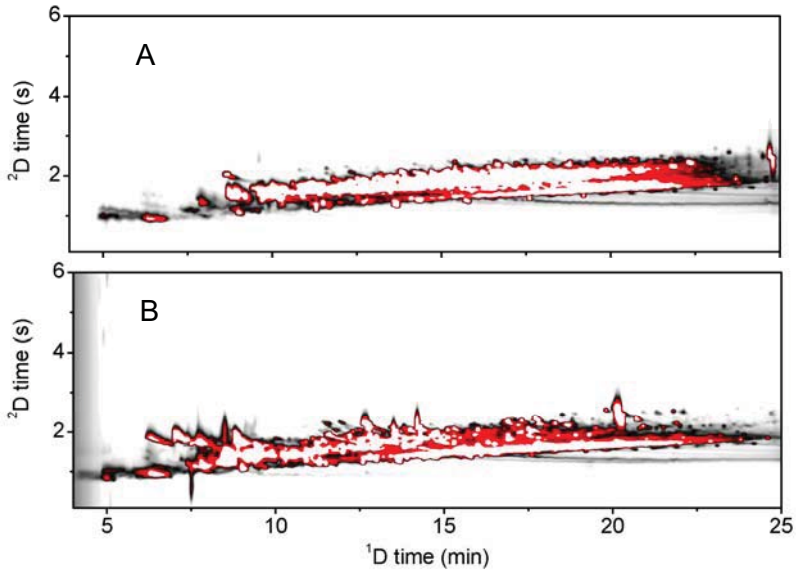
Figure 3.8 2D plot of volatiles in heroin sample Ha analysed by SPME on BP20/DB-1 column set



BPX5/BPX50

The separation of volatiles in cocaine samples on NP–P column sets was initially conducted on the BPX5/BPX50 column set (see Figure 3.9A). While slightly increased peak signal for components was obtained when compared with BPX50/BPX5 column set, and significantly more components detected than in case of BP20/DB-1, the components are located in a rather narrow band on the 2D plot along the 1D 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. Similar 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 (see Figure 3.9B)

Figure 3.9 2D plot 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

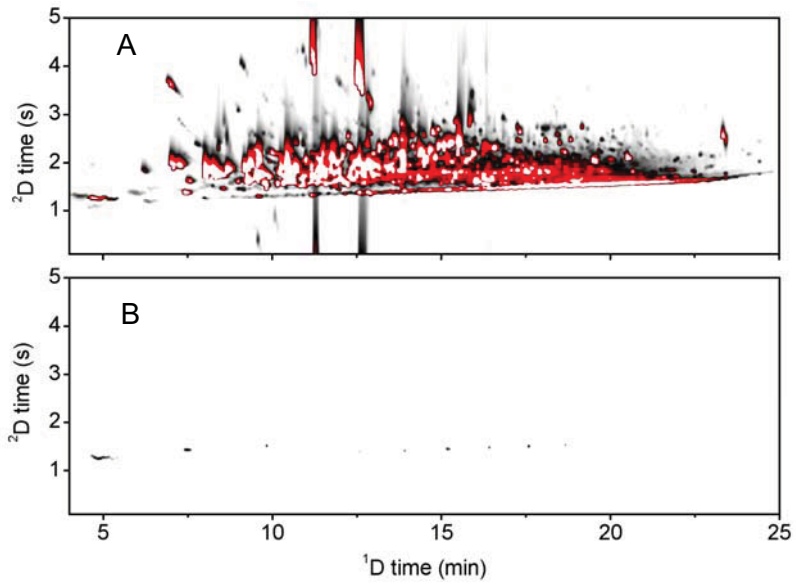


BPX5/BP20

BPX5/BP20 was the second NP–P column set tested for volatiles in cocaine and heroin. It has shown better spread of components on the 2D space, as well as good peak shape (see Figure 3.10). This column set revealed the highest number of separated volatiles in cocaine and heroin samples compared with 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 versus strong H-bonding and weak ϖ- and van der Waals bondings of the BP20 column, is more discriminative towards heroin and cocaine volatile constituents.

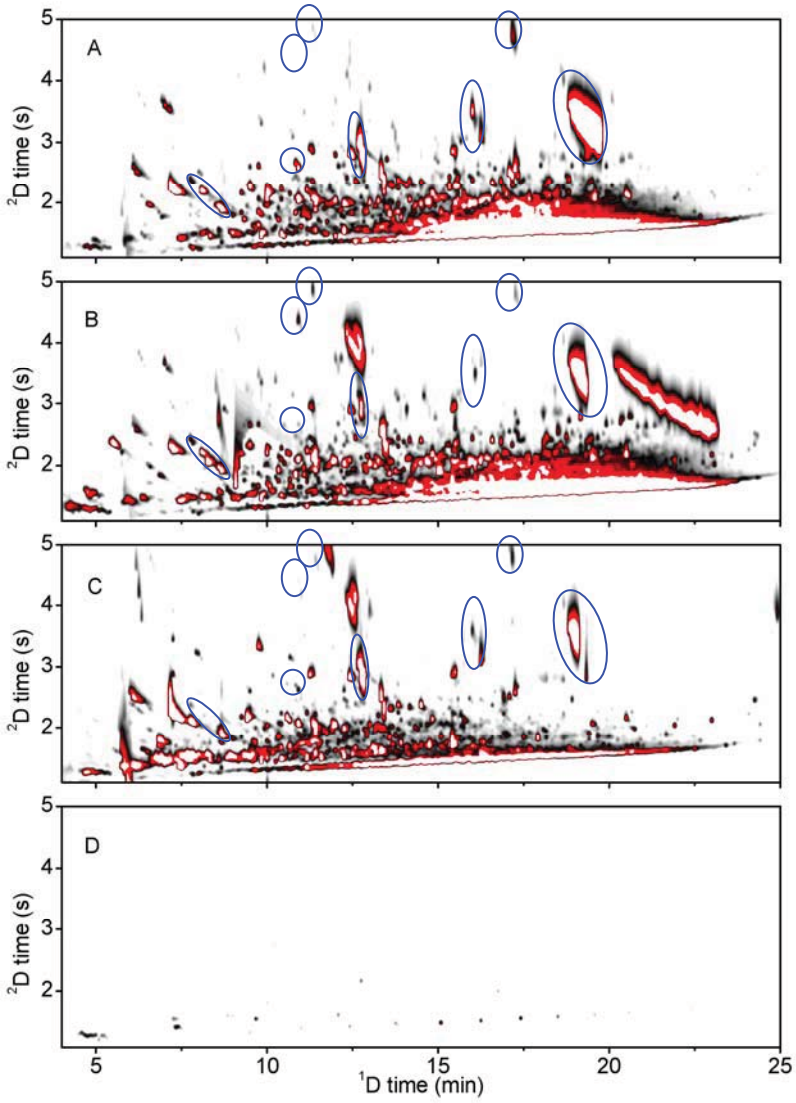
A sample GC×GC-FID profile of volatiles and semi-volatiles in cocaine is shown in Figure 3.10 along with a 2D plot of a blank, where only a few siloxanes are detected as background components. However, unambiguous identification of the components is necessary in order to avoid comparison of samples based on non-origin related markers. Unfortunately, a high number of reference components are required for GC×GC-FID to identify all components. Because of this, all further experiments were conducted on this column set, including the application employing a FPD.

Figure 3.10 Representative 2D plot of (A) cocaine sample Cc analysed by SPME on BPX5/BP20 column set at 0°C modulation temperature and 5 s modulation period, along with (B) a 2D plot of a blank, analysed under the same conditions



By contrast with heroin volatile profiles of samples Ha, Hb and Hc on BPX50/BPX5 (given in Figure 3.6), profiles on BPX5/BP20 generated more individual components and could serve as reliable sample comparisons based on much more individually separated components. A closer look at the heroin profiles in Figure 3.11 can distinguish sample Ha from sample Hb and the differences are easily noticeable at the circled regions. Differentiation of these two samples on the other column set was almost impossible. The largest spot in sample Hb at 1tR of 20–23 minutes is due to the high abundant peak of caffeine, which in fact is not a good component for sample comparison.

Figure 3.11 Representative 2D plots of heroin samples Ha (A), Hb (B) and Hc (C) analyzed 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



BPX5/BP20 with FPD

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

Cocaine and heroin volatiles profiling on GC×GC-TOFMS

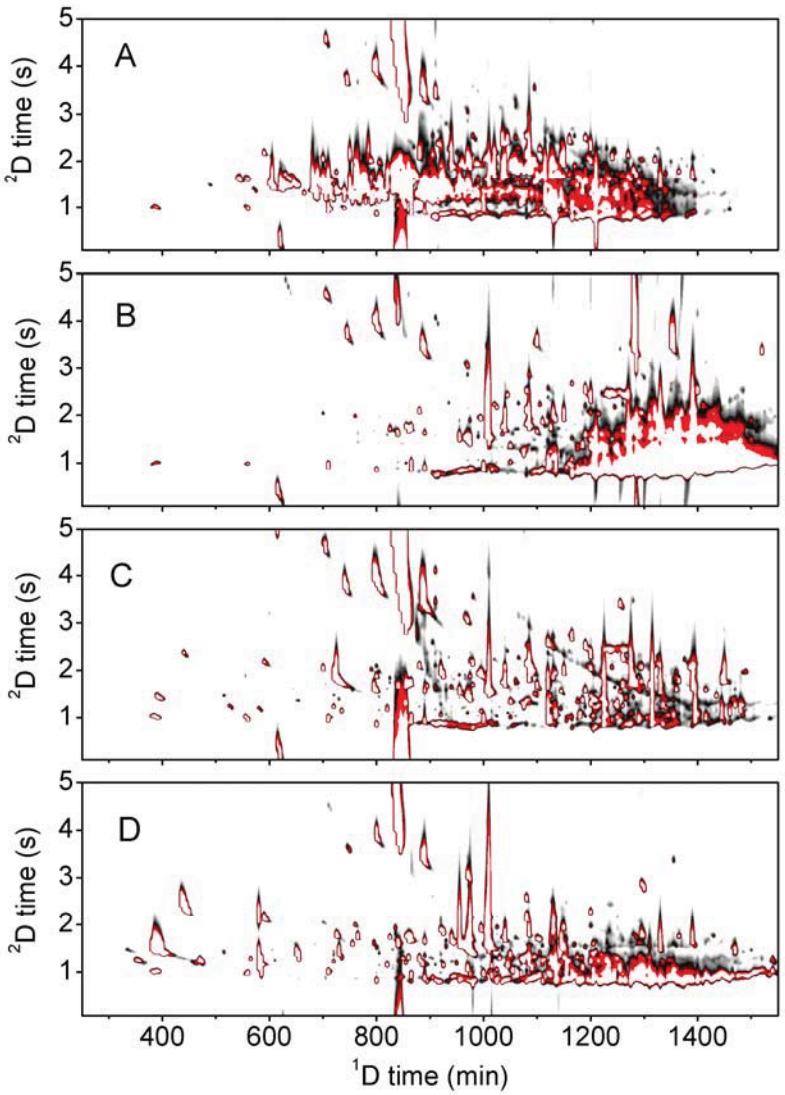
GC×GC-TOFMS separation and identification were conducted on BPX5/BP20 column set under the same conditions as in GC×GC-FID experiments, with two differences: 1.4m 2D column was replaced with 1m long 2D column of the same type and helium was used as a carrier gas instead of hydrogen. The reason was to avoid any wraparounds and still to keep good spread of the components on 2D plots.

Identification and location of components on 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 and TOFMS as a detector. 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 derivatisation (ie truxillines). Head space SPME is generally not efficient in sampling higher molecular weight components, components which show low vapour pressure and polar components on polydimethylsiloxane/divinylbenzene. However, GC×GC-TOFMS chromatograms of volatiles and semi-volatiles in four different cocaine samples (see Figure 3.12) have revealed a large number of components, some of them as potentially good markers for profiling purpose.

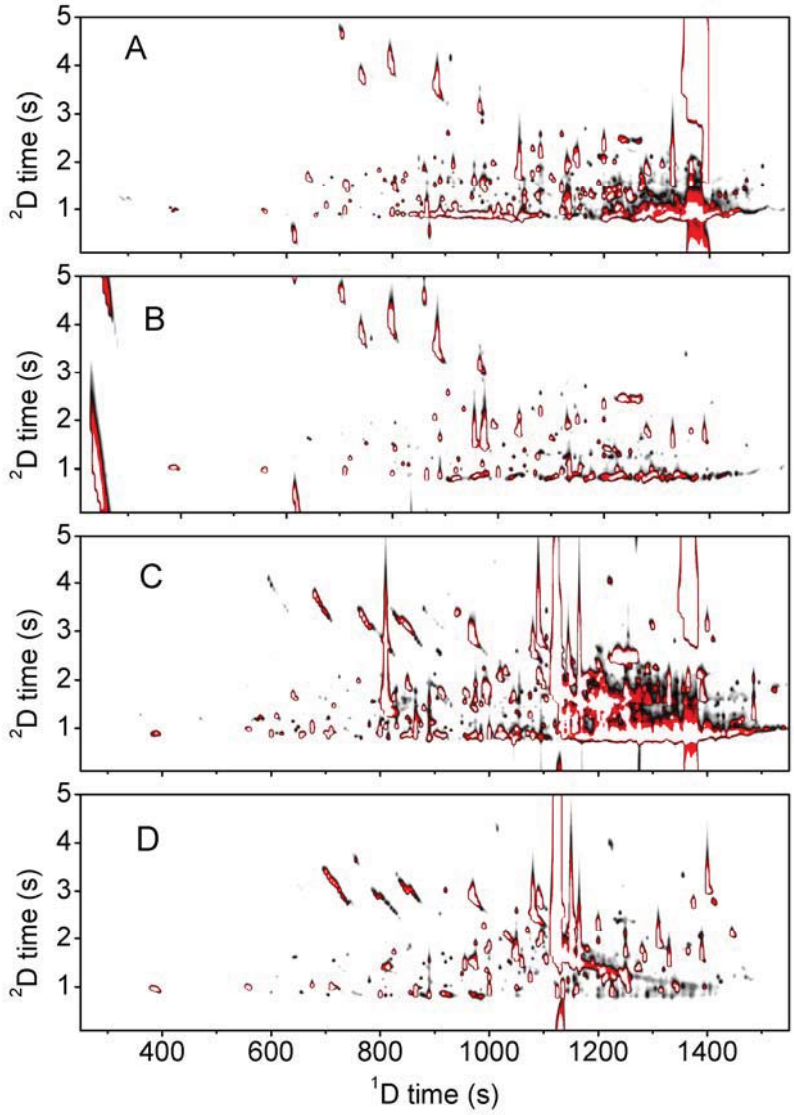
Good spread of the volatiles on this column set has also been obtained for heroin samples. Figure 3.13 shows different profiles of different heroin samples seized in Australia. It is well known that highly purified heroin samples from SEA (the majority of heroin samples seized in Australia come from this region) have simple organic impurity profiles making them unsuitable for sample-to-sample discrimination. However, volatile profiles of these samples are of reasonable complexity and represent a good base for selecting many markers for sample discrimination. The huge peak at 1tR of 1350 s in Figure 3.13A and Figure 3.13C is due to the heavy sample adulteration with caffeine.

Figure 3.12 Representative GC×GC-TOFMS 2D plots of cocaine samples (A) Ca, (B) Cb, (C) Cc and (D) a cocaine sample seized in Australia, analysed on BPX5/BP20 column set. Visually different profiles have been obtained for different samples



By contrast with the previous methods for volatile profiling (Dujourdy & Besacier 2008), where components eluting up to acetophenone (RMM 120) have been 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 (see Table 3.1) and the list of selected components for heroin profiling consisted of 17 components (see Table 3.2).

Figure 3.13 Representative GC×GC-TOFMS 2D plots of heroin samples (A) JP, (B) JP1, (C) JP2 and (D) JP3 seized in Australia, analysed 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, ecgonidine 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 (PMK, 1-methyl pyrrole, 6-caprolactam) or heroin sample constituents (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 the country of seizure trivial. Highly purified heroin samples, supposed to originate from SEA (samples seized in Australia), lack many of the components selected for profiling making classification between SEA and SWA samples trivial. However, the lack of markers in these samples make differentiation of samples within a region difficult and probably sample amount increasing is seeing as possible solution.

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, trimethyl benzenes, 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, xylene isomers, phenyl acetate, benzaldehyde etc are detected in heroin samples but not taken for profiling purpose.

The lowest boiling point solvents such as methanol, ethanol, acetone, diethyl ether, 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 (70 minutes in total) even though unlikely is not excluded. However, such a confirmation requires an extensive research.

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 that originated from that batch or particular supply chain. Two pesticides (γ-lindane and trifluraline) have been detected at different level in all three cocaine samples from Macedonia and in one sample from Australia, which could be possible good batch markers if they originate from the coca crop treatment. Their presence in the samples has been confirmed by GC-accTOFMS with an average mass accuracy of less than five ppm.

Table 3.1 Selected 23 components for cocaine profiling with their retention times, ions used for area calculation and similarity against NIST05 and Wiley7 MS libraries

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

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

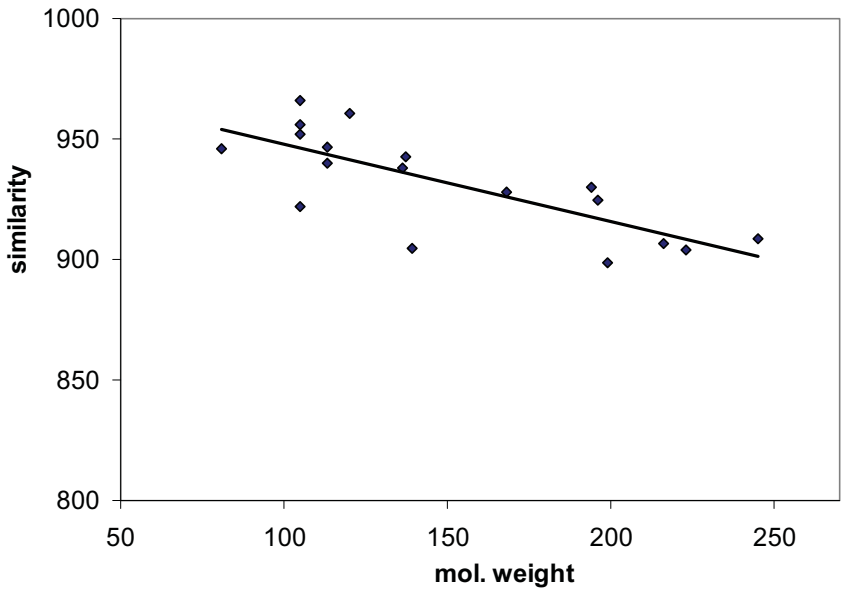
Once the full mass spectra of the matrix are retained, cutting agents and other non-target components can be easily included in the list if such is needed. On average 400 components out of 1,400–1,700 detected in total have been tentatively identified in the samples with similarity above 800 and S/N threshold of 100. 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.

Table 3.2 Selected 17 components for heroin profiling with their retention times, ions used for area calculation and similarity against NIST05 and Wiley7 MS libraries

	Name	1tR	2tR	Similarity	Quant. ion (m/z)
1	4-cresol acetate	770	1.76	941	150
2	3-cresol acetate	805	1.76	904	150
3	1,2,4-trimethoxybenzene	975	2.02	945	168
4	3,5-dimethoxybenzaldehyde	1060	2.18	918	166
5	3,4-dimethoxybenzaldehyde	1090	2.44	926	166
6	4-methoxyquinoline	1135	2.34	850	159
7	3,4-dimethoxybenzyl acetate	1165	2.14	835	210
8	Xanthene isomer 1	1195	1.64	-	181
9	Xanthene isomer 2	1205	1.74	-	181
10	Xanthene isomer 3	1215	1.76	-	181
11	Unknown 197 isomer 1	1210	1.20	-	197
12	Unknown 197 isomer 2	1215	1.26	-	197
13	Unknown 197 isomer 3	1250	1.32	-	197
14	meconine	1275	3.78	885	194
15	hydrocotarnine	1320	1.92	840	220
16	Acetyl codeine	1395	3.44	827	341
17	trifluralin	1200	1.26	810	306

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. 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 our previous conclusion (Mitrevski et al. 2008; Mitrevski, Wilairat & Marriott 2010a) 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, as calculated for the components given in Table 3.1, is shown in Figure 3.14. The average similarity of selected cocaine profiling components (excluding 2 outliers) was 925 and the average similarity for the identified heroin components was 880. The lower average similarity for heroin components is probably contributed to by their higher average molecular mass (data not given) when compared with cocaine components. The high similarity of TOFMS spectra to the commercial library entries excludes the need for TOFMS dedicated in-house library, one which was necessary in our study on sterols and anabolic agents (Mitrevksi et al. 2008; Mitervski, Wilairat & Marriott 2010a, 2010b).

Figure 3.14 The average similarity of TOFMS spectra of selected volatiles in cocaine samples against NIST05 and Wiley7, versus their molecular weight



Conclusion

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 importantly, discrimination between samples from the same country (region). This is especially valuable for highly purified heroin samples usually originating from SEA region, where the lack of impurities in the profiles prevents their discrimination.

Volatile and semi-volatile component profiles are complex, containing 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 strong ϖ-ϖ^* 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.

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

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Appendix

The following publications and reports were prepared by Professor Marriott and PhD student Mr Blagoj Mitrevski during the period of the NDLERF Grant. Mr Mitrevski was primarily responsible for the illicit drugs profiling work and was assigned the task of undertaking the NDLERF project. However, he held an IPRS grant and under this grant it was decided that two parallel studies would be conducted, namely:

- steroids analysis; and
- illicit drugs analysis.

For completeness, studies in both these areas will be reported here, since this represents aspects of learning the technology that was developed for steroids and then later applied to illicit drugs analysis.

Refereed journals

1. Blagoj Mitrevski, J. Thomas Brenna, Y. Zhang and Philip J. Marriott
   A new paradigm in drugs analysis: comprehensive two-dimensional gas chromatography for steroid analysis
   Chemistry in Australia, 74 (Nov) (2007) 3-5 ERA Ranking -
2. Shin Miin Song, Blagoj Mitrevski and Philip J. Marriott
   Chemical Fingerprinting of Illicit Drug Seizures—2D Profiling Using Novel GC Methods
   Chemistry in Australia, 75 (April) (2008) 20-22 ERA Ranking -
3. Blagoj S. Mitrevski, J. Thomas Brenna, Y. Zhang, Philip J. Marriott
   Application of Comprehensive Two-dimensional Gas Chromatography to Sterols Analysis
   Journal of Chromatography, A, 1214 (2008) 134–142; ERA Ranking A+
4. Blagoj Mitrevski, Konstantinos Kouremenos and Philip J. Marriott
   Accelerating analysis for metabolomics, drugs and their metabolites in biological samples using multidimensional methods in gas chromatography
   Bioanalysis I(2) (2009) 367-391, ERA Ranking -
5. Jacqui Adcock, Mike Adams, Blagoj Mitrevski, and Philip Marriott
   Peak modeling approach to accurate assignment of first-dimension retention times in comprehensive twodimensional chromatography
   Analytical Chemistry, 81 (2009) 6797-6804 ERA Ranking A+
6. Blagoj S. Mitrevski, Prapin Wilairat and Philip J. Marriott
   Improvement of Separation and Identification of Anabolic Agents in Doping Control: GC´GC-TOFMS as a New Tool
   Accepted for publication in Journal of Chromatography A. 2010 ERA Ranking A+
7. Blagoj S. Mitrevski, Prapin Wilairat, Philip J. Marriott.
   Comprehensive Two-Dimensional Gas Chromatography – Time-of-Flight Mass Spectroscopy Method Performance Against World Anti-doping Agency Identification Criteria
   Accepted for publication in Analytical and Bioanalytical Chemistry. 2010 ERA Ranking B.
8. Blagoj S. Mitrevski, Philip J. Marriott (additional authorship TBA).
   Illicit Drug Profiling by Using Comprehensive Two-Dimensional Gas Chromatography and Chemometric Analysis
   Manuscript In Preparation, Jan 2010

Conference presentations

Philip Marriott and Blagoj Mitrevski

Design of a comprehensive two-dimensional gas chromatography (GC×GC) system for drugs analysis

Invited Keynote Lecture. Asianalysis Jeju, Korea, 7-9 November 2007

Philip J. Marriott, Tin Tran, Blagoj Mitrevski

Studies in comprehensive two-dimensional gas chromatography / mass spectrometry

Invited Lecture, ANZSMS22 Biennial Mass Spectrometry Conference, Sydney, 27-30 January 2009

Blagoj Mitrevski and Philip Marriott

Separation and identification of endogenous sterols by using comprehensive two-dimensional gas chromatography

Singapore International Chemistry Conference 5 (SICC5) and 7th Asia Pacific International Symposium on Microscale Analysis and Separation (APCE 2007). 17-19 December 2007

Blagoj Mitrevski, Philip Marriot and Prapin Wilairat

Application of GCxGC-TOFMS for detection and confirmation of anabolic substances in doping control

Oral Presentation, 16th Annual Topics in Research and Development Meeting, 6-9 December 2008, Macquarie University, Sydney

Blagoj Mitrevski, Philip Marriott and Prapin Wilairat

Application of GCxGC-TOFMS for detection and confirmation of anabolic substances in doping control

Poster presentation, ACROSS Symposium, University of Tasmania, Hobart, 8-10 December 2008,

J. T. Brenna, Y. Zhang, H.J. Tobias, G.L. Sacks, B.S. Mitrevski, P.J. Marriott

Urinary Steroidomics by Comprehensive Two Dimensional Gas Chromatography-TOFMS (2D GC×GC-TOFMS) and Isotope Ratio Mass Spectrometry (IRMS). Instrumentation and Application to Sports Antidoping

ASMS 2009. Sanibel Meeting, Lipidomics and Lipids in Mass Spectrometry. January 23 - 26, 2009

TradeWinds Island Grand Hotel, St. Petersburg Beach, Florida

Blagoj Mitrevski, Prapin Wilairat and Philip J. Marriott

GCxGC-TOFMS improves the separation and identification of anabolic agents in doping control

TIAFT Confence, Geneva, Switzerland August 2009

Other Reports

Marriott, Philip J, Mitrevski, Blagoj
Australian research tackles drug cheats
Television News segment broadcast on Australia Network News, 25 November 2009.
www.australianetworknews.com

Travel

Mr. Blagoj Mitrevski presented his most recent research data in WADA Anabolic Agents analysis at The International Association of Forensic Toxicologists (TIAFT) held in Geneva, Switzerland in August 2009. In association with this visit, he also returned to his home in Macedonia to consult with his former laboratory which services the analysis of street capture drugs, and which had supplied him with samples of drugs, the analysis of which were reported in the previous report.

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